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Welcome by the editors

We are delighted to edit the extended abstracts of the IPWG workshop on "Multidisciplinary approaches toward phytoplasma-associated diseases detection and management" held in Lucknow, India. Phytoplasma associated diseases are a major limiting factor to quality and productivity of many economically important agriculture crops worldwide. Annual losses due to phytoplasma diseases in many crops under the pathogen favorable conditions, favored by climate change, always led to disastrous consequences to farming communities.

The IPWG group, born in Bologna, Italy, in 2007 is the forum for sharing information and strength and/or built new and more intense scientific interactions among participants. It is formed by worldwide scientists working on several aspects of these phloem-limited microorganisms and their interaction with plant and insect hosts to increase and disseminate the knowledge about phytoplasma-associated diseases worldwide. The group is regularly organizing meetings every four years in a different country, and it has successfully organized meetings in Italy (2007), Germany (2011), Mauritius (2015), Spain (2019) and Oman (2023).

In the frame of this working group activity the IPWG 2025 workshop held in Lucknow, India at the ICAR-Indian Institute of Sugarcane Research, Lucknow from 25-28, February 2025 has an expected attendance of more than 100 scientists from 16 countries worldwide. The meeting sessions will be focused on omics, new detection tools, interactions, country status, epidemiology and control. Integrated disease management and diseases of quarantine importance will also be discussed together with sessions on phytoplasma diseases in sugar crops, palms and citrus.

The extended abstracts cover several aspects of phytoplasma research and are really imperative and valuable in illustrating the research and development needs in phytoplasma field. Different aspects of phytoplasma-associated diseases are presented. These topics will provide an authoritative scientific backdrop for informed discussions and debates on the achievements in phytoplasma research and their practical applications helping to share a global vision on phytoplasma research.

The presentations of the workshop are included in this issue of Phytopathogenic Mollicutes, which is a half yearly official publication of the Technology Society of Basic & Applied Sciences (TSBAS) and is published by Indianjournals.com, New Delhi, India. This issue contains 87 extended abstracts. We are sure that this issue of Phytopathogenic Mollicutes will be helpful to update the knowledge on phytoplasma research at a global level and for harnessing and understanding the full potential of this unique group of microorganisms. All the papers published in this issue have been reviewed and accepted by the IPWG Scientific Committee. Online information concerning these IPWG proceedings are available at the following web addresses:

- Indian Journals website: www.indianjournals.com
- IPWG website: www.ipwgnet.org

We want to thank the contributors for their diligence and timely submissions of extended abstracts. We apologize for errors that could have arisen during the editing process despite our careful attention. We also thank all the workshop participants, the IPWG scientific committee members and Fabio Montanari, for their consistent support in revising, composing and bringing out this issue of the Journal in time for the workshop.

Besides the IPWG 2025 workshop, a training program on "Phytoplasma outbreaks: collect, preserve, identify phytoplasmas and their insect vectors" was also scheduled in February 22-23, 2025. There are 24 training delegates registered from 4 countries. The training will cover a field trip, molecular detection of phytoplasmas in plants and insects and utilization of bio-informatic tools for phytoplasma appropriate identification.

We welcome everyone attending the IPWG 2025 workshop in Lucknow and wish them a comfortable stay and meaningful interactions with the international colleagues.





Assunta Bertaccini and Govind Pratap Rao
Editors-in-Chief

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Contents

Omics based research [Omics]

Insights into phytoplasma genomes of Indian origin 1-2
Kiran Kirdat, Sneha Verma, Srinibas Behara, Ramachandran Sundararaj and Amit Yadav

Characterization of the membrane protein of onion yellows phytoplasma that has a domain of Deg/HtrA family protease 3-4
Hidemasa Oda, Ai Endo, Takahiro Sambe, Kensaku Maejima, Shigetou Namba, Yasuyuki Yamaji and Kenro Oshima

Unveiling the 3D structure of phytoplasma effectors: a streamlined protocol for SAP11CaPM expression and purification 5-6
Mattia Tabarelli, Amelie Blabl, Alberto Ceccon and Katrin Janik

Transcriptome analysis of sesame phyllody phytoplasma: towards identification of resistance-imparting genes 7-8
Hemavati Ranebennur, Celia Chalam Vasimalla, Govind Pratap Rao, Kirti Rawat, Manoj Kumar Yadav, Shivani Gupta, Ashutosh Rao and Rashmi Yadav

CRISPR/Cas9-mediated generation of tomato RAD23 knockouts for studying phytoplasma-induced symptoms 9-10
Junichi Inaba, Bo Min Kim, Yan Zhao and Wei Wei

"Flavescence dorée" phytoplasma strains genetic variability: a comparative genomics approach 11-12
Zahra Golestani Hotkani, Giulia Calia, Diego Micheletti, Alberto Gelmetti, Alessandro Cestaro, Matteo Brilli and Mirko Moser

New tools for phytoplasma detection [New detection tools]

Artificial intelligence in phytoplasmology: image-based disease detection models for phytoplasma infection in flower crops 13-14
Kizhalot Prabha, Kadukothanahalli Veerabhadraiah Shivakumar, Sapkal Saurabh, Govind Pratap Rao and Kuchimanchi Venkataramana Prasad

Leveraging artificial intelligence and big data to advance phytoplasma disease detection and crop health management 15-16
Wei Wei, Jonathan Shao and Yan Zhao

Development of a multilocus sequence typing method for accurate identification of 16SrV phytoplasma strains via Oxford nanopore 17-18
Stefano Costanzo, Sam Grinstead, Yan Zhao and Wei Wei

Oxford nanopore sequencing using metabarcoding as detection and identification tool for '*Candidatus* Phytoplasma' and '*Ca*. Liberibacter' taxa 19-20
Ellen A. Everaert, Kris De Jonghe, Maaike Heyneman, Yoika Foucart, Dieter Slos and Annelies Haegeman

PhenoTruck^{AI}: a mobile laboratory for hyperspectral and molecular detection of phytoplasma quarantine diseases like "flavescence dorée" 21-22
Wolfgang Jarausch, Bonito Thielert, Markus Michel, Patrick Menz, Miriam Runne, Gesa Götte, Sebastian Warnemünde and Sylvia Wagner

Molecular variation of '*Candidatus* Phytoplasma ziziphi' isolated during co-cultivation from micropropagated jujube witches' broom infected shoots 23-24
Anton Zakurin, Jidong Li, Francesco Pacini and Assunta Bertaccini

A colorimetric LAMP based assay for rapid screening of sandalwood spike phytoplasma 25-26
Donald James, Adharsh Tharayil Sunder, Meenu Muralikrishnan, Preethu Thekkepurath, Murali Revanasiddappa and Suma Arun Dev

Recombinase polymerase amplification assay: a simplified and rapid approach for detection of fastidious plant pathogens 27-28
Virendra Kumar Baranwal

Evaluation of a hybridization probe-based multilocus sequence typing technique for identification of diverse phytoplasmas 29-30
Harvinder Bennypaul, Daniel Sanderson, Tim Dumonceaux and Christine Hammond

Comparative study of phytoplasma DNA extraction methods 31-32
Ida Bashkirova, Aleksey Shvartsev, Yuri Shneyder and Irina Smirnova

A novel approach for detection of sandal spike infection in *Santalum album* 33-34
Tresa Hamalton and Sreedevi Cigatagere Nagaraj

Satellite-based detection of apple proliferation and pear decline 35-36
Ali Al Masri, Katrin Kohler, Jukka Höhn, Miriam Runne and Wolfgang Jarausch

Phytoplasma-plant-insect vector interactions [Interactions]

Insect vector transmission of phytoplasmas and citrus greening bacteria in diverse agricultural crops of India 37-38
Govind Pratap Rao

Leafhoppers as vectors of phytoplasma diseases 39-40
Naresh M. Meshram, Akash Nikoshe and Dilip Kumar Ghosh

Molecular and morphological characterization of leafhoppers as vectors of sesame phyllody in Andhra Pradesh, India 41-42
Gopisetty Sandhya, Madem Gurivi Reddy, Govind Pratap Rao, Reddypogu Sarada Jayalakshmi Devi and Duddu Bharathi

A new invader in Germany: *Scaphoideus titanus*, the vector of "flavescence dorée" in grapevine 43-44
Barbara Jarausch, Lars Askani, Sanela Kugler, Wolfgang Jarausch, René Fuchs, Olaf Zimmermann, Kerstin Zikeli, Miriam Runne and Jürgen Gross

Monitoring of alternative insect vectors of "flavescence dorée"-related phytoplasmas in different wine-growing regions in Germany 45-46
Barbara Jarausch, Sandra Biancu, Sanela Kugler, Wolfgang Jarausch and Michael Maixner

Biology and phytoplasma detection of the newly described *Bamboosella dimorpha* leafhopper in grapevine yellows-affected vineyards 47-48
Nicola Mori, Enea Guerrieri, Francesco Sanna, Flavio Serina, Sara Torcoli and Fabio Quaglino

First report of a psyllid vector of '*Candidatus* Phytoplasma pruni' (strain 16SrIII-J) 49-50
Tomás Llantén, Sebastián Cabrera, Javiera Fuentes, Camila Gamboa, Constanza González, Alan Zamorano and Nicola Fiore

Insights into phytoplasma lineages: evaluating single-locus and multilocus trees from next-generation sequencing data 51-52
Syeda Wardah Noor, Christopher H. Dietrich and Valeria Trivellone

Seasonal dispersal of *Cacopsylla bidens* and its role in the epidemiology of '*Candidatus* Phytoplasma pyri' in mixed-crop systems 53-54
Rakefet Sharon, Maor Tomer and Annat Zisovich Charitt

First identification of *Cacopsylla bidens* as a vector of '*Candidatus* Phytoplasma pyri', the agent associated with pear decline 55-56
Maor Tomer, Annat Zisovich Charitt and Rakefet Sharon

Deciphering the impact of phytoplasma presence on structure and biochemical composition of brinjal crop 57-58
Apoorva Srivastava and Smriti Mall

Biophysical properties of trichome in sesame phyllody 59-60
Kailash Narayan Gupta, Vivek Chouksey and Anand Kumar Vishwakarma

A review on phytoplasma induced biochemical responses in plants 61-62
Desireddy Vindyasree, Madem Gurivi Reddy, Reddypogu Sarada Jayalakshmi Devi, Boya Hussainammagari, Chaitanya and Manyam Pradeep

Association of phytoplasmas with tapping panel dryness syndrome of *Hevea brasiliensis*: a pathological perspective to unresolved mystery? 63-64
Shaji Philip, Ann Tom, Edwin Prem, Ramyasree Puramerimadathil, Ruksana Kuttickal Sajeed, Alphonsa Mathew and Sainamole Kurian

Country status of phytoplasma diseases [Country status]

Phytoplasmas diversity in India 65-66
Smriti Mall, Ragini Vishwakarma, Km Preeti and Mithilesh K. Gupta

Phytoplasma disease prevalence in Namsai, Arunachal Pradesh, India: an exploratory study 67-68
Jutimala Phookan and Manoj Kumar Kalita

Molecular characterization and symptom assessment of phytoplasma infection in king chilli across Assam, India 69-70
Shabiba Jahan Hazarika, Dibya Sree Dutta and Manoj Kumar Kalita

Assessment of pear decline prevalence and phytoplasma Infection in major commercial orchards of Al-Mafraq, Jordan 71-72
Asem H. Abu Alloush, Kristi D. Bottner-Parker, Fabio Quaglino and Wei Wei

Identification of 16SrII-D phytoplasmas in sesame and groundnut crops in Tirupati district of Andhra Pradesh, India 73-74
Kudithipudi Suhas, Madem Gurivi Reddy, Bommu Veera Bhaskara Reddy and Karanam Venkatachalapathi Hari Prasad

Unveiling the hidden threat: a comprehensive study on groundnut phytoplasma in the major growing regions of Andhra Pradesh, India 75-76
Periyamaya Devar Pusalapuram, Reddypogu Sarada Jayalakshmi Devi, Harikishan Sudini, Manda Jhonson, Ampagouni Prasanna Rajesh

Preliminary identification of phytoplasmas infecting flower crops in Andhra Pradesh, India 77-78
Dasari Meghanath, Kalleti Arunodhayam, Madem Gurivi Reddy, Chitrapu Ruth, Mude Raja Naik, Nagarapu Sai Aswani and Karuba Gopal

Multigene characterization of phytoplasmas associated with flat branch disease in sweet cherry and their effects on some fruit properties 79-80
Kishan Lal Kumawat, Sajad Un Nabi, Shreenath, Wasim Hassan Raja, Mahendra Kumar Verma and Govind Pratap Rao

Morpho-pathological and molecular characterization of a '*Candidatus* Phytoplasma australasiaticum' strain from linseed 81-82
Lakshman Prasad, Shashank Kumar Yadav, Davender Singh, Sushma Sagar, Balram Jat, Mahender Singh Saharan, Govind Pratap Rao and Vikender Kaur

Identification of phytoplasma disease infection in forest trees in Jammu region, India 83-84
Ashok Kumar Singh, Deepak Kumar and Govind Pratap Rao

Status and challenges of sandal spike disease in Indian forests: an analysis 85-86
Sundararaj Ramachandran, Athulya Ramakrishnan, Narasimha Murthy and Amit Yadav

Threat of tamarisk phytoplasmas in man-made tamarisk forest for agricultural crops and natural resources plants in Iran 87-88
Seyyed Alireza Esmaeilzadeh-Hosseini, Ghobad Babaei and Assunta Bertaccini

Rice orange leaf phytoplasma: a reemerging disease in coinfection with rice tungro virus in the Philippines 89-90
Gilda Jonson, Ivy Lituañas and van Schepler-Luu

Growing prevalence of phytoplasma diseases in Ayodhya region, India 91-92
Sushil Kumar Singh, Harshita Yadav, Apoorva Srivastava and Smriti Mall

An update status of phytoplasma diseases in chickpea 93-94
Madem Gurivi Reddy, Govind Pratap Rao, Kudithipudi Suhas and Reddypogu Sarada Jayalakshmi Devi

An update status of recently emerging '*Candidatus* Phytoplasma omanense' strains in Iran 95-96
Seyyed Alireza Esmaeilzadeh-Hosseini, Ghobad Babaei, Abbas Paydar Ardakani and Assunta Bertaccini

Presence of '*Candidatus* Phytoplasma asteris' in red gram and horse gram in Ananthapuramu district of Andhra Pradesh, India 97-98
Vemana Kuruba, Kirti Rawat, Sarada Jayalakshmi Devi Reddypogu, Hemavati Ranebennur, Shivani Gupta and Govind Pratap Rao

Molecular detection of '*Candidatus* Phytoplasma australasiaticum' and '*Ca*. P. trifolii' in grapevine in India 99-100
Shivani Gupta, Mailem Y. Shimray, Jayesh Gehlot, Abhinav Tiwari, Kirti Rawat, Hemavati Ranebennur, Nitika Gupta, Somnath K. Holkar, Virendra K. Baranwal, Govind P. Rao and Susheel K. Sharma

Identification of '*Candidatus* Phytoplasma trifolii' associated with mango phyllody in Kerala, India 101-102
Sajeena Abdulmajeed, Shivani Gupta, Dhanya Mannananil Krishnankutty, Makeshkumar Thangaraj, Muthulakshmi Lajapathy Jeeva, Syamala Swayamvaran Veena, Ayisha Rehman and Govind Pratap Rao

Severe recurrence of tomato big bud disease associated with 16SrII phytoplasmas in Andhra Pradesh, India 103-104
Shivani Gupta, Sesha Kiran Kollipara, Tirukoillur Mohanbabu Hemalatha, Hemavati Ranebennur and Govind Pratap Rao

Identification of a '*Candidatus* Phytoplasma citri' strain associated with phyllody disease of cumin 105-106
Manish Kumar, Ramesh and Govind Pratap Rao

Emerging phytoplasma threat: plum orchards under siege in Himachal Pradesh, India 107-108
Abhinav Thakur, Anil Handa and Aman Chauhan

Multilocus gene characterization of phytoplasmas associated with weeds in chickpea crops in Andhra Pradesh, India 109-110
Desireddy Vindyasree, Madem Gurivi Reddy, Manyam Pradeep, Boya Hussainammagari Chaitanya, Madduri Subrahmanya Venkata Chalam and Reddypogu Sarada Jayalakshmi Devi

Epidemiology and control of phytoplasma-associated diseases [Epidemiology and control]

Evaluating the threat of phytoplasma disease emergence in agroecosystems and natural habitats 111-112
Valeria Trivellone, Roxana Jafari Haddadian and Christopher H. Dietrich

Emerging hosts of '*Candidatus* Phytoplasma trifolii': a molecular insight into *Luffa acutangula*, *Momordica dioca* and *Lawsonia innermis* from Northeast India 113-114
Manoj Kumar Kalita and Dibya Sree Dutta

Biological and molecular features of '*Candidatus* Phytoplasma prunorum' associated with a severe outbreak in apricot 115-116
Marco Gagliardi, Romolo Laurita, Roberto Montalbetti, Vittorio Colombo, Nicola Mori, Francesco Pacini and Assunta Bertaccini

Influence of weather parameters on sesame phyllody 117-118
Vivek Chouksey, Kailash Narayan Gupta and Anand Kumar Vishwakarma

Identification of resistant germplasm in sesame against phyllody disease 119-120
Kailash Narayan Gupta, Anand Kumar Vishwakarma, Rajani Bisen, Rashmi Yadav, Govind Pratap Rao and Sanjay Kalia

Epidemiology of brinjal little leaf phytoplasma in Assam, India: genetic diversity and seed transmission 121-122
Dibya Sree Dutta, Rontung Kalita and Manoj Kumar Kalita

Combatting pear decline in Flemish orchards: a comprehensive approach to disease detection and management 123-124
Ellen Everaert, Tim Belien, Gertie Peusens, Serge Remy, Dany Bylemans and Kris De Jonghe

Further study on the potential of *Frateuria defendens* as a biocontrol agent against phytoplasmas 125-126
Lilach Iasur-Kruh, Suzana Moussa and Vered Naor

Integrated disease management and diseases of quarantine importance [Management]

Presence of "flavescence dorée" phytoplasma in grapevine lignified canes and effectiveness of different sanitation methods 127-128
Nadia Bertazzon, Chiara De Gregorio, Olesya Kalinina, Francesco Rampino, Veronica Sofia Bottarel, Cezar Dumitrescu, Luisa Filippin and Elisa Angelini

Testing for '*Candidatus* Phytoplasma phoenicium' and peach latent mosaic viroid presence in Russia 129-130
Yuri Shneyder, Elena Karimova, Tatiana Zhivaeva, Evgeniya Lozovaya, Ida Bashkirova and Yuri Prikhodko

Evaluation of sesame germplasm against the phyllody disease 131-132
Pankaj Sharma, Jaswinder Kaur, Amandeep Singh, Anna Goyal, Priya, Tanisha Gupta and Prabhjpdt Singh Sandhu

Management of phyllody of sesame 133-134
Vivek Chouksey, Kailash Narayan Gupta and Anand Kumar Vishwakarma

Phytoplasma diseases of sugar crops [Sugar crops]

Fastidious, phloem-limited bacteria, pathogens of sugar beet 135-136
Bojan Duduk, Jelena Stepanovic, Nikola Stanojevic, Emil Rekanovic, Nina Vuckovic, Nataša Duduk and Ivana Vico

Identification of '*Candidatus* Phytoplasma solani' in sugar beet in the Voronezh region of the Russian Federation 137-138
Yuri Prikhodko, Maria Pruchkina, Tatiana Zhivaeva, Yuri Shneyder, Olga Stognienko and Elena Gerr

Molecular identification of phloem-restricted bacteria in insects and sugar beet crops in Italy 139-140
Luca Guerrini, Anton Zakurin, Francesco Pacini, Nicola Mori, Sri Tej Mateeti, Giovanni Campagna, Massimo Zaghi, Maria Fattori and Assunta Bertaccini

Multigene characterization of a 16SrII-D phytoplasma associated with sugarcane in Oman 141-142
Ali M. Al-Subhi, Azza K. Al-Aamri, Rashid A. Al-Yahyai and Abdullah M. Al-Sadi

Identification of diverse phytoplasmas in sugarcane yellow canopy syndrome 143-144
Francesco Pacini, Eleonora Satta, Samanta Paltrinieri and Assunta Bertaccini

Mechanized sett treatment: an innovative technology to deliver various agro-inputs for the management of sugarcane diseases including the grassy shoot 145-146
Palaniyandi Malathi, Rasappa Viswanathan and Amalraj Ramesh Sundar

Grassy shoot of sugarcane in India: impact to cane cultivation, diagnostics and sustainable disease management 147-148
Rasappa Viswanathan

Diagnostics of sugarcane grassy shoot phytoplasma for a healthy seed nursery programme in sugarcane 149-150
Kadirvel Nithya, Ramaswamy Manimekalai, Kalimuthu Kaverinathan, Ramasamy Nithyanantham and Rasappa Viswanathan

Seed treatment: suitable approaches for establishing nursery of sugarcane free of phytoplasma associated diseases 151-152
Nilesh Singh, Madhupriya Pandey, Pankaj Kumar Rai, Sant Sharan Tripathi and Ajay Kumar Tiwari

A multiplex PCR assay for rapid differentiation of sugarcane phytoplasmas reveals co-infection in samples from Thailand 153-154
Weerakorn Saengsai, Benjawan Ruttawat and Suchirat Sakuanrungsirikul

Antagonist bacteria in action: a strategy for managing sugarcane white leaf disease 155-156
Weerakorn Saengsai, Benjawan Rattawat, Sineenat Phomthirat, Chamaiporn Kaiyaphai, Raweewan Chuekittisak, Nattapat Khumla and Suchirat Sakuanrungsirikul

Phytoplasma diseases of citrus crops [Citrus crops]

Mixed infection of phytoplasmas, "huanglongbing" and citrus tristeza virus in major citrus species at New Delhi, India 157-158
Surabhi Mitra, Govind Pratap Rao, Eapen Punnapuzha Koshy and Shailendra Kumar Srivastava

First report of '*Candidatus* Phytoplasma aurantifolia' associated with witches' broom disease of grapefruit in Oman 159-160
Mohammed Al-Sadrani, Emran Al-Jahwari and Ali M. Al-Subhi

CRISPR/Cas-based detection of *Spiroplasma citri* for citrus stubborn disease management 161-162
Justin Shih and Wei Wei

Phytoplasma diseases of palm trees [Palm trees]

Current and future molecular diagnostics of palms phytoplasmas 163-164
Ramaswamy Manimekalai, Smita Nair, Govind Pratap Rao, Madhavan Kochu Babu, Virendra Kumar Baranwal and Karthikeyan Gandhi

Search for coconut lethal yellowing alternate hosts among coconut associated weeds and cultivated crops in Mozambique 165-166
Amide Bangali, Marília Mazivele, Nils Hogberg and João Bila

Detection, epidemiology and management of phytoplasma diseases of palms 167-168
Vinayaka Hegde, Merin Babu and Arulappan Josephrajkumar

Comparative symptomatology of root (wilt) and mid whorl yellowing diseases of coconut in Kerala, India 169-170
Amitha Paul, Joy Michal Johnson, Sinijadas K, Radhika Nurani Subramanian, Radhakrishnan Nanjil Venkata Chalam, Usha Chacko Thomas, Swapna Alex and Manju Ramakrishnan Nair Vimala Devi

Phytoplasma diseases of oil palm 171-172
Madhavan Kochu Babu, Govind Pratap Rao, Kochubabu Sumi and Ramaswamy Manimekalai

Transmission studies on spear rot disease of oil palm 173-174
Madhavan Kochu Babu, Govind Pratap Rao, Kochubabu Sumi and Ramaswamy Manimekalai

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Omics

Insights into phytoplasma genomes of Indian origin

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Abstract

The genomes of phytoplasma strains of Indian origin associated with economically significant crops and weeds, were sequenced and analyzed over the past few years, revealing diversity in coding sequences and effector proteins. Mobile genetic elements and horizontally acquired genes highlighted their adaptive evolution. Comparative analyses revealed host-specific adaptations and co-evolutionary traits. These findings provide valuable insights into phytoplasma pathogenicity, host interaction mechanisms, and genome-guided strategies for sustainable disease management.

Keywords: India, host-pathogen interaction, genome adaptation

Introduction

Phytoplasmas are a unique group of obligate, phloem-inhabiting plant pathogens with remarkably reduced genomes ranging between 500 and 900 kilobases (Oshima *et al*., 2004). These bacteria, classified under the class Mollicutes, lack cell wall and essential metabolic pathways, relying on host cellular machinery for survival. This parasitic nature underpins their pathogenicity, manifesting as symptoms such as phyllody, witches' broom, yellowing, and sterility across a wide range of economically significant crops and weeds (Rao *et al*., 2017).

In India, where agriculture is a cornerstone of the economy, phytoplasma-associated diseases pose a major threat to food security and rural livelihoods. Legume crops, sugarcane, brinjal, and sesame have reported significant yield losses due to phytoplasma infections (Kirdat *et al*., 2023a). Advances in genome sequencing have provided valuable insights into the biology, evolution, and host-pathogen interactions of these enigmatic bacteria. Efforts have focused on sequencing and analysing the genomes of key phytoplasma strains of Indian origin, including those associated to peanut witches' broom, sugarcane grassy shoot, and brinjal little leaf diseases.

Materials and Methods

Symptomatic tissues exhibiting phytoplasma infection symptoms were collected from *Parthenium hysterophorus* (strains PR08 and PR34), sugarcane (*Saccharum officinarum*, strain SCGS), *Cynodon dactylon* (strain LW01) Sesame (*Sesamum indicum*, strain SS02) and sandalwood (*Santalum album*, strain SW86) as described (Kirdat *et al*., 2020, 2023b, 2024; Ranebennur *et al*., 2022). Phytoplasma titer was confirmed using quantitative PCR. The genomic DNA was extracted and sequenced using Illumina and Oxford Nanopore Technology (ONT) sequencing platforms. Quality sequence reads were processed for metagenomic binning (MetaBAT2) (Kang *et al*., 2015) and hybrid assembly (Unicycler) (Wick *et al*., 2017), followed by quality checks of the assembled genomes. The curated genome sequences were deposited in GenBank database and annotated using NCBI Prokaryotic Genome Annotation Pipeline (Zhao *et al*., 2012).

Results and Discussion

The complete genomes of parthenium phyllody phytoplasma strains PR08 and PR34 were obtained, with PR08 ('*Candidatus* Phytoplasma australasiae= australasiaticum') comprising 588 kb assembled into a single circular chromosome (GenBank accession number CP097206) and PR34 comprising 614 kb, also assembled into one circular chromosome (GenBank accession number CP097207). PR08 contained 468 coding sequences (CDS), while PR34 contained 474 CDS, with genome GC content of 24.3% and 24.1%, respectively (Kirdat *et a*l., 2023b). These genomes provided critical insights into the genetic basis of phyllody disease, including potential effector proteins and genes associated with host adaptation. The sugarcane grassy shoot phytoplasma ('*Ca*. P. sacchari', SCGS) produced a draft genome sequence of 505 kb, assembled into 29 scaffolds (GenBank accession number VWXM01000000) with 404 CDS and a GC content of 19.9% (Kirdat *et al*., 2020).

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Similarly, the *C. dactylon* ('*Ca*. P. cynodontis', LW01) genome, spanning 483 kb and assembled into 21 scaffolds (GenBank accession number VWOH01000000), contains 425 CDS with a GC content of 20.5%, possibly reflecting its adaptation to Bermudagrass as a host (Kirdat *et al*., 2020). The draft genome of sesame phyllody phytoplasma, strain SS02 (GenBank accession number JAHBAJ020000000), consisted of 553,228 bp, assembled into 60 contigs, with a GC content of 23.55% and a completeness of 95.21%. Annotation revealed 465 coding sequences, 17 tRNA genes, and three rRNA genes (Ranebennur *et al*., 2022). The *S. album* ('*Ca*. P. asteris, SW86) genome consisted of 554 Kb, assembled into 20 scaffolds (GenBank accession number JAGVSK010000000), and harboured 436 CDS with a GC content of 28.5%. This genome uniquely encodes duplicated cation-transporting ATPases and effectors like SAP11 and SAP05, linked to in the pathogenicity and severity of sandal spike disease (Kirdat *et al*., 2024).

Genome sequencing of Indian phytoplasma strains, including PR34, PR08, SCGS, SW86, and SS02, has revealed a diverse repertoire of effector proteins and other pathogenesis-related factors critical to their virulence and adaptability. Effector proteins such as SAP11, SAP05, SAP54, and TENGU, identified in strains like SW86, modulate host transcription, ubiquitin-mediated degradation, and auxin pathways, causing flower abnormalities, witches' broom, and stunted growth. Comparative analyses have also highlighted strain-specific effector profiles. PR34 targets for resistant crop development and advanced disease management strategies. Genome sequencing thus serves as a cornerstone for decoding phytoplasma virulence and enhancing agricultural sustainability.

The genomes of the studied phytoplasmas exhibit significant plasticity, heavily influenced by mobile genetic elements such as group II introns. These introns are prevalent in the genomes of parthenium phyllody phytoplasma strains PR34 and PR08. Strain PR34 harbours 23 group II introns, constituting 4% of its genome, while PR08 contains 17 copies. Their absence in most of the phytoplasmas underscores notable genomic diversity. Group II introns in PR34 are strategically located, often flanked by effector protein genes such as SAP, suggesting their role in facilitating horizontal gene transfer and genome rearrangements. These rearrangements likely contribute to the evolution of pathogenicity and host specificity. The presence of both complete and truncated copies of group II introns in PR34 underscores their dynamic role in shaping genome structure over time.

The sequencing of Indian phytoplasma genomes has provided transformative insights into their pathogenicity, taxonomy, and evolutionary dynamics. Notably, the SCGS genome highlights 14 novel secreted proteins, emphasizing its possible distinct virulence strategies. These advancements not only deepen the understanding of phytoplasma-host interactions but also offer actionable targets for disease management. By leveraging genomic insights, Indian research has laid a solid foundation for sustainable agricultural practices, ensuring crop resilience and food security.

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Omics

Characterization of the membrane protein of onion yellows phytoplasma that has a domain of Deg/HtrA family protease

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Abstract

Since phytoplasma resides intracellularly within a host plant cell and lacks a cell wall, its membrane proteins may have important roles in the host-phytoplasma interaction. DegQ-like protein of onion yellows phytoplasma (OY-DegQ) is a membrane protein that contains a Deg/HtrA family serine protease domain and shows partial similarity to *Escherichia coli* DegQ. To examine the protease activity of OY-DegQ, the soluble domain of OY-DegQ was purified with glutathione S-transferase and its function was analyzed. As a result, the degradation of a substrate protein was observed, suggesting that OY-DegQ functions as a protease.

Keywords: phytoplasma, serine protease, enzymatic activity, plant disease

Introduction

Phytoplasma is a plant pathogenic bacterium associated with severe damages to many plant species in the world (Maejima *et al*., 2014). Since phytoplasma resides intracellularly within a host plant cell and lacks a cell wall, its membrane proteins are exposed to the host cytoplasm and have important roles in the host-phytoplasma interaction (Arashida *et al*., 2008; Oshima, 2021). DegQ-like protein of onion yellows phytoplasma (OY-DegQ) is a membrane protein that contains a Deg/HtrA family serine protease domain and shows partial similarity to *Escherichia coli* DegQ (Kolmar *et al*., 1996). OY-DegQ has several unique features not found in DegQ from other bacteria. First, it is more than two times as large as DegQ of other bacteria (Figure 1). Second, it has two transmembrane regions although others DegQ had none of them. Third, its serine protease domain is presumed to be exposed to the plant cytoplasm. Therefore, OY-DegQ is likely to play important roles in the interaction between phytoplasma and the host cells, however, the function of phytoplasma DegQ is still unclear. This study was aimed to purify the soluble domain of a recombinant phytoplasma DegQ protein and analyze its function.

Materials and Methods

The soluble region of the *degQ* gene from the onion yellows phytoplasma ('*Candidatus* Phytoplasma asteris' OY-W strain) was inserted into pGEX-4T-1. The constructed plasmid was used to transform *E. coli* BL21 strain. The transformed BL21 strain was cultured in LB/amp liquid medium, and after adding IPTG (1 mM), cultured at 15°C to induce expression of the fusion protein with glutathione S-transferase (GST). GST-OY-DegQ protein was extracted from the cells by sonication, and the extracted protein solution was separated into soluble and insoluble proteins by centrifugation.

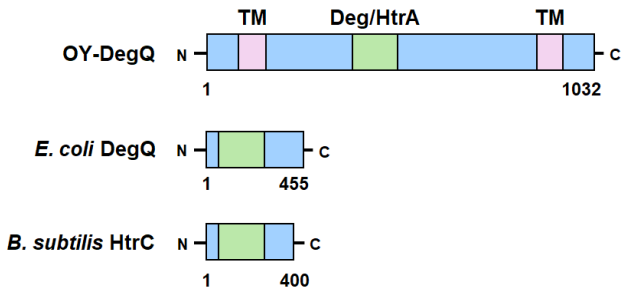


Figure 1. Primary structures of Deg/HtrA family proteins. OY-DegQ has two transmembrane regions (TM, pink boxes) and a central Deg/HtrA serine protease domain (green box). OY-DegQ is more than twice as long as *Escherichia coli* DegQ and *Bacillus subtilis* HtrC. The numbers under each protein indicate the number of amino acids.

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The soluble protein fraction was applied to a glutathione sepharose column to purify GST-OY-DegQ. The obtained GST-OY-DegQ was incubated with substrates (casein) at 37°C for 8 hours, and then the proteins were separated by SDS-PAGE and protease activity was assayed by observing whether the substrate was fragmented.

Results

To examine the protease activity of OY-DegQ, the purified GST-OY-DegQ fusion protein reacted with the substrate casein and was subjected to SDS-PAGE. As a result, no degradation of the substrate protein was observed before incubation, but after the GST-DegQ-OY fusion protein reacted with casein at 37°C for 8 hours, multiple bands, resulting from the degradation of casein, were observed (Figure 2). On the other hand, no degradation of the substrate was observed when GST reacted with casein at 37°C for 8 hours. These results suggest that DegQ of the OY phytoplasma functions as a protease.

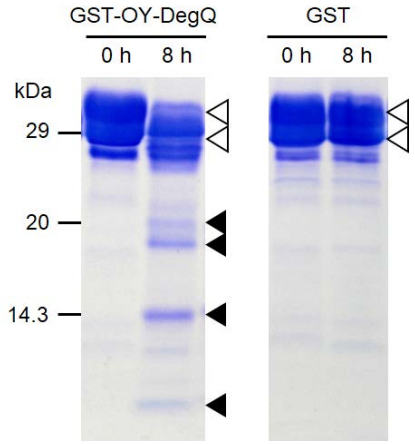


Figure 2. Degradation of casein protein by OY-DegQ. A band (black triangle) representing the degradation product of the substrate casein protein (open triangle) was observed only after incubation with GST-OY-DegQ for 8 hours.

Discussion

In this study, it was analyzed the function of phytoplasma DegQ and suggested that it acts as a protease. Deg/HtrA family proteases are one important group of these proteolytic enzymes, which are ATP-independent serine endopeptidases found in all domains of life, including Bacteria, Archaea and Eukarya (Schuhmann *et al*., 2012). Deg proteins from *E. coli* are protein quality control enzymes, acting as a protease and degradating irreversibly damaged proteins, or as a chaperone, thereby assisting with refolding of denaturated proteins (Clausen *et al*., 2011). *E. coli* DegQ functions in the periplasm, the space between the inner and outer membranes. On the other hand, phytoplasma DegQ is a membrane protein with two transmembrane domains and is fixed on the cell membrane. Furthermore, because phytoplasma lacks an outer membrane, its DegQ is likely to degrade some host proteins, which might contribute to infectivity and/or pathogenicity.

Since phytoplasmas colonize plants and insects intracellularly, secreted proteins and membrane proteins play important roles. In recent years, functional analysis of several secreted proteins has progressed, and their effects on the host are becoming clearer (Kitazawa *et al*., 2022; Oshima *et al*., 2023), but there are still a few reports on the function of membrane proteins (Suzuki *et al*., 2006; Neriya *et al*., 2014). Further elucidation of the functions of membrane proteins and their interactions with the host will likely lead to a better understanding of host control by phytoplasmas.

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Omics

Unveiling the 3D structure of phytoplasma effectors: a streamlined protocol for SAP11$_{CaPM}$ expression and purification

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Abstract

SAP11-like proteins, a well-characterized class of phytoplasma effectors, are known to interact with TCP plant transcription factors, leading to significant changes in plant phenotype and metabolism. Understanding the molecular mechanisms underlying these interactions is essential. This study focuses on the expression and purification of the SAP11 effector of '*Candidatus* Phytoplasma mali' (SAP11$_{CaPM}$) in *Escherichia coli*. The protocol includes the expression of the fusion protein with maltose binding protein for improved solubility and facilitated purification. Following affinity purification, the fusion protein was cleaved using factor Xa, and SAP11$_{CaPM}$ was separated via cation exchange chromatography. This approach provides sufficiently pure protein quantity for nuclear magnetic resonance studies.

Keywords: phytoplasmas, SAP11-like, NMR, protein purification

Introduction

SAP11-like proteins were among the first phytoplasma effectors to be identified and are likely the best-characterized to date (Sugio *et al*., 2011). Their ability to bind and destabilize members of the plants transcription factors family Teosinte branched, Cycloidea, Proliferating cell factors (TCP), significantly altering plant phenotype and metabolism, has been documented in several species (Janik *et al*., 2017; Pecher *et al*., 2019; Al-Subhi *et al*., 2021; Chen *et al*., 2022). Despite the extensive research over the past 15 years, several questions about the precise molecular mechanisms through which SAP11-like proteins exert their functions are still unanswered. A crucial step in determining their mode of action, revealing their active sites would be the experimental (and not only *in silico* prediction) characterisation of their three-dimensional structure. This could be achieved using Nuclear Magnetic Resonance (NMR) technology. NMR is a powerful tool to investigate the three-dimensional structure and dynamic properties of proteins at atomic resolution, providing insights into folding, interactions, and conformational changes under physiological conditions. However, successful NMR studies require the protein to be at a high degree of purity and concentration (approximately 1 mL at 2-3 mM) and isotopically labeled, typically with ^{15}N and ^{13}C isotopes.'

Here it is described the expression of '*Candidatus* Phytoplasma mali' SAP11 (SAP11$_{CaPM}$) in *Escherichia coli* in minimal media via the pMAL-c5x expression plasmid and the subsequent purification. Using this plasmid, the protein of interest can be expressed as a C-terminal fusion product with the Maltose Binding Protein (MBP), separated by a Factor Xa protease recognition site. This approach leverages MBP for two key advantages: achieving high-level expression of the target protein in the soluble fraction and enabling its isolation without requiring a specific antibody. Following incubation with Factor Xa protease, the two fragments are separated, yielding the target protein with a high degree of purity and concentration.

Materials and Methods

The coding sequence of SAP11$_{CaPM}$ was subcloned into the vector pMAl-c5x and transformed into *E. coli* strain BL21(DE3). The cells were first cultured in LB medium and subsequently transferred to M9 minimal medium (Table 1) as described by Marley *et al.* (2001). The expression of the MBP-SAP11$_{CaPM}$ fusion protein was induced with IPTG at a final concentration of 0.3 mM and carried out for 20-24 hours at 20°C. The cells were then harvested and resuspended in an aqueous buffer, containing Tris 20 mM, NaCl 150 mM and protease inhibitors. The cell suspension was then subjected to lysis through a cycle of freeze-thaw and 8 cycles of sonication at 60-80 W 30 seconds. The total cell lysate was processed using an ÄKTA Go chromatography system (Cytiva). First, it was loaded onto a HiTrap MBP 5 ml column

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(Cytiva) for affinity chromatography, and the MBP-fusion protein eluted with 10 mM maltose. The purified fraction was then incubated with Factor Xa protease at a fusion:protease ratio of approximately 200:1 (w/w) for 22 hours at 20°C. Subsequently the resulting protein fragments, consisting of MBP and $SAP11_{CaPM}$, were separated using a HiTrap SP HP 5 ml cation exchange column (Cytiva), in Tris buffer 50 mM, pH 7.4, eluting through a NaCl gradient ranging from 50 mM to 400 mM approximately.

Table 1. Composition of M9 minimal media.

Component	Concentration
Na_2HPO_4	6.5 g/l
KH_2PO_4	3 g/l
NaCl	0.5 g/l
$^{15}NH_4Cl$	1.1 g/l
U-^{13}C-U-^{1}H-D-Glucose	4.0 g/l
$MgSO_4 \cdot 7H_2O$	246.5 mg/l
CaCl, $2H_2O$	14.7 mg/l
Carbenicillin	100 mg/l
MEM vitamins (100x)	10 ml/l

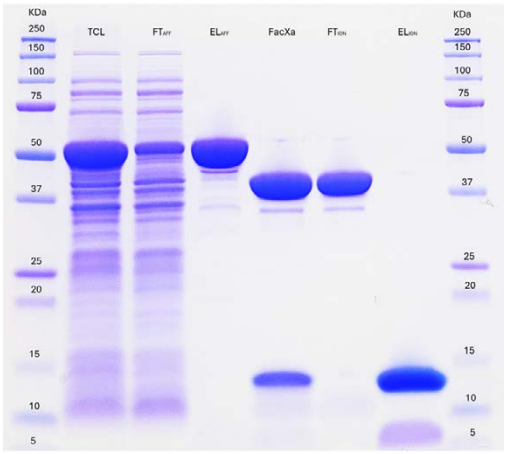


Figure 1. SDS-PAGE analysis of the expression and purification of the fusion protein, performed on Bio-Rad Any kD™ acrylamide gel and stained with Imperial™ Protein Stain. Approximately 20 µg of protein were loaded per lane, with molecular weights indicated on the sides based on the Precision Plus Protein™ Dual Xtra Prestained Protein Standard. TCL: total cell lysate; FTaff: flow-through from MBP affinity purification; ELaff: eluate from MBP affinity purification; FacXa: MBP eluate fraction after incubation Factor Xa; FTion: flow-through from cation exchange chromatography; ELion: eluate from cation exchange chromatography.

Results

The results of the expression and purification are shown in the SDS-PAGE in Figure 1. As evidenced by the intense band at approximately 55 kDa in the TCL column, corresponding to the total cell lysate, the fusion protein is successfully expressed (expected MW 53086 Da), with a yield of approximately 75 mg per liter of culture.

In FT_{AFF} and EL_{AFF} columns the two fractions corresponding respectively to flow-through and eluate fractions of the MBP affinity purification are shown, highlighting a good recovery rate of the fusion protein, with only a low amount of contaminants. The FacXa column represent the eluate fraction subjected to incubation with the FactorXa protease where the two intense bands corresponding to MBP (42,725 Da) and $SAP11_{CaPM}$ (10,361 Da) are visible. The last two columns correspond to the two fractions resulting from the cation exchange separation, with MBP appearing in the flow-through (FT_{ION}) and $SAP11_{CaPM}$ in the eluate (EL_{ION}).

Discussion

Despite being costly, experimental technologies for the determination of the three-dimensional structure of proteins, such as NMR, provide a precision still unmatched by computational methods (Terwilliger *et al.*, 2024). For instance, AlphaFold's prediction of SAP11-like proteins is generally poor and limited to ordered regions. The prediction of $SAP11_{CaPM}$, for instance, is associated to a modelling score of 0.39, which lies significantly below the threshold for a prediction that would likely resemble the true structure. Following this protocol, it is possible to produce effector proteins in sufficient quantity and quality for NMR analysis, with a reasonable investment of time and resources. Should the NMR analysis yield promising results, the same protocol could be adapted to study other effector proteins.

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Omics

Transcriptome analysis of sesame phyllody phytoplasma: towards identification of resistance-imparting genes

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Abstract

Phyllody disease, associated with phytoplasmas, is a major biotic stress affecting sesame production, causing significant yield losses. High-throughput transcriptome sequencing was used to identify potential resistance-associated genes. Total RNA was extracted from resistant and susceptible sesame plants under controlled conditions. Transcriptome sequencing was performed on the Novaseq 6000 platform, generating about 37.5 GB of raw data. Transcriptome assembly utilized Trinity-v2.15.0, yielding 88,372 ORFs, with 69,847 sequences matching to known species using BLAST-X against the NCBI database. Differential expression analysis identified genes with >2.0 log-fold change and FDR <0.01 between resistant and susceptible genotypes. Genes implicated in stress responses, hormone signalling, and pathogen defence pathways were significantly upregulated in resistant genotypes. These findings contribute to understanding the molecular basis of resistance against phyllody disease and provide a foundation for breeding disease-resistant sesame varieties.

Keywords: *Sesamum indicum*, sequencing, expression analysis

Introduction

Sesame (*Sesamum indicum*), an important annual oilseed crop, is primarily grown in tropical and subtropical regions, particularly in Africa and Asia (Kabak and Donson, 2017). It is cultivated mainly for its valuable oil, which is widely used in cooking and animal feeds, and for its numerous health benefits (Jayaraj *et al*., 2020). In 2019, global sesame production reached approximately 64.5 million tons from 12.82 million hectares, accounting for about 12% of the world's vegetable oil consumption (MALR, 2020). One of the primary threats to sesame production is phyllody, a phytoplasma-associated disease, recognized as a major constraint on sesame cultivation in both Asia and Africa. In India, one of the world's top sesame producers, phyllody incidence can range from 10% to 100%, with losses in yield reaching 80% (Rao, 2021). In sesame, phytoplasma infection manifests through various physiological and biochemical disruptions. These include reduced photosynthetic efficiency due to decreased leaf area and chlorophyll content, impaired carbohydrate transport, and altered genetic expression (Singh *et al*., 2020). Infected plants often show symptoms such as leaf yellowing, stunting, and poor seed development, ultimately leading to reduced yields and oil quality. As the demand for sesame oil continues to rise, addressing this issue has become critical to ensuring sustainable sesame production.

Recent advances in molecular biology offer new opportunities to combat this challenge by identifying the genetic basis of resistance to phyllody disease. In this context, high-throughput transcriptome sequencing has emerged as a powerful tool to explore the complex gene expression profiles of plants under stress conditions. This study utilizes high-throughput transcriptome sequencing to identify potential resistance-associated genes in sesame.

Materials and Methods

Field-grown sesame plants were classified into resistant (IC557244) and susceptible (WB+PHY) genotypes based on observed responses to phytoplasma-associated phyllody disease under controlled glasshouse conditions. Leaf, stem, and flower tissues were collected for transcriptome analysis. Total RNA was extracted from various tissues using TRIzol reagent (Invitrogen), followed by DNase treatment to remove

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genomic DNA contamination. RNA quality and integrity were assessed using a Nanodrop spectrophotometer and Agilent Bioanalyzer 2100 with the Plant RNA Nano Chip. Only samples with RNA integrity numbers (RIN) above 7.0 were processed further (Figure 1).

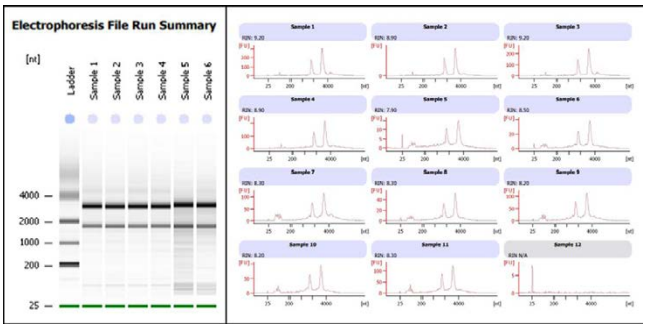


Figure 1. Gel electrophoresis and quality analysis over Agilent Bioanalyzer using 2100 expert Plant RNA Nano Chip.

Transcriptome sequencing was performed using the Novaseq 6000 platform (Illumina) with paired-end (2 x 150 bp) sequencing. Adapter trimming was performed using Trimmomatic, and transcriptome assembly was conducted with Trinity-v2.15.0, using a k-mer value of 25 and a minimum contig length of 200. Reads from resistant and susceptible genotypes were mapped to the assembled transcriptome using RSEM. Differential expression analysis was conducted using edgeR, with significance thresholds set at log2 fold change >2.0, p-value <0.01, and FDR <0.01. Open reading frames (ORFs) were predicted using FGeneSH, and functional annotation was performed using BLAST-X against the NCBI nr database. Gene ontology (GO) enrichment analysis was performed to identify biological processes and pathways associated with resistance.

Results

A total of about 37.5 GB of high-quality raw sequencing data was generated. The assembly yielded 88,372 ORFs, with 69,847 sequences showing significant similarity to known proteins in the NCBInr database. Tandem duplication events were identified in 2,782 genes, potentially contributing to trait enhancement in resistant genotypes (Table 1).

Table 1. Transcriptome-assembly report.

Feature	Value
Total sequences	258,554,712
Total assembled transcripts	128,142
Total assembled genes	86,306
% GC in assembled transcripts	39.88
N50 value of contigs	1,401
Mean contig lenght /cutoff value 200 bp)	467
Longest size of contig (bp)	15,499

Comparative analysis revealed significant upregulation of 1,245 genes and downregulation of 987 genes in resistant genotypes compared to susceptible ones. Key upregulated genes included those involved in pathogen recognition, hormonal pathways (jasmonic acid and salicylic acid signalling), and secondary metabolite biosynthesis (Figure 2). GO enrichment analysis highlighted processes such as defence response, oxidative stress management, and hormonal regulation. Resistance-associated pathways included MAPK signalling, phenylpropanoid biosynthesis, and WRKY transcription factor-mediated stress signalling.

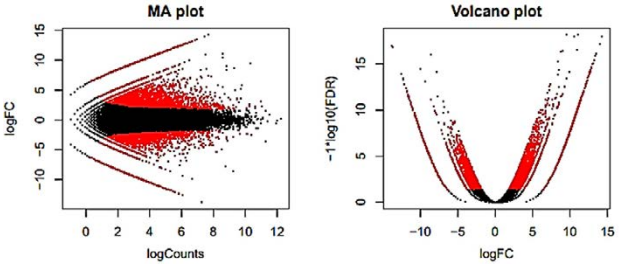


Figure 2. Visual representation of the number of genes showing significant changes in gene expression in a comparative set of conditions.

Discussion

The transcriptomic analysis provides valuable insights into the molecular mechanisms underlying resistance to phytoplasma-associated phyllody disease in sesame. Genes involved in hormonal signalling (*e.g.*, jasmonic acid and salicylic acid pathways) and secondary metabolite production were significantly upregulated in resistant plants, corroborating their roles in pathogen defence. Tandemly duplicated genes observed in the resistant genotype may contribute to enhanced stress tolerance by amplifying beneficial traits. The identified resistance-associated genes and pathways can be targeted for functional validation and breeding programs to develop phytoplasma-resistant sesame varieties. Future work should focus on the validation of candidate genes using RT-qPCR and genome editing approaches such as CRISPR-Cas9 to establish their functional roles in disease resistance.

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Omics

CRISPR/Cas9-mediated generation of tomato RAD23 knockouts for studying phytoplasma-induced symptoms

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Abstract

Phytoplasma infection often induces abnormal flower development, such as phyllody and cauliflower-like inflorescence, leading to compromised fruit development and significant crop yield reduction. In *Arabidopsis*, the phytoplasma effector SAP54 interacts with RAD23 proteins to induce floral abnormalities, partially alleviated in *rad23* mutants. To investigate whether a similar mechanism may occur in tomatoes, in this study, CRISPR/Cas9 technology was employed to generate knockout tomato mutants of *SlRAD23C* and *SlRAD23D*. Target-specific guide RNAs (sgRNAs) were designed to disrupt the coding sequences of *SlRAD23C* and *SlRAD23D*. PCR screening and sequencing confirmed successful deletions in the targeted regions, leading to truncated protein products in mutant lines. These mutants will be further analyzed for their response to potato purple top phytoplasma-induced flower deformation to develop tomato lines less susceptible to phytoplasma-related reproductive reduction.

Keywords: flower malformation, phyllody, guide RNAs (sgRNAs)

Introduction

Phytoplasmas are bacterial pathogens that infect various plants, associated with abnormal flower development and leading to significant economic losses in crops. One remarkable symptom of phytoplasma infection is phyllody, where flower parts such as petals and stamens are transformed into leaf-like structures, disrupting normal flower formation and severely impacting fruit production. Studies have identified the effector protein SAP54 (also known as phyllogen) (Iwabuchi *et al*., 2020), produced by phytoplasmas, as a key factor in phyllody. SAP54 interacts with RAD23 proteins, particularly RAD23C and RAD23D, which are involved in the plant's ubiquitin-proteasome system, a mechanism responsible for protein degradation. By interacting with these proteins, SAP54 promotes the degradation of specific floral regulators, leading to phyllody.

In *Arabidopsis*, knocking out RAD23C and RAD23D has been shown to disrupt SAP54's ability to induce phyllody, highlighting the importance of these proteins in the process (MacLean *et al*., 2014). This suggests a potential approach to develope resistance by targeting RAD23 genes. Tomato plants, which are also susceptible to phytoplasma-induced phyllody, experience symptoms such as big bud (BB, a form of phyllody), associated with potato purple top (PPT) phytoplasma. In this study, the CRISPR/Cas9 gene editing was used to generate knockout mutants of *SlRAD23C* and *SlRAD23D* in tomatoes. The goal is to explore the interaction between SAP54 and RAD23, aiming to mitigate phytoplasma-induced symptoms and enhance understanding of phytoplasma pathogenesis mechanisms.

Materials and Methods

The full-length sequences of the *SlRAD23C* (Solyc04g007120) and *SlRAD23D* (Solyc02g063130) genes were obtained from the NCBI database. Two sgRNAs were designed for each gene: sgRNA-C1 and sgRNA-C2 targeting *SlRAD23C*, and sgRNA-D1 and sgRNA-D2 for *SlRAD23D* (Figure 1). These sgRNAs were cloned into CRISPR/Cas9 vectors and introduced into *Agrobacterium tumefaciens* strain GV3101, which facilitated the generation of transgenic tomato lines of the Moneymaker cultivar *via* *Agrobacterium*-mediated transformation. Cotyledons from tomato seedlings were co-cultivated with *Agrobacterium* carrying the CRISPR/Cas9 constructs, followed by transfer to selective media containing kanamycin and timentin for regeneration. The regenerated plants were subsequently moved to the soil for further analysis.

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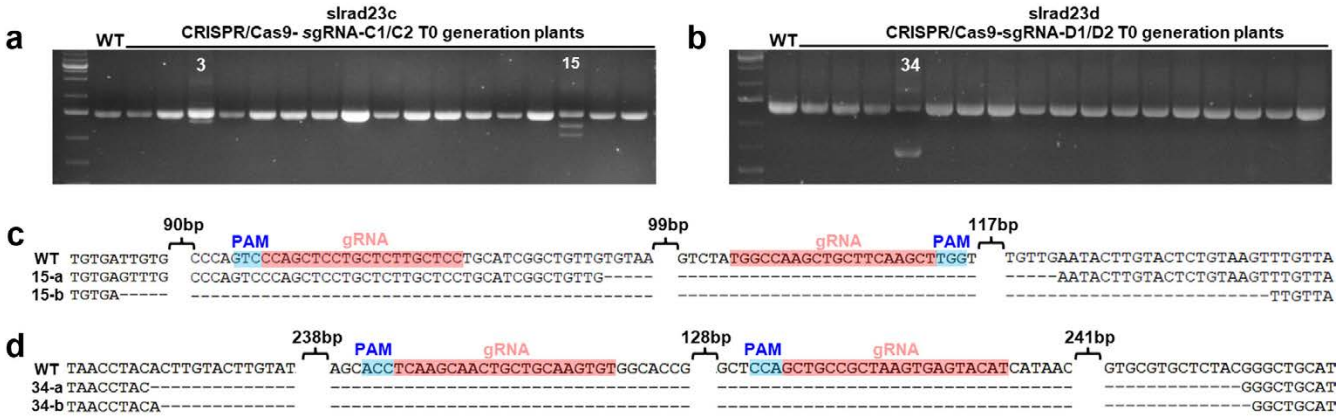


Figure 1. CRISPR/Cas9-mediated mutations in tomato *SlRAD23C* and *SlRAD23D* genes in T0 transgenic plants. In (a) and (b), screening of T0 transgenic plants for mutations in *SlRAD23C* (a) and *SlRAD23D* (b) using CRISPR/Cas9 with gene-specific guide RNAs. Flanking primers were used to amplify the regions containing the sgRNA target sites. In (c) and (d), validation of DNA deletions in transgenic lines slrad23c-15 (c) and slrad23d-34 (d).

Genomic DNA from the transgenic plants was extracted, and PCR with gene-specific primers was employed to detect mutations in *SlRAD23C* and *SlRAD23D*. Sanger sequencing of the PCR products was then conducted to confirm and characterize nucleotide sequence alterations by aligning with reference sequences to pinpoint specific deletions or insertions induced by the CRISPR/Cas9 system.

The confirmed slrad23c and slrad23d mutant lines were propagated for further analysis. T0 generation plants were cultivated in controlled conditions to obtain T1 seeds. The T1 and subsequent generation plants will be analysed for homozygous mutations and tested for resistance to PPT phytoplasma infection, with a focus on the suppression of BB symptoms.

Results and Discussion

The sgRNAs targeting *SlRAD23C* and *SlRAD23D* were successfully cloned into CRISPR/Cas9 vectors and introduced into tomato plants. PCR screening identified reduced-size PCR products in two slrad23c lines (slrad23c-3 and slrad23c-15) and one slrad23d line (slrad23d-34) (Figure 1a and b). Sequencing confirmed deletions in the target genes, with slrad23c-15 displaying 255 bp and 412 bp deletions and slrad23d-34 having a 691 bp deletion (Figure 1c and d). These deletions would result in truncated, non-functional SlRAD23C and SlRAD23D proteins.

Despite the genetic alterations, phenotypic analysis of the T0 plants showed no observable differences from wild-type plants under standard growth conditions. This suggests that *SlRAD23* genes may not be essential for basic vegetative development. However, the focus of these knockouts is their potential impact on reproductive development, specifically under phytoplasma-induced stress. The mutants are being propagated to the T1 and subsequent generations to investigate their response to PPT phytoplasma infection and their ability to reduce or suppress floral deformation and BB symptoms associated with the disease.

Further context is provided by previous research identifying the full-length PPT-SAP54 sequence in the genome of PPT phytoplasma (Wei *et al*., 2023). Comparative analysis with other SAP54 sequences revealed that PPT-SAP54 belongs to the Phyl-A family, which includes pathogens such as aster yellows witches' broom (AYWB), and onion yellows (OY) phytoplasmas. The conserved N64 residue in PPT-SAP54, which regulates PHYL1 activity, suggests that this effector protein is functional and likely plays a key role in inducing phyllody/BB.

The confirmed *RAD23* gene knockouts, alongside the presence of an active SAP54 effector in PPT phytoplasma, set the stage for exploring the interaction between *SlRAD23* knockouts and the pathogen's ability to induce phyllody. The T1 and later generations will be critical for assessing whether the absence of these genes affects the plant's reproductive response under phytoplasma infection. Understanding this relationship may offer valuable insights into the molecular mechanisms behind phytoplasma-induced phyllody. It could contribute to developing tomato varieties with reduced susceptibility to phytoplasma-induced reproductive issues.

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Omics

"Flavescence dorée" phytoplasma strains genetic variability: a comparative genomics approach

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Abstract

"Flavescence dorée" (FD) is a severe and epidemic grapevine yellows disease associated with the "flavescence dorée" phytoplasma transmitted by the leafhopper *Scaphoideus titanus*, affecting most of the major European viticultural regions. Control of FD relies only on insecticides against *S. titanus* and uprooting of the infected plants. No fully resistant grapevine cultivar is available thus, understanding phytoplasmas pathogenicity and genomics is crucial for developing alternative defense strategies. This research describes the genome sequencing of four "flavescence dorée" strains, detected in different symptomatic grapevine varieties collected in the Trentino province (north Italy), using Oxford nanopore long-read sequencing. A comparative analysis of all available phytoplasmas genomes with these four "flavescence dorée" phytoplasmas was performed, providing insights into the genomics of these "flavescence dorée" strains.

Keywords: phytoplasma genomics, grapevine, epidemiology

Introduction

Phytoplasmas are wall-less, intracellular bacteria that are associated with often lethal diseases in numerous plant species worldwide, including grapevines (Hemmati *et al.*, 2023). In grapevines (*Vitis* sp.), diverse phytoplasma ribosomal groups are linked to grapevine yellows, with "flavescence dorée" (FD) associated with the "flavescence dorée" phytoplasma from the 16SrV ribosomal group, being particularly devastating. FD is a quarantine disease in the European Union (Cruz *et al.*, 2019). High-throughput genome sequencing has emerged as a valuable tool for characterizing phytoplasmas and facilitating comparative genomic analyses (Tan *et al.*, 2021). This study was aimed to reconstruct high-resolution sequences of "flavescence dorée" phytoplasma genome strains using Oxford nanopore long-read sequencing. The goal was to generate complete circular and annotated chromosomes of the "flavescence dorée" phytoplasmas and identify both conserved features among phytoplasmas and specific characteristics unique to the strains through comparative genomics and phylogenetic analyses with a large set of genes.

Materials and Methods

In the summer of 2023, symptomatic leaves from Chardonnay, Pinot Gris, and Pinot Noir grapevine varieties were collected in Trentino province from distant (20-30 km) vineyards. Following collection, sample preparation and DNA extraction were conducted using the Macherey-Nagel Midi Kit according to the manufacturer's protocols. The extracted samples were then tested for phytoplasma presence by FD-specific PCR. The quality and quantity of the DNA were assessed to ensure suitable material for subsequent analyses. A total of 600-1000 ng of DNA from PCR-positive samples was used for library preparation with Kit V14 (SQK-LSK114), following the manufacturer's instructions. The resulting library was sequenced on a P2 Solo device using a flow cell R10.4. Sequences generated were base called using the ONT MinKNOW proprietary software. Long reads were processed through the PLANTOGEN-A pipeline (https://github.com/Giulia-Calia/PLANTOGEN-A/tree/mai).

The completeness of the draft genomes was evaluated using BUSCO v5.2.2. Genome annotation was conducted with Bakta v1.9.1 to identify genomic features and functional

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elements. Functional characterization of the annotated genome was performed using the BlastKoala and KEGG databases to assess metabolic pathways and functional capabilities. Comparative genomic analyses were conducted with all available phytoplasma genomes from the NCBI database. The studied strains were clustered by gene similarity, and phylogenetic trees were constructed through core gene concatenation using Roary, AliView, IQ-TREE2, and FigTree.

Functional characterization of the annotated genome was performed using the BlastKoala and KEGG databases to assess metabolic pathways and functional capabilities. Comparative genomic analyses were conducted with all available phytoplasma genomes from the NCBI database. The studied strains were clustered by gene similarity, and phylogenetic trees were constructed through core gene concatenation using Roary, AliView, IQ-TREE2, and FigTree.

Results

The analysis produced genome assemblies of "flavescence dorée" strains consisting of a circular chromosome ranging from 653,775 to 654,297 bp, with a GC content of 20.74% and an average coverage of 30 for long reads. These strains were classified as belonging to the 16SrV-D and FD2 phytoplasma strain clusters. The sequence of the map gene was found to be 100% identical to the described genotype M54.

The completeness of the draft assemblies, as estimated by BUSCO, ranged from 93.4% to 94.7%. The assembled genomes exhibited collinearity and functional consistency when compared to the available reference genome (Debonneville *et al*., 2022). However, all strains presented single nucleotide polymorphisms (SNPs) and insertions/deletions (indels). An inversion of a 365,541 bp region was identified in the Chardonnay strain (Figure 1), with gene orientation and function remaining consistent with homologous regions in other strains. Interestingly, genes from the core set of PMUs were present, though they were distributed sparsely across the circular genome.

Comparative analysis of gene content between "flavescence dorée" and other phytoplasma genomes retrieved 74 orthologous proteins shared by all phytoplasma genomes under analysis. Multiple sequence alignments of homologous genes were prepared using MAFFT v7.525. The phylogenetic tree, constructed based on the concatenated alignment of these proteins, indicated that the genomes under study clustered closely with '*Candidatus* Phytoplasma rubi' and '*Ca*. P. ziziphi'.

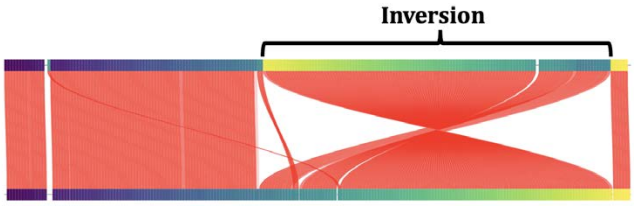


Figure 1. FastANI output showing ANI percentages between FD phytoplasma reference genome (bottom) and FD phytoplasma strain from Chardonnay cultivar (top)

Discussion

Phytoplasmas present unique challenges for genome sequencing due to their low genomic complexity and the contamination of host DNA, which often results in incomplete assemblies. In this study, it was possible to successfully assemble the genomes of four FD phytoplasma strains, providing valuable insights into their genomic characteristics.

Among the 151 markers in the BUSCO database of mollicutes conserved genes, seven are absent in all FD phytoplasma strains. Functional classification of the assembled genomes revealed significant genes related to metabolism and genetic information processing, corroborating previous findings in phytoplasma genomics. Notably, the absence of common transposon-like structures, or functional potential mobile units (PMUs), suggests that these strains may have followed a distinct evolutionary trajectory with reduced genome plasticity compared to other phytoplasmas. Furthermore, no homologs of the well-characterized effector proteins SAP05, SAP11, SAP54, and TENGU were detected in any of the strains. Phylogenetic analysis demonstrated a strong genetic affinity among the studied "flavescence dorée" strains and those of '*Candidatus* Phytoplasma rubi' and '*Ca*. P. ziziphi'.

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New detection tools

Artificial intelligence in phytoplasmology: image-based disease detection models for phytoplasma infection in flower crops

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Abstract

In floriculture, the economic products are the flowers; phytoplasma infection in domestic cultivations results in economic loss to the farmer. Farmers often lack the expertise to identify the infection due to the lack of awareness about the diseases. Similarly, in nursery plants, phytoplasma infections are often confused as novel plant types. Thus, an interactive expert system can help in appropriate identification of phytoplasma presence in flower crops. As a preliminary step towards it, an image-based disease prediction model for phytoplasma infection in China aster has been developed. The accuracy per class for healthy and infected was 91% and precision, recall and F1 score are observed to be 0.91 respectively. Such technologies are the need of the hour which can help in improving the accuracy and speed of disease deetection, allowing farmers to respond quickly and effectively to disease outbreaks.

Keywords: machine learning, image-based detection, phyllody

Introduction

Algorithmic and computational advances in the field of machine learning (ML) have paved the way for advances in natural language processing, image recognition, robotics, data processing and many other fields with no exception in plant sciences and microbiology. Though artificial intelligence applications are in the initial phase in phytoplasma research, many significant breakthroughs have been achieved. Algorithm that identifies effectors screening the genomes are one among them (Carreón-Anguiano *et al*., 2023; Calia *et al*., 2024).

Advances in molecular biology has revolutionized the detection of phytoplasma diseases in laboratory conditions. From nested PCR, diagnostics has advanced to "All in One Dual technology" for simultaneous detection of multiple phytoplasma using Crisper assays (Feng *et al*., 2023). In field conditions disease detection often rely on field surveys and visual inspection, which are prone to human error unless done by an expert. It is difficult for an expert to visit each and every field for disease identification. In this decade, the advent of ML algorithms has offered a promising solution to this problem, enabling rapid and accurate identification of infections through trained expert models remotely through web interfaces, unmanned aerial vehicles (UAV) (Plata *et al*., 2021; Narmilan *et al*., 2022) and smart phone applications.Phytoplasmas are a major threat in flowering plants as they interfere with the reproductive phase of the plants and inhibit the formation of flowers resulting in phyllody. In floriculture, the economic parts are flowers, phytoplasma infection in domestic cultivations results in economic loss to the farmer who lack the expertise to identify the infection often due to the lack of awareness about the disease. Similarly, phytoplasma infections in nursery plants are often confused as novel plant types due to the virescence symptoms. Thus, an interactive expert system, where phytoplasma infection can be rightly identified is imperative in flower crops. Keeping this in view, a preliminary attempt was made to develop an image-based disease prediction model and deploying it in web interface for phytoplasma infection in China aster (*Callistephus chinesis*) of Asteraceae family.

Materials and Methods

Phytoplasma infected and healthy aster plants from the experimental fields of ICAR-DFR were used as source of input images. Images of symptoms in infected and healthy plants of China aster were collected using a digital camera 50 MP (OIS, PDAF) with f/1.8 aperture. Approximately 150 images were pre-processed by trimming and resizing.

Transfer learning of Convolutional Neural Network (CNN) architecture based pretrained TensorFlow.js model

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was done using the collected symptomatic and asymptomatic images. Extraction of features from plant images was done using the pre-trained model and fine tuning was done using the dataset in hand. The two classes of samples trained were healthy and infected based on symptoms. An 85% percent of the samples was used to train the model and 15% as test samples. Training was done with a batch size of 16 and epoch of 50. After training the model classified the images into healthy and infected classes. The model's performance was evaluated using metrics like accuracy and precision. The process workflow is given in Figure 1.

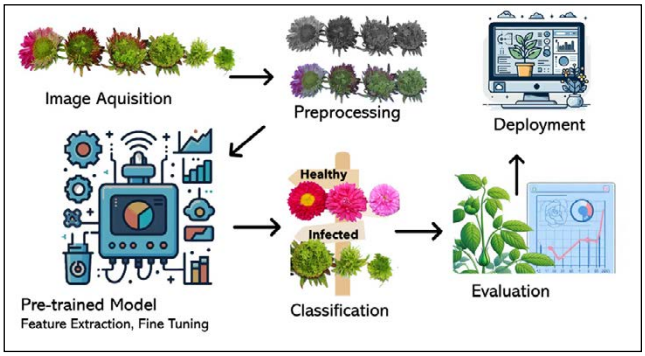


Figure 1. Flow diagram for transfer learning process pipeline for development of disease detection model.

Results

After training the model, it classified the test images into healthy and infected classes. The model was evaluated for performance. The accuracy per class for healthy and infected was 91% and precision, recall and F1 score are observed to be 0.91 respectively. This indicates that the model maintains a good balance between precision and recall, suggesting that it is not biased towards either false positives or false negatives.

Discussion

Timely detection and right identification are the key in management of pathogen infection in plants. The same is applicable in phytoplasma infections. The symptom database, *i*PhyDSDB, with nearly 35,000 phytoplasma DNA sequences identifying 945 plant species associated with phytoplasma diseases is a major resource for remote identification of phytoplasma symptoms. This database search also requires at least some basic knowledge about phytoplasma symptoms. In case of marginal farmers without prior information about the disease, it will be difficult to access the right information.

An image-based search will be more beneficial and easier for the nonexpert without any prior knowledge of disease to identify the phytoplasma infections. Machine vision assisted object identifier models are helping in such disease diagnostics. The model developed in this study has shown good performance matrix values indicating the robustness in identifying the training data. Successful deployment of deep learning architectures depends on good quality dataset. The task of specific data collection for the problem at hand, here phytoplasma infection, is challenging and requires significant effort. Even after accurately training the model with dataset, it was found 'Overfit' as the model distinguished the training samples accurately, the performance on predicting test samples were not absolutely accurate. The constraint may be the limited number of good quality training samples representing all different features of the infection or healthy or host plants classes in the dataset. As it has been observed that a large, verified dataset of images of diseased and healthy plants is required to develop accurate image classifier for disease identification (Prabha *et al*., 2020). Another observation while testing the model is that it classifies only healthy and infection features, and it doesn't distinguish the host plant from China aster to chrysanthemum or marigold or any other. Thus, this indicates the need for increasing the number of classes where host plants identification features also to be extracted and trained.

For accurate detection of disease, it is not just plant and disease images, tall the components of disease such as time and environment need to be taken into consideration. Incorporation of contextual data, like favourable weather conditions, soil properties and agronomic practices will improve the accuracy of detection.

The developed model in this study is preliminary and more classes and fine tuning will further improve it. Such technologies are the need of the hour which can help in improving the accuracy and speed of disease detection, allowing farmers to respond quickly and effectively to disease outbreaks.

Acknowledgements

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New detection tools

Leveraging artificial intelligence and big data to advance phytoplasma disease detection and crop health management

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Abstract

Phytoplasmas are minute, cell wall-less bacteria that infect various economically important crops worldwide, leading to significant agricultural losses. Traditional diagnostic methods are often time-consuming, require specialized expertise, and thus limit timely intervention. This study addressed these challenges by combining two complementary approaches. The first is devising an artificial intelligence (AI)-based diagnostic system utilizing Convolutional Neural Networks (CNNs) trained on extensive image datasets, enabling rapid, accurate detection of phytoplasma infections with promising accuracy. The second approach involves the construction of a comprehensive image and symptom database that allows farmers to compare crop symptoms for early disease identification. The image database serves as an initial online-accessible tool for proactive crop management while the AI model is being further optimized. Eventually, the AI diagnostic model and image database will be integrated, forming a scalable, powerful solution aimed at advancing phytoplasma disease management and enhancing crop protection strategies.

Keywords: machine learning, real-time diagnostics, convolutional neural networks

Introduction

Phytoplasmas are bacteria that infect a wide range of economically significant crops such as cherries, strawberries, and grapevines, associated with diseases like little cherry, strawberry phyllody, and grapevine yellows. These infections lead to substantial agricultural losses, reducing both crop quality and yield. Early detection is crucial for preventing the spread of these diseases; however, traditional diagnostic methods are labor-intensive and require specialized equipment, making them inaccessible to many farmers. In this study, an artificial intelligence (AI)-based system is explored for phytoplasma disease detection. Using convolutional neural networks (CNNs), the system is designed to analyze plant images and provide rapid and early disease detection.

In the meantime, to support farmers and growers in recognizing diseases in their crops, it was developed an image and symptom database named Phytoplasma Disease and Symptom Database (*i*PhyDSDB, Wei *et al.*, 2024a). This database contains a comprehensive collection of plant symptom images and associated data. It serves as a critical backup tool, allowing the users to compare symptoms observed in their crops with those in the database, helping to early identify potential infections. The offer of this interim solution it is aimed to empower farmers and gain recognition while the AI-based system undergoes further development. Ultimately, the combination of the AI model and this robust database will provide an integrated, scalable approach to managing phytoplasma diseases and safeguarding crop production.

Materials and Methods

To develop the AI-based detection system, it was conducted a case study with tomato plants infected by potato purple top (PPT) phytoplasma. It was assembled a dataset of several thousand images, evenly split between healthy and infected plants, with an additional testing set (20% of the dataset) to validate model performance on unseen data. Five CNN architectures were trained using transfer learning: four pre-trained models (VGG-16, Google Inception v3, NASNet, and DenseNet201) and one custom model developed specifically for phytoplasma detection. The pre-trained models utilize

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weights from ImageNet, allowing them to adapt general image recognition capabilities to the dataset. In contrast, the custom model was trained from scratch to capture unique morphological symptoms of infection. All models were implemented in Tensorflow with data augmentation to support robust training.

Phytoplasma nucleotide sequences were retrieved from the NCBI database using "phytoplasma" as a keyword and organized in Excel. Host plant information was gathered and standardized using resources like Plants of the World Online and the USDA PLANTS Database. Phytoplasma symptom data were added, and duplicates were filtered to create a finalized list of susceptible plants. Images of infected plants were sourced online, with URLs embedded in the *i*PhyDSDB built using MySQL with a PHP, HTML, and CSS front end for interactive querying.

Results

In this study, four pre-trained models and one custom model were employed. Pre-trained models are efficient but may lack flexibility in identifying the subtle morphological features associated with the phytoplasma presence. In contrast, the custom model, while requiring more data and training time, offers specificity in detecting the unique symptom patterns of phytoplasma infection (Table 1).

Table 1. Key distinctions between pre-trained models and custom models in terms of performance, flexibility, and data requirements.

Aspect	Pre-trained models using transfer learning	Custom models
Training time	Shorter, as pre-trained weights are used	Longer, as training starts from scratch
Data requirements	Less data needed	Large amounts of data are needed
Performance	Generally good, especially with limited data	Potentially higher with enough data
Flexibility	Less flexible due to fixed architecture	Highly flexible, tailored to the task
Ease of use	Easier, especially with existing frameworks	Requires more expertise in model design
Risk of overfitting	Lower, especially with pre-trained feature extraction	Higher, especially with limited data

The pre-trained CNN models, such as VGG-16, Google Inception v3, NASNet, and DenseNet201, showed strong performance when applied to the tomato dataset. These models benefited from their prior training on large general-purpose image datasets and were fine-tuned for phytoplasma symptom detection, resulting in high accuracy rates. The custom CNN model, specifically designed for this study, also performed well, though its accuracy was slightly lower than the pre-trained models. Despite this, the custom model offered unique insights into the specific visual markers of phytoplasma infections (Wei *et al*., 2024b).

By employing ensemble learning techniques, such as majority voting and weighted voting, the system's overall reliability and accuracy were improved. The ensemble method combined the predictions of multiple models, mitigating the potential weaknesses of any single model. This approach not only enhanced the robustness of the system but also reduced the risk of false positives and false negatives, ensuring more consistent results across a wide range of image inputs.

The *i*PhyDSDB was created to compile symptom images for nearly 1,000 plant species infected by phytoplasmas, with image links available for 372 species. Some species lacked images due to undocumented symptoms or asymptomatic infections. The *i*PhyDSDB site, (https://plantpathology.ba.ars.usda.gov/iphydsdb/) provides specific host information, symptom descriptions, and image links. It features an advanced search tool for symptoms or plant hosts with both common and scientific names, making it a valuable resource for plant pathology professionals, educators, and researchers (Wei *et al*., 2024a).

Discussion

This case study demonstrates that an AI-based detection system holds significant potential for detecting phytoplasma diseases like potato purple top in tomato plants. It was identified the potential of ensemble learning strategies, where the predictions from multiple models are combined to enhance the overall accuracy and reliability of detection. By combining the strengths of various models, it is expected to further improve detection accuracy and ensure that the system remains robust across different datasets. Building on the success of this model for PPT phytoplasma in tomatoes, this approach is now extended to other crops affected by different phytoplasma diseases.

Complementing the AI detection system is the *i*PhyDSDB, a valuable resource developed to assist farmers, growers, and other users with symptom comparison, identification, and diagnosis. Expanding the database across multiple plant species will strengthen the AI model's diagnostic capabilities and support a more comprehensive approach to disease management.

To further advance practical applications, there is the aim to transition towards AI model deployment through a user-friendly web-based interface or mobile app. This tool will offer real-time diagnostic support, empowering users to manage phytoplasma-related diseases more efficiently and enhancing overall agricultural disease management practices.

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New detection tools

Development of a multilocus sequence typing method for accurate identification of 16SrV phytoplasma strains via Oxford nanopore

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Abstract

The elm yellows phytoplasma group (16SrV) includes diverse members associated with devastating plant diseases on a broad host range across Europe, Asia and Americas. To enable the detection and precise identification of a number of 16SrV phytoplasma strains, it was developed and assessed a multilocus sequence analysis approach that combines targeted PCR pre-amplification of DNAs with nanopore sequencing. Three housekeeping genes (16S rRNA; *secY*; *map)* were amplified from each sample and sequenced simultaneously using a single-use Flongle Flow Cells. Furthermore, it was developed a bioinformatic analysis pipeline to run BLAST searches of assembled reads against a curated DNA sequence database of 16SrV phytoplasmas. Preliminary results on selected DNA samples suggest that this method can produce sufficient reads and consensus sequence information across the three selected markers to allow accurate determination of phytoplasma strains identity in less than two hours. Moreover, by barcoding the amplicons produced during library preparation, it was possible to process up to 24 different samples in one sequencing experiment therefore reducing the overall cost per sample. Owe to the improved R10.4.1 Flow Cell and the high sequencing depth, consensus sequences produced by this method were at least as accurate as those obtained by Sanger sequencing and allowed differentiation of closely related strains of group 16SrV and the identification of mixed infections.

Keywords: grapevine, "flavescence dorée", nanopore sequencing, diagnostics

Introduction

The elm yellows group (16SrV) represents a major cluster of phytoplasmas. Phytoplasma strains belonging to this group are associated with different plant diseases in elm, alder, grapevine, cherry, blackberry, peach and other plant species. A few of these strains, associated with a devastating grapevine yellows disease known as "flavescence dorée", are exotic to the United States of America (USA) and quarantine pathogens in many countries. To ensure the safety of American agricultural production, and international trade, specific and sensitive diagnostic tools are needed for the early and rapid detection of exotic phytoplasmas. Oxford Nanopore Technology (ONT) is a powerful sequencing platform with many practical applications, including specific and sensitive pathogen detection. In this study the feasibility of nanopore high-throughput sequencing technology was examined as a diagnostic tool to achieve fast and accurate detection and identification of a number of 16SrV phytoplasmas.

Materials and Methods

For this study, a total of 24 phytoplasma strains, representing five 16SrV subgroups: 16SrV-A (3), 16SrV-C (15), 16SrV-D (4) and 16SrV-E (2), were analysed. For eight of them, originating from the USA, genomic DNA was extracted from infected leaf tissue using a Qiagen DNeasy Plant Mini Kit. The remaining strains were obtained as genomic DNA from phytoplasma collections. A schematic representation of the followed procedure is shown in Figure 1. Three *loci*, corresponding to the 16S rRNA, *secY* and *map* genes, were chosen for multilocus sequence typing (MLST) of the phytoplasma strains. The amplification reactions were performed using RepliQa HiFi ToughMix (Quantabio, Beverly, MA). Previously designed PCR primers (Table 1) were used to amplify DNA fragments of the three *loci* in singleplex reactions. PCR cycling parameters consisted of 38 cycles of 98°C for 10 seconds, 55°C for 5 seconds and 68°C for 10 seconds. The amplicons from each strain were purified and submitted for Sanger sequencing. After Qubit

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quantification, the amplicons were pooled in equimolar amounts for library preparation. The DNA library was prepared using the Ligation Sequencing gDNA-Native Barcoding Kit 24 V14, following the ONT protocol. Approximatively, 10 fmol of the final library was loaded onto an R10.4.1 Flongle Flow Cell for sequencing. Raw data was generated by MinKNOW™ software and basecalling was carried out using Dorado v0.8.1 with Super-accurate model. The filtered and trimmed FASTQ reads were either mapped to a reference sequence derived from the "flavescence dorée" phytoplasma strain CH genome (Debonneville *et al.*, 2022; GenBank accession number CP097583) using minimap2 v2.22-r110 (Li, 2018) or *de novo* assembled using Flye version 2.9.2 (Kolmogorov *et al.*, 2019). A curated database of group 16SrV DNA sequences from the three targeted genes was established based on those available in NCBI. A bioinformatic analysis pipeline was developed to perform BLAST alignment of the ONT assembled sequences with those from the curated database.

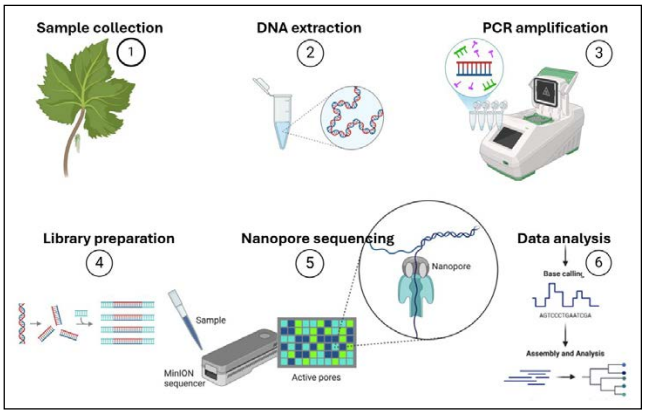


Figure 1. Schematic representation of the nanopore-based diagnostic procedure.

Table 1. Primer sets used for MLSA analysis of the selected group 16SrV phytoplasma strains.

PCR primer pair	Target gene	Approximate amplicon size	Reference
P1A/16S-SR	16S rRNA	1,573 bp	Lee *et al.*, 2004
FD9-F5/MAP-R1	*map*	876 bp	Arnaud *et al.*, 2007
FD9r/FD9f2L	*secY*	1,325 bp	Arnaud *et al.*, 2007

Results and Discussion

Currently, most of the phytoplasma identification protocols are based on 16S rRNA gene sequences. However, the 16S rRNA gene sequences among group 16SrV phytoplasmas are highly conserved, making it difficult to differentiate the various strains. To overcome this obstacle, a MLST scheme was developed with enhanced resolving power based on three genes: 16S rRNA, *secY* and *map*, with the latter two being more genetically variable. After *in silico* evaluation of several PCR primers, three primer sets were selected (Table 1) to amplify regions of the targeted genes in all phytoplasma strains of group 16SrV. Using these primers, amplicons of the expected sizes were produced from the 24 DNA samples tested. Genomic DNAs from healthy plant samples were used as controls. On average, a 3-hour sequencing run with 10 samples generated 160,000 PASS reads (92% of total output), with 16,000 reads/barcode. After assembly, consensus sequences were used as BLAST queries against the curated database to identify regions of similarity and calculate the statistical significance of the matches. The results are ranked based on E-values. Due to the improved R10.4.1 Flow Cell and the resulting high sequencing depth (about 200x), this method produced consensus sequences that were as accurate as those obtained by Sanger sequencing. It also allowed the differentiation of closely related strains in group 16SrV and the identification of mixed infections. The introduction of this methodology should provide an efficient diagnostic tool which may be deployed at port of entry to prevent the movement of exotic phytoplasma species. The preliminary results obtained in this study indicates that this methodology shows promise for rapid and accurate detection of phytoplasma strains that are challenging to identify using conventional methods.

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New detection tools

Oxford nanopore sequencing using metabarcoding as detection and identification tool for '*Candidatus* Phytoplasma' and '*Ca*. Liberibacter' taxa

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Abstract

This study explores the potential of Oxford Nanopore Technology (ONT) using metabarcoding for the detection and identification of the phloematic bacteria '*Candidatus* Phytoplasma' and '*Candidatus* Liberibacter'. The research involved determining target taxa, selecting candidate barcodes, establishing reference databases and developing and assessing primers. Furthermore, PCR assays, library preparation protocols, and bioinformatics pipelines were tested and optimized. The results demonstrate the capability of ONT metabarcoding for a reliable detection of these pathogens while also highlighting the challenges, particularly the critical need for well-curated reference databases to enhance taxonomic assignments.

Keywords: phytoplasma, liberibacter, diagnostics

Introduction

The phloematic bacteria comprise two important taxa, '*Candidatus* Phytoplasma' and '*Ca.* Liberibacter' that are both including several pathogenic organisms posing significant threats to crops worldwide. Rapid and accurate detection and identification of these pathogens are essential for an effective disease monitoring and control. However, several factors make the detection of phloematic bacteria particularly difficult. The phytoplasma identification is complex, comprising numerous (16S rRNA gene-based) '*Ca.* Phytoplasma' species, ribosomal groups, subgroups and strains. Although the '*Ca.* Liberibacter' taxon consists of four relevant pathogenic taxa, some of them features complex genetic lineages or haplotypes, further challenging detection and identification. The '*Ca.* Phytoplasma' taxon also have a broad host range, associated with hundreds of cultivated herbaceous and woody plants. Additionally, '*Candidatus*' species from both taxa are associated with slowly developing, similar and diverse symptoms and can coexist within the matrix, making symptom-based diagnosis difficult (Ermacora and Osler, 2019; Katsir *et al*., 2018). Together, these factors complicate the detection and identification of these pathogens substantially. Currently, phytoplasma identification relies on 16S rRNA gene PCR sequencing requiring amplicon length of 1,500 bp. When resolution is insufficient, additional metabarcoding or whole genome average nucleotide identity (ANI) analysis is recommended (Bertaccini *et al*., 2022; Wei and Zhao, 2022). The '*Ca.* Liberibacter' species identification is also based on the 16S ribosomal region, while in addition, '*Ca.* Liberibacter solanacearum' haplotyping typically involves SNP analysis in the 16S, 16S-23S, and 50S regions (Nelson *et al*., 2011). Consequently, reliably detecting and identifying bacteria from both groups requires multiple and often nested PCR assays. Moreover, in a diagnostic setting where time is critical, if species from both groups are anticipated, technicians may need to run several assays in parallel making the process both time-consuming and costly.

Oxford nanopore technology (ONT) using a metabarcoding approach, could offer a better alternative by multiplexing PCR assays to reduce time without compromising accuracy and reliability. The aim of this study is therefore to explore the potential of this technology for detecting and identifying '*Ca.* Phytoplasma' and '*Ca.* Liberibacter' species.

Materials and Methods

The first step in developing a metabarcoding ONT strategy involved *in silico* work, which included identifying target taxa, selecting candidate barcodes, developing and assessing primers, and establishing reference databases. Candidate barcodes were selected based on three criteria: i) availability of sequencing information in public databases, ii) sufficient taxonomic resolution, and iii) existence of conserved flanking regions for primer development. Reference databases for the selected barcodes were established using

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type strain sequencing data (for '*Ca.* Phytoplasma') or strains with known haplotypes (for '*Ca.* Liberibacter') to ensure high-quality databases. Finally, the literature was screened for existing primers, and new primers were developed for each barcode. Primers that successfully passed the *in silico* evaluation proceeded to wet laboratory evaluation, where the most effective set was ultimately chosen. In the next step, barcode PCR assays were optimized to determine optimal annealing temperatures and primer concentrations. This was achieved using an unspiked mock sample comprising nine bacterial species, along with uninfected plant samples, spiked samples, and infected samples. Subsequently, the library preparation protocol and the bioinformatics workflow were developed and optimized. Finally, various flongle platform metabarcoding runs were set up to evaluate the potential of this ONT metabarcoding strategy, testing the whole phytoplasma and liberibacter collection, single and mixed infected samples, and serial dilutions in both simplex and multiplex barcode PCR assays.

Results

It was carried out a categorization of the '*Ca.* Phytoplasma' species according to their relevance for Belgium, identifying 20 '*Ca.* Phytoplasma' species as extremely relevant and 10 as relevant. In contrast, all four liberibacter taxa were included in the analysis. Based on literature reviews, 13 potential barcodes were identified for phytoplasmas on the following genes: 16S rRNA, *rplV*, *groEL*, *secA*, *secY*, *tuf*, *rpoB*, *map*, *inmp*, *leuS*, *stamp*, *mboII* and *vmpI*. Further refinement based on the three selection criteria led to the choice of 16S rRNA, *rplV*, *secA* and *tuf*. Due to the limited availability of sequence information, the number of potential barcodes for '*Ca.* Liberibacter' was constrained. Ultimately, the barcodes were selected based on recommendations from EPPO (2024), specifically 16S, 16S-23S and 50S. Afterwards, reference databases were established, and primers were selected for each barcode. Barcode PCR assays were optimized leading to a selection of optimal primer concentrations and annealing temperatures. A library preparation protocol and a bioinformatics workflow were set up and optimized. Finally, the results of the different flongle platform metabarcoding runs are available.

Discussion

The first flongle platform runs showcased the potential of metabarcoding through nanopore sequencing while also highlighting several challenges. The most significant obstacle was the absence of well-constructed, complete, and curated reference databases. Without this, the reliability of taxonomic assignments remains questionable, affecting the validity of the results.

Finally, in an attempt to improve taxonomic assignment it was moved away from fixed thresholds for each barcode across all '*Candidatus*' species. Instead, it is suggested a flow diagram tailored to the specific needs of each barcode and phytoplasma ribosomal group.

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New detection tools

PhenoTruck^{AI}: a mobile laboratory for hyperspectral and molecular detection of phytoplasma quarantine diseases like "flavescence dorée"

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Abstract

"Flavescence dorée" (FD) is an epidemic quarantine disease of grapevine in Europe efficiently spread by the leafhopper *Scaphoideus titanus*. Promptly uprooting infected stocks and insect control are mandatory. Systematic monitoring of FD has also to be conducted in FD-free areas. However, in these regions the non-quarantine disease "bois noir" (BN), which induces similar symptoms in grapevine as FD, is widespread. Therefore, fast and reliable detection methods for FD monitoring in the field had to be developed. The concept of the PhenoTruck® is based on three axes: large-scale screening of vineyards using remote sensing by drones (UAVs), hyperspectral screening of leaf samples for phytoplasma presence and molecular identification of FD phytoplasmas in a mobile laboratory. The latter is a special vehicle with 4-wheel drive which allows autonomous laboratory work direct at the field. Drone image data are automatically processed and sample strategies developed. One compartment of the mobile laboratory is equipped with a dual hyperspectral camera system (VNIR+SWIR, wavelength range from 400 – 2,500 nm). The spectra of leaf samples are automatically analyzed for phytoplasma presence. Using machine learning technologies, phytoplasma-infected and healthy leaves as well as phytoplasma symptoms and other biotic and abiotic stress factors could be distinguished. In addition, the spectral discrimination of FD- and BN-infected leaves was achieved. The molecular identification of FD-infections was done by improved LAMP assays. The concept of the mobile laboratory PhenoTruck® is open for any other plant disease detection.

Keywords: grapevine, "bois noir", Palatinate grapevine yellows, disease monitoring, LAMP, UAV, machine learning

Introduction

"Flavescence dorée" (FD) is a severe disease in southern European grapevine growing regions. It is associated with 16SrV group -C and -D phytoplasmas which are epidemically transmitted by the grapevine leafhopper *Scaphoideus titanus*. While the leafhopper is of American origin and has been introduced in South-western Europe, the phytoplasma is of European origin and can be found widespread in alders in Western Europe and in *Clematis vitalba* in Eastern Europe (Malembic-Maher *et al.*, 2020). The FD phytoplasma can be transmitted occasionally from alder to grapevine by the invasive leafhopper *Orientus ishisdae* (Jarausch *et al.*, 2023). FD is constantly spreading northwards and has been blocked so far by the Alpine barrier. German grapevine growing areas are regarded as FD-free so far. One single FD-infected grapevine plant has been detected in 2020 (Jarausch *et al.*, 2021), which was immediately eradicated. It was putatively transmitted from infected alder nearby. FD is regulated as quarantine pest in the European Union (EU, 2019/2072) and a systematic monitoring of FD is required also in FD-free regions. However, this monitoring is hampered by the widespread presence of "bois noir" (BN) disease, associated with '*Candidatus* Phytoplasma solani', and the occasional occurrence of "Palatinate grapevine yellows" (PGY), another 16SrV phytoplasma. Both diseases are not epidemic but induce in grapevine similar symptoms like FD. Therefore, fast and reliable detection methods for FD monitoring in the field have to be developed. The concept of the PhenoTruck® is based on three axes: large-scale screening of vineyards using remote sensing by drones (UAVs), hyperspectral screening of leaf samples for phytoplasma infections and molecular identification of FD in a mobile laboratory right on the field's edge (Figure 1).

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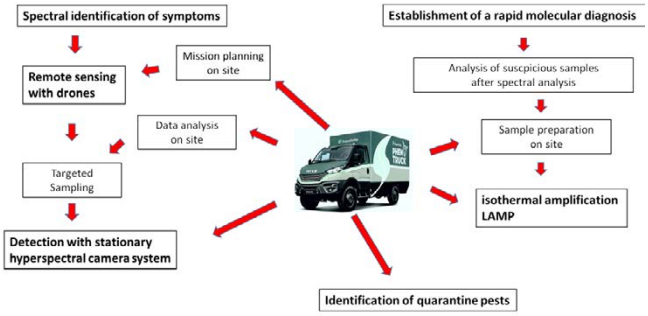


Figure 1. Concept of the PhenoTruck®.

Materials and Methods

The study was conducted in 2023 in 11 vineyards with 5 white varieties and in 10 vineyards with 5 red varieties in Palatinate in Southwest Germany and in six vineyards in Trentino, Northern Italy. In Germany, samples were taken from leaves with the symptoms of BN, PGY, virus infection, "esca" disease, insect damage, branch break and Mg-deficiency. In Trentino, leaves with symptoms of FD and BN were compared. Healthy leaves of all varieties served as control. In summary, more than 3,000 grapevine-leaves were tested. Phytoplasma infections of FD/PGY and BN were confirmed by molecular methods. LAMP detection of FD and BN was done according to Kogovsek *et al.* (2015, 2017) with improvement of the total nucleic acid extraction using a kit-based method. The hyperspectral measurements were done with the two hyperspectral push broom cameras Hyspex VNIR-1600 and Hyspex SWIR-320m-e to cover a spectral range from 400 nm up to 2,500 nm (Thielert *et al.*, 2024). The processing of hyperspectral data was done with a machine learning (ML)-pipeline using the Fraunhofer MATLAB-based HawkSpex®Flow. The best model to predict a specific symptom was selected by an AutoML-approach as described by Thielert *et al.* (2024). The remote sensing of vineyards was achieved with the multispectral drone DJI P4 with sensors in blue (450 nm), green (560 nm), red (650 nm), red edge (730 nm) and near infrared (NIR; 840 nm). High quality GNSS-information by RTK was used for georeferencing. The 3-D-point clouds and orthorectified reflectance maps were processed by an automated photogrammetry-pipeline. Symptomatic plants were identified using the Fraunhofer HawkSpex®Flow and index-based algorithms to generate a disease map usable for sampling strategies.

Results and Discussion

The PhenoTruck® is a mobile laboratory with two compartments mounted on a small 4-wheel drive truck. The truck is technically equipped to be autonomous in the field. One compartment uses the hyperspectral camera system described above for imaging and detection of phytoplasma infected leaves. In addition, drone flight missions and index-based analyses on site are supported. The second laboratory is equipped for molecular analyses. A prototype of the PhenoTruck® has demonstrated its usability in a case study conducted in Trentino to compare FD- and BN-infected grapevine leaves by hyperspectral and molecular analyses. Both diseases could be distinguished with 80% accuracy in the VNIR and SWIR range (Thielert *et al.*, 2024). Under practical conditions, phytoplasma-induced symptoms must be distinguished from other biotic or abiotic factors that cause similar symptoms. These different stress factors were analyzed with samples from German vineyards by lab-based hyperspectral imaging. Phytoplasma-infections could be distinguished from other stress factors more easily in the red varieties than in the white ones. The accuracy of distinction was 88% vs. 83% in the VNIR-range and 91% vs. 88% in the SWIR-range. BN and PGY could be distinguished in a white variety with accuracies of 78% (VNIR) and 84% (SWIR). Specific indices were developed to identify symptomatic grapevines in the multispectral drone data. These were used to establish disease maps for sampling strategies. Further work is needed to distinguish phytoplasma symptoms from other stress factors. A streamlined high quality DNA extraction protocol from grapevine leaves was developed to enable a reliable FD- or BN-detection by LAMP or qPCR.

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New detection tools

Molecular variation of '*Candidatus* Phytoplasma ziziphi' isolated during co-cultivation from micropropagated jujube witches' broom infected shoots

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Abstract

The aim of the study was to isolate and cultivate '*Candidatus* Phytoplasma ziziphi' (16SrV-B) from micropropagated jujube shoots exhibiting witches' broom symptoms. As a result, liquid cultures containing '*Ca.* P. ziziphi' along with at least one bacterial species, *Pseudomonas*, were obtained. Subsequently, colonies were grown on solid agar media, in which the presence of '*Ca.* P. ziziphi' alongside the predominant *Pseudomonas* species was confirmed using PCR methods. It was found that over time, the phytoplasma cell cultures tends to accumulate DNA changes that significantly distinguish them from the original isolate. It is possible that these variations may provide advantages to particular phytoplasma strains allowing them to more efficiently multiply in artificial nutrient media.

Keywords: isolation, phytoplasma, detection, primers specificity, 16S rRNA gene, sequencing

Introduction

Jujube is a native and economically important fruit tree of China also cultivated in Central and West Asia and northern Africa. The jujube industry is seriously suffering from the jujube witches' broom (JWB) disease that has destroyed about 3 to 5% of jujube trees in the world and is associated with the presence of '*Candidatus* Phytoplasma ziziphi' (Wang *et al.*, 2022). JWB infection modifies the developmental processes of jujube trees, which then show a wide range of symptoms, such as virescence, phyllody, witches' broom, stunting and general decline (Lee *et al.*, 2009). Phytoplasmas, are highly dependent on metabolic compounds obtained from their host's cells (Namba, 2019) and this makes their cultivation in artificial media extremely difficult. The aim of the study was to detect, identify, and isolate '*Ca.* P. ziziphi' in a artificial medium previously allowing phytoplasma isolation from infected periwinkle and grapevines (Contaldo *et al.*, 2016).

Materials and Methods

The material used was from a micropropagated jujube cultivar Huizao from jujube orchards in Xinzheng County, Henan Province, China. Small shoot *in vitro*, no more than 3 mm in length and 1 mm in diameter were used (Figure 1). The liquid medium for cultivation used was a modified CB (Contaldo *et al.*, 2016). This solution after autoclaving was supplemented with 0.22 µm filter-sterilized 25 µg/ml ampicillin. For the solid medium the addition of 12 g/l agar before autoclaving



Figure 1. Micropropagated jujube shoot infected by '*Ca.* P. ziziphi' (left) and in liquid culture (center); colonies (right).

was done. All isolation and passage operations were conducted under sterile conditions. To confirm the presence of phytoplasma DNA, PCR followed by nested PCR was conducted using universal primers for phytoplasma detection fU5/rU3 (Lorenz *et al.*, 1995) M1/M2 ($16R_{758F}$/$16R_{1232R}$) (Gibb *et al.*, 1995) and R16F2n/R2 (Gundersen and Lee, 1996). The amplification conditions for all primer sets were: 35 cycles of 94°C for 1 minute (2 minutes for the initial denaturation), 50°C for 2 minutes, and 72°C for 3 minutes; followed by 1 cycle at 72°C for 10 minutes for final elongation. For nested PCR, 1 ì l of PCR amplicon was used as a template at the original concentration or diluted 1:30. Fast digest *Tru*I and *Tsp509*I restriction enzymes were used for RFLP analysis according

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to the described manufacturer's protocol. Bidirectional sequencing of PCR products was performed using the same primers that were used for amplification: fU5/rU3-M1/M2 primer pairs in direct and nested PCR for the detection of '*Ca.* P. ziziphi', while R16F2n/R2-M1/M2 pairs for amplification of *Pseudomonas* species.

Results

'*Ca.* P. ziziphi' was detected in both liquid and solid nutrient media. However, the phytoplasma presence was accompanied by *Pseudomonas* species. The presence of the phytoplasma in the cultures was confirmed by amplification of the 16S ribosomal RNA gene using nested PCR with primers fU5/rU3 followed by M1/M2, RFLP analysis with *Tru*1I and sequencing. Moreover, the use of nested PCR with primers R16F2n/R2 followed by M1/M2 allowed to detect *Pseudomonas* species. Bioinformatics analysis showed that the R16F2n/R2 primer has 2 SNPs that prevent the amplification of '*Ca.* P. ziziphi' sequences. During the cultivation in liquid medium and in the colonies obtained on solid medium, it was found that over time, the phytoplasma cultures tends to accumulate DNA changes that significantly distinguish them from the original isolated strain. After two months of cultivation, changes in 4-5 positions on the 481-482 fragment amplified by the M1/M2 primer, were detected. Hence, the similarity to the original sequence increased from 98.98% to 99.18%. Moreover, these variation mainly occur at identical positions in different colonies (Figure 2).

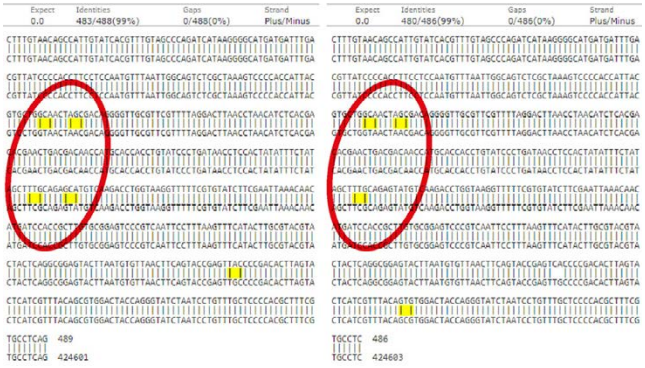


Figure 2. BLAST of amplicons sequences from the colony obtained from liquid culture isolated in 2024 (left) and 2023 (right) with the reference '*Ca.* P. ziziphi' genome.

Discussion

As a result of the work, several interesting findings were obtained. For the isolation of phytoplasma into culture, it is not necessary to have a large amount of plant material. By following the protocol used in this work, it is possible to obtain a live culture even from a unique sample not exceeding 3 mm in length and 1 mm in width. During the cultivation in liquid medium and colonies appearance on solid agarized medium, it was found that over time, phytoplasma cultures begin to accumulate DNA changes that significantly distinguish them from the original isolated strain. The variations in sequences mainly occur at identical positions in different colonies.

It is possible that these variations provide advantages to the isolated phytoplasma strains allowing them to more efficiently multiply in the artificial nutrient media. The accumulation of changes, on the one hand, may complicate their long-term cultivation. It makes determining the taxonomic affiliation of phytoplasmas in cultures more difficult, and it may lead to changes in their properties, particularly a reduction or loss of pathogenicity, which may also hinder further research. On the other hand, in one of the late-emerging colonies obtained from the initial tube, a sequenced sample matched the original 100% so factors helping in isolating the appropriate strain should be further elucidated. Since phytoplasmas, being obligatory parasites, lack a significant part of the metabolic apparatus and depend on nutrients obtained from the host organism, their cultivation in pure culture is difficult. In this work, the success of phytoplasma cultivation was likely due to the presence of at least two organisms in the culture '*Ca.* P. ziziphi' and *Pseudomonas* species. The dominant *Pseudomonas* bacteria very likely played the role of a supplier of missing substances for '*Ca.* P. ziziphi' growth. It could be hypothesized that '*Ca.* P. ziziphi' was not cultivated in the nutrient medium and that the positive PCR results were due to the presence of residual phytoplasma DNA from the plant tissue. However, two factors allow this to be excluded: i) during co-cultivation with other bacteria, as bacteria release metabolites that degrade external DNA and readily absorb nucleotides for their own needs, all residual phytoplasma DNA would have been hydrolyzed and assimilated by the dominant bacteria; ii) the accumulation of mutations, mainly in the same positions, directly indicates that '*Ca.* P. ziziphi' is alive and subject to some kind of selective pressure from the environment. Thus, this work convincingly demonstrated that '*Ca.* P. ziziphi' phytoplasmas can be cultivated, although in co-cultivation with other bacteria. At the same time, the work raises several additional questions, particularly whether pure cultures can be obtained and how to reduce the selective pressure of the medium to maintain genome stability in cultures.

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New detection tools

A colorimetric LAMP based assay for rapid screening of sandalwood spike phytoplasma

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Abstract

In this study it was developed a colorimetric loop mediated isothermal amplification-based assay for detection of phytoplasmas associated with sandal spike disease in the East Indian sandalwood trees, offering a promising alternative for rapid and accurate detection. This sensitive molecular tool will provide a reliable diagnostic approach for sandal spike disease, facilitating research on its transmission dynamics and landscape epidemiology, while enabling rapid screening of high-quality planting material - key measures for mitigating the disease spread and conserving the economically valuable sandalwood trees.

Keywords: SSD, loop mediated isothermal amplification assay, detection, *Santalum album.*

Introduction

The East Indian sandalwood (*Santalum album* L.) is a tropical evergreen tree prized both culturally and economically for its fragrant heartwood and essential oil. The sandalwood trees are declining due to illegal felling and the spread of sandal spike disease (SSD), the most damaging disease affecting sandalwood trees. It is associated with the presence of phytoplasmas and poses a significant challenge to sandalwood cultivation. Despite extensive research, there is currently no effective cure for SSD, and hence further spread has to be contained to prevent rapid decline of sandalwood. Since transmission of SSD phytoplasma through seeds has been established, the rapid screening of SSD phytoplasma in planting material is vital for controlling its spread (Kirdat *et al*., 2022). Several methods including immunological and molecular techniques such as PCR, nested PCR, and quantitative PCR have been reported for SSD detection. However, these methods have limitations in terms of time, specificity, and sensitivity. Loop-mediated isothermal amplification (LAMP) is a molecular technique that offers several advantages over traditional methods (Tomlinson *et al*., 2010). LAMP is highly specific, rapid and capable of amplifying DNA under isothermal conditions in less than an hour and it is suitable for field applications. LAMP enables rapid analysis of sandalwood spike disease incidence, and screening of quality planting material, making it essential for reducing disease spread. LAMP can be also used for rapid screening of putative insect vectors, alternate and/or collateral hosts thus helping in landscape epidemiology studies for supporting long-term conservation efforts.

Materials and Methods

The leaves of healthy sandalwood trees were collected at the KFRI Main Campus. The leaves of diseased (SSD infected) plants were collected from the SR-52 region of Sandalwood Reserve Forest, Marayoor Division, Kerala with the help of an expert forest pathologist. The plant genomic DNA was extracted using a modified CTAB method. Prior to LAMP primer evaluation, nested PCR was carried out for confirmation using primers P1 and P7 followed by R16F2n and R16R2 (Duduk *et al*., 2013). Primers for LAMP were designed using the NEB LAMP primer tool based on partial sequences of the 16S rRNA region of SSD phytoplasma obtained from NCBI GenBank (accession number EF050071) (Table 1). DNA extracted from both SSD-infected (test) and non-infected (control) plants, along with a non-template control, was utilized in a LAMP assay conducted with the Warmstart® Colorimetric LAMP Kit (LAMP 2X Master Mix with UDG, catalogue number M1804S; New England Biolabs) following the manufacturer's protocols. A 25 µl reaction mixture containing WarmStart Colorimetric LAMP Master Mix with *Bst* 2.0 polymerase was incubated at 65°C for 30 minutes in a thermal cycler. After incubation, the color change in the reaction mixtures was observed and recorded.

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Table 1. Sandal spike phytoplasma LAMP assay primers designed using partial 16S rRNA gene (GenBank accession number EF050071).

Primers	Sequences
F3 - Forward outer primer	5'-TCGAAGGTACCCGAAAAACC-3'
B3 - Backward outer primer	5'-TCCCCACCTTCCTCCAATT-3'
FIP -Forward inner primer	5'-TGCACCACCTGTGCAACTGATAACCAGGTCTTGACATGCTTC-3'
BIP -Backward inner primer	5'-TGTTGGGTTAAGTCCCGCAACGCTTGCTAAAGTCCCCACCAT-3'
LF - Loop forward	5'-CCTCCACTGTGTTTCTACAGCTTTG-3'
LB - Loop backward	5'-AGCGCAACCCTTATTGTTAGTTACC-3'

For further confirmation, the LAMP products were loaded onto a 0.8% agarose gel, and agarose gel electrophoresis was performed following standard protocols.

Results

DNA was extracted from randomly selected test leaf samples collected from SR-52 region which included spike infected and non-infected sandal plants. A total of 4 good quality test DNA samples were randomly selected for screening LAMP primer efficiency. Prior to the assessment of LAMP primers, a nested PCR was conducted, which demonstrated that only one of the four samples tested, specifically Test Sample 2 (T2), produced a positive result. Given the sensitivity of LAMP, T1 and T2 samples were subsequently selected for the LAMP assay. Additionally, a negative control (NC) was included, comprising DNA from a healthy sandalwood tree located at the KFRI Main campus identified by an expert pathologist as without sandal spike symptoms. The two test samples (T1 and T2) used showed positive reaction, *i.e.* a color change from pink to yellow, whereas the non-template control (without DNA) and a negative control (SSD non-infected sample DNA) shown no color change indicating the absence of SSD phytoplasma (Figure 1).

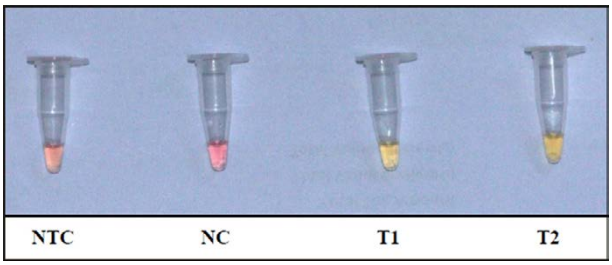


Figure 1. Colorimetric SSD LAMP assay showing positive result in test samples T1 and T2 (pink to yellow color change) (NTC- non-template control, NC- negative control and T- test samples).

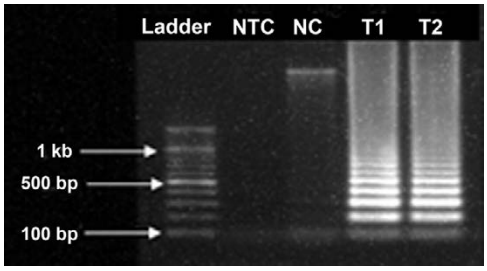


Figure 2. 1% agarose gel of LAMP results showing positive results as ladder like multiple bands: NT, non-template control, NC, negative control, T1 and T2, tested samples.

The LAMP products were analyzed using agarose gel electrophoresis to examine the distinctive ladder pattern typical of loop-mediated isothermal amplification products. When the LAMP PCR products were run on a 1% agarose gel, positive samples displayed the characteristic ladder-like multiple band progression of LAMP amplicons, while no bands were observed in the non-template controls (Figure 2).

Discussion

Nested PCR confirmed the presence of phytoplasmas in symptomatic plants, with only sample T2 testing positive. However, the LAMP assay detected positive results for both samples T1 and T2, indicated by a pink to yellow color change, highlighting that it was having an enhanced sensitivity. Analysis of LAMP PCR products on a 1% agarose gel showed the expected characteristic ladder bands in both samples confirming the colorimetric results. This LAMP diagnostic tool provides a rapid assay for detecting SSD in planting material, helping to reduce its spread. It can also facilitate quick assessments of SSD in potential insect vectors and alternative hosts in the Marayoor reserve.

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New detection tools

Recombinase polymerase amplification assay: a simplified and rapid approach for detection of fastidious plant pathogens

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Abstract

Plant pathogens threaten global food security, by causing substantial yield losses in several economically important crops. The early detection and identification of pathogens is of utmost importance to control the spread of such plant pathogens and the emergence of new diseases, reducing the associated agricultural losses. Traditional detection methods are time-consuming and require specialized equipment. Recombinase Polymerase Amplification (RPA) is an isothermal amplification technique which offers a rapid and sensitive detection of plant pathogens directly from plant samples. By eliminating the need for thermal cycling and DNA extraction, RPA simplifies the detection process and enables on-site pathogen detection. The present study highlights the potential of RPA as a powerful tool for early detection and effective disease management of fastidious pathogens infecting commercially important crops.

Keywords: crop pathogen, RPA, simplified tissue extraction, on-site detection

Introduction

Plant pathogens such as viruses, bacteria and phytoplasmas pose a serious threat to global food security by affecting the quality and reducing the yield up to 40% of economically important crops leading to huge economic losses (Savary *et al*., 2019; Baldi and La Porta, 2020; Venbrux *et al*., 2023). Recombinase polymerase amplification (RPA) is a rapid, isothermal nucleic acid amplification technique that has emerged as a powerful tool for the rapid and accurate detection of fastidious plant pathogens like greening bacteria, viruses, viroid and phytoplasmas. This technique involves the use of a DNA recombinase, primers, single stranded DNA (ssDNA)-binding proteins, nucleotides and a strand-displacement enzyme (Piepenburg *et al*., 2006; Lobato and O'Sullivan, 2018). By targeting specific genetic sequences of various plant pathogens, RPA assays can accurately identify and quantify the presence of harmful microorganisms in plant samples. This technology has been successfully used for the detection of a wide range of pathogens affecting economically important crops such as banana and citrus (Kapoor *et al*., 2017, 2020; Diksha *et al*., 2024; Gupta *et al*., 2024). RPA-based diagnostic tools have the potential to significantly improve disease management strategies. By enabling rapid and accurate detection of pathogens, these tools empower farmers and researchers to implement timely intervention measures significantly minimizing the crop losses and protecting agricultural productivity.

Material and Methods

For the template preparation in RPA based assays, crude plant extracts are prepared by crushing the plant tissues (leaves, midrib and veins) in different extraction buffers using mortar and pestle. Different extraction buffers like CTAB 10%, different ratios of NaOH:EDTA, General Extraction Buffer (GEB, Agdia, USA), CTAB extraction buffer, 0.1 M phosphate buffer (pH 7.4), Mackenzie buffer, 2X lysis buffer (GCC Biotech; West Bengal, India) and Sterile Distilled Water (SDW) are used for the optimization of the assay. Further, the primers are designed using Primer3plus software (https://www.bioin formatics.nl/cgi-bin/primer3plus/primer3plus.cgi/), as per TwistAmp® reaction kit guidelines (https://www.twistdx. co.uk). The primers are designed from the highly conserved region within a gene. After standardization of the suitable primer pair giving specific amplification for the pathogen. Other reaction conditions such as incubation temperature and time are also optimized. Then the sensitivity of the assay is determined by using a serial dilution (10-fold) of the template. The specificity of the primers is assessed by cross-reaction assays using

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templates from the related pathogens. Finally, the assay is validated by large scale screening of the symptomatic and asymptomatic samples suspected to be infected with the target pathogen.

Results and Discussion

RPA has emerged as a promising tool for rapid and sensitive detection of various plant pathogens. The use of crude plant extracts further enhances the practicality of this technique for on-site diagnosis. Studies revealed that the crude extract prepared in either GEB buffer/ NaOH:EDTA/ phosphate buffer, are the most appropriate buffers for the amplification in RPA based assays, showing higher efficiency and clarity in results (Kapoor *et al*., 2017, 2020; Diksha *et al*., 2024; Gupta *et al*., 2024).

The use of a constant temperature in RPA ranging from 37-42°C, eliminates the need for thermal cycling. Dispensing of DNA purification significantly simplifies the detection process (Lobato and O'Sullivan, 2018; Oliveira *et al*., 2021). Further, with a short reaction time usually 25-30 minutes, provides RPA an additional advantage over other molecular techniques, making it one of the fastest techniques for nucleic acid amplification (Ivanov *et al*., 2021). Since RPA shows high sensitivity, it has the ability to detect the presence of the pathogen even at a very lower titre. It requires minimal equipment's and can be performed in the resource-limited laboratories or even in field settings. The amplified products can be analyzed with the help of agarose gel electrophoresis or with naked eyes using fluorescent dyes or lateral flow assay (Srivastava *et al*., 2019; Diksha *et al*., 2024).

The assay has been extensively used for detection of several plant viruses, viroids and citrus greening bacterium. The successful application of RPA based assays for detecting *'Candidatus* Liberibacter asiaticus', one of the agents associated with citrus greening disease, demonstrates the potential of this technology for managing diseases (Diksha *et al*., 2024). The RPA-based diagnostic assay holds promise for phytoplasma detection and would be highly useful for epidemiology and management of diseases associated with the presence of difficult plant pathogens like phytoplasmas.

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New detection tools

Evaluation of a hybridization probe-based multilocus sequence typing technique for identification of diverse phytoplasmas

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Abstract

Phytoplasmas are specialized plant pathogenic bacteria that can cause devastating losses in crops and natural ecosystems. Correct identification of '*Candidatus* Phytoplasma' species and strains is essential for managing the diseases associated with the presence of these pathogens. Currently available PCR-based assays are often incapable of unambiguously identifying phytoplasma strains. This study evaluated a recently reported probe-based multilocus sequence typing technique for identification of diverse phytoplasmas. Results proved the efficacy of this technique in specifically identifying '*Candidatus* Phytoplasma' species and strains of tested phytoplasmas.

Keywords: '*Candidatus* Phytoplasma', hybridization probes, multilocus sequence typing

Introduction

Phytoplasmas are specialized plant pathogenic bacteria that can cause devastating losses in crops and natural ecosystems. Currently phytoplasmas are divided into 40 ribosomal groups and over 90 subgroups based on RFLP analysis of PCR-amplified 16S rRNA genes. However, under this scheme it has been recognized that 16S rRNA-encoding gene sequences alone are not sufficient for defining strains, and that other more variable gene sequences should also be considered for their differentiation (Martini *et al*., 2007).

To increase possibility of resolution, there have been ongoing attempts to supplement the 16S rRNA based system with additional sequences from genes such as *rp*, *tuf*, *secY*, *secA*, *nusA*. These additional molecular markers demonstrate a much-improved resolving power in the delineation of ecological strains of phytoplasmas leading to the proposition of multiple gene-based systems for differentiation of phytoplasmas (Lee *et al*., 2010).

It was recently reported a method for the simultaneous, PCR-free determination of DNA sequences for a set of taxonomic markers for different phytoplasma groups (Pusz-Bochenska *et al*., 2022). This hybridization-based approach involves using a set of probes to capture phytoplasma markers from infected plants or insects, then assembling the individual gene sequences including the 16S rRNA gene, *tuf*, *secY*, *secA*, *nusA*, and *rp*. Here it is presented an evaluation of the efficacy and specificity of this method using reference phytoplasma positive samples.

Materials and Methods

Plant material infected with '*Candidatus* Phytoplasma mali', '*Ca*. P. pruni', '*Ca*. P. solani', were maintained in apple, cherry, and periwinkle respectively. DNA was extracted from 1.0 g leaf petiole and midrib tissue using the method of Angelini *et al*. (2007) . Hybridization probes to capture phytoplasma markers from infected plants were designed as described (Pusz-Bochenska *et al*., 2022). The DNA from samples was prepared for hybridization as described earlier. The procedure for hybridization generally followed the protocol provided by IDT (version 4, May 2019). The amount of phytoplasma DNA in the samples was measured using 16S qPCR (Bennypaul *et al*., 2023) to ensure that the hybridization had worked as expected. Captured libraries were sequenced using a 500 cycle DNA nano kit from Illumina, with 25% PhiX as described previously (Pusz-Bochenska *et al*., 2022).

Results

Leaf petiole and midrib samples from apple, cherry, and periwinkle, singly infected with '*Ca*. P. mali', '*Ca*. P. pruni', '*Ca*. P. solani' respectively, were used in this study. Quality checks, adaptor trimming and mapping of sequence reads were done as described previously (Pusz-Bochenska *et al*., 2022). Among all three tested samples, the number of reads mapping to each target marker ranged from a minimum of 1,792 to a maximum of 56,639 (Table 1).

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Table 1. Number of reads mapping to the targeted markers, and target assembly lengths of phytoplasmas investigated.

Sample	*16S*	*secY*	*secA*	*tuf*	*nusA*	*rp*
'Ca. P. mali' (16SrX-A)	56,639 (**2305**)	1,792 (762)	8,639 (1923)	11,447 (**1182**)	3,349 (**1077**)	5,561 (**1651**)**
'Ca. P. pruni' (16SrIII-A)	52,955 (1566)	9,597 (**1248**)	23,952 (**2538**)	17,218 (**1206**)	8,260 (**1143**)	11,394 (**1748**)***
'Ca. P. solani' (16SrXII-A)	46,629 (**2525**)	9,787 (612)	21,521 (1773)	16,324 (888)	10,419 (**1113**)	16,073 (**626**)*

* = 1 ORF; ** = 2 ORFs; *** = 3 ORFs; numbers in brackets show length of assembled sequence in bp, assemblies containing complete open reading frames (ORF) are shown in bold.

The 16S rRNA gene was the best represented target in the data for all three samples. Of the six markers considered, full ORF sequences were obtained in three, four, and five cases for '*Ca.* P. solani', '*Ca.* P. mali' and '*Ca.* P. pruni' infected samples, respectively. A phylogenetic tree of concatenated sequences of *secA*, *secY*, *tuf*, and *nusA* from each sample was constructed using maximum likelihood method (GTR Model) (Figure 1).

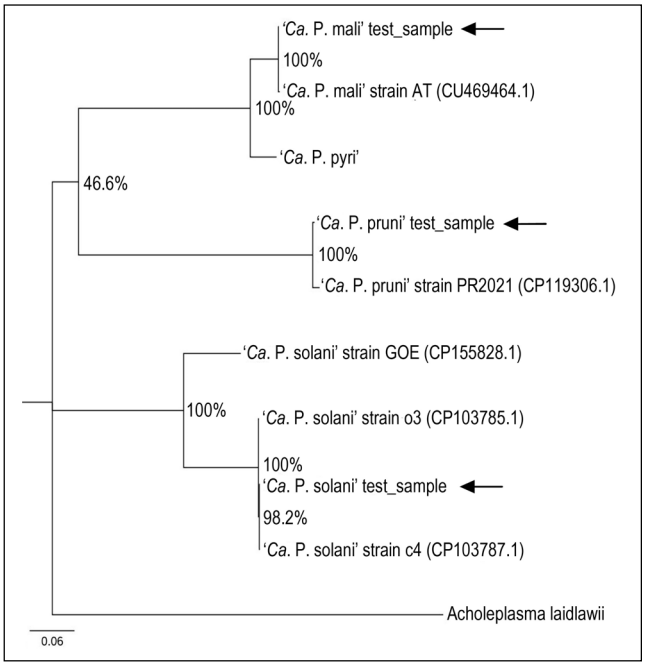


Figure 1. Phylogenetic tree constructed from concatenated sequences of *secA*, *secY*, *tuf*, *nusA* genes of phytoplasmas by maximum likelihood method, with *Acholeplasma laidlawii* used as the outgroup. The numbers on the branches represent the bootstrap support obtained from 1,000 replicates. Arrows indicate test samples.

The corresponding concatenated sequences of the above four markers from closely related '*Ca.* Phytoplasma' species and/or strains were assembled from sequences available in the NCBI database. The concatenated sequence obtained from apple was identified as belonging to '*Ca.* P. mali' as it clustered with the reported AT strain of '*Ca.* P. mali'. Similarly, the phytoplasma in the cherry sample was identified as '*Ca.* P. pruni' due its placement in the same clade as '*Ca.* P. pruni'. The concatenated sequence from periwinkle clustered with sequences belonging to various '*Ca.* P. solani' strains, identifying it belonging to the latter species and most closely genetically related to strain c4 (GenBank accession number CP103787). These results show that specific, strain-level identification of '*Ca.* Phytoplasma' species is possible with this hybridization probe-based MLST technique.

Discussion

Various PCR-based assays are available for detection and identification of phytoplasmas (Bennypaul *et al*., 2023). Most of these methods target one gene (*i.e.*, 16S rRNA) which often do not provide sufficient resolution for regulatory purposes. A study conducted by Seemüller and Schneider (2004) found that the 16S rRNA-encoding gene sequences of strains of phytoplasmas associated with apple proliferation, pear decline, and European stone fruit yellows were nearly identical, and thus not sufficient for identification under the 16S rRNA gene sequence classification scheme, but differentiable by RFLP method on the same sequence (Heinrich *et al*., 2001). By combining sequence information from multiple markers, the evaluated hybridization probe-based technique provides '*Ca.* Phytoplasma' species and strain resolution of diverse phytoplasmas.

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New detection tools

Comparative study of phytoplasma DNA extraction methods

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Abstract

The study of phytoplasmas and methods of their detection is an important aspect of determining the phytosanitary condition of the crops in a territory and to help the use of uninfected planting material by agricultural producers. For the study of phytoplasmas it is necessary to carefully and correctly prepare the sample to be analysed with appropriate DNA extraction procedures. Sample preparation consists in extracting fragments of tissues from suitable plant material mainly consisting of phloematic tissues. A comparative study and validation of methods of DNA extraction of phytoplasmas on the example of vegetative parts of the pear tree was carried out. It is shown that the reagent kits CytoSorb and SORB-GMO-B minimize the testing time and are not inferior to the traditional method based on CTAB extraction.

Keywords: '*Candidatus* Phytoplasma pyri', plant tissue, nucleic acid, plant quarantine

Introduction

The Russian Federation imports a large amount of crop planting material, including from countries in which dangerous pathogens are present. Plant protection specialists as well as agricultural producers should understand the possible risk associated with the danger of introduction of quarantine pathogens and dangerous diseases and assess the potential damage in case of entry of infected plant material. Phytoplasmas pose a high risk if they enter and infect economically important crops (Shneyder *et al*., 2017, Karimova *et al*., 2019). At the same time, their diagnostic methods are rather limited due to the biology of the pathogens. Evaluation and selection of the best kits for their testing will help to maintain the phytosanitary welfare of the country.

The concentration of phytopathogenic microorganisms in plant material is subject to significant variations and depends on the season, growing conditions, and infection background; therefore, it is important to obtain DNA of the pathogen to be tested in sufficient concentration and purity for its accurate detection. Currently, a large number of commercial reagent kits for extraction of microbial DNA from various types of samples is available. The aim of this study was to search, validate and compare methods for phytoplasmas DNA extraction from infected plant material using reagent kits of Russian production and select the most effective ones. In the experiment, it was compared the extraction of phytoplasma DNA using locally produced reagent kits with the extraction method of Doyle and Doyle (PM 7/62(3), 2020).

Materials and Methods

Phytoplasma DNA extraction using the Doyle and Doyle method is considered to be a standard method, but it is labour-intensive and resource-consuming. In addition to the standard method, 3 reagent kits from LLC Syntol (Russia) for phytoplasma DNA extraction from plant material were used CytoSorb, SORB-GMO-B, and FitoSorb.

In the preparation of samples for extraction, a BagMixer 400 W homogeniser (Interscience, France) was used with the compatible BagFilter homogenization bags. Given the unequal distribution of the pathogen in the plant parts, composite samples from leaves, shoots and root system were collected for testing (Blinova *et al*., 2020).

Plant material of *Pyrus communis* L. naturally infected with '*Candidatus* Phytoplasma pyri' with a medium level of infection was used in the experiments. Preparation of samples for studies according to the modified Doyle and Doyle technique consisted in the extraction of tissue fragments from plant material using grinding and CTAB (cetyl-trimethylammonium bromide) buffers.

The commercial kits tested were different in their principle of operation: CytoSorb is based on lysis of plant

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extract in the guanidine hydrochloride (GuHCl) followed by sorption of nucleic acids on silicon particles. In the SORB-GMO-B kit, lysis is performed using CTAB followed by deproteinization with chloroform (CHCl), and by DNA precipitation on silicon sorbent with diatomaceous earth. DNA extraction with the FitoSorb kit is performed using extraction buffer (included in the kit) with further lysis of plant extract with guanidine thiocyanate (GuSCN) and precipitation of nucleic acids on magnetic sorbent.

The quality of '*Ca.* P. pyri' DNA extraction was assessed by quantitative PCR with the reagent kit '*Ca.* P. pyri'-RV (LLC Syntol, Russia) on the CFX-96 PCR machine (Bio-Rad, USA) by regression analysis. Primers and probes used were those designed for the *dihydrolipoamide acetyltransferase* (*aceF*) gene (Bashkirova *et al.*, 2022). The extraction of phytoplasma DNA was performed in 4-times repetitions to obtain a more accurate result (Table 1).

Table 1. Extraction of '*Ca.* P. pyri' DNA by different methods.

Reagent set and threshold cycle (Ct) by FAM fluorescence channel (specificity)				
Samples	Doyle & Doyle	CytoSorb	SORB-GMO-B	FitoSorb
'*Ca.* P. pyri' (1)	26.9	28.6	31.9	38.7
'*Ca.* P. pyri' (2)	27.1	29.5	31.9	39.2
'*Ca.* P. pyri' (3)	27.2	28.5	31.5	39.2
'*Ca.* P. pyri '(4)	25.9	29.6	31.0	39.8
NIC (1)	N/A	N/A	N/A	N/A
NIC (2)	N/A	N/A	N/A	N/A
AV ± SE	26.8 ± 0.4	29.1 ± 0.3	31.6 ± 0.2	39.2 ± 0.3

NIC – Negative extraction control; N/A – not available; AV – average value; SE – standard error

Results

The commercial kit '*Ca.* P. pyri'-RV (LLC Syntol, Russia) allows the detection of a specific fragment of the phytoplasma *aceF* gene by the FAM fluorescence channel, with simultaneous internal control (IC) reaction – HEX channel. The use of the traditional Doyle and Doyle technique was adopted as a reference and allowed the detection of phytoplasma DNA in all the positive tested samples. The average value of the threshold cycle was 26.8. The data obtained with the HEX detection channel indicated no inhibition of the reaction and no false negatives, with a median threshold cycle value of 31.2.

For the CytoSorb reagent kit, the Ct value was 29.1, which was slightly lower than the values of samples extracted according to the Doyle and Doyle method and can be also recommended for the extraction of phytoplasma DNA. The Ct value for IC was 32.1, and no inhibition of the reaction and false negative results were observed.

When using the SORB-GMO-B kit, the threshold cycle value was 31.6, indicating a decrease in sensitivity. Reaction inhibition and false negative results were absent; the growth of the IC fluorescence curve was observed at cycle 32.

The FitoSorb kit showed the lowest efficiency of phytoplasma DNA extraction. Thus, an increase in the fluorescence signal for the specific reaction channel was observed at 39.2 cycles, which is equal to single copies of the pathogen. In addition, a slight inhibition of the reaction (threshold cycle 32.9) was detected for the HEX channel, which may be due to insufficient washing of the magnetic sorbent from secondary metabolites.

When comparing the results of DNA extraction of '*Ca.* P. pyri' using the Doyle and Doyle technique and domestic reagents, it can be concluded that the CytoSorb and SORB-GMO-B kits allow faster extraction of phytoplasmas DNA but are slightly lower in the amount of nucleic acid.

Discussion

For the detection and identification of difficult microorganisms such as phytoplasmas, it is very important to use validated DNA extraction methods. High-quality DNA extraction is one of the important factors for obtaining reliable results in molecular diagnostic of plant pathogens. The obtained experimental results indicate that some of the tested reagent kits can be used for extraction of phytoplasma DNA. Comparative testing of all reagent kits allows to conclude that the most effective kits for phytoplasma DNA extraction are CytoSorb and SORB-GMO-B (LLC Syntol, Russia), however the samples testing negative with these methods should be retested using the traditional Doyle and Doyle method to be sure that they are free from '*Ca.* P. pyri' for propagation purposes.

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New detection tools

A novel approach for detection of sandal spike infection in *Santalum album*

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Abstract

Santalum album, known as east Indian sandalwood, is an economically important tree species for its valuable heartwood and essential oil. One of the most common phytoplasma infection is the sandal spike disease (SSD), associated with spike-like symptoms . In this study, genomic DNA was extracted from symptomless and SSD infected sandalwood leaves. Phytoplasma detection was done by nested PCR, using two sets of universal 16S rRNA gene primers P1/P7 and R16F2n/R16R2, and by a single step PCR using sandal spike specific primer along with an internal control primer set. Expected amplicons of about 1.8 kb and 1.2 kb, respectively, were visualized from infected samples with nested PCR. With the single step PCR, amplicons of 300 bp corresponding to the internal control were observed in all the sandalwood samples, and amplicons of 500 bp were obtained only from a number of symptomatic samples.

Keywords: sandalwood, spike disease, phytoplasma, nested PCR, DNA

Introduction

Phytoplasmas are prokaryotes without a cell wall and require cell hosts for their survival (Lee *et al*., 2010). Globally, several phytoplasma diseases have been reported in many important ornamental, agriculture and forestry species (Da Silva, 2016). They are mainly transmitted by insect vectors, and also by dodders and grafting (IPPC, 2016). Sandalwood, known as a royal tree, is also susceptible to phytoplasma infection, exhibiting characteristic symptoms such as internode and leaf size reduction, stiffening and discoloration, and spike like inflorescence at the advanced stage of the disease (Sundaraj *et al*., 2022). Earlier detection methods for phytoplasma included symptomatic expression, followed by transmission microscopy, fluorescence staining and serological assays. Recently, DNA based technology allowed detection and characterization of phytoplasmas (Rao *et al*., 2011) on the 16S rRNA gene. The development of universal primers allow amplifying all known phytoplasmas by PCR (Arhens and Seemüller, 1992) such as primer P1/P7 and R16F2n/R16R2 (Deng and Hiruki, 1991, Schneider *et al*., 1995; Gundersen and Lee, 1996). Further, the latest development of a single step PCR based assay has facilitated the rapid detection of SSD (Muthulakshmi *et al*., 2022).

Materials and Methods

Phytoplasma infected (SD1-6) and symptomless (SWH1-3) leaf samples of sandalwood were collected from the field. A tissue cultured sandalwood (SWTC) leaf sample was used as a negative control. Using leaves stored at -20°C, DNA was extracted from 500 mg tissue following the enrichment protocol described by Arhens and Seemüller (1992), after crushing with liquid nitrogen. The lysate extracted with chloroform: isoamyl alcohol (C:I) was treated with 5 µl RNase-A (20 mg/ml) for 30 minutes at 37°C, and then 5 µl proteinase-K (20 mg/ml) at 37°C for 40 minutes, followed by extraction with C:I and DNA pelleting with ice-cold propanol, and then washing twice with 70% ethanol. The air-dried DNA sample was suspended in 50 µl of autoclaved MilliQ water and checked for purity and quantity by agarose gel electrophoresis and UV-Vis spectrophotometry. Nested PCR for phytoplasma detection was performed using primers P1/P7 and R16F2n/R16R2 and BSA as enhancer, at 94°C for 90 seconds, and 35 cycles of 94°C for 30 seconds and 55.5°C for 50 seconds; 72°C for 1 minute, and 72°C final extension for 10 minutes for the nested reaction. Single step PCR was performed using the spike specific and internal control primer sets *i.e*., SSD1 and SSES2 (Muthulakshmi *et al*., 2022) at 94°C for 3 minutes, and 40 cycles of 94°C for 1 minute; 62°C for 1 minute; 72°C for 2 minutes, and 72°C final extension for 10 minutes. PCR amplifications were visualized with electrophoresis in agarose gel (1.5%) stained with ethidium bromide.

Results

DNA was obtained from all samples with concentration of 43 ng – 209 ng. Faint bands of about 1.8 kb were observed

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only for samples SD3 and SD5 after PCR with P1/P7 primers, whereas amplicons of about 1.2 kb were visualized from all infected sandalwood samples after nested PCR with R16F2n/R16R2 primers. No bands were observed in symptomless and tissue cultured samples, confirming the phytoplasma presence only in infected samples (Figure 1).

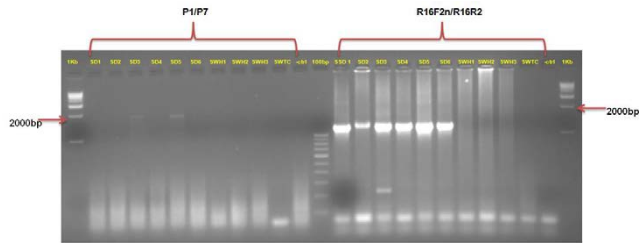


Figure 1. Amplicons using universal primer sets in nested PCR.

After the single step PCR, a single bright PCR band of 300 bp was observed in all the samples for the internal control primer set SSES2. A single bright PCR band of 500 bp was visualized in four (SD1, SD2, SD3 and SD5) symptomatic sandalwood samples, with no amplification from the symptomless samples using the SSD1 primer set (Figure 2).

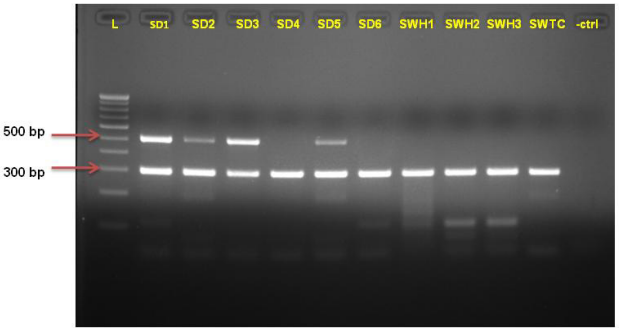


Figure 2. Amplicons of single step PCR using SSD and internal control primer sets.

Discussion

Successful PCR detection require proper sampling and reliable DNA extraction method, due to uneven distribution of phytoplasmas in the host tissue, and other factors like the phytoplasma strain and environmental conditions. Detection of SSD before symptom expression, was reported using ELISA (Da Silva, 2016). Khan (2004) reported two SSD phytoplasma strains of the subgroup 16SrI-B using RFLP analysis after nested PCR. However, there is very little knowledge about the different strains of phytoplasmas associated with sandalwood diseases. A single step PCR-based detection of sandal spike phytoplasma developed by Muthulakshmi *et al.* (2022) was adopted in this study. Results showed amplification for all the symptomatic and symptomless sandalwood samples for the internal control primer at 300 bp. However, amplicons at 500 bp were only visualized in four infected samples out of six, though the same samples gave amplification in the nested PCR assay. This indicates that the detection of phytoplasmas is highly dependent on their concentration in the tissues (IPPC, 2016). Therefore, there is a need to advance the detection technique using real-time monitoring of the amplification, which could also help in detecting the phytoplasma load in the infected sample. The absence of 500 bp amplicons for SSD1 primers in two samples despite amplification during the nested PCR, could also be due to the presence of other phytoplasmas in the samples. This study validates both nested and single step PCR assays for the detection of phytoplasma infection in sandalwood. Additional studies on the 16S rRNA gene along with other non-ribosomal genes like *secY*, *secA* or ribosomal protein genes can aid in delineation of phytoplasma groups and subgroups, and closely related distinct strains in sandalwood.

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New detection tools

Satellite-based detection of apple proliferation and pear decline

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Abstract

Apple proliferation (AP) and pear decline (PD) are important diseases of fruit trees in Europe that are difficult to control. Timely uprooting of infected trees is a successful measure to limit the spread of the diseases and is therefore mandatory in some production areas. Large-scale monitoring strategies are required. AP and PD induce in infected trees an early leaf reddening in late summer or early autumn that is suitable to be monitored by remote sensing. Very high-resolution satellite images covering the region of reference orchards were analysed with machine learning methods to identify apple and pear orchards and AP- and PD-infected trees. High accuracy of apple and pear orchard identification was possible with values from 0.98 to 0.99. Symptomatic trees were identified in multispectral data of the WorldView satellites with a spatial resolution of 15 cm x 15 cm. Vegetation indices which combine information on the biomass and pigment proved to be suitable for detecting both AP and PD at tree level. Vitality maps and diagnostic maps are two targeted products out of the current study. While the diagnostic map reveals trees with phytoplasma-induced symptoms such as red leaves at a specific time point, the vitality map also causally records other vitality parameters of the trees in a time series.

Keywords: '*Candidatus* Phytoplasma mali', '*Ca.* P. pyri', remote sensing, multispectral analyses

Introduction

Apple proliferation (AP) and pear decline (PD) are among the economically most important diseases of apple and pear in Europe. Different psyllid vectors transmit '*Candidatus* Phytoplasma mali' and '*Ca.* P. pyri' very efficiently. Therefore, timely uprooting infected trees is an efficient strategy to limit the spread of the diseases. In some apple growing regions, it is even mandatory. As AP is transmitted on a regional scale, large-scale monitoring strategies are required. Ground-based monitoring by experts is laborious, time-consuming, expensive and nevertheless error prone. Therefore, automated remote sensing by satellites would be of great benefit.

While AP induces specific symptoms, PD does not. However, premature leaf reddening in early autumn is highly correlated with both diseases (Jarausch *et al.*, 2023, 2024). This symptom was used to evaluate the feasibility of detecting AP- or PD-infected trees in reference orchards by high-resolution multispectral satellite images.

Materials and Methods

The study was conducted in two fruit growing regions of Palatinate in Southwest Germany. Reference orchards of apple and pear with high infection rates of AP and PD were selected as described in Jarausch *et al.* (2024). AP-infection was confirmed either by specific symptom recording or PCR testing. PD-infected as well as healthy, asymptomatic trees were exclusively confirmed by PCR (Jarausch *et al.*, 2024).

Satellite data with different spectral and spatial resolutions were used with the aim of detecting the reddening symptoms of AP and PD in the apple and pear orchards. PlanetScope (PS) imagery (3 m spatial resolution) was analysed to identify apple and pear orchards as a first step of the digital disease identification process. Machine Learning (ML) methods like Random Forest (RF) were applied to classify land use of orchards and vineyards with the PS data. The temporal prognosis of the leaf reddening determined the start of the satellite monitoring in the years 2019-2022. Images from the PlanetScope and SkySat satellites (Planet Labs PBC) as well as WorldView-2 and -3 (Maxar Technologies Inc.) were specifically acquired from the satellite operating companies. The AP pathosystem was investigated with very high spatial resolution satellite imagery of WorldView-3 (0.31 m in panchromatic and 1 m in multispectral bands). For PD detection, SkySat images with 0.50 m resolution were combined with drone data to georeferentiate the trees in the orchard (Kohler *et al.*, 2023; Thielert *et al.*, 2023). New high spatial resolution satellite images of WorldView-3 (0.15 m in multispectral bands) were used to identify PD-infected trees. Since the timing of detection of phytoplasma-induced

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reddening is important (Jarausch *et al.*, 2023, 2024), the recording windows for the satellite data were end of September to mid-October for apple and mid-August to mid-October for pear. The images provided data from visual (Blue, Green, Red) and near-infrared (RedEdge, NIR) spectral channels. Different indices for growth parameters were analysed: NDVI (Normalized Difference Vegetation Index), SIPI (Structure Intensive Pigment Index), MCARI2 (Modified Chlorophyll Absorption Ratio Index 2) and MSAVI (Modified Soil Adjusted Vegetation Index).

Results and Discussion

The results of this study demonstrate that high-resolution satellite images can effectively detect and classify different orchard types with very high accuracies. Two different fruit growing areas were analysed with a time series of 6-8 satellite images during the vegetation period from March until October for the detection of apple and pear orchards in 11 land use classes. Testing different machine learning algorithms indicated that classification by Random Forest was the best option with high detection accuracies between 0.98 and 0.99 for the different orchard types. This good detection performance was achieved very constantly over the years in 2018, 2019, and 2020.

Ten AP-symptomatic and ten asymptomatic apple trees were precisely GPS-mapped in a reference orchard and localised in satellite images of WorldView-3. The reflection spectra of the infected trees showed significantly increased values in the RedEdge at 722.7 nm (n = 10, $P < 0.05$) and in the NIR bands at 824 nm and 913.6 nm.

PD-infected and asymptomatic pear trees were analysed in a highly PD-infected orchard. WorldView-3 satellite images acquired at the ultra-fine spatial resolution of 15 cm x 15 cm were employed. Using drone imagery pear trees were precisely georeferenced in the satellite images. This hybrid approach has the potential to help in tree-level health condition detection while overcoming the possible limitations of using lower-resolution imagery (Gago *et al.*, 2021). A comparison of drone and satellite images (Figure 1) showed that it is possible to detect PD-infected trees in satellite images. Vegetation indices were tested to distinguish the symptomatic from the asymptomatic pear trees (Figure 2). NDVI, SIPI and MCARI2 are particularly suitable for this purpose. The difference in the mean values was statistically significant between the input groups (n = 46; p < 0.05). These results support recent data research by Knauer *et al.* (2024) in indicating the potential for the detection of apple proliferation disease.

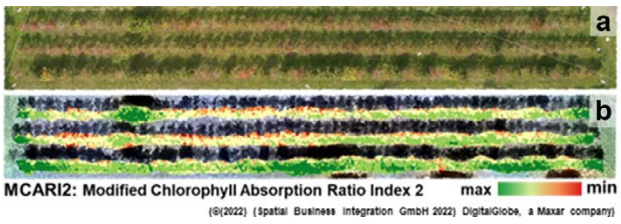


Figure 1. Pear orchard in the MCARI2 based WorldView-3 HD satellite image (15 cm) (b) and corresponding drone image (a).

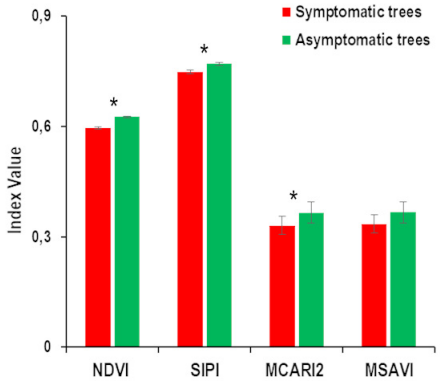


Figure 2. Vegetation indices of symptomatic versus asymptomatic pear trees from WorldView-3 satellite image from September 22, 2022 (each mean of n=46 with standard deviation, * statistically significant $p = 0.05$).

The satellite images from WorldView-3 with a very high spatial resolution were best suited for the localization and observation of tightly grown trees in rows. With lower spatial resolution, e.g. 50 cm x 50 cm in SkySat images, the localization of the trees could be simplified by GPS measurements or by merging satellite and drone images. Alternatively, it could be more suitable to provide the information about the symptoms not by tree but by zone, due to the orchard cultivation system and the difficulty in identifying single trees. High-resolution remote sensing, together with machine learning methodologies, represents an important advance in a sector of agriculture where precision farming techniques are increasingly being adopted for better management and monitoring of orchard health.

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Interactions

Insect vector transmission of phytoplasmas and citrus greening bacteria in diverse agricultural crops of India

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Abstract

The history of phytoplasma associated diseases and citrus greening/"huanglongbing" (HLB) presence in India is about 130 and 300 years old, respectively. Different species of leafhoppers, planthoppers and psyllids are known as natural/putative vectors of different strains of phytoplasma/ HLB strains identified in India. Among them, leafhoppers are the major group of insects responsible for transmission of several phytoplasma strains in brinjal, sesame, sugarcane, rice, wheat, bamboo, chickpea, fruits and ornamentals. Species of *Cofana unimaculata*, *Empoasca indicus*, *Hishimonus phycitis*, *Mukaria splendida*, *Orosius albicinctus* and *Recilia dorsalis* are reported as potential vector species in the transmission of 16SrI, 16SrII, 16SrVI, 16SrXI and 16SrXIV groups of phytoplasma strains. Moreover *Stephanis typica*, *Laodelphax striatellus* and *Sogatella furcifera* are reported as vectors of 16SrXI, 16SrVI and 16SrXIV phytoplasma strains in coconut root wilt, rapeseed phyllody, wheat chlorotic streak and stunt under field conditions. However, only one species of psyllid, *Diaphorina citri* is reported transmitting the HLB bacterium in India.

Keywords: leafhopper, planthopper, psyllid, natural vector, putative vector, HLB

Introduction

Phytoplasmas are phloem restricted bacteria without cell walls and are transmitted by phloem-sap feeding Hemiptera families such as Cicadellidae, Delphacidae, Cixiidae, Derbidae, Psyllidae and rarely by Pentatomidae and Tingidae (Marzachì *et al*., 2004; Viraktamath *et al*., 2023). The 16SrI and 16SrII phytoplasma groups are the prevalent and widespread in India followed by 16SrVI, 16SrXI and 16SrXIV. Sesame phyllody, brinjal little leaf, tomato big bud, sugarcane grassy shoot, sandal spike, bamboo witches' broom, coconut root wilt, arecanut yellow leaf and many ornamentals (chrysanthemum, marigold, jasmine, rose, seasonal flowers), legumes (chickpea, lentil, pigeon pea) and temperate and tropical fruits (stone fruits, mango, papaya, sapota) diseases are the major phytoplasma threats in India causing serious economic losses every year (Rao, 2021). "Huanglongbing" (HLB, citrus greening) is one of the most devastating diseases of citrus which has affected the global citrus industry including India during last decades (Ghosh *et al*., 2022).

Phytoplasmas and HLB are mainly spread by vegetative propagation, grafting and insect species belonging to the families Cicadellidae (leafhoppers), Fulgoridae (planthoppers) and Psyllidae (psyllids). These insect species can transmit phytoplasmas and HLB efficiently because of their specialized piercing and sucking mouthparts (Weintraub, 2007). A phloem-feeding habit is thus necessary but insufficient for phytoplasma transmission. Therefore, host range of phytoplasma/HLB is mainly dependent upon the feeding habits of the insect vectors (Weintraub *et al*., 2019). Updated information on natural and putative vectors identified for different phytoplasma groups and citrus greening bacterium in India are reported.

Results and Discussion

More than 20 species of leafhoppers, two species of planthoppers and one psyllid species are reported as putative or natural vectors for several phytoplasma strains and citrus greening bacterium in India (Rao, 2021; Ghosh *et al*., 2022). Among insects, leafhoppers are the major group reported associated with transmission of several phytoplasma strains in brinjal, sesame, sugarcane, rice, wheat, bamboo, ornamentals, chickpea, vegetables, temperate and tropical fruits and ornamentals. The major leafhopper vector species are *Circulifer haematoceps*, *Cofana unimaculata*, *Deltocephalus vulgaris*, *Empoasca devastans*, *E. motti*, *Exitianus indicus*, *Hishimonus phycitis*, *Maiestas portica*, *Mukaria splendida*, *Orosius albicinctus*, *Pyrilla perpusilla*, and *Recilia dorsalis*. They are reported performing efficient natural transmission of phytoplasma strains associated with diseases of several phytoplasma crops in India (Rao, 2021; Ravi *et al*., 2022; Reddy *et al*., 2021, Ranebennur *et al*., 2022; Kumar *et al*., 2024) (Table 1).

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Table 1. Insect species reported as vectors of phytoplasmas and HLB in India.

Disease and agent	Phytoplasma ribosomal group/subgroup	Insects
Sesame phyllody	16SrII-A, 16SrII-C	*Circulifer haemotoceps, Orosius albicinctus*
Brinjal little leaf	16SrVI-D	*Hishimonus phycitis, Empoasca devastans*
Sugarcane grassy shoot / white leaf	16SrXI	*Deltocophalus vulgaris*
Rice orange leaf	16SrXI	*Recilia dorsalis*
Toria phyllody	16SrVI	*Laodelphax striatellus*
Coconut root wilt	16SrXI	*Proutista moesta*
Spear rotof oil palm	16SrXI	*Stephanitis typica*
Toria phyllody	16SrVI	*Laodelpax striatellus*
'*Candidatus* Liberibacter asiaticus'	-	*Diaphorina citri*

Among the leafhopper species, *H. phycitis, E. indicus*, and *O. albicinctus* are the more likely potential leafhopper species playing a dominant role in transmission of 16SrI, 16SrII, 16SrVI, 16SrXI and 16SrXIV groups of phytoplasma strains. However, only three planthopper species, *Stephanis typica, Laodelphax striatellus* and *Sogatella kolophon*, are reported to transmit '*Ca.* P. trifolii', *Ca.* P. oryzae and '*Ca.* P. cynodontis' strains in toria, coconut and wheat under field condition (Azadvar *et al*., 2011; Kumar *et al*., 2024; Kochu Babu and Nair, 2000). Moreover, only one psyllid species, *Diaphorina citri* is reported to transmit the HLB in citrus orchards all over India (Ghosh *et al*., 2022).

S. typica, a lacewing bug, belonging to family Tingidae is also reported as transmitting phytoplasmas in the 16SrXI group associated with spear rot disease of oil palm in India (Kochu Babu and Nair, 2000). The presence of phytoplasmas belonging to 16SrI, -II, -V, -VI, -XI and -XIV groups have been reported in *E. indicus, P. perpusilla, M. portica, C. unimaculata* collected from wheat fields (Kumar *et al*., 2024); *E. motti* and *S. kolophon* collected from soybean field harboured 16SrI group phytoplasmas (Kumar *et al*., 2015); *O. albicinctus, Amrasca bigutella bigutella* collected from chickpea fields were reported as infected with 16SrI, 16SrII and 16SrVI groups (Reddy *et al*., 2021) and *H. phycitis, Empoasca sp., E. indicus, C. unimaculata, Amritodus atkinsoni,* and *Kolla paulula* collected from various ornamentals fields in India tested also positive for phytoplasma presence (Rihne *et al*., 2022). However further studies are required to prove the transmission ability of these insect species.

Phytoplasmas can also be harboured by weeds, which act as natural reservoirs facilitating their spread by insect vectors to economically important plants and thereby increasing economic losses. More than 34 weed species are reported to be the hosts of phytoplasmas belonging to five ribosomal groups (16SrI, 16SrII, 16SrV, 16SrVI and 16SrXIV) (Rao, 2021). Weed species can have a profound effect on vector distribution and infection levels. Hence, the weed hosts tested positive for phytoplasma strains in India may represent important sources of natural reservoirs and transmit the phytoplasma/HLB through the reported insect vector species.

Habitat management is an important agro-ecological approach in integrated pest management of phytoplasmas and HLB diseases. Since, limited studies have been performed on epidemiological aspects of phytoplasma vector transmission in India (Reddy *et al*., 2021), the epidemiological models for prediction of phytoplasma and vector spread may provide an insight information to disease epidemiology and control.

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Phytopathogenic Mollicutes
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Interactions

Leafhoppers as vectors of phytoplasma diseases

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Abstract

Leafhoppers (Cicadellidae) are critical agricultural pests and vectors of economically significant plant pathogens, including phytoplasmas, spiroplasmas and viruses. These pathogens cause devastating diseases resulting in substantial crop losses worldwide. In India, key vector species include *Nephotettix virescens*, *Cicadulina bipunctata*, and *Orosius albicinctus*. Despite their importance, vector-pathogen associations remain poorly understood for the majority of species. The subfamily Deltocephalinae, which accounts for 63% of known vector species, plays a central role in pathogen transmission. Tribes such as Macrostelini, Deltocephalini, and Chiasmini harbour numerous vector species capable of spreading mollicutes and plant viruses. Phylogenetic studies reveal the polyphyletic nature of Deltocephalinae, with vectors distributed across multiple lineages, suggesting a substantial number of undiscovered vectors. Feeding preferences, such as xylem or phloem sap ingestion, further influence vector competency and pathogen transmission dynamics. Research has largely focused on agroecosystems and species associated with economically important crops, leaving natural habitats and tropical ecosystems underexplored. The lack of systematic surveys and phylogenetic analyses limits the understanding of the full extent of leafhopper-pathogen interactions. Identifying new vector species, especially in under-studied ecosystems, is critical as many pathogens and vectors may emerge in human-dominated landscapes due to habitat alteration and climate change. Comprehensive studies integrating taxonomy, phylogenetics, and pathogen transmission dynamics are essential for uncovering the evolutionary relationships influencing vector competency. Such insights will facilitate the development of predictive models for identifying potential vector species and designing effective management strategies to mitigate leafhopper-borne diseases in agricultural systems.

Keywords: Cicadellidae, Deltocephalinae, vectors, plant disease

Introduction

The leafhopper family (Cicadellidae) constitutes one of the largest groups of insects in the order Hemiptera, with approximately 35,000-45,000 estimated species worldwide, though only about 22,600 have been formally described (Dietrich, 2004). These small but highly diverse insects are significant in agriculture and horticulture due to their dual impact: direct feeding damage and their role as vectors of critical plant pathogens (Nielson, 1979). Leafhopper-transmitted pathogens include phytoplasmas, spiroplasmas, and viruses, which cause devastating plant diseases such as rice tungro, sesame phyllody, potato purple top roll, sandal spike, and ragi streak. These diseases lead to considerable economic losses globally, especially in tropical and subtropical agricultural regions. Understanding leafhoppers as vectors is vital for devising effective management strategies to mitigate crop losses.

Morphological Characteristics and Identification

Leafhoppers can be distinguished from other Auchenorrhyncha by the presence of two or more rows of spines on their hind tibiae and a pronotum that does not extend over the abdomen. In contrast, their close relatives, the Membracidae, have a pronotum that extends over the abdomen. They are often confused with planthoppers (Delphacidae) but can be differentiated by the position and structure of their antennae. In leafhoppers, the antennae are positioned between the eyes, whereas in planthoppers, they are located beneath the eyes. Additionally, the second segment of the antenna (pedicel) in leafhoppers is small and lacks externally visible sensillae, unlike the enlarged pedicel with prominent sensillae seen in planthoppers. Leafhoppers also lack the movable large spur at the apex of the hind tibia that is characteristic of planthoppers.

Leafhoppers as Vectors of Plant Pathogens

The major leafhopper vectors in India are listed in Table 1. These species transmit pathogens such as rice tungro, little leaf of brinjal, sesame phyllody, and witches' broom in potato. Among them, Deltocephalinae emerges as the most significant subfamily, accounting for 63% of all vector species

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worldwide (Nielson, 1979). Tribes within Deltocephalinae, such as Macrostelini, Chiasmini, and Deltocephalini, host numerous vectors of mollicutes and plant viruses. The random distribution of vector competency across multiple lineages within this subfamily suggests the existence of many yet-undiscovered vectors. This highlights the need for systematic phylogenetic surveys to uncover their diversity and role in disease transmission. While most leafhopper lineages feed on phloem sap, some groups exhibit specialized feeding behaviour. For instance, Cicadellinae predominantly feed on xylem, while Typhlocybinae specialize in mesophyll feeding. These preferences influence vector competency, as certain pathogens are adapted to specific plant tissues. Phylogenetic analyses have revealed evolutionary relationships among subfamilies, such as the close association between Cicadellinae and Evacanthinae (Zahniser and Dietrich, 2013). The phylogeny revealed a closed relationship between Cicadelinae and Evacanthinae (Galetto *et al*., 2011). Such studies emphasize the need for integrative approaches combining morphology, phylogenetics, and molecular tools to refine the understanding of vector biology.

Table 1. Leafhopper vectors of plant-pathogens in India.

Leafhopper vector*	Pathogen transmitted
Deltocephalinae	
Hishimonus phycitis (Distant)	Little leaf of brinjal phytoplasma
Orosius albicinctus (Distant)	Phyllody of sesame phytoplasma Phyllody of gram phytoplasma Potato purple top roll phytoplasma
Nephotettix virescens (Distant)	Rice tungro virus
Nephotettix nigropictus (Stål)	Rice yellow dwarf phytoplasma Rice tungro virus
Maiestas dorsalis (de Motschulsky)	Rice yellow dwarf phytoplasma Rice stunt phytoplasma
Cicadulina bipunctata (Melichar)	Orange leaf virus
Cicadulina chinai Ghauri	Ragi streak virus Ragi mottle streak virus
Coelidiinae	
Calodia kirkaldyi Nielson (=*Coelidia indica* auct)	Sandal spike phytoplasma
Ulopinae	
Mesargus albimaculatus (Distant)	Sandal spike suspected Purple top roll of potato
Typhlocybinae	
Alebroides nigroscutellatus (Distant)	Witches' broom of potato, phytoplasma

*Based on Nielson (1979)

Some groups of Membracoidea known to include smaller numbers of vectors of plant pathogens are Aphrodinae, Coelidiinae, Iasiinae, Idiocerinae, Macropsinae and Typhlocybinae. Aphrodinae is sister to the lineage comprising Neocoelidinae and Deltocephalinae. Coelidiinae, an agroecosystem, includes only one known vector (Dietrich, 2013). Iassinae (*sensu lato*) includes tribes Gyponini (=Scarinae) and Iasiinae, each of which includes one known forests and savannas, and are uncommon in agroecosystems. Idiocerinae, Macropsinae, and Megopthalminae (=Agalinae) are closely related members of a large lineage that also includes Membracidae and other treehoppers. Idiocerinae and Macropsinae are restricted to woody hosts, but many megopthalmines feed on herbaceous plants. Taxonomic challenges persist in leafhopper identification and traditional taxonomy, reliant on morphological features, often proves insufficient due to overlapping characteristics and variability between life stages. Detailed phylogenetic studies have redefined groups like Deltocephalinae, which was found to be polyphyletic, leading to the recognition of its monophyletic clades (*e.g.,* Acinopterini, Fieberiellini). These advancements not only clarify evolutionary relationships but also aid in identifying potential vectors.

Conclusion and Future Directions

The majority of current research focuses on economically significant crops, neglecting wild plants and weeds, which can act as reservoirs for pathogens. Additionally, anthropogenic activities, including habitat modification and climate change, are driving leafhopper vectors into new environments, posing emerging threats to agriculture. Comprehensive studies of leafhopper-pathogen interactions in diverse ecosystems are critical to predicting and managing future outbreaks. Phylogenetic insights into vector competency, feeding preferences, and pathogen specificity can guide the development of targeted pest control strategies. Systematic surveys and screening of underexplored species are essential to uncover new vectors and understand their ecological roles. Furthermore, the integration of genomic tools with traditional taxonomy will enhance ability to track pathogen evolution and vector adaptation.

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Phytopathogenic Mollicutes
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Interactions

Molecular and morphological characterization of leafhoppers as vectors of sesame phyllody in Andhra Pradesh, India

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Abstract

Sesame phyllody is one of the major biotic constraints causing economic yield losses. It is mainly transmitted by leaf hoppers. In 2021 "kharif" season, a survey was conducted in phyllody infected sesame fields at S. V. Agricultural College, Tirupati, India and insect samples were collected for morphological and molecular identification. Four diverse types of leaf hoppers were identified based on morphological features and multiple sequence alignment of the *COI* gene, out of which *Hishimonus phycitis* was identified as a potential vector for the sesame phyllody phytoplasma.

Keywords: *Hishimonus phycitis*, *secA*, *COI* gene

Introduction

Phytoplasmas are phloem-limited bacteria; therefore, only phloem-feeding insects can acquire and transmit the pathogen. All confirmed vectors of phytoplasmas belong to the order Hemiptera including leaf hoppers, planthoppers and psyllids. Their host range is dependent upon their feeding habits (Weintraub *et al*., 2019). Among biotic stresses, sesame phyllody (SP) is one of the major constraints causing economic yield losses (Rao and Nabi, 2015). The major disease symptoms of SP are flower virescence, witches' broom, phyllody, shoot tip fasciation, leaf and flower bud proliferation, sterility of flowers, flattening of the shoot apex (Rao and Nabi, 2015; Yadav *et al*., 2022). Different leaf hopper species were reported as vectors for SP phytoplasmas, *e.g., Orosius orientalis* in Turkey (Ikten e*t al*., 2104), *O. albicinctus* and *Circulifer haematoceps* in Iran (Salehi *et al*., 2016) and *Hishimonus phycitis* in India (Rao and Nabi, 2015).

Materials and Methods

To find out the potential vector for sesame phyllody phytoplasmas in Andra Pradesh, insects were collected from the infected sesame fields at S.V Agricultural College Farm, Tirupati by using yellow sticky traps at weekly intervals. They were preserved in 70% ethanol for DNA extraction and PCR assays. Collected leaf hoppers were separated based on morphological features, stored in plastic vials at 4ºC in 70% ethanol until the final identification to species level at the Division of Entomology, IARI, New Delhi for identification based on morphological characteristics to the species level (Reddy *et al*., 2020). Total genomic DNA was extracted using a modified CTAB method. Molecular identification of the leaf hopper species was performed on the partial mitochondrial cytochrome c oxidase subunit I gene (*COI* or *COXI*) according to Folmer *et al*. (1994). The extracted DNA was amplified for phytoplasma 16S rDNA with universal primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by nested primer pair R16F2n/R16R2 (Gundersen and Lee, 1996). Amplification of gene *secA* was also performed with primer pairs: SecAfor1/SecArev3 followed by nested PCR with SecAfor5/ SecArev2 (Hodgetts *et al*., 2008; Bekele *et al*., 2011).

Results

Four major leaf hopper species were morphologically identified as *Hishimonas phycitis*, *Orosius albicinctus*, *Cofana spectra*, *Empoasca* sp. (Figure 1). PCR assay for *COI* gene produced the expected DNA fragment of about 658 bp in the four leaf hopper species that were sequenced, edited and deposited in GenBank under the accession numbers OP102538 for *H. phycitis*, OP108442 for *O. albicinctus*, OP108440 for *C. spectra* and OP113529 for *Empoasca* sp. In nested PCR analysis for phytoplasma presence a 1.25 kb fragment was amplified from *H. phycitis* and from *O. albicinctus*, *C. spectra*

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and *Empoasca* sp. collected during "kharif" season and from a SP phytoplasma positive sample (GenBank accession number MT420677). The DNAs from the *H. phycitis* positive in the 16S rRNA gene, was also positive with *secA* gene. Pairwise sequence comparison of 1.2 kb amplicon of R16F2n/R2 sequences of *H. phycitis* (GenBank accession number OP070927) showed 100% sequence identity with '*Candidatus* Phytoplasma australasiae = australasiaticum' and to several phytoplasmas in the 16SrII group. The comparison of 840 bp partial sequences of *secA* gene (GenBank accession number OP058671) showed 99.63% to 99.83% sequence similarity with strains of 16SrII group. Phylogenetic analysis of both gene sequences confirmed the identification. In the virtual RFLP the restriction site maps showed that *H. phycitis* phytoplasma profile was identical to the one of 16SrII-D subgroup (GenBank accession number Y10097) with similarity coefficient of 1.



Figure 1. From left *Hishimonas phycitis, Orosius albicintus, Cofana spectra* and *Empoasca* sp.

Discussion

Orosius orientalis, *Exitianus indicus*, *Circulifer haematoceps*, *Empoasca motti* were reported as potential vectors of sesame phyllody phytoplasma in Iran, India and Turkey (Ikten *et al*., 2014; Salehi *et al*., 2016; Gogoi *et al*., 2019). In India *O. orientalis*, *H. phycitis* and *E. indicus* were reported as potential vector species for SP phytoplasma in several states (Nabi *et al*., 2015; Gogoi *et al*., 2019; Kalita *et al*., 2018; Yadav *et al*., 2022). In the present study, *H. phycitis* from Chittoor district was found positive for phytoplasma 16SrII-D subgroup, which was also detected in plants with SP disease. This phytoplasma may therefore play a crucial role in transmission of SP phytoplasma in Chittoor and nearby districts. The information on insect vectors which harbour 16SrII-D subgroup phytoplasmas, that is the major group of phytoplasmas identified as associated with sesame phyllody, is important to monitor the leafhopper population in and around sesame fields for proper management of the phyllody disease. Sesame phyllody could be managed either by using resistant cultivars, insecticides against leafhoppers or by following crop hygiene practices. Developing cultivars and varieties with durable resistance to the vector and to the disease is reported as another management tool and should be an integral component of the sesame breeding program (Rao and Nabi, 2015). Some of the cultural methods, particularly rotation and sowing dates could also be fruitfully applied considering that it was reported that the severity of phyllody is influenced by the time of sowing (Rao and Nabi, 2015; Phookan *et al*., 2020).

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Interactions

A new invader in Germany: *Scaphoideus titanus*, the vector of "flavescence dorée" in grapevine

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Abstract

In the frame of a national survey program, a monitoring for the presence of *Scaphoideus titanus* was conducted in the southern part of Baden-Württemberg, Germany. Yellow sticky traps were installed in different vineyards in a zone of about 20 km north to south close to the Rhine Valley from July until October 2024 with a two-week change. At the beginning of August, the first specimens were detected and identified as *S. titanus* and until the end of September, about 500 further *S. titanus* were collected on the traps. In total, 436 individuals were analysed for phytoplasma infection with different molecular methods. So far, no "flavescence dorée" phytoplasma presence could be confirmed. However, some suspicious cases made a reliable diagnostic difficult. Therefore, a quantitative PCR assay was developed to check the quality of *S. titanus* DNA extraction.

Keywords: monitoring, transit, yellow sticky traps, 16SrV phytoplasmas

Introduction

"Flavescence dorée" (FD) is the most devastating yellows disease on grapevine in Europe and its associated agent, the "flavescence dorée" phytoplasma is epidemically spread by the Nearctic leafhopper *Scaphoideus titanus* Ball (Malembic-Maher *et al.*, 2020). So far, neither the disease nor the vector was present in Germany. Selective surveys have been carried out in the past to monitor the appearance of *S. titanus* in different wine-growing regions of Germany (B. Jarausch, unpublished). New EU regulations and national monitoring programs made systematic monitoring for *S. titanus* mandatory. As the invasion of this leafhopper to Germany was expected at the Southwestern borders to France and Switzerland, the State Institute of Viticulture and Enology in Freiburg has been monitoring this region since 2007. Since 2015, these investigations have also been carried out as part of the national monitoring of harmful organisms in collaboration with the Biological Diagnosis and Plant Health Department at the Center for Agricultural Technology Augustenberg (LTZ) and the monitoring was further intensified when *S. titanus* was first reported from the nearby Alsace region in France in 2016 (Kuntzmann *et al.*, 2016). In 2024, an intensive monitoring was carried out in the upper Rhine valley in the district of Lörrach at the Southwestern part of Baden-Württemberg.

Materials and Methods

The monitoring was conducted with yellow sticky traps (Aeroxon) placed inside vineyards as well as at their borderline with special focus on gone-wild *Vitis* plants. Traps were first installed in vineyards at proximity of motorway service stations, business parks and camping sites. After the first findings of *S. titanus*, the area was extended to adjacent regions (Figure 1). In total, 37 traps were installed and changed bi-weakly from the beginning of July until the end of October. All leafhopper specimens were morphologically identified, and total nucleic acids (TNA) were extracted from individuals of *S. titanus* with a CTAB-based method according to Jarausch *et al.* (2023). Different PCR detection methods were applied to identify FD phytoplasmas. A first screening of all insect individuals was carried out with the universal phytoplasma detection assay developed by Christensen *et al.* (2004). Suspicious results were then tested with the duplex quantitative PCR assay of Pelletier *et al.* (2009) to identify

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16SrXII ("bois noir") phytoplasmas or 16SrV (FD) phytoplasmas. Distinction between different 16SrV group phytoplasmas (*e.g.*, Palatinate grapevine yellows or FD) was attempted by sequencing the *map* gene marker or the *vmp*A-R1 gene marker according to Malembic-Maher *et al.* (2020).

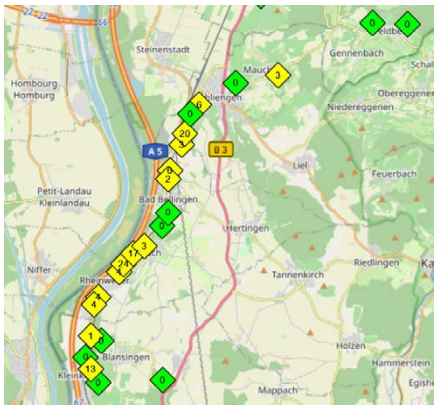


Figure 1. Trap sites of WBI in Southern Baden and number of *S. titanus* captures on September1st 2024 presented in the online tool VitiMeteo (www.vitimeteo.de).

In order to check the quality of TNA-extraction from *S. titanus* individuals extracted from yellow sticky traps, a quantitative PCR assay based on the *COII* gene of *S. titanus* was developed. The available COII sequences of European specimens of *S. titanus* (Papura *et al.*, 2012) were used to select the primers ScaphoCOIIfor (5'-TCGAATACTTCTTGAAGGGCA-3') and ScaphoCOII rev (5'-ATTGATACGGCCAGGCGATC-3'). Quantitative PCR amplification was achieved with a SYBR Green™ protocol according to Jarausch *et al.* (2023) with the following cycling conditions: initial denaturation for 15 minutes at 95°C followed by 40 cycles of 15 seconds 95°C and 45 seconds at 66°C. Melting curve analysis ranged from 60°C–90°C, specific products had a melting temperature at around 78°C. Various species of leafhoppers, psyllids and Drosophilidae were used for specificity tests.

Results and Discussion

In August 2024, adult individuals of *S. titanus* were detected for the first time in Germany on yellow sticky traps in two different vineyards in the Southwest of Baden-Württemberg (Askani *et al.*, 2024). Since then, more than 500 individuals have been captured until the end of October on a total of 21 different trap sites. The highest captures were recorded from the middle of August until the middle of September with a maximum of 28 *S. titanus* per trap. The monitoring data can be observed on www.vitimeteo.de. A total of 436 *S. titanus* has been tested individually by different PCR methods for the presence of "flavescence dorée" phytoplasmas. So far, no infection could be confirmed. The qPCR assay of Christensen *et al.* (2004) and the duplex qPCR assay of Pelletier *et al.* (2009) both showed some suspicious results, which could not be confirmed by *map* gene marker analysis. The *vmp*A-R1 marker gene analysis yielded inconclusive results for two samples.

The reliability of "flavescence dorée" phytoplasma detection in the insect vector is of significant importance for the risk assessment in the so far FD-free area. As the DNA of insects captured on sticky traps might be degraded prior TNA extraction or the TNA extraction might be impaired, a qPCR quality assay for *S. titanus* DNA was developed. The primers were selected in the available sequences of the mitochondrial *COII* gene. They amplify a specific product of *S. titanus* that cannot be seen with extracts of 18 different leafhopper species, 5 different psyllid species or 4 different *Drosophila* species which might be present on the sticky traps. First data of the quality check of the phytoplasma-tested *S. titanus* individuals revealed no problems with TNA extraction. Cq values ranged between 13 and 25. In 2023, no specimens of *S. titanus* were found on the sampling sites and it is not clear how long the leafhopper is already present in South Baden. However, it is suspected that it was introduced via transit traffic, since the trap locations are close to the highway and rail freight traffic. So far, no FD-infected grapevine has been detected. Official control measures against overgrown rootstock grapevines and insecticide treatments have started.

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Interactions

Monitoring of alternative insect vectors of "flavescence dorée"-related phytoplasmas in different wine-growing regions in Germany

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Abstract

"Flavescence dorée" (FD) is a threatening disease of grapevine associated with phytoplasmas of the 16SrV ribosomal group and is epidemically spread within vineyards by the Nearctic leafhopper *Scaphoideus titanus*. However, recent studies demonstrated that the spread of FD is not only related to this pathosystem but may include alternative vectors and reservoir plants. Alders have been shown harbouring different 16SrV-strains that can be transmitted from alder to alder by *Orientus ishidae* and *Allygus* spp., and by *O. ishidae* also from alder to grapevine. In all cases the Map-FD2 strain M38 was transmitted. Therefore, a monitoring for both leafhopper species collected on alders has been conducted over 5 years in different wine-growing regions in Germany to investigate their occurrence and infection status. *O. ishidae* individuals naturally infected with strain M38 were captured in all regions except Mosel. *Allygus* spp. were also infected with strain M38 but harboured in addition several other non-epidemic strains. Both species captured in Württemberg were able to transmit strain M38 to alder test plants. The results indicate that the widespread occurrence of *O. ishidae* infected with the epidemic "flavescence dorée" strain M38 represents a risk for the spread of "flavescence dorée" in regions that are so far free from FD.

Keywords: *Alnus glutinosa*, 16SrV phytoplasmas, *Oncopsis alni*, *Orientus ishidae*, *Allygus* spp., transmission

Introduction

In Europe, "flavescence dorée" (FD) is epidemically spread by the Nearctic leafhopper *Scaphoideus titanus* Ball. Only recently, it was shown that the FD phytoplasma is widespread in some reservoir plants, mainly black alders (Malembic-Maher *et al.*, 2020). However, recently published studies indicated that the FD epidemiology is not restricted to the grapevine-*S. titanus* pathosystem but may involve additional vectors and reservoir plants (Rizzoli *et al.*, 2021; Rigamonti *et al.*, 2023). Alternative vectors, although not capable of transmitting the "flavescence dorée" phytoplasma from grapevine to grapevine, provide a continuous inoculum of the phytoplasma within the vineyard, albeit sporadically (Rigamonti *et al.*, 2023; Jarausch *et al.*, 2023). Malembic-Maher *et al.* (2020) demonstrated that some "flavescence dorée"-related strains are disseminated from alders to grapevine by the Macropsinae *Oncopsis alni* but are not further transmitted by *S. titanus* while the Deltocephalinae species *Allygus* spp. and *Orientus ishidae* transferred strains from alders to broad beans that proved to be compatible with *S. titanus*. During transmission trials, Jarausch *et al.* (2023) showed that both species were capable of infecting alder seedlings but only *O. ishidae* was able to transmit 16SrV phytoplasmas directly to grapevine. In addition to the results presented in Jarausch *et al.* (2023), the monitoring of the described alternative vector species was extended on alder stands adjacent to vineyards to other wine-growing regions in Germany. It was determined the infection status, the *map* genotype and the transmission capacity to evaluate the risk of spreading FD-related phytoplasmas in other FD free wine-growing regions. FD-related phytoplasmas are widespread in alders. The invasive leafhopper *O. ishidae* has the potential to transmit these phytoplasmas from alder to grapevine. This represents a risk for FD-free regions like Germany when the main FD-vector *S. titanus* occurs. Therefore, the objective of the present study was to explore different German wine-growing regions for the presence and FD-phytoplasma infection rates of the potential alternative vector species *O. ishidae* and *Allygus* spp. In addition, the vector of the non-epidemic variant of grapevine 16SrV-phytoplasmas Palatinate grapevine yellows (PGY), *Oncopsis alni*, was included in the study.

Materials and Methods

Sampling of Cicadellidae species was done in all major wine-growing regions in Germany (Table 1) from 2017 to 2021.

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Table 1. 16SrV phytoplasma-infection rate (number of positive/total number of tested) and *map* genotypes of alternative insect vector species in different regions.

Region	N° sites	*Allygus* spp.	Map-types	*Orientus ishidae*	Map-types	*Oncopsis alni*	Map-types
Mosel	10	24/39 (62%)	M43,M44,M52,M53	0/15		16/43 (37%)	M43,M45,M53,M101
Nahe	3	35/37 (95%)	M38,M53	14/19 (74%)	M38	4/6 (67%)	M45,M53
Rheinhessen	13	59/168 (35%)	M13,M15,M38,M39,M47,	7/33 (21%) M52,M53,M78,M121	M38	13/35 (37%)	M38,M48,M52,M53
Pfalz	19	29/74 (39%)	M13,M38,M47,M53	19/54 (35%)	M38	13/87 (15%)	M39
Franken	9	27/71 (38%)	M38	3/36 (8%)	M38	14/17 (82%)	M53,M78
Baden	6	0/2		1/14 (7%)	nd	0/1	
Württemberg	9	23/120 (19%)	M13,M38,M45,M48,M49,	31/122 (25%) M58,M78	M38, M38*	18/44 (41%)	M48,M52,M110

*genetic variant of M38 differing by one SNP represented by six strains (reference strain 6953); nd, not determined.

The insects were collected from alder trees adjacent to the vineyards from July to September. The sampling sites were selected by geo data-based monitoring tools. The total nucleic acid of morphologically determined individual insects was extracted with a CTAB protocol. The 16SrV phytoplasmas were detected by PCR using group-specific primers fAY/rEY (Jarausch *et al*., 2023). The phytoplasmas detected in PCR positive samples of insects were characterized by amplification of *map* gene by nested PCR and sequencing (Arnaud *et al*., 2007). Transmission trials with groups of *Allygus* spp. and *O. ishidae* were conducted as dual choice approaches with *Alnus* and *Vitis* test plants according to Jarausch *et al*. (2023).

Results and Discussion

The results are shown in Table 1. The infection rates varied considerably among the different wine-growing regions being the highest for *Allygus* spp. and *O. ishidae* in the region of Nahe. Although *O. ishidae* was captured in all regions, no (Mosel) or only a few individuals (Franken and Baden) were infected with 16SrV phytoplasmas. Only the Map-FD2 genotype M38 was transmitted by *Allygus* spp. or *O. ishidae* to alder and grapevine test plants in the study conducted in Palatinate (Jarausch *et al*., 2023). This could be confirmed in transmission trials with *Allygus* spp. and *O. ishidae* individuals captured in Württemberg. Two out of three alder test plants in trials with *O. ishidae* and one out of two alder test plants in trials with *Allygus* spp. became infected with strain M38. No transmission to *Vitis* test plants occured in these trials. FD strain M38 was also found in *Allygus* spp. in the different wine-growing regions, except for Mosel. However, this species also harboured several other non-epidemic FD strains like *O. alni*, the vector of PGY (Maixner *et al*., 2000). All PCR-positive individuals of *O. ishidae* were infected with strain M38 or a genetic variant of M38, differing for a single SNP, that was discovered at one sampling site in Württemberg. These results indicate that the strain M38 poses a potential risk as inoculum for FD emergence in Germany when transmitted from alder to grapevine and if the main vector *S. titanus* is present.

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Phytopathogenic Mollicutes
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Interactions

Biology and phytoplasma detection of the newly described *Bamboosella dimorpha* leafhopper in grapevine yellows-affected vineyards

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Abstract

The aim of this study was to investigate the biology and the possible role in grapevine yellows epidemiology of the newly described alien species *Bamboosella dimorpha* in northern Italy regions. The results showed that the bamboo feeding leafhopper is present in the vineyard surroundings where *Phyllostachys* spp. plants are growing. The flight dynamics confirming its ability to held three generations per year. *B. dimorpha* can survive a couple of days on grapevine leaves. The specimens collected using sweeping nets from bamboo plants located in vineyard borders carried "flavescence dorée" phytoplasma. Further investigations are needed to evaluate the ability of *B. dimorpha* to transmit this phytoplasma .

Keywords: phytoplasma vectors, "flavescence dorée" epidemiology, *Phyllostachys* spp.

Introduction

Bamboosella dimorpha Sanna, Holzinger & Callot (Cicadellidae, Deltocephalinae, Mukariini) is a newly described bamboo feeding leafhopper likely native to south-eastern Asia. In Europe the first report was in 2011 in the botanical garden in Strasbourg (France) then, it was found in Italy in 2016, in Switzerland in 2017 and in Slovenia in 2023 (Sanna *et al*., 2024). The leafhopper body is 5-6 mm in length, uniformly brownish yellow and flattened dorso-ventrally (Figures 1 and 2).



Figure 1. Adults of *Bamboosella dimorpha*. Male on the left, female on the right (Photo F. Sanna).

In the European countries *B. dimorpha* completes up to three generations per year, one in late spring and two between late summer and winter and overwinters mainly in the egg stage. Adults are mainly present from June to mid-July and from September to December, with some specimens surviving until spring (Sanna *et al*., 2024). Recently, during the monitoring of insect vectors in grapevine yellows-affected vineyards some *B. dimorpha* individuals were captured. The aim of this study was to investigate the biology and the role in grapevine yellows epidemiology of the alien species, newly described *B. dimorpha*, in northern Italy.

Materials and Methods

The survey was carried out since 2022 in Franciacorta, a grape-growing area of the Lombardy Region (Northern Italy) famous worldwide to produce sparkling wine. In 20 grapevine yellows-affected vineyards the presence of *B. dimorpha* was monitored by checking yellow sticky traps. The traps were positioned according to line transect, sampling from the surrounding wooded areas to the centre of the vineyard. In each vineyard from one to three transects were established. The traps were placed from late June to the first week of October and replaced weekly. Alive specimens captured with sweep net and mouth aspirator were used for grapevine survival bioassay or stored at -20°C

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for molecular analysis. To evaluate the survival capacity on grapevine, adults (male and female) were caged in small Magenta boxes with filter containing a grapevine cultivar Chardonnay leaf inserted on an agar-water medium. Considering that *B. dimorpha* nymphs and adults generally inhabit the upper surfaces of *Phyllostachys* spp. leaves, where they remain closely appressed to the leaf surface (Figure 2), the survival bioassay was carried out confining the specimens to either the upper or lower surfaces of grapevine leaves. About molecular analyses, nucleic acids extracted from the collected insects were employed as templates for *map* and *stamp* genes amplification by nested PCR (Arnaud *et al.*, 2007; Fabre *et al.*, 2011), followed by sequencing and bioinformatics analyses (Rigamonti *et al.*, 2023), to detect phytoplasmas associated with "flavescence dorée" (FD) and "bois noir" (BN), respectively.



Figure 2. Adults of *Bamboosella dimorpha* on the upper side of a bamboo (*Phyllostachys* spp.) leaf.

Results

B. dimorpha specimens were found in 3 out 20 investigated vineyards with 179 and 470 minimum and maximum captures respectively. According with its ecology, the leafhopper was found in the site where bamboo plants were present in the surroundings. Regarding the distribution of the captures in the vineyard, only 13% of adults was captured on the traps placed on the edge rows. Flights peaks were registered at the beginning of July and at the middle of October, confirming the ability to *B. dimorpha* to held at least two generations per year. Specimens confined within laboratory cages demonstrated the ability to survive under controlled conditions. The bamboo feeding leafhopper was able to survive on grapevine 3-5 days on the upper side and 2-3 days on the lower side of the leaves.

The specimens collected using sweeping nets from bamboo plants located in vineyard in the surroundings carried "flavescence dorée" phytoplasmas (average infection rate: 4.2%), while '*Candidatus* Phytoplasma solani' was not detected in the analysed insects.

Discussion

The newly described bamboo feeding leafhopper *B. dimorpha* is present in the vineyard surroundings where *Phyllostachys spp.* plants are growing, sometimes with high population density. Considering that it can (i) colonize the rows of grapevine at the edges, (ii) survive a couple of days on grapevine leaves, (iii) carry "flavescence dorée" phytoplasmas, further investigations are needed to evaluate the ability of *B. dimorpha* to transmit "flavescence dorée" phytoplasmas or other phytoplasmas from bamboo to bamboo and from bamboo to grapevine.

Acknowledgements

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Interactions

First report of a psyllid vector of '*Candidatus* Phytoplasma pruni' (strain 16SrIII-J)

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Abstract

Mallow psyllid (Hemiptera: Psyllidae) individuals have been captured on *Malva* sp. plants infected with '*Candidatus* Phytoplasma pruni' (strain 16SrIII-J), in pear orchards in Libertador Bernardo O'Higgins region, Chile. The mallow psyllid transmitted '*Ca.* P. pruni' to periwinkle plants, being this the first report of 16SrIII-J transmission by a psyllid. *COI* and mitochondrial genome sequences indicate that mallow psyllid individuals captured in the pear orchards would belong to a new genus.

Keywords: mallow psyllid, pear orchards, *Russelliana* sp., transmission trials

Introduction

'*Candidatus* Phytoplasma pruni' (strain 16SrIII-J) in Chile is infecting many herbaceous and woody plant species, not only of agronomic interest. Two Cicadellidae species, *Paratanus exitiosus* Beamer 1943 and *Bergallia valdiviana* Berg 1881, have been demonstrated to be vectors of the 16SrIII-J phytoplasma. In addition, five other leaf hopper species have been identified as potential vectors (Montano *et al*., 2024). In 2023, the presence of phytoplasma 16SrIII-J was detected in psyllid individuals captured on *Malva* sp. plants in pear orchards in the Libertador Bernardo O'Higgins region of Chile. Transmission trials were carried out to clarify whether the mallow psyllid is a vector of the phytoplasma 16SrIII-J.

Materials and Methods

The capture of mallow psyllid adults was carried out for 8 months between October 2023 and May 2024, every 15 days. The insects were captured using an entomological sweeping net and 100 sweepings directed to *Malva* sp. plants present inside the pear orchards. At the end of each capture, the insects were observed with a LEICA S6D binocular magnifier. Those that presented typical mallow psyllid morphology (Figure 1) were used for transmission trials to periwinkle plants obtained from *in vitro* micropropagation and kept in 300 ml glass jars with 60 ml of Murashige and Skoog culture medium. A maximum of 20 individuals were added to each jar. During the transmission tests, the jars were kept in a growth chamber at 25°C, with a 16/8 hours (light/dark) photoperiod. During the transmission tests the plants were observed daily to remove dead insects and store them in 70% alcohol at -20°C. At the end of the trial, after 7 days, the plants were transferred to a substrate of peat and perlite in a 2:1 ratio and placed in a growth chamber under the same temperature and photoperiod conditions mentioned above, to observe the appearance of symptoms. After three months from the start of the transmission test, molecular analyses were carried out, on a monthly basis, to detect phytoplasmas in plants and insects.



Figure 1. Adults of the mallow psyllid: (a) and (c) lateral view; (b) dorsal view; (d) ventral view.

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PCR with primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and nested PCR with R16F2n/R2 primers on the 16S rRNA gene (Gundersen and Lee, 1996), were performed as described by Schaff *et al*. (1992). Amplicons from nested PCRs were sequenced and *in silico* restriction fragment length polymorphism (RFLP) analyses were performed using *Rsa*I, *BstU*I and *Hha*I restriction enzymes. Molecular identification of the insects was carried out through amplification and sequencing of the cytochrome-C-oxidase subunit 1 (*COI*) gene (Vrijenhoek, 1994) and the mitochondrial DNA.

The transmission tests performed were 113, using a total of 1,353 insects. The presence of 16SrIII-J phytoplasma was detected in 7 (6.2%) periwinkle plants (Table 1). The same phytoplasma was detected in the insect specimens that were fed on these plants. Positive periwinkle plants only showed yellowing and never phyllody.

Table 1. Results of transmission trials with mallow psyllid to periwinkle plants.

Transmission Trial (TT)	TT start date	Months after the start of TT when in the plant 16SrIII-J was detected
TT34	November 30, 2023	3
TT35	November 30, 2023	6
TT36	November 30, 2023	6
TT40	November 30, 2023	7
TT50	December 27, 2023	7
TT56	January 24, 2024	7
TT58	January 24, 2024	7

Amplifications of the *COI* gene region showed nucleotide sequence identities of approximately 83% with the closest sequence available in GenBank, that was the one of the psyllid *Russelliana solanicola*. According to Zhang and Bu (2022), in the *Insecta* order, to belong to the same species, the percentage of identity of the *COI* gene sequence between two individuals, must be equal or greater than 97%. The same result was obtained with the analysis of mitochondrial DNA with 80.24% of nucleotide identity between the mallow psyllid and the closest sequence available in GenBank, again *R. solanicola*.

Discussion

This the first report of the phytoplasma 16SrIII-J transmission by a psyllid. The low transmission efficiency of phytoplasma 16SrIII-J can be explained by low genetic compatibility between the vector and the pathogen (Weintraub and Beanland, 2006), especially considering that the most efficient vectors of phytoplasmas 16SrIII-J are leafhoppers. An additional factor that could be contributing to the low transmission efficiency of phytoplasma 16SrIII-J is the latency period (LP). A prolonged LP could limit the time in which the phytoplasma establishes and multiplies within the vector, which would result in a lower concentration of the pathogen available to be transmitted (Alma *et al*., 2019). The absence of phyllody has been unusual, so an analysis has been carried out to determine the characteristics of the SAP54 effector present in the phytoplasma strain 16SrIII-J transmitted by the mallow psyllid (data not shown). The results indicate absence of the effector, and this could explain the lack of phyllody symptoms in plants (Gamboa, 2019), suggesting important differences between 16SrIII-J strains transmitted by leafhoppers and psyllids.

Molecular identification indicates that the mallow psyllid individuals captured in the pear orchards, would belong to a new genus close to *Russelliana*. This data needs to be confirmed with a morphological analysis of the individuals.

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Phytopathogenic Mollicutes
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Interactions

Insights into phytoplasma lineages: evaluating single-locus and multilocus trees from next-generation sequencing data

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Abstract

Phytoplasmas detected in insects collected from natural areas provide opportunities to discover new strains and identify potential novel vectors. Integrating these strains into existing phytoplasma phylogenies, which are primarily based on agroecosystem data, enhances the understanding of evolutionary relationships and supports the development of a more robust classification framework. In this study, it was analyzed insect samples collected from natural habitats using both single-locus and multilocus approaches to gain a comprehensive understanding of phytoplasma strain diversity. The results reveal notable differences in tree topologies, with congruent outcomes observed only for samples clustering with reference strains from well-studied phytoplasma groups (*e.g.*, 16SrI). These findings emphasize the importance of multilocus analyses in resolving complex evolutionary histories, particularly for prokaryotic taxa with limited genomic resources. This study highlights the value of multilocus approaches in identifying novel phytoplasma strains and their potential insect vectors, offering insights that can inform future ecological and agricultural research.

Keywords: phytoplasma diversity, potential insect vectors, natural habitats, housekeeping genes

Introduction

Analyzing the presence of phytoplasmas in leafhoppers and other phloem-feeding Hemiptera collected from natural areas provides a unique opportunity to discover new or understudied phytoplasma strains and identify novel potential vectors (Trivellone *et al.*, 2021, 2022). Incorporating these newly identified strains into existing phytoplasma phylogenies, can enhance the understanding of evolutionary relationships among lineages. Furthermore, this may contribute to a more robust classification of phytoplasmas overall (Glaeser and Kämpfer, 2015). To delineate a new '*Candidatus* Phytoplasma' species, a 16S rRNA gene sequence identity threshold of 98.65% has been proposed (Bertaccini *et al.*, 2022; Wei and Zhao, 2022). However, relying solely on the genetic divergence of a single locus may yield inaccurate or inconclusive results (Johnson *et al.*, 2019), particularly for certain phytoplasma lineages, such as those within the 16SrXI and 16SrXIV groups. To address these limitations, when whole-genome data are available, a genome-wide average nucleotide identity (ANI) threshold of 95% has been recommended. Furthermore, if both thresholds fail to distinguish between strains, a multilocus sequence analysis (MLSA) approach can be employed. By comparing single-locus and multilocus phylogenetic reconstructions preliminary results are presented here from a new next-generation sequencing approach to evaluate differentiation ability for phytoplasma strains detected in insects collected from wild habitats.

Materials and Methods

A total of 96 leafhopper specimens were analyzed individually using quantitative PCR to verify the presence of phytoplasmas and further characterized using a multilocus approach (Trivellone *et al.*, 2023). The insect specimens represented different species collected from various natural areas worldwide (722 sites in 50 countries). DNA was extracted, and all phytoplasma-positive samples were analyzed using a new anchored hybrid enrichment (AHE) probe kit containing about 58,000 DNA probes targeting 178 candidate loci shared among 50 partial or full phytoplasma genomes, to obtain additional DNA sequence data (Trivellone *et al.*, 2022). The quality of the probe design was verified using available phytoplasma genomic resources following the procedures of Lemmon *et al.* (2012). Single-locus phylogenetic trees were constructed for the *groEL*, *groES*, *grpE*, *rplB*, *rplO*, *rplP*, *rplV*, *rpsC*, *rpsS*, *secA*, *secY*, and *tuf* genes. A multilocus tree was constructed by concatenating sequences from a subset of samples and genes. Reference sequences were obtained from GenBank. Phylogenetic analysis was carried out with sequences aligned using Concatenator-0.2.1 and analyzed by maximum

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likelihood using MEGA 11 (www.megasoftware.net) with default parameters and bootstrapping with 1,000 replicates.

Results

All 96 screened leafhopper specimens tested positive for the bacterial 16S rRNA gene using qPCR. AHE assembly yielded phytoplasma 16Sr sequences belonging to eight ribosomal groups (16SrI, -II, -III, -IV, -VII, -XI, -XII, -XIV), and a total of 12 housekeeping genes were retrieved. Single-locus phylogenetic analysis was conducted on the 12 genes using a subset of 15 samples. Phylogenetic trees constructed from single-locus (SecA) and multilocus datasets (GroEL, GroES, GrpE, rplB, rplP, rplO, rplV, rpsC, rpsS, SecA, SecY, and tuf) revealed notable differences in the resolution and clustering of '*Candidatus* Phytoplasma' species and strains. Phylogenetic analysis of the *secA* gene sequences identified a well-supported clade comprising '*Ca.* P. sacchari', '*Ca.* P. oryzae' and three samples collected from different regions (Mexico, Australia, and Mongolia). A second clade included two samples from the United States of America (USA), aligning with '*Ca.* P. pruni' strains. A third clade consisted of one sample from the USA and one from Mexico, both clustering with reference strains of '*Ca.* P. asteris,' although with a weak bootstrap support (38) (Figure 1). In the multilocus phylogenetic tree, the clustering of the WE05 sample with '*Ca.* P. asteris'-related strains was well-supported (bootstrap value: 71) and consistent with the topology of the *secA* phylogenetic tree (data not shown).

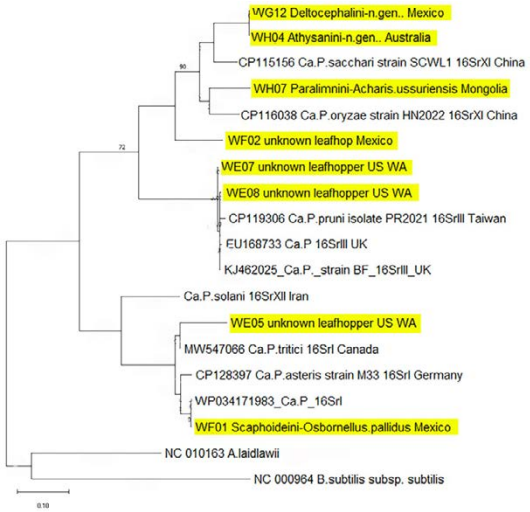


Figure 1. Phylogenetic tree of the *secA* gene sequences inferred by maximum likelihood bootstrap values >70% (1,000 replicates) shown. The tree includes 8 insect samples, 11 reference strains from and 2 outgroups (*Acholeplasma laidlawii* and *Bacillus subtilis*).

Discussion

The use of single-gene analyses underscores the importance of incorporating multiple loci to resolve evolutionary relationships in phytoplasmas. In this study, the *secA* phylogenetic tree and other single-locus trees provided an initial overview of broad clustering patterns; however, it was constrained by low bootstrap support. Such limitations are inherent to single-gene analyses, as they capture only a narrow window of genetic diversity and are influenced by locus-specific biases (Martini *et al.*, 2019). Leafhopper samples collected from Australia, USA and one from Mexico represented all undescribed insect species. Interestingly, *Acharis ussuriensis* collected in Mongolia and found positive for a '*Ca.* P. oryzae' strain had previously tested positive in samples from China, where three new phytoplasma strains belonging to groups 16SrI and 16SrXIV were identified (Inaba *et al.*, 2023). While single-gene methods remain useful for initial assessments, multigene approaches provide greater accuracy, reliability, and statistical support.

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Interactions

Seasonal dispersal of *Cacopsylla bidens* and its role in the epidemiology of '*Candidatus* Phytoplasma pyri' in mixed-crop systems

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Abstract

'Candidatus Phytoplasma pyri', associated with pear decline, was identified in northern Israel pear, nectarine, and almond crops. The psyllid *Cacopsylla bidens* was found to vector this pathogen in pears and found year-round in pear orchards. It was found seasonally in pears' surrounding crops. Molecular testing revealed 22–36% of psyllids found in pears, nectarine, almond, and cherry plots carried the pathogen. Findings highlight the vector's seasonal movement between crops, emphasizing the need for integrated management to control the pear decline in mixed-crop systems.

Keywords: psyllid, vector, epidemiology, management

Introduction

Analyzing the presence of phytoplasmas in leaf hoppers and *'Candidatus* Phytoplasma pyri' belongs to the apple proliferation (AP) phytoplasma group (16SrX) which includes three '*Candidatus* Phytoplasma' species: '*Ca.* P. mali' (16SrX-A), '*Ca.* P. prunorum' (16SrX-B) and '*Ca.* P. pyri' (16SrX-C). These wall lacking bacteria are associated with diseases named apple proliferation, European stone fruit yellows, and pear decline, respectively (Seemüller and Schneider, 2004) and are genetically very similar (Bertaccini *et al.*, 2014). Pear decline (PD), associated with the presence of *'Ca.* P. pyri', is one of the most devastating diseases of *Pyrus communis* (pear) in Europe and North America (Seemüller *et al.*, 2011) as well as South America, Middle East, Chile and Taiwan. Recently, '*Ca.* P. pyri' was also detected in almond orchards in Jordan (Alloush *et al.*, 2023).

In Israel, phytoplasma symptoms were found in pears, almonds, and nectarine. In 2017 observations were made of nectarine (*Prunus persica*) orchards in northern Israel, where trees displayed leaf discoloration, early defoliation, and overall tree decline symptoms. In addition, in 2020 peach trees from south of the Golan were identified positive for '*Ca.* P. pyri' (Zisovich *et al.*, 2022). Those trees displayed yellow leaf discoloration, lack of vigor, and reduced tree size. A year later in 2021, almond trees *(Prunus dulcis)* displayed reduced tree size, leaf discoloration, and degeneration of the almond kernel while on the tree. Molecular testing confirmed the '*Ca.* P. pyri' presence in all three crops that are cultivated in proximity in northern Israel creating a patchy ecosystem.

Recently *Cacopsylla bidens*, the pear psyllid, was identified as a vector of *'Ca.* P. pyri' that induces PD and a PD associated disease described as peach yellow leaf roll (PYLR) in California (Purcell and Suslow, 1984), reported to be present in peach in other areas of the world such as Spain, Argentina and recently Israel (Amparo *et al.*, 2015; Fernandez *et al.*, 2017; Zisovich *et al.*, 2022). This study focused on the dispersal patterns of *C. bidens* and its role in the epidemiology of PD.

Materials and Methods

Three geo-climatic areas in northern Israel within a 25 km radius of one another were chosen: Upper Galilee (situated on calcareous rock, about 400 m above sea level), Southern Golan Heights (situated on partially calcareous and partially basaltic rocks, about 300m above sea level) and Northern Golan Heights (situated on basaltic rocks, about 1000 m above sea level). Pear orchards are cultivated in all three areas. The chosen pear plots were located with adjoining plots of nectarine in Upper Galilee, adjoining plots of nectarine and Almond in Southern Golan Heights, and adjoining plots of nectarine and cherry in Northern Golan Heights. The presence of the pear psyllid in the plots was monitored every two to three weeks throughout 2022 using sticky yellow traps. Every month, samples of the psyllids from the traps of each plot were tested for the presence of *'Ca.* P. pyri'.

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Table 1. Number of psyllids/trap/2-3 weeks monitored in the three geo-climatic areas during the seasons in the diverse crop's plots.

		Number of psyllid/traps in the crops			
		Pear	Nectarine	Almond	Cherry
Upper Galilee					
	Spring	11	3	-	-
	Summer	2	0	-	-
	Autumn	12	0	-	-
	Winter	72	16	-	-
Southern Golan Heights					
	Spring	6	6	3	-
	Summer	16	0	0	-
	Autumn	52	1	0	-
	Winter	50	9	3	-
Northern Golan Heights					
	Spring	10	6	-	3
	Summer	21	0	-	1
	Autumn	206	1	-	3
	Winter	30	4	-	7

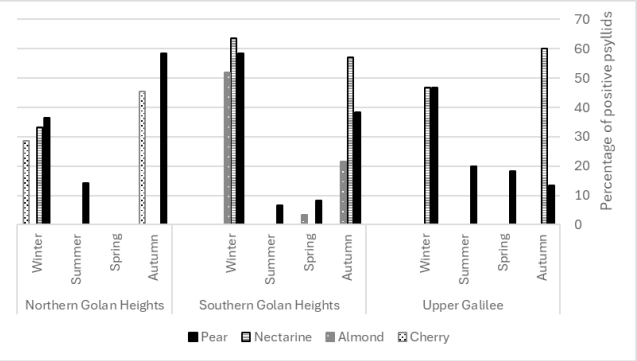


Figure 1. Percentage of psyllids tested positive for phytoplasma by crops in the geo-climatic areas during the seasons.

Results

A total of 16,267 psyllids were collected in the pear plots, 1,887 in the nectarine plots, 721 in almond plots, and 465 in cherry plots. In Upper Galilee, most of them were found in the winter, and in the Golan Heights during the autumn and the winter (Table 1). Psyllids were found in pear plots throughout the year but in the other crops, they were not found in the summer and only a few in autumn.

Pear psyllids carrying the phytoplasma were found in all the crops in all the geo-climatic areas (Figure 1). In pears 36% of the 113 tested psyllids, in nectarine 34% of the 73, in almonds 29% of the 62 and in cherry 22% of the 41 that were tested. One of the pear cultivars was more susceptible, and almost all the plots were infected with the phytoplasma. Of the psyllids collected from those plots, 70% carried the phytoplasma (data not shown).

Discussion

Results indicated that while *C. bidens* predominantly inhabited pear orchards, it was also observed in nectarine, almond, and cherry plots, particularly during the winter, early spring, and late autumn. Molecular analyses confirmed the presence of *'Ca.* P. pyri' in *C. bidens* populations from all the crops. Interestingly, the percentage of carrying psyllid did not differ dramatically between pears, nectarine, and almonds and was around 30%. The main factor seems to be the timing of psyllids' arrival to the plots. It is suggested that the psyllid coordinates with the early appearance of leaves in the surrounding crops when the pear is still dormant.

These findings demonstrate the critical role of *C. bidens* in the epidemiology of *'Ca.* P. pyri' across multiple host crops. The seasonal movement of this vector between crops highlights the need for integrated management strategies to control its spread and mitigate the impact of pear decline in mixed-crop systems.

Acknowledgments

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Interactions

First identification of *Cacopsylla bidens* as a vector of '*Candidatus* Phytoplasma pyri', the agent associated with pear decline

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Abstract

This study confirms *Cacopsylla bidens* as the vector of '*Candidatus* Phytoplasma pyri', associated with pear decline in northern Israel. Transmission trials showed that *C. bidens* can transmit the phytoplasma to pear trees, with higher success observed in autumn due to increased phytoplasma prevalence in psyllids. Findings emphasize the vector's critical role in the pear decline epidemiology and the importance of management strategies to control its spread.

Keywords: phytoplasma, epidemiology, infectious disease, molecular detection

Introduction

'Candidatus Phytoplasma pyri' belongs to the apple proliferation (AP) phytoplasma group (16SrX) which includes three '*Candidatus* Phytoplasma' species: '*Ca.* P. mali' (16SrX-A), '*Ca.* P. prunorum' (16SrX-B) and '*Ca.* P. pyri' (16SrX-C). These wall lacking bacteria are associated with diseases named apple proliferation, European stone fruit yellows, and pear decline, respectively (Seemüller and Schneider, 2004) and are genetically very similar (Bertaccini *et al*., 2014). Pear decline (PD), associated with the presence of *'Ca.* P. pyri', is one of the most devastating diseases of *Pyrus communis* (pear) in Europe and North America (Seemüller *et al*., 2011) as well as South America, Middle East, Chile and Taiwan. Recently, '*Ca.* P. pyri' was also detected in almond orchards in Jordan (Alloush *et al*., 2023). In Israel, phytoplasma symptoms were found in pears, almonds, and nectarine. In 2017 observations were made of nectarine (*Prunus persica*) orchards in northern Israel, where trees displayed leaf discoloration, early defoliation, and overall tree decline symptoms. In addition, in 2020 peach trees from south of the Golan were identified positive for '*Ca.* P. pyri' (Zisovich *et al*., 2022). Those trees displayed yellow leaf discoloration, lack of vigor, and reduced tree size. A year later in 2021, almond trees *(Prunus dulcis)* displayed reduced tree size, leaf discoloration, and degeneration of the almond kernel while on the tree. Molecular testing confirmed the '*Ca.* P. pyri' presence in all three crops that are cultivated in proximity in northern Israel creating a patchy ecosystem.

The phytoplasmas enclosed in the apple proliferation group are vectored primarily by psyllids (Jarausch and Jarausch, 2010). In Israel, the pear psyllid *Cacopsylla bidens* is the main pest in pear orchards (Shaltiel-Harpaz *et al*., 2014) and in 2021 '*Ca.* P. pyri' was identified in *C. bidens*. In this study the ability of *C. bidens* to transmit '*Ca.* P. pyri' to pear trees was tested and verified.

Materials and Methods

Transmission trials were conducted on potted pear plants under quarantine conditions in a net house at the Northern R&D Research Station. A total of 16 plants was used in October 2021, and 8 plants were used in August 2022. Prior to the trials, all plants were tested for the absence of phytoplasmas using PCR and confirmed to be negative.

Collection and preparation of psyllids – *C. bidens* individuals were collected from pear orchards of the Eden strain that is highly infected with '*Ca.* P. pyri' (Figure 1). A subset of these psyllids was tested for phytoplasma presence before the experiments. The remaining psyllids were caged on healthy, potted pear plants at a density of 10–14 psyllids per plant.

Inoculation access period (IAP) - The psyllids were allowed a 14-day IAP on the plants. Ten plants were used in 2021 and five in 2022 for inoculation treatments. Five plants in 2021 and three in 2022 served as untreated controls.

Post-inoculation testing - After the IAP, psyllid groups were tested for the presence of phytoplasmas using PCR.

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A subset of the amplified DNA was sent for sequencing to confirm the phytoplasma identity.

Plant testing - Plant tissues were tested for phytoplasma presence one month after the IAP and again after 6–8 months using PCR (Figure 2).

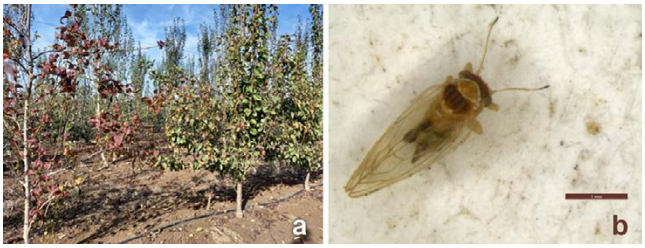


Figure 1. Infected pear plot (a) from where *Cacopsylla bidens* was collected to the laboratory (b) for the transmission experiments.



Figure 2. Transmission experiment- netted bags with psyllids on healthy potted plant (a). Symptomatic pear tree after 6 months from insect inoculation (b).

Results

In the initial sample of psyllids tested by PCR for phytoplasma prior to the experiment, 70% were found to carry the phytoplasma. Following the inoculation period during the experiment conducted in October 2021, the phytoplasma was detected in all but one of the psyllid groups employed. The potted plant associated with the group that tested negative for phytoplasma was included with the control plants.

One month after the October 2021 transmission experiment, PCR testing of the leaves did not yield any positive results for phytoplasma presence. However, eight months post-experiment, 4 out of 10 inoculated plants (40%) were found to be positive for phytoplasma.

A repeated experiment conducted in August 2022 showed one infected plant out of five tested (20%). In both experiments, all control plants consistently tested negative for phytoplasmas.

Discussion

The results demonstrated that *C. bidens* successfully acquires phytoplasmas from infected pear trees and can transmit the phytoplasma to pear plants. The transmission experiments confirmed its role as a vector, with the experiment conducted in October yielding a higher success rate compared to the one performed in August. This difference may be attributed to the higher proportion of psyllids carrying phytoplasma observed during autumn.

While *C. bidens* effectively acquired and transmitted the phytoplasma, successful transmission was confirmed only to pear plants, and detection required more than six months post-inoculation. These findings establish *C. bidens* as a vector of '*Ca.* P. pyri' and underscore its significance in the epidemiology of pear decline in Israel. Future studies will focus on exploring the potential for *C. bidens* to transmit the phytoplasma to other host plant species.

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Interactions

Deciphering the impact of phytoplasma presence on structure and biochemical composition of brinjal crop

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Abstract

Phytoplasma infection significantly affects the morphology and biochemical profile of brinjal, a key vegetable crop. This study compares healthy and phytoplasma-infected brinjal samples, revealing substantial morphological changes in infected plants, such as reduced leaf and fruit size, shorter stems and stunted growth. Biochemical analysis indicated increased levels of total phenolic content, total flavonoid content, and antioxidant activity, suggesting that phytoplasma infection triggers stress-related secondary metabolites. These insights enhance the understanding of phytoplasma-host dynamics and underscore the importance of developing effective management strategies to protect brinjal crops.

Keywords: morphological changes, secondary metabolites, phytoplasma-host interactions, BLL

Introduction

Brinjal or eggplant (*Solanum melongena* L.), a member of the Solanaceae family, is a key vegetable crop in tropical and subtropical regions and is widely grown in India, the world's second-largest producer. Known for its rich nutritional profile, brinjal is high in amide proteins, water-soluble sugars, vitamins and minerals (Naeem and Ugur, 2019). However, brinjal is vulnerable to biotic stresses, particularly brinjal little leaf (BLL) disease associated with phytoplasmas, which are cell-wall lacking bacteria that infect phloem tissues. BLL leads to severe morphological changes, stunted growth, and reduced yield. Seven phytoplasma ribosomal groups (16SrI, 16SrII-D, 16SrIII-J, and -U; 16SrV, 16SrVI-A and -D, 16SrIX-C, and 16SrXII-A) have been identified associated with brinjal worldwide (Kumar *et al*., 2017; Snehi *et al*., 2021). Phytoplasma infections alter host morphology and biochemistry, reducing productivity. Studying these changes provides insights into phytoplasma-induced stress responses, aiding disease management.

Materials and Methods

Brinjal plants with severe little leaf symptoms were collected from agricultural fields in Bhatpaar Rani village, near Deoria, Uttar Pradesh, India, in February 2024 (Figure 1). Morphological data were recorded for symptomatic plants, and various parameters were compared between symptomatic and healthy plants, with percentage reductions calculated to assess the impact of infection. For genomic DNA extraction, 100 mg of leaf midrib was processed using the CTAB method (Ahrens and Seemüller, 1992). PCR was performed with universal primers P1/P7 (Deng and Hiruki 1991; Schneider *et al*., 1995), and nested PCR with R16F2n/R16R2 (Gundersen and Lee, 1996), followed by electrophoresis in 1% agarose gel for UV visualization. Biochemical quantification of Total Phenolic Content (TPC), Total Flavonoid Content (TFC), and antioxidant activity was conducted for both infected and healthy plants.



Figure 1. Symptomatic brinjal plant.

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Dried extracts from a Soxhlet apparatus were dissolved to 1 mg/ml, vortexed, and tested. For TPC, 0.1 ml of extract was mixed with 2.5 ml of 10% folin-ciocalteu reagent and 2.0 ml of 7.5% sodium carbonate, incubated in the dark for 1.5 hours, and absorbance was recorded at 765 nm. TPC was calculated as mg Gallic Acid Equivalents (GAE)/g sample using a gallic acid standard curve. TFC was measured by mixing 1 mg extract with methanol, 10% $AlCl_3$, 1 M potassium acetate, and distilled water, incubated for 30 minutes, and absorbance measured at 415 nm, expressed as mg Quercetin Equivalents (QE)/g sample. Antioxidant activity was evaluated by DPPH assay, and the results were used for comparative analysis.

Results

Molecular detection via nested PCR assay showed positive bands of expected size of 1.2 kb indicative of phytoplasma presence (data not shown). The morphological study revealed significant reductions in plant structure like leaf and fruit size, stem length and plant height in symptomatic brinjal plants (Table 1). Biochemical quantification data of secondary metabolites depicted TPC to be 0.769 mg GAE/g and TFC to be 2 mg QE/g higher in infected leaves of brinjal as compared to the healthy ones. However antioxidant activity was lowered by 36.11% in phytoplasma infected brinjal leaves (Table 2).

Table 1. Comparison of various morphological parameters of phytoplasma infected brinjal plants.

S. No.	Morphological character	Healthy brinjal plant	Infected brinjal plant	Percentage reduction
1	Plant height	125 cm	65 cm	48%
2	Leaf length	21.87 cm	3.2 cm	85.3%
3	Leaf width	13.5 cm	1.5 cm	88.8%
4	Stem length	61.9 cm	20 cm	67.7%
5	Fruit size	23.4 cm	4.6 cm	80.3%

Table 2. Biochemical quantification and percentage change.

S. No.	Parameters	Healthy plant	Infected plant	Change (%)
1	Total phenolic content (mg GAE/g)	4.871	5.64	15.78
2	Total flavonoid content (mg QE/g)	36.0	38.0	5.56
3	Antioxidant activity-IC50 (mg/ml)	6.539	4.178	36.11

Discussion

This study demonstrates the substantial impact of phytoplasma infection on morphology and biochemistry of brinjal plants. Structurally, infected plants exhibited severe reductions in height, leaf size, fruit size, and stem length, with some parameters reduced up to 88% compared to healthy plants. These changes reveal the extensive impact of BLL disease on cellular integrity and overall development of infected plants, that is consistent with previous findings showing symptoms like stunted growth and leaf abnormalities due to phloem disruption (Bertaccini, 2022). Biochemically, the elevated levels of TPC and TFC in infected plants indicate a stress-induced metabolic response. The 15.78% and 5.56% increases in TPC and TFC reflect an upregulation of secondary metabolites as a defence response, a similar phenomenon is reported in studies of phytoplasma-infected brinjal crops (Chauhan *et al*., 2024), where increased phenolic levels correlated with susceptibility and increased plant stress responses. Despite the increased phenolic and flavonoid content, the antioxidant activity decreased by 36.11% in infected plants. The reduction in the antioxidant activity may indicate a depletion of the plant's capacity to effectively counteract the oxidative stress.

The combined effects of morphological disruption and altered biochemical pathways underscore the importance of the disease and suggest effective management strategies to protect brinjal crops from the adverse effects of phytoplasma infection.

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Interactions

Biophysical properties of trichome in sesame phyllody

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Abstract

This study investigates biophysical properties of the trichome of sesame plants showing phyllody in the four genotypes of sesame GT-10, TKG-308, VRI-1, and RT-351. Trichome density, type, length, and width were examined using microscopic techniques. The results revealed two main types of trichomes: glandular and non-glandular. Healthy plants showed significantly higher trichome density, length, and width compared to diseased plants. The highest trichome density was recorded in healthy plants in GT-10 (29.23/mm). GT-10 also exhibited both glandular and non-glandular trichomes, unlike other genotypes, which predominantly had non-glandular trichomes. The findings indicate that phyllody infection affects the trichome development of sesame plants, with reduced trichome size and density in diseased plants. These results could aid in developing new insect-resistant cultivars through breeding programs incorporating biophysical traits.

Keywords: sesame disease, phytoplasma, microscopy, breeding

Introduction

The most important sesame diseases are phyllody, root and stem rot and powdery mildew (Gupta *et al*., 2018). Sesame phyllody became epidemic in India, mostly in peninsular regions, with an incidence of about 75% (Manjunatha *et al*., 2012). Sesame phyllody cause up to 80% yield loss (Salehi *et al*., 2017). Phytoplasmas have been transmitted by plant sap sucking insects such as planthoppers, psyllids and leafhoppers; they are pleomorphic and have a small genome. In plants, they are confined to the phloem tissue and spread by passing through the pores of the sieve plates that separate the phloem sieve tubes. Leafhoppers prefer the soft part of the leaf to suck the cell sap, therefore avoid the harder part or the thick veins near the petiole and prioritized the front and middle portions of the leaf for cutting and subsequent processing since they are softer.

Materials and Methods

Trichome density was examined in leaves of four selected genotypes in both healthy and diseased plant. Plants that were 30 to 45 days old were used to collect leaf samples. The leaf samples were cut into one square centimeter pieces to prepare them for covering with a cover slip. The leaf segments were placed in a glass vial with a 2 cm diameter and 15 ml of distilled water. The glass vial was then allowed to stand for a short while before 15 ml of 70% ethanol was added, allowed to stand for another minute, and then poured out. This was done to get rid of contaminants that might have adhered to the leaf surface. To the previously mentioned glass vials with a clean leaf segment, 99% ethanol was added. For the double boiling process, the same glass vials were put in a beaker that had already been partially filled with distilled water. The beaker was then placed on a hot plate at 70 to 80°C for 15 to 20 minutes, or until the ethanol turned dark green. After carefully and slowly draining off the ethanol, 15 ml of new, 99% ethanol was added, sealed in a beaker and set over a hot plate to boil. This process was repeated to eliminate the chlorophyll. Following the yellowing of the leaf segments, the ethanol in the vials was drained and 95% lactic acid was added. The vials were then sealed with cotton swabs and heated in the same way, between 75 and 85°C, for about 20 minutes, or until the leaf segments were almost translucent. After removing the glass vials from the beaker, the hot distilled water was drained out and replaced with cool water, allowing the glass vials to cool and become easier to handle. Slides were cleaned with 70% ethanol before two drops of lactic acid were applied. A cleared leaf segment was then carefully placed on the slide with the use of a camel hairbrush, being careful not to break or create bubbles. On a leaf segment that was laying above the slide, a drop of lactic acid was introduced, and it was then covered with a cover slip that had no air bubbles. Using a binocular microscope with a 100 x magnification,

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Table 1. Trichome density in phyllody infected plants.

Genotypes	Type of trichome	Trichomes density / microscopic field		Trichomes length (mm)		Trichome width (mm)	
		Diseased	Healthy	Diseased	Healthy	Diseased	Healthy
TKG- 308	Non-glandular	10.33	24.2	24.2	0.071	0.075	0.018
GT-10	Non-glandular and glandular	21.66	29.33	29.23	0.132	0.168	0.036
VRI-1	Non-glandular	11.33	20.10	20.10	0.65	0.88	0.021
RT-351	Non-glandular	11.33	18.12	18.12	0.107	0.122	0.022

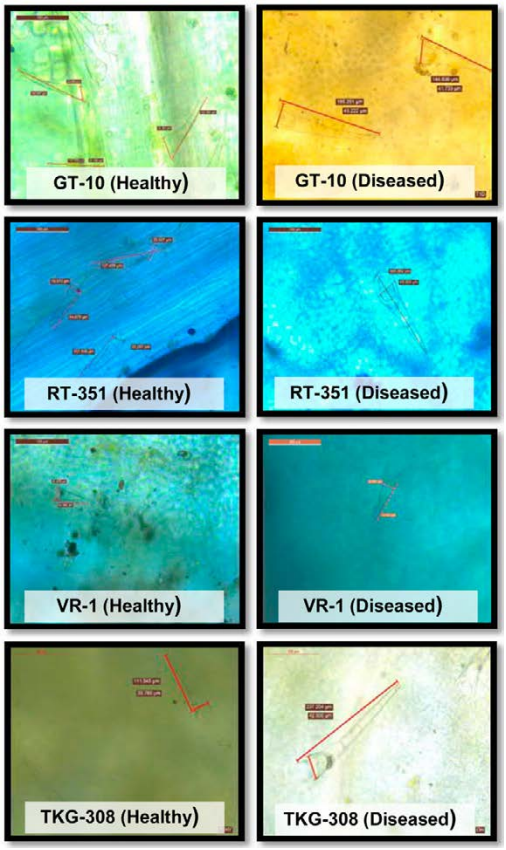


Figure 1. Comparison of trichome density, length and width between diseased and healthy preparations from sesame leaves.

10 x objective lens and 10 x/22 eyepiece lens, the slides were examined. Trichome numbers were counted inside a single, randomly selected microscopic field, measuring 4.5 mm.

Results

Among the selected genotypes the trichome density (Table 1) ranged from 18.22 to 29.23 mm in healthy plants (h) and 10.33 to 22.66 mm in diseased plants (d). Trichomes density was lower in diseased leaves in comparison to healthy leaves, the maximum trichome density was observed in genotype GT-10 (d – 21.66 and h – 29.23), TKG-308 (d – 10.33 and h – 24.2), VRI -1 (d – 11.33 and h – 20.10) and RT-351 (d – 11.33 and h- 18.12). Among all the genotypes glandular and non-glandular type of trichomes were found, in particular non-glandular type of trichome was found in all the genotypes except GT-10 in which both glandular and non-glandular type of trichome were found. Trichome density of both types was higher in healthy plants compared to diseased plants among all the genotypes (Figure 1). The highest trichome length was observed in genotype GT-10 (d – 0.132, h - 0.168) followed by RT-351 (d – 0.107, h- 0.122), VRI-1 (d – 0.65, h- 0.88) and TKG-308 (d – 0.071, h- 0.075). Among all the genotypes trichome width was higher in healthy plants in comparison to diseased plants. The highest trichome width was observed in genotype GT-10 (d – 0.36, h - 0.40) followed by RT-351 (d – 0.22, h - 0.26), Prachi (d – 0.023, h - 0.024), VRI-1 (d – 0.021, h- 0.043) and TKG-308 (d – 0.018, h - 0.020).

Discussion

Biophysical properties of sesame such as trichomes were studied in five genotypes. The comparison between healthy and diseased plants of the same genotype allows to verify that the maximum trichome density, length and width were observed in healthy plants in comparison to diseased plants.

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Interactions

A review on phytoplasma induced biochemical responses in plants

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Abstract

Phytoplasma-infected plants exhibit symptoms such as witches' broom, phyllody, virescence, yellowing induced by the phytoplasma effector proteins. It has been revealed that phytoplasmas are capable of modulating biochemical responses of plants involved in defense mechanisms. Phenolic compounds, free amino acids in source leaves, protein contents, some secondary metabolites, and polyamines were found to increase, while chlorophyll content and photosynthetic activity and the diversity of chemical compounds were reduced as a consequence of phytoplasma infection. This review focuses on the phyllody disease pathosystem.

Keywords: phyllody, secondary metabolites, disease, plants

Introduction

Phytoplasmas are classified under the Phylum Mycoplasmatota, Class Mollicutes, Family Acholeplasmataceae, Order Acholeplasmatales, Genus '*Candidatus* Phytoplasma' (IRPCM, 2004). Phytoplasma-associated diseases are causing significant economic losses on diverse cultivated crops (Bertaccini and Lee, 2018). Phytoplasmas have a serious impact on vegetable crops, cassava and sugarcane in different parts of the world (Kumari *et al*., 2019; Rao *et al*., 2018). A wide range of symptoms are associated with the presence of phytoplasmas in the diseased plants and may vary depending on the strain, host, time of infection, age of the plant and environmental conditions (Bertaccini and Lee, 2018). Infected plants show a wide range of symptoms due to imbalance of plant hormones or growth regulators. Phytoplasmas are mainly spread between plants by phloem-feeding insects and their host range is dependent upon feeding habits of the insect vectors (Weintraub *et al*., 2019).

Phytoplasmas harm infected plants by affecting various physio-biochemical and metabolic processes and altering genetic expression, with the biosynthesis and accumulation of carbohydrates being the most highly affected processes. Phytoplasma-induced harms also include stomatal closure, photosynthetic impairment due to declining leaf area, and photosynthetic pigments, leading to limited transportation of photo-assimilates to sink organs, causing yield and quality weakness. One of the main effects of phytoplasma infection is the decrease in plant productivity caused by inhibition of photosynthesis.

The phytoplasmas show a variety of side effects that proposes significant aggravations in the ordinary adjust of hormones and the activity of antioxidant enzymes characterized by stunting of the plants and change of the floral parts into leafy structures bearing no container and seeds. Phytoplasma infection disturbs the operation and formation of the photosynthate, disrupts sugar metabolism and, in particular, stimulates the abnormal accumulation of starch in the chloroplasts. The increase in sugar content temporarily benefits phytoplasma proliferation; however, the excessive damage to the structure and basal metabolism of the plants significantly reduced their subsequent photosynthetic products. After being infected by phytoplasmas, the host plant exhibits increased respiration, cell wall enhancement, and synthesis of some defense complexes; more carbohydrates and free amino acids are essential for these metabolic activities to provide energy to the host plant.

Plants express various antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), peroxidase

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(POD), and polyphenol oxidase (PPO), to mitigate oxidative damage by catalytically scavenging free radicals and reactive oxygen species (ROS). In phytoplasma-infected plants, total phenols, phenylalanine ammonia-lyase (PAL) and POD activities were significantly higher in leaves of several infected species such as mungbean, lettuce and sesame (Hameed *et al*., 2017; Ahmed *et al*., 2022; Akkurak *et al*., 2022). Amino acid, protein, total phenolics and H_2O_2 contents, antioxidant enzyme activity of brassica plants increased considerably in phytoplasma infected plants (Yaseen *et al*., 2020). Maize plants infected by bushy stunt phytoplasma showed more accumulation of protein contents (Junqueira *et al*., 2004). In contrast to these results, a decreased level of soluble protein contents was observed in different phytoplasma infected plants like apple proliferation infected apple trees (Bertamini *et al*., 2002).

Pathogen attack can activate the overabundance of ROS in cells, which can cause oxidative stress and severe physiological damage. This was reported in apple, apricot, and grapevine by Musetti *et al*., (2005). Phytoplasma infection impaired the amino acid translocation in host plants (Carginale *et al*., 2004). High amino acid concentration was also observed in source and sink leaves of *Catharanthus roseus* infected by ash yellows and in source leaves of tobacco plants infected by apple proliferation (Lepka *et al*., 1999). Phenolic contents increased up to three-fold in "stolbur" infected tomato plants (Favali *et al*., 2001).

However, contrary to these results, a reduction in protein contents was also reported in phytoplasma infected tomato and corn plants. This reduction in protein contents might indirectly correlate with chloroplast damage in phytoplasma infected leaves which results in reduced activity of the major soluble protein in leaves that is RuBPC and hence CO_2 fixation (Bertamini *et al*., 2002). In sweet orange, primary metabolites including chlorophyll a, b, total chlorophyll contents, soluble sugar were significantly reduced as well as the chlorophill a/b ratio. Meanwhile, carotenoid, hydrogen peroxide and malondialdehyde were lower in the phytoplasma infected plants while proline and soluble protein were higher in the phytoplasma-infected plants. The secondary metabolites including phenolics, glycine betaine and anthocyanin were increased, while ascorbic acid was decreased in the phytoplasma-infected plants. All the antioxidative enzymes: ascorbate peroxidase, superoxide dismutase, catalase, and peroxidase activities were found to be increased in the phytoplasma-infected plants (Rasool *et al*., 2020).

Conclusion

Alterations in level of phenolic compounds, total soluble proteins, PAL, POX, CAT, PPO, total chlorophyll, primary and secondary metabolites in phytoplasma infected plants severely damage the physiological and biochemical processes which leads to the appearance of symptoms characteristic to specific host-phytoplasma interaction. The elevated level of different biochemical parameters in diseased plants indicates that the reaction of plants to phytoplasma infection is induced rather than constitutive.

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Phytopathogenic Mollicutes
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Interactions

Association of phytoplasmas with tapping panel dryness syndrome of *Hevea brasiliensis*: a pathological perspective to unresolved mystery?

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Abstract

Tapping panel dryness is a serious disorder impairing the synthesis of latex in the laticifer cells of the soft bark region of para rubber, *Hevea brasiliensis*. Decades of research did not yield conclusive evidence for the causative factor of this disorder. Soft bark region of the bark is rich in latex vessels and phloem sieve tubes. Semi- and ultrathin sections of this region demonstrated blockage of sieve tubes and shrunken latex vessels. Presence of pleomorphic bodies in the size range of 400-2,000 nm was depicted in scanning electron microscopy images. Confocal laser scanning electron microscopy with DAPI staining compared the healthy and diseased soft bark region. Diseased sample exhibited fluorescence in the sieve tubes with thick callose deposition. Presence of phytoplasmas in tapping panel samples dryness affected bark samples was confirmed by amplification and sequencing of 16S rRNA gene region with universal primers. The sequence of the phytoplasma from clone RRII 105 was submitted to NCBI with accession number KP025809.

Keywords: para rubber, DAPI, callose, tapping panel dryness

Introduction

Latex is the cytosol of laticifers present in the soft bark region of *Hevea brasiliensis*. It is the main source of natural rubber across the globe, which is harvested from the barks of *H. brasiliensis* by tapping. Natural rubber is a major raw material for automobile industries and its production plays a significant role in the economy of rubber growing countries. Tapping panel dryness as the name suggests is the lack of flow of latex through tapping panel due to blockage of latex biosynthesis in the laticifers of soft bark. Several investigations on physiology, biochemical and molecular aspects of the disease did not yield any conclusive evidence. Hence, the malady remained in the status of physiological disorder of unknown cause.

Transcriptomic studies revealed activation of genes involved in programmed cell death and defense signalling (Nie *et al*., 2023). The current study involving anatomical and molecular investigation into the soft bark tissues of healthy and tapping panel dryness (TPD) affected bark samples gives a new perspective of pathological influence in inducing the syndrome.

Materials and Methods

Soft bark tissues from healthy and TPD affected trees were collected for anatomical study and molecular characterisation. Semi- and ultrathin sections of inner soft bark region were subjected to microscopic studies. Transverse and longitudinal sections of 30 µm thickness were stained with Congo red and O-dianisidine. Similarly, sections were subjected to a confocal laser scanning electron microscopic (SEM) analysis. DNA binding DAPI (4′2 ,6-diamidino-2-phenylindole) stain was used to detect the presence of microbes in the transverse and longitudinal sections of soft bark region. DNA was extracted from the inner soft bark region of healthy and diseased *H. brasiliensis* (Kirkpatrick *et al*., 1987). Amplification of the 16S rRNA gene region through nested PCR was done using the R16mF2/R16mR1 and R16F2n/R16R2 primer sets (Gundersen and Lee, 1996).

Results

TPD affected trees exhibited complete drying up of the tapping panel (Figure 1). Light microscopy analysis revealed complete panel blockage of sieve plates of TPD affected samples. Thick deposition of callose as stained by Congo red was visible in TPD affected samples, whereas sieve pores of healthy samples were not clogged (Figure 2). Fluorescence imaging via DAPI staining in in sieve tubes of TPD affected sample indicates presence of phytoplasma-like structures

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(Figure 3). SEM analysis revealed pleomorphic bodies in the range of 400-2,000 nm size (Figure 4). Amplification of a 1,215 bp fragment on the 16S rRNA gene with universal primers was only achieved in TPD affected samples but also in unopened trees affected by TPD (Figure 5). The sequence submitted to NCBI with the accession number KP025809 showed 98.98% identity with 16S rRNA gene of the *Ternstroemia gymnanthera* witches' broom phytoplasma strain TGWB2 from China.

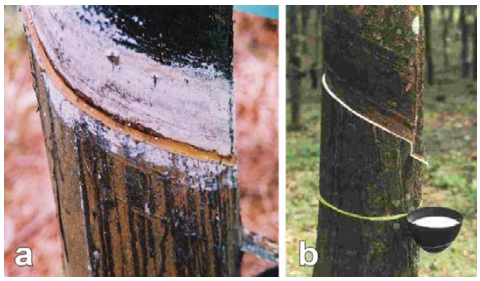


Figure 1. Tapping panels of rubber tree latex flow in healthy tree (a) and dried panel in TPD affected trees (b).

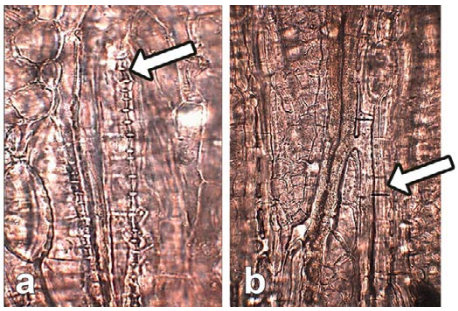


Figure 2. Sieve tubes in soft bark region: opened pores of sieve tubes in healthy bark (a); callose deposited sieve tubes in TPD affected bark (b).

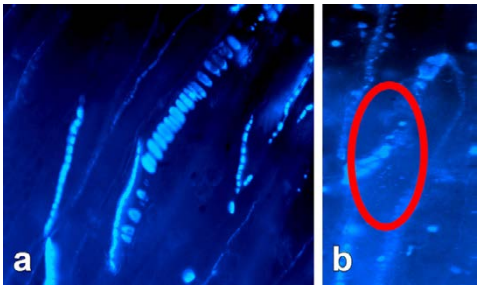


Figure 3. Confocal laser SEM images of sieve tubes: lack of fluorescence in healthy bark (a); fluorescence in TPD affected bark (b).

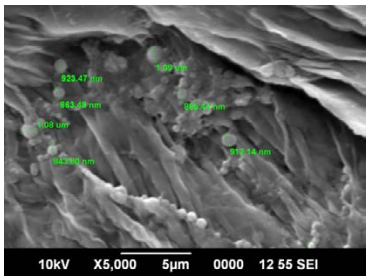


Figure 4. SEM image of pleomorphic bodies in the sieve tubes of TPD affected bark.

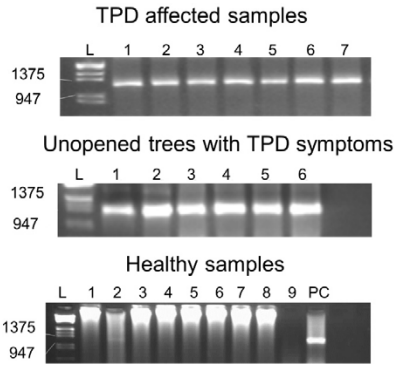


Figure 5. Gel image of 16S rRNA gene amplification results L: ladder, PC: positive control.

Discussion

Tapping panel dryness syndrome is a severe disorder affecting productivity of rubber tree, *H. brasiliensis*. Callose deposition in the sieve tubes and presence of microbe-like structures in anatomical studies aligns with previous studies of programmed cell death defense signalling in TPD affected samples (Nie *et al*., 2023). Sieve tubes are in close association with latex vessels of *H. brasiliensis*. They both originate from fusiform initials of vascular cambium (Sivan *et al*., 2011). Hence, pathogenic invasion into the sieve tubes could block the sieve pores to further spread. This could trigger defense signalling and programmed cell death in nearby cells of the tissue. Presence of phytoplasmas confirmed through molecular detection hints at the pathogenic perspective of the syndrome. Phytoplasma can traverse through phloem tissue through sieve pores (Wang *et al*., 2024). Blockage of sieve tubes and thereby lack of supply of nutrients to laticifers could be the reason for blockage of latex biosynthesis in TPD affected rubber trees.

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Phytopathogenic Mollicutes
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Country status

Phytoplasmas diversity in India

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Abstract

In India, 190 plant species have been associated with phytoplasma diseases that are affecting a number of crops important to agriculture, including ornamentals, fruits, vegetables, trees. In India eleven ribosomal groups of phytoplasmas were identified, the majority of which have been reported from the country's Northeast region followed by southern part. In Central and Western India, very few phytoplasmas have been identified. While phytoplasmas were primarily confined to the Eastern and Southern regions, it is now evident that phytoplasmas are distributed in all states across India. The most common group of phytoplasmas is the 16SrI (aster yellows) followed by 16SrII group, together they were associated to over 58 diseases in India.

Keywords: diversity, phytoplasma ribosomal groups, 16S rRNA gene

Introduction

More than 190 plant species are known to be associated with phytoplasmas in India. The first reports started from Kerala district in 1874 when coconut root wilt disease was reported (Rao, 2021). Coconut root wilt, sandal spike, brinjal little leaf, grassy shoot of sugarcane, rice yellow dwarf, sesame phyllody, white leaf of *Cynodon dactylon* are diseases reported since longtime in India (Vasudeva and Sahambi, 1955; Varma *et al*., 1969). However only with the development of molecular tools more than 150 phytoplasmas have been identified and well documented in India (Kumari *et al*., 2019; Tiwari *et al*., 2023; Karthikeyan *et al*., 2024; Srivastava and Mall, 2024). Based on 16S rRNA gene analysis the presence of 11 phytoplasma ribosomal groups was identified across the country in a variety of plant hosts such as vegetables, ornamental species, sugarcane crops, oil and cereals, palm, trees, fodder and weeds (Rao, 2021). Recently draft genome sequences of phytoplasmas associated with sugarcane grassy shoot (16SrXI) and Bermudagrass white leaf disease (16SrXIV) were submitted from India broadening the understanding about phytoplasma genomics (Kirdat *et al*., 2020). The present study aimed to present a current status of phytoplasmas diversity and geographical distribution in India.

Materials and Methods

For comparison and phylogenetic analysis, phytoplasma sequences from India were obtained from the NCBI GenBank databases. The BLASTn program was used to compare the 16S rDNA sequences. A 1,000 bootstrap dataset was used to produce a phylogenetic tree using MEGA 11 software (Tamura *et al*., 2021).



Figure 1. Geographical distribution of phytoplasma ribosomal groups in India.

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Results and Discussion

The diversity of phytoplasmas showed a wide geographical distribution especially in Northeastern region to Southern parts of the country (Figure 1).

In the Northeastern region the most diverse phytoplasma groups were detected such as 16SrI, -II, -V, -VI, -IX and -XIV followed by the Southern parts of the country where 16SrI, -II, -IV and -XI were present. However phytoplasma diversity was also reported from western parts with 16SrI, -II, -VI, -XI groups and central part of India with 16SrV and 16SrVI groups. There was a remarkable increase in the incidence of phytoplasma diseases in last decade from entire regions of the country. However, '*Candidatus* Phytoplasma asteris', '*Ca.* P. aurantifolia=citri', '*Ca.* P. palmae , '*Ca.* P. ulmi', 'Ca. P. trifolii', '*Ca.* P. phoenicium', '*Ca.* P. oryzae', '*Ca.* P. cynodontis', respectively representing 16SrI, 16SrII, 16SrIV, 16SrV, 16SrVI, 16SrIX, 16SrXI and 16SrXIV groups are the main '*Ca.* Phytoplasma' species detected in diverse plant species in India (Figure 2).

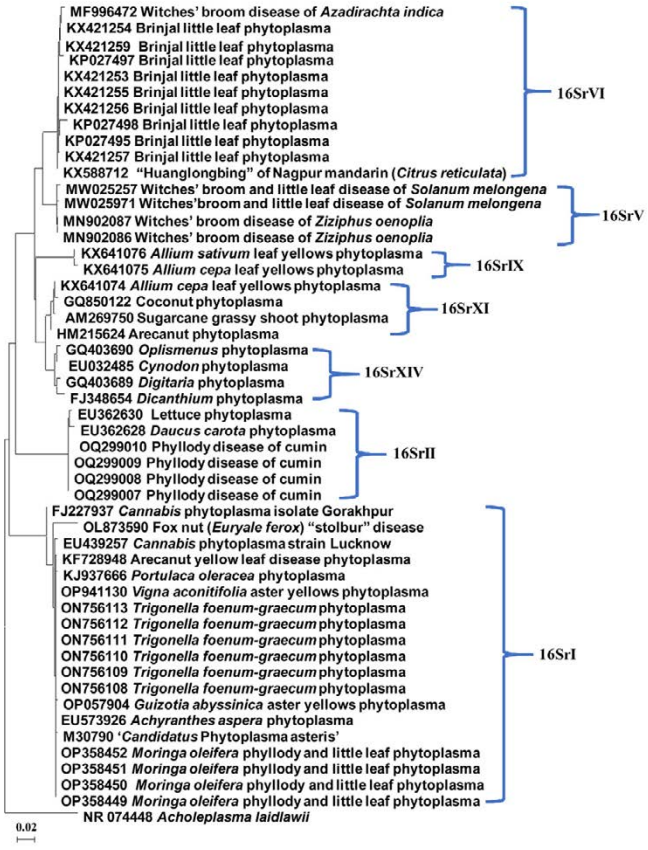


Figure 2. Phylogenetic tree built by using MEGA 11 software to depict the molecular diversity of phytoplasmas in India.

Sesame phyllody is one of the devastating diseases associated with 16SrI-B and 16SrII-C and -D subgroups. Interestingly the 16SrI-B phytoplasmas are dominant in sesame crops of North India while 16SrII phytoplasmas are mainly reported from South India. '*Ca.* P. asteris' (16SrI) represents the prevalent phytoplasma, exhibiting a broad host range followed closely by the 16SrII group, both of which have been reported in nearly every region of India. Over the past decades, the diversity of phytoplasmas in India, as noted by Mall *et al.* (2011), was recorded as 45 diseases. However, the update situation reveals an increase to 190 diseases, indicating not only a rise in the number of host plants but also a significant expansion in the variety of phytoplasma ribosomal groups identified throughout the country. Previously, phytoplasmas were primarily confined to the Eastern and Southern regions; however, after ten years, it has been become evident that phytoplasmas are now reported from all states across India. The current status of phytoplasmas is alarming, necessitating a greater emphasis on developing effective control and management strategies.

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Phytopathogenic Mollicutes
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Country status

Phytoplasma disease prevalence in Namsai, Arunachal Pradesh, India: an exploratory study

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Abstract

A roving survey was carried out in Namsai district of Arunachal Pradesh, India for prevalence of phytoplasma diseases and symptom presence. The phytoplasma infected plants were identified based on the symptomatology and confirmed by PCR analysis. The important crops grown in Namsai were surveyed and different crops exhibiting symptoms were observed and photographic evidence were collected for further studies. Symptoms of suspected phytoplasma infection was observed in potato (*Solanum tuberosum*), sesame (*Sesamum indicum*), garden pea (*Pisum sativum*), marigold (*Tagetes* spp.), hemp (*Cannabis sativa*) and henna plant (*Lawsonia inermis*). The symptoms mainly included yellowing of leaves, phyllody, virescence, little leaves, witches' broom, flower virescence and stem flattening.

Keywords: epidemiology, symptoms, hosts, disease

Introduction

Namsai is a foothill district of Arunachal Pradesh, India. It was the 18th district of the state carved from Lohit district. The expanding host range and escalating incidence of phytoplasmas have garnered significant concern, necessitating investigations into their host range. Phytoplasmas are obligate prokaryotic wall less, pleomorphic bacteria with size of 200-800 nm and a very small genome of 680-1600 kb. They multiply in plant phloem tissues and insect haemolymph. Phytoplasmas are transmitted by phloem feeding leafhoppers and planthoppers (Bertaccini *et al*., 2014). The global spread of phytoplasma diseases in plant species has accelerated significantly over the past few decades, fuelled by the growing international trade and distribution of plant materials. Host range of phytoplasmas in this region of the country has been poorly studied hence this study is a preliminary work to get an idea regarding the abundance of phytoplasmas in different crop species.

Materials and Methods

A roving survey was carried out in the five circles of Namsai district, Arunachal Pradesh *i.e*., Chongkham, Lathaw, Piyong, Namsai and Lekang during 2023-2024. The district is located in between latitude 27°30' to 27°55'N and longitude 95°52' to 96°20'E (Figure 1).

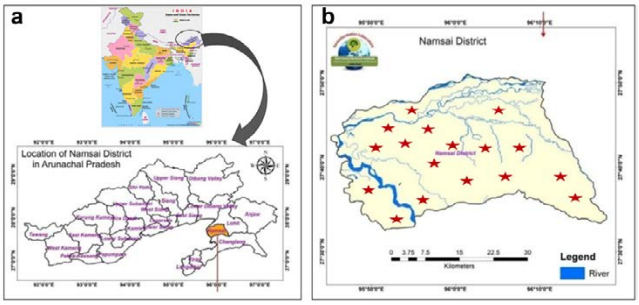


Figure 1. (a) Location of Namsai; (b) Surveyed areas of Namsai (from Hazarika *et al*., 2021).

The average annual temperature is 22.8°C. Precipitation here averages 2,728 mm (Hazarika *et al*., 2021). The symptomatic plants were observed, it was taking photographic evidence, and the samples were collected for further analysis. Total DNA from all plants was extracted by following the protocol of Kollar *et al*. (1990). For PCR, primer pairs P1/P6 was used (Deng and Hiruki, 1991; Smart *et al*., 1996). The PCR products were resolved on 1.5% agarose gel in 1X Tris EDTA (TAE) containing ethidium bromide (Sambrook and Russell, 2001).

Results

During the survey, different phytoplasma associated symptoms were observed in different crops. The crops

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exhibiting symptoms of phytoplasma included: potato (*Solanum tuberosum*), sesame (*Sesamum indicum*), garden pea (*Pisum sativum*), marigold (*Tagetes* spp.), hemp (*Cannabis sativa*) and henna plant (*Lawsonia inermis*). Table 1 shows the list of crops affected by phytoplasma in different circles of Namsai district.

The symptoms observed were mostly phyllody, yellowing of the leaves and flower virescence in sesame, stunting and reduction in leaf size and witches' broom in potato, stem flattening in henna plants, little leaf and witches' broom in marigold and hemp and yellowing of leaves and bushy growth in garden pea plants (Figure 2). Furthermore, stem flattening of henna is the first report of phytoplasmas in henna plants (*Lawsonia inermis*) in this region. The presence of phytoplasmas was confirmed by PCR analysis (Figure 3).

Table 1. List of crops affected by phytoplasma in Namsai district.

Name of the Circle	Crops affected by phytoplasma
Chongkham	Potato (*Solanum tuberosum*), sesame (*Sesamum indicum*)
Lathaw	Sesame (*Sesamum indicum*), garden pea (*Pisum sativum*), marigold (*Tagetes* spp.)
Piyong	Henna plant (*Lawsonia inermis*), potato (*Solanum tuberosum*), marigold (*Tagetes* spp.)
Namsai	Garden pea (*Pisum sativum*), hemp (*Cannabis sativa*), marigold (*Tagetes* spp.)
Lekang	Marigold (*Tagetes* spp.)

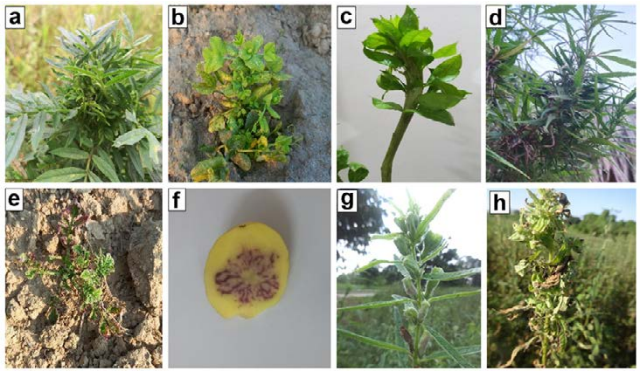


Figure 2. Symptomatic crops, (a) marigold, (b) garden pea, (c) henna plant, (d) hemp, (e) potato, (f) potato tuber, (g) sesame phyllody and (h) flower virescence in sesame.

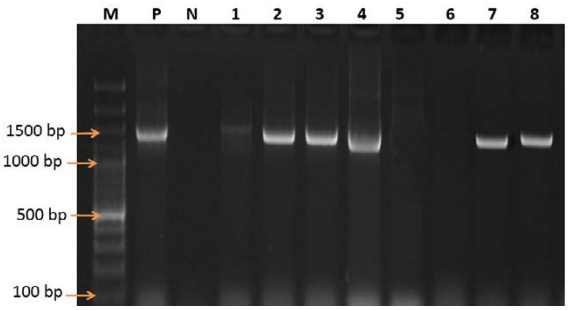


Figure 3. Results of PCR analysis in agarose 1.5% gel. M. 100 bp ladder; P. positive control; N. negative control; 1. marigold; 2. garden pea; 3. henna plant; 4. hemp; 5-6. blank; 7. potato 8. Sesame.

Discussion

Phytoplasmas are associated with diseases in several commercial plants causing serious economic losses all over the world. Similar symptoms of the plants have been reported from different parts of the world as well, like, marigold little leaf (Raj *et al*., 2011), bushy plants of garden pea (Singh *et al*., 2024), witches' broom of hemp (Rasoulpour *et al*., 2017), sesame phyllody (Sertkaya *et al*., 2007; Phookan *et al*., 2020). The host range of phytoplasmas has been increasing hence proper studies should be carried out to apply effective management strategies.

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Country status

Molecular characterization and symptom assessment of phytoplasma infection in king chilli across Assam, India

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Abstract

This study presents phytoplasma infections in king chilli (*Capsicum chinense*) across the major growing districts in Assam, India, from 2022 to 2024. Symptoms observed included little leaf, witches' broom, and severe stunting, with disease incidences of 19.34% in Golaghat, 17.09% in Jorhat, and 13.32% in Sivasagar. Phylogenetic tree analysis and virtual RFLP analysis using *i*PhyClassifier indicated that the phytoplasma present in king chilli is classified within the 16SrVI-D subgroup. These findings highlight the significant phytoplasma threat to king chilli cultivation in Assam.

Keywords: '*Candidatus* Phytoplasma trifolii', 16SrVI-D, disease, molecular identification

Introduction

The king chilli (*Capsicum chinense* Jacq.), or ghost pepper/ "bhut jolokia", is a natural hybrid of *C. chinense* and *C. frutescens*, primarily grown in Assam and Northeast India. Culturally significant and extremely hot, it ranks as the world's seventh hottest chilli, with a Scoville rating of 1,001,304 and capsaicin content of 3–5% (Borgohain and Devi, 2007). So far, six phytoplasma groups and five subgroups have been globally identified associated with diseases in chilli (Kumari *et al*., 2019). Despite the common occurrence of phytoplasma-related diseases, there has been lack of comprehensive molecular research on phytoplasmas specifically affecting king chilli in Northeast India. Therefore, this study aims to examine symptomatology and conduct molecular characterization of phytoplasmas detected in king chilli in Assam.

Materials and Methods

A roving survey was conducted in the major king chilli growing districts of Assam namely, Jorhat, Sivasagar, and Golaghat. During the survey, samples suspected of phytoplasma infection were collected along with asymptomatic samples, and details such as symptoms and disease incidence were recorded.

To investigate phytoplasma presence, total DNA was extracted from both symptomatic and asymptomatic plant samples using a CTAB protocol. The DNAs were analyzed by PCR using P1/P6 primers (Deng and Hiruki, 1991; Smart *et al*., 1996) and were Sanger sequenced. The highest-quality consensus sequence was deposited in GenBank. Subsequently, the partial sequence was subjected to phylogenetic and *in silico* RFLP analyses with *i*PhyClassifier tool (Zhao *et al*., 2009) for ribosomal subgroup identification.

Results

During the survey conducted from 2022 to 2024 in the major king chilli-growing districts of Assam, India, various phytoplasma-associated symptoms were observed, including little leaf, witches' broom, curling and cupping of leaves, shortened petioles and internodes, stem flattening, severe stunting, and, ultimately, necrosis and death of the plants (Figure 1). The disease incidence varied across the surveyed districts (Table 1), with the highest incidence of 19.34% recorded in Golaghat district, followed by Jorhat at 17.09%. The lowest incidence was observed in Sivasagar district, with 13.32%.

All symptomatic samples collected during the survey produced an amplicon of 1.5 kb confirming the presence of phytoplasmas in them. The PCR products were Sanger sequenced in both directions, and the best quality consensus sequence was deposited in GenBank (accession number ON819760). BLASTn analysis indicated that the 16S rDNA sequence of king chilli phytoplasma shared 99.52% sequence identity with '*Candidatus* Phytoplasma trifolii'. A phylogenetic tree (Figure 2) and virtual RFLP analysis using *i*PhyClassifier of the 16S rRNA gene sequence indicated that the phytoplasma associated with king chilli is classified within the 16SrVI-D subgroup, exhibiting a similarity coefficient of 1.00.

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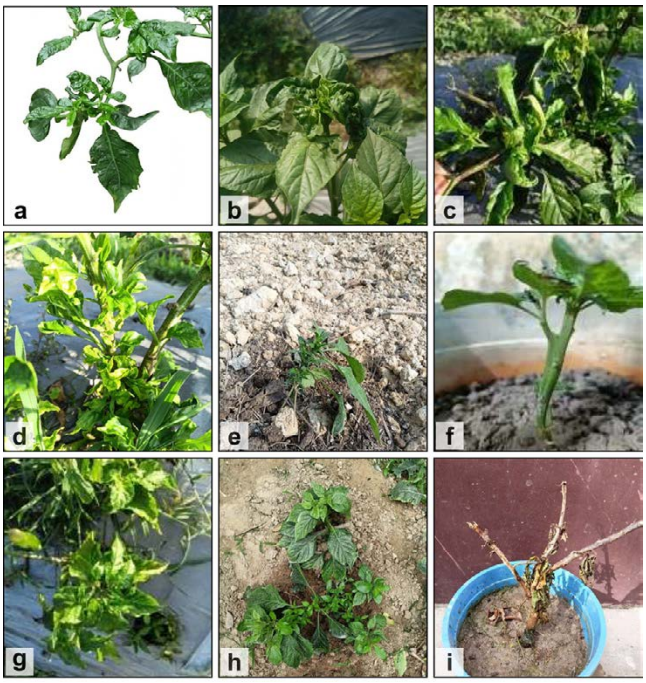


Figure 1. Symptoms in infected king chilli: (a) little leaf; (b, c) curling and cupping of leaves; (d) shortened petiole (e) shortened internode; (f) stem flattening; (g) witches' broom (h) stunting; (i) necrosis and death.

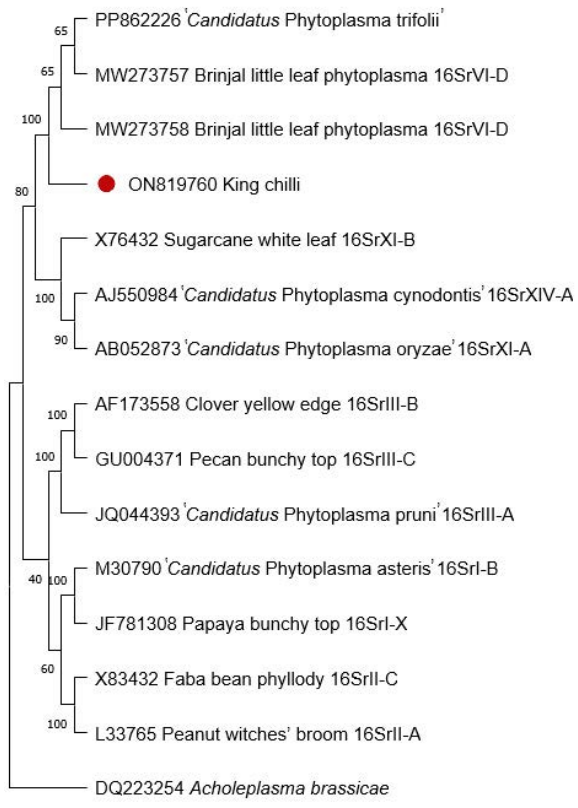


Figure 2. Phylogenetic tree constructed by maximum likelihood method of a 16S rRNA gene sequence from the king chilli and selected phytoplasmas from GenBank. *Acholaeplasma brassicae* was used as the outgroup.

Table 1. Incidence of the phytoplasma infection in king chilli in the surveyed farmers' fields.

District	Latitude and Longitude	Disease incidence (%)	Mean	Symptoms observed*
Jorhat	26.735838°N 93.980463°E	15.23	17.09	RL, SP, SI, CC, SF
	26.837657°N 94.247296°E	18.96		RL, ST, SI, CC, SP, WB
Sivasagar	26.807678° N94.57375°E	26.69		RL, SP, CC
	26.807732°N 94.57378°E	7.65	13.32	RL, SP, CC
	26.8372°N 94.4996°E	11.63		RL, SP, SI, CC
Golaghat	26.484803°N 93.929995°E	25.45	19.34	RL, CC, ST, SP
	26.511378°N 93.940826°E	13.23		RL, CC, ST, SP, SI

*ST- stunted growth, RL- reduced leaf size, SP- shortened petioles, SI-shortened internode, CC- curling and cupping, WB- witches' broom, SF- stem flattening.

Discussion

This study identified '*Ca.* P. trifolii', 16SrVI-D as being associated with the little leaf symptoms observed in king chilli across all three surveyed districts, where incidence rates ranged from 13–19%. Given king chilli's economic significance as a cash crop in the Northeastern region, further research is needed to investigate key aspects such as primary insect vector identification and biology, disease epidemiology, transmission dynamics, environmental factors, and potential resistance mechanisms. Such insights will be essential for developing effective management strategies to protect the yield, quality, and sustainability of king chilli cultivation.

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Country status

Assessment of pear decline prevalence and phytoplasma infection in major commercial orchards of Al-Mafraq, Jordan

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Abstract

Pear decline, associated with '*Candidatus* Phytoplasma pyri' (16SrX-C), is one of the most destructive phytoplasma diseases of pear worldwide. In 2023, a four month survey was conducted in commercial pear orchards across the Al-Mafraq region, Jordan. Pear decline-like symptoms, including leaf curling, reddening and stunted growth, were observed with a disease incidence ranging from 70% to 95%. A total of 200 symptomatic and 13 asymptomatic samples were collected from pear trees in four locations. Molecular detection using nested PCR with specific primers targeting the phytoplasmas in 16SrX group revealed a 100% infection rate in all tested symptomatic samples. No phytoplasma presence was detected in asymptomatic samples. The results highlight the widespread presence of '*Ca.* P. pyri' in commercial pear orchards in Al-Mafraq, indicating the need for their further molecular characterization and the development of management strategies to mitigate the disease's impact.

Keywords: '*Candidatus* Phytoplasma pyri', *Cacopsylla bidens*, epidemiological survey

Introduction

Pear decline (PD), associated with '*Candidatus* Phytoplasma pyri', impacts pear production globally, particularly in Mediterranean regions. PD symptoms, including leaf reddening, yellowing, and dieback, lead to severe declines in tree health and fruit yield, with serious implications for orchard productivity (Seemüller and Schneider, 2004).

In Jordan, pear production plays an important role in the country's agricultural economy, contributing to family consumption and local markets. Although recent studies have confirmed the presence of '*Ca.* P. pyri' in non-commercial orchards (Abu Alloush *et al.*, 2024), there are limited data on the prevalence of this pathogen in commercial pear farms, where large-scale outbreaks could result in significant economic losses. This preliminary study aims to address this knowledge gap by conducting a focused epidemiological survey of commercial pear orchards in the Al-Mafraq region, Jordan's primary pear-producing area. The objective is to assess PD prevalence and confirm the presence of *'Ca.* P. pyri' through molecular detection techniques. These findings will provide essential baseline data, forming the foundation for future research and guiding effective management strategies to mitigate the disease's impact on Jordan's pear industry.

Materials and Methods

A four-month-long survey (September–December 2023) was conducted in four commercial pear orchards across four locations in the Al-Mafraq region, Jordan: Um-Alqotain, Al-Saiedieh, Albustaneh, and Sama Al-Sarhan (Figure 1).

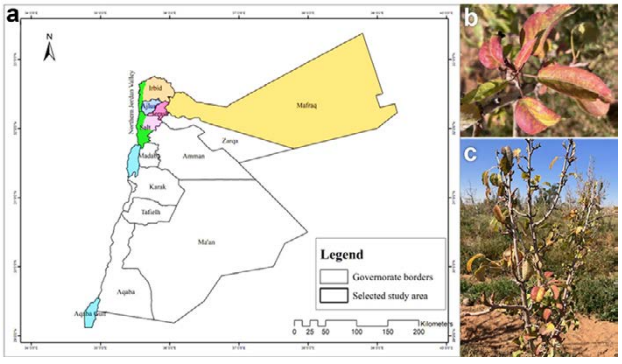


Figure 1. Map of Jordan highlighting the surveyed areas (a) and symptomatic pear trees (b and c). In panel a, the green area represents the Ajlun Governorate, where previous studies identified pear decline (PD) associated with '*Candidatus* Phytoplasma pyri' (16SrX-C). The yellow area indicates the Al-Mafraq Governorate, the focus of the current study, which aims to assess PD incidence and spread in commercial pear farms. Panel b shows typical leaf reddening, while panel c illustrates decline symptoms both associated with PD infection.

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Table 1. Pear decline (PD) disease prevalence and PCR-based detection in commercial pear orchards in the Al-Mafraq Region, Jordan.

Locations	Surveyed orchard (hectare/number of pear trees) * Same grower	Number of observed diseased pear trees in 100 trees (randomly picked)	Incidence rate	Number of asymptomatic samples tested (PCR-positive rate)	Number of symptomatic samples tested (PCR-positive rate)
Um-Alqotain	Rakan Al-Hayes (3 ha/3,000)	95	95%	50 (100%)	3 (0%)
Al-Saiedieh	Aljabaly* (5 ha/6,000)	95	95%	50 (100%)	3 (0%)
Albustaneh	Aljabaly* (8 ha/23,000)	85	85%	70 (100%)	4 (0%)
Sama Al-Sarhan	Farhan AlSaeid (1 ha/3,000)	70	70%	30 (100%)	3 (0%)
Total	4 orchards (17 ha/35,000)	345/400	86.25%	200 (100%)	13 (0%)

A total of 200 symptomatic samples was collected from pear trees exhibiting typical pear decline symptoms such as leaf curling, reddening, and stunted growth, and 13 asymptomatic samples were collected. Plant tissues (flowers, fruits, petioles, and midribs) were subject to DNA extraction using the 2.5% CTAB method. Nested PCR detection of '*Ca.* P. pyri' was performed using primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by fO1/rO1 (Lorenz *et al.*, 1995).

Results

Field surveys conducted across four commercial orchards in Al-Mafraq revealed a high incidence of pear decline symptoms. The most observed symptoms included leaf curling, reddening, stunted growth, and dieback, with varying severity across locations. A total of 200 symptomatic samples was collected from trees displaying these symptoms, along with 13 asymptomatic samples for comparison. All collected symptomatic samples were subjected to nested PCR using the primers P1/P7 and followed by fO1/rO1 specific to the 16SrX group, particularly '*Ca.* P. pyri'. The PCR results confirmed infection in all tested symptomatic samples, with a 100% positive rate. None of the asymptomatic samples tested positive for the 16SrX phytoplasmas, indicating a clear association between the presence of symptoms and phytoplasma infection.

The PCR detection results were consistent across all four surveyed locations. The infection rates, based on the number of symptomatic samples tested, ranged from 70% to 95%, depending on the orchard. The overall incidence rate for the surveyed orchards in in the Al-Mafraq Region was 86.25%. However, no significant differences were observed in the PCR confirmation of infection across different farms, with all symptomatic samples testing positive. Approximately 20% of the PCR amplicons were sequenced, confirming the presence of '*Ca.* P. pyri' and related strains (data now shown).

Discussion

This preliminary study highlights the significant presence of '*Ca.* P. pyri' (16SrX-C) in commercial pear orchards in Al-Mafraq, Jordan. The high presence of pear decline disease emphasizes the need for effective management strategies in the region's commercial farming systems. Early detection is essential for timely intervention to limit the spread of the disease.

The slight variation in incidence rates between locations suggests that environmental or management factors may influence the disease spread. Further research is needed to investigate these influences and tailor interventions accordingly. As this was a preliminary survey, future studies should expand geographically and temporally, covering different regions and seasons to account for variations in disease prevalence. Investigating the role of *Cacopsylla bidens* in transmission is also essential to understand its possible impact on the spread of PD in Jordan.

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Country status

Identification of 16SrII-D phytoplasmas in sesame and groundnut crops in Tirupati district of Andhra Pradesh, India

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Abstract

Phyllody, stunting and little leaf symptoms were observed in sesame and groundnut crops in experimental plots of Regional Agricultural Research Station, Tirupati District of Andhra Pradesh during the "kharif" season of 2024. The presence of phytoplasmas was confirmed in symptomatic samples using nested PCR assays with phytoplasma-specific universal primer pairs (P1/P7 and R16F2n/ R16R2) and *rp* multilocus gene primers. Sequence analysis of 16S rRNA, *rp* genes and virtual RFLP analysis indicated that phytoplasmas affecting both groundnut and sesame are members of the group 16SrII '*Candidatus* Phytoplasma australasiae=australasiaticum', subgroup 16SrII-D. This information is critical to support further development of targeted strategies to control phytoplasma diseases.

Keywords: phyllody, groundnut little leaf, *rp* gene, '*Candidatus* Phytoplasma australasiae=australasiaticum', virtual RFLP analysis

Introduction

Sesame or gingelly (*Sesamum indicum* L.), Pedaliaceae, is an economically significant oilseed crop, known for its high oil yield and nutrient density. India holds the world second position in sesame cultivation, with an area of 1.73 million hectares and a production of 0.78 million tons, accounting for 12.4% of global sesame production. Major diseases include *Alternaria* leaf spot, powdery mildew and phyllody (Kolte, 2018; Rajpurohit, 1993; Rao *et al*., 2015). Phyllody is a very serious disease with the potential to reduce yields up to 80% (Kumar and Mishra, 1992). Groundnut (*Arachis hypogaea* L.), Fabaceae, is a vital oilseed crop known for its high protein content. India ranks world's second-largest groundnut producer totaling 10 million tons and is first world's producer by cultivation area. Major diseases include *Sclerotium* rot, "tikka" leaf spot, viral diseases such as PNBV (peanut bud necrosis virus) and PSNV (peanut stunt necrosis virus), and phytoplasmas, whose occurrence has increased in the last years posing a threat for the crop.

Materials and Methods

A phytoplasma survey was performed in sesame and groundnut crops in the experimental plots of Regional Agricultural Research Station, Tirupati during the "kharif" season of 2023 to 2024. Leaves were collected from ground nut and sesame plants showing symptoms of phyllody, shortening of internodes and little leaf and also from asymptomatic plants. DNA was extracted from collected leaves using the CTAB method (Ahrens and Seemüller, 1992) and stored at -20^{0}C for further use. The extracted DNA was used as a template in PCR with phytoplasma 16S rDNA primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), and nested PCR using R16F2n/R16R2 (Gundersen and Lee, 1996), which yielded amplicons of about 1.8 kb, and 1.2 kb, respectively. Amplification of the ribosomal protein (*rp*) gene with primer pairs rp(II)F/rp(I)R1A and rp(II)F2D rp(I)R1A in nested reaction (Martini *et al*., 2007) was performed to support phytoplasma identification. Amplicons were cloned and sequenced; sequences were assembled using BioEdit 7.2, aligned with ClustalW and used to construct a phylogenetic tree with the neighbour joining method (MEGA 7.0). *In silico* RFLP with the *i*PhyClassifier online tool (Zhao *et al*., 2009) was used for phytoplasma subgroup identification.

Results

Little leaf and stunting in ground nut (Figure 1) and phyllody in sesame (Figure 2) showed an incidence of 4-12%. Amplicons of about 1,390 bp and 1,240 bp were consistently

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obtained for the 16S rRNA and *rp* genes, respectively for both symptomatic groundnut and sesame samples. No amplification was detected in symptomless plants collected from non-symptomatic fields. BLASTn analysis of the 16S rRNA partial gene sequences of phytoplasmas from both sesame (GenBank accession number PQ056968) and groundnut (GenBank accession number PQ097752) showed a 99.92% identity to '*Candidatus* Phytoplasma australasiae=australasiaticum' (GenBank accession number Y10097) and 100% sequence identity with the sequences of phytoplasmas associated with soybean witches' broom (GenBank accession number OQ361779), sunflower phyllody (GenBank accession number MK421430) and faba bean phyllody (GenBank accession number. KP869129). Similarly, BLASTn analysis of the *rp* gene of the sesame strain (GenBank accession number PQ062534) and groundnut strain (GenBank accession number PQ062535) showed 99.57% to 100% sequence identity with those of sweet potato witches' broom phytoplasma (GenBank accession number EF193376), alfalfa witches' broom phytoplasma (GenBank accession number EF193371), peanut witches' broom phytoplasma (GenBank accession number OR861615) and other phytoplasma strains of the 16SrII group. The virtual RFLP analysis of the R16F2n/R2 sequence using 17 restriction enzymes was performed for further confirmation of phjytoplasma identity. Comparison of the virtual RFLP profiles confirmed that they were identical to those of reference strain of the 16SrII-D subgroup (GenBank accession number Y10097) with similarity coefficient 1.00. These results confirmed that the phytoplasmas associated with sesame and groundnut diseases belong to 16SrII-D subgroup.



Figure 1. Stunting and little leaf symptom of groundnut; healthy plant is on left.



Figure 2. Phyllody symptom in sesame; healthy plant is on left.

Discussion

Phytoplasma diseases cause severe economic losses to many oil crops including sesame, peanut, mustard, soybean, sunflower, linseed, and canola in Asia (Vemana *et al.*, 2023). It appears that 16SrI and 16SrII phytoplasma groups are prevalent in different Asian countries. Sandhya *et al.* (2024) reported 16SrI-B and 16SrII-D phytoplasma subgroups associated with sesame phyllody in Andhra Pradesh. Similarly, the 16SrII-D subgroup was associated with groundnut little leaf and excessive shoot proliferation disease in Andhra Pradesh. Further research is needed to identify and characterize the specific strains within the 16SrII group in different geographical regions of India and understand how severely they impact different oilseed and hosts like sesame and groundnut. This knowledge would help developing specific strategies to reduce 16SrII-D phytoplasmas impact in the Indian agroecosystems.

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Country status

Unveiling the hidden threat: a comprehensive study on groundnut phytoplasmas in the major growing regions of Andhra Pradesh, India

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Abstract

Groundnut is one among India's most significant food and cash crops and it is also an oilseed crop. It is affected by several fungal, bacterial and viral diseases, which plays a significant impact in lowering its yields. Among the many diseases that affect groundnut, phytoplasma associated diseases were once thought to be rather unimportant. However, the disease's incidence has been increasing recently and it was likely posing a threat to groundnut farming. An investigation was carried out to identify the symptoms and disease incidence of groundnut phyllody, little leaf, witches' broom in Andhra Pradesh. A roving survey was conducted across five major groundnut growing districts to assess the incidence of phytoplasmas in the groundnut cultivations, *i.e.*, Anantapur, Kadapa, Kurnool, Sri Satya Sai and Tirupati districts in 2023 "kharif" season. Regarding the symptomatology of phytoplasma of groundnut there is great confusion with peanut bud necrosis, peanut stem necrosis, peanut clump disease, and peanut stunt disease as they share some similar symptoms.

Keywords: phyllody, little leaf, witches' broom, peanut, disease

Introduction

Groundnut (*Arachis hypogaea* L.) belongs to the family Fabaceae also known as earthnut and peanut. It is believed to have originated in the southern Bolivia to northern Argentina region of South America. It is popularly known as the unpredictable legume and energy capsule. In India, groundnut is regarded as the "king of oilseeds". It is one among India's most significant food and cash crops, as an oilseed crop, it contains 40% to 53% oil and 24–36% protein. Groundnut is produced on 327 lakh hectares worldwide and have a productivity of 1,648 kg per hectare, yielding 539 lakh tonnes all over the world (FAO, 2021). Groundnut is affected by several fungal and viral diseases in India, which plays a significant impact in lowering yields. There have been reports of more than 55 diseases affecting groundnut (Ghewande *et al.*, 1983; Subrahmanyam *et al.*, 1985). Among the many diseases that affect groundnut, phytoplasma-related diseases were once thought to be rather unimportant. However, the disease's incidence has been increasing recently and was likely posing a threat to groundnut farming. Phytoplasmas are obligate wall less intracellular pathogens of plants that belong to class mollicutes. They are surrounded only by a membrane, pleomorphic in shape with an average diameter of 0.2- 0.8 µm. Phytoplasma contains one circular double stranded DNA molecule and are associated with diseases in over 1,000 plant species worldwide, including agricultural, horticultural, ornamental, trees and weed plants. Among oil seeds sesame, soyabean, brassicas are reported infected by different phytoplasma strains. In groundnut crop in India the phytoplasma presence was also recently reported (Mitra *et al.*, 2020). There is little information available on groundnut phytoplasma infection in India. So, an attempt was made to study the disease incidence and symptomology of groundnut phytoplasmas in the major groundnut growing districts of Andhra Pradesh.

Materials and Methods

A roving survey was conducted in farmer's fields and groundnut experimental plots at the research station in major groundnut growing districts of Andhra Pradesh (Anantapur, Kadapa, Kurnool, Sri Satya Sai and Tirupati) during the 2023 "kharif" season. Three mandals in two districts and two mandals in three districts were surveyed which included at least one village from each mandal.

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Table 1. Incidence of groundnut phytoplasma diseases in the major groundnut growing districts of Andhra Pradesh.

District	Village	Location	N. of observed fields	Symptoms	Disease incidence (%)
Sri Satya Sai	Kadiri	Research plots, ARS fields	3	Little leaf, phyllody, witches' broom, pegs with negative geotropism	21
	Malakavemula	Farmer fields	3	Witches' broom, little leaf	20
	Cherlapalli	Farmer fields	3	Little leaf, pegs with negative geotropism	18
Anantapuram	Rekulagunta	Research plots, ARS fields	3	Witches' broom, little leaf, pegs with negative geotropism	16
	Muttala	Farmer fields	3	Witches' broom, pegs with negative geotropism	15
	Vepalaparthy	Farmer fields	3	Little leaf, pegs with negative geotropism	12
Kurnool	Hampa	Farmer fields	3	Little leaf, pegs with negative geotropism	11
	Banavasi	KVK fields, farmer fields	3	Little leaf, phyllody	8
Kadapa	Utukuru	Research plots, ARS fields	3	Little leaf, pegs with negative geotropism	6
	Chinnalebaka	Farmer fields	3	Witches' broom, little leaf	4
Tirupati	Perur	Research plots, ARS fields	3	Little leaf, phyllody, witches's broom, pegs with negative geotropism	3
	Perur	Wetland farm	3	Little leaf, phyllody, pegs with negative geotropism	1

In each groundnut field, a plot area of 3 x 3 m was selected and the total number of healthy and symptomatic groundnut plants showing phytoplasma suspected symptoms were recorded calculating the percentage of disease incidence. Three spots were randomly selected in each field and the mean of disease incidence was calculated in individual field/experimental plots by averaging the incidence of three spots at each location by using the formula:

$$\text{Percent disease incidence} = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Results

The predominant symptoms observed in diseased groundnut plants were little leaf, phyllody, witches' broom (proliferation of axillary buds and shortening of internodes that leads to bushy appearance), inverted "v" shaped drying from the tip of the leaf, bright chlorotic spots, stem splitting, negative geotropism of pegs with malformed pods or absence of pod formation (Figure 1). These symptoms are as similar to those reported for peanut little leaf disease reported to occur in other countries (Martini *et al*., 2018). Disease incidence in the fields of groundnut in Andhra Pradesh ranges from 18-21% in Sri Satya Sai district, 12-16% in Anantapur district, 8-11% in Kurnool district, 6-4% in Kadapa districts and 1-3% in Tirupati districts (Table 1). The infected varieties include K6, K9, TAG-24, Lepakshi and in all the districts the major symptoms were little leaf, phyllody, witches' broom and negative geotropism of pegs with absence or malformed pods.



Figure 1. Symptoms in groundnut infected by phytoplasmas.

Discussion

The disease incidence was higher in Kadiri (18-21%) region of Sri Satya Sai district and lower in Perur region (1-3%) of Tirupati district and characteristic symptoms were also recorded such as little leaf phyllody, phyllody, witches' broom, pegs with negative geotropism. The molecular characterization of phytoplasma in infected groundnut samples are performed for confirmation of phytoplasma presence. Future epidemiological studies will be aimed at identification of the weeds and insect vector to manage this economically important disease in India.

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Country status

Preliminary identification of phytoplasmas infecting flower crops in Andhra Pradesh, India

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Abstract

In 2022-2023, phytoplasmas were identified in all the major flower crops of the Andra Pradesh state. The key symptoms identified were witches' broom, phyllody, little leaf, virescence, stunting and yellowing of leaves. A nested polymerase chain reaction assay was performed using universal and *secA* gene specific primers for detection and characterization of phytoplasmas from the symptomatic tissues. Phylogenetic analysis revealed that marigold phytoplasma strains belong to the 16SrI group, while those infecting crossandra, China aster, chrysanthemum, rose, and jasmine belong to the 16SrII group, associated with aster yellows and peanut witches' broom phytoplasmas, respectively. The restriction fragment length polymorphism profiles generated through *i*PhyClassifier confirmed the phytoplasma identification.

Keywords: flower crops, aster yellows, peanut witches' broom

Introduction

Loose flower production is commercially important in India, and Andhra Pradesh state is a prominent contributor to this industry. Phyllody is the most important phytoplasma disease in many flower-growing areas of India, causing severe losses to the flower industry. While phytoplasma diseases of flower crops have been reported extensively in India from the past two decades, their occurrence in Andhra Pradesh has remained largely unstudied. Phytoplasma symptoms have now been observed in Andhra Pradesh in numerous flower crops such as marigold, crossandra, rose, jasmine, China aster and chrysanthemum. Phytoplasmas-associated diseases of these flower crops have been reported from several parts of the world (Bellardi *et al*., 2018). The aim of the current study was to investigate the distribution of phytoplasma diseases in the major flower-growing areas of Andra Pradesh and to identify and classify them through PCR assays, sequencing and RFLP analysis.

Materials and Methods

During 2022 and 2023, extensive surveys were conducted throughout the year in the farms and nurseries of important flower growing areas of Andhra Pradesh to verify the presence of phytoplasmas. Symptomatic leaf samples were collected from all the major flower crops (Table 1, Figure 1).



Figure 1. Symptoms of phytoplasma infection in flower crops: (a) China aster little leaf and (b) phyllody; (c and d) *Chrysanthemum* phyllody; (e) rose phyllody; (f and g) marigold witches' broom (h, i and j), phyllody (k) virescence (l) dwarf; (m) crossandra little leaf (n) jasmine little leaf.

DNA was extracted from the symptomatic leaf samples using CTAB method (Ahrens and Seemüller, 1992). PCR analyses were performed using P1/P7, R16F2n/R2 and SecAfor2/Rev3 primers (Deng and Hiruki, 1001; Schneider *et al*., 1995, Gundersen and Lee, 1996, Hodgetts *et al*., 2008) for the detection

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Table 1. Samples tested in PCR.

	Symptom tested	Variety	Location
Marigold	Phyllody	Ashtagandha	Annamayya
	Little leaf/ phyllody	Supreme yellow	Tirupati
	Little leaf/ phyllody	Begitall yellow	Chittoor
	Witches' broom	Victor 2	Kadapa
	Little leaf/ stunting	Tennis ball plus	West Godavari
	Virescence	Orange glory	Ananthapur
	Witches' broom	Ashoka yellow	Ananthapur
Crossandra	Little leaf	Pileru local	Annamayya
Jasmine	Little leaf/ wire stem	*Jasminum sambac*	Tirupathi
China aster	Phyllody	Arka kamini	Tirupati
	Little leaf	Violet cushion	Chittoor
Chrysanthemum	Phyllody	Red gold	Ananthapur
	Phyllody	Bullet white	Chittoor
Rose	Phyllody	Arka savi	Annamayya

of phytoplasmas. RFLP analysis was performed in the *i*PhyClassifier using sequences from the nested-PCR amplified products. The partial 16S rDNA nucleotide sequences of identified phytoplasmas were compared using the BLAST algorithm with the sequences available in GenBank. Phylogenetic analyses were carried out using MEGA7 software.

Results

Although all the collected plant samples showed clear symptoms of phytoplasmas, only a few plants tested positive for their presence. PCR products amplified with R16F2n/R16R2 and SecAfor2/Rev3 primers were obtained from marigold plants with witches' broom symptoms, rose, chrysanthemum and China aster plants with phyllody symptoms, and crossandra and jasmine with little leaf symptoms. These symptoms were mainly observed during autumn and early spring seasons. The sequence analysis of the 16S rRNA gene confirmed the presence of '*Candidatus* Phytoplasma asteris' in marigold and '*Ca.* P. australasiae=australasiaticum' in rose, chrysanthemum, China aster, crossandra, and jasmine. The virtual RFLP patterns derived from the R16F2n/R2 fragment of marigold phytoplasma strains are identical (similarity coefficient 1.00) to the reference strain of 16SrI-B subgroup (GenBank accession number M30790), while for rose, chrysanthemum, China aster, crossandra and jasmine the phytoplasma strains are 16SrII-D subgroup (GenBank accession number Y10097).

Discussion

Extensive survey in ornamentals revealed that witches' broom symptoms were observed in marigold plants positive for '*Ca.* P. asteris', while phyllody symptoms were identified in rose, chrysanthemum, China aster and little leaf symptoms in crossandra and jasmine plants infected by '*Ca.* P. australasiae=australasiaticum'. Aster yellows phytoplasma was reported in most parts of the country and its negative impact on flower production was evidenced. Across India, in various flower crops are reported phytoplasma-induced diseases due to strains of 16SrI and 16SrII groups (Rao *et al*., 2011). Moreover, 42 different ornamental plant species are known to be susceptible to these two phytoplasma ribosomal subgroups (Chaturvedi *et al*., 2009). Among those the flower crops are hosts for 16SrI, 16SrII and 16SrIII phytoplasma which induces symptoms of phyllody, witches' broom and little leaf (Esmailzadeh Hosseini *et al*., 2011; Rojas-Martinez *et al*., 2003; Panda *et al*., 2021; Raj *et al*., 2011). Work on finer classification of the identified phytoplasmas is in progress

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Country status

Multigene characterization of phytoplasmas associated with flat branch disease in sweet cherry and their effects on some fruit properties

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Abstract

Cherry trees occupy a prime position among temperate fruit crops all over the world with a high nutritional value and abundant phenolic compounds contributing greatly to health benefits. Symptoms of flat branching characterized by flat, highly compressed branches with a rough corrugated surface, twisted at the tip, were observed in some cherry cultivars at ICAR-CITH experimental field. The phytoplasma presence was confirmed through multigenic approach which shows presence of '*Candidatus* Phytoplasma asteris' and '*Ca.* P. trifolii' strains. The incidence of flat branch and witches' broom symptoms associated with '*Ca.* P. trifolii' varied from 5.8 to 25% in cultivars Bigarreau Nepoleon (Double), Bigarreau Noir Grossa and CITH-Cherry-9. However, incidence of leaf rolling, swollen nodes and bud proliferation associated with '*Ca.* P. asteris' was 7.5% in cultivar Stella and 10% in Sunburst, respectively. The multigene characterization of sweet cherry phytoplasma strains confirmed the validity of these molecular markers for identification of phytoplasmas that are enclosed in 16SrI and 16SrVI groups, respectively. During the study it was observed that flattened branches produce very little annual growth and subsequently affected spur production during next year. The negative effect of branch flattening was observed on set, growth, and retention of fruits. It delayed phenological events related to fruit development and maturity. Moreover, compared to normal branches, the flat branches produce low quality fruits in terms of size, weight, color, and biochemical properties.

Keywords: *Prunus avium*, flat branching, multigene characterization, vascular system, fruit quality

Introduction

Sweet cherry (*Prunus avium* L.) is a stone fruit belonging to the Rosaceae family. Sweet cherries are believed to have originated near the Caspian and Black Seas on the European and Asian borders and are now commercially grown in many countries around the world. Cherries grow best under temperate conditions and Kashmir valley alone contributes about 90% of the total cherry production of the country. A flat branching (FB) was observed in Bigarreau Noir Grossa cultivar first during mid spring of 2016-17 and since then it was observed in other genotypes CITH-Cherry-9, CITH-Cherry-6 and CITH-Cherry-8, Bigarreau Nepoleon, CITH-C-14 in subsequent years at ICAR-Central Institute of Temperate Horticulture, Srinagar in Jammu and Kashmir. The symptoms of FB were closely matching the symptoms associated with phytoplasma presence in other crops such as *Tinospora cordifolia* and *Impatiens balsamina* (Seif and Gruppe, 1985; Somashekhara *et al.*, 2015). Several strains of phytoplasmas have severe effect on both quality and yield of cherry (Kirkpatrick *et al.*, 2011; Wang *et al.*, 2014; Wright *et al.*, 2021). Based on the above information, the effects of flat branching associated with phytoplasma infections was studied in cherry fruits.

Materials and Methods

The study was carried out in a cherry orchard located at ICAR-Central Institute of Temperate Horticulture, Srinagar, Jammu and Kashmir, Union territory of India (33.58'N, 74°48'E and 1644 m above sea level) to find out the effect of flat branching on phenological events, set, growth, retention and quality of fruits. The soil of the experimental field is clay loam in texture with medium-to-low fertility status having pH 6.93 and 0.37 dSm-1 EC. The cherry genotypes CITH- Cherry selections, CITH-Cherry-9, CITH-Cherry-6 and CITH-Cherry-8, CITH-C-14, Bigarreau Nepoleon (Double) Bigarreau Noir Grossa (Mishri) were selected for the study. DNA was extracted from the sample using a CTAB method (Ahrens and Seemüller, 1992). Nested PCR was carried out to

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amplify 16S rRNA, *secA, rp, tuf* and *secY* genes with phytoplasma-specific primers in all symptomatic sweet cherry cultivars. Bioinformatic analysis was carried out for assessing phenotypic parameters of fruits like fruit retention, diameter, firmness and colour that were also recorded. Biochemical parameters as total soluble sugars (TSS), sugars and phenolic content and DPPH assays were measured to verify the presence of biochemical changes associated with the phytoplasma presence in cherry.

Results

The symptoms observed were flat, highly compressed branches with a rough corrugated surface, and twisted at the tip (Figure 1). During 2019–2021 other symptoms were observed such as leaf roll, swollen nodes, and witches' broom in five cultivars of sweet cherry. Phytoplasma presence was confirmed by amplifying 16S rRNA, *secA, rp, tuf* and *secY* genes with phytoplasma-specific primers in all symptomatic sweet cherry cultivars in nested PCR assays. Pairwise sequence comparison, phylogeny and virtual RFLP (16S rRNA gene) analysis confirms the presence of '*Candidatus* Phytoplasma trifolii' in the samples from flat branch and witches' broom symptoms. The disease incidence varied from 5.8% to 25% in cultivars Bigarreau Nepoleon (Double), Bigarreau Noir Grossa and CITH-Cherry-9. Moreover, leaf rolling, swollen nodes and bud proliferation was associated with '*Ca.* P. asteris' presence and show incidence of 7.5% in cultivar Stella and 10% in Sunburst, respectively.



Figure 1. Flat branching in cherry: (a) branch in initial stage of flattening, (b) advanced stage in flattening, (c) fully flattened cherry branch, (d) comparison of a healthy and a flat branch, (e) cross-section of a healthy branch and (f) cross-section of a flat branch.

The fruit length, width, thickness and geometric mean diameter were comparatively higher in fruits harvested from normal branches in all the genotypes. Flat branches produced very little annual fruiting shoot growth compared to normal branches. Irrespective of the genotype, fruits harvested from flat branches recorded low TSS content (12.61 to 14.01°B) compared to fruits obtained from normal branches (13.08 to 15.61°B). The total color difference between fruit harvested from flat branches compared to normal branches at rectangle coordinates were 8.75, 6.14, 10.10 and 7.07 for CITH-C-6, CITH-C-8, CITH-C-9 and BNG, respectively. Anthocyanin content revealed that fruits harvested from normal branches had significantly higher ($p<0.01$) content of anthocyanin (129.34-213.98 mg/100g pulp) compared to fruits harvested from flat branches (112.41-200.35 mg/100g pulp).

Discussion

The multigene characterization of sweet cherry phytoplasma strains confirmed the validity of these molecular markers for identification of phytoplasmas enclosed in 16SrI and 16SrVI groups. The presence of phytoplasmas in sweet cherry is the first report from India. During the study it was observed that flattened branches produce very little annual growth and subsequently affected spur production during the next year. The negative effect of branch flattening was observed on fruit set, fruit growth, and fruit retention. It delayed phenological events related to fruit development and maturity. Moreover, compared to normal branches, the flattened branches produce inferior quality fruits in terms of size, weight, color, and biochemical properties.

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Country status

Morpho-pathological and molecular characterization of a '*Candidatus* Phytoplasma australasiaticum' strain from linseed

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Abstract

Linseed has rapidly emerged as a novel host to phytoplasmas in various parts of the world particularly in Indian subcontinent recently. The expansion of phytoplasma incidence on linseed and its geographical distribution is an alarming threat amid climatic fluctuations. Morpho-pathological and molecular analysis of the '*Candidatus* Phytoplasma' strain associated with linseed phyllody and stem fasciation was undertaken. Phytoplasma like bodies were observed in phloem tissues through transmission electron microscopy. For the molecular characterization, the presence of phytoplasmas in symptomatic samples was confirmed by nested PCR-based 16S rRNA and *secA* genes. The sequences of the 16S rRNA and *secA* genes identified '*Candidatus* Phytoplasma australasiaticum' presence in the symptomatic samples.

Keywords: flax, geographical locations, *Linum usitatissimum*, phyllody, phytoplasmas

Introduction

Linum usitatissimum L., commonly known as linseed or flax, is one of the key oil and fiber yielding crop species having prodigious applications in the food, textile, paint and pharmaceutical sectors. Numerous biotic stresses are the major impediments in achieving yield stability and sustainability in linseed production amid the rising global demands (Kaur *et al*., 2023). Phytoplasma-associated maladies have been reported in diseases of major crops. Flax phyllody was first documented in Canada in 1957, where it caused an epidemic that resulted in significant yield losses (Saharan and Mehta, 2005). Akhtar *et al*. (2013) identified a strain wrongly identified as '*Candidatus* Phytoplasma aurantifolia' in plants of flax showing phyllody and stem fasciation in Pakistan. In India, the first report of phytoplasma infection in flax was by Biswas *et al*. (2014) who identified '*Ca*. P. asteris' (16SrI-B) in flax plants with phyllody and stem fasciation symptoms. Ajithkumar *et al*. (2021) reported linseed to be a new host for '*Ca*. P. cynodontis' (16SrXIV-A) and recently, Yadav *et al*. (2024) reported '*Ca*. P. australasiaticum' (16SrII-D) in the linseed germplasm accessions from North India. The study of a phytoplasma strain associated with phyllody and stem fasciation in linseed from a new geographical location in India is reported here.

Materials and Methods

The linseed germplasms were sown at ICAR-NBPGR, experimental farm, IARI, New Delhi (28°38'53.7"N, 77°09' 05.4"E at 218 m above sea level) as per the methodology and experimental details reported in Kaur *et al*. (2023). Disease incidence for each plot of symptomatic accession was recorded by counting the number of symptomatic plants and dividing them by total number of plants in the plot (Prasad *et al*., 2024). The observations were recorded upon attainment of physiological maturity (80% of the plants with browning of capsules) in three replicates.

Infected tissue was excised from the representative plants of symptomatic as well as asymptomatic accessions in three replicates. Total genomic DNA was extracted using 0.5 g of tissue following a CTAB method (Doyle and Doyle 1987). Qualitative and quantitative analysis of DNA was carried out spectrophotometrically using Nanodrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Scientific™). An amount of 50 ng/µl DNA was subsequently used as a template amplification with phytoplasma universal primer combination P1/P7 (Deng and Hiruki 1991; Schneider *et al*., 1995) followed by R16F2n/R16R2 (Gundersen and Lee, 1996) using TaKaRa LA Taq® DNA polymerase (Takara Bio). The PCR product was diluted 1: 30 to be used as template for nested PCR with primer pair R16F2n/R16R2. To further confirm the phytoplasma identity the conserved partial housekeeping gene *secA* was amplified and Sanger sequenced. Representative 16S rRNA gene sequences were retrieved from the NCBI GenBank database for phylogenetic comparisons and identified using *i*PhyClassifier online tool

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(Zhao *et al.*, 2009). The presence of phytoplasmas in the sieve tubes of phloem tissue of symptomatic plant was observed by stereo binocular microscope followed by transmission electron microscopy (TEM) as per protocol by Musetti *et al.* (2023). Six samples from each asymptomatic or symptomatic plant were cut into ultrathin sections stained and then observed under TEM JEM-1011 (JEOL USA,) operated at 100 kV.

Results and Discussion

Disease incidence from 10 to 20% was recorded in different plots in plants 45 days after sowing. The symptomatic plants exhibited flattened fasciated stem axis having sparsely distributed leaves with the reduced area, twisted inflorescence with leaves closely appressed at apex, malformed flowers and capsules. The overall yield reduction was up to 68.9%. The samples produced phytoplasma-specific amplification of expected size only from symptomatic plants and control. No amplification was obtained in the asymptomatic samples collected from the same field. Sequence analysis of the PCR products was showing 100% identity among themselves. The 16S rRNA and *secA* gene sequences analysis indicated that the detected strains had 99.93% identity with '*Ca.* P. australasiaticum' (Yadav *et al.*, 2024). The comparative virtual RFLP profiling was found to be identical (similarity coefficient 1.00) to the reference pattern of 16Sr group II, subgroup D (GenBank accession number Y10097) and phylogenetic analysis based on the *secA* gene also confirmed this identification (data not shown). Electron microscopy of ultrathin sections of fasciated stems and leaf tissue displayed the presence of numerous wall-less spherical to oval pleomorphic phytoplasma like bodies (Figure 1). Xue *et al.* (2018) reported that the disruption in the grana and stroma lamellae structure of chloroplasts may lead to impaired photosynthetic efficacy leading to aberrant carbohydrate accumulation in the infected plants. The reported findings showed that the presence of phytoplasmas leads to various ultrastructural changes which may be attributed to the accumulation of sugars and starch, thereby disturbing sieve tube functionality (Musetti *et al.*, 2023). Therefore, intervening the genes encoding enzymes involved in phytoplasma adherence to the cell membrane may be a potential route to check disease spread. The present study highlights the incidence of phytoplasma in linseed from northern India.

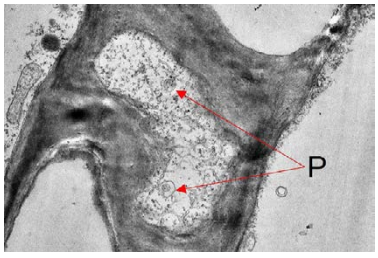


Figure 1. Transmission electron microscopy image of the phloem tissue of a phytoplasma infected linseed plant showing the presence of pleomorphic bodies (P).

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Country status

Identification of phytoplasma disease infection in forest trees in Jammu region, India

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Abstract

Forest trees always have been of great importance to mankind since prehistoric times. They are important, valuable and necessary to the existence of life. Phytoplasmas are important group of plant pathogens which drastically damage growth and commercial value of forest trees. During a survey of different districts of Jammu and Kashmir during 2022-2023, suspected phytoplasma symptoms were observed on *Cassia fistula* ("amaltas") and *Toona ciliata* ("tooni") growing in roadsides and forests. The majority of the symptoms recorded were leaf yellowing, witches' broom, stunting, phyllody and proliferation of shoots. Total DNA was extracted from the symptomatic trees and phytoplasma presence was confirmed by nested PCR assays using universal primer pairs P1/P7 and R16F2n/R16R2 in both "amaltas" and "tooni" trees. The preliminary analyses indicated the presence of '*Candidatus* Phytoplasma asteris' in these two forest trees of Jammu region, India.

Keywords: *Toona ciliata*, phyllody, peduncle, *Cassia fistula*

Introduction

"Toona" (*Toona ciliata*), commonly known as red cedar or toon (tun), is a genus in the mahogany family, Meliaceae native from Afghanistan to India. It is an important timber tree. It provides a valuable hardwood used for furniture, ornamental panelling, ship building, and musical instruments like the sitar, "rudra veena" and drums (Singh *et al*., 2021) "amaltas" (*Cassia fistula*) is widely grown as an ornamental plant for landscapes, grown in tropical and subtropical areas. It is also known as golden shower due to its yellow flower. In "ayurveda", this tree is known as "aragvadh", meaning disease killer and the fruit pulp is purgative and also ingredient of some mass-produced herbal laxative, flower extract has an antiaging property and used in a variety of cosmetic (Mwangi *et al*., 2021). Phytoplasmas associated with 700 plant diseases worldwide, belongs to class *Mollicutes* and inhabit the sieve tubes of infected plants and haemolymphs of insects (Bertaccini *et al*., 2014). Many important phytoplasma diseases have been reported on trees and ornamentals plant causing severe losses worldwide (Rao *et al*., 2020).

Material and Methods

Surveys were conducted during the years 2022-2023 across the major forest growing areas in Jammu and Kashmir, India. Bushy appearance of subbranch due to shorten internodes and reduction in leaf size (phyllody-like structure) were seen on *T. ciliata* trees in Digiana park of Jammu. Moreover, flat peduncle and pedicel of inflorescence and unopened buds of flower of *C. fistula* were randomly observed at Kalidhar hills of Jammu. The symptomatic leaves and young growing shoots and infected samples were collected. The disease incidence of phytoplasma suspected symptoms in *T. ciliata* and *C. fistula* was recorded on the basis of visual observation. Five to seven symptomatic leaves from both tree species were collected for phytoplasma detection and molecular characterization. The total genomic DNA was extracted by CTAB method (Ahrens and Seemüller, 1992). PCR amplification was performed employing primer pair P1/P7 followed by nested primers R162Fn/R16R2 (Gundersen and Lee, 1996). Furthermore, phytoplasma primer pairs secAfor1/secArev3 followed by secAfor2/secArev3 were employed for *secA* gene analysis (Hodgetts *et al*., 2008). The amplified 16Sr RNA gene fragments of *T. ciliata* and *C. fistula* phytoplasmas were purified, cloned and sequenced using the M13Fwd/M13Rev universal primer pair in both directions at Biologia India, Bangalore. Phylogenetic analysis was performed using Maximum-likelihood method in MEGA 11 with 1,000 bootstrap replications. *Acholeplasma palmae* 16Sr RNA gene sequence was used as outgroup sequence in phylogeny comparison.

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Results and Discussion

Bushy appearance of subbranch were observed in *T. ciliata* trees due to shorten internodes and reduction in leaf size (phyllody-like structure) in Digiana park of Jammu region (Figure 1). Flat peduncle and pedicel of inflorescence and later on, unopened flower buds of *C. fistula* were randomly observed at Kalidhar hills of Jammu. The incidence of the disease in the two species was recorded from 13% in growing region of Jammu during the surveys.



Figure 1. Phytoplasma symptoms of *T. ciliata* exhibiting bushy appearance (witches' broom) of subbranch due to shorten internodes and reduction in leaf size (phyllody-like structure).

DNA fragments of approximately 1.25 kb and 480 bp were amplified from all symptomatic samples with primer pair R16F2n/R16R2 and secA2/secArev3, respectively. However, no amplifications were observed from DNA extracted from asymptomatic plant tissues samples from both tree species from all the surveyed areas. The specific amplicon was sequenced bi-directionally and sequences were assembled. Representative 16S rRNA and *secA* genes nucleotide sequences amplified from the symptomatic *T. ciliata*, and *C. fistula* leaf samples were deposited in GenBank database under accession numbers OP801671 and OP801672, respectively. BLASTn analysis revealed that 16S rRNA gene sequences of both tested phytoplasma strains shared 100% identity with '*Candidatus* Phytoplasma asteris' (16SrI-B). The phylogenetic analyses of 16S rRNA gene sequences of *T. ciliata* and *C. fistula* phytoplasma strains in the study showed clustering with 16SrI phytoplasma group strains belonging to 16SrI-B subgroup.

To date, 11 phytoplasma ribosomal groups (16SrI, -II, -III, -V, -VI, -IX, -XII, -XIV, -XIX, -XXI, -XXX) and 48 subgroups have been reported to be associated with trees and desert trees in Asia (Marcone *et al*., 2022). Earlier, 16SrV and 16SrI-B phytoplasmas were reported from north-western Himalayan states of India (Shreenath *et al*., 2022; Gupta *et al*., 2023). In the present study, the occurrence of 16SrI-B subgroup phytoplasma strain in *T. ciliata* and *C. fistula* from Jammu and Kashmir indicated the wider spread of phytoplasma disease at newer locations, which is alarming and needs immediate attention towards planning suitable management strategy. Considering the broad host range of '*Ca.* P. asteris' and capability of transmission through insect vectors and weeds in India (Rao, 2021), further studies are suggested to work on epidemiology of tree phytoplasma strains to avoid further spreading of the disease in other states and in other important crops of the Country.

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Country status

Status and challenges of sandal spike disease in Indian forest: an analysis

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Abstract

Sandalwood is predominantly found in the forests of southern India, with this region accounting for 90% of the sandalwood distribution in the country. The primary cause of declining sandalwood resources in southern India is infection by the '*Candidatus* Phytoplasma asteris' (16SrI-B), a member of the aster yellows group of phytoplasmas. This study provides a brief overview of the current status of sandal spike disease in the primary forest habitats of Kerala, Karnataka, and Tamil Nadu, as well as the challenges involved in managing this disease.

Keywords: *Santalum album*, phytoplasma, aster yellows

Introduction

Phytoplasmas are a large, diverse group of obligate, cell wall-less bacterial pathogens classified within the class Mollicutes (Lee *et al*., 2000; Kirdat *et al*., 2023). These fastidious prokaryotes are strictly dependent on their hosts and are associated with various symptoms like yellowing, witches' broom, stunting, little leaf, phyllody, and others (Rao *et al*., 2017). Phytoplasmas are primarily transmitted by phloem feeding insects such as leafhoppers, planthoppers and psyllids (Weintraub and Beanland, 2006), parasitic plants (Akhtar *et al*., 2009), vegetative propagation (Bertaccini *et al*., 2014) and in a few cases through seeds and seedlings (Kirdat *et al*., 2022).

These complex pathogens make controlling the related diseases challenging, especially within India's diverse agriculture and forest ecosystems. Phytoplasma diseases pose severe threats to forestry in India, affecting various tree species and impacting biodiversity, timber production, and ecosystem health. Across India, various forest species, including *Acacia catechu*, *Pongamia pinnata*, *Eucalyptus*, *Cedrela toona*, bamboo, *Gmelina* sp., *Santalum album*, and *Ziziphus oenoplia*, are vulnerable to phytoplasma infection (Sundararaj *et al*., 2022). Of these, sandal spike disease (SSD) represents the most significant threat to sandalwood cultivation in India (Sundararaj *et al*., 2020).

The SSD is known to be associated with aster yellows phytoplasmas and is prevalent in the states of Karnataka, Tamil Nadu, and Kerala (Mondal *et al*., 2020). SSD results in the loss of approximately 1% to 5% of sandalwood trees annually. Considering the economic value of sandalwood, this study aimed to analyze the status of SSD in the major sandalwood-producing states of India.

Materials and Methods

Extensive surveys were conducted in sandalwood forest habitats across Kerala, Karnataka, and Tamil Nadu from 2017 to 2024 to assess the status of SSD. Study areas were selected based on the abundance of sandal trees, and in each location, 100 sandalwood trees were evaluated in three replicates for SSD symptoms, with dead trees recorded. Trees were screened for phytoplasma presence using nested PCR with P1 and P7 primers followed by R16.100F and R16.1386R primers (Kirdat *et al*., 2022). Phytoplasma 16S rRNA gene amplification in symptomatic samples confirmed the presence of phytoplasma associated with SSD. Data on SSD incidence and mortality were collected and expressed as percentages.

Results

Table 1 provides detailed data on SSD incidence and mortality across study areas. SSD incidence in sandalwood reserves in Marayoor, Kerala, ranged from 0.33% to 16.57%, with mortality rates from 0.33% to 10.52%. The reserve SR 52 exhibited the highest SSD incidence (16.57%), and mortality (10.52%) compared to other sandal reserves. The lowest incidence was observed in VSR 2 and NSR 2, while SR 51 had no trees left as they were uprooted due to SSD mortality. In Karnataka, SSD incidence ranged from 11% to 70%, with up to 15% mortality. Sulikere recorded the highest incidence (70.0%), while Bannerghatta National Park had the highest

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mortality (15%). In Tamil Nadu, two forest ranges, Vellimalai and Satyamangalam, were surveyed and it was reported 12% and 8% of symptomatic trees, respectively, with mortality rates of 4% and 1%.

Table 1. Incidence and mortality of SSD infected trees in Kerala, Karnataka and Tamil Nadu, India.

State	Study area	Symptomatic trees (%)	Dead trees (%)
Kerala	Nachivayal Sandal Reserve 1	0.00	0.00
	Nachivayal Sandal Reserve 2	0.80	1.06
	Sandal Reserve 51(SR 51)	*	*
	Sandal Reserve 52(SR 52)	16.57	10.52
	Sandal Reserve 54(SR 54)	15.69	8.00
	Kanthaloor Sandal Reserve 1 (KSR 1)	9.25	6.40
	Kanthaloor Sandal Reserve 2 (KSR 2)	7.48	4.00
	Vannanthura Sandal Reserve 1(VSR 1)	3.12	2.52
	Vannanthura Sandal Reserve 2 (SR 2)	0.00	0.00
Karnataka	Bannerghatta National Park	45.00	15.00
	Sulikere, Bangalore	70.00	0.00
	Chamundi Hills	12.00	3.00
	MM Hills- Curve	11.00	4.00
	Ponnachi-MM Hills	14.00	7.00
	Koulihallakere Dam, BRT range	47.00	2.00
	Mavuthur B Beat BRT range	57.00	7.00
Tamilnadu	Vellimalai	12.00	4.00
	Satyamangalam	8.00	1.00

*No trees were found in the study area

Discussion

These findings reveal a significant presence of SSD in the major sandalwood habitats, with infection rates reaching 70% in certain areas, such as Sulikere in Karnataka, highlighting the pervasive threat of SSD in south India's sandalwood forests. The high incidence rates observed underscore the widespread and severe impact of phytoplasmas on sandalwood, particularly in forests where disease management practices are limited. Such infection levels pose a considerable risk to the long-term viability of sandalwood populations, which are already in decline due to illegal harvesting and habitat fragmentation.

The silent spread of SSD through vertical transmission, particularly via infected seeds and seedlings, presents a major challenge for sandalwood conservation (Kirdat *et al*., 2022). This mode of transmission means that even without insect vectors, SSD can propagate as infected plant material is used to establish new plantations. When these infected seedlings are introduced to disease-free areas, they risk becoming new reservoirs for SSD, thereby expanding the disease's range and threatening future sandalwood production. Addressing this issue requires both identifying potential insect vectors to understand disease dynamics and implementing rigorous standards for seedling certification. By ensuring that only disease-free plant materials are distributed, particularly in commercial nurseries, it can be limited the inadvertent spread of SSD and protect sandalwood populations. An integrated management approach that includes monitoring, vector control, and certification will be essential for sustaining valuable sandalwood resources in India.

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Country status

Threat of tamarisk phytoplasmas in man-made tamarisk forest for agricultural crops and natural resources plants in Iran

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Abstract

Over the past two decades, symptoms of tamarisk witches' broom, yellowing, and little leaf were observed with extensive distribution up to 100% in some areas in man-made tamarisk forest in rural deserts of central regions of Iran in Chah Afzal (Yazd province). Using direct and nested polymerase chain reaction on the phytoplasma ribosomal and non-ribosomal genes followed by RFLP and phylogenetic analyses revealed the presence of '*Candidatus* Phytoplasma asteris' (16SrI-B subgroup), '*Ca.* P. australasiae=australasiaticum' (16SrII-D subgroup), and '*Ca.* P. trifolii' (16SrVI-A subgroup). Based on the results, tamarisk represent a relevant source of infection for the agricultural crops and natural resources plants. Since phytoplasma strains detected on the tamarisk trees are similar to important phytoplasma diseases on different plant species in this area, it is possible that tamarisk trees have an important role in the epidemiology of phytoplasma disease and cause great damages. Threat of tamarisk witches' broom phytoplasma diseases in man-made tamarisk forest for agricultural crops and natural resources plants is serious and management of the disease at the earliest opportunity is recommended.

Keywords: '*Candidatus* Phytoplasma asteris'; '*Ca.* P. australasiae=australasiaticum; '*Ca.* P. trifolii'; *Tamarix aphylla*

Introduction

Phytoplasmas that are cell wall-less prokaryotes belong to the class Mollicutes and associated to plant diseases worldwide, transmitted mainly by leafhoppers and psyllids, causing devastating losses in more than one thousand plant species (Bertaccini *et al.*, 2014). Based on the restriction fragment length polymorphism (RFLP) and sequence analysis of the 16S rRNA gene, phytoplasmas are classified in '*Candidatus* Phytoplasma' species and in ribosomal groups, respectively (Bertaccini *et al.*, 2022). Over the past two decades, symptoms of tamarisk witches' broom, yellowing, and little leaf were observed with extensive distribution in some areas in man-made tamarisk forest planted as forestry for anti-desertification and used as windbreak. Considering the phytoplasma strains detected in tamarisk (Esmailzadeh Hosseini *et al.*, 2017; 2024) prevention methods are reported.

Materials and Methods

Total DNAs extracted from symptomatic and symptomless plants were subjected to direct and nested PCR using P1/P7, R16mF2/R16mR2 and R16F2n/R16R2 primer pairs under published conditions. Samples with sterile H_2O were used as negative controls (Esmailzadeh Hosseini *et al.*, 2024). Virtual RFLP, phylogenetic and DNA homology analyses of partial 16S ribosomal sequences and non-ribosomal genes (*tuf* and *secA*) were used to identify the detected phytoplasmas.

Results

Tamarisk witches' broom, yellowing, and little leaf were observed with extensive distribution up to 100% in some areas in man-made tamarisk forest in rural deserts of central regions of Iran in Chah Afzal (Yazd province) (Figures 1 and 2).

Using direct and nested polymerase chain reaction on the phytoplasma ribosomal and non-ribosomal genes (*tuf* and *secA*) followed by RFLP and phylogenetic analyses revealed the presence of '*Candidatus* Phytoplasma asteris' (16SrI-B), '*Ca.* P. australasiae=australasiaticum' (16SrII-D), and '*Ca.* P. trifolii' (16SrVI-A). Further amplifications were performed on selected phytoplasma- positive samples on *tuf* and *secA* genes, and the produced sequences indicated the presence of mixed phytoplasma infection in some of the samples (Esmaeilzadeh-Hosseini *et al.*, 2024).

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Figure 1. Witches' broom, yellowing, and severe proliferation in *Tamarix aphylla* affected by the phytoplasma disease.



Figure 2. Infected tamarisk trees around the pistachio gardens in Yazd province as the potential threat to pistachio gardens.

Discussion

Tamarisk represents a relevant source of infection for the agricultural crops and natural resources plants. The phytoplasmas identified in symptomatic tamarisk trees were detected in Iran in a wide range of agricultural, forest, and rangeland symptomatic plant species (Esmaeilzadeh-Hosseini *et al*., 2023) such as 16SrI-B: *Allium cepa*, *Vitis vinifera* and *Rosa canina* yellows; *Eruca sativa*, *Lactuca sativa* and *Sonchus oleraceus* phyllody; *Eucalyptus camaldulensis* little leaf; *Morus alba* and *Tragopogon dubius* witches' broom; *Prunus armeniaca* decline; *Solanum tuberosum* purple top; *Ziziphus jujube* witches' broom and yellowing; 16SrII-D: *Artemisia sieberi* witches' broom and little leaf; *Beta vulgaris* subsp. *esculenta* and subsp. *vulgaris*, *Medicago sativa*, *Peganum harmala*, *Petroselinum crispum*, *Petunia hybrida*, *Taraxacum officinale* and *Taverniera cuneifolia* witches' broom; *Catharanthus roseus*, *Calendula officinalis*, *Cosmos bipinnatus*, *Cucumis sativus*, *Cucurbita pepo* and *Heliopsis helianthoides*, *Solanum melongena*, *Sesamum indicum*, *Vicia faba* and *Zinnia elegans* phyllody; *Capsicum annuum* big bud; *Prunus dulcis* dieback; *Punica granatum* and *Saccharum officinarum* little leaf; *Solanum lycopersicum* leaf roll; *Sophora alopecuroides* dwarfing and yellowing; 16SrVI-A: *Brassica napus*, *Juniperus procumbens*, *Medicago sativa* and *Pinus brutia* witches' broom; *B. oleracea* yellows; *C. officinalis*, *C. roseus*, *C. sativus*, *S. indicum*, *S. melongena* and *Vigna unguiculata* phyllody; *Citrullus lanatus* and *Cota tinctoria* witches' broom; *Erigeron canadensis* leaf malformation and witches' broom; *Glycine max* bud proliferation and aborted seed pods; *Phoenix dactylifera* yellowing; *S. lycopersicum* big bud, flower virescence, phyllody and little leaf; *S. alopecuroides* dwarfing and yellowing; *V. vinifera* yellows; *Zea mays* yellowing and dwarfing; *Z. jujube* witches' broom and yellowing. Diseased tamarisk trees may play an important role in the epidemiology of economically relevant phytoplasma diseases. Tamarisk witches' broom in man-made tamarisk forests is serious and management of the disease at the earliest is recommended. The research about this disease is at the beginning, therefore methods for preventing and reducing its damages are reported: i) planting of tamarisk trees in the man-made forest area should be avoided; ii) avoid creating gardens and fields in the vicinity of artificially planted tamarisk; iii) when symptoms are firstly observed in an area free of infection, the trees must be uprooted and destroyed; iv) according to the high disease spread in front of the impossibility of eradication and re-implantation due to high costs, the symptomatic plant must be severely pruned and the pruned branches should be destroyed; v) while tamarisk tree cuttings from symptomatic plants sprouted up to 90.3% but with 15–25 days' delay compared to the asymptomatic ones and showed internode shortening and witches' broom, while the branch cuttings from asymptomatic plants had normal growth and sprouted up to 97.8% (Esmaeilzadeh-Hosseini *et al*., 2024), it must be forbidden to take cuttings from tamarisk trees in infected area.

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Country status

Rice orange leaf phytoplasma: a reemerging disease in coinfection with rice tungro virus in the Philippines

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Abstract

Rice orange leaf disease associated with the presence of rice orange leaf phytoplasma (ROL) transmitted by a new vector *Nephotettix virescens* became a reemerging disease in coinfection with rice tungro virus (RTV) in the Philippines. The occurrence of ROL and RTV mixed infection was monitored in rice growing areas and the results confirmed that ROL and RTV mixed infections were significantly spreading in the field. Insect transmission data indicated that an individual viruliferous insect transmits both ROL and RTV at the same time. These findings may represent a potential threat to rice production if not properly addressed.

Keywords: bacilliform virus, transmission, *Nephotettix virescens*, epidemic

Introduction

Rice orange leaf (ROL) disease in the Philippines was first identified to be associated with a mycoplasma-like organism (Hibino *et al*., 1987) later identified as '*Candidatus* Phytoplasma asteris' 16SrI-B subgroup (Jonson *et al*., 2020). *Recilia dorsalis* was the known vector of the phytoplasma associated with this disease, but later *Nephottetix cincticeps* was also found to transmit it in South China (Li *et al*., 2015) and recently *Nephottetix virescens* which is the vector of rice tungro virus (RTV) was also reported to transmit ROL in the Philippines (Jonson *et al*., 2020). This seemed alarming since, rice tungro disease (RTD) is the most damaging virus disease with 30 to 100% yield losses in Asia (Azzam and Chancellor, 2002). Since the detection of virus and phytoplasma coinfection is a potential threat to rice production and require attention to develop new rice varieties with improved resistance to both pathogens the occurrence of ROL and RTV mixed infection was monitored in rice growing areas in the Philippines.

Materials and Methods

Symptomatic second youngest leaf samples suspected to be infected with ROL phytoplasmas and rice tungro disease were collected in farmer's fields in different provinces and in the International Rice Research Institute (IRRI) rice fields in the Philippines during 2021-2024. In each province the sample collection was performed in 1-4 municipalities. Total DNA was extracted from 322 samples consisting of 1.0 g of symptomatic leaf tissues using a CTAB method and the final concentration was adjusted to 50 ng/µl using sterile distilled water. ROL phytoplasma and rice tungro bacilliform virus (RTBV) (indicative of the presence of rice tungro disease) were detected from extracted DNA by nested PCR and PCR respectively, following published procedures (Jonson *et al*., 2020; Ong *et al*., 2021).

Newly emerged adults of *N. virescens* were confined in mixed infected sources for 15 days acquisition access period (AAP). A total of 120 viruliferous *N. virescens* was used in two trials to inoculate TN1 (Taichung native 1) rice variety susceptible to the RTD using one insect per seedling. After AAP individual viruliferous insects and single 14-day-old asymptomatic TN1 rice seedling were enclosed in a tube for 24 h inoculation access period (IAP). The symptoms assessment was performed at 14 days post inoculation (DPI) when the second youngest leaf from each inoculated plant was sampled and tested for the presence of ROL phytoplasmas and RTBV as described above.

Table 1. Occurrence of ROL phytoplasma coinfection with RTBV in different provinces of the Philippines in 2021-2024.

			Number (%) of infected plants		
Year	Province	Total samples	ROL phytoplasmas	RTBV	Coinfection
2021	5	41	ND	ND	26 (63.0)
2022	5	30	ND	ND	6 (20.0)
2023	3	139	18 (12.0)	52 (37.4)	69 (49.6)
2024	10	112	28 (25.0)	42 (10.7)	42 (37.5)

ND, no data

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Table 2. Insect transmission from individual viruliferous *Nephotettix virescens* fed on rice plants coinfected by ROL phytoplasma and rice tungro disease.

	Total number of insects	Number (%) of inoculated TN1 seedlings			
Trial		ROL phytoplasma	RTBV	coinfection	Not infected
Trial 1	80	0	40 (50.0)	36 (45.0)	4 (5.0)
Trail 2	40	15 (37.5)	5 (12.5)	3 (7.5)	17 (42.5)

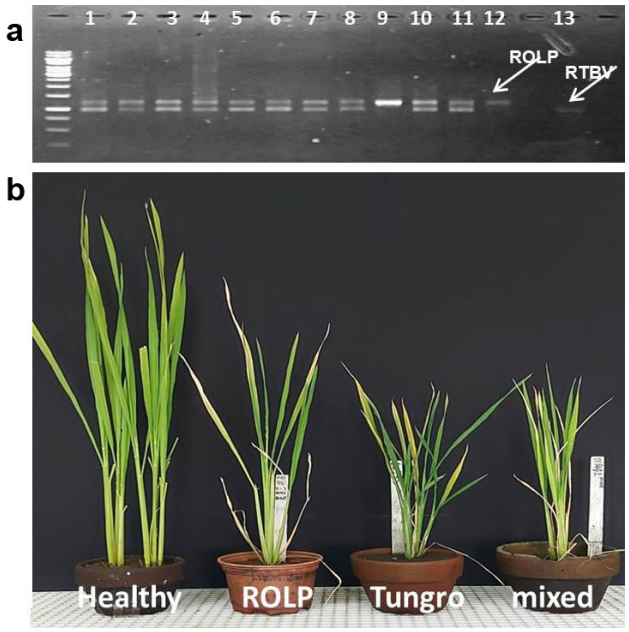


Figure 1. Detection and symptomatology of insect inoculated TN1 rice seedlings fed on mixed infected disease source. (a) Multiplex PCR to detect ROL phytoplasma (1.2 kb) and RTBV (1 kb); (b) symptoms of ROL phytoplasma, tungro disease and mixed infected plants after 14 DPI.

Results

The 322 symptomatic leaf samples collected and tested showed that co-infection was the highest infection situation detected, and it was consistently present in all the four consecutive years of the monitoring (Table 1). This is also indicating infection spreading in the surveyed rice fields. The molecular detection results confirmed that mixed infections consistently occurred in rice fields (Figure 1a).

Data on insect transmission fed on mixed infected rice plants showed that individual viruliferous insects can transmit both the ROL phytoplasma and RTD at the same time. ROL phytoplasma-infected plants were moderately stunted with yellow-orange leaves discoloration and reduced tillers compared to healthy plants and generally showed mild symptoms while tungro disease and mixed infection showed both severe symptoms (Figure 1b).

The detection of RTBV was used to verify the rice tungro disease presence in the two insect inoculation trials. Both trials showed the presence of mixed infection transmission however the rate of infection differed from each other with lower number of infected plants in the trial 2 (Table 2).

Discussion

In 2021, ROL phytoplasma coinfection with tungro disease was initially observed in farmer's fields in the Philippines. In early 2023-2024, ROL phytoplasma and tungro disease coinfection became consistently present at high incidences in most of the rice growing areas in the country and also at IRRI field plots. The overall data indicated that this coinfection is evident and predominantly spreading in the rice fields. The transmission study shows that a single N. *virescens* can transmit both phytoplasma and virus at the same time. The two insect transmission trials, however, exhibited inconsistent rates of infections when compared to each other. This result may be attributed to a different pathogen load in each disease source used for the transmission. Although the symptoms of infected plants varied according to the pathogen detected, by symptoms alone, it is difficult to distinguish the rice plant co-infected. This complicates the screening for tungro disease resistance that must be therefore validated also against the presence of these mixed infections to appropriately contain further rice yield losses.

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Country status

Growing prevalence of phytoplasma diseases in Ayodhya region, India

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Abstract

Phytoplasmas are emerging plant pathogens causing significant yield losses. The Ayodhya region in India has become a hotspot for '*Candidatus* Phytoplasma australasiaticum' (16SrII-D). In recent years molecular assays identified this phytoplasma in 18 plant species from Ayodhya region with many findings being first report in the world. This highlights the host adaptability of these phytoplasmas and emphasizes the need for effective management strategies.

Keywords: field survey, molecular diagnostics, agricultural impact

Introduction

Phytoplasmas are emerging as significant pathogens, associated with severe diseases in a wide range of crops and ornamental plants. The Ayodhya region in India has become a hotspot for phytoplasma research, particularly with the frequent detection of '*Candidatus* Phytoplasma australasiaticum' (16SrII-D). These phytoplasmas are associated with diverse symptoms, including yellowing, flat stem, witches' broom, stunting, and phyllody, leading to substantial losses in crop yield and quality. Recent studies from the region have revealed first-time global reports of multiple phytoplasma infections in important medicinal and commercial plants.

Materials and Methods

Comprehensive field surveys were conducted across the Ayodhya region to identify symptomatic plants. Molecular tools such as nested PCR were employed for phytoplasma detection using phytoplasma-specific primers targeting 16S rRNA, *secA*, *secY*, *tuf*, and *rp* genes. The amplified products were sequenced, and the sequences were analyzed using bioinformatics tools to confirm identity and compare them with global databases.

Results

The studies identified '*Ca.* P. australasiaticum' (16SrII-D) as the most abundant phytoplasma in Ayodhya. A total of 18 plant species have been recorded to show phytoplasma presence in Ayodhya and surrounding regions (Table 1). The most frequent symptoms observed were witches' broom, phyllody, flat stem and yellowing (Figure 1).

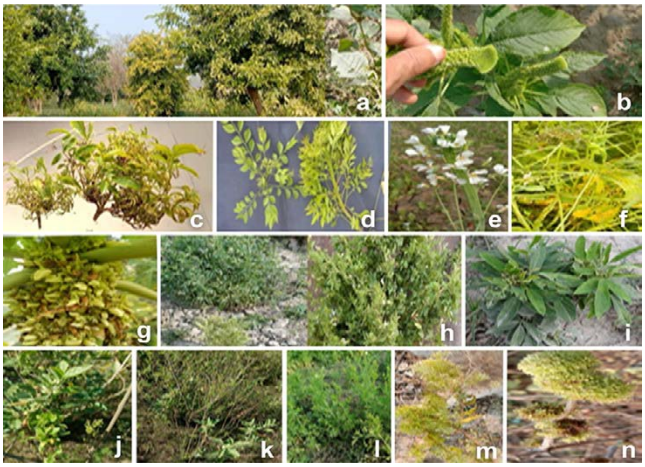


Figure 1. Various phytoplasma symptomatic plants from Ayodhya region: (a) *Aegle marmelos*, (b) *Amaranthus hypochondriacus*, (c) *Tecoma stans*, (d) *M. koenigii*, (e) and (f) radish, (g) papaya, (h) lentil, (i) pigeon pea, (j) and (k) ashwagandha, (l) black cumin, (m) and (n) ber.

Discussion

The findings highlight the widespread prevalence and host adaptability of '*Ca.* P. australasiaticum' (16SrII-D) in Ayodhya. Its ability to infect a wide range of hosts, from medicinal plants like *Aegle marmelos* and *Withania somnifera* to economically significant crops such as papaya and spinach, underscores its agricultural and ecological impact. The

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Table 1. List of plants reported to be associated with phytoplasmas from Ayodhya (Uttar Pradesh) and surrounding regions.

Plant species	Symptoms	Location	Phytoplasma	GenBank accession number	References
Aegle marmelos (Rutaceae)	Yellowing and stunting	Ayodhya	16SrII-D	PP415872/PP415873	Singh *et al.*, 2024a
Amaranthus hypochondriacus (Amaranthaceae)	Inflorescence malformation	Ayodhya	16SrII-D	ON611696/ON611697	Singh *et al.*, 2022b
Cajanus cajan (Fabaceae)	Witches' broom	Ayodhya	16SrIX-C	KY439872	Rao *et al.*, 2018
Cannabis sativa (Cannabaceae)	Witches' broom	Ayodhya	16SrII-D	OQ518445	Srivastava *et al.*, 2023
Carica papaya (Caricaceae)	Flower malformation	Faizabad	16SrII-D	MW533152/MW533153	Panda *et al.*, 2021
Impatiens balsamina (Balsaminaceae)	Flat stem, little leaf, phyllody	Ayodhya	16SrII-D	MW077131/MW077132	Panda *et al.*, 2021
Lens culinaris (Fabaceae)	Witches' broom	Faizabad	16SrVI-D	KY439869	Rao *et al.*, 2018
Luffa cylindrica (Cucurbitaceae)	Phyllody	Ayodhya	16SrII-D	OQ549913/OQ549914	Rao *et al.*, 2023
Mangifera indica (Anacardiaceae)	Witches' broom	Ayodhya	16SrII-D	OQ124148	Contaldo *et al.*, 2022
Moringa oleifera (Moringaceae)	Leaf yellowing and stunting	Ayodhya	16SrI-B	OP358449/OP358450	Singh *et al.*, 2023a
Murraya koenigii (Rutaceae)	Leaf yellowing and flat stem	Gorakhpur/ Ayodhya	16SrII-C/16SrII-D	OP984130OP984129	Dubey *et al.*, 2022
Mustard	Flat stem, phyllody, virescence	Gorakhpur/ Faizabad	16SrXI-B/16SrVI-D	KY378647	Rao *et al.*, 2016
Nigella sativa (Ranunculaceae)	Witches' broom	Ayodhya	16SrVI-D	PP859450/PP981036	Gupta *et al.*, 2024
Papaver somniferum (Papaveraceae)	Phyllody and stunting	Ayodhya	16SrII-D	ON212384/ON212385	Singh *et al.*, 2023b
Raphanus sativus (Brassicaceae)	Flat shoot, shoot proliferation	Ayodhya	16SrII-D	-	Singh *et al.*, 2022a
Spinacia oleracea (Amaranthaceae)	Witches' broom	Ayodhya	16SrII-D	OQ518444	Srivastava *et al.*, 2023
Tecoma stans (Bignoniaceae)	Little leaf, witches' broom	Ayodhya	16SrII-D	OQ119726/OQ119727	Mitra *et al.*, 2023
Withania somnifera (Solanaceae)	Little leaf, declining	Uttar Pradesh	16SrVI-D	PP859450/PQ002484	Singh *et al.*, 2024b

detection of phytoplasmas in ornamental plants, including *Tecoma stans* and *Impatiens balsamina*, further indicates their potential threat to biodiversity. The growing abundance of phytoplasmas in Ayodhya region not only enhance the understanding of phytoplasma-host interactions but also stress the urgent need for effective disease management strategies to protect crops and ensure agricultural sustainability.

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Country status

An update status of phytoplasma diseases in chickpea

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Abstract

Chickpea is an essential pulse crop; however, diseases and pests pose significant challenges to chickpea production, especially in tropical and subtropical areas. Chickpea stunt, associated with the presence of phytoplasmas, is particularly damaging in many chickpea-growing countries. The 16SrII-D subgroup phytoplasma strain is associated with chickpea stunt disease in India, Pakistan, Oman, Australia, Myanmar, and Sudan. This is an overview of phytoplasma diseases linked to chickpea stunt, focusing on their geographical distribution, identification, epidemiology, and impact.

Keywords: CpS disease, detection, diversity, epidemiology, management

Introduction

Chickpea is a cool-season crop primarily cultivated in arid and semi-arid regions worldwide under rain-fed conditions (Millan *et al*., 2006). India is the leading producer globally, of the world's chickpea production, followed by Australia, Pakistan, Myanmar, Ethiopia, Turkey, and Iran (Merga and Haji, 2019). While Israel records the highest chickpea productivity at 4,770.82 kg/ha, India's productivity remains comparatively low at 935.34 kg/ha (Merga and Haji, 2019).

Chickpeas provide a rich source of plant-based protein and contribute to biological nitrogen fixation. However, chickpea productivity in developing countries falls short of the global average due to various abiotic and biotic stresses, including drought, low temperatures, fungal diseases, and viral infections. Significant diseases affecting chickpeas include *Ascochyta* blight, *Botrytis* gray mold, *Fusarium* wilt, dry root rot, stunt disease, phyllody, and witches' broom, which can severely reduce yields. This review provides up-to-date insights on key phytoplasma diseases affecting chickpea.

Phytoplasma diseases in chickpea

Phytoplasmas are unique, wall-less prokaryotic pathogenic bacteria, first identified in Japan and initially termed mycoplasma-like organisms due to their similarities with animal mycoplasmas. These pleomorphic organisms, surrounded by a single membrane and measuring 200 to 800 nm in diameter, possess a small genome of approximately 680–1600 kb. Phytoplasmas are classified within the Phylum Tenericutes, Class Mollicutes, Family Acholeplasmataceae, and Genus '*Candidatus* Phytoplasma' (IRPCM, 2004). They are associated with diseases in over 1,000 plant species, affecting agriculture, horticulture, ornamental plants, trees, and weeds (Rao *et al*., 2018). Legumes, valued for their proteins, carbohydrates, and minerals, are grown primarily for seeds, silage, and soil enhancement through green manure. Phytoplasma infections have been linked to reduced yield and quality in crucial legume crops (Bertaccini *et al*., 2014; Rao *et al*., 2017). So far, six phytoplasma groups (16SrI, -II, -V, -VI, -IX, -XII, -XIV) have been reported to infect key legume crops worldwide, including pea, broad bean, mung bean, cowpea, pigeon pea, lentil, soybean, chickpea, and faba bean (Rao *et al*., 2017; Al-Subhi *et al*., 2018; Reddy *et al*., 2021a). In India, phytoplasma diseases have sporadically been reported in mung bean, soybean, cowpea, chickpea, and pigeon pea (Rao *et al*., 2017).

Chickpea phyllody, associated with a strain of the 16SrII-D subgroup phytoplasma, has been reported in several countries, including India, Pakistan, Oman, Australia, Myanmar, and Sudan (Reddy *et al*., 2021a). Phyllody symptoms include stunted growth, abnormal leaf-like structures in place of flowers (phyllody), flower sterility, and the development of axillary shoots with small leaves, resulting in a bushy appearance. In India, chickpea phyllody was first reported in Tamil Nadu, with subsequent reports from Haryana, confirmed by electron microscopy observations (Ghanekar *et al*., 1988). In the past this disease has been documented across the major chickpea-growing regions, including Haryana, Karnataka, Uttar Pradesh, Maharashtra, Andhra Pradesh, and New Delhi. Average chickpea yield losses of 10–

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15% due to phyllody have been reported in India (Ghanekar *et al*., 1988), with disease incidences ranging from 3% to 6% in various parts of Karnataka (Ramappa *et al*., 2008). Recently, chickpea phyllody incidence has increased and is now observed throughout all India's chickpea-growing regions (Shreenath *et al*., 2020; Reddy *et al*., 2021a).

Two phytoplasma subgroups (16SrII-D and 16SrII-C) have been identified in chickpea stunt (CpSD) and phyllody samples from various states in India. Phylogenetic analyses of 16S rRNA sequences have revealed a close relationship between these chickpea strains and members of the peanut witches' broom (16SrII) group (Reddy *et al*., 2021a). Further molecular characterization of chickpea little leaf and phyllody associated with CpSD has been documented, employing genes like *secA*, *rp*, *secY*, *imp*, and *tuf* to confirm the identification of phytoplasma strains in symptomatic chickpea plants (Reddy *et al*., 2021a). Additionally, weeds such as *Cleome viscosa*, *C. sparsiflora*, *Parthenium hysterophorus*, *Phyllanthus niruri*, and *Catharanthus roseus* have been identified as hosts for the 16SrII-D phytoplasma subgroup associated with CpS disease (Reddy *et al*., 2021a).

Historically, *Orosius orientalis* was identified as the primary leafhopper vector for chickpea phyllody (Ghanekar *et al*., 1988). Recently, however, two other leafhopper species, *Exitianus indicus* and *Empoasca* spp., were identified as potential vectors of the 16SrII-D strain (Reddy *et al*., 2021a). Understanding the alternate hosts and vectors of 16SrII-D phytoplasmas is essential for effective monitoring of weed and leafhopper populations in chickpea fields to manage phytoplasma diseases. Reddy *et al*. (2021a) documented genetic diversity and mixed infections of chickpea chlorotic dwarf virus (CpCDV) and phytoplasma strains in chickpea stunt disease in India, emphasizing the importance of innovative management strategies. Reddy and Rao (2019) developed a multiplex PCR protocol to identify phytoplasma subgroup strains 16SrII-C and -D using specific primers (Reddy *et al*., 2021a). Mixed infections of CpCDV and phytoplasmas in chickpea stunt disease have also been identified. A duplex PCR assay has been developed to detect such mixed infections in CpSD samples, employing CpCDV coat protein-specific primers (MCPF/R) and *tuf* gene nested primers for phytoplasmas in a single reaction.

For the epidemiology of CpSD Reddy *et al*. (2021b) developed a model based on insect vector populations and temperature variations to predict the spread of chickpea stunt disease in India. Two models, the beta model and Briere's model, were used to assess the effect of temperature on leafhopper populations, with the beta model found to be a better predictor of leafhopper abundance. A spatial analysis indicated that the western and Indo-Gangetic plains are suitable for vector growth all the year-round, except in northern hill zones. This predictive model could inform crop planning and disease management strategies.

Discussion

CpSD remains a significant threat to chickpea production worldwide since it is prevalent in India, Africa, and the Middle East, and the emergence of new strains and recombinations has expanded its host range and led to epidemics. Phytoplasmas are also associated with CpSD, with significant implications for chickpea cultivation in both developing and developed regions. Given chickpea's importance as a global pulse crop, ongoing research is crucial to understand the biology, ecology, and epidemiology of these diseases. More systematic studies on resistance screening, vector management, and alternate plant host species are essential for advancing chickpea breeding programs and disease management strategies.

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Country status

An update status of recently emerging '*Candidatus* Phytoplasma omanense' strains in Iran

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Abstract

So far, phytoplasmas enclosed in 16SrI, 16SrII, 16SrIII, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 16SrXIV, 16SrXXIX and 16SrXXX ribosomal groups were identified in Iran. Among the identified phytoplasma strains, due to the distribution and severity of the damage, new emerging phytoplasma strains in 16SrXXIX group are a new and very important threat to agricultural products. These identified '*Candidatus* Phytoplasma omanense' strains reported from Iran were associated with diseases such as: *Prunus persica* and *P. domestica* yellowing and decline (Mehriz, Yazd province); *Sophora alopecuroides* dwarfing and yellowing (Damaneh and Fereydun Shahr, Isfahan province); *Diospyros kaki* yellowing, reddening, die-back, and decline (Mehriz, Yazd province) all in 16SrXXIX-A subgroup; *Convolvulus arvensis* witches' broom and dwarfing (Bafgh, Yazd province) in 16SrXXIX-B subgroup and 16SrXXIX (subgroup not determined) *Cressa cretica* witches' broom (Ardakan, Yazd province).

Keywords: phytoplasma diseases, ribosomal groups, economic damage

Introduction

Phytoplasmas are members of class Mollicutes inhabiting the phloem of a wide range of plant species belonging to different families and are associated with different destructive plant diseases. They are transmitted mainly by leafhoppers and psyllids. Currently at least 49 '*Candidatus* Phytoplasma' species are available based on 16S rRNA gene sequences (Bertaccini *et al*., 2022). '*Ca.* P. omanense' (Cassia witches' broom phytoplasma group, 16SrXXIX) was described in Oman infecting *Cassia italica* and associated with witches' broom (Al-Saady *et al*., 2008). The 16SrXXIX group comprises phytoplasma strains distributed in three subgroups named as 16SrXXIX-A, Cassia witches' broom (CaWB), 16SrXXIX-B, Iranian bindweed witches' broom and dwarfing (RBiWB) and XXIX-B(C), almond witches' broom yellowing and dieback (AL163) (Table 1).

Materials and Methods

Based on molecular studies (direct and nested PCR, real and virtual RFLP, phylogenetic and 16S ribosomal DNA and non-ribosomal genes comparison) in samples from various symptomatic plant species collected in different geographic areas of Iran and reported in previous studies, an update status of new emerging '*Ca.* P. omanense' strains in Iran are reported.



Figure 1. *Diospyros kaki* yellowing, reddening, die-back, and decline; Mehriz (Yazd province, Iran).

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Table 1. '*Candidatus* Phytoplasma omanense' (GenBank accession number EF666051) strains detected in Asia.

Ribosomal subgroup	Diseases	Geographic distribution	GenBank accession number
16SrXXIX-A	*Prunus persica and Prunus domestica* yellowing and decline	Mehriz, Yazd province, Iran	MF142457
16SrXXIX-A	*Sophora alopecuroides* dwarfing and yellowing	Damaneh and Fereydun Shahr, Isfahan province	-
	Diospyros kaki yellowing, reddening, die-back, and decline	Mehriz, Yazd province, Iran	-
	Vitis vinifera yellows	Lebanon	-
16SrXXIX-B	Iranian bindweed witches' broom and dwarfing (RBiWB	Bafgh, Yazd province, Iran	KY047493
16SrXXI-B(C)	Almond witches' broom yellowing and dieback (AL163)	Jordan	OL873126
Not determined	*Cressa cretica* witches' broom	Ardakan, Yazd province, Iran	-



Figure 2. *Prunus persica* decline; Mehriz (Yazd province, Iran) being removed from the field by the farmer.



Figure 3. *Sophora alopecuroides* dwarfing and yellowing; Damaneh and Fereydun Shahr (Isfahan province, Iran) (right) compared to healthy one (left).



Figure 4. Small leaves, shortened internodes, proliferation of axillary buds, bushy growing and witches' broom habit in *Cressa cretica* (right) in Ardakan (Yazd province, Iran) compared to a healthy one (left).



Figure5. *Convolvulus arvensis* witches' broom and dwarfing; Bafg (Yazd province, Iran) (right) compared to a healthy one (left).

Results

The identified phytoplasmas enclosed in 16SrXXIX group reported from Iran were 16SrXXIX-A subgroup: *Prunus persica* and *Prunus domestica* yellowing and decline (Mehriz, Yazd province); *Sophora alopecuroides* dwarfing and yellowing (Damaneh and Fereydun Shahr, Isfahan province); *Diospyros kaki* yellowing, reddening, die-back, and decline (Mehriz, Yazd province); 16SrXXIX-B subgroup: *Convolvulus arvensis* witches' broom and dwarfing (Bafgh, Yazd province) and 16SrXXIX (subgroup not determined) *Cressa cretica* witches' broom (Ardakan, Yazd province) (Figures 1-5).

Discussion

So far, phytoplasmas enclosed in 16SrI, 16SrII, 16SrIII, 16SrVI, 16SrVII, 16SrIX, 16SrX, 16SrXI, 16SrXII, 16SrXIV, 16SrXXIX and 16SrXXX ribosomal groups were identified in Iran (Esmaeilzadeh-Hosseini *et al*., 2023). The detection of '*Ca*. P. omanense' in Iran seems to be wider than initially described especially considering its distribution in arid areas in middle East, north Africa and several southern Mediterranean areas and due to the spread and severity of the damage, cassia witches' broom phytoplasma associated diseases are a threat to agricultural products in Iran.

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Country status

Presence of '*Candidatus* Phytoplasma asteris' in red gram and horse gram in Ananthapuramu district of Andhra Pradesh, India

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Abstract

In 2022 symptoms of little leaf, malformation of inflorescence and witches' broom were observed in pigeon pea (red gram) and horse gram in the Ananthapuranum district of Andra Pradesh in India. DNA was amplified from symptomatic samples of red gram and horse gram by nested PCR assays using primers that target the 16Sr RNA gene. No DNA amplifications were observed the non-symptomatic samples. Sequence comparison, phylogeny and virtual RFLP analysis of the 16Sr RNA gene sequences of phytoplasmas identified confirmed the presence of a '*Candidatus* Phytoplasma asteris' (16SrI-B) strain. Association of '*Ca.* P. asteris' with horse gram witches' broom and little leaf is a new world report.

Keywords: phytoplasma, little leaf, legume, malformation

Introduction

Pigeon pea (*Cajanaus cajan*) known as red gram or "arhar" or "tur") and horse gram (*Macrotyloma uniflorum*), are annual/perennial legumes from the family Fabaceae. Being consumed on a large scale in South Asia, pigeon pea is a major source of protein for the population of the Indian subcontinent. India ranks first in red gram global production with 42.8 lakh tonnes cultivated under 48.24 lakh hectares and producing 887 kg/hectare in 2020-21 (agricoop.nic.in). In Andhra Pradesh, red gram is grown as a sole crop and intercrop for groundnuts. Horse gram is well known for its hardiness, soil adaptability and drought tolerance. Its grain is commonly grown for animal feed and for human consumption. The total area under horse gram cultivation is about 5.07 lakh ha and production of 2.62 lakh tonnes producing 516 kg/ha. Recently, phytoplasma infection has increased in red gram and horse gram, thus the occurrence of phytoplasma symptoms at Ananthapuramu district in Andhra Pradesh, India was investigated.

Materials and Methods

A survey was conducted in fields of Rayalaseema region comprising Ananthapuramu and Sri Sathya Sai districts during the "kharif" season 2022. Red gram plants (variety LRG 41) and horsegram (variety PHG9) exhibiting symptoms of witches' broom and little leaf were collected along with asymptomatic plants. DNA was extracted from leaf tissues using the CTAB method (Ahrens and Seemüller, 1992). The aster yellows phytoplasma strain (GenBank accession number CP035949) maintained in the glasshouse was used as a positive control and symptomless plants maintained in greenhouse were used as negative control. PCR was performed under reported conditions (Rao *et al*., 2014). Nested PCR was employed to determine phytoplasma presence using universal primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) followed by R16F2n/R2 (Gundersen and Lee, 1996). Ten microliters of both direct and nested PCR products were analyzed via electrophoresis on a 1.0% agarose gel, stained with GoodView™ nucleic acid stain and visualized under a UV Gel Doc transilluminator. The 1.25 kb amplicon was purified from the gel using the Wizard® SV Gel and PCR Clean-up System (Promega, Madison, USA) following the manufacturer's protocol. The purified PCR products were then ligated into the pGEM®T vector and cloned into *Escherichia coli* (DH5-á). The cloned 16S rRNA gene fragments were subsequently sequenced at Agri Genome, Kerala, India.

Results

During the survey, typical symptoms of little leaf, malformation of inflorescence and witches' broom were

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observed in red gram and horse gram in different fields (Figures 1 and 2). The observed incidence was 5-10% causing considerable yield loss. Nested PCR and gel electrophoresis results showed that all the symptomatic plants produced the expected length bands of 1.2 kb. The PCR products were purified, cloned and sequenced. Nucleotide BLAST analysis of 16S rRNA gene sequences of redgram revealed a 100% of identity with '*Candidatus* Phytoplasma asteris' (16SrI-B) from *Catharanthus roseus* aster yellows phytoplasma (GenBank accession number CP035949) from South Africa and that of horsegram revealed a 100% of sequence identity with *Clarkia unguiculata* aster yellows phytoplasma (GenBank accession number ON870386) from New Delhi. The *i*PhyClassifier tool (Zhao *et al*., 2009) classified the 16S rRNA gene sequences into 16SrI-B subgroup, with a similarity coefficient of 1.0. Phylogenetic analysis through MEGA11 showed that the phytoplasma strains clustered with '*Ca*. P. asteris' (Figure 3).

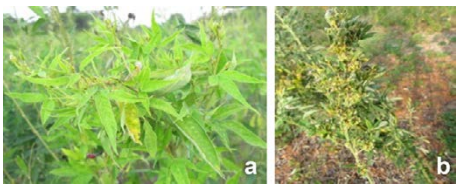


Figure 1. (a) Red gram plant showing little leaf and witches' broom and (b) malformation of inflorescence.



Figure 2. (a) Horse gram witches' broom symptoms and (b) little leaf symptoms.

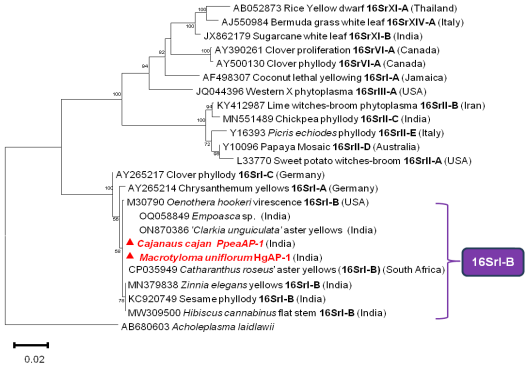


Figure 3. Phylogenetic relationship among the identified red gram (*Cajanaus cajan*) and horse gram (*Macrotyloma uniflorum*) phytoplasmas strains, based on sequences of the 16Sr RNA gene, rooted with *Acholeplasma laidlawii,*

Discussion

Pigeon peas have been reported affected by phytoplasma diseases worldwide associated with symptoms of witches' broom, little leaf, flower malformation and stunting of whole plant. '*Ca*. P. asteris' (Raj *et al*., 2006) and '*Ca*. P. aurantifolia' (Vijay Kumar *et al*., 2008; Gangadhara *et al*., 2022) have been reported in pigeon pea. In horse gram a phytoplasma in subgroup 16SrII-D has been reported from Tirupati, Andhra Pradesh (Reddy *et al*., 2024). Farmers are leaving the stubbles of crops in the field after harvest, and it could be the source of survival of the phytoplasma and vector occurring in nature. This is the first report of a '*Ca*. P. asteris' (16SrI-B subgroup) strain in horse gram. The identification of this phytoplasma strain in horse gram suggests that regular surveillance and monitoring of Fabaceae is needed to protect key agricultural crops from disease outbreaks, and further epidemiological studies are required in areas growing legumes.

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Country status

Molecular detection of '*Candidatus* Phytoplasma australasiaticum' and '*Ca.* P. trifolii' in grapevine in India

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Abstract

Grapevine cultivation is one of the most remunerative farming enterprises in India. Grapevine phytoplasma-associated disease has been noticed in several districts of Maharashtra, Jammu and Kashmir and Northeastern state of India in recent years. Field surveys in 2023-2024 across five major states including Maharashtra, Andhra Pradesh, Telangana, Mizoram and Karnataka revealed the incidence of grapevine phytoplasma disease ranging from 12-18% on the basis of phytoplasma symptoms. These were yellowing, reddening and rolling of leaf margins and were observed in different grapevine varieties. The phytoplasma presence was assayed employing universal and specific phytoplasma nested primer pairs, P1/Tint and 3F/3R which yielded 1.3 kb amplicons from all the symptomatic grapevine samples collected. The BLAST analysis of 16Sr RNA gene sequences shared higher sequence identity with '*Candidatus* Phytoplasma australasiaticum' and '*Ca.* P. trifolii'. The phylogeny confirmed the presence of strains related to '*Ca.* P. australasiaticum' (16SrII-D) and '*Ca.* P. trifolii' (16SrVI-D).

Keywords: phytoplasma, 16SrVI-D subgroup, 16SrII-D subgroup, 16S rRNA gene, yellows

Introduction

Grapevine is a major commercial fruit crop globally and is highly susceptible to economically important phytoplasma diseases. India ranks fourth in the global grape production contributing both to the domestic market and international exports (FAO, 2023). During a two-year survey conducted across five wine-growing regions in India, phytoplasma infection rates were found to be significantly high, ranging from 12% to 18%. Therefore, the present study aims to identity the phytoplasma strains detected in the commercial grapevine varieties.

Material and Methods

Surveys were conducted from September to November 2023 and from March to June 2024 across five major grapevine growing states in India (Table 1). The disease incidence of symptomatic grapevines in five vineyards was recorded. In each surveyed vineyard five to seven symptomatic leaves were collected together with asymptomatic samples. The total genomic DNA was extracted by CTAB method (Ahrens and Seemüller, 1992). PCR amplification was performed employing primer pair P1/Tint (Smart *et al.*, 1996) followed by nested primers 3F/3R (Manimekalai *et al.*, 2010). The amplified 16Sr RNA gene fragments were purified and sequenced after cloning using the M13Fwd/M13Rev universal primer pair in both directions at Biologia India, Bangalore. Phylogenetic analysis was performed using Maximum-likelihood method in MEGA 11 with 1,000 bootstrap replications. *Acholeplasma palmae* 16S rRNA gene sequence was used as outgroup in phylogeny.

Results and Discussion

Grapevine leaf samples collected from different major grapevine growing states of India (Maharashtra, Andhra Pradesh, Telengana, Karnataka and Mizoram) showed symptoms including marginal yellowing, reddening and rolling of leaves.

Moreover, upward rolling of leaves and reduced internodal length of canes were recorded from Telangana. In the surveyed areas, the incidence of the disease ranged from 12-18% in the popular grapevine varieties.

DNA fragments of approximately 1.3 kb were amplified from all symptomatic grapevine leaf samples with primer pair 3F/3R, while no amplifications were observed from asymptomatic grapevine from all the surveyed areas.

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Table 1 Samples of grapevine collected during the surveys.

Sample	Location	Variety	Symptoms (leaf)
HG235	Nashik, Maharashtra	Danaka	Yellowing
HG326	ICAR-NRCG, Pune, Maharashtra	Manjari Kishmish	Yellowing
HG412	Ananthpur, Andhra Pradesh	Manik Chaman	Yellowing
HG428	Mudugurki, Devanahalli, Bangalore rural, Karnataka	Dilkush	Yellowing
HG488	Rayalapur, Medchal, Telangana	Thompson seedless	Upward rolling and reduced intermodal length
HG490	Rayalapur, Medchal, Telangana	Super Sonaka	Upward rolling and reduced intermodal length
HG492	Rayalapur, Medchal, Telangana	Manik Chaman	Upward rolling and reduced intermodal length
HG493	Rayalapur, Medchal, Telangana	Danaka	Reduced intermodal length
HG494	Rayalapur, Medchal, Telangana	Danaka	Upward rolling and reduced intermodal length
HG497	Rayalapur, Medchal, Telangana	Manik Chaman	Upward rolling and reduced intermodal length
HG499	Yadaram, Shamirpet, Rangareddy, Telangana	Sarita seedless	Upward rolling and reduced intermodal length
HG501	Yadaram, Shamirpet, Rangareddy, Telangana	Sarita seedless	Upward rolling and reduced intermodal length
HG504	Yadaram, Shamirpet, Rangareddy, Telangana	Manik Chaman	Upward rolling and reduced intermodal length
HG512	Jaganguda, Shamirpet, Rangareddy, Telangana	Sarita seedless	Upward rolling
HG538	Thumkunta, Shamirpet, Rangareddy, Telanagana	Manik Chaman	Yellowing on margin, reduced internodal length
HG556	VengSang, Champhai Block, Mizoram	Bangalore blue	Yellowing like mosaic
HG559	VengSang, Champhai Block, Mizoram	Thompson seedless	Yellowing
HG574	Bethlehem, Champhai, Mizoram	Bangalore blue	Yellowing on margin
HG576	Seduh Hholan, Champhai	Bangalore blue	Yellowing on margin



Figure 1. Phylogenetic analysis of '*Ca.* P. australasiaticum' and '*Ca.* P. trifolii' from grapevine identified in the present study.

The specific amplicon was sequenced bi-directionally and sequences were assembled. BLASTn analysis revealed that 16S rRNA gene sequences of the grapevine phytoplasma strains shared up to 99% identity with '*Candidatus* Phytoplasma australasiaticum' and '*Ca.* P. trifolii'. The phylogenetic analyses showed the closer clustering of these sequences with those of phytoplasma strains belonging to 16SrII-D and 16SrVI-D subgroups (Figure 1).

Phytoplasmas classified into eleven ribosomal groups were detected in grapevine across various regions worldwide (Angelini *et al*., 2018). In India a phytoplasma strain in grapevine was confirmed as group 16SrXI-B from Baramati area of Pune district of Maharashtra (Rao *et al*., 2020). In the present study, the occurrence of 16SrII-D and 16SrVI-D subgroups strain in grapevine in Nashik, Pune, Ananthpur, Bangalore, Rayalapur, Rangareddy, Champhai areas of Maharashtra, Andhra Pradesh, Telengana, Karnataka and Mizoram states indicated the wider spread of phytoplasmas in this fruit species at newer locations, which is really alarming and needs immediate attention towards planning suitable management strategy.

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Country status

Identification of '*Candidatus* Phytoplasma trifolii' associated with mango phyllody in Kerala, India

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Abstract

Symptoms of little leaf, proliferation of young shoots and witches' broom of suspected phytoplasma infection were observed in 8-year-old mango trees of the variety Kalappady at Central Tuber Crops Research Institute, Thiruvananthapuram, Kerala in India. The leaf samples were assayed for the presence of phytoplasmas using nested PCR assays. Phytoplasma presence was detected in the symptomatic mango leaf samples by polymerase chain reaction with specific primer pairs amplifying the 16S rRNA gene. Pairwise sequence comparison and phylogenetic analysis of 16S rRNA gene sequences of mango phytoplasma strain indicates its relatedness with phytoplasmas in the clover proliferation group. *In silico* RFLP analyses using the *i*PhyClassifier of the 16S rDNA sequence of the mango phytoplasma strain allowed its classification into the 16SrVI-D subgroup. This is first report of '*Candidatus* Phytoplasma trifolii' in mango trees in Kerala.

Keywords: fruit, phytoplasma, 16SrVI-D subgroup, 16Sr RNA gene.

Introduction

Mango (*Mangifera indica* L.; family Anacardiaceae), known as "king of fruits", is a fleshy tropical stone fruit and almost half of the world's mangoes are cultivated in India. Though India is the largest producer country of mangoes, it accounts for less than 1% of the international mango trade. The crop is cultivated in an area of 76,574 ha with a production of 475,224 tonnes of fruits and productivity of 6,206 kg/ha in Kerala state (Agricultural Statistics, 2023). Many important phytoplasma diseases have been reported on fruit crops causing severe yield losses worldwide (Rao *et al*., 2020). Phytoplasma diseases, due to their widespread occurrence and severe impact, are becoming a challenge on several fruit crops in India (Mitra *et al*., 2023). So far three phytoplasma ribosomal groups (16SrI, 16SrII and 16SrV) were identified in mango worldwide. During a survey of mango orchards in 2024, severe incidence of little leaf, shoot proliferation along with witches' broom symptoms were recorded in the variety Kalappady at Central Tuber Crops Research Institute, Sreekaryam, Thiruvananthapuram, Kerala, India. In the present study, an attempt was made to identify the phytoplasma strain associated with the disease.

Material and Methods

During a survey in February 2024 across the mango orchards of Central Tuber Crops Research Institute, Sreekaryam, Thiruvananthapuram (Latitude 8°32'42.74"N, Longitude 76°54'53.72"E), symptoms of phytoplasma were observed in the mango variety Kalappady. Five leaf samples of the symptomatic mango trees along with healthy leaf samples were collected for molecular analyses. The total genomic DNA was extracted utilizing DNA extraction kit. Amplification of phytoplasma 16S ribosomal DNA was performed employing the primer pair P1/Tint (Deng and Hiruki, 1991; Smart *et al*., 1996) followed by the nested primers 3F/3R (Manimekalai *et al*., 2010) as described. The amplified 16S rRNA gene fragments of phytoplasmas were purified and sequenced using the M13Fwd/M13Rev universal primer pair in both directions at Biologia India., Bangalore. Phylogenetic trees were constructed using Neighbor joining method in MEGA 11 with 1,000 bootstrap replications. *Acholeplasma palmae* 16Sr RNA gene sequence was used as outgroup sequence. Computer-simulated analysis of the 16S rRNA gene sequence was performed using the *i*PhyClassifier to determine the phytoplasma ribosomal subgroup and the similarity coefficient was calculated (Zhao *et al*., 2009).

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Figure 1. Phytoplasma symptoms in mango; (a) little leaf (b) shoot proliferation along with witches' broom.

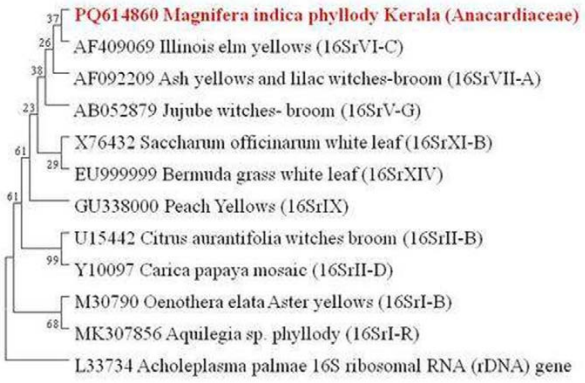


Figure 2. Phylogenetic tree inferred by Neighbor joining method for 16Sr RNA gene analysis of the mango phytoplasma strain.

Results and Discussion

In February 2024 little leaf, shoot proliferation as well as witches' broom (Figure 1) were observed in 8 years old mango tree Kalappady variety. DNA fragments of approximately 1.3 kb were amplified from all the five symptomatic leaf samples collected from the symptomatic infected mango trees (data not shown). However, no amplifications were observed from the DNA extracted from asymptomatic mango leaf tissues. One representative sample of 16S rRNA gene amplified from the symptomatic mango leaf was sequenced and deposited in GenBank under the accession number PQ614860. BLASTn analysis of this 16Sr RNA gene sequence revealed that it shared 99.26% sequence identity with '*Candidatus* Phytoplasma trifolii'. The phylogenetic analyses of 16Sr RNA gene sequence of mango phytoplasma strains in the study showed clustering with strains of clover proliferation phytoplasma group closely related with 16SrVI-D subgroup (Figure 2). The *in silico* RFLP analysis of 16S rRNA gene sequence confirmed the mango strain as enclosed in 16SrVI-D subgroup with a similarity coefficient of 1.0.

Phytoplasmas associated plant diseases have a wide geographic distribution in India under which more than 200 plant species are listed to be hosts of phytoplasmas classified in different ribosomal groups and subgroups (Rao, 2021). The presence of phytoplasmas in 16SrI and 16SrII groups in mango was reported from Egypt, Pakistan and Guadeloupe and India (Kazmi *et al*., 2007; El-Banna and El-Deeb, 2007; Castaneda *et al*., 2018; Kirdat *et al*., 2019; Debnath *et al*., 2020). In this work, the 16Sr RNA gene identification of the 16SrVI phytoplasma detected in mango clarified its closer genetic relatedness to '*Ca*. P. trifolii'. The occurrence of 16SrVI-D subgroup phytoplasma strain in mango from Kerala is a new world report. Considering the economic importance of mango as a major fruit crop in all states and the wide occurrence of '*Ca*. P. trifolii' in many crops in India (Rao, 2021), additional studies are required to assess if '*Ca*. P. trifolii' represents a threat to mango orchards and to identify potential insect vectors. Nevertheless, given its detection in mango with severe symptoms without flowering and fruiting, the '*Ca*. P. trifolii' may become an economic problem for mango production in India.

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Country status

Severe recurrence of tomato big bud disease associated with 16SrII phytoplasmas in Andhra Pradesh, India

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Abstract

In recent years tomato big bud (TBB), a phytoplasma-associated disease has been noticed as alarming in several districts of Andhra Pradesh, India. Field surveys in mid-2024 across the Tirupati and Annamaya districts revealed an incidence of tomato big bud disease ranging from 32-100% with phyllody symptoms in tomato hybrid variety, Saaho (TO 3250). The phytoplasma presence was investigated employing universal phytoplasma specific nested primer pairs, from TBB symptomatic samples from both Tirupati and Annamaya districts. The BLAST analysis of 16Sr RNA gene sequences of TBB phytoplasma strains from both the surveyed districts showed 97.39-97.47% sequence identity with '*Ca.* P. australasiaticum' reference strain. The phylogenetic analysis of 16S rRNA gene sequences of detected TBB strains confirmed the presence of 16SrII-related phytoplasma strains.

Keywords: outbreak, 16Sr RNA gene, PCR, sequencing, phylogeny

Introduction

Solanaceous vegetable crops are susceptible to infection by various phytoplasma diseases, among which tomato big bud (TBB) is one of the most destructive impacting tomato crops across different regions of India (Kumari *et al.*, 2019). As an important vegetable crop, tomato holds significant economic importance in India, stands second worldwide in both area and production (FAO, 2024). Phytoplasma diseases, due to their widespread occurrence and severe impact on crop productivity and quality, are recognized as a major agricultural challenge worldwide and in India, leading to severe economic losses (Rao, 2021). During survey of tomato crops in 2022 and 2023, severe incidence of tomato witches' broom and big bud were recorded in the popular hybrid tomato variety, Saaho (TO 3250), in Pudipatla village at Tirupati and Billurivaripalle village at Annamayya districts of Andhra Pradesh. In the present study, an attempt was made to molecularly characterize the phytoplasma strains associated with the disease.

Materials and Methods

Regular surveys were made during "kharif" season (July-October) in 2024 across the two major tomato growing districts, Tirupati and Annamayya of Andhra Pradesh state in India. The incidence of phytoplasma suspected symptoms in tomato crops was recorded on visual observation in different fields. Five leaf and young shoot samples from each of the surveyed Pudipatla village at Tirupati district and Billurivaripalle village at Annamayya district along with healthy samples, were collected for molecular characterization of the associated phytoplasmas.

The total genomic DNA was extracted by CTAB method from symptomatic TBB samples (Ahrens and Seemüller, 1992). PCR amplification was performed employing primer pair P1/Tint (Deng and Hiruki, 1995; Smart *et al.*, 1996) followed by nested primers R16F2n/R16R2 (Gundersen and Lee, 1996). The amplified 16Sr RNA gene fragments of tomato phytoplasmas were purified and sequenced using the M13Fwd/M13Rev universal primer pair in both directions at Biologia India, Bangalore. Phylogenetic analysis was performed using Maximum-likelihood method in MEGA 11 with 1,000 bootstrap replications. *Acholeplasma palmae* 16Sr RNA gene sequence was used as outgroup sequence to root the phylogeny tree.

Results and Discussion

In July-October "kharif" season of 2024, typical phytoplasma associated symptoms of severe phyllody and witches' broom

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were observed in tomato fields of Andhra Pradesh at Pudipatla village at Tirupati district (Figures 1a and b) along with malformed inflorescences with sterile buds at Billurivaripalle village at Annamayya district (Figures 1c-e).

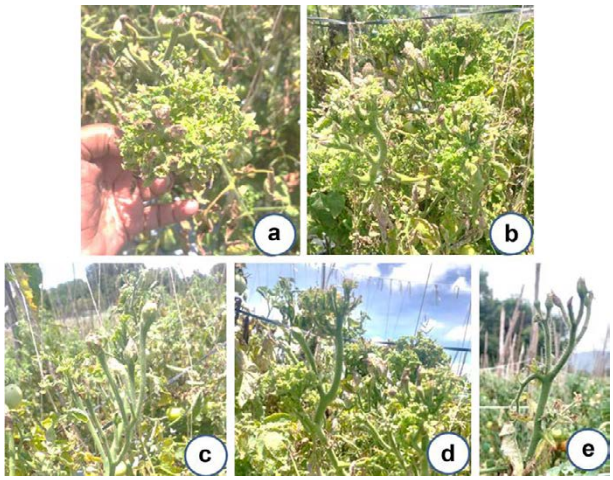


Figure 1. Phytoplasma symptoms in tomato plants. Phyllody along with witches' broom symptoms at Pudipatla village (a, b); big bud symptoms with malformed inflorescence and sterile vegetative structures without flowers and fruit at Billurivaripalle village (c, d, e).

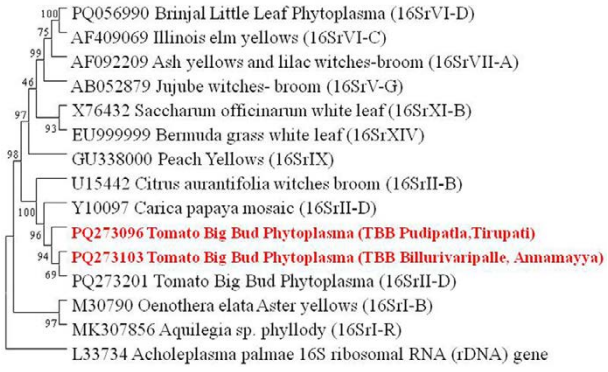


Figure 2. Phylogenetic tree inferred by maximum likelihood method (ML) for 16Sr RNA gene analysis of tomato big bud (TBB) phytoplasma strains from Andra Pradesh.

The incidence of the disease was recorded from 32 to 100% in different fields. Maximum incidence of the disease was recorded in hybrid variety Saaho (TO 3250) in both districts. DNA fragments of approximately 1.25 kb were amplified from TBB and phyllody symptomatic plants with primer pair R16F2n/R16R2 (data not shown). However, no amplifications were observed from DNA extracted from asymptomatic tomato plant tissues from both the surveyed districts. One representative 16Sr RNA gene nucleotide sequence amplified from the symptomatic tomato samples from each of the two districts was deposited in GenBank database, under the accession numbers PQ273096 (Tirupati) and PQ273103 (Annamaya). BLASTn analysis revealed that 16Sr RNA gene sequences of both strains shared respectively 97.47% and 97.39% identity with '*Candidatus* Phytoplasma australasiaticum' (GenBank accession number Y10097). The phylogenetic analyses of 16Sr RNA gene sequences of TBB strains in the study showed clustering with 16SrII phytoplasma group (Figure 2).

Various phytoplasma strains infecting tomato crops have been reported from different parts of the world (Kumari *et al*., 2019). Among these, the TBB disease has gained prevalence in several countries and has been found associated with different phytoplasmas (16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI and 16SrXI) (Martini *et al*., 2018). The phytoplasma strains associated with tomato big bud in India were confirmed as '*Ca.* P. australasiaticum' subgroup 16SrII-D from Uttar Pradesh, Haryana and Kadiri district of Andhra Pradesh (Singh *et al*., 2012; Reddy *et al*., 2021). In the present study, the occurrence of 16SrII group phytoplasma strains associated with TBB in other two new districts of Andhra Pradesh indicated the wider spread of TBB disease, which is alarming and needs immediate attention towards planning suitable management strategy. Considering the broad host range of '*Ca.* P. australasiaticum' and related strains and their capability of transmission through insect vectors and weeds in India (Rao, 2021), further studies are suggested to understand the epidemiology of TBB disease in the country.

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Country status

Identification of a '*Candidatus* Phytoplasma citri' strain associated with phyllody disease of cumin

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Abstract

Cumin is a key export spice of India, widely used in cooking and as herbal medicine. Since 2019, phytoplasma symptoms, including yellowing, phyllody, witches' broom, and seed elongation, have been observed in cumin crop at the Agricultural Research Station in Mandor, and farmers field of Jodhpur, Barmer and Nagaur districts of Rajasthan. Symptoms appeared 70-75 days after sowing, with disease incidence increasing from 0.25% to 10.6% in 2022. Screening of 80 cumin genotypes, GC 4, MCU 73, MCU 105, and MCU 32 showed low disease incidence (0.5-1.5%), while MCU 78 had the highest incidence (10.6%). Phytoplasma presence was confirmed in 80 cumin symptomatic germplasm accessions by employing phytoplasma specific primers. Pairwise sequence identity and phylogeny of 16S rRNA gene sequences of CuPP-Jod strains in the study indicated the presence of '*Candidatus* Phytoplasma citri' (16SrII-C subgroup). Chickpea, lentil and weeds growing in and nearby cumin fields were also presenting phytoplasma symptoms and were also analysed for phytoplasma presence. Phylogenetic and virtual RFLP analysis of 16S rRNA gene sequences confirmed the identity of cumin and chickpea phytoplasmas as '*Ca.* P. citri'.

Keywords: disease, yellowing, witches' broom, phytoplasma, 16S rRNA

Introduction

Cumin (*Cuminum cyminum* L.) member of the family Apiaceae (Umbelliferae) is a key export spice of India, widely used in cooking and as herbal medicine. Originating from the Mediterranean and near eastern regions (Edison and Kallupurackal, 1989; Nabhan, 2014), cumin is extensively cultivated in countries such as Uzbekistan, Tajikistan, Turkey, Morocco, Egypt, India, Syria, Mexico, Pakistan, and Chile (Azeez, 2008). India, being the world's largest producer, consumer, and exporter of cumin seed, has an annual production of 7.95 lakh tonnes over 10.87 lakh hectares in 2020-21. Cumin cultivation in India is predominantly concentrated in Rajasthan and Gujarat, contributing to over 90% of the country's total production, with Rajasthan alone cultivating it in an area of 6.09 lakh hectares with an annual production of 3.18 lakh tonnes (Anonymous, 2022). In 2020-21, cumin exports reached 0.299 million tons, valued at 33,280 million rupies. Since 2019, phytoplasma-like symptoms, including yellowing, phyllody, witches' broom, and seed elongation, have been observed in cumin crop at the Agricultural Research Station in Mandor, and farmers field of Jodhpur, Barmer and Nagaur districts of Rajasthan. Keeping in mind the importance of the crop an attempt has been made to identify the phytoplasmas associated with cumin phyllody and their natural reservoirs in the region.

Materials and Methods

During 2019 to 2023, surveys were made and phytoplasma symptoms in cumin along with weeds and other crop plants such as chickpea were collected from Jodhpur, Barmer and Nagaur districts of Rajasthan, India. Incidence of disease and types of symptoms were recorded in 80 genotypes of cumin. To detect the presence of phytoplasmas in symptomatic cumin samples, genomic DNA was extracted from cumin genotypes, chickpea plants and one asymptomatic cumin plant using the Qiagen DNeasy plant mini kit (Germany). The extracted DNA was amplified using nested PCR assays with universal phytoplasma detection primers for 16S rRNA gene (P1/P7 and R16F2n/R2) (Deng and Hiruki, 1991; Schneider *et al.*, 1995; Gundersen and Lee, 1996). The amplicons of about 1.25 kb in the 16S rRNA gene were amplified from all symptomatic cumin and chickpea plant samples and positive control of brinjal little leaf. PCR amplified products from two selected positive samples (CuPPJod-01 and CuPPJod-02) and one chickpea sample (CpPJod-01) of 16S rRNA gene, were sequenced from both

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ends. Further 16S rRNA gene sequences were subjected to phylogenetic analysis and virtual RFLP analysis using 17 restriction enzymes through *i*Phyclassifier (Zhao *et al*., 2009).

Results

The symptoms related to phytoplasma infection were first recorded after 70-75 days of sowing in the month of January of the years 2019 to 2022. The major symptoms recorded were yellowing, phyllody, witches' broom and deformed elongated seeds. Disease incidence was recorded as 0.25-10.6% from 2019 to 2022, using quadrate method. Screening of 80 cumin genotypes, GC4, MCU73, MCU105, and MCU32 showed lower incidence (0.5-1.5%), while MCU78 had the highest incidence (10.6%). Phytoplasma presence was confirmed in 80 cumin symptomatic germplasm accessions by employing phytoplasma primer pairs P1/P7 and R16F2n/R2 in nested PCR followed by sequencing. The 1,245 bp sequences were deposited in GenBank under the accession numbers PQ632161-63. These sequences showed 100% sequence identity with each other and 99.12% identity with '*Candidatus* Phytoplasma aurantifolia=citri' reference strain (GenBank accession number U15442). The chickpea, lentil and weeds (*Phyllanthus niruri* and *Asphodelus tenuifolius* L., *Melilotus indica* L.) growing in and nearby cumin fields with phytoplasma symptoms were also analyzed for phytoplasma presence. Phylogenetic and virtual RFLP analysis of 16S rRNA gene sequences further classified the cumin and chickpea, phytoplasmas as '*Ca.* P. citri' (16SrII-C subgroup) (Figure 1).

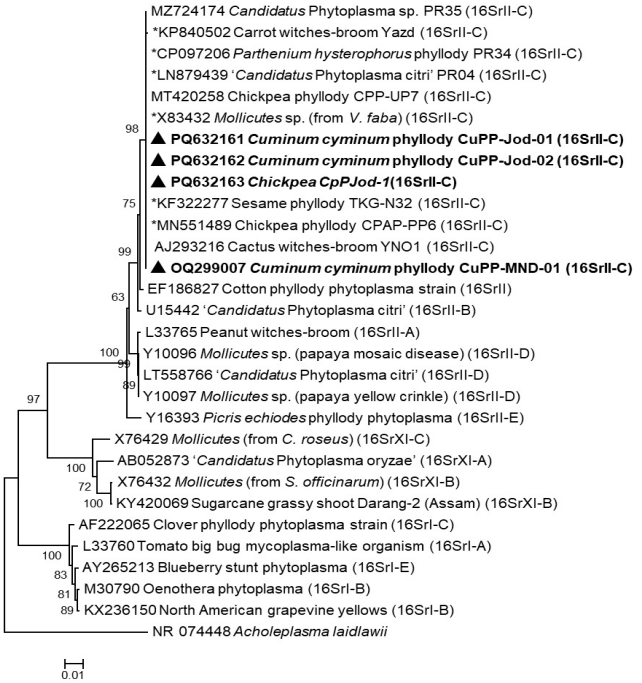


Figure 1. Neighbour Joining phylogenetic tree based on 16S rRNA gene showing the phylogenetic relationship among the cumin phyllody and chickpea phytoplasma strains with reported phytoplasma strains. The phylogenetic tree was constructed in Mega X with 1,000 bootstraps.

Discussion

Phytoplasma diseases are a major constraint for production of many economically important field crops including medicinal plants and spices (Rao, 2021). Phytoplasmas belonging to the 16SrII-C group infecting fennel, cumin and small cardamom has been reported from India (Rao, 2021; Kumar *et al*., 2024). In the present study, these phytoplasmas has been identified and characterized in 80 cumin genotypes/accessions and chickpea in Jodhpur in the state of Rajasthan. Earlier 16SrII-C subgroup phytoplasmas has been reported from various crops and weeds in India (Rao *et al*., 2021). The findings of the present study have economic and epidemiological implications and needs immediate attention to reduce export losses due to phytoplasma disease in cumin and to prevent the potential spread to other crops.

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Country status

Emerging phytoplasma threat: plum orchards under siege in Himachal Pradesh, India

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Abstract

Plum is considered to be one of the most important deciduous stone fruits of the temperate region belonging to the family *Rosaceae* and the genus *Prunus*. Plum trees exhibiting different symptoms like necrosis, stunting, yellowing, leaf reddening, severe inward longitudinal rolling of leaves, brittle leaves, abnormal thickening of the midribs and primary veins which produce tiny chlorotic leaves were collected. DAPI staining was done, and total DNA was extracted from infected samples and analyzed by PCR using the phytoplasma universal primer pairs P1/P7 followed by nested PCR with primer pairs R16F2n and R16R2. The results indicated that the symptomatic plum trees are infected with '*Candidatus* Phytoplasma ziziphi' strains. This is the first report of this phytoplasma in plum in India.

Keywords: disease, molecular detection, DAPI staining, PCR

Introduction

In India, the state of Himachal Pradesh ranks second in production of plum. Plum trees exhibiting different symptoms like necrosis, stunting, yellowing, leaf reddening, severe inward longitudinal rolling of leaves, brittle leaves, abnormal thickening of the midribs and primary veins which produce tiny chlorotic leaves were observed (Figure 1). However, there have been no previous reports of phytoplasma-associated diseases in plum trees in India. Therefore, detecting and identifying the pathogens that affect plum production is of utmost importance. To address this issue, preliminary studies were aimed at establishing the possible phytoplasma presence in plum plants showing these symptoms. The survey was performed based on symptomatology, histopathological detection using DAPI based fluorescence microscopy and molecular detection.



Figure 1. Symptoms in plum. From left: reddening and curling of leaves; yellowing symptoms in leaves; partial infection in a tree.

Material and Methods

To confirm the presence of phytoplasmas, symptomatic plum trees were selected and infected leaves exhibiting the above described symptoms were collected during autumn (August to November) season of 2023 from Shimla and Sirmaur districts of Himachal Pradesh, India.

Fluorescent microscopy studies were conducted using DAPI test (EVOS® Cell Imaging System, Carlsbad, California, USA) on symptomatic and asymptomatic samples collected from the same orchards. About 300 mg of leaf midribs from symptomatic and asymptomatic trees were subjected to CTAB extraction method (Ahrens and Seemüller, 1992) and for phytoplasma detection, PCR amplifications were carried out using universal primer pairs P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed in nested PCR by internal primers R16F2n and R16R2 (Gundersen and Lee, 1996). Further, the PCR products were sequenced and assembled using Bio Edit software (Hall, 1999) and submitted in the NCBI. Furthermore, phylogenetic trees were constructed using the MEGA 11 developed by Tamura *et al.* (2021).

Results and Discussion

Results on the fluorescent microscopic studies by employing DAPI test showed the distinctive blue fluorescence upon binding minor groove of DNA in the sieve tube elements of the sections of phloem tissues prepared from shoot, leaf petioles and mid veins of a symptomatic trees whereas sec-

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tions of phloem preparation from the asymptomatic trees did not show the same fluorescence in sieve tube elements (Figure 2).

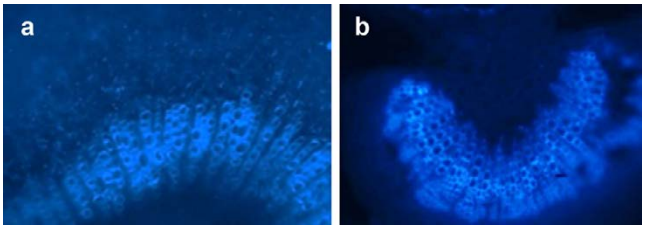


Figure 1. Fluorescent microscopy studies: (a) healthy control; (b) fluorescent phytoplasma bodies are visible in the sieve tube elements of the leaf midrib of a sample from a symptomatic plant.

Gel electrophoresis showed PCR products of the expected length (about 1,240 bp) only in symptomatic plum samples after nested amplification (data not shown). After sequencing of the obtained 16S rRNA gene amplicons it was possible to confirm that in the symptomatic leaves there were phytoplasmas. The phytoplasmas detected in plum with reddening of leaves along with mid veins and curling of leaves, yellowing symptoms and in some cases also showing only partial infection of the canopy were identified as members of elm yellows, 16SrV-B ('*Candidatus* Phytoplasma ziziphi') ribosomal subgroup (Jung *et al*., 2003).

'*Ca*. P. ziziphi' infection in plum was previously reported in China and include little leaves, branches with shorten internodes, and witches' broom (Gao *et al*., 2020) that are partially similar to those detected in the present work. Although '*Ca*. P. ziziphi' was already reported in India to infect peach (Khan *et al*., 2013) and jujube (Khan *et al*., 2008) this is the first identification of the phytoplasma in plum. However the phytoplasma is reported to infect other stone fruits and it was identified in cherry in Italy (Paltrinieri *et al*, 2005) and in China (Zhu *et al*., 1997; Wang *et al*., 2014). Moreover, in India the presence of '*Ca*. P. asteris' was reported in apricot plants showing decline symptoms in Jammu and Kashmir (Rao *et al*., 2020).

Phytoplasma infection in stone fruits worldwide is mainly associated with the presence of '*Ca*. P. pruni' (X-disease, 16SrIII-A) in the American continent and '*Ca*. P. prunorum' (European stone fruit yellows, 16SrX-B) in the European crops (Fiore *et al*., 2018). The finding of this phytoplasma in plum is therefore alarming and should be taken into due consideration to avoid outbreaks related to both the presence of possible, still unknow, insect vectors and to the use of infected material for grafting or producing propagation materials.

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Country status

Multilocus gene characterization of phytoplasmas associated with weeds in chickpea crops in Andhra Pradesh, India

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Abstract

Suspected phytoplasma symptoms of leaf chlorosis, witches' broom and little leaf were recorded in *Cleome viscosa* and *Croton sparsiflora* weeds near chickpea fields in Andhra Pradesh. The symptomatic weed species were analyzed for phytoplasma presence through nested PCR assays by utilizing universal phytoplasma specific primer pairs, P1/P7 and R16F2n/R16R2 and resulted positive. The phytoplasma identification was further achieved by using five genes *secA*, *rp*, *imp*, *tuf* and *secY*. The results of the multilocus gene amplification indicates that phytoplasmas belonging to 16SrII group were present in the tested materials. The presence of phytoplasmas in weeds near chickpea fields poses a risk for disease transmission through phloem-feeding insect vectors, such as leafhoppers and planthoppers, which are known to transmit phytoplasmas over short and long distances.

Keywords: molecular characterization, PCR, multilocus analyses, sequencing

Introduction

Phytoplasma are obligate prokaryotic wall-less bacteria, pleomorphic, with size varying from 200 to 800 nm and possess a very small genome of about 680-1,600 kbp. Phytoplasmas are associated with over 600 diverse plant diseases worldwide, mainly transmitted by phloem-feeding insects, especially leafhoppers and planthoppers (Bertaccini *et al*., 2014). Besides many economic crop species, several weeds are reservoirs of phytoplasmas and play an important role as natural alternative/collateral hosts (Blanche *et al*., 2003; Joomum *et al*., 2007; Pasquini *et al*., 2007; Harrison and Oropeza, 2008; Mall *et al*., 2010). Early detection of these phytoplasmas in weeds is very important to avoid the possibility of further spread of phytoplasma diseases to cultivated crops.

Material and Methods

Symptomatic *Cleome viscosa* and *Croton sparsiflora* were collected near chickpea fields in SPSR Nellore district, Andhra Pradesh (Figure 1). Total nucleic acid was extracted by modified cetyl trimethyl ammonium bromide (CTAB) method and used for PCR amplification by using phytoplasma universal primers designed to amplify 16S rDNA. PCR amplifications were conducted using phytoplasma specific universal P1 (Deng and Hiruki, 1991) and P7 (Schneider *et al*., 1995) amplifying about 1,800 bp fragment. Further, PCR products were used in nested PCR amplification using universal primer pair R16F2n and R16R2 (Gundersen and Lee, 1996). Amplification and sequencing of five genes *sec*A, *rp*, *sec*Y, *imp* and *tuf* were employed by the utilization of primer pairs: SecAfor1/SecArev3 followed by nested PCR primers SecAfor5/ SecArev2 (Hodgetts *et al*., 2008; Bekele *et al*., 2011); rp(II)F/rp(I)R1A followed by seminested PCR primers rp(II)F2/ rp(I)R1A (Martini *et al*., 2007), SecYF1(II)/SecYR1(II) followed by semi-nested PCR primers SecYF2 (II)/SecYR1(II) (Lee *et al*., 2010), TUF-II-F1/ TUF-II-R1 followed by semi-nested PCR primers TUF-II-F2/ TUF-II-R1) and IMP-II-F1/IMP-II-R1 followed by semi-nested PCR primers IMP-II-F2/IMP-II-R1 (Al-Subhi *et al*., 2018). The pair-wise sequence comparison analysis was done through BLAST analysis. The sequence data of each sample was edited, aligned and assembled with CLC Genomics Workbench 12.0.

Results

Both symptomatic weed species, *C. viscosa* and *C. sparsiflora* yielded about 1.8 kb product in PCR with P1/P7 universal primer pair.

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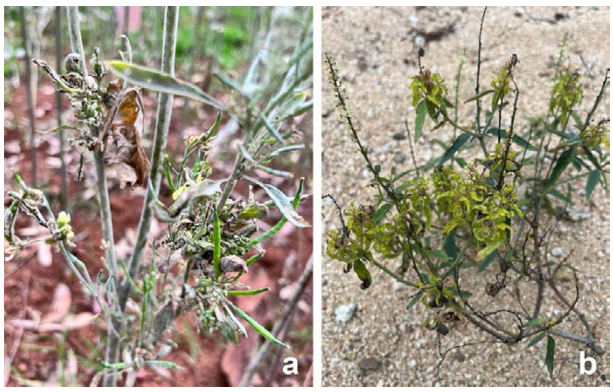


Figure 1. (a) Witches' broom symptom in *Cleome viscosa* and (b) yellowing of leaves in *Croton sparsiflora*.

These products were further processed for nested PCR assays with R16F2n/R16R2 primer pair, which yielded specific amplicons of about 1.2 kb. PCR products of about 1,390 bp and 1,290 bp with direct rp(II)F/rp(I)R1A and semi-nested rp(II)F2/rp(I)R1A primers; about 840 bp and 600 bp with SecAfor1D/SecArev3 primer pair followed by SecAfor5D/SecArev3; about 1.7 kb amplicons with direct secYF1(II)/secYR1(II) and semi-nested secYF2(II)/secYR1(II) primer pairs; about 1,094 bp amplicon size with semi-nested TUF-II-F2/TUF-II-R1 primer and 717 bp with semi-nested IMP-II-F2/IMP-II-R1 primer pairs were amplified from weed species. The results of the multilocus gene amplification indicates that phytoplasmas belonging to 16SrII group were present in the tested materials.

Discussion

This study highlights the role of weed species, *C. viscosa* and *C. sparsiflora*, as collateral hosts of 16SrII phytoplasmas, which could pose a threat to nearby cultivated crops such as chickpea. The successful amplification of phytoplasma DNA from symptomatic weeds using both universal and multilocus gene primers confirms their association with phytoplasma-related diseases hosts (Blanche *et al*., 2003; Mall *et al*., 2010). This highlights the ecological significance of weeds in maintaining phytoplasma populations and potentially exacerbating their spread to economically important crops. The observed witches' broom in *C. viscosa* and yellowing of leaves in *C. sparsiflora* are consistent with phytoplasma-associated symptoms reported in other plant host species. These symptoms arise from the disruption of hormonal and metabolic pathways due to phytoplasma colonization in the phloem, which impairs nutrient transport and plant growth (Omar *et al*., 2014). The presence of phytoplasmas in weeds near chickpea fields poses a risk for disease transmission through phloem-feeding insect vectors, such as leafhoppers and planthoppers, which are known to transmit phytoplasmas over short and long distances. This underscores the need for integrated pest and weed management strategies to minimize the impact of phytoplasma diseases on crop productivity.

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Epidemiology and control

Evaluating the threat of phytoplasma disease emergence in agroecosystems and natural habitats

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Abstract

Outbreaks of phytoplasma diseases annually cause billions of dollars in crop losses worldwide. A few efforts have been made to predict disease outbreaks and management continues to focus primarily on reducing pathogen spread following an outbreak. This study leverages machine learning to assess the global risk of emerging phytoplasma diseases using data from the literature on previous phytoplasma outbreaks in agroecosystems, combined with newly documented occurrences of phytoplasma-positive insects (potential vectors) in natural areas worldwide. By applying supervised machine learning on these datasets, key predictors of vector-host-phytoplasma interactions were identified and their importance in facilitating disease outbreaks was evaluated. The model highlights critical differences between two types of ecosystems and establishes a foundation for predicting new phytoplasma-host associations. These findings pave the way for targeted interventions to mitigate the risk of future outbreaks.

Keywords: vector-phytoplasma associations, machine learning, pathogen biodiversity, emerging plant diseases

Introduction

Outbreaks of plant diseases affecting agriculture have, until very recently, been considered rare and unpredictable. Thus, disease management continues to focus on reducing pathogen spread following an outbreak. Proactive strategies, such as landscape-level prediction of disease emergence incorporating data on natural vegetation and its interfaces with crops have rarely been attempted; however they should be feasible given sufficient information on vegetation cover, climate, and previously documented associations between pathogens and their hosts (including insect vectors). Here it was outlined a new approach for predictive modeling of disease emergence in phytoplasmas, one of the most widespread and important groups of vector-borne plant pathogens, which annually cause billions of dollars in yield losses to agriculture worldwide (Kumari *et al.*, 2019). This approach leverages available landscape, socio-economic and climatic data, combined with data on associations between phytoplasmas, their insect vectors and plant hosts from agroecosystems and natural habitats to identify areas at risk of disease outbreaks. By using two databases created by the team at the University of Illinois, this study provides preliminary results of a modeling approach to predict emerging phytoplasma diseases.

Materials and Methods

Relevant data (coordinates, country, crop type, infected insects, rate of infection, phytoplasma strain, year) were compiled from 838 publications, yielding a database of 317 unique occurrences of phytoplasma-insect associations. About 42% specimens tested (135) were phytoplasma positive.

About 2,000 insect specimens representing distinct species and collected in various natural areas worldwide (722 sites in 50 countries) were analyzed using qPCR to quantify phytoplasma cells. Phytoplasma-positive samples were further characterized using a multi-locus approach (Trivellone *et al.*, 2023). About 13% specimens (265) were positive.

Environmental variables known or hypothesized to affect phytoplasma outbreaks, such as temperature, precipitation, population density, altitude, cropland proportion, land cover type, and environmental performance index were selected. Bioclimatic (average temperature -˚C and precipitation -mm) and elevation (m above sea level) data‘ at 30 arc seconds (approximately 1 km^2) resolution were downloaded from Worldclim 2.1 (Fick and Hijmans, 2017). Global raster data were downloaded from the Socioeconomic Data and Applications Center (SEDAC) website. There were considered 6 predictors: UN WPP-Adjusted Population Density, 2000 and 2020 (CIESIN, 2018),

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human footprint (Venter *et al.*, 2016), Terrestrial Biome Protection (global weights) /Biodiversity/Ecosystem Vitality (Wolf *et al.*, 2022), Food Insecurity Hotspots Data Set, v1 (2009 – 2019) (CIESIN, 2020), Global Agricultural Lands in 2000 (Ramankutty *et al.*, 2012), Landcover 2000 and 2050 (Millennium Ecosystem Assessment, 2005).

Data were analyzed using five supervised machine learning algorithms (Gradient Boosting Machine, Random Forest, Neural Network, General Linear Model, Recursive Partitioning) to build a final model that calculates the probability of infection of a potential insect vector (phytoplasma positive/negative) given the values of the most important selected variables. The models were trained using 20% of the data and the remainder of the data was used to test model accuracy. Classification accuracy and result reliability was evaluated using the Kappa statistic (comparison of observed to expected accuracy), with values ranging from -1 to 1 (<0 indicated that the classification is no better than a random classification). The models were fitted using the R-package caret (Kuhn, 2008).

Results

The most important variables driving positivity rate in known and potential insect vectors were precipitation, temperature, population density, proportion of cropland (for both habitat types), altitude (for agroecosystems) and environmental performance index (for natural habitats) (Figure 1). The top five predictors were used to train each classification model. The class imbalance ratio (proportion of negative samples) was calculated for agroecosystems and natural habitat datasets (0.75 and 6.5, respectively) and a correction was applied for the highly imbalanced natural habitat dataset. For both datasets, Random Forest performed better than the other algorithms (k= 0.30, for agroecosystems and k= 0.51, for natural habitats). For agroecosystems, sites in lowlands with higher population density were associated with higher probability of infections. For natural habitats, higher infection probability was associated with dry conditions and lower proportion of crop lands.

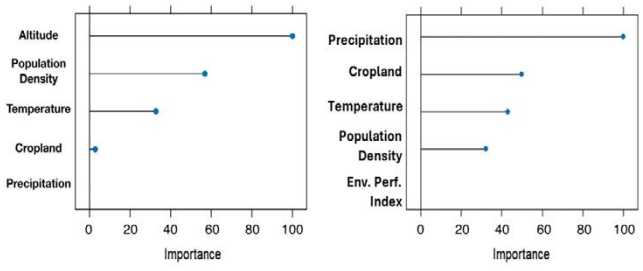


Figure 1. Top 10 variables by importance estimated using random forest to predict phytoplasma-infected insects in agroecosystems (left) and natural habitats (right).

Discussion

Data compiled from literature on phytoplasma outbreaks in agroecosystems were used combined with data from intensive screening of insect specimens collected from natural areas worldwide, to create a classification model predicting the probability of phytoplasma-positive insect vectors in different habitat types based on climatic, socio-economic and landscape characteristics. Although the models performed accurately on the test data, and data on recorded positive samples in natural habitats may be adequate, it was only possible to include 317 records from 47 sites in agroecosystems. Addition of data from agroecosystems, particularly in under sampled areas of the tropics, may increase the predictive power of the model. Next steps include adding more records from tropical regions, and from plant infections, using infection rate as the continuous variable to estimate the severity of outbreaks. Another step will be to evaluate a dataset explicitly incorporating interfaces between natural areas and agroecosystems to estimate the risk of spillover.

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Epidemiology and control

Emerging hosts of '*Candidatus* Phytoplasma trifolii': a molecular insight into *Luffa acutangula*, *Momordica dioca* and *Lawsonia innermis* from Northeast India

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Abstract

During April 2023, suspected symptoms of phytoplasma infection, such as little leaf, curling, and cupping were observed on *Luffa acutangula* and *Momordica dioca* plants, while stem flattening was observed in *Lawsonia inermis* plants in Jorhat district of Assam, India. To investigate the presence of phytoplasmas, leaf samples collected from both symptomatic and asymptomatic plants were subjected to DNA extraction and PCR assays. The presence of phytoplasmas was confirmed in symptomatic plants, while asymptomatic plants tested negative. Sequencing and phylogenetic analysis of the 16S rRNA gene revealed that the phytoplasma strains infecting these plants are '*Candidatus* Phytoplasma trifolii' strains. Using *in silico* RFLP analysis, the phytoplasma strains were further identified as 16SrVI-D subgroup. This is the first report of '*Ca.* P. trifolii' strains, subgroup 16SrVI-D in *Luffa acutangula*, *Momordica dioca*, and *Lawsonia inermis* worldwide.

Keywords: '*Candidatus* Phytoplasma', little leaf, stem flattening, phytoplasma, 16SrVI-D

Introduction

Luffa acutangula (ridge gourd) and *Momordica dioca* (spiny gourd), both from the Cucurbitaceae family, are widely cultivated vegetables having therapeutic properties, such as antifungal, antioxidant, and antidiabetic effects. *Lawsonia inermis* ("henna") from the Lythraceae family also holds industrial and medicinal value due to compounds found in various plant parts. Phytoplasma diseases pose a significant threat to these plants, affecting yield and quality of their products (Bertaccini *et al*., 2014).

Phytoplasmas belonging to seven groups (16SrI, 16SrII, 16SrIII, 16SrVI, 16SrVIII, 16SrIX, 16SrXII) associated with cucurbit diseases have been reported worldwide (Kumari *et al*., 2019). Similarly, 19 phytoplasma ribosomal groups, each comprising various subgroups, have been reported to infect medicinal plants and among them, eight (16SrI, 16SrII, 16SrV, 16SrVI, 16SrXI, 16SrXII, 16SrXIV, and 16Sr XXIV) are reported from India (Marcone *et al*., 2016). Notably, subgroup 16SrI-B phytoplasmas are frequently identified in Europe, North America, and Asia (Marcone *et al*., 2016).

Phytoplasma diseases significantly impact the yield, quality, and lifespan of vegetable and medicinal plants. Despite numerous reports of phytoplasma diseases affecting various cucurbits and medicinal plants in India, there is no record of phytoplasmas in *L. acutangula*, *M. dioca*, and *L. inermis* globally. In this study, an attempt was made to identify the phytoplasmas present in symptomatic plants of these species.

Materials and Methods

In April 2023, during field surveys in the Jorhat district of Assam, India, phytoplasma-suspected symptoms were observed on *L. acutangula*, *M. dioca*, and *L. inermis* plants. To investigate the possibility of phytoplasma being associated with the symptoms, total DNA was extracted from five symptomatic and two non-symptomatic plants of each species using a CTAB protocol. The DNAs were analysed by direct and nested PCR using P1/P6 (Deng and Hiruki, 1991) and R16F2n/R16R2 primers (Gundersen and Lee, 1996), respectively. PCR products were Sanger sequenced in each direction, and the consensus sequences were deposited in GenBank. Partial sequences were further subjected to *in silico* RFLP analysis with *i*PhyClassifier tool (Zhao *et al*., 2009) for ribosomal subgroup identification.

Results

Typical phytoplasma-associated symptoms such as little leaf, curling, and cupping of leaves were observed on *L. acutangula*

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and *M. dioca* plants. Additionally, stem flattening was observed in the *L. inermis* plant in Jorhat (26.7247°N, 94.1936°E) (Figure 1). The incidence of these symptoms was about 3-5%, based on visual observations.



Figure 1. Phytoplasma associated symptoms in cucurbits and medicinal plants: (a) *Luffa acutangula*; (b) *Momordica dioca*; (c) *Lawsonia inermis*.

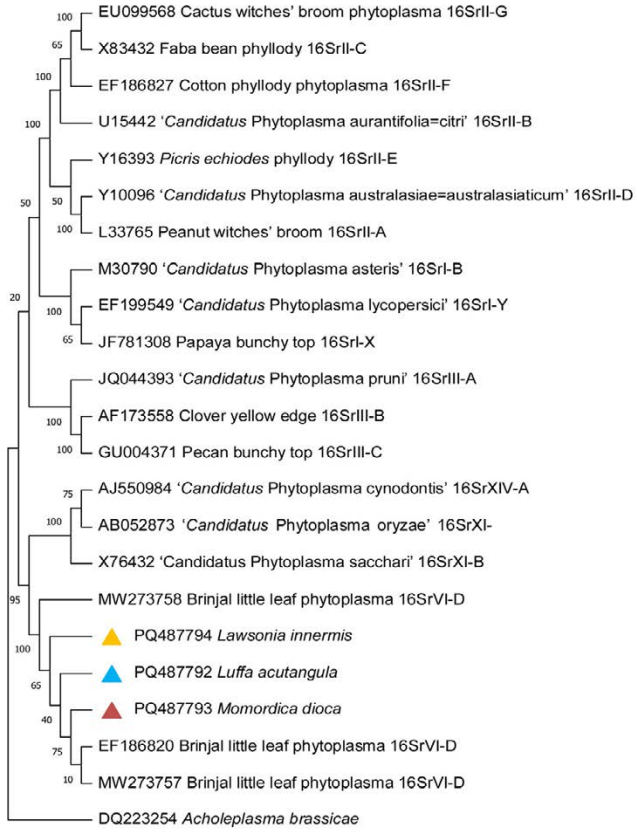


Figure 2. Phylogenetic tree constructed by maximum likelihood method of 16S rRNA gene sequences from the phytoplasmas detected in *Luffa acutangula*, *Momordica dioca*, *Lawsonia inermis* and selected phytoplasma from GenBank database. *Acholaeplasma brassicae* was used as outgroup.

All symptomatic samples collected during the survey yielded an amplicon size of approximately 1.5 kb from PCR. Additionally, a nested-PCR assay produced DNA fragments of about 1.25 Kb from the same samples. No amplification was observed in DNAs from asymptomatic plant samples. The PCR products were Sanger sequenced in both directions, and the consensus sequences were deposited in GenBank with the accession numbers PQ487792 (*L. acutangula*), PQ487793 (*M. dioca*), and PQ487794 (*L. inermis*). Blast analysis of these sequences demonstrated 99.14% sequence identity with '*Candidatus* Phytoplasma trifolii', 16SrVI-A (GenBank accession number AY390261). Further, phylogenetic tree analysis (Figure 2) and *i*PhyClassifier-based virtual RFLP analysis of the 16S rRNA gene sequence revealed that the phytoplasmas associated with these three plant species belongs to the 16SrVI-D subgroup, with a similarity coefficient of 1.00 with the reference strain of this subgroup.

Discussion

Phytoplasma-associated plant diseases are widely distributed in India, posing risks to vegetable and medicinal plants due to economic losses and impacts on phytochemical production. Around 150 plant species in India host various phytoplasma ribosomal groups (Rao *et al*., 2020). This study reports for the first time, the presence of 16SrVI-D phytoplasma subgroup in *L. acutangula*, *M. dioica*, and *L. inermis* from Assam, India. Previously, 16SrVI-D has been found in solanaceous and medicinal crops in Assam, often spreading via leafhopper vectors (Kalita *et al*., 2019; Dutta *et al*., 2022, 2024). Further studies on insect vectors and epidemiology are essential to control the spread of these phytoplasmas.

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Epidemiology and control

Biological and molecular features of '*Candidatus* Phytoplasma prunorum' associated with a severe outbreak in apricot

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Abstract

European Stone Fruit Yellows (ESFY) outbreaks have recently become quite common in stone fruits, especially apricots, where the disease can severely damage production, resulting in economic losses. Since there is scarce knowledge about the disease, this study evaluated some biological and molecular aspects of '*Candidatus* Phytoplasma prunorum' (16SrX-B) associated with ESFY by analyzing 30 symptomatic plants from a severely infected apricot orchard in Italy. In particular, resistance induction by Plasma Activated Water (PAW) was evaluated and genetic variability in the phytoplasma strains was searched through a multigenic study. In addition, the presence of potential insect vectors was investigated and cultivation trials of phytoplasma and other possible bacterial agents in artificial media were conducted. The results showed that PAW treatments did not affect the presence of phytoplasmas, nor the symptom severity in plants, and despite the different symptomatology observed in the orchard, no '*Ca.* P. prunorum' variants were identified. Moreover, despite the growing number of symptomatic plants in the orchard, none of the potential vector species analyzed were positive for phytoplasmas. Finally, cultivation trials showed that it was not possible to isolate '*Ca.* P. prunorum' on the artificial medium, however, *Pseudomonas* species were isolated and identified.

Keywords: 16SrX-B, ESFY, *Cacopsylla pruni*, plasma activated water, apricot

Introduction

'*Candidatus* Phytoplasma prunorum' (16SrX-B) is the phytoplasma associated with European Stone Fruit Yellows (ESFY) disease, which is spread mainly in Europe, but also in Egypt, Turkey, Iran, and Tunisia. It is one of the most dangerous diseases for stone fruits, especially apricots, and can lead to significant economic losses (Koncz *et al.*, 2024).

'*Ca.* P. prunorum' is transmitted through propagation material and the psyllid *Cacopsylla pruni*, the only vector known until now (Koncz *et al.*, 2024; Marcone *et al.*, 2022). The defense against the pathogen is made exclusively by prevention methods, such as using healthy nursery material, reducing insect vector populations, removing the symptomatic plants, or pruning the rootstock suckers (which can easily attract the vectors) (Marcone *et al.*, 2010). Since there is lack of effective defense methods, this study evaluated the efficacy of Plasma Activated Water (PAW) as a sustainable resistance inducer. PAW is derived from the interaction of water and cold atmospheric-pressure plasma, generated by different sources. Such interaction enriches the liquid in reactive oxygen and nitrogen species (RONS), mainly hydrogen peroxide (H_2O_2), nitrite (NO_2^-), and nitrate (NO_3^-), lowers the pH and increases oxidation-reduction potential and conductivity, overall increasing its biological activity (Thirumdas *et al.*, 2018). Some authors observed that the RONS generated in PAW, are capable of inducing the synthesis of phenylalanine ammonia-lyase, a key enzyme in the phytoalexins production, and the activation of the systemic acquired resistance, demonstrating that PAW can effectively induce a resistance response in plants infected by bacteria (Perez *et al.*, 2019) and phytoplasmas (Zambon *et al.*, 2019; Laurita *et al.*, 2020). Considering the different symptomatology observed in the orchard, and the rising number of symptomatic plants, previous studies assessed the presence of '*Ca.* P. prunorum' strain variants (Kison and Seemüller, 2001; Martini *et al.*, 2010) and hypothesized the presence of other potential insect vectors. Therefore a strain characterization was performed, and new potential vectors were searched for. Cultivation trials were also conducted to isolate the phytoplasma and other bacteria using the artificial substrate designed for isolating and growing mainly grapevine phytoplasmas (Contaldo *et al.*, 2016).

Materials and Methods

Thirty apricot plants cultivar Faralia grafted on Myrabolan

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from and orchard in Italy, were analyzed in this study. The samples collected from each plant consisted of leaf veins from symptomatic leaves and phloem tissue from small branches. The potential insect vectors were captured with yellow chromotropic traps positioned in the orchard from March to July. The species identified and analyzed were *Fieberiella florii*, *Japananus hyalinus*, *Platymetopius* sp., and *Reptalus* sp. The DNA extraction was performed with a CTAB method.

PAW was generated using a hybrid cold plasma source, consisting of a dielectric barrier discharge source (DBD) and a corona discharge source, both connected to a micro-pulsed high-voltage generator (AlmaPulse, AlmaPlasma s.r.l.). A total volume of 6 l of tap water was treated using the DBD reactor operating at 20 kV and 5 kHz for 45 seconds, and the corona source operating at 14 kV and 4 kHz for 5 minutes, repeating the process 6 times, for a total of 34.5 minutes of treatment. The amounts of RONS produced were 5 mg/L for H_2O_2, 500 mg/L for NO_3^-, and 80 mg/L for NO_2^-. The treatments on plants were performed by drilling a hole in the stem's basal part, injecting 20 ml of liquid inside, and immediately sealing them with grafting putty. Three treatments were performed in April, June and July 2023. Overall, 17 plants were treated with PAW and 13 with tap water.

The isolation was performed by cutting leaf veins and branches' phloem tissue (previously sterilized in sodium hypochlorite 40%) inside liquid CB media (CBl) (Contaldo *et al*., 2016) containing ampicillin 50 µg/ml and incubating it at 25°C until the observation of microbial growth. Then, a drop of CBl was transferred and distributed into solid CB media (CBs) and incubated at 25°C in microaerophilic conditions until the growth of colonies. On CBs different antibiotics (ampicillin 50 µg/ml, kanamycin 0.5 µg/ml, cefalexin 0.5 µg/ml, and 5-fluorouracil 0.5 µg/ml) were added both individually and in combinations.

Phytoplasma detection in plant and insect material and colonies was performed by amplifying the 16S ribosomal gene, followed by RFLP analysis, using 16SrX group phytoplasma-specific primers and enzymes, respectively. The presence of genetic variants in the '*Ca*. P. prunorum' strain was assessed by amplifying the non-ribosomal genes *secY*, *aceF*, *leu*, and *secA* with specific primers, followed by RFLP analysis with specific enzymes.

Results

PAW treatments did not affect the presence of phytoplasmas in apricots: the treated plants showed the same symptomatology as the controls, and it was possible to detect '*Ca*. P. prunorum' both before and after treatments. Despite the different symptomatology observed in the orchard, '*Ca*. P. prunorum' variants were not identified for any of the genes analyzed. Moreover, despite the rising number of symptomatic plants and the absence of *C. pruni*, none of the insect species analyzed tested positive phytoplasmas. Finally, cultivation trials showed that it was not possible to isolate '*Ca*. P. prunorum' on CB medium, but among the bacterial endophytes isolated, *Pseudomonas* sp., and *P. graminis* were identified after 16S ribosomal gene sequencing.

Discussion

The negative results of PAW treatments could be due to several factors, including excessive PAW dilution in old and oversized plants, non-optimal treatment mode, the necessity to repeat treatments over years, or excessive susceptibility of the plants' rootstock-graft combination. Since no variants of '*Ca*. P. prunorum' were found, new variants could be searched for in other genes or with other methods (*e.g*., sequencing) or the differences in the symptomatology could be due to the synergy with other pathogens or different endophytic populations (*e.g*., *Pseudomonas* sp., and *P. graminis*) which could modulate the severity of the infection. The increase in symptomatic plants could be due to vector species different from the one analyzed or long period of latent infection that was present in the nursery material. Finally, cultivation trials were not successful probably due to the high endophytic competitive populations present within the apricot trees at the time of sampling or the non-optimal growth environment provided by the compounds and/or antibiotics of the artificial media.

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Epidemiology and control

Influence of weather parameters on sesame phyllody

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Abstract

Phyllody is an important disease in the sesame growing area of Madhya Pradesh, India. This study carried out impact of weather parameters-temperature, relative humidity, and rainfall-on the incidence of phyllody disease and the population of its insect vector. It was verified that minimum temperature, relative humidity, and rainfall were significantly negatively correlated with disease incidence and leafhopper population, while maximum temperature showed a positive, though statistically non-significant, correlation. The highest disease incidence (22.2%) and leafhopper population (5.8 per leaf) occurred during week 45 when the temperatures were moderate, but the relative humidity was low. This indicates that specific weather conditions, particularly lower humidity and higher minimum temperatures, might favour the disease spread and the insect vector population. The study concludes that effective management of sesame phyllody disease could benefit from monitoring these key weather parameters to predict disease outbreaks and implement timely interventions.

Keywords: phytoplasma disease, management, disease incidence

Introduction

The most important sesame diseases are phyllody, root/stem rot and powdery mildew. Sesame phyllody is epidemic in India, mostly in peninsular regions, with an incidence of about 75% (Manjunath *et al*., 2012: Gupta *et al*., 2018). A virus was hypothesized as the causative agent of the disease, later confirmed as mycoplasma-like organisms (MLO) now called phytoplasmas (Das and Mitra, 1998), which play a leading role in causing significant crop losses. Phytoplasmas are transmitted by insects that feed on plants such as planthoppers, psyllids and leafhoppers, they are pleomorphic bacteria and have a small genome. In plants, they are confined to the phloem tissue and spread by passing through the pores of the sieve plates that separate the phloem sieve tubes. Kumar *et al*. (2009) revealed that the maximum occurrence of phyllody disease is transmitted by crops grown in the 35–36 metrological week due to the seasonal prevalence of sesame-sap-feeding insects, as well as the peak infestation of the leafhopper vector, *Orosius albicinctus*, in the 36th metrological week. Cagirgan *et al*. (2013) verified the effect of climatic variability on the incidence of sesame phyllody and the disease's symptoms in a Mediterranean setting. This study unequivocally demonstrated the influence of climatic variability in the disease severity. Herein the disease symptoms are described and further details are reported on impact of insect vector population and weather parameters on the occurrence and progression of phyllody disease in India.

Materials and Methods

In the Experimental plot of the Department of Plant Pathology, AICRIP on Sesame and Niger, College of Agriculture, JNKVV Jabalpur, observations were made from 02-10-2023 to 20-11-2024, covering 8 meteorological weeks during "kharif" 2023. This was done to investigate the relationship between meteorological parameters and the natural occurrence of sesame phyllody. The incidence and population of leafhoppers were correlated with weather data such as rainfall (mm), relative humidity (%), and maximum and minimum temperature (°C). The percentage of disease incidence was also determined to correlate leafhopper population and phyllody disease presence.

Results

The impact of weather and climatic parameters on disease incidence percentages and vector populations was calculated. The highest leafhopper population of 5.8/leaf was found to be present at 45 standard weeks, along with the highest disease incidence of 22.2%. Minimum vector populations of 3.2 per leaf and minimum disease incidence of 12.3% were found at 40 standard weeks. Minimum temperature, relative humidity and rainfall (Figures 1, 2

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and 3) were shown to have a negative significant correlation with the exception of maximum temperature, which was found to be negatively but not statistically correlated with the percentage of disease incidence. Disease incidence was positively correlated with the maximum leafhopper population. Relative humidity was negatively significant connected, although temperature, minimum temperature and rainfall were not significantly correlated. Relative humidity has a negative connection with both the percentage of disease incidence and the number of plant leafhoppers (-0.713 and -0.728, respectively). The highest temperature was negatively correlated (although not significantly) with plant leafhopper and disease incidence. A multiple correlation between the disease incidence and a group of four independent variables (weather parameters) was responsible for the disease in the cropping season under study. The coefficient of multiple determinations (R2) was 0.989%.

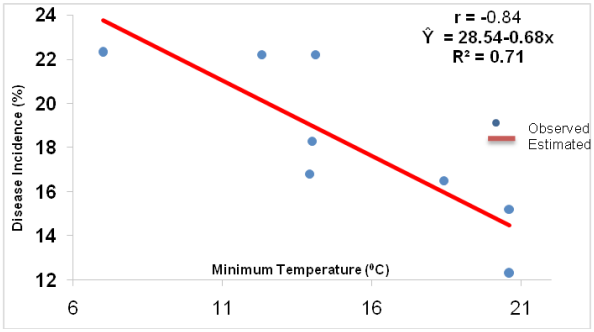


Figure 1. Regression of minimum temperature on phyllody incidence of sesame.

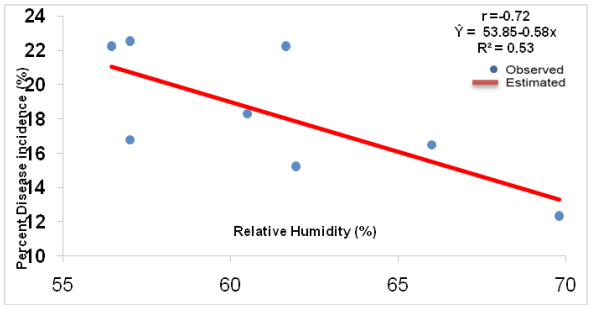


Figure 2. Regression of relative humidity on phyllody incidence of sesame.

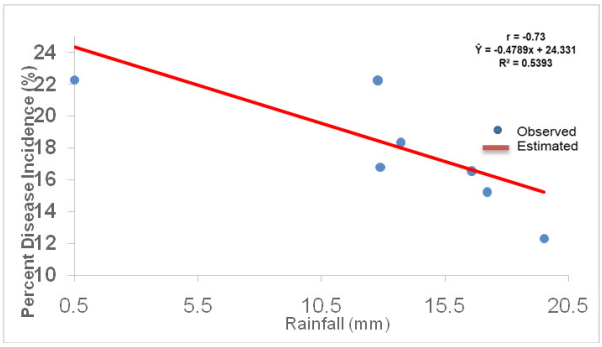


Figure 3. Regression of rainfall on phyllody incidence of sesame.

Discussion

The results of analyzing the four weather factors showed that the percentage of disease incidence was negatively correlated with the minimum temperature, relative humidity and rainfall, but not with maximum temperature. The percentage of disease incidence and leafhopper population positively correlated with each other. Similar findings about the effect of weather parameters in relation to the incidence of phyllody disease was reported by Kumar *et al.* (2009).

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Epidemiology and control

Identification of resistant germplasm in sesame against phyllody disease

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Absract

Sesame is an ancient oilseed crop. It plays an important role in the oilseed economy of the world. The export of sesame is increasing day to day and having bright future for its export potential. The crop is affected by many pests and diseases. One of the important pests is the leafhopper *Orosius albicinctus*, which transmits the phyllody diseases associated with phytoplasmas. Phyllody is an important disease of sesame, the affected plants become stunted, and the floral parts being modified into leafy structure which results in non bearing of fruits and seeds causing yield loss up to 33.9%. The investigation was undertaken to screen a large number of germplasms against phyllody disease in sesame. A field experiment was conducted to find out resistant germplasm against phyllody during the "kharif" cropping seasons in 2020 (3,701 accessions), 2021 (1,475 accessions), 2022 (85 accessions) and 2023 (67 accessions). Seeds were sown in augmented plot design with resistance and susceptibility check after every twelfth row. The incidence of phyllody was recorded individually by counting the number of infected and healthy plants at random by quadrate selection in each row and the percentage of disease incidence was calculated. Observations on disease incidence, test weight and yield gm/plant were recorded. Among the 5,328 accessions tested only less than 10% of the screened germplasm was found resistant to phyllody disease.

Keywords: phytoplasma, incidence, screening

Introduction

Sesame (*Sesamum indicum* L.), a member of the Pedaliaceae, is one of the most ancient oil seed crops used by humans. It is one of the most important oil seed crops cultivated in the 12.84 million hectares worldwide, producing more than 6.74 million tons of seed. India, Sudan, Myanmar, Nigeria and China are the major contributors with more than 78% of the world production (FAO, 2022). Sesame seed is a rich source of protein (20%) and edible oil (50%) and contains high amounts of saturated fatty acids (47% oleic acid and 39% linolenic acid) (Uzun *et al*., 2008). Sesame oil has excellent stability due to the presence of the natural antioxidants sesamoline, sesamin and sesamol and it is widely used for different purposes (Shyu and Hwang, 2002). The crop has low productivity (0.53 tons/ha) due to its cultivation in sub-marginal lands with improper agronomic practices and non-availability of high yielding varieties lacking inbuilt resistance to biotic and biotic stresses. Sesame phyllody (Figures 1 and 2) can cause a yield loss of up to 34% or even 100% in case of severe incidence (Sarwar and Haq, 2006). Phytoplasmas are associated with devastating diseases in a wide variety of plant species (Bertaccini and Lee, 2018). These sieve tubes inhabiting pathogens spread in nature by sap sucking leafhopper, planthoppers and psyllids. Leafhoppers (*Orosius albicinctus*, *O. orientalis*, *Hishimonus phycitis* and *Cirulifer haematoceps*) were reported as vectors in transmission of one or more phytoplasmas associated with phyllody in sesame (Ozdemir, 2017). Apart from the insect vectors, sesame phyllody can be transmitted by dodder and graft inoculation (Figue 3). Periwinkle is used as an effective indicator. Hence the following investigation was planned and undertaken to manage phyllody disease in sesame by finding resistant germplasm.

Materials and Methods

A field experiment was conducted at the Research Farm of Project Co-ordinating Unit, Sesame and Niger, JNKVV., Jabalpur during the "kharif" cropping seasons in 2020 (3,701 accessions), 2021 (1,475 accessions), 2022 (85 accessions) and 2023 (67 accessions). Seeds were sown in augmented

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plot design with resistance and susceptibility check after every 12th row. The incidence of phyllody was recorded individually by counting the number of infected and healthy plants at random by quadrate selection in each row and the percentage of disease incidence was calculated. Observations on disease incidence, test weight and yield gm/plant were recorded.



Figure 1. Healthy sesame field.



Figure 2. Sesame field infected with phyllody.



Figure 3. Left insect vector of phyllody, right dodder transmitted phyllody.

Results

Among the 3,701 accessions of sesame, most of the lines was susceptible and only in 63 accessions it was found resistance under natural field condition against phyllody, and disease reaction was below less than 10% during the year 2020. Among the 1,475 accessions of sesame, most of the lines were susceptible and only 58 were found resistant under natural field condition against phyllody and the disease reaction was below less than 10% during the year 2021. Among the 85 accessions of sesame, most of the lines were susceptible and only 66 accessions were found resistant under natural field condition during the year 2022. Among the 67 accessions of sesame, most of the lines were susceptible and only 21 accessions were found resistant under natural field condition against phyllody, and the disease reaction was below less than 10% during the year 2023.

Discussion

Resistance of sesame accessions was evaluated according to disease incidence, test weight and yield. Accessions with less than 10% disease incidence were considered as resistant. Four-year data concluded that among the 5,328 accessions only in 208 accessions it was found resistance to phyllody under natural field condition. The disease incidence was always below 10%. Similar results were reported by Sarwar and Haq (2006) for evaluation of sesame germplasm for genetic parameters and disease resistance. Sesame phyllody could be therefore managed by using resistant cultivars (Akhtar *et al*., 2013).

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Epidemiology and control

Epidemiology of brinjal little leaf phytoplasma in Assam, India: genetic diversity and seed transmission

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Abstract

A roving survey across five agro-climatic zones of Assam was conducted to assess the incidence of brinjal little leaf (BLL) disease and verify the presence of genetic diversity of phytoplasma strains associated. The highest disease incidence was recorded in the Upper Brahmaputra Valley Zone (18.73%) and the lowest in the Hill Zone (1.25%). Molecular analysis of the 16S rRNA gene sequences from this study revealed that the phytoplasmas infecting brinjal are '*Candidatus* Phytoplasma trifolii' strains. *In silico* RFLP analysis further identify these strains as enclosed in the 16SrVI-D subgroup. Seed transmission studies confirmed that BLL phytoplasma is seed-transmissible, showing a 100% disease incidence in seedlings grown from seeds collected in symptomatic mother plants, verified through symptomatology, PCR, and 16S rRNA gene sequence comparison.

Keywords: brinjal, '*Candidatus* Phytoplasma', 16SrVI-D, molecular identification

Introduction

Brinjal (*Solanum melongena* L.), commonly known as eggplant, is a member of the Solanaceae family and serves as a significant vegetable crop cultivated year-round across various regions of India, excluding higher altitudes. In Assam, it is grown on approximately 18,308 hectares, yielding around 3.25 million metric tons. One of the major threats to brinjal cultivation is the brinjal little leaf (BLL) disease, associated with phytoplasma presence, which limits production. Genetically diverse phytoplasmas are responsible for the BLL disease. So far, phytoplasmas from seven different groups have been recognized to infect brinjal worldwide (Rao and Kumar, 2017; Snehi *et al*., 2021). In recent years, there has been a notable increase in research on phytoplasma diseases, including new insights into BLL disease globally and in India. However, Assam lacks systematic studies and a comprehensive understanding of BLL disease epidemiology, despite sporadic observations of symptoms. This knowledge gap hinders the development of sustainable disease management practices tailored to the region. To address this issue, the current investigation was initiated.

Materials and Methods

A roving survey was conducted from 2021 to 2024 to study BLL disease incidence across five agro-climatic zones of Assam: North Bank Plains Zone (NBPZ), Upper Brahmaputra Valley Zone (UBVZ), Central Brahmaputra Valley Zone (CBVZ), Lower Brahmaputra Valley Zone (LBVZ), and Barak Valley Zone (BVZ). In each zone, symptomatic brinjal plants were observed, disease incidence percentages were calculated, and samples were collected for molecular analysis. DNA was extracted from symptomatic and non-symptomatic plants using a CTAB protocol, followed by analysis through direct and nested PCR with P1/P6 (Deng and Hiruki, 1991) and R16F2n/R16R2 primers (Gundersen and Lee, 1996), respectively. PCR products were sequenced in both directions and consensus sequences were deposited in GenBank. Partial sequences were further subjected to *in silico* RFLP analysis using the *i*PhyClassifier tool (Zhao *et al*., 2009) for 16Sr subgroup identification.

For seed transmission studies, plants with prominent BLL symptoms were uprooted during survey, transported to the Department of Plant Pathology, Assam Agricultural University, Jorhat, and analyzed for phytoplasma presence. Mature fruits from BLL-infected plants were dried; seeds were collected, sown, and seedlings were grown under insect proof conditions. Molecular detection and identification by sequencing was performed at various growth stages to confirm phytoplasma presence.

Results

Typical phytoplasma-associated symptoms were observed in brinjal plants during the survey such as little leaf, stunted

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growth, flower virescence, phyllody, witches' broom and curling and cupping of leaves. The incidence of BLL disease varied across Assam's five agroclimatic zones (Figure 1). The highest incidence was recorded in the UBVZ (18.73%), followed by the CBVZ (12.72%). The lowest incidence was in the HZ at 1.25%.

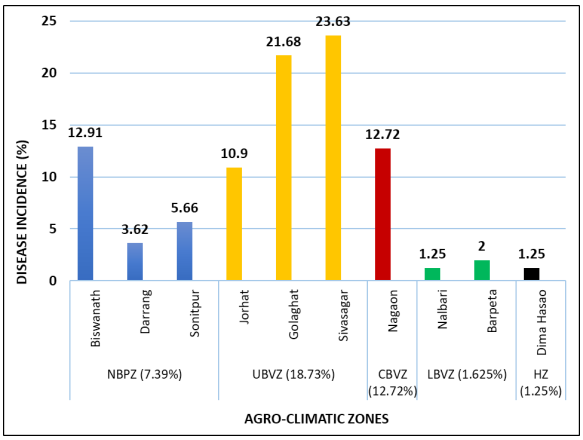


Figure 1. Brinjal little leaf disease incidence in different agro climatic zones of Assam.

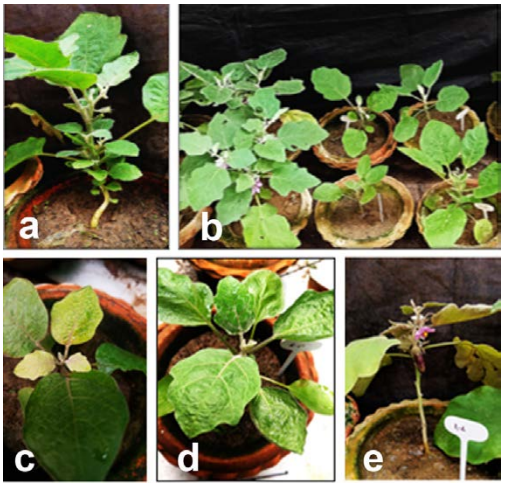


Figure 2. Seed transmission trials: (a) stunted leaves; (b) stunted plant; (c) yellowing; (d) leaf curling; (e) mummification of fruits.

Total genomic DNA from 198 brinjal samples, healthy and suspected of BLL phytoplasma infection, was extracted across five agro-climatic zones in Assam. PCR with universal primer pairs P1/P6 and nested primer pairs R16F2n/R16R2 confirmed phytoplasma presence in symptomatic plants, yielding 1.5 kb and 1.25 kb DNA bands, respectively (data not shown). The PCR products were Sanger sequenced in both directions, and the consensus sequences were deposited in GenBank with the accession numbers MW261864, MW261862, MW261866, PP514746, and PP524729. BLASTn analysis of these sequences demonstrated over 99% sequence identity with '*Candidatus* Phytoplasma trifolii'. Further, *i*PhyClassifier-based virtual RFLP analysis of the 16S rRNA gene sequence revealed that the BLL phytoplasma sequences generated in this study belong to 16SrVI-D subgroup. The BLL phytoplasma was confirmed to be transmitted by seeds, with symptoms appearing 28–30 days after sowing (Figure 2). PCR analysis detected the pathogen in all symptomatic plants, indicating a 100% transmission rate. Consistently, across various study stages—seed, seedling, 60 days post-transplanting, and fruiting—PCR assays produced expected amplicons of 1.5 kb and 1.25 kb. BLASTn analysis of Sanger sequences from PCR products of both mother plants and seedlings showed 100% identity between the two phytoplasma strains detected in mother plants and seedlings, respectively (GenBank accession numbers MW261863 and MW261865), confirming the phytoplasma seed transmission.

Discussion

The results of the study confirmed that the BLL phytoplasma sequences generated in this study belong to '*Ca.* P. trifolii' (16SrVI-D subgroup), indicating that the 16SrVI-D subgroup is the most widespread group infecting brinjal cultivation in Assam, India. The first evidence of phytoplasma seed transmission was reported in alfalfa from Oman, with subsequent studies confirming this in rapeseed, tomato, corn and very recently also in carrot and brinjal (Satta *et al*., 2019, 2020; Darabakula *et al*., 2024). This study confirmed that BLL phytoplasma is transmissible via seeds to progeny, providing conclusive evidence of seed transmission in brinjal. These findings underscore the importance of seed health management in preventing the spread of phytoplasma diseases.

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Epidemiology and control

Combatting pear decline in Flemish orchards: a comprehensive approach to disease detection and management

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Abstract

Pear decline, associated with the presence of *'Candidatus* Phytoplasma pyri', significantly impacts pear production in Flanders, causing symptoms like reduced fruit quality and stunted growth. This study aims to develop sustainable control strategies by examining factors that drive symptom expression, including co-infections, stress conditions, and varietal compatibility. High-throughput sequencing will be used to detect pear decline presence and identify potential co-infections across Flemish orchards. This will be complemented with drone assisted remote sensing in the field. Additionally, a vector control strategy focused on *Cacopsylla* populations will be explored. Expected outcomes include refined detection tools, symptom management through targeted practices, and validated control measures, paving the way for a sustainable approach to mitigate pear decline disease damages.

Keywords: '*Candidatus* Phytoplasma pyri', control, vector, disease monitoring, remote sensing, stress factors

Introduction

Recent years have seen a significant rise in the impact of pear decline associated with the presence of '*Candidatus* Phytoplasma pyri' on pear production in Flanders. Fruit from infected trees often remains small, lacks color, and has diminished flavor. Leaf symptoms typically emerge late in the summer, around September to October, at which point affected trees exhibit characteristic early yellow to red discoloration. Additional symptoms may include stunted growth, dieback of shoots, reduced leaf size and quantity, premature leaf drop in June and July, and increased flower drop. Notably, the disease can also remain latent, with symptoms not readily apparent (Gazel *et al*., 2007). This raises crucial questions about the factors that might trigger or suppress symptom manifestation, including infections by other pathogens, varietal susceptibility, rootstock or intermediate stem compatibility, drought or heat stress, nutritional deficiencies, and issues related to irrigation practices.

'*Ca.* P. pyri' can be spread through the propagation process in nurseries when infected starting material is used for grafting or oculating. However, in practice, this phytoplasma primarily depends on phloem-feeding insect vectors - specifically, pear psyllids - for its transmission, spread, and survival. In Belgium, *Cacopsylla pyri*, and to a lesser extent *Cacopsylla pyricola*, are important vectors (Belien *et al*., 2010). Once trees are infected, there is no curative treatment available. Therefore, prevention through effective screening and control of planting material, as well as existing orchards, is crucial in managing this disease. An important question remains: is it necessary to uproot all infected trees, or can it can be implemented some form of management for those orchards that are already infected? Is it possible to manage symptom expression in these trees to ensure that production and quality remain unaffected? Additionally, could other pathogens be involved, suggesting that adjusting control measures for pear decline might be beneficial? It can also be limited a further spread through a tailored vector control strategy? Addressing these questions requires more research and the development of effective control measures for practical application.

The aim of this study is therefore to address these questions and to develop and implement a sustainable and effective control strategy against pear decline.

Materials and Methods

The first step toward an effective and sustainable control strategy for pear decline involves expanding the understanding of the problem. This study will screen Flemish pear orchards using high-throughput sequencing to detect pear decline and potential co-infections, including viruses, bacteria, and other pathogens that may contribute

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to symptom expression. Techniques such as total RNA Illumina sequencing, metabarcoding with Oxford nanopore sequencing, quantitative PCR and LAMP analysis will be optimized and implemented. Additionally, the potential of drone assisted remote sensing, using RGB and multispectral cameras, will be tested and validated for rapid, large-scale detection of pear decline-infected pear trees based on diagnostic results. Whenever early tree discoloration is effectively confirmed by the growers, the soil map will also be reviewed to identify any potential link with anomalous soil types in the plot. In the second part, the aim is to gain insights into the causal stress factors driving increased expression of decline symptoms, which adversely affect pear production. The stress conditions to be evaluated include climatic factors (such as heat and drought), variety and rootstock selection, soil type and structure, and cultivation techniques, among others. Based on these insights, targeted and knowledge-based management strategies will be developed and tested under field conditions. The study begins with an extensive monitoring and inventory effort within Flemish pear cultivation, drawing on inspiration from previous phytoplasma-related research on other crops, both domestically and internationally. In the third step a vector control strategy will be elaborated. Primary focus will get to *C. pyri* and to a lesser extent also to *C. pyricola*. Methods under evaluation are stem injection of plant protection products, autumn control of adult pear psyllids using physical plant protection products, such as Siltac/Stix and kaolin treatments, among others. In the last step, practical control measures against pear decline are developed, validated and implemented under field conditions.

Expected Results

It is anticipated gaining a comprehensive understanding of the current pear decline situation in Flanders, facilitated by an efficient detection method developed through remote sensing and optimized sequencing technologies. These tools will enhance the capacity for ongoing disease monitoring, particularly in the context of co-infections by phytoplasmas and other pathogens like other bacteria and viruses. The project is expected to reveal insights into the variability within phytoplasma populations, which will possibly aid in understanding symptom development.

Furthermore, it is aimed to establish an effective, field-tested control strategy that prioritizes precision management of pear psyllid populations, minimizing the risk of phytoplasma transmission. This strategy will incorporate targeted cultivation practices, designed to mitigate decay symptoms by addressing key stress factors. Finally, there is the aim to validate the efficacy of alternative pesticides against pear decline phytoplasma infections and to assess the potential of combining tolerant rootstock and inter-stem varieties to reduce susceptibility.

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Epidemiology and control

Further study on the potential of *Frateuria defendens* as a biocontrol agent against phytoplasmas

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Abstract

Frateuria defendens isolated from the gut of phytoplasma insect vector is being studied as a possible bio-control agent to control yellows disease. Its low survival as endophyte in the plant tissue suggests exploiting its secreted antibacterial metabolites. Different isolates of *F. defendens* were tested for their inhibitory activity. The best isolate was introduced to melon plants and its cells were found in the plant sap 5 and 10 days post application. Sap, obtained from *F. defendens*- treated plants, inhibited the growth of *Spiroplasma melliferum* (a model mollicute) and altered the endophytic community of the plants. The results point at *F. defendens* as a candidate to develop bio-control measures against the yellows disease.

Keywords: inhibition activity, bacteria, management, *Spiroplasma melliferum*

Introduction

Frateuria defendens was isolated in Israel from the gut of the phytoplasma vector *Hyalesthes obsoletus* (Iasur-Kruh *et al*., 2018) Previous studies have shown its ability to penetrate and move in the plant vascular system of various plant species including melon and reduce yellows symptoms in grapevine and periwinkle suggesting that it may serve as a biocontrol agent to control phytoplasmas and other mollicutes (Lidor *et al*., 2018). However, its survival inside the plant tissue as an endophyte is short-lived (Naor *et al*., 2019) suggesting exploiting its anti-mollicute potential in a different way to use it as a biocontrol measure. The objectives were to study the inhibitory activity of *F. defendens* secreted metabolites using *Spiroplasma melliferum* and melon plants as model.

Materials and Methods

F. defendens isolates were grown on a rich medium (Tractenberg and Gilad, 2001) for 10 days before separating the crude filtrate by centrifugation and filtration (0.22 µm). Inhibitory activity of *F. defendens* isolates was assessed optically (O.D. 595 nm) by the growth of *S. melliferum* culture in a medium supplemented with *F. defendens* filtrate and phenol red as a marker of growth (red-no growth, yellow-culture growth). To test the inhibitory activity of *F. defendens* in the plant sap, young melon seedlings in a growth chamber (28°C, 16/8 day/night photoperiod) were used. Plant sap was extracted from stem pieces using a syringe 10 days after *F. defendens* cells were applied by foliar spray. The presence of *F. defendens* in the sap was confirmed by PCR analysis. The changes in the endophytic community in the plant were assessed by Next-Generation Sequencing (NGS). Raw sequence data was processed using the R package 'dada2' (version 1.28.0) (Callahan *et al*., 2016).

Results

F. defendens isolates were obtained only from insects captured in the fall in two regions during 2013 and 2020. The rate of the inhibitory activity of *F. defendens* isolates was inconsistent and varied from none to high (Figure 1). *F. defendens* isolated from 2013 showed the highest inhibitory activity, probably it secreted more antimicrobial substances than the other clones and was used for further studies.

Melon plants are known for their high amount of phloem sap which can be extracted from the stems (Figure 2). *F. defendens* cells were applied to young melon seedlings to test their inhibitory activity *in planta*. The presence of *F. defendens* was confirmed in the plant sap, and its inhibitory activity was retained for 5 days post-application but not after 10 days. These results imply that *F. defendens* secretes secondary metabolites *in planta*. Introducing *F. defendens* into melon plants altered its microbiome community (Figure 3). The relative abundance of different bacterial genera was different in treated compared to non-treated plants. These changes were more pronounced 5 days post-application than

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after 10 days. Interestingly, the relative abundance of *Herbaspirillum* (Figure 3, light brown), which is known as a beneficial endophyte (Alves *et al.*, 2015), was enhanced in the treated plants after 5 days and declined almost to its initial rate 5 days later.

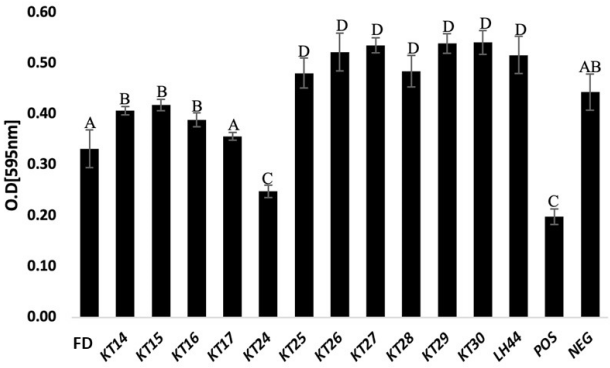


Figure 1. The filtrates inhibitory test of *F. defendens* isolates. Inhibition of *S. melliferum* in the presence of filtrate containing secondary metabolites secreted by *F. defendens* clones (FD, KT14-29, LH44). Growth was measured optically according to the change of red color. Negative control (NEG) no growth (red solution): OD~0.4; positive control (POS) culture growth (yellow solution): OD~0.2.



Figure 2. Extracting plant sap from stems of melon plants 5 and 10 days following *F. defendens* application by root dip or foliar spray.

Discussion

F. defendens has shown a direct and indirect inhibitory activity against mollicutes. The inhibition activity of the crude filtrate suggests that *F. defendens* secretes secondary metabolites that act together to inhibit the culture growth of a mollicute species. Although plant tissue is not its favorite residing niche, its ability to secrete secondary metabolites with antimicrobial activity *in planta* and *in vitro* makes it a suitable candidate to be exploited as a biocontrol agent. However, the constraints to conduct big-scale trials must be overcome before the go/no go stage.

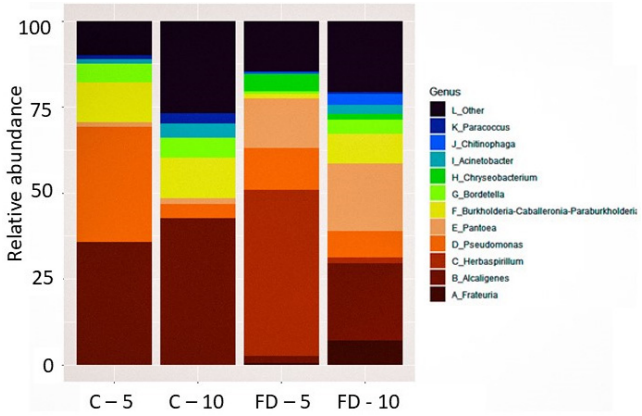


Figure 3. Relative abundance of major endophytic genera found in melon plants 5- and 10-days post *F. defendens* application. No application: C - 5, C -10; *F. defendens* applications: Fd - 5 , Fd – 10.

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Management

Presence of "flavescence dorée" phytoplasma in grapevine lignified canes and effectiveness of different sanitation methods

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Abstract

Control of propagation materials in nursery for the presence of "flavescence dorée" (FD) phytoplasma, a quarantine pathogen of grapevine, is of the greatest importance to avoid long-distance spreading of FD. Three different sanitation methods were tested on Glera variety scions. Hot water treatment proved to be the best, though a few FD-infected grafted plants were found in one trial. At the same time, PCR analyses of mature canes, suitable for grafting, from infected plants in vineyard showed that lignified canes with symptomatic leaves were very infected (97.3%), while also lignified canes with asymptomatic leaves from the same Glera plants harboured FD phytoplasma (from 9.8 to 26.0%).

Keywords: detection, grapevine yellows, hot water treatment, multiplication material, thermotherapy

Introduction

"Flavescence dorée" (FD) is a quarantine disease of grapevine, widely spread in most European grapegrowing countries, associated to the presence of phytoplasmas belonging to 16SrV-C and -D ribosomal subgroups. It is transmitted by a leafhopper of North American origin, *Scaphoideus titanus* Ball. FD is associated with very serious damages to grapevine and wine production, till the death of the infected plants. Control is mandatory and includes mainly preventative measures, such as insecticide treatments against the vector, rouging of infected grapevine plants and planting healthy materials.

Control of multiplication materials in nursery is of the greatest importance to avoid long-distance spreading of the diseases. The European Regulation EU 2021/2285 made mandatory the hot water treatment of grapevine multiplication material in certain conditions in the areas where FD is present. The aims of the present work were to evaluate: i) the prevalence of the phytoplasma in the 1-year-old mature canes from FD-infected plants of Glera variety, very common in North of Italy; ii) the effectiveness of different methods to eliminate the pathogen in nursery.

Materials and Methods

In summer 2022 and 2023, visual surveys were carried out in two severely infected vineyards with more than 80% of symptomatic plants, previously checked with PCR analyses for FD presence. Vineyards were grown with Sylvoz training system. Canes with symptomatic leaves were labelled for a total of 260 plants of Glera in 2022 and 400 in 2023. In December 2022 and 2023, mature canes suitable for grafting, were collected and divided in two types: A) with symptomatic leaves; B) with asymptomatic leaves from symptomatic plants. Mature canes from PCR-tested healthy plants were collected as a negative control.

In January 2023 and 2024 secondary phloem was obtained by cortical scraping in 248 samples of 1-year-old lignified collected canes, and used for subsequent RNA extraction (Casarin *et al.*, 2023). Total RNA was reverse transcribed by M-MLV reverse transcriptase, and quantitative PCR for FD detection was carried out (Angelini *et al.*, 2007). In 2023 and 2024 early spring, canes were cut to obtain scions to be grafted on healthy SO4 rootstock. Unluckily, mature canes with symptomatic leaves were only a few in winter 2022-2023, and completely absent in winter 2023-2024 and were not included in the treatments. In 2023, 2,500 rootstocks and 2,500 scions (type B) were divided into 5 treatments: 1) thermotherapy by hot water, 50°C for 45 minutes, as suggested by EPPP PM 10/18 (TT); 2) peracetic acid 1% (product D50) for 60 minutes, as optimized in a previous trial (A. Angelini *et al.*, unpublished) (PA); 3) acid water, using a protocol previously established (Lucchetta *et al.*, 2013) (AW); 4) untreated (NT); 5) healthy as a negative control (H). Given the results, in 2024 only TT treatment was applied, using 5,000 potentially infected scions from

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Glera (type B) treated and untreated, together with the healthy control (500 grafted plants). Graftings were then planted in randomized blocks with eight replicates in an isolated area, and regular visual surveys were carried out during the two summers. Leaves from suspected symptomatic plants were collected, DNA extracted and PCR-analysed for FD presence (Angelini *et al*., 2001; 2007). Dead plants were also counted.

Results

The PCR analyses in 1-year old canes could detect the presence of FD phytoplasma at different extent in the two types of canes: mature canes with symptomatic leaves (A) were very infected, almost 100% of the tested samples, while mature canes with asymptomatic leaves from the same infected plants (B) showed a lower infection rate, very different in the two years, thought the sampling and methodology were the same (Table 1).

Table 1. Results of PCR analyses carried out in different type of 1-year old mature canes from cv Glera. A: canes with symptomatic leaves; B: canes with asymptomatic leaves from symptomatic infected plants; H: canes from healthy plants.

Year	Variety	Types of cane	No. tested	FD-infected (%)
2023	Glera	A	37	97.3
2023	Glera	B	50	26.0
2023	Glera	H	50	0.0
2024	Glera	B	61	9.8
2024	Glera	H	50	0.0

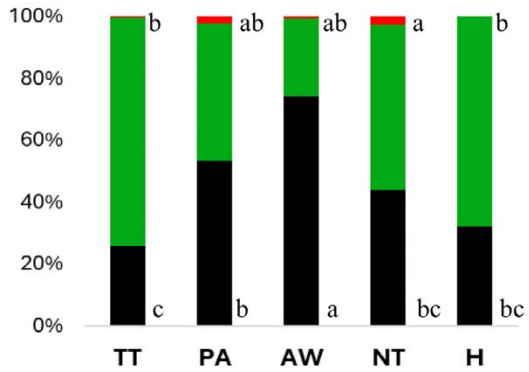


Figure 1. Percentage of dead (black), asymptomatic (green) and infected and symptomatic (red) plants in the 2023 trial. TT: hot water; PA: peracetic acid; AW: acid water; NT: untreated; H: healthy. Different letters correspond to statistical difference ($P<0.05$).

In 2023 the treatments differently affected the yield and FD-infection rate of the plants (Figure 1). Common yield values in nursery varied between 60 and 90%, as in TT, NT and H theses. The number of dead plants in the AW thesis was significantly higher than in the other ones. The number of symptomatic and FD-infected plants (PCR tested) was low in every thesis, absent only in the H thesis. In details, infected plants were 0.20%, 2.38%, 0.79% and 2.78% in the TT, PA, AW and NT theses, respectively. The number of infected plants in NT thesis was statistically higher than TT and H theses, that were not statistically different between them. PA and AW theses showed intermediate values. In 2024 H and TT thesis did not show symptomatic plants, while four symptomatic FD-infected plants (PCR tested) were identified in the untreated thesis (0.16%).

Discussion

The prevalence of FD-infected samples was much higher in lignified canes with symptomatic leaves (type A), as expected, but interestingly also many mature canes with asymptomatic leaves (type B) from the same infected plants were infected in 2023 (26%). In 2024 the prevalence of FD-infected type B canes was approximately three times lower. Thus, it can be hypothesized that there was a strict correlation with the annual seasonal conditions. Moreover, in autumn 2023 the mature canes with symptomatic leaves were practically absent in vineyard; as lignification in 2022 started quite soon compared to autumn 2023, probably most of the FD-infected canes did not achieve lignification. Further work focused to check the FD presence in the asymptomatic leaves from the infected canes is ongoing, but preliminary results are negative. Evaluating both the yield and the sanitation effect, the treatments with peracetic acid and acid water seem not to be suitable as a nursery practice. The hot water treatment confirmed to be the best choice, though the presence of a few infected plants in the 2023 trial arises some questions that should be studied deeper.

In conclusion, this work confirmed that the possibility of obtaining FD-infected grafted plants is very low even using clearly symptomatic plants, which is not the common practise in nursery, where annual surveys and uprooting of infected plants in mother plots and nurseries are mandatory.

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Management

Testing for '*Candidatus* Phytoplasma phoenicium' and peach latent mosaic viroid presence in Russia

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Abstract

In previous studies, diagnostic methods for '*Candidatus* Phytoplasma phoenicium' and peach latent mosaic viroid (PLMVd) were verified, allowing to detect these pathogens in planting material with high efficiency. In the present study, the best diagnostic methods tested were applied to control the planting material of samples from 124 stone fruit trees of domestic and imported origin collected during monitoring at All-Russian Plant Quarantine Center. PLMVd was detected in several samples of peach fruits of foreign origin. However, all samples of domestic origin tested free from both '*Ca.* P. phoenicium' and PLMVd, confirming the official notification of National Plant Protection Organization of the Russian Federation.

Keywords: almond witches' broom phytoplasma, PLMVd, stone fruit crops, plant quarantine

Introduction

Horticulture is the most important agricultural sector in Russia. Due to the diversity of soil and climatic conditions on the territory of the country, more than 60 species of various fruit and berry crops are grown. The area under orchards in Russia is about 500 thousand hectares and in recent years has been increasing by about 10% annually (www.fao.org). Industrial fruit growing in Russia is the most developed sector in the Central, Southern, Volga and North Caucasian federal districts. For the further development and increase in fruit production, it is necessary to intensify and improve the industry by using modern technologies of orchard cultivation, fruit production and storage. The main requirement for increasing the area under fruit crops is the production of sufficient quantity of quality planting material free from infectious pathogens. The control of phytoplasmas, viroids and viruses is very difficult and effective treatments to reduce the presence of these pathogens are not available or are under development (Smirnova *et al*., 2016). Viruses, viroids and phytoplasmas are mainly detected by serological and molecular methods used in diverse combinations to confirm positive samples. In particular the detection of phytoplasmas and viroids is possible only by molecular methods, which often makes it difficult to confirm the pathogen presence.

'*Candidatus* Phytoplasma phoenicium' associated with almond witches' broom and peach latent mosaic viroid (PLMVd), were studied in this work. '*Ca.* P. phoenicium' is a very dangerous pathogen in the countries where it is reported such as Lebanon and Iran (Verdin *et al*., 2003). The main hosts of '*Ca.* P. phoenicium' are peach (*Prunus persicae*), nectarine (*Prunus persica* var. nucipersica), almond (*Prunus amygdalis*) and apricot (*Prunus armeniaca*). Fruits quality declines in affected plants, yield reduction may reach 70-100%, mass death of trees is observed 3-4 years after the first symptoms appear. The most significant damage to which '*Ca.* P. phoenicium' was associated in almond, peach and nectarine production is reported in Lebanon and Iran, with severe social and economic consequences (EPPO, 2024b).

PLMVd hosts are peaches and hybrids of peach with almond and plum. Other stone fruit crops have also been reported as hosts of PLMVd: apricot, almond, plum (*Prunus domestica*) and cherry (*Prunus avium*). PLMVd is widely distributed in Mediterranean countries. The occurrence of this viroid on peach varies from 15 to 85% in different countries of this area. The distribution of PLMVd is also very wide in peach plantations in the USA - up to 60%, China - up to 65%, Japan - up to 90% (Serra *et al*., 2017).

'*Ca.* P. phoenicium' and PLMVd are mainly transmitted by vegetative propagation, therefore, sensitive and specific methods are needed to detect their presence even in a latent form and during the period unfavorable for the symptom expression, when their concentration is minimal.

Materials and Methods

The Euphresco project 2017-F-234 set up reliable protocols for the identification of '*Ca.* P. phoenicium' (Bianco *et al*., 2021)

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selecting among the best phytoplasma diagnostic methods previously published. These methods are also recommended in the EPPO diagnostic protocol 7/150 '*Candidatus* Phytoplasma phoenicium' (EPPO, 2021). In the present study, the diagnostic methods standardized after adaptation to domestic PCR reagents and their optimisation were used to test host plant samples of Russian and imported origin.

DNA extraction from plant samples for phytoplasma testing was performed using the DNeasy Plant Mini Kit (Qiagen). The PCR with primers AW16sF/AW23sR was applied for the detection and identification of '*Ca*. P. phoenicium' (Jawhari *et al*., 2015). Optimised PCR conditions were as follows: 95°C for 5 minutes; 35 cycles (30 seconds at 95°C, 30 seconds at 52°C and 1 minute at 72°C); 72°C for 7 minutes. The expected amplicon size is 492 bp.

RNA extraction from plant samples for testing the presence of viroid was carried out with the commercial kit Proba-NK (Agrodiagnostika, Russia). To screen the presence of PLMVd, it was used the Reagent kit for peach latent mosaic viroid (Agrodiagnostika), with the preliminary reverse transcription carried out in a separate tube using a reagent kit for reverse transcription (Agrodiagnostika), which includes the random primers for RT-PCR. For confirmatory test it was used the conventional one-tube RT-PCR with primers PLMVd-P3F and PLMVd-P4R designed and tested earlier at All-Russian Plant Quarantine Center for identification of peach latent mosaic viroid (Prikhodko *et al*., 2021) in combination with the OneStep RT-PCR kit (Qiagen). PCR conditions were 50°C for 30 minutes 95°C for 15 minutes; 40 cycles (30 seconds at 95°C, 45 seconds at 60°C and 1 minute at 72°C); 72°C for 5 minutes. The expected amplicon size is 252 bp.

Results

It was tested a total of 124 samples of shoots and fruits of stone fruit plants collected during monitoring from All-Russian Plant Quarantine Center in 7 regions of the Russian Federation, 12 samples of peach seedlings from Italy and 9 peach fruits from Turkey (Table 1).

Table 1. Summary of tested samples by region with the number of infected samples.

Region	Number of samples tested and positive detected in brackets	
	'Ca. P. phoenicium'	PLMVd
Primorsky Krai	30 (0)	30 (0)
Moscow Oblast	20 (0)	20 (0)
Republic of Crimea	15 (0)	15 (0)
Krasnodar Krai	17 (0)	17 (0)
Stavropol Krai	15 (0)	15 (0)
Volgograd Oblast	10 (0)	10 (0)
Tula Oblast	15 (0)	5 (0)
Turkey	11 (0)	11 (9)
Italy	12 (0)	2 (0)

PCR results showed that all samples were free from '*Ca*. P. phoenicium'. Among the tested plant and fruit samples 9 peach fruits were positive to PLMVd that was confirmed by sequencing results (data not shown). All other stone fruit plants originating from the Russian Federation and Italy were free from PLMVd.

Discussion

All designed primers and commercial kits for detection and identification of '*Ca*. P. phoenicium' and PLMVd that were used in this study showed high efficiency in the process of testing plant material of stone fruit plants. In some samples of the plant material collected before, PLMVd was detected (Prikhodko *et al*., 2021). The tested plants previously collected on the territory of the Russian Federation, as well as seedlings of stone fruit plants of imported origin, were free from '*Ca*. P. phoenicium' and PLMVd, which confirms the data of the Russian National Plant Protection Organization. Testing imported planting material of host plants for the two pathogens under investigation, especially from countries where they were reported, should be continued as it is important to maintain the phytosanitary safety of the Russian Federation.

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Management

Evaluation of sesame germplasm against the phyllody disease

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Abstract

Sesame is one of the important oilseed crops of India and suffers from many diseases. Among them phyllody is one of the most important causing severe crop and yield losses. To identify resistant sources, a set of 1,477 germplasm lines were evaluated. The germplasm set was sown in 3 m row in two replications along with susceptible check in paired row after every fifth entry. To attract the insect vector, one of the rows of the susceptible check was given top cutting at full vegetative stage. Out of the total accessions sown, 949 accessions were observed free from phyllody disease and 55 genotypes proved to be resistant with 0.1–10% incidence of phyllody. Notably, 81 lines were found to be moderately resistant with 30.1-40% disease incidence and 29 lines were found to be susceptible to phyllody corresponding to 40.1-50% disease incidence. However, some germplasm demonstrated significant susceptibility, with 16 accessions categorized as highly susceptible (>50% disease incidence). This screening effort would provide valuable insights for breeding programs aiming to develop phyllody-resistant cultivars.

Keywords: *Sesamum indicum*, phytoplasma, resistance, germplasm screening

Introduction

Sesame (*Sesamum indicum* L.), referred to as "til," is one of the oldest oilseed crops in the world and a significant crop farmed in India. It is frequently referred to as the "Queen of Oilseeds" as its oil content can reach up to 63%, far higher than that of other oilseed crops like groundnut (45%–56%), sunflower (45%), rapeseed (40%), and soybean (20%) (Teklu *et al*., 2021). Sesame seeds are abundant in proteins (19%–25%) and fatty acids (45%–55%). Important nutrients like magnesium, phosphorus, calcium, iron, and zinc are also abundant in sesame seeds that are rich in vitamins B and E and have strong antioxidant qualities (Mostashari *et al*., 2024). Sesame production in India is extremely impoverished due to low harvest index, disease susceptibility, seed bursting, and erratic growing plant behaviors (Rao *et al*., 2015). Sesame is continuously threatened by several diseases, but the most commercially significant disease affecting sesame is phyllody. Earlier the disease was suspected to be caused by a virus or due to environmental factors (Rhind *et al*., 1937). The first evidence of phytoplasma association to the phyllody disease was reported by Cousin *et al*. (1971). The phytoplasmas inhabit the nutrient-rich phloem tissue and thrive in the form of extremely small (400–900 nm diameter) spherical, oval, or elongated cells in sieve elements, with a genomic size of only 680–1,600 kb. They are transmitted by phloem-sucking insects and are associated with diseases that impact hundreds of plant species (Weintraub and Beanland, 2006). Phytoplasmas cause considerable damage to diverse economically important plants. The phytoplasmas associated with sesame phyllody are transmitted by the leafhoppers *Orosius albicinctus* Distant (Abraham *et al*., 1977) and *Hishimonus phycitis* Distant (Nabi *et al*., 2015). Managing sesame phyllody is essential as the disease leads to significant crop losses and compromises both the quality and yield of sesame. Screening sesame genotypes against phyllody is essential to identify reliable sources of resistance.

Materials and Methods

The screening of sesame germplasm was conducted at Punjab Agricultural University, Ludhiana to find out the sources of resistance against sesame phyllody. The test genotypes were sown during the second week of July with a spacing of 30 cm 10 cm. To find out the source of resistance, a total of 1,477 germplasm lines were screened under natural field conditions using a randomized complete block design. A susceptible check (RT 346) was also sown. Observations on disease incidence and severity were recorded at 60, 75, 90 and 110 days after sowing. Five plants from each germplasm were randomly selected and based on the reaction of the genotypes against disease, they were graded into different categories using a specific disease rating scale (Table 1).

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Table 1. Reaction of sesame germplasm to phyllody.

Disease incidence %	Disease reaction	Number of genotypes	% of genotypes
0	Highly resistant	949	64.2
0.1-10%	Resistant	55	3.7
10.1-20%	Moderately resistant	81	5.5
20.1-30%	Tolerant	53	3.6
30.1-40%	Moderately Susceptible	34	2.3
40.1-50%	Susceptible	29	2.0
>50%	Highly Susceptible	16	1.1
Non germinated	-	260	17.6
Total number of genotypes screened	-	1,477	-

The observations recorded for field screening of phyllody (scoring scale 0-6) was by using the following formula.

$$\text{Disease incidence (\%)} = \frac{\text{Plants infected in a row } 100}{\text{Total number of plants in a row}}$$

Results

The development of cultivars with durable resistance to phyllody should be an integral component of sesame breeding program. The analysis of 1,477 sesame genotypes screened for disease resistance revealed substantial variation in disease incidence and resistance levels. Significantly, 949 lines genotypes showed high resistance, indicating a robust genetic basis for disease resistance in these samples. The resistant to moderately resistant groups included 136 genotypes (9.2%), with disease incidence varying from 0.1% to 20%. On the other hand, a smaller group of genotypes showed various degrees of susceptibility. Only 34 genotypes (2.3%) were moderately susceptible (30.1–40% disease incidence), while 29 (2.0%) and 16 (1.1%) genotypes were classified as susceptible or highly susceptible, with disease incidences between 40.1% and greater than 50%, respectively. Additionally, 260 lines did not germinate, which reduced the number of viable genotypes evaluated for disease response to 1,217 genotypes.

Discussion

Overall, the data highlights a promising reservoir of resistant and highly resistant genotypes, providing a valuable foundation for breeding efforts focused on enhancing disease resistance in sesame crops. The analysis of sesame genotypes screened for disease resistance reveals a substantial majority (64.2%) of highly resistant genotypes with no disease incidence, which might be having escaped the disease and are subject to reconfirmation. The additional presence of resistant to moderately resistant genotypes (9.2%) provide further breeding options, as these lines show low to moderate levels of disease incidence (up to 20%), which could still support robust crop performance with manageable disease levels. On the other hand, only a small fraction of genotypes (5.3%) showed moderate to high susceptibility, suggesting that highly vulnerable lines are relatively less within the screened population. The 260 non-germinated lines (17.6%) slightly reduced the pool available for disease assessment, which could indicate the need to address factors affecting germination in future screenings. Overall, the data offers a strong genetic foundation for advancing disease resistance in sesame through targeted breeding programs, with the diverse range of resistance levels providing valuable opportunities for creating durable, high-yield sesame varieties.

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Management

Management of phyllody of sesame

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Abstract

Sesame phyllody is an important disease in the sesame growing area of Madhya Pradesh, India. Studies were carried out to evaluate the efficacy of various insecticides and botanical pesticides. Among the eight treatments performed the results showed that all significantly reduced both disease incidence and leaf hopper populations compared to the untreated control. Imidacloprid was the most effective, achieving a 68.75% reduction in disease incidence, lowest leaf hopper population (1.11 per leaf), highest seed yield (5.12 q/ha) and a benefit :cost ratio of 1.54 was recorded.

Keywords: disease, phytoplasma, incidence, epidemiology

Introduction

In India this sesame is commonly known as "till". Sesame seeds are a rich source of protein and edible oil, protein usually ranges between 20 and 26% and oil content usually range from 45 to 50% and they also contain high amounts of saturated fatty acids (47% oleic acid and 39% linolenic acid) and some natural antioxidants like sesamolin, sesamin and sesamol, sesame oil has very good stability (Moazzami *et al*., 2006). The most important sesame diseases are phyllody, root and stem rot, *Phythopthora* blight, *Alternaria* leaf spot, *Cercospora* leaf spot and powdery mildew (Gupta *et al*., 2018). Sesame phyllody causes up to 80% yield loss (Salehi *et al*., 2017) and is associated with phytoplasmas transmitted by insect vectors, particularly leaf hoppers. The most sustainable and viable method for managing phyllody and its vector can be an integrated strategy by combining cultural, host-plant resistance, biological, physical and chemical methods (Kumari *et al*., 2019). Among the different management practices, producing clean propagating materials, effective insect vector control, weed management and stimulation of plant defences are practically important. Identification of alternative control strategies against these diseases, such as bio-control practices of phytoplasma strains could also provide effective tools for reducing phytoplasma spread in an environmentally sustainable approach to management. Breeders should identify the plant varieties showing resistance to the phytoplasma diseases from different regions for the breeding programs to offer improved hybrid varieties for replanting practices in India.

Materials and Methods

The experiment was carried out at Department of Plant pathology JNKVV, Jabalpur and Research field during "kharif" 2023. The field trial was conducted using a randomized block design with eight treatments (imidacloprid 17.8% SL (T1), thiamethoxam 25% WG (T2), diafenthiuron 50% WP (T3), spinosad 45% SC (T4), lambda cyhalothrin 5% EC (T5), neem oil (T6), tobacco leaf extract (T7) and control (T8) with three replications. A TKG-308 sesame variety was used in this experiment. Recommended doses of fertilizers were given with light watering to improve seedling germination. Two sprays of insecticide and botanicals were given. The first spraying was done after the appearance of the disease and subsequently two at 15 days interval.

Results

Among all the treatments T1 (imidacloprid spraying at 17.8 SL at 5 ml/l) was found the most effective with the lowest disease incidence (6.28%), minimum leaf hopper population (1.11) and maximum percentage of disease control (68.75%). The second-best treatment was T2 (thiamethoxam 25% WG) with 1.13/leaf hopper/leaf population, 7.59% disease incidence and 62.23% disease control. T7 (tobacco leaf extract spray) was the less effective among all the treatments with a 1.41 leaf hopper/leaf, disease incidence of 14.52% and minimum percentage of disease control of 27.76%. T8 (the untreated control group) had the maximum leaf hopper population (1.72 leaf hopper/leaf) and the highest disease incidence of 20.1%.

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Table 1. Efficacy of insecticides against sesame leafhoppers and sesame phyllody ("kharif" 2023).

$T_{no.}$	Treatments	Leafhopper/leaf	Incidence (%)	% control	Yield (q/ha)	B:C ratio
T_1	Imidacloprid 17.8% SL	1.45	6.28 (14.47)	68.75	5.12	1.54
T_2	Thiamethoxam 25% WG	1.46	7.59 (15.94)	62.23	5.01	1.49
T_3	Diafenthiuron 50%WP	1.50	9.88 (18.26)	50.84	4.62	1.14
T_4	Spinosad 45% SC	1.51	11.2 (19.54)	44.27	4.25	1.07
T_5	Lambda cyhalothrin 5% EC	1.46	9.24 (17.65)	54.02	4.75	1.36
T_6	Neem oil	1.53	13.32 (21.38)	33.73	4.15	1.06
T_7	Tobacco leaf extract	1.55	14.52 (22.34)	27.76	4.01	1.005
T_8	Control	1.64	20.1 (26.56)	-	3.69	0.86
	Sem+_	0.034	0.908			
	CD 0.5%	0.105	2.78			

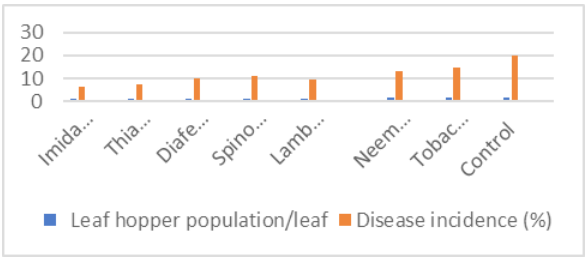


Figure 1. Efficacy of insecticides against sesame leafhopper and sesame phyllody.

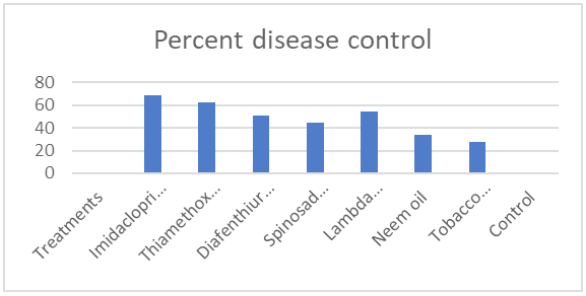


Figure 2. Effect of insecticides on percentage of disease control of sesame phyllody**.**

Results on leafhopper population and disease control indicate that two foliar sprays of imidacloprid 17.8 SL at 15-day interval was effective in managing the sesame phyllody disease and reducing the leafhopper population of sesame with 68.75% of disease control (Table 1, Fgures 1 and 2).

Discussion

To study the effect of spraying insecticides and botanical pesticides on leafhopper their populations were recorded. It was observed that all insecticides treatments significantly decreased the leafhopper population compared to the other treatments. However, all treatments reduced the leafhopper population as compared with control. The minimum leafhopper population (1.11 leafhopper/leaf) was observed in spraying of imidacloprid 17.8 SL as compared with other treatments. All the treatments were found more effective in reducing the disease incidence over the control. The disease incidence recorded after the second spray ranged from 6.28 to 14.52% while in the untreated control it was 21.13%. However, among all the treatments the spray of imidacloprid 17.8 SL was showing the low mean of disease incidence (7.27%) while the maximum disease incidence recorded (22.3%) was observed in the untreated control. Similar findings about the efficacy of insecticides in relation to the incidence of phyllody disease were reported by Dey *et al.* (2005); Gupta *et al.* (2023), Iqbal *et al.* (2013), Esmailzadeh Hosseini *et al.* (2015) and Markad *et al.* (2018). They reported that the sesame phyllody vector leafhopper was successfully managed by spraying insecticides, which resulted to be effective for the management of the phyllody disease.

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Sugar crops

Fastidious, phloem-limited bacteria, pathogens of sugar beet

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Abstract

Sugar beet, Europe's sole sugar source, faces significant threats from two phloem-limited pathogens: '*Candidatus* Arsenophonus phytopathogenicus' and '*Candidatus* Phytoplasma solani'. These are associated with syndrome "basses richesses" and rubbery taproot disease (RTD), respectively. Surveys across Central Europe revealed a predominance of '*Ca.* P. solani', including a novel 16SrXII-P subgroup strain in eastern Germany, while '*Ca.* A. phytopathogenicus' dominated in Western Europe. Genetic analysis showed minimal pathogen diversity within the Pannonian Plain but higher variability in other regions. The cixiid *Reptalus quinquecostatus* was confirmed as the vector of the epidemic '*Ca.* P. solani' strain (tuf-D) detected in the RTD outbreaks, which also increased susceptibility to *Macrophomina phaseolina*. These findings underscore the need for improved monitoring and management of sugar beet pathogens across Europe.

Keywords: '*Candidatus* Phytoplasma solani, '*Ca.* Arsenophonus phytopathogenicus', rubbery taproot disease, syndrome "basses richesses"

Introduction

Sugar beet is the only source of sugar in Europe, cultivated across nearly three million hectares (FAOSTAT). In Europe, two phloem-limited bacteria, '*Candidatus* Arsenophonus phytopathogenicus' and '*Candidatus* Phytoplasma solani' (also known as "stolbur" phytoplasma) are associated with two diseases, syndrome "basses richesses" (SBR) and rubbery taproot disease (RTD), respectively. These diseases pose substantial challenges to sugar beet production in Europe (Sémétey *et al.*, 2007; Curcic *et al.*, 2021a; Duduk *et al.*, 2024). Both SBR and RTD share non-specific symptoms, such as yellowing and proliferation of leaves. However, distinct symptoms differentiate the two diseases: SBR is characterized by vascular tissue discoloration and deformation of young leaves, while RTD manifests as necrosis of older leaves, wilting, rubbery taproots, and plant decline (Figure 1).

Recently, a novel '*Ca.* P. solani' related strain has been described in sugar beet in Germany and classified under the 16SrXII-P subgroup (Duduk *et al.*, 2023). '*Ca.* A. phytopathogenicus' belongs to γ-proteobacteria and is believed to have evolved from insect symbionts to colonize sugar beet, potato and onion. Phytoplasmas are wall less bacteria infecting hundred of plant species worldwide. This review synthesizes findings from surveys and experiments conducted over a five-year period (2019–2023) to investigate the prevalence and characteristics of sugar beet infections by these phloem-limited bacteria in Europe.



Figure 1. The complete loss of yield and abandoned sugar beet field due to RTD, eventually ploughed unharvested.

Materials and Methods

Initially the presence of phytoplasmas and γ-proteobacteria in sugar beet was investigated in regions of the Pannonian Plain (Serbia and Slovakia), where epidemic outbreaks of

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RTD have been documented, and in Germany, where both pathogens are present. Studies on RTD were initiated in 2019 at four locations in northern Serbia, known for their history of RTD occurrence. Additional research sites across the Pannonian Plain, particularly in Slovakia, were also included.

To distinguish epidemic strains from non-epidemic ones, pathogen characterization was conducted using variable, epidemiologically relevant non-ribosomal genes. Epidemiological studies carried out in 2020 and 2021 targeted potential reservoir plants and vectors. Transmission experiments were performed using both single-plant inoculations and field trials. Given the susceptibility of RTD-affected sugar beets to rot, the potential association between RTD and fungal root rot was also investigated. Finally in 2023 an extensive survey was conducted encompassing Central Europe from the Wallachian and Pannonian plains in south to Poland and Germany in the Northern European plain to the north. The analyses included samples from East Europe (Russia) and West Europe (France) for comparison.

Results

Molecular diagnostics confirmed the presence of both '*Ca.* P. solani' and '*Ca.* A. phytopathogenicus' across Central Europe, with a notable predominance of the phytoplasma.

These pathogens were detected in all surveyed regions within the Pannonian and Wallachian Plains, but were absent in Bohemia, consistent with observed disease symptoms. In eastern Germany, a specific strain of '*Ca.* P. solani'-related phytoplasma was identified as the dominant bacterium, in contrast to France, where '*Ca.* A. phytopathogenicus' is typically prevalent. This German strain was classified into a novel subgroup, designated as 16SrXII-P. In Poland, both pathogens were detected, although their occurrence was limited. The 16S rDNA sequences of '*Ca.* P. solani' 16SrXII-A exhibited high conservation. However, multi-locus analyses of two more variable *loci*, the *tuf* and *stamp* genes revealed distinct patterns of variability across different regions. In Central Europe, particularly within the Pannonian Plain, the *tuf* and *stamp* sequences showed minimal variability, contrasting with the high variability observed in Eastern and Western Europe, which are associated with epidemic and sporadic occurrences, respectively. The insect vector *Reptalus quinquecostatus* was identified as responsible for transmitting the epidemic strain (tuf-D) of '*Ca.* P. solani' 16SrXII-A, which is linked to the widespread RTD outbreaks in Serbia and Slovakia. Additionally, sugar beet plants affected by RTD were found to be more susceptible to *Macrophomina phaseolina* agent of charcoal root rot, exacerbating the losses associated with the phytoplasma presence.

Discussion

In contrast to France, where phytoplasma play a relatively minor role, phytoplasmas are the dominant pathogens over '*Ca.* A. phytopathogenicus' in Central Europe, particularly the Pannonian Plain and eastern Germany (Sémétey *et al.*, 2007; Curcic *et al.*, 2021b). The cixiid *R. quinquecostatus* was confirmed as the vector responsible for transmitting the epidemic strain of '*Ca.* P. solani', thereby driving the epidemic outbreaks of RTD. While *M. phaseolina* has been considered the most significant root pathogen of sugar beet in Serbia, it is regarded as a minor pathogen of weakened plants in other regions. These findings suggest that *M. phaseolina* exacerbates the yield losses associated with the presence of '*Ca.* P. solani', which may explain the differing perspectives on its importance as a pathogen in Serbia versus other regions.

These findings emphasize the importance of further research into the interactions between vectors, pathogens, and plant hosts, as well as the ecological/agronomical factors driving these disease outbreaks. This is crucial for effective management of these severe diseases. The results obtained suggest that greater emphasis should be placed on monitoring the bacteria infections in sugar beet across Europe.

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Sugar crops

Identification of '*Candidatus* Phytoplasma solani' in sugar beet in the Voronezh region of the Russian Federation

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Abstract

The purpose of the research was to study the genetic characteristics of 9 strains of the "stolbur" phytoplasma ('*Candidatus* Phytoplasma solani') detected in sugar beet plants in the Ramonsky and Ertilsky districts of the Voronezh region. The strains were detected by quantitative PCR. Confirmatory tests were carried out with nested-PCR using universal primers for phytoplasmas P1/P7 and fU5/rU3. The obtained amplification products were sequenced. Seven strains were found to be '*Ca.* P. solani' having more than 99% identity with the 16S rRNA gene sequences of the reference strain of this '*Ca.* Phytoplasma' species and containing also DSB1 and DSB2 sequences unique to '*Ca.* P. solani'. Two strains were not identical to '*Ca.* P. solani', however the amplicons sequenced were too short for the evaluation of their genetic relationship with 'Ca. P. solani', however they could represent genetic variants of this phytoplasma.

Keywords: "stolbur" phytoplasma, PCR, primers, sequencing, leafhopper

Introduction

The sugar beet is an important agricultural crop and is cultivated in the Russian Federation on an area of more than 1 million ha. Sugar beet is infected by several phytoplasmas belonging to groups 16SrI, 16SrII, 16SrIII and 16SrXII. Among them, '*Candidatus* Phytoplasma solani', subgroup 16SrXII-A is characterized by high harmfulness and high damage (Curcic *et al.*, 2021).

Materials and Methods

In 2022-2024, sugar beet plants with symptoms of leaf chlorosis of different intensity, sometimes combined with deformation, wrinkling and changes in leaf shape, were observed in several districts of Voronezh Region. Necrosis on the leaves has been found less frequently and in some cases plant death has been observed. Thirty-three plants with typical symptoms were tested by ELISA and RT-PCR for Beet yellows virus (BYV), Beet mosaic virus (BtMV), Beet necrotic yellow vein virus (BNYVV), Beet black scorch virus (BBSV), Beet soil-borne virus (BSBV) and *Polerovirus* spp. Screening tests for the presence of "stolbur" phytoplasma were carried out by quantitative PCR with the reagent kit '*Ca.* P. solani'-PB (Sintol, Russia). Confirmation tests for the presence of "stolbur" phytoplasma were performed by PCR with universal primers to phytoplasmas P1/P7 (Deng and Hiruki, 1991; Schneider *et al.*, 1995) followed by fU5/rU3 (Lorenz *et al.*, 1995) in nested-PCR. The obtained amplicons were sequenced using a modified Sanger method. BioEdit 7.0.5.3 software and BLASTN 2.12.0+ program was used to analyse the results.

Results

No viruses were detected by ELISA and RT-PCR carried out to detect BBSV, BNYVV, BSBV, BtMV, BYV and *Polerovirus* spp. in the tested samples. Eighteen samples out of 33 tested positive for '*Ca.* P. solani' with Sintol qPCR kit. In a subsequent nested-PCR test, 9 positive samples were found, which were named as Ram-8, Ram-9, Ram-13, Ram-15, Ram-15, Ram-16, Ram-20, Ram-21, Ram-27 and ERt-31. The size of the amplification products obtained in nested-PCR ranged from 829 to 848. All the sequences obtained were consistent with the 16S rRNA gene regions of numerous strains of '*Ca.* P. solani' and closely related strains deposited in the NCBI GenBank.

According to BLASTN 2.12.0+ analysis, the identified strains Ram-8, Ram-9, Ram-13, Ram-15, Ram-21, Ram-27, and Ert-31 clearly belong to the '*Ca.* P. solani', because according to available guidelines (Quaglino *et al.*, 2013), their nucleotide sequences had >99% identity with the 16S rRNA gene sequences of numerous strains of this '*Ca.* Phytoplasma' species and contained DSB1 and DSB2 sequences unique to the '*Ca.* P. solani'.

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Table 1. Identity percentages of obtained sequences to '*Ca*. P. solani' strains from Voronezh region, Russian Federation.

Strain	Presence of unique sequences		Identity with '*Ca*. P. solani' at NCBI	
	DSB1	DSB2	Identity percentage	Number of strains with maximal identity
Ram-8	+	+	100%	96
Ram-9	+	+	99.64%	108
Ram-13	+	+	100%	108
Ram-15	+	+	100%	108
Ram-16	-	-	100%	108
Ram-20	-	-	99.15-99.27%	2
Ram-21	+	+	100%	108
Ram-27	+	+	100%	108
Ert-31	+	+	100%	109

The identity of strains Ram-8, Ram-13, Ram-15, Ram-21, Ram-27, and Ert-31 with '*Ca*. P. solani' was 100% and for strain Ram-9 it was 99.64%. The absence of unique DSB1 and DSB2 sequences was found for strain Ram-16, despite its 100% identity with '*Ca*. P. solani' available strains for the sequence compared. The Ram-20 strain also does not have these unique sequences. In addition this strain only showed identity over 99% with two '*Ca*. P. solani' strains. Compared to 65 others '*Ca*. P. solani' the identity of the Ram-20 strain was 98.18%. In addition, the nucleotide sequence identity of strain Ram-20 was 98.42% compared to one of the bindweed yellows phytoplasma ('*Ca*. P. convolvuli') and 98.30% compared to '*Ca*. P. fragariae', '*Ca*. P. australiense' and closely related strains belonging to group 16SrXII phytoplasmas.

Leafhopper caught on glue traps in sugar beet fields in Voronezh region were also tested for the presence of '*Ca*. P. solani' by qPCR that was detected in *Hyalesthes obsoletus* Signoret, *Pentastiridius leporinus* L. and *Neoaliturus fenestratus* H.F.S. In all tested leafhopper *Psammotettix striatus* L., *Eupteryx atropunctata* Goese, *Circulifer haematoceps* Mulsant, Rey and *Empoasca affinis* Nast. this phytoplasma was not detected.

Discussion

The identification of "stolbur" phytoplasmas (16SrXII-A) common in sugar beet in the Voronezh region of the Russian Federation was achieved. All the studied strains were identified as '*Ca*. P. solani' and the two strains not 100% identical to '*Ca*. P. solani' needs further evaluation on whole 16S rRNA gene sequence to be appropriately evaluated (Bertaccini *et al*., 2022) as possible genetic variants of the same phytoplasma. The main species of leafhoppers that are carrying "stolbur" phytoplasma in Voronezh region were identified and are the same reported in other sugar beet phytoplasma infected areas of Europe (Bressan *et al*., 2008).

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Phytopathogenic Mollicutes
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Sugar crops

Molecular identification of phloem-restricted bacteria in insects and sugar beet crops in Italy

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Abstract

Ruberry taproot disease and "basses richesses" syndrome are seriously damaging the sugar beet production in Europe. The study evaluated the presence of '*Candidatus* Phytoplasma' and '*Ca.* Arsenophonus phytopathogenicus' in insects and sugar beet fields in Italy. '*Ca.* P. solani' was found in several insect species, in some cases for the first time; moreover in three insect species, bacteria of the genus *Arsenophonus* were found having high 16S rRNA gene identity to those detected in sugar beet. Group 16SrIX phytoplasmas were identified for the first time in two sugar beet plants. These data suggest further investigation to understand the possible correlation of the presence of phloem-restricted bacteria with the reduction in yield and sugar polarization.

Keywords: "basses richesses", rubbery taproot, '*Candidatus* Arsenophonus phytopathogenicus', '*Ca.* Phytoplasma solani'

Introduction

Sugar beet (*Beta vulgaris* subsp. *vulgaris*) is the second largest sugar crop with more than 4.5 million hectares under cultivation worldwide. Some diseases are recently worrying the sugar beet sector in Europe. The rubbery taproot disease (RTD) is associated to '*Candidatus* Phytoplasma solani' and transmitted by different species of cixiids: *Hyalesthes obsoletus*, *Reptalus quinquecostatus*, *R. cuspidatus* and P*entastiridius leporinus* (Kosovac *et al.*, 2023). This disease is mainly present in the Balkan area and the most common symptoms are yellowing and necrosis of older leaves, while the taproot shrivels and becomes rubbery and may also show rot, for which a correlation with the fungus ascomycete *Macrophomina phaseolina* has been suggested (Duduk *et al.*, 2023b). The "basses richesses" syndrome (SBR) is associated to '*Ca.* P. solani' and to the phloem-inhabiting bacterium '*Ca.* Arsenophonus phytopathogenicus' and transmitted by *P. leporinus* (Duduk *et al.*, 2023a). To date, the presence of SBR has been largely detected in Switzerland, France, Germany and Serbia. This syndrome causes a reduction of the sugar content in the taproot leading to considerable economic damage (Gatineau *et al.*, 2002).

Materials and Methods

From 2022 to 2024, monitoring was carried out in 13 sugar beet fields distributed between Veneto and Emilia-Romagna regions to verify the presence of these bacteria in both plants and insects. The insects were captured with four chromotropic yellow sticky traps for each field, placed at the edges and changed every three weeks; then they were identified to the species. The sugar beet samples showed symptoms attributable to the diseases under study. Using the CTAB buffer extraction technique (Angelini *et al.*, 2001), nucleic acids were extracted from the insects and from 1 g of leaf veins or taproot vascular tissues of sugar beet samples. Nested PCR amplification was performed on 16S rRNA gene using 1 µl of the 1: 30 diluted DNA with R16F2n/R16R2 primers followed by $16S_{758f}$(M1)/$16S_{1232r}$ (M2) (Gundersen and Lee, 1996; Gibb *et al.*, 1995). The amplicons were subjected to RFLP analysis with *Tru*1I enzyme. For the detection of '*Ca.* A. phytopathogenicus' PCR amplification on 16S rRNA gene was performed using 1 µl of DNA with Fra4/Fra5 primers (Zreik *et al.*, 1998). All amplicons were sequenced in both senses with primers used for amplification and assembled sequences were compared with those at NCBI database.

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Results

The monitoring conducted on insects captured in sugar beet fields mainly investigated the population of leafhoppers with less extent for ciixids. More than 30 different species were identified and analyzed for the presence of both bacteria. In Table 1 are reported the insects resulted positive for the presence of bacteria of the genus *Arsenophonus* and in Table 2 are listed those in which the presence of '*Ca.* P. solani' was identified. Sequencing of the amplicons obtained for *Arsenophonus* bacteria showed the presence of some polymorphisms which resulted in different identity percentages with the available sequences of *'Ca.* A. phytopathogenicus' (GenBank accession number MT139648); however, all of them shared more than 99% identity with this reference strain, but also showed high homology with the endosymbiont bacteria of the genus *Arsenophonus*. Among the sugar beet samples collected two of those resulting positive showed a *Tru*I RFLP profile referable to 16SrIX phytoplasmas. The sequencing of these amplicons confirmed the presence of a phytoplasma strain related to '*Ca.* P. phoenicium'.

Table 1. Insect species positive for '*Ca.* A. phytopathogenicus'.

Insect species	Positive samples	Total samples
Hebata decipiens	1	31
Laodelphax striatellus	9	24
Zyginidia pullula	1	20

Table 2. Insect species positive for '*Ca.* P. solani'.

Insect species	Positive samples	Total samples
Anaceratagallia laevis	2	18
Macrosteles sexnotatus	1	5
Austroagallia sinuata	1	10
Neoaliturus fenestratus	1	9
Hebata decipiens	4	31
Psammotettix zelleri	2	20
Hephathus freyi	1	2
Reptalus artemisiae	1	8
Hyalestes sp.	1	7
Thamnotettix zelleri	1	5
Laodelphax striatellus	5	24
Zyginidia pullula	3	20

Discussion

The detection of several insect samples positive to '*Ca.* P. solani' shows the high presence of the phytoplasma in the surveyed area, some positive insects are known to be the vectors in sugar beet such as *H. obsoletus* and *R. artemisiae*, while others are only carrier; moreover *H. freyi* and *Z. pullula* have never been reported positive for this phytoplasma. Only in *L. striatellus*, the presence of endosymbiont bacteria of the genus *Arsenophonus* had already been reported (Li *et al.*, 2018). In some samples, higher homology to *'Ca.* A. phytopathogenicus' sequence (GenBank accession number MT139648) was observed when compared to the sequence of endosymbiont bacteria. This is suggesting therefore a possible phytopathogenic effect of these strains that may play a role in the decline in average sugar content at harvest in sugar beet in Italy during the recent years. The detection of group 16SrIX phytoplasmas in sugar beet is the first report worldwide for this species. These phytoplasmas are associatated with extensive damages in almond, peach, and nectarine in Asian countries and were scattered identified in weed and ornamental host species also in Italy; therefore, it will also be necessary to further investigate their possible role in the sugar beet sugar content decline. Extensive testing to verify the presence of the other reported phloem inhabiting bacteria in sugar beets in Italy are in progress.

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Sugar crops

Multigene characterization of a 16SrII-D phytoplasma associated with sugarcane in Oman

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Abstract

Sugarcane is an economically important monocot plant belonging to Poaceae, the grass family. It is reported to develop sugarcane white leaf disease symptoms associated with phytoplasmas. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA, *tuf* and *imp* genes of phytoplasmas applying direct and nested PCR techniques. The PCR assays from all symptomatic plants produced amplicons in both direct and nested reactions. Sequence homology results on BLAST and phylogenetic analysis of the 16S rRNA, *tuf* and *imp* genes sequences indicate that the sugarcane phytoplasma from Oman is a member of the 16SrII-D subgroup that shares 100% sequence identity to '*Candidatus* Phytoplasma australasiae=austalasiaticum'. This is the first report of a phytoplasma from the 16SrII-D subgroup infecting sugarcane plants in the world.

Keywords: disease, sugarcane white leaf, SCWL, molecular identification, Oman

Introduction

Sugarcane (*Saccharum officinarum*) cultivation is limited in Oman due to its arid climate, which is unsuitable for water-intensive crops like sugarcane. As a result, sugar and related products are primarily imported to satisfy domestic demand. Locally produced sugar and sugar-based products, including raw sugar and derivatives, are used in traditional foods, such as Omani "halwa" and various other dishes, highlighting their importance in the country's culinary heritage. Phytoplasmas affecting sugarcane such as those associated with white leaf (SCWL) and grassy shoot (SCGS), are phloem-restricted bacterial pathogens. These pathogens induce diverse symptoms, including stunting, leaf whitening, excessive tillering, dwarfing and reduced sucrose content (Zhang *et al*., 2023a). Phytoplasmas linked to SCWL and SCGS diseases are classified within the 16SrXI group that includes subgroups 16SrXI-B, 16SrXI-D, and 16SrXI-F (Viswanathan *et al*., 2011; Abeysinghe *et al*., 2016; Yadav *et al*., 2017). Multilocus sequence typing has demonstrated that SCWL and SCGS phytoplasmas belong to two distinct populations of '*Candidatus* Phytoplasma sacchari' (Zhang et al. 2023b). In one-year-old sugarcane fields grown in farms at Bahla (N: 22°992'77.93", E: 57°292'97.83"), and Samail (N: 23°192'06.83", E: 58°42'43.53"), Al Dakhlia, Oman, symptoms of SCWL, which are classical symptoms of phytoplasma diseases were observed.

Materials and Methods

Leaf samples from two symptomatic and two asymptomatic sugarcane plants were sampled for total nucleic acid DNA extraction using the Doyle and Doyle (1987) method with some modifications. Polymerase chain reaction (PCR) was used with specific primers for the phytoplasma 16S rRNA gene P1/P7 (Deng and Hiruki 1991; Schneider *et al*., 1995) and the primer pair R16F2n / R16R2 (Gundersen and Lee, 1996) in the nested PCR. The phytoplasmas detected in the two infected samples were further characterized by using specific direct and nested primer pairs of the *imp* and *tuf* genes. Ready-to-Go PCR beads (Pharmacia Biotech, Sweden) were used in PCR amplification reactions with 25 µl reaction volumes and 10 pmoles of each primer. The positive PCR products of the three genes were purified and sequenced at Macrogen (South Korea). Multiple sequence alignments of the 16S rDNA, *imp* and *tuf* genes with 16S rDNA, *imp* and *tuf* genes from phytoplasma GenBank sequences (NCBI, Bethesda, MD, USA) (http://ncbi.nlm.nih.gov/BLAST) were conducted using Clustal W (Thompson *et al*., 1994) and phylogenetic trees were made according to the neighbor-joining (NJ) method using MEGA 6.0 software (Tamura *et al*., 2013). The bootstrap values are expressed as percentages of 1,000 replications and a Kimura two-parameter model (Kimura, 1980). The trees of 16S rDNA and *tuf* genes were rooted using *Bacillus subtilis*.

Results

The direct and nested PCR reactions of the 16S rRNA, *tuf* and *imp* genes showed positive results from the symptomatic sugarcane plants as well as the positive control samples. The

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healthy sugarcane plants and the water samples (negative controls) did not show amplification.

The 16S rRNA sequences of the two positive sugarcane phytoplasma strains were deposited in GenBank under the accession numbers PQ677117 and PQ677118. The DNA sequences of the 16S rRNA, *tuf* and *imp* genes were 100% identical to each other. The BLAST search at NCBI of the 16S rRNA, *tuf* and *imp* genes showed that sugarcane phytoplasmas from this study had a 100% identity with the corresponding sequences of '*Candidatus* Phytoplasma australasiae=australasiaticum' (16SrII-D). It is evident from the results of the phylogenic trees of the 16S rRNA, *tuf* and *imp* genes that phytoplasma from Oman clustered with alfalfa witches' broom phytoplasma from Oman with 99% bootstrap, which is a member of the 16SrII-D subgroup (Figure 1 and data not shown).

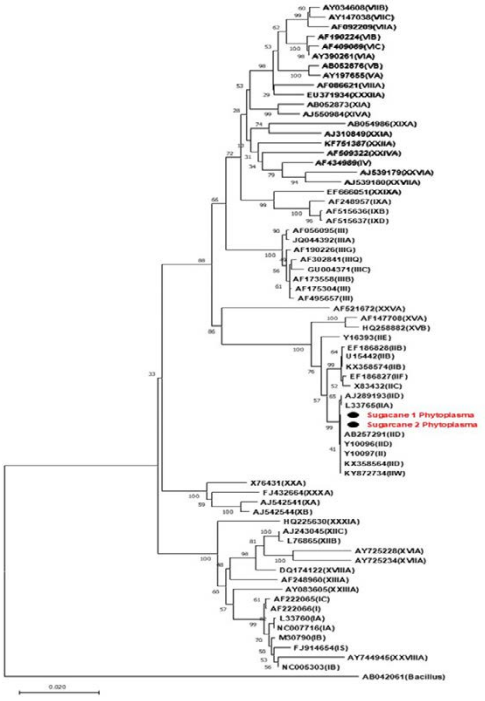


Figure 1. Phylogenetic tree constructed by neighbor-joining (NJ) method of 16S rRNA gene sequences from sugarcane phytoplasma.

Discussion

Sugarcane showed typical SCWL symptoms similar to those described in Asia (Nithya *et al*., 2023). The results of the two phytoplasma strains included in this study showed that they have the same DNA sequences of the 16S rRNA, *tuf and imp* gene sequences. Phylogenetic analysis clustered these sugarcane phytoplasmas in the same subclade with 16SrII-D subgroup phytoplasma strains. Based on blast results of DNA sequences and phylogenetic analyses, the sugarcane phytoplasma from Oman is a member of the 16SrII-D subgroup and a strain of '*Ca*. P. australasiaticum'. This is the first report of a phytoplasma from the 16SrII-D subgroup naturally infecting sugarcane plants worldwide.

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Sugar crops

Identification of diverse phytoplasmas in sugarcane yellow canopy syndrome

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Abstract

Sugarcane is cultivated in tropical and subtropical regions for its high rate of sucrose accumulation, ease of propagation via vegetative stem cuttings and multiple harvests from a single planting. The Australian sugarcane industry is recently facing a new threat know as yellow canopy syndrome (YCS). Samples exhibiting the yellowing symptoms were collected in Queensland, and molecular analyses were conducted to verify the presence of phytoplasmas. The tested samples resulted positive to the presence of '*Candidatus* Phytoplasma brasiliense', '*Ca.* P. aurantifolia=citri' and '*Ca.* P. asteris' by preliminary RFLP analyses followed by amplicon sequencing. Despite the large presence of phytoplasmas in the symptomatic samples tested further investigation are needed to confirm a correlation between the YCS and the detected bacteria.

Keywords: sugarcane, YCS, molecular detection, RFLP analysis, sequence analysis

Introduction

The sugarcane yellow leaf syndrome (YLS), characterized by yellowing of the midrib and lamina, was first reported in the East Africa (Rogers, 1969) and later in Hawaii, South Africa (Cronjé *et al*., 1998) and Cuba (Peralta *et al*., 1999). It is presently widely reported in most sugarcane-growing countries from all continents. It can cause losses from 30% up to over 60% in the susceptible varieties (Schenck *et al*., 1997; Comstock *et al*., 1994, 1998). Symptoms of YLS have been attributed to the presence of a luteovirus or phytoplasmas in Hawaii, Brazil, Australia, South Africa, Cuba, the USA and Mauritius (Schenck *et al*., 1997; Vega *et al*., 1997; Cronjé *et al*., 1998; Arocha *et al*., 1999; Scagliusi and Lockhart, 2000; Tran-Nguyen *et al*., 2000; Aljanabi *et al*., 2001). Phytoplasmas have been consistently associated with YLS, but latent infections also occur (Bailey *et al*., 1996; Cronjé *et al*., 1998; Arocha *et al*., 2000; Aljanabi *et al*., 2001). Other sugarcane diseases associated with the presence of phytoplasmas include white leaf (SCWL) and grassy shoot (SCGS) in southeast Asia, Ramu stunt disease in Papua New Guinea, and green grassy shoot (SCGGS) in India. The yellow canopy syndrome (YCS) is a sporadic phenomenon characterized by the golden-yellowing of the mid-canopy in sugarcane observed in Australia mainly during the peak growing period in summer from December to March. The symptom of YCS is a distinct yellowing of the leaves, clearly distinguished from other potential causes of leaf discoloration, including drought stress, phytotoxicity, insect damage, known diseases, nutrient deficiencies, and natural maturation processes. First observed in 2011, YCS has since spread to all major sugarcane growing regions in Australia, becoming a critical concern for the industry. Over the past few decades, the Australian sugarcane industry has experienced a decline in yields with an increasing threat of YCS. This syndrome leads to considerable yield losses (Marquardt *et al*., 2016). The severity of the syndrome can be modulated by altering the sink strength in the plant, and after that sugarcane can exhibit recovery. Despite ruling out several potential causes, including known sugarcane pathogens, the precise etiology of YCS remains unidentified.

Materials and Methods

A total of 36 symptomatic sugarcane samples were collected in 2018 and the total genomic DNA was extracted using CTAB protocols from leaves, stalks and roots. The 1 to 30 dilutions of DNA in sterile distilled water were used as template for detection through nested PCR assays. PCR analyses were performed in a final reaction volume of 25 µl containing 12.5 µl of Safe-Green 2X DreamTaq PCR master mix, 10.5 µl of sterile deionized filtered water, 0.5 µl of each primer at 20 pmol/µl (final concentration 0.4 µM), 1 µl of DNA template. One microliter of 1:30 diluted PCR product was used as template for nested PCR assays. Primers pairs R16m1/m2 (Gundersen and Lee, 1996) were employed to amplify the ribosomal operon, followed by fU5/rU3 (Lorenz *et al*., 1995) and $R16_{758f}$ (=M1)/$R16_{123r}$(=M2) (Gibb *et al*., 1995) for the nested amplification. The obtained PCR products were visualized after electrophoresis in 1% agarose gel in TA buffer (tris-acetate 1.04 M, EDTA 0.002 M) and staining in ethidium

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bromide, under UV transilluminator. The phytoplasma identification was conducted through RFLP analysis in 6.7% polyacrylamide gel, using *Tru*I endonuclease according to manufacturer's instructions. Obtained amplicons were sequenced by Macrogene (Milan, Italy) on both strands, using the same primers employed for the amplification. Raw sequences were assembled and checked using Pregap4 and Gap4 software from the Staden package (Staden *et al*., 1998) and were compared using BLAST tool to the sequences present in the NCBI database.

Results

RFLP analysis allowed to identify the presence of profiles referable to phytoplasmas in the 16SrXV group in 22 samples, 16SrII in two samples and 16SrI in two samples (Figure 1). In one sample, a possible mixed infection of 16SrII and 16SrI groups was detected. The sequencing results confirmed the identity of the phytoplasma identified as '*Ca*. P. brasiliense' (16SrXV), '*Ca*. P. aurantifolia' (16SrII) and '*Ca*. P. asteris' (16SrI). Out of the 36 samples tested, 26 resulted positive for phytoplasma presence (Table 1).

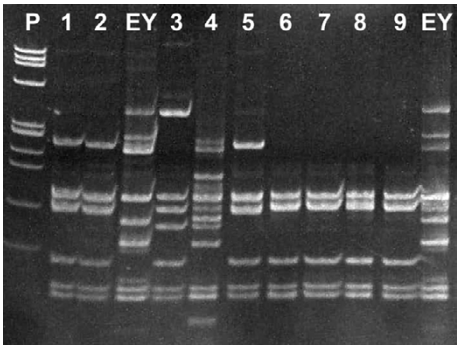


Figure 1. RFLP of fU5/rU3 amplicons digested with *Tru*1I from sugarcane samples (1 to 9). EY, '*Ca*. P. ulmi' positive control; P, marker phiX174 DNA digested with *Hae*III.

Table 1. Results of phytoplasma detection in sugarcane samples.

	Positive samples	Identity to type strain
'*Ca*. P. brasiliense'	22/26	100% (345 bp)
'*Ca*. P. citri=aurantifolia'	2/26	99.92% (1,267 bp)
'*Ca*. P. asteris'	2/26	-

Discussion

The identification of '*Ca*. P. brasiliense' and '*Ca*. P. citri=aurantifolia' in sugarcane is consistent with the phytoplasma detection in YLS in other sugarcane growing areas worldwide. The presence of these phytoplasmas should be taken into consideration also as a possible threat to other cultivated plants together with their possible association to major yield losses. The use of healthy and uninfected plant propagation material remains the first condition to avoid the spread of these bacteria. The study of possible alternative hosts and insect vectors for the detected phytoplasmas, as well as the selection of possible resistant cultivars require further research.

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Sugar crops

Mechanized sett treatment: an innovative technology to deliver various agro-inputs for the management of sugarcane diseases including the grassy shoot

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Abstract

As sugarcane is an annual crop with ratooning behavior and propagated by vegetative means, pathogen infection in the stalks is transmitted to main field through the seed cane and that leads to disease epidemics. Earlier studies with various fungicides indicated that overnight soaking is pre-requisite for the management of primary inoculum of sett and soil borne fungal pathogens. Furthermore, rapid sett treatment with sett treatment device showed its equal efficacy as overnight conventional soaking along with more practical solutions. In continuation, application of sett treatment device for the delivery of physical, chemical and biological agents was evaluated to envisage the mechanized sett treatment for integrated disease management in sugarcane. Results of the present study indicated that the sett treatment device can be effectively used for treating setts with physical (hot water), chemical (nutrients, pesticides) and biological (microbes) either individually or in combination for disease management and improvement of crop growth. In this respect, efficacy of all the three treatments had been proven for the management of red rot, smut and grassy shoot diseases. Additionally, their efficacy was proven in improving varietal performance in popular varieties under field conditions.

Keywords: sett treatment device, agro inputs, phytoplasma disease

Introduction

Effective management of any stress is associated with practicability of management practice. It is well known that, among the delivery methods for improvement of crop growth, seed treatment plays key role for easy, effective, economical and rapid delivering of agrochemicals and/or microbes. In earlier studies, it was demonstrated the advantages of mechanized sett treatment over conventional treatment and proved its efficacy in managing sugarcane fungal diseases in healthy nursery programs (Malathi *et al*., 2017, 2021). In continuation, it was demonstrated the applicability of sett treatment device for treating setts with physical (hot water), chemical (nutrients) and biological (microbes) agents, either individually or in combination for fungal and phytoplasma disease management and improvement of crop growth in popular sugarcane varieties. The study indicated that the mechanized treatment has several advantages as it is simple, economical, ecofriendly, effective in delivering many inputs with uniformity and amenable for large scale application. Since this multi-use device has a potential to improve sugarcane growth and crop yield, the technology was commercialized, and the licenses were issued through Agrinnovate. Furthermore, a process patent has been awarded for this invention (Patent - 3323/CHE/ 2011).

Materials and Methods

In the present study, all the experiments were mainly focused on utilizing sett treatment device (STD) for disease management and additionally other inputs such as fungicides, insecticides, nutrients and microbes have been evaluated for various purposes. The principle involved in STD is vacuum infiltration by creating a negative pressure followed by absorption of the chemicals inside the setts. This method of sett treatment was performed at prescribed vacuum level (150-250 mmHg) and duration (15-30 minutes) in the newly fabricated large size units of different shapes: square and cylindrical types as standardized earlier in the prototype (Malathi *et al*., 2017).

Results

As varietal degeneration due to non-fungal pathogens is an important phenomenon, selection of healthy seed material and eradication of accumulated pathogens in routine planting material by thermotherapy – hot water/ hot air

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treatment is recommended. Since this treatment will be highly helpful for maintenance and rejuvenation of germplasm and breeding stocks, the newly identified mechanized system of sett treatment has been validated with hot water instead of plain water and along with fungicide and nutrients. *Saccharum officinarum* (IJ-76-419) and the prominent cultivar Co 86032 were evaluated with various treatments including hot water alone and its combination with fungicide and nutrients. Observations up to 90 days showed that the treatments were not inhibitory to germination, instead their growth and tillering were improved as in plain water with other ingredients.

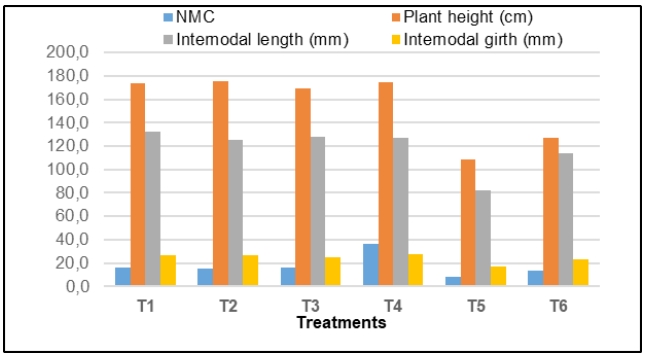


Figure 1. Efficacy of mechanized sett treatment with hot water alone or in combination with vacuum against grassy shoot diseaase in CoV 9210.

Based on earlier standardization, for grassy shoots disease (GSD) management hot water treatment has been validated by using completely infected CoV92101 stalks. Single bud setts were subjected to various treatments including recommended practice of hot water treatment at 50°C for 1 hour and 52°C for 30 minutes without vacuum were compared with hot water treatment in the sett treatment device for 15 minutes. Treated setts were planted in the field and pits and observations were taken on germination, disease incidence, number of millable canes, plant height, number of nodes, internodal length and girth of stalks. Results clearly indicated that there was no significant difference on germination among treatments including control, while there was significant improvement in yield attributes such as number of millable canes, cane height, internodal length and girth of stalks. However difference in the number of nodes between control and treatments was also insignificant. With the improvement of yield attributes, there was significant reduction in disease incidence in all the treatments as compared to control and it is interesting to note that there was no disease in the pits with treated setts from the device (Figure 1). However 10% of the clumps shown GSD in the ratoon crop. Under field conditions also, significant reduction in disease incidence could be observed with increase in temperature level by using completely infected canes.

Discussion

Hot water treatment or hot air treatment are used in quarantine for eradication of insects, mites, nematodes, fungi, bacteria, phytoplasmas and viruses. The basic principle involved is that treatment temperature should be sufficiently high to kill the associated pest/pathogen but not the host. Using phytoplasma free stock through tissue culture method is currently recommended for the sugarcane farmers. However, in Indian condition, appearance of GSD in tissue cultured plantlets is noticed and also it is an expensive approach and there is insufficient tissue culture seedling for distribution during the planting season. Alternative treatments such as thermotherapy and antibiotic were introduced to sugarcane stocks for controlling sugarcane grassy shoot phytoplasma and showed positive results (Pliansinchai *et al*., 1997). However, there is no effective hot water system available under Indian conditions. Hence these treatments can be validated in large scale under field conditions for the management of both fungal and non-fungal diseases.

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Phytopathogenic Mollicutes
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Sugar crops

Grassy shoot of sugarcane in India: impact to cane cultivation, diagnostics and sustainable disease management

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Abstract

Grassy shoot of sugarcane associated with sugarcane grassy shoot phytoplasma is a serious disease in India and many other countries in the Asian region. The disease causes severe losses to cane production and productivity in India, where it is also associated with low ratoon productivity. None of the varieties under cultivation or parents in breeding blocks is resistant to the disease. Hence the disease must be controlled through various management strategies. Heat treatment offers partial inactivation of the phytoplasmas in the planting materials, hence healthy seed nurseries established through meristem culture combined with molecular detection is found to be the viable solution to effectively manage the disease.

Keywords: SCGS phytoplasma, economic impact, healthy propagation materials, detection

Introduction

The grassy shoot disease (GSD) was first observed in 1949 in the sugarcane cultivar Co419 in Ahmadnagar district of Maharashtra in India. Later, the diseases referred with different names like new chlorotic disease, albino disease, yellowing disease, bunchy disease or leafy tuft were brought under the common name grassy shoot (SCGS). A disease with similar symptoms of chlorotic leaves accompanied by excess tillering referred as white leaf disease (WLD) occurs in many Asian countries like Vietnam, Thailand, Indonesia and Sri Lanka (Nithya *et al*., 2020). Both GSD and WLD now are made as synonymous, and the diseases are confined to Asian countries. GSD incidence and severity increased at an alarming rate in many Asian countries, and more profoundly in India where severe outbreaks were widely reported in all the states (Viswanathan, 2000). The etiology of the disease has been studied in detail and the associated phytoplasma was molecularly identified and characterized including its complete genome.

Economic importance

The disease is mainly transmitted through infected planting materials. Planting infected setts cause conversion of growing shoots into chlorotic ones with extremely high number of tillers (Figure 1). These chlorotic tillers never form millable cane, leading losses in cane production and yield. Most of the varieties under cultivation in India exhibit varying intensities of grassy shoot. In a moderately infected crop, up to the 40% reduction in sugarcane yield was reported (Tiwari *et al*., 2012). About 50-75% plant crop infection resulted in 100% failure in millable cane production in the ratoon crop (Viswanathan, 2000, 2001). SCGS-phytoplasma infection can cause reductions of 35% in stalk height, 15% in stalk girth, 50-60% in internode length. Very high yield losses are due to GSD/WLD disease initial stages all over in the Asian countries, particularly when the planting material is obtained from infected sources, or when the disease transmission occurs during the early stages of the crop growth.



Figure 1. GSD affected sugarcane clump shows lack of cane formation amidst healthy canes in plant crop.

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Figure 2. Chlorotic sprouts in ratoons leads to poor crop establishment due to death of the diseased tillers.

More reports have come from India on the economic impact caused by the disease on popular varieties. Losses of 5 to 70% in plant crop and up to 100% in ratoon crops have been recorded in different states (Nasare *et al*., 2007, Tiwari *et al*., 2012; Viswanathan *et al*., 2011). Occasionally, due to severe occurrences of the disease, huge crop losses to cane production are encountered.

Many varieties faced extinction from cultivation due to the severe incidence of GSD or neglect seed health. For example, the popular cv CoS767, widely cultivated in the sub-tropical India went out of cultivation due to severe GSD during the last decade (Tiwari *et al*., 2012; Viswanathan, 2016). The crop severity increases in the ratoons due to build-up of higher pathogen titre in the stools. Chlorotic sprouts emerge from the infected stools immediately after plant cane harvest in the ratoons (Figure 2). Soon, the lean and lanky sprouts die and causes poor crop establishment and ultimately crop losses increase in the ratoons.

Management

Lack of healthy seed program has been attributed to the disease perpetuation and severity. Although practicing multiple ratoons brings better dividends to cane cultivation, GSD severity hinders it due to the severe expression of the disease in the ratoons. Detailed studies taken at different centers revealed absence of host resistance to the SCGS-phytoplasmas. Hence managing the disease through host resistance is not possible and it is necessary to apply other management strategies. Molecular diagnostics play a key role in detecting the pathogen and to index tissue culture seedlings for freedom from phytoplasmas (Viswanathan *et al*., 2018).

Conclusion

In the 1970s and 1980s, substantial works were carried out on managing the disease using aerated steam and moist hot air therapies, however, complete eradication of the pathogen from the planting material is not achieved through heat therapy. Heat treatment offers only partial inactivation of the pathogen in the setts and the disease appears in the late growth stages in the plant crop and in the ratoons (Viswanathan, 2016). Recently, healthy nursery system through meristem culture combined with molecular detection proved to be the best strategy to manage the disease (Viswanathan *et al*., 2018). Single bud nurseries derived through the disease-free mother plants have come to play in different states to quick distribute healthy planting materials in the country. It is necessary to adopt such nurseries in war footing to spread healthy planting materials and sustain cane and sugar yield in India (Viswanathan, 2023). In spite of substantial research works done over the decades, insect vector(s) involved in secondary transmission of the disease is(are) not clear and this warrants detailed investigation. Recently SCGS-phytoplasmas infection in sorghum was found (Nithya *et al*., 2024), hence existing collateral / alternate host plants in the ecosystem need to be studied. Detailed studies on host-pathogen interaction are needed to identify the potential pathogenicity/ host targets to manage the disease through genetic manipulation.

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Sugar crops

Diagnostics of sugarcane grassy shoot phytoplasma for a healthy seed nursery programme in sugarcane

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Abstract

Among the biotic stresses, the grassy shoot disease is a major production constraint to farmers and sugar industries in most of the Asian countries. The disease is associated with the presence of '*Candidatus* Phytoplasma sacchari' detected by sequencing of amplified samples and by symptom presence under field conditions. In recent years, many diagnostic tools like LAMP, RPA, CRISPR-Cas and LFIA have been developed for plant pathogen detection. In LAMP assay, all the positive samples collected from different places had shown multiple bands through electrophoresis. Under the colorimetric assay, all positive samples had shown fluorescent green color, and the negative sample was non fluorescent. Similarly, in the RPA assay all the samples had shown the expected amplification of 200 bp under isothermal conditions at 37-42°C. Quantitative PCR assay was found sensitive to detect the pathogen even at 10^{-1}ng/µl in comparison with LAMP and RPA. Recently, clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nuclease-based plant pathogen detection is gaining momentum in molecular detection. Development of onsite field diagnostics like LFIA, and RPA-dipstick assays are important for the selection of healthy planting materials and for the healthy seed nursery program for the supply of disease-free planting materials to the farmers. However, major challenges in the onsite field diagnostics would be the sensitivity which may vary in comparison with the detection technique used, because of crude sap usage in place of purified nucleic acids.

Keywords: SCGS phytoplasma, onsite-field diagnostics, disease management

Introduction

Sugarcane (*Saccharum* hybrid) is one of the most important commercially grown cash crops cultivated in more than 100 countries and contributing more than 80% of world sugar requirement. India is the second largest producer of sugarcane after Brazil and this crop is cultivated in 4.6 million hectares of land area by around 6 million farmers and it provides raw material to more than 500 sugar industries, "gur" and "khandsari" units in India (Anonymous, 2024). Because of the growing demand of biofuel and green energy in the recent years, this crop is being seen as a viable alternative for production of renewable energy. The crop is affected by various biotic and abiotic stresses and among the biotic stresses, the grassy shoot disease is a major constraint in most of the Asian countries such as Bangladesh, China, India, Malaysia, Myanmar, Nepal, Pakistan, Sri Lanka, Sudan, Thailand, and Vietnam (Rishi and Chen, 1989; Wongkaew *et al*., 1997; Rao *et al*., 2008, 2014; Viswanthan *et al*. 2005, 2011; Nithya *et al*. 2020). In India, it is widely reported in all the sugarcane growing places and causes losses of 5 to 50% in plant crop and more than 50% in ratoon crop (Viswanathan and Rao, 2011). The disease is associated with the presence of '*Candidatus* Phytoplasma sacchari' (16SrXI group). Since sugarcane is a vegetatively propagated crop, the pathogen is primarily spread through stem cuttings and by secondary transmission through phloem sap feeding insect vectors. In recent years, many diagnostic tools like Loop mediated Isothermal amplification assay (LAMP), Recombinase polymerase amplification (RPA) assay, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas and Lateral flow Immunodiagnostic Assay (LFIA) have been developed but it is important to use these diagnostics under onsite field conditions.

Materials and Methods

SCGS samples were collected from the major sugarcane growing places of India: Tamil Nadu, Andhra Pradesh, Haryana, Uttar Pradesh, Bihar, and Maharashtra. Moreover, symptomatic and asymptomatic samples were collected from different varieties at ICAR-SBI, Coimbatore (Figure 1). All the samples were stored in -80°C until further processing. Total DNA was extracted by following a CTAB method, purified and quantified using a nano spectrophotometer. Nested PCR assay was followed using the

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universal P1/P7 and R16F2n/R16R2 primers. The positive samples were cloned into pGEMT easy vector and sent for sequencing. The ribosomal nucleotide sequence of SCGS phytoplasma from GenBank was downloaded and aligned using Bioedit ClustalW multiple alignment tool. The most conserved regions of the aligned sequences were used to design the LAMP primers using Primer ExplorerV4 software. Two sets of LAMP primers including outer and inner primers were designed and LAMP assay was standardized. Ten microliters of products were loaded and analysed for the presence of multiple bands through electrophoresis on 2.5 % agarose gel stained with ethidium bromide. To assess the sensitivity of the LAMP primers in comparison with quantitative PCR assay, standard curve was prepared with plasmids containing SCGS phytoplasma PCR fragments and the phytoplasma titre was calculated. Similarly, RPA primers were designed and standardized under isothermal condition at various temperatures.



Figure1. Sugarcane grassy shoot disease symptom.

About 70-80% of the samples collected from different places of India as well as from different varieties had shown the expected amplification of 1.8 kb in PCR using P1/P7 primers and in nested PCR all the samples had shown the expected amplification of 1.2 kb which was cloned, the colony PCR results confirmed the presence of inserts. Positive sequences were submitted to GenBank (accession numbers from MN904464 to MN904480). In the LAMP assay, all the positive samples had shown multiple bands through electrophoresis on 2.5% agarose gel stained with ethidium bromide. Under the colorimetric assay, all positive samples had shown fluorescent green color under UV light and the negative sample was non fluorescent. Similarly, in the RPA assay all the samples had shown the expected amplification of 200 bp under isothermal conditions at 37-42°C. Quantitative PCR assay was found sensitive to detect the pathogen until at 10^{-1}ng/µl in comparison with LAMP and RPA. Also, not much significant difference was found in the pathogen titre from the symptomatic samples and the asymptomatic sample collected nearby infected clumps.

Discussion

In phytoplasma detection, the isothermal amplification assays such as LAMP and RPA have been considered as an alternative approach to the nested PCR assay because of their simple rapid and reliable results. Recently, CRISPR(Cas) nuclease-based plant pathogen detection is gaining momentum and has been successfully used for the '*Ca.* P. trifolii' (Wheatley *et al.*, 2022). The qPCR assay could detect the mosaic and yellow leaf viruses even at 10^{-3} to 10^{-1} ng/µl of sample dilutions and sensitive over the RT-LAMP and RT-PCR assay (Anandakumar *et al.*, 2018, 2020). Similarly, in case of sugarcane yellow leaf disease no significant difference was found in the pathogen titre from symptomatic and asymptomatic samples (Nithya *et al.*, 2023). Development of onsite field diagnostics is important for the selection of healthy planting materials and for the healthy seed nursery program for supply disease-free planting materials to the farmers. Major challenges in the onsite field diagnostics would be the assay sensitivity. So, field diagnostics by following standard procedures would play a significant role in healthy material selection.

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Sugar crops

Seed treatment: suitable approaches for establishing nursery of sugarcane free of phytoplasma associated diseases

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Abstract

The supply of disease-free seed material forms the basis for higher yield production. Hence, a clean seed program is of prime importance. To eliminate the phytoplasmas associated with sugarcane grassy shoot, white leaf and yellows leaf thermotherapy is one of the proven e approaches to inactivate the bacteria residing inside the tissues of seed canes. On the other hand, the varieties that have soft buds or bud bulging issue can be treated with streptocycline (at 0.01%) in cold water (CWT at 15ºC). This is also effective in preventing the infection and enhanced the rate of germination as compared to hot water treatment (HWT) (at 50ºC for 2 hours). In addition, treatment with systemic fungicide like carbendazim at 0.1% in CWT is recommended to prevent entry of soil pathogens into the setts.

Keywords: grassy shoot, white leaf, yellow leaf, disease free nursery, hot water treatment, cold water treatment

Introduction

The sugarcane seed programme aims to produce disease and pest free, genetically pure nuclear seed material of approved released varieties under standard system of cultivation so that maximum yield and sugar recovery could be achieved by the cane growers and sugar industry. Diseases are one of the major constraints in the profitable cultivation of sugarcane because vegetative propagation favours the accumulation of pathogens inside sugarcane tissues. Along with the seed canes cultivation, disease causing pathogens are also introduced into new areas. Slow accumulation of different pathogens over a period of time makes minor disease of major importance in due course of time. Several epidemics due to red rot, smut, wilt, grassy shoot, ratoon stunting, leaf yellows and leaf scald occurred in the past indicated that the disease infected seed cane played significant role in the build-up of pathogens and further spread of the disease (Viswanathan and Rao, 2011). Among the sugarcane diseases, the white leaf (SCWL), grassy shoot (SCGS), and leaf yellows (SCYL) associated with phytoplasmas result in severe reduction in yield and quality (Tiwari *et al*., 2012; Kumar *et al*., 2016; Rao, 2021). Earlier studies from Uttar Pradesh state of India, reported more than 50% incidence of SCGS and WLD in several parts of eastern and western Uttar Pradesh and up to 40% reduction in qualitative and quantitative parameters in affected sugarcane varieties (Tiwari *et al*., 2012; Iqbal *et al*., 2015; Rao *et al*., 2017). The transmission of the disease in fields occur through insect vectors and propagating infected sugarcane setts (Rao *et al*., 2014; Tiwari *et al*., 2017).

As supply of disease-free seed material form the basis for higher sugarcane production, clean seed programme is of prime importance. To get rid of SCGS, WLD or SCYLP diseases associated with phytoplasmas in the 16SrXI group ('*Candidatus* Phytoplasma sacchari'), the thermotherapy is suggested as proven effective approach to eliminate/inactivate the phytoplasmas residing inside the tissues of cane stalks. In heat therapy, the pathogens present in seed canes are inactivated or eliminated at set temperatures of 50ºC for two hours without any side effects on host tissues, however in some cases, effects on germination have been observed (Tiwari and Rao, 2023). Another approach to prevent phytoplasma from sugarcane setts is treating the canes in cold water (CWT) (15ºC, tap water) with HCl at 500 ppm (Kaewmanee and Hanboonsong, 2011) or streptocycline at 0.01% (A. K. Tiwari *et al*., unpublished). So, keeping in view the importance of phytoplasma associated diseases in sugarcane, a regular feature set treatment programme is going on in producing, developing and distributing phytoplasma free canes by using how water treatment (HWT)/CWT in Uttar Pradesh.

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Material and Methods

In a regular feature mode, the nurseries are raised in autumn and spring season of sugarcane planting by using HWT at 50ºC for 2 hours and CWT at 15ºC for two hours with streptocycline at 0.01% (A. K. Tiwari *et al*. unpublished). Initially 1 ha area is selected for planting after HWT/CWT treatments. The sugarcane varieties having soft bud tissues CoS 13235, CoSe 13452, Co 15023 are used in CWT and varieties with hard buds CoLk 14201, CoS 17231, CoS 09232 are treated with HWT. Both HWT and CWT are done by dipping two budded sugarcane setts in water with antibiotic along with a systemic fungicide (carbendazim at 0.1%) (A. K. Tiwari *et al*., unpublished). The treated setts are planted in prepared fields plots of 1 ha area, and germination percentage along with disease incidence are observed by regular surveys monitoring and roughing is done for symptomatic clumps. The visually disease-free planting materials are again used in next year planting at research farms by following CWT along with the systemic fungicide and antibiotic addition as describe above.

Results and Discussion

The seed programme is being implemented to produce and supply disease free seeds of maximum quality to farmers through breeder seeds. This program is a continuous process in India, and every year CWT/HWT is being implemented and results in disease free (SCGS, WLD and YLD) seed material for the supply to the growers.

The HWT on varieties CoLk 14201, CoS 17231, and CoS 09232 was found effective towards controlling the phytoplasma disease symptoms (SCGS, WLD, YLD) and bacterial symptoms (LSD) after two years of consecutive treatments. In general, thermotherapy results in softening of buds, hence, care should be taken while handling the treated setts to reduce the bud damage in transportation especially in varieties having soft bud tissues and of bulging nature. However, CWT with antibiotic can be adopted which significantly improves the germination (more than 58%) and also eliminates the phytoplasma associated symptoms of diseases in varieties CoS 13235, CoSe 13452 and Co 15023. Regular use of HWT/CWT has helped in eliminating phytoplasma diseases at seed farms of Sugarcane Research and Seed Multiplication Center, Gola, Kheri, Uttar Pradesh (A. K. Tiwari *et al*., unpublished).

By following the CWT and HWT at SRS, Gola, on an average more than 15 thousand quintal disease free breeder seeds are available for distribution every year and interestingly in last five years, it was not recorded any incidence of SCGS, WLD or YLD diseases due to regular monitoring of treatment of setts through CWT/HWT and roughing symptomatic clumps. It has been advisable to soak seed cane in hot water which is not only a useful control measure for the management of phytoplasma associated disease, but also useful in stimulating the growth and yield of canes. On the other hand, the varieties that have soft buds or bud bulging nature should be treated with CWT with streptocycline which not only prevent the phytoplasma associated diseases but also enhance the rate of germination. It is also suggested that combination of treatment with carbendazim at 0.1% in CWT that is preventing the easy entry of soil pathogens into the setts.

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Sugar crops

A multiplex PCR assay for rapid differentiation of sugarcane phytoplasmas reveals co-infection in samples from Thailand

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Abstract

Sugarcane white leaf (SCWL), sugarcane grassy shoot (SCGS) and sugarcane green grassy shoot (SCGGS) are the most devastating sugarcane diseases in Thailand that cause significant losses to the crop production and the sugar industries. SCWL and SCGS phytoplasmas were found to be closely related based on 16S rDNA gene analysis while SCGGS was distantly related to them. So far, differentiation of these phytoplasmas have been based on Sanger sequencing which is time consuming and costly. This study aimed to develop a rapid and simple method for simultaneous identification of these phytoplasmas by multiplex-PCR targeting the 16S rDNA gene and to investigate the incidence of co-infections. Three sets of primers were designed to produce the target amplification products at 340 bp, 230 bp and 120 bp respectively to SCWL, SCGS and SCGGS. The investigation in 20 asymptomatic samples revealed two samples showing mixed infection of SCWL and SCGS. These results thus indicated that this multiplex-PCR method provides a simple, rapid and reliable differentiation method for the three phytoplasma diseases in sugarcane in Thailand. Notably, this is the first report to detect co-infection of the two phytoplasmas. The sensitivity of this method thus demonstrated substantial potential as an effective tool for screening of sugarcane plants prior to propagation as well as providing a reliable method to avoid misinterpretation of the analysis in ambiguous samples.

Keywords: molecular detection, sequencing, diseases, molecular differentiation

Introduction

The sugarcane white leaf (SCWL) and sugarcane grassy shoot (SCGS), recognised as white leaf disease, and sugarcane green grassy shoot (SCGGS) are the three devastating diseases in sugarcane industry in Thailand since decades (Figure 1) (Sakuanrungsirikul *et al.*, 2013).



Figure 1. Sugarcane plants showing white leaf (SCWL), grassy shoot (SCGS) and green grassy shoot (SCGGS) symptoms.

These diseases are cane-borne with the two leafhoppers *Matsumuratetrix hiroglyphicus* and *Yamatotettix flavovittatus* reported to transmit SCWL and SCGS diseases, respectively (Matsumoto *et al.*, 1969; Hanboonsong *et al.*, 2002). However, transmission vector of SCGGS in Thailand is still unidentified. Distinctive symptoms of these two diseases are that SCWL infected plants show white leaf while SCGS shows white leaf with multiple shootings. Sugarcane infected with SCGGS disease shows multiple shootings but no white leaf symptom that make it difficult to identify the infected plant when the infection is low. The SCWL disease is mostly found in the northeastern planting area while SCGS can be found in many regions, but the higher incidence is in the central and eastern regions. SCGGS disease is mostly found in the western region, but the incidence is lower than the one of the first two diseases (Sakuanrungsirikul *et al.*, 2013). Many effective methods are available for screening and detecting phytoplasma infection in sugarcane, however, discrimination of the phytoplasmas associated with the three sugarcane diseases is based on sequencing methods that are costly, time consuming and complicate. The objective of this study was to develop a rapid and simple method for simultaneous identification of these phytoplasmas by multiplex-PCR and to investigate the possible presence of co-infections.

Materials and Methods

Sugarcane plants showing symptoms associated with phytoplasmas were collected in the regions of Thailand where the presence of the three diseases was confirmed by previous studies. Plants showing SCWL, SCGS and SCGGS

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were collected from fields during October 2022 and September 2023. Samples were homogenised and total DNA was extracted as described in Sakuanrungsirikul *et al.* (2013). Primers were designed using the BioEdit Sequence Alignment Editor Software version 7.0. The primers were developed for detection through multiplex PCR targeting the 16S rRNA gene. A total of three primer pairs were designed, each specifically targeting phytoplasma associated with the three diseases targeted in this study. The thermal cycling condition were 5 minutes at 94°C followed by 35 cycles for 30 seconds at 94°C, 30 and 40 seconds at 55°C and 1 minute at 72°C with final extension for 7 minutes at 72°C, PCR products were purified using the GF-1 PCR Clean-up Kit (Vivantis, Malaysia) and sequenced by ABI 3500 in Khon Kaen Field Crops Research Center. The sequences were assembled using DNA Base V.4 and aligned with appropriate sequences. A phylogenetic tree was constructed using the neighbour joining method with MEGA 7.0 (Kumar *et al.*, 2016) using 1,000 bootstrap replications with CrustalW software (Hall, 1999).

Results

The phytoplasma specific primer pairs amplified the target DNA and generated an expected amplicons of 340 bp, 230 bp and 120 bp (Figure 2), respectively from the symptomatic plants by multiplex PCR. The assay showed primers specificity to target sites without cross-reaction with phytoplasmas in grass and other pathogens tested, and high detection sensitivity at the lowest of picogram plant DNA sample.

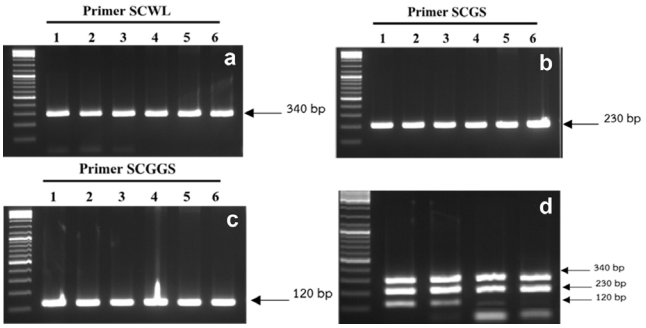


Figure 2. Detection of phytoplasma DNAs (lanes 2-7) using multiplex-PCR primers. (a) SCWL, (b) SCGS, (c) SGCCS, (d) amplicons of 3 phytoplasma in one-step multiplex-PCR.

Amplification was obtained also from asymptomatic plants. These results were confirmed by the multiplex PCR assay and expected length fragments were again successfully amplified with primer pairs SCWL, SCGS and SCGGS, respectively used in a single tube reaction. For this assay the concentration of template DNA was increased to 75 ng/µl and the primer concentration was reduced to 0.5 µl; the annealing temperature was optimized at 55°C. The multiplex PCR amplicons of sugarcane samples were directly sequenced using forward and reverse primers, Partial 16S rRNA gene sequences showed that SCWL strain had 99% identity with GenBank accession number KJ18021, SCGS displayed 97% identity a '*Candidatus* Phytoplasma sacchari' strain (GenBank accession number MT510185), and SCGGS exhibited 95% identity with sugarcane green grassy shoot strain SUK22-4 from Thailand (GenBank accession number KF908793).

Discussion

The incidence of phytoplasma infection observed solely through white leaf symptoms is an unreliable indicator of infection and does not accurately reflect the severity of SCWL infection. Therefore, the incidence of SCWL infection must be accurately detected using molecular methods. This research demonstrated that the three phytoplasmas were differentiated by separating the DNA fragments based on their sizes through the amplification of target DNA. Phytoplasmas are unevenly distributed and often present in low titers in the phloem tissues of infected plants, making their detection quite tricky and challenging. PCR assays using P1/P7 primers may fail to yield visible DNA products; therefore, nested PCR is recommended to increase the sensitivity and specificity of the reaction (Bertaccini *et al.*, 2019; Reddy *et al.*, 2019). For multilocus gene-specific amplification (Martini *et al.* 2019), an additional PCR cycle is typically required, which takes time and necessitates additional consumables. Consequently, the multiplex PCR-based method developed in this study could be recommended for detecting phytoplasmas in plants and insects. The developed protocol and can serve as a fast and sensitive assay that may be further developed for other phytoplasma strains.

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Sugar crops

Antagonist bacteria in action: a strategy for managing sugarcane white leaf disease

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Abstract

Sugarcane white leaf disease (SCWL) is one of the most threatening diseases affecting sugarcane cultivation in Thailand. Current disease elimination techniques remain ineffective as the pathogen is seed-borne. Interestingly, sugarcane infected with SCWL phytoplasmas exhibits fewer symptoms when cultivated under favourable conditions compared to adverse environments, highlighting a relationship between plant health and stress tolerance. This study aimed to investigate integrated management practices to enhance the growth and survival of SCWL-infected sugarcane. Antagonistic bacteria were isolated from soil samples collected from sugarcane fields in Khon Kaen, Thailand. Experiments using *Streptomyces* spp. No. 8 and *Bacillus subtilis* No. 15 as soil drenches in sugarcane infected with phytoplasmas below a critical threshold showed statistically significant improvements in plant height, root fresh weight, and brix levels compared to untreated controls. Field evaluations using disease-free sugarcane cuttings treated with hot water and *B. subtilis* No. 15 confirmed the effectiveness of this management method. Seedlings were grown in a greenhouse with PGPR-3 biofertilizer and received weekly foliar sprays of *B. subtilis* No. 15 before being transplanted into fields enriched with organic and PGPR-3 biofertilizers and treated bimonthly with *B. subtilis* No. 15. The results showed that planting disease-free sugarcane increased the survival rates to 100%, stalks per plant by 55%, height by 28%, and brix levels by 11% compared to disease-free and latent-infected plants. Ten months after planting, no white leaf symptoms were observed in cuttings with phytoplasma content at 9 copies/µl. In infected cuttings managed with this method, no symptoms appeared, despite phytoplasma levels reaching 174 copies/µl. In contrast, infected cuttings without management exhibited 58% white leaf symptoms, with phytoplasma content exceeding 23,800 copies/µl. These findings support the conclusion that biological control of phytoplasma diseases in sugarcane using antagonistic bacteria is a promising and sustainable alternative to chemical control.

Keywords: phytoplasma, diseases, sugarcane management, biological control

Introduction

Sugarcane white leaf (SCWL) disease was first discovered in Thailand in 1956. Shortly after its discovery, it was considered a disease that only occurred locally in the northeastern and northern areas of Thailand, although the damage to sugarcane crops was severe in the places where it occurred. Recently, however, SCWL disease has spread rapidly, now affecting almost all of northeastern Thailand and also occurring in many sugarcane production areas in northern and central Thailand. According to the estimates from the department of agriculture, the loss of sugarcane revenue caused by SCWL disease amounts to approximately 30–40 million USD per year (Hanboonsong *et al.*, 2016), and the total damage could be as much as 10 times the yearly damage if one includes the effect on related industries. In addition, SCWL disease has spread to other countries in Southeast Asia, causing fear of increased economic damage. The severity of the disease varies with the climate each year and by area. In the initial stage of the disease, there may be a decrease in growth rate, pale green of the leaves, and subsequently, whitening and death of those leaves. At present, there are no effective treatments for SCWL disease. In severe cases, it can lead to plant death or render the crop unsuitable for ratooning in subsequent seasons. The widespread transmission of sugarcane white leaf disease mainly occurs by the two ways through which the SCWL disease spreads. The first is through insect vectors. The two known insect vectors are the leafhoppers, *Matsumuratettix hiroglyphicus*

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and *Yamatotettix flavovittatus*, of which *M. hiroglyphicus* is considered the most important in terms of the insect's density in the sugarcane field and disease transmission efficacy. The second is through transmission when sugarcane infected with SCWL disease is used as seedcane (Hanboonsong *et al.*, 2006). Currently, the outbreak of sugarcane white leaf disease is intensifying due to the expansion of sugarcane planting areas, which has led to a shortage of healthy cuttings. Infected cuttings are often unknowingly propagated or planted in disease-free areas, combined with a lack of monitoring and understanding regarding the onset and spread of this disease. The use of nitrogen-fixing bacteria to enhance the growth of sugarcane affected by white leaf disease has shown promising results in past research. It was found that sugarcane infected with white leaf disease at a pathogen level of approximately 100 copies/µl can exhibit white leaf symptoms under stress conditions, such as environmental factors, soil quality, and drought (Saengsai *et. al.*, 2024).

Materials and Methods

In the laboratory *Bacillus subtilis* No.15 and *Streptomyces* No.8 were cultured in NB and AGMB media. IAA production was measured using the Salkowski method, the *nifH* gene expression was also verified according to Saengsai *et al.* (2024).

In greenhouse the efficiency of antagonist bacteria for control of SCWL disease was evaluated with select sugarcane cuttings of phytoplasma-symptomatic variety KK3, harboring approximately 100 copies/µl of phytoplasma (Sakuanrungsirikul *et al.*, 2013). The sugarcane setts were soaked with a bacterial suspension at the concentration of 10^8 CFU ml^{-1} for 1 hour and the evaluation of antagonist bacteria efficiency for control SCWL disease was performed.

In the field sugarcane seedlings were transplanted at the farmer's field in Pakho, Kumphawapi, Udon Thani, Thailand. The sugarcane was sprayed with *B. subtilis* No. 15 with a concentration of 10^8 CFU ml^{-1} once a month. The data collected on the growth and brix value were recorded. Statistical analysis was performed comparing with no treated controls.

Results

In the laboratory, antagonistic bacteria were isolated from soil samples collected from sugarcane fields in Khon Kaen, Thailand. Among the isolates, two showed the highest IAA production after seven days of culture *Streptomyces* spp. No. 8 and *B. subtilis* No. 15. These isolates were used as soil drenches in sugarcane infected with phytoplasmas below critical threshold. Treated plants showed statistically significant improvements in plant height, root fresh weight, and brix levels as compared to untreated controls. A qPCR analysis revealed a substantial reduction in phytoplasma content in treated plants from 100 copies/µl to 0.5 – 10 copies/µl. In the field *B. subtilis* No. 15 was applied to SCWL infected sugarcane with phytoplasma concentration below critical level using cell suspensions of the antagonist bacteria by soaking and spraying. The plants showed increased survival rates to 100%, increase of stalks per plant by 55%, height by 28%, and brix levels by 11% as compared to non-treated controls. Analysis of the phytoplasma concentration using qPCR revealed a significant reduction of the pathogen titre from 23,800 copies/µl to 174 copies/µl.

Discussion

The report by Sakuanrungsirikul *et al.* (2013) stated that sugarcane infected with SCWL disease at a pathogen level of approximately 100 copies/µl indicates the level where the disease can induce symptoms of white leaf. The effective antagonistic bacteria used to strengthen sugarcane with SCWL disease show that an initial pathogen level of about 100 copies/µl indicates a level of surveillance at which planting is possible but requires good field management. This study used selected bacterial treatments by soaking and spraying to reduce the pathogen concentration to a level acceptable for propagation in the field. Management approaches for SCWL disease, such as increasing the use of healthy cuttings, pruning SCWL-infected plants, and enhancing irrigation and biofertilizer application in sugarcane fields, showed significant effects on increasing yield and quality, and in addition a reduction of the incidence of SCWL disease, however they did not completely eliminate the phytoplasmas (Nguyen *et al.*, 2023).

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Citrus crops

Mixed infection of phytoplasmas, "huanglongbing" and citrus tristeza virus in major citrus species at New Delhi, India

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Abstract

A study was conducted in Delhi to identify mixed infections in important citrus cultivars using PCR and RT-PCR assays using specific primers for phytoplasmas, '*Candidatus* Liberibacter' species (HLB), and citrus tristeza virus (CTV) to test pomelo, kinnow mandarin, and kagzi kalan lemon cultivars from different fields. Citrus samples from symptomatic leaf tissues were examined and 16S rRNA, *omp*, and *coat protein* (CTV) genes verified by sequence comparisons, confirmed the presence of mixed infection with amplification of the expected length fragments of about 1.25 kb, 809 bp and 672 bp, respectively. Additionally, the investigation verified that 16SrI-B subgroup phytoplasmas were present in pomelo, while 16SrII-C subgroup was detected in kinnow mandarin and 16SrII-D subgroup in kagzi kalan lemon. These results were confirmed using virtual restriction fragment length polymorphism. The presence of '*Ca.* L. asiaticus' identified by phylogenetic analysis was detected in all tested samples. Genetic diversity was also discovered in the CTV isolates that were separated into three genogroups based on the *coat protein* gene.

Keywords: citrus greening, HLB, CTV, molecular detection, phylogenetic analysis

Introduction

Citrus, a major fruit tree crop in over 130 countries, is facing decline due to biotic stresses from viruses and mollicutes, as well as inadequate management practices. Citrus trees are susceptible to various pathogens, including citrus exocortis viroid, citrus tatter leaf virus, citrus psorosis virus, citrus ringspot virus, and citrus yellow vein clearing virus. Recent reports of citrus leaf blotch virus and citrus vein enation virus in Korea highlight the challenges faced by growers (Bester *et al*., 2021; Mitra *et al*., 2022; Kwak and Kil, 2022). Transmission of citrus viruses involves vectors like aphids, nematodes, grafting, and propagation (Kim *et al*., 2024). In India and abroad, phytoplasmas, "huanglongbing" (HLB), and citrus tristeza virus (CTV) are prevalent pathogens infecting citrus orchards and causing significant yield losses. This study aims to investigate the mixed infection of phytoplasmas, '*Candidatus* Liberibacter' species associated with HLB, and virus in citrus decline disease in the Delhi region (Ghosh *et al*., 2019; Kokane *et al*., 2020; Noorizadeh *et al*., 2022; Mitra *et al*., 2022).

Materials and Methods

A 2021-22 survey involved extracting symptomatic and asymptomatic leaf samples collected from pomelo, kinnow, and kagzi kalan lemons from IARI research fields in New Delhi, India. DNA/RNA were extracted by kits and PCR and RT-PCR assays for bacteria and virus detection, respectively were performed using specific primers for the 16S rRNA gene (R16mF2/ R16mR2 followed by nested primers R16F2n/ R16R2) for phytoplasmas (Gundersen and Lee, 1996), *omp* gene (omp1218f/omp2026r) for '*Ca.* Liberibacter' species (Deng *et al*., 2008), CTV-*cp* gene (BC24/BC25) for virus (Korkmaz *et al*., 2008). The PCR products were purified and cloned, then outsourced for sequencing. Contigs were compared in BLASTn (https://www.ncbi.nlm.nih.gov/) and submitted to GenBank database. Sequence assembly and construction of phylogenetic trees were performed. Partial sequences from the 16S rRNA gene were subjected to *in silico* RFLP analysis with the *i*PhyClassifier tool (Zhao *et al*., 2009).

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Results

The symptomatic citrus trees showed yellowing, witches' broom, and chlorotic streaks. The amplification of the genes was achieved in all samples, but not in asymptomatic samples. Phytoplasma strains pomelo Delhi (PDP-3,-4), kinnow mandarin Delhi (KNDP-1,-2) and kagzi kalan lemon Delhi (KKLDP-2,-4) showed 100% sequence identity among themselves and 99.98-99-81% with strain of *Eucalyptus* little leaf and leaf yellowing, chickpea phyllody and sesame phyllody (GenBank accession numbers PQ816569, PQ816570, PQ642030, PQ642031, PQ642032, PQ642033, MZ678361, MT420257 and KY420071). The virtual RFLP analysis of 16S rRNA gene of PDP strains showed the presence of phytoplasmas in 16SrI-B subgroup. However, KNDP and KKLDB strains showed patterns referable to 16SrII-C and 16SrII-D subgroups respectively (data not shown). Moreover, samples PDB-3, PDB-4, KNDB-1, KNDB-2, KKLDB-1 and KKLDB-2 (GenBank Accession numbers PQ824583, PQ824584, PQ655500, PQ655501, PQ824585, and PQ824586) showed 99.81-99.55% nucleotide sequence identity with '*Ca.* L. asiaticus' strains (Figure 1) and 100-99.76% among themselves. The study reveals intra-farm divergences in CTV isolates suggesting that genetic variability may result from recombination events between divergent CTV sequences despite previous phylogenetic analysis indicate genogroup incongruity (Sharma *et al.*, 2012).

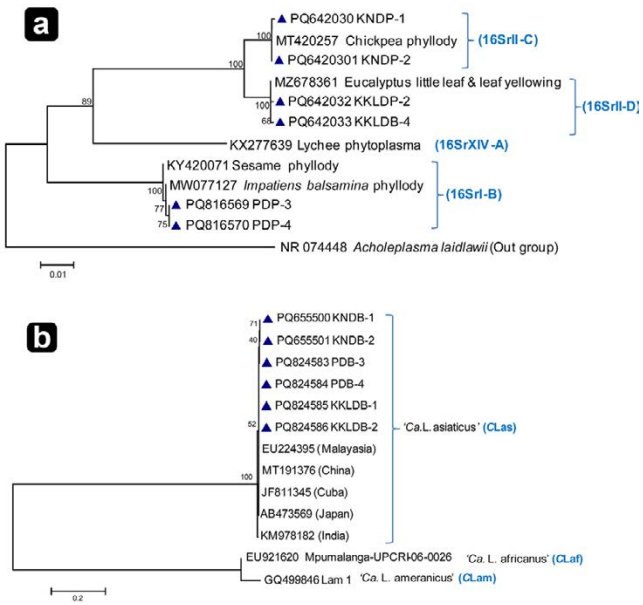


Figure 1. Phylogeny construction using neighbour joining method with software MEGA 7 (Kumar *et al.,* 2016) with 1,000 bootstrap replications. Strains of both phytoplasmas (a) and HLB (b) were compared with selected strains. Sequences of *Acholeplasma laidlawii* for 16S rRNA gene, '*Ca.* L. africanus' (CLaf) and '*Ca.* L. americanus' (CLam) for *omp* gene were used as outgroups to root the respective trees.

Discussion

Citrus decline disease, associated with the presence of viruses, phytoplasmas, and HLB, affects citrus varieties in India, negatively impacting older trees and hindering profitable business. Further research is needed to develop duplex or multiplex PCR assays and produce healthy materials for grafting (Ghosh *et al.*, 2019; Kokane *et al.*, 2020; Mitra *et al.*, 2022).

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Citrus crops

First report of '*Candidatus* Phytoplasma aurantifolia' associated with witches' broom disease of grapefruit in Oman

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Abstract

This study investigates the presence of '*Candidatus* Phytoplasma aurantifolia' in grapefruit (*Citrus paradisi*) affected by witches' broom disease in the Al Sharqia Governorate of Oman during 2023. The infected tree shows symptoms typical of phytoplasma infection, including leaf and stem proliferation, clustering, stunted growth, and yellowing. DNA extracted from symptomatic samples was analyzed using PCR targeting the SAP11 and *tuf* genes, alongside universal phytoplasma markers targeting the 16S, intergenic 16S–23S, and a small part of the 23S rDNA region. The BLAST analysis of the *tuf*, SAP11, and 16S rRNA genes sequences and phylogenic tree of 16S rRNA genes revealed 100% identity with strains of '*Ca.* P. aurantifolia' from Oman. This represents the first report of this phytoplasma affecting grapefruit in Oman, expanding the pathogen's know host range.

Keywords: citrus, SAP11, WBDL, grapefruit, phytoplasmas

Introduction

Witches' broom disease of lime (WBDL), associated with '*Candidatus* Phytoplasma aurantifolia', belonging to the subgroup 16SrII-B is a major citrus disease in Oman and surrounding areas like Iran and UAE. This pathogen primarily affects acid lime (*Citrus aurantifolia*), the fourth cultivated crop in Oman. The disease is characterized by abnormal proliferation of shoots, yellowing of leaves, shortened internodes, and eventual dieback of branches, which can culminate in tree death (Figure 1). Oman was converted by this disease from main exporter in the region to be an importer. Now local production of Omani acid lime covers only the 30% of the total local consumption. The decline in production reaches the 25% from its peak in the nineties, the import value for the lime only, cost 81.62 million USD in 2023. Historically, WBDL has been detected in lime trees, and grapefruit (*Citrus paradisi*) is not previously documented as a host of this phytoplasma in Oman. This paper reports the presence of symptomatic grapefruit trees in the Al Sharqia Governorate, Oman, and the molecular evidence of phytoplasma presence.

Materials and Methods

Samples ware collected from symptomatic branches of grapefruit symptomatic trees in Al Sharqia governorate during 2023. Symptoms observed included leaf and stem proliferation, clustering, stunted growth, and yellowing. The collected samples were conserved in plastic, sealed and sent to Ghadafan Agriculture Station laboratory for analysis. Total DNA was extracted from plant tissues using a standard CTAB protocol. PCR amplification was performed using multiple primer sets targeting SAP11, *tuf*, and 16S rRNA genes. Amplified products were sequenced, and BLAST analysis was conducted to confirm the pathogen's identity.



Figure 1. Typical symptom of WBDL in grapefruit in the field.

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Results

Symptoms typical of phytoplasma infection were observed in grapefruit trees. PCR amplification and sequencing of 16S rRNA gene confirmed the presence of '*Ca.* P. aurantifolia' in all symptomatic grapefruit samples. The amplified sequences of *tuf*, SAP11, and 16S rRNA *loci* were 100% identical to the sequences of phytoplasmas deposited in GenBank under accession numbers MG774493, KX358622, EF186828 from previous WBDL studies. The 16S rRNA phylogenetic tree cluster grapefruit strain with deposited strains from Omani lime witches' broom phytoplasmas (Figure 2).

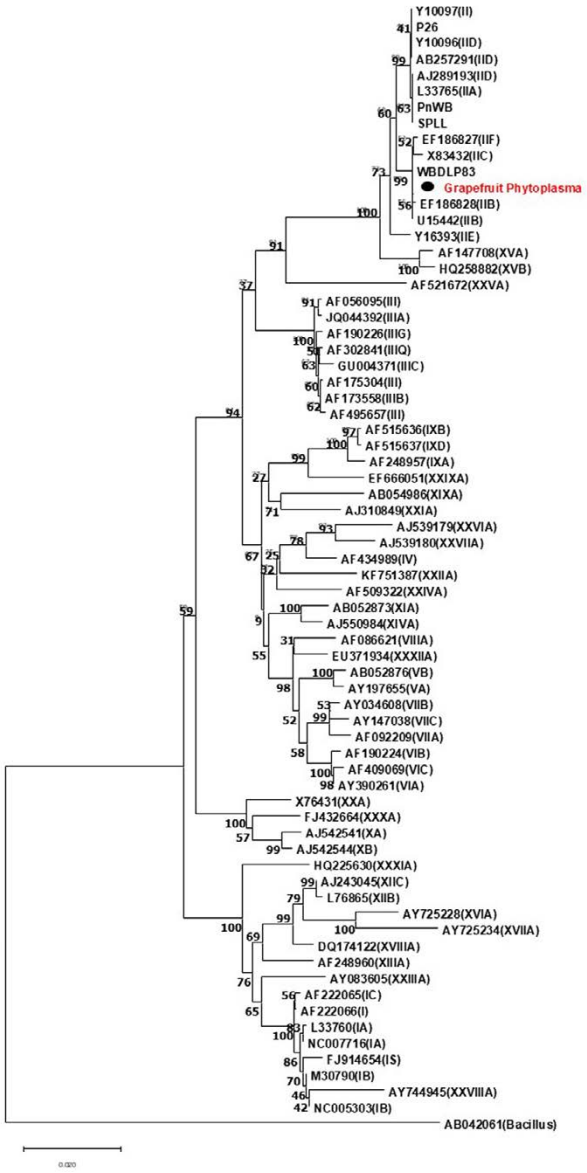


Figure 2. Phylogenetic tree constructed by neighbor-joining (NJ) method analysis of 16S rRNA gene sequences from described grapefruit phytoplasmas.

Discussion

The presence of '*Ca.* P. aurantifolia' in grapefruit highlight the pathogen's capability to infect a citrus species other than lime with the same typical symptoms (Garnier *et al.*, 1991). This host expansion aligns with previous findings suggesting the phytoplasma's adaptability to new hosts within citrus orchards (Al-Subhi *et al.*, 2019).

The 100% genetic identity of the *tuf*, SAP11, and 16S rRNA genes with previously reported Omani strains underscores the conserved nature of the population of this phytoplasma. Such genetic stability suggests that '*Ca.* P. aurantifolia' is highly adapted to the local citrus environment in Oman (Al-Abadi *et al.*, 2016; Al-Ghaithi *et al.*, 2018; Al-Subhi *et al.*, 2019). The 16S rRNA gene phylogenetic analysis clustered grapefruit phytoplasma strains in the same subclade with WBDL phytoplasma samples belonging to 16SrII-B subgroup, '*Ca.* P. aurantifolia' (Zreik *et al.*, 1995). This is the first report of a phytoplasma from the 16SrII-B subgroup naturally infecting grapefruit trees in Oman.

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Citrus crops

CRISPR/Cas-based detection of *Spiroplasma citri* for citrus stubborn disease management

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Abstract

Spiroplasma citri, the causal agent of citrus stubborn disease, remains a challenging plant pathogen to control, causing substantial economic loss in global citrus production. Rapid and efficient detection assays are essential for timely and effective mitigation interventions. This research focused on developing a CRISPR/Cas-based detection system using the DETECTR method to create a point-of-care assay for *S. citri*. Preliminary results demonstrated the successful design of RPA primers, crRNAs, and a protocol for detecting *S. citri* at a concentration as low as 1 fM in two hours. Further optimization will be performed to enhance field applicability, including developing lateral flow strips and enabling direct, equipment-free, detection in the field.

Keywords: DETECTR, field-deployable, lateral flow strips

Introduction

Spiroplasmas are phloem-limited, cell wall-free bacteria that colonize arthropods and plants, acting as causal disease agents under certain conditions. One example is *Spiroplasma citri*, which infects citrus crops, causing citrus stubborn disease (CSD) (Bové *et al*., 2003). The disease symptoms include irregular, small, and green fruit, as well as reduced leaf size, bunching of branches, dieback and leaf drop. As a result, fruit production can be reduced up to 52%, making CSD a prevalent challenge in some citrus-growing regions. Controlling CSD remains difficult due to several biological and ecological factors. Over 60 plant species can act as reservoirs for *S. citri*, and latent infection periods can last up to a year before symptoms are visible. Moreover, bacterial titers fluctuate with the plant over time and across different seasons, further complicating both detection and management. Presently, *S. citri* detection relies primarily on PCR-based assays (Yokomi *et al*., 2008; Wang *et al*., 2015). While these methods are highly sensitive and specific, they require delivering samples to testing laboratories. This paradigm requires considerable time and forms technological barriers to resource-limited citrus production areas. These limitations highlight the need for alternative, thermocycler-free, point-of-care detection methods to better protect the global multibillion-dollar citrus bioeconomy. In recent years, CRISPR/Cas technologies have been adapted into nucleic acid detection systems for pathogens such as SARS-CoV-2 and phytoplasmas (Chen *et al*., 2018). For DNA detection, these methods operate by mixing the sample with the site-specific nuclease Cas12a, its sample-specific guiding crRNA, and an oligo DNA reporter with a quenched signal molecule. Upon recognition and cleavage of the target DNA by the Cas12a/crRNA complex, the nuclease gains the ability to non-specifically cleave oligonucleotide reporters, releasing the quenched signal. This reaction enables detection via hand held spectrophotometric devices or visual inspection using UV light. For even simpler field accessibility, lateral flow strips can be used, where biotin-labelled oligos trigger signal generation through streptavidin-antibody conjugation, eliminating the need for specialized equipment. Thus, this project endeavoured to develop similar CRISPR/Cas-based detection assays for *S. citri*.

Materials and Methods

Spiralin sequences of *S. citri* were obtained from NCBI and aligned via Clustal Omega. PCR was performed using the Takara Hot Start Long Amp kit with modified extension temperature at 68°C for 5 minutes per cycle. RPA was carried out using the RPA Liquid Basic kit from TwistDx following the manufacturer's instructions. For the CRISPR-Cas12a detection system, key reagents included the LbaCas12a enzyme kit, crRNAs and FAM-reporter oligos, all obtained from IDT (Iowa, USA). The final reaction mixture contained 50 nM of each component in a 1x concentration of NEBuffer 2.1. The digest assay was prepared using 1.8 µg of DNA mixed with the CRISPR-Cas12a detection system without any FAM-reporter. The reaction was incubated at 37°C for 1 hour, then 1 µl of NEB proteinase K was added and incubated for 10 minutes.

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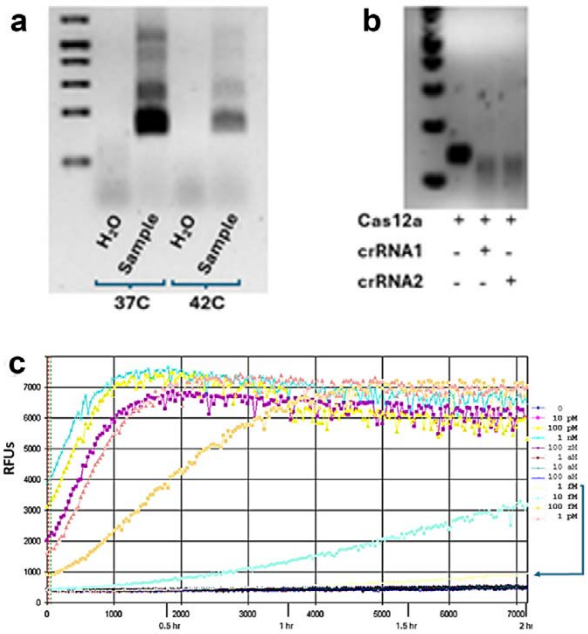


Figure 1. Optimization of RPA, crRNAs, and DETECTR assay: (a) RPA efficiency is higher at 37°C compared to 42°C using ScF2/ScR2 primers; (b) Cas12a digestion shows similar effectiveness with both crRNAs at 1 hour; (c) fluorescence over time for each concentration over 2 hours. The Y-axis represents relative fluorescent units (RFUs).

The resulting products were analysed via electrophoresis in a 3% agarose gel. For the DETECTR assay, 2 µl of the RPA product was added to a final reaction volume of 20 µl. The mixture was transferred to black 384-well plates and analysed using a FLUOstar+ Galaxy fluorescent plate reader. The reader was set with a gain of 130 and maintained at 37˚C. Fluorescent readings were taken every 2 minutes for a total of 2 hours, and relative florescent units were recorded. Three replicates of each sample were performed and averaged to ensure accuracy and reproducibility.

Results

The spiralin sequences from *S. citri* and other *Spiroplasma* species were aligned to determine species-specific polymorphisms, which were used to design both RPA primers and crRNAs. Initial PCR testing of the RPA primers indicated the ScF2/ScR2 primer combination produced the most specific and abundant amplification. The column-purified PCR product was used to evaluate the RPA reaction with ScF2/ScR2 primers. Results showed that amplification at 37˚C produced at least twice as much product compared to 42˚C (Figure 1A). The purified PCR product was also used to assess the efficiency of two crRNAs for Cas12a digestion. After 1 hour of incubation, both crRNAs demonstrated similar digestion efficiency, indicating comparable performance (Figure 1B). The PCR product was serially diluted from 1 nM down to 100 aM, with water serving as negative control. Each dilution was subjected to RPA amplification for 20 minutes, followed by the Cas12a DETECTR assay. Results showed that the 1 nM, 100 pM, 10 pM, and 1 pM samples reached saturation in relative fluorescence unit within 30 minutes. The 100 fM sample achieved saturation by 60 minutes. These samples exceeded the detection threshold at the start of the assay. In contrast, 10 fM and 1 fM samples required 20 and 80 minutes, respectively to cross the detection threshold. Samples at concentration < 100 aM and negative control were not detectable throughout 2 hours (Figure 1C).

Discussion

The resulting fluorescence-time graph indicates a promising assay for the detection of *S. citri*. The lowest detectable concentration, 1 fM, could yield positive results in less than two hours. This detection timeline is compared to other DETECTR-based assays for phytoplasmas and other pathogens (Wheatley *et al*., 2021, 2022), suggesting that this approach holds strong potential as an effective *S. citri* detection method. Future work will focus on increasing the assay's sensitivity, with a target detection limit approaching 1 aM. This will be achieved by optimizing RPA amplification, which includes increasing amplification time and testing alternative or more specific RPA primers. Additionally, adjustments to the concentration of fluorophore, and Cas12a/crRNA may improve sensitivity and time required to reach detectable fluorescence thresholds. Upon optimizations, the assay will be applied to crude plant/vector DNA extracts to test sensitivity in the presence of high genomic DNA and potential inhibitors of the RPA or Cas12a processes. This would demonstrate *in situ* viability of the technology. Lastly, lateral flow strips will be developed and tested using both purified test DNA and crude extracts to establish the assay's efficacy as a portable, equipment-free detection method.

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Palm trees

Current and future molecular diagnostics of palms phytoplasmas

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Abstract

Initial molecular diagnostic tools involved PCR amplification, often requiring nested PCR due to the low titre of phytoplasmas in infected palm and other species host tissues. This was followed by advanced techniques such as quantitative PCR assays and digital PCR, which offer enhanced sensitivity and accuracy. The development of isothermal diagnostic platforms, including the loop-mediated isothermal amplification assay, has further expanded the toolkit for phytoplasma detection in diverse hosts. The integration of CRISPR-based diagnostics holds immense potential for rapid, specific, and sensitive detection of phytoplasmas. CRISPR platforms, with their programmability and precision, could enable point-of-care detection and multiplexed assays for simultaneous identification of multiple pathogens.

Keywords: LAMP, CRISPR, ddPCR, root wilt, arecanut yellow leaf

Introduction

Phytoplasma diseases in palms were first reported as early as the 18th century, with symptoms ranging from mild to highly lethal, often resulting in palm mortality. These pathogens affect at least 39 palm species, with coconut and date palms being the most susceptible. Coconut palms alone are afflicted by over 11 distinct diseases, among which lethal yellowing-type diseases are prevalent and highly transmissible. Phytoplasma-related diseases in date palms include white tip die back in North Africa (Cronjé *et al*., 2000), yellowing in Kuwait (Al-Awadhi *et al*., 2002), lethal decline in Texas (Harrison *et al*., 2002), and "al-wijam" disease in Saudi Arabia (Alhudaib *et al*., 2007). These diseases pose significant threats to palm cultivation, underscoring the need for early detection and effective management strategies.

Phytoplasmas are obligate bacteria classified under the class *Mollicutes*, characterized by a pleomorphic shape due to the absence of cell wall. These organisms have a small genome size. A distinctive feature of the phytoplasma genome is its high repetitive DNA content, comprising 14–23% of the genome, organized into potential mobile units (PMUs). These PMUs, unique to the phytoplasma genome, include *tra5* insertion sequences (ISs), phage integrase genes, and genes involved in replication and transcription. They are often flanked by inverted repeats, further underscoring their role in genome mobility and adaptation. Characterization of the pathogen is possible through sequencing and analyzing the highly conserved genomic regions of 16S ribosomal DNA and other conserved gene sequences (*tuf*, 23S rRNA genes and the 16S/23S rRNA intergenic spacer). Molecular diagnostic techniques like PCR, quantitative PCR and RFLP analysis are currently used for diagnosis. The loop mediated isothermal amplification (LAMP) assay has been applied for the detection of phytoplasma from several hosts (Nair *et al*., 2016b).

Molecular Diagnostics in Palms

Molecular detection techniques for coconut root wilt disease and arecanut yellow disease have been developed using nested PCR with specifically designed primers (Manimekalai *et al*., 2010a; 2010b). Semi-nested primers 1F7-7R3/1F7-7R2 efficiently amplified a 490 bp region of the phytoplasma 16S rDNA, enabling the effective detection of phytoplasmas in yellow leaf-diseased arecanut samples. TaqMan qPCR, utilizing FAM as fluorescence emitter and TAMRA as

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quencher, has also proven efficient for detecting palm phytoplasmas (Nair *et al*., 2015, 2016a). Digital PCR enables sensitive and absolute quantification of nucleic acids by partitioning DNA samples into numerous single, parallel PCR reactions (Morcia *et al*., 2020). Additionally, LAMP has become a popular tool for detecting phytoplasmas. LAMP can generate up to 10^9 copies of a target sequence within an hour, offering high specificity and speed. Positive LAMP reactions can be identified through agarose gel electrophoresis, displaying a ladder-like pattern due to the varying numbers of inverted repeats in LAMP products (Figure 1). Alternatively, colorimetric detection can be achieved by adding the metal indicator hydroxy naphthol blue (HNB) (Figure 2). Other isothermal amplification platforms, such as recombinase polymerase amplification (RPA) and helicase-dependent amplification (HDA), also serve as methods for pathogen detection.

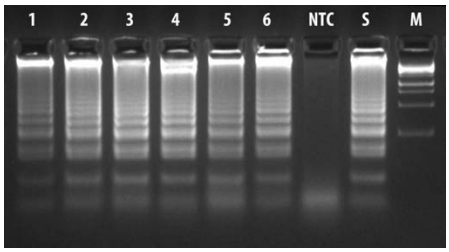


Figure 1. Detection of LAMP amplicons by agarose gel electrophoresis.

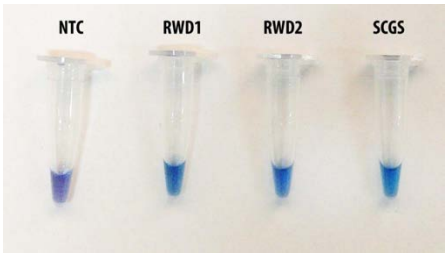


Figure 2. Colorimetric analysis of LAMP products using HNB dye. The positive samples show blue color and the NTC remain violet. RWD1, RWD2 – root wilt diseased coconut samples, NTC – no template control (Nair *et al*., 2016b).

The advent of next-generation sequencing (NGS) technologies has revolutionized plant pathogen detection. High-throughput next generation sequencing and single-molecule long-read third-generation sequencing have enabled precise identification of pathogens and simultaneous detection of multiple pathogens. Single- molecule sequencing technologies are paving the way for the development of portable, on-site pathogen detection devices (Nezhad, 2014). Zikeli *et al*. (2018) demonstrated the application of NGS using the Illumina MiSeq platform for the simultaneous detection of viruses, viroids, and phytoplasmas in grapevine and fruit tree samples. Similarly, Chalupowicz *et al*. (2018) reported the use of the Oxford nanopore single-molecule sequencing platform for detecting and identifying various plant pathogens, including phytoplasmas. The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) nuclease-based nucleic acid detection has been used as a novel molecular diagnostics technology. CRISPR-Cas system has been developed for the detection of '*Candidatus* Phytoplasma trifolii' (Wheatley *et al*., 2022).

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Palm trees

Search for coconut lethal yellowing alternate hosts among coconut associated weeds and cultivated crops in Mozambique

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Abstract

The worldwide coconut phytoplasma disease management is achieved by the early detection followed by immediate eradication of the infected plants along with replanting with resistant varieties if available. Knowledge of alternate hosts is crucial for effective disease management based in eradication of the phytoplasma host plants. This study aims to search potential coconut lethal yellowing alternate hosts among herbaceous species growing or cultivated within the coconut farms. The study was conducted in Zambezia province of Mozambique, the region where coconut lethal yellowing is endemic. Potential alternate hosts were screened by PCR. *Phragmites australis*, *Psidium guajava*, *Smilax kraussiana* and *Tephrosia purpurea* were PCR positive with at least two different primers. This a is a great contribution toward sustainable integrated coconut lethal yellowing management.

Keywords: phytoplasma, epidemiology, PCR, sequencing

Introduction

Coconut production represents an important source of income for most rural household, and coconut lethal yellowing disease outbreaks continue to spread in affected areas compromising the income and livelihood of the people (Bila *et al*., 2015a), which include the current and potential investors in the sector. Currently, disease management is based on early detection and immediate destruction of infected plants along with replanting using tolerant or better, resistant varieties. Knowledge of alternate hosts is crucial for an effective disease management. In Côte d'Ivoire, the coconut lethal yellowing phytoplasma was detected in alternate hosts of Poaceae, Verbenaceae, Plantaginaceae, Phyllanthaceae, and Cyperaceae (Arocha-Rosete *et al*., 2016). Bila *et al*. (2015b) identified *Elaeis guineensis* and *Borassus aethiopum* palm species as coconut lethal yellowing alternate hosts in Mozambique. This study aims to continue the search for lethal yellowing phytoplasma presence among herbaceous species growing or cultivated within coconut farms.

Materials and Methods

Samples were collected in the hot and rainy season in the districts of Namacurra, Nicoadala and Quelimane from Zambézia province, the most affected site in Mozambique (Figure 1).



Figure 1. Coconut farm with infected palms (left); Infected coconut tree used as central point for potential alternate hosts samples collection (right).

The collected plants were identified at species or genus level in the field with assistance of botanist experts using their expertise and field manual. The plants that could not be identified in the field were taken to the herbarium laboratory for identification. Samples were collected in the vicinity of coconut lethal yellowing symptomatic plants, within 10 meters. placed in labeled plastic bags and transported to the laboratory in a cool box for testing. DNA extraction was performed using the protocol developed by Harrison *et al*. (2002), with minor modifications (Bila *et al*., 2015b). Before PCR, all DNA from the same species was grouped in a single tube, thus forming 83 composite samples out of the 200 samples collected. A PCR was performed to detect the specific DNA fragment of the phytoplasma, using the following primer pairs, Phyto14 F and Phyto14 R, G183/ASKR and RhodeF/

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RhodeR (Mazivele *et al.*, 2018), designed to target the lethal yellowing phytoplasma from Mozambique, West Africa and Est Africa, respectively.

Results

The PCR results using Phyto14F/R primers pair (Figure 2) revealed that the phytoplasma DNA was amplified in eight composite samples namely *Phragmites australis*, *Prnnisetum purpureum*, *Psidium guajava*, *Saccharum officinarum*, *Smilax kraussiana*, *Stenotaphrum secundatum*, *Tephrosia purpurea* and *Waltheria indica*, from five botanical families specifically Poaceae, *Myrtaceae*, *Smilacaceae* Fabaceae and Malvaceae. *Phragmites australis* and *Psidium guajava* were also amplified with primer pair RhodeF/R designed to ampilify Tanzanian lethal decline phytoplasmas, while *P. australis*, *S. kraussiana* and *T. purpurea* were co-amplified with primer pair G813/AKSR designed to amplify '*Candidatus* Phytoplasma palmicola' (Figure 3).

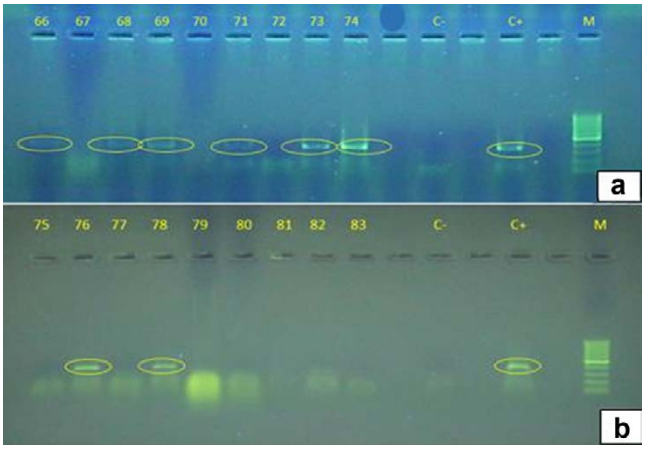


Figure 2. PCR result with Phyto14F/R primer pair, numbers are samples, C- is a negative control (water), C+ is the positive control, and M is the 1 kb marker. PCR results with RhodeF/R (a) and with G813/AKSR primer pairs (b).

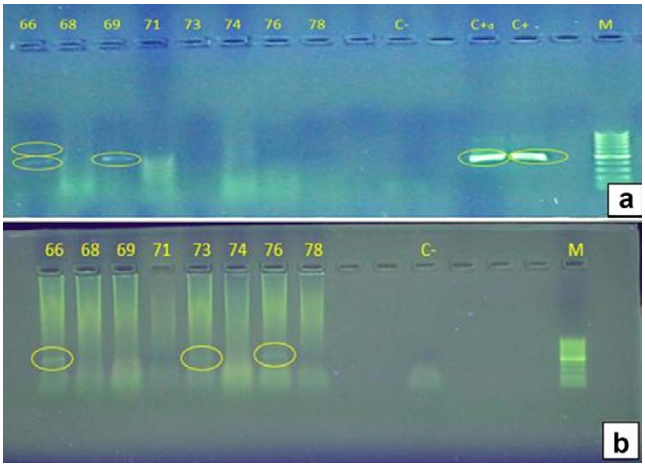


Figure 3. PCR result with RhodeF/R primes, numbers are samples, C- is a negative control (water), C+ is the positive control, and M is the 1 kb marker. PCR results with RhodeF/R (a) and with G813/AKSR primer pairs (b).

Discussion

Among the families mentioned above as being potential hosts in the present work, Poaceae stands out from the others for presenting a greater number of alternative hosts (4), which is equivalent to 50% of the potential host species identified in the present study. According to Arocha and Jones (2010), the Poaceae family has the largest reported number of host plant species for phytoplasmas worldwide. All families identified as potential hosts in the present work show the greatest abundance in the number of species collected per family, which suggests that their abundance in coconut production fields increase the probability of being potential alternative hosts. *P. purpureum*, *S. officinarum*, *S. secundatum* and *W. indica* were only amplified by Phyto14F/R, while *P. australis* samples were positive for the three primers pairs used. The fact that the potential alternate hosts were selectively detected by three different primers confirms the high diversity of phytoplasmas potentially associated with coconut lethal yellowing in Mozambique. The results from this study will be confirmed by sequencing the PCR products.

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Phytopathogenic Mollicutes
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Palm trees

Detection, epidemiology and management of phytoplasma diseases of palms

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Abstract

Phytoplasma diseases pose a significant threat to the palms worldwide, affecting over 50 palm species across the continents. The rise in reports of palm phytoplasma diseases from various regions and the expanding host range of existing phytoplasmas affecting new palm species is a major concern. Significant advancements have been made in the molecular characterization and diagnostics of palm phytoplasmas. There is an urgent need for multi-faceted strategies to protect farmers from the risks and contain the spread of the disease in new outbreak areas. The development of affordable, sensitive, and reliable on-farm diagnostic kits, identification of insect vectors, and breeding programs aimed at developing disease resistant varieties are essential to mitigate the risks for farmers.

Keywords: *Mollicutes*, Arecaceae, plant pathogen

Introduction

Palms, part of the Arecaceae family, are deeply woven into the culture, cuisine, and traditions of many rural communities, often serving as a vital income source for farmers. However, the growth and productivity of palms face challenges from both biotic and abiotic stresses. One of the significant threats is phytoplasma diseases, which have impacted palm cultivation for over a century, harming the economies of many countries. These diseases have been identified in more than 50 palm species across different continents. The rise in reports of palm phytoplasma diseases from various regions and the expanding host range of phytoplasmas is of major concern worldwide.

Current Scenario of Palm Phytoplasma Diseases

The phytoplasma diseases have been reported across a range of economically vital palm species. While some phytoplasma diseases are lethal and widely spread, others merely weaken palm health or are restricted to specific areas. Among these, the most infamous is lethal yellowing that impacted several countries in the Caribbean area (Harrison *et al*., 2002). Notable diseases in Africa include "kaincope" in Togo, "awka" wilt or bronze leaf wilt in Nigeria, "kribi" in Cameroon and Cape St. Paul Wilt in Ghana and Cote d'Ivoire. A lethal decline is reported from Tanzania, Mozambique, Equatorial Guinea and Kenya (Bertaccini *et al*., 2023). The "bogia" coconut syndrome emerging from Papua New Guinea poses an imminent risk to coconut farming in Oceania (Kelly *et al*., 2011).

In Asia, phytoplasma diseases of coconut palms have been reported in India, Sri Lanka, Indonesia and Malaysia. This includes root (wilt) disease, lethal wilt disease and "tatipaka" from India, "weligama" coconut leaf wilt from Sri Lanka, "kalimantan" wilt in Indonesia and coconut yellow decline in Malaysia (Rao *et al*., 1956; Solomon *et al*., 1983; Sitepu *et al*., 1988; Wijesekara *et al*., 2008; Nejat *et al*., 2009, Babu *et al*., 2021). Among these the first tree are non-lethal whereas the latter are fatal to palms. "Weligama" causes about 40 to 60% reduction in yield and the crop loss due to root (wilt) disease was estimated as 968 million nuts. Arecanut (*Areca catechu*), another important commercial plantation crop, is suffering for yellow leaf disease that is the most serious phytoplasma malady affecting its cultivation in Asia reported from India (Menon, 1963), China (Lin *et al*., 2023) and Sri Lanka. It considerably reduces production and quality of the betel nut.

Across the globe, nine phytoplasma ribosomal groups have been identified in palms: 16SrI, -II, -IV, -VII, -XI, -XII, -XIV, -XXII, and -XXXII. Among the cultivated species, the coconut palms are infected by the greatest number of these phytoplasmas, followed by date palm and oil palm. Specifically, coconut palms host several phytoplasmas such as '*Candidatus* Phytoplasma asteris' (16SrI), '*Ca*. P. palmae' (16SrIV-A, -B, -E and -D), '*Ca*. P. cocostanzaniae' (16SrIV-C), '*Ca*. P. fraxini' (16SrVII), '*Ca*. P. palmicola' (16SrXXII-A and -B), '*Ca*. P. oryzae' (16SrXI-A), '*Ca*. P. cynodontis' (16SrXIV-A), '*Ca*. P. malaysianum' (16SrXXXII-B) and '*Ca*. P. noviguineense' (Bertaccini *et al*., 2022). Several species of ornamental palms are reported as hosts of distinct groups of phytoplasmas. Most of the ornamental palms used for landscaping in America were found to be susceptible to a straiin of '*Ca*. P. palmae' belonging to the 16SrIV-D subgroup (Bahder *et al*., 2019).

Several hemipteran insects have been reported as putative vectors of palm phytoplasmas based on PCR results,

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but their vector status has not yet been established by transmission trials. The most appropriate method for determining the vector role of an insect species is the cage transmission test. For palm-phytoplasma pathosystems, this approach has several logistical constraints as it is very difficult to cage and maintain perennial woody palms for a long time. Hence, only very few insects have been identified as phytoplasma vectors.

The primary diagnosis of phytoplasma diseases of palms is through symptoms. The association of phytoplasmas with the disease is usually established by microscopy or molecular detection techniques, however, a lack of consistency in detection was observed when a large number of palm samples were analyzed (Hegde *et al*., 2016) very likely for the low titre of phytoplasma and their uneven distribution in palms.

The lack of curative treatments for phytoplasma diseases highlights the urgent need to develop multi-faceted strategies. The slow down approach, which involves surveillance, eradication, and containment, may be considered a prime strategy when a phytoplasma disease emerges in a new area. Measures must be taken to confine the disease to a limited area and create a buffer zone around the affected region to slow the spread. Periodic surveillance, diagnosis, and eradication of phytoplasma-infected palms must be continued systematically within the diseased zone and buffer zone. This helps to slow the disease's progression. Though the surveillance, eradication, and quarantine measures have helped to restrict the disease's spread, isolated cases have been reported in a few places quite far from the boundary. Similarly, the systematic implementation of surveillance and removal programs has successfully contained the "tatipaka" disease in India. Resistant varieties form the most viable and cost-effective option in managing phytoplasma diseases, especially for perennials like palms. Coconut breeders around the world have identified some palm varieties/accessions with varying degrees of resistance/tolerance to phytoplasma diseases. Green dwarf coconuts were found to be sources of resistance to phytoplasmas. In India also green dwarfs and their hybrids were found promising against the root wilt disease. Systematic evaluation trials have led to the release of Kalpasree (Chowghat Green Dwarf), Kalparaksha (Malayan Green Dwarf) Kalpasankara (Chowghat Green Dwarf x West Coast Tall) and Kalpavajra for cultivation in areas where the disease is prevalent (Thomas *et al*., 2012).

Conclusion

In recent years, significant strides have been made in the identification of phytoplasmas infecting palm trees. However, despite advances in molecular biology, the production of affordable, sensitive, and reliable on-farm diagnostic kits for farmers is still a distant dream. The precise identification of insect vectors, along with the characterization of the phytoplasma they carry and effective transmission trials, are necessary but remains a challenge. An intense focus on designing and validating sustainable, location-specific palm-based cropping systems is essential to mitigate risks for farmers.

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Palm trees

Comparative symptomatology of root (wilt) and mid whorl yellowing diseases of coconut in Kerala, India

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Abstract

Root (wilt) disease is a one and half century-old devastating disease of coconut producing flaccidity, yellowing of leaves and necrosis. In recent years another disease of coconut, is spreading in southern Kerala, the mid whorl yellowing disease. The primary symptoms of the latter are drastically different, like rapid mid whorl yellowing, abnormal shedding of nuts and flowers, drying of inflorescence and spadix and lethal decline similar to the lethal yellowing disease of coconut.

Keywords: phytoplasma, disease, lethal yellowing

Introduction

The coconut palm (*Cocos nucifera* L., family Arecaceae) is also known as "kalpavriksha" due to its multifaceted role in human livelihood. It is a tropical perennial plantation crop widely being cultivated in Kerala, India. The state ranks top in its area (767,800 ha) and second in the production (5,628.42 million nuts) in India. However, the productivity is much below (7,042 nuts/ha) the national average (9,018 nuts/ha) and also that of the neighbouring states (CDB, 2024). The primary reasons for the low productivity are the extreme climatic conditions, senescent palms, suboptimal input utilization, and the prevalence of various pests and diseases, notably phytoplasma-associated diseases, which resulted in substantial crop losses. Phytoplasmas are bacteria that colonise the phloem tissues of plants and are associated with yellowing-like diseases in palms, which are often lethal (Dery *et al*., 2005). The earliest observation of a lethal yellowing disease of coconut was in 1834 from the Grand Cayman island in the Caribbean (Eden-Green, 1995). Around 50 years later, a non-lethal, debilitating disease of coconut, known as root (wilt) (RWD), was noticed in India. This disease emerged in the southern Kerala and led to the loss of approximately 6 million palms. It is currently distributed in southern districts of Kerala and nearby districts of Tamil Nadu. The disease causes up to 35% reduction in yield with losses up to 80% in severe cases. The annual loss due to root (wilt) disease is estimated to be around 968 million nuts per year (Manimekalai *et al*., 2010). In recent years the occurrence of another yellowing similar to the lethal yellowing disease of coconut, characterised by rapid mid whorl yellowing, abnormal shedding of nuts and flowers, drying of inflorescence and spadix, was found in Thiruvananthapuram and nearby districts of Kerala. The affected palms often die within a short period (Anju, 2011; Paul, 2023). However, the etiology of the disease has not yet been resolved. The present study aims to identify the detailed symptomatology of mid whorl yellowing disease (MWYD) and to compare the symptoms of MWYD and RWD of coconut.

Materials and Method

A roving survey on the incidence of RWD and MWYD was carried out in the major coconut-growing areas of the Ernakulam, Kottayam, Alappuzha, and Thiruvananthapuram districts of Kerala, India. The symptoms of both diseases were recorded at all the surveyed locations. The survey covered around 100 palms from each area.

Results

The presence of RWD was observed in all the surveyed locations. Meanwhile, a severe incidence of MWYD was observed in Thiruvananthapuram, followed by random occurrence in Alappuzha and Kottayam districts. The RWD-infected palms predominantly produced abnormal inward

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bending of leaflets (flaccidity) in all the leaves in outer and middle whorls (Figure 1a). The spindle leaf of infected palms showed the presence of brown spots. In some cases, yellowing of leaves (especially the outer whorl) and marginal necrosis of leaflets was also observed (Figures 1b and 1c). The inflorescence necrosis and reduction in the number of mature, immature and tender nuts were observed in RWD-infected palms (Figure 1d). The roots collected from the diseased palms showed necrosis progressing upwards from the tip. Even though most of the palms showed a reduction in yield, certain well-managed coconut fields could tackle the disease effects by nutrient application. The superimposition of other fungal diseases like leaf rot disease was found to be frequently present in RWD-affected palms (Figures 1e and 1f).

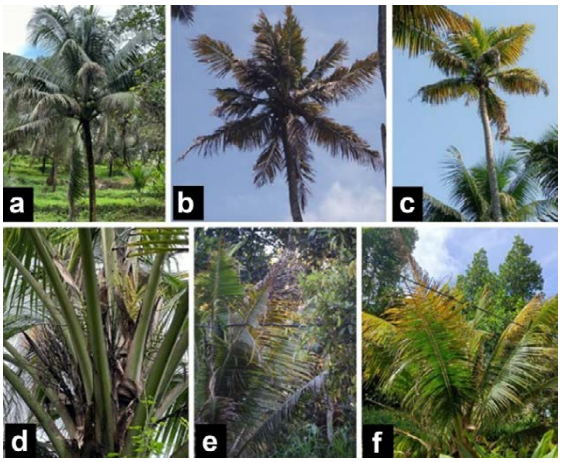


Figure 1. Flaccidity symptoms in outer and middle whorl leaves (a), yellowing and marginal necrosis of leaves (b and c), leaf rot infection in RWD infected palms (d and e), inflorescence necrosis (f).

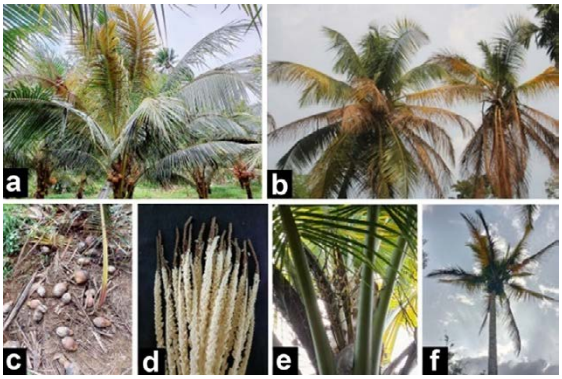


Figure 2. Yellowing of middle whorl (a and b), abnormal nut fall (c), necrosis of inflorescence in unopened spathe (d), necrosis of buttons (e), yellowing of crown.

Symptoms MWYD of coconut were observed in both tall and dwarf varieties (Figure 2a and 2b). The first visible symptom was the profuse shedding of young and tender coconuts (Figure 2c). But along with the nut fall, a progressive necrosis starting from the rachilla tips was also seen. The newly emerging inflorescence with a few or no female flowers showed necrosis of spikelets from the tip downwards either before or just after opening (Figure 2d). In the initial stages of disease progress, no foliar yellowing was observed in any of the whorls. As the disease progressed the yellowing initiated in the tip leaflets of the middle whorls and progressed towards the basal leaflets of a few or all the leaves in the middle whorl of the palm crown. The middle whorl yellowing can be recognised from far distance due to its intensified reddish-yellow colour. Gradually the palms became barren within a short span of four to eight months. The size of the crown was reduced and the growth of the palm was highly retarded. Severe cases resulted in the decay of the spear leaf, crown death, and production of bare trunk resembling telephone poles.

The symptoms of MWYD are drastically different from those of the RWD in coconut palms. In case of MWYD the characteristic symptoms of RWD like flaccidity, necrosis of root tissues and association of other fungal pathogens were absent. RWD is a debilitating disease, instead MWYD incidence will eventually leads to the death of palms. In comparison to RWD, the incidence and severity of mid whorl yellowing are much less, but this disease will be a big threat for coconut growers of the state due to its lethal nature.

Discussion

The MMYD depicts the characteristic symptoms of lethal yellowing disease (LYD) like abnormal nut fall, inflorescence necrosis, yellowing of leaves and lethal nature (Eden-Green, 1995). Even though certain facts like sporadic manifestation in individual palm trees rather than affecting the entire plantation uniformly, gradual shedding of mature and immature nuts unlike sudden shedding of all the nuts and characteristic middle and/or inner whorl yellowing of leaves make MWYD different from LYD. Considering all the facts, it can be speculated that the variations in MWYD symptoms compared RWD and LYD may be imparted by a variant strain of reported phytoplasmas.

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Palm trees

Phytoplasma diseases of oil palm

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Abstract

The phytoplasma-associated diseases significantly impact oil palm and related palm species, including spear rot disease (SRD), oil palm stunting, root (wilt) disease (RWD) of coconut, and yellow leaf disease (YLD) of arecanut. SRD is associated with phytoplasmas belonging to 16SrXI-B and 16SrIV-A groups. They are transmitted by insect vectors such as *Proutista moesta* and *Stephanitis typica* and by dodder (*Cassytha filiformis*). Cross-transmission studies have revealed a common phytoplasma strain linking SRD, RWD, and YLD, indicating a shared epidemiological pathway among the phytoplasmas associated with these diseases. While *P. moesta* is a common vector among the three palm species, *S. typica* plays a specific role between coconut and oil palm. Molecular studies and microscopy have confirmed the presence of phytoplasmas in infected palms and insect vectors. Lethal wilt, another phytoplasma-associated disease of oil palm in Colombia, associated with 16SrI-B subgroup phytoplasmas, underscores the global prevalence of these pathogens. There is a need for stringent phytosanitary measures and further research to prevent the spread of phytoplasma diseases in oil palms.

Keywords: spear rot disease, oil palm stunt, Malaysian oil palm phytoplasma, lethal wilt, lethal yellowing

Introduction

Oil palm (*Elaeis guineensis* Jacq.) is susceptible to a wide range of microorganisms, including fungi, bacteria, phytoplasmas, viruses, and viroids. Among these, phytoplasma-associated diseases include spear rot disease, oil palm stunt, lethal wilt, lethal yellowing, and Malaysian oil palm phytoplasma disease. Mycoplasma-like organisms (MLOs = phytoplasmas) were first reported in oil palm in the West New Britain area of Papua New Guinea. However, there is no evidence from this initial observation to suggest that MLO occurrence in Papua New Guinea poses significant economic or phytosanitary concerns to this species (Jones and Turner, 1979 unpublished; Turner, 1981).

Spear rot disease (SRD), first recorded in 1989 (Kochu Babu, 1989), has been observed in as many as 28,677 oil palms in Kerala and Karnataka, India. This disease is associated with phytoplasmas belonging to the 16SrXI-B and 16SrIV-A groups and is transmitted by *Proutista moesta* Westwood (Order: Homoptera, Family: Derbidae) and *Stephanitis typica* (Order: Heteroptera, Family: Tingidae) (Kochu Babu, 1993; Sumi, 2015). Characteristic symptoms include chlorosis of the youngest whorl of fronds and rotting of the spear in the early stages. As the disease progresses, chlorotic fronds develop necrosis, dry out, snap, and hang from the crown. Notably, the lower fronds remain green and unaffected at the onset, distinguishing SRD from other wilt diseases. Newly emerging fronds are often short, chlorotic, rudimentary, and prone to rotting (Kochu Babu, 1993). The interrelationship between phytoplasma associated with coconut root (wilt) disease (RWD), arecanut yellow leaf disease (YLD), and oil palm spear rot disease (SRD) has been established through transmission studies and molecular analyses (Kochu Babu, 1993; Sumi, 2015, Sumi *et al*., 2014, 2015). While these diseases are associated with the same phytoplasmas, the symptoms vary across palm species. The overall SRD incidence of 5.49% during the crop's economic life span suggests a slow-spreading and non-devastating disease with an erratic and patchy pattern of spread. Cross-transmission studies provide further evidence of phytoplasma interrelationships among palm species. Transmission from SRD-affected oil palm to coconut using *P. moesta* and dodder, resulted in flaccidity symptoms within 26–28 months (Anonymous, 1995). Transmission from YLD-affected arecanut to oil palm using dodder, lead to SRD symptoms within 8–11 months (Kochu Babu *et al*., 2004). Transmission from YLD-affected arecanut to coconut seedlings using *P. moesta*, resulted in symptoms within 15–16 months (Anonymous, 1995). Transmission from RWD-affected coconut to healthy oil palm using *P. moesta* and *S. typica*, produced SRD symptoms within 13–23 months (Anonymous, 1995; Nair and Kochu Babu, 2000).

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Further epidemiological studies on phytoplasma diseases in palm species across India are essential to deepen the understanding of their molecular interrelationships and epidemiology. Notably, the detection of phytoplasma DNA in embryos of RWD-affected coconut palms (Manimekalai *et al*., 2014) and lethal yellowing-affected palms (Oropeza *et al*., 2017) highlights the need for research on SRD-affected oil palm embryos. This is particularly critical as two oil palm seed gardens are located in SRD hotspots, and the transportation of oil palm sprouts to disease-free states warrants strict phytosanitary measures. Azadvar *et al*. (2012) reported the presence the 16SrI-B subgroup '*Candidatus* Phytoplasma asteris' in oil palm stunting disease in clonally propagated oil palms imported from Costa Rica and planted in Tadikalpudi, Eluru district, Andhra Pradesh. These palms exhibited significant reduction in frond length, resulting in a small crown and a stunted appearance. However, this disease has not been observed since then. In Mozambique, coconut lethal yellowing phytoplasma was reported also in oil palm. Additionally, the transmission of phytoplasma from root (wilt) disease (RWD)-affected coconut to healthy arecanut by *P. moesta* resulted in typical yellow leaf disease (YLD) symptoms (Anonymous, 1996). Evidence suggests that RWD, YLD, and spear rot disease (SRD) are likely associated with the same strain of phytoplasma, with cross-infections occurring in nature through the common vector *P. moesta* (Anonymous, 1995). *P. moesta* is a common vector facilitating phytoplasma transmission among the three palm species, while *S. typica* may act as a vector between coconut and oil palm, as it has not been reported in arecanut. Since *S. typica* breeds on coconut, migratory populations of this insect, after acquiring phytoplasmas from coconut, might transmit the pathogen to oil palm (Bila *et al*., 2015).

Lethal wilt, a major disease affecting oil palm in Colombia, has an incidence of up to 30% in several commercial plantations. Symptoms include leaf discoloration, with lower leaves turning brown and hanging downward like a collapsed umbrella. Álvarez and Claroz (2003) reported the presence of a 16SrI group phytoplasma. Further studies by Álvarez *et al*. (2014) revealed that the phytoplasma, identified as '*Ca*. P. asteris,' belonged to ribosomal subgroup 16SrI-B but was molecularly distinguishable from other aster yellows phytoplasma strains. Insects associated with the disease were identified from the families Cicadellidae and Membracidae.

Conclusion

The association of phytoplasmas with oil palm spear rot disease and related palm diseases like RWD and YLD highlights the complexity of these diseases epidemiology and interrelationship. Cross-transmission studies have established a shared phytoplasma strain among the three diseases, with *P. moesta* and *S. typica* identified as vectors. This emphasizes the critical role of vector management in disease control. The global occurrence of phytoplasma diseases, such as lethal wilt in Colombia, further demonstrates the importance of international phytosanitary measures. Detection of phytoplasma DNA in embryos of RWD-affected coconuts and lethal yellowing cases warrants further investigation into embryo transmission in SRD-affected oil palms. Given that oil palm seed gardens are located in hotspots of these diseases, the safe transportation of sprouts to disease-free regions is imperative. Future studies should focus on understanding the molecular diversity of phytoplasmas, vector-pathogen interactions, and effective disease management strategies to mitigate their impact on oil palm cultivation.

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Palm trees

Transmission studies on spear rot disease of oil palm

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Abstract

Spear rot disease (SRD) of oil palm has been consistently associated to phytoplasma presence in the phloem tissues of affected palms. This study aimed to investigate the etiological role of phytoplasmas in SRD and to explore the interrelationships between SRD, root wilt disease (RWD) in coconut and yellow leaf disease (YLD) in arecanut, all of which are known to be phytoplasma-associated diseases. Transmission studies were conducted using putative insect vectors, such as *Proutista moesta* and *Stephanitis typica*, and the vegetative vector, dodder (*Cassytha filiformis*), to assess the potential for cross-transmission between these diseases. The research demonstrated successful transmission of phytoplasmas from SRD-affected oil palms to healthy oil palm seedlings, healthy arecanut, and healthy coconut, as confirmed through symptom manifestation and light and electron microscopy. Additionally, transmission of RWD and YLD through these vectors was also observed, supporting the hypothesis of a shared phytoplasma association across these diseases. Phytoplasmas were identified in the salivary glands of *P. moesta* after prolonged acquisition and inoculation periods. These findings highlight the importance of insect vectors and dodder in facilitating the spread of phytoplasmas across different palm species. The results also underscore the need for cautious management of oil palm cultivation in regions affected by RWD and YLD, particularly with respect to the movement of oil palm sprouts from infected areas to other regions, to prevent further spread of these devastating diseases.

Keywords: SRD, RWD, YLD, *Proutista moesta*, *Stephanitis typica*, dodder

Introduction

Spear rot disease of oil palm (SRD) is a lethal and infectious malady (Kochu Babu, 1989, 1999) that was constantly associated with mycoplasma like organisms (MLOs= phytoplasmas) in phloem tissues. The primary inoculum of SRD was suspected to originate from root wilt disease of coconut (RWD) and yellow leaf disease of arecanut (YLD) (Kochu Babu and Ramachandran Nair, 1993, Kochu Babu *et al*., 2004). To determine the etiological role of phytoplasmas in SRD and their interrelation with RWD and YLD, transmission studies were conducted using putative vectors *Proutista moesta* Westwood (planthopper) and *Stephanitis typica* Dist. (tingid), as well as dodder (*Cassytha filiformis* L.). Transmission was confirmed through light microscopy (LM), electron microscopy (EM) and successful reproduction of symptoms.

Materials and Methods

Dodder laurel (*Cassytha filiformis* L., Lauraceae), raised from seeds and established on periwinkle (*Catharanthus roseus*), was used as a bridge vector. It was first connected to SRD-affected oil palms and then transferred to healthy oil palm seedlings maintained under insect-proof conditions. Freshly emerged adults of *P. moesta* from empty bunch refuge were kept enclosed in nylon net were released onto young leaves of SRD-affected palms for acquisition access period (AAP) followed by inoculation access period (IAP) on older leaves of the same palms (Kochu Babu and Ramachandran Nair, 2000). After a combined AAP+IAP period of 35 days, the vectors were recaptured and introduced to 8-month-old healthy oil palm seedlings. Similarly, *S. typica* underwent an AAP+IAP period of 17 days before being recaptured and released onto healthy oil palm seedlings. Transmission of the disease was assessed through LM, EM, and observation of symptom manifestation in the host plants.

Results and Discussion

Three out of the ten oil palm seedlings that were connected to SRD-infected dodder plants during different periods exhibited typical SRD symptoms within 10–13 months. The presence of phytoplasmas in the donor palms, *C. filiformis*,

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and recipient palms was confirmed using Dienes' staining. Transmission was further validated by detecting phytoplasmas under electron microscopy (EM). Although none of the 28 oil palm seedlings inoculated with *P. moesta* developed visible SRD symptoms within 10 months, light microscopy (LM) revealed indications of phytoplasma presence in four seedlings. In a related study, two out of four oil palm seedlings bridged to yellow leaf disease affected arecanut palms that were connected with YLD-infected arecanut palms expressed typical SRD symptoms within 8–11 months, with phytoplasmas confirmed through EM. Phytoplasmas were observed in the salivary glands of *P. moesta* subjected to AAP+IAP period of over 31 days on SRD palms. Transmission studies conducted using *P. moesta*, *S. typica*, and dodder laurel among SRD, YLD, and RWD showed phytoplasma transmission evidenced by LM, EM, and symptom reproduction.

SRD-affected oil palm connected with healthy oil palm using dodder resulted in symptom manifestation within 10-18 months, and infection of healthy arecanut connected with SRD-affected oil palm (Anonymous, 1995; Kochu Babu, 2001). RWD-affected coconut to oil palm using *P. moesta* exposed to healthy oil palm resulted in symptom development within 17-23 months (Anonymous, 1995), while *S. typica* caused symptoms in 13 months (Nair and Kochu Babu, 2000). RWD-affected coconut exposed to healthy arecanut using *P. moesta* caused symptoms of yellow leaf disease (YLD) within 32 months (Anonymous, 1996). YLD-affected arecanut exposed to healthy coconut seedlings using *P. moesta* led to symptoms of flaccidity within 15-16 months (Anonymous, 1995).

Summary and Conclusion

Cross-transmission studies among SRD, RWD, and YLD show that the phytoplasmas are common to these diseases and are transmitted by *P. moesta* and *S. typicus* (Kochu Babu, 1993). This interrelationship has been further confirmed through molecular assays (Sumi, 2015, Sumi *et al*., 2014, 2015, 2023). These findings suggest that oil palm cultivation in areas affected by RWD and YLD should be approached with caution, particularly regarding the transportation of oil palm sprouts from affected regions (Kerala) to other states in India.

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