INVESTIGATIONS ON A PHYTOPLASMA DISEASE OF CHERRY IN HIMACHAL PRADESH

Thesis

by

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(H-2021-35-D)

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CERTIFICATE-I

This is to certify that the thesis titled, "**Investigations on a phytoplasma disease of cherry in Himachal Pradesh**" submitted in partial fulfillment of the requirement for the award of the degree of **Doctor of Philosophy Plant Pathology** in the discipline of **Plant Protection** to Dr. Yashwant Singh Parmar University of Horticulture & Forestry, (Nauni) Solan (HP) – 173 230 is a bonafide research work carried out by **Mr. Aman Chauhan (H-2021-35-D)** son of Sh. Bir Singh Chauhan, under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Place: Nauni, Solan
Dated: 26.06.25

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CERTIFICATE – II

This is to certify that the thesis titled, "**Investigations on a phytoplasma disease of cherry in Himachal Pradesh**" submitted by **Mr. Aman Chauhan (H-2021-35-D)** son of Sh. Bir Singh Chauhan to the Dr. Yashwant Singh Parmar University of Horticulture & Forestry, (Nauni) Solan (HP) – 173 230 India in partial fulfillment of the requirement for the degree of **Doctor of Philosophy Plant Pathology** in the discipline of **Plant Protection** has been approved by the Advisory Committee after an oral examination of the student in collaboration with an External Examiner.

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Dated: **(Aman Chauhan)**

CONTENTS

LIST OF ABBREVIATIONS

%	:	Percent
°C	:	Degree Celsius
µg	:	Micro gram
µl	:	Micro liter
amsl	:	Above mean sea level
AP	:	Apple proliferation
AT	:	Adenine - thymine
AT+GC	:	Adenine - thymine + guanine - cytosine
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base pair
C	:	Chlorosis
CABI	:	Centre for Agriculture and Bioscience International
CLY	:	Cherry lethal yellows
cm	:	Centimeter
CTAB	:	Cetyltrimethyl ammonium bromide
cv.	:	Cultivar
DAPI	:	4,6 diamidino-2-phenylindole
DNA	:	Deoxy ribonucleic acid
dNTP	:	Deoxy nucleotide triphosphate
dsDNA	:	Double stranded deoxy ribonucleic acid
EDTA	:	Ethylenediamine tetra acetic acid
ELISA	:	Enzyme linked immunosorbent assay
EPPO	:	European and Mediterranean Plant Protection Organization
ESFY	:	European stone fruit yellows
et al.	:	Co-workers
EtBr	:	Ethidium bromide
fig.	:	Figure
GTPs	:	Graft transmissible pathogens
ha	:	Hectare
i.e.	:	that is
IEM	:	Immunosorbent Electron Microscopy
IR	:	Inward Rolling
JWB	:	Jujube witches' broom
kb	:	Kilo bases
kbp	:	Kilo base pair
LR	:	Leaf Reddening
LT	:	Leaf Tattering
Ltd.	:	Limited
M	:	Molar
m	:	Meter
MEGA	:	Molecular Evolutionary Genetic Analysis
mg	:	Milligram
$MgCl_2$	:	Magnesium chloride
ml	:	Milliliter
MLOs	:	Mycoplasma like organisms
mM	:	Millimolar
mRNA	:	Messenger ribonucleic acid

MT	:	Metric tons
MVS	:	Mid Vein Swelling
NaCl	:	Sodium chloride
NCBI	:	National Centre for Biological Information
ng	:	Nano gram
ng/µl	:	Nano gram per micro litre
NGS	:	Next Generation Sequencing
NHB	:	National Horticulture Board
nm	:	Nano meter
No.	:	Number
OD	:	Optical density
PCDO	:	Progeny Cum Demonstration Orchard
PCR	:	Polymerase Chain Reaction
PD	:	Pear decline
PDC	:	Per cent disease control
PDV	:	Prunus dwarf virus
pM	:	Pico Molar
PVP	:	Polyvinyl pyrrolidone
PYLR	:	Peach yellow leaf roll
qPCR	:	Quantitative polymerase chain reaction
rDNA	:	Recombinant deoxy ribonucleic acid
RFLP	:	Restriction Fragment Length Polymorphism
RHR&TS	:	Regional Horticulture Research And Training Station
RNA	:	Ribonucleic acid
rpm	:	Rotation per minute
rRNA	:	Ribosomal ribonucleic acid
S	:	Scorching
SEM	:	Scanning Electron Microscopy
sp.	:	Species
TAE	:	Tris-acetate-EDTA
Taq	:	*Thermus aquaticus*
TE	:	Tris-EDTA
TEM	:	Transmission Electron Microscopy
Tm	:	Melting Temperature
USA	:	United States of America
UV	:	Ultra violet
V	:	Volt
v/v	:	Volume by volume
var.	:	Variety
viz.	:	Namely
w/v	:	Weight by volume
WX	:	Western X- disease
Y	:	Yellowing

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Chapter – 1

INTRODUCTION

Cherry (*Prunus avium* L.) is a fleshy drupe and is an important fruit grown in temperate regions belonging to family Rosaceae. The sweet cherry and sour cherry both originated in the area between the Black and Caspian Seas in Europe and Asia Minor comprising most of the present day Turkey. The cultivated forms are of the species sweet cherry (*Prunus avium* L.) to which most cherry cultivars belong and the sour cherry (*Prunus cerasus* L.) which is mainly used for cooking. Generally, cherries flourish in deep, well-drained, loamy soils with a requirement of cooler climate for successful cultivation between 2,000 to 2,700 m above mean sea level with 1,000 to 1,500 hours chilling period essentially needed during winters (Chadha 2019). Cherry ripens first among all the stone fruits and have a huge demand in the late spring and early summer.

World production of cherry was recorded to be 2.76 million metric tons in 2022 from an area of 454.66 thousand hectare. Turkey is the leading producer of cherry with the production of 0.65 million metric tons that constitutes 23.7 per cent of the total produce worldwide, followed by Chile (0.44 MMT), Uzbekistan (0.22 MMT) and United States of America (0.21 MMT) (Anonymous 2022). In India, because of prevalent temperate conditions in northern region, the production of cherry at commercial scale is confined to hilly areas of Kashmir, Himachal Pradesh and Uttarakhand. The total area under cherry cultivation during 2022 was 3.55 thousand ha with the production of 10,934.4 metric tons (Anonymous 2022). In Himachal Pradesh, cherry is commercially cultivated in Narkanda, Kotgarh, Baghi, Matiana, Kumarsain and Thanadhar regions of district Shimla contributing to more than 90 per cent of the total cherry production. The total area under cultivation is 0.55 thousand hectare with a production of 765 MT during the year 2023-24 (Anonymous 2024). However, during the recent surveys conducted in cherry orchards, the plants were found to be severely affected by a new disease resulting in reddening of leaves, wilting of branches over time, which ultimately lead to the death of whole plant and is causing severe economic losses to the growers. The pattern of infection seems to be systemic in nature and is probably a resultant of a phloem restricted pathogen which could possibly be phytoplasma.

Plant diseases of phytoplasma etiology fall in the category of diseases usually referred to as ‘yellows group’ of diseases which were earlier thought to be caused by viruses or virus-

like agents. Non helical wall-less bacteria that colonize the plant phloem were first reported almost 50 years ago when Doi et al. (1967) detected mycoplasma-like bodies in a mulberry plant showing yellows symptoms by electron microscopy. About twenty-five years later, it was proposed to rename these mycoplasma-like organisms because they had been shown by molecular analyses not to be phylogenetically related to the genus *Mycoplasma*.

One symptom that seems to be common in phytoplasma disease of cherry is the presence of undersized leaves, off-coloured fruits and a general decline in the production. For this reason the term 'little cherry' is often applied to these diseases. Symptoms on infected trees, varies with variety and rootstock used. Fruits of sweet cherry on Mazzard rootstock gives the most diagnostic symptoms, while cherry on Mahaleb rootstock shows wilt and decline symptoms. Disease trees appear normal during most of the growing season, but in the late summer and early fall the leaves show on an orange-red colour along the basal portion of the midrib. Retarded growth and die back occur in older infected trees. Fruits fail to mature, remain small, are conical in form, hang on the tree after normal cherries have dropped and fail to develop normal colour of the variety concerned. The symptoms on chokecherries (wild cherries) are same for both eastern and western X-disease except for the absence of rosette effect on western choke cherry. The most striking symptoms on the chokecherry is the appearance of the red and yellow foliage in late summer before there is any evidence of normal fall colouration.

The trivial name phytoplasmas was proposed by B.B. Sears and B.C. Kirkpatrick to the International Committee on Systematic Bacteriology (ICSB) Subcommittee on the Taxonomy of *Mollicutes* and subsequently used (Sears and Kirkpatrick 1994). Later, the ICSB Subcommittee on the Taxonomy of *Mollicutes* agreed that the phytoplasmas could be assigned a provisional taxonomic status of *Candidatus*. Phytoplasmas lack rigid cell walls, are surrounded by a single unit membrane, and are sensitive to the antibiotic tetracycline (Doi et al. 1967). They are mainly classified on the basis of 16S RNA gene sequence restriction fragment length polymorphism and classified into 35 ribosomal groups, nearly 300 subgroups and 49 '*Candidatus* Phytoplasma' species (Lee et al. 1998; IRPCM 2004; Bertaccini 2022; Bertaccini and Lee 2018). They inhabit the phloem sieve elements of infected plants and the gut, haemolymph, salivary gland and other organs of sap-sucking insect vectors (Kirkpatrick 1992). The genome of the phytoplasmas has a size of 530-1,350 kilo basepairs and a G+C content of 23-29 percent.

As of now, six groups of phytoplasma were reported on cherry from all over the world including *Ca.* P. asteris (16SrI) from Poland (Cieślińska and Smolarek 2015; Cieślińska and Smolarek 2019) , Turkey (Caglayan et al. 2013), Iran (Zirak et al. 2021), Italy (Paltrinieri et al. 2001) and China (Gao et al. 2011), *Ca.* P. aurantifolia (16SrII) from Iran (Zirak et al. 2010), *Ca.* P. pruni (16SrIII) from USA (Uyemoto and Kirkpatrick 2011), *Ca*. P. ziziphi (16SrV) from China (Wang et al. 2018) and Italy (Paltrinieri et al. 2008), *Ca.* P. prunorum (16SrX-B) from Czech Republic (Navratil et al. 2001) and Poland (Cieślińska and Smolarek 2019), *Ca*. P. pyri (16SrX-C) from Poland (Cieślińska and Smolarek 2015) and Italy (Paltrinieri et al. 2001) and *Ca*. P. solani (16SrXII-A) from Italy (Paltrinieri et al. 2001; Paltrinieri et al. 2008). However, in India phytoplasma strains have been documented to infect some stone fruits such as peach and plum (Rao 2021; Shreenath et al. 2022) but not reported so far in cherry cultivars.

The development of reliable method for detection of phytoplasma in infected tissues is still one of the biggest challenge to study this elusive pathogen. Due to the absence of proper culturing of most of the species of genus *Candidatus* phytoplasma, it has a direct impact on available information for development of improved detection methods. The phytoplasma detection relies on diagnostic techniques such as observation of symptoms, insect or dodder/graft transmission to host plant and DAPI staining together with electron microscopy. Stains are also differentiated and identified by their biological properties such as similarity in symptoms induced in infected plants and insect vector ranges. However, these methods are laborious, time consuming and have less specificity. An alternative approach which involve molecular tools based on gene sequences are fast emerging as the appropriate technique to detect the phytoplasma. Different variants of PCR (nested PCR, quantitative PCR) techniques have been developed which are highly effective methods of phytoplasma detection within plants and vectors. These techniques increase both sensitivity and specificity in detection and are capable of detecting multiple phytoplasma present in infected tissue in case of mixed infection (Bertaccini et al. 2019).

In Himachal Pradesh, there has not been organized and comprehensive surveys of orchards of cherry growing regions. Therefore, there is a need to survey the cherry orchards of Himachal Pradesh to determine the severity of disease and identifying the phytoplasma-free healthy planting material for propagation. The present investigation thus aims to detect

the presence of phytoplasma in infected cherry plants by using DAPI staining and molecular techniques under the following objectives:

OBJECTIVES:

1. To survey cherry growing regions of Shimla district for recording the incidence of disease.
2. To characterize the associated phytoplasma on the basis of fluorescent microscopy and PCR based molecular techniques.
3. To screen the available germplasm of cherry for source of resistance against phytoplasma.

Chapter – 2

REVIEW OF LITERATURE

2.1 Discovery and Taxonomy of Phytoplasmas

For a long time, many "yellows"-type diseases in plants—characterized by flower abnormalities, yellowing, and witches'-broom—were believed to be caused by viruses. This assumption persisted until 1967, when a team of Japanese scientists, using electron microscopy, observed small, pleomorphic, cell wall-less microorganisms within the phloem sieve elements of diseased plants (Doi et al. 1967). These microorganisms, morphologically resembling animal and human mycoplasmas, were initially termed *mycoplasma-like organisms* (MLOs).

The first 16S rRNA gene sequence from an MLO was reported in 1989 and revealed significant phylogenetic differences from animal mycoplasmas. Consequently, B.B. Sears and B.C. Kirkpatrick proposed the term *phytoplasma* to the International Committee on Systematic Bacteriology (ICSB) Subcommittee on the Taxonomy of Mollicutes, which was subsequently adopted (Sears and Kirkpatrick 1994). The word *phytoplasma* is derived from Greek: *phyto* (plant) and *plasma* (formed or molded), highlighting their plant association.

Due to extensive reductive evolution, phytoplasmas have lost many genes essential for independent life, making them entirely dependent on their plant hosts for nutrients. As a result, axenic culture of phytoplasmas remains unachieved. Traditional bacterial taxonomy, reliant on phenotypic characterization, is inapplicable to phytoplasmas, given their non-culturable nature. Furthermore, symptoms, host range, and vector specificity proved unreliable for classification due to variability. Thus, genotypic, culture-independent methods—especially DNA sequencing of the 16S rRNA gene—became essential tools for phytoplasma taxonomy, evolutionary studies, and diversity analysis.

Initially, the absence of a comprehensive classification system meant many phytoplasma identities remained unresolved. Techniques such as 16S rRNA gene sequencing, RFLP analysis of PCR-amplified 16S rDNA, 16S-23S spacer region analysis, serological methods, and biological property assessments gradually enabled broader phytoplasma detection and characterization. The first RFLP analysis of PCR-amplified 16S rDNA with

multiple restriction enzymes was conducted by Lee et al. (1994) and Schneider et al. (1993), leading to the classification of phytoplasmas into 10 major groups and 15 subgroups. Seemüller et al. (1998) later expanded this to 20 groups based on additional phytoplasma strains.

The 'Candidatus' status for provisional species was introduced by the International Code of Nomenclature of Bacteria (ICNB) in 1995, facilitating classification using structural, metabolic, reproductive, and genomic information (Murray and Schleifer 1994; Murray and Stackebrandt 1995). In 2004, the International Research Programme on Comparative Mycoplasmology (IRPCM) Phytoplasma/Spiroplasma Working Team formalized the provisional genus *'Candidatus* Phytoplasma' and provided guidelines for describing new species, emphasizing near-full-length (>1200 bp) 16S rRNA sequences with less than 97.5% identity to any existing species (Firrao 2004). Based on 16S rRNA sequencing a number of new '*Candidatus'* species were reported in last few years indicating the great biodiversity. Recently, two new Candidatus species were proposed *Ca*. Pytoplasma wodyetine and *Ca*. Phytoplasma noviguineense by using the same gene sequence (Naderali et al. 2017; Miyazaki et al. 2018)

Recent revisions (Bertaccini et al. 2022) updated these standards:

- Required 16S rRNA gene sequence length: >1500 bp
- Sequence identity threshold: 98.65%
- Introduction of whole-genome Average Nucleotide Identity (ANI) criteria
- Use of multilocus sequence analysis (MLSA) for further differentiation if ANI and 16S rRNA thresholds are surpassed.

Currently, there are 37 recognized phytoplasma groups and 48 named *Candidatus* species (Zhao and Davis 2016).The present classification of phytoplasma is (Bertaccini et al. 2022):

Taxonomic Position:

- **Kingdom:** Bacteria
- **Phylum:** Mycoplasmatota
- **Class:** Mollicutes
- **Order:** Acholeplasmatales

- **Family:** Acholeplasmataceae
- **Genus:***Candidatus* Phytoplasma

2.2 Economic Importance

Phytoplasmas are major constraints on crop production worldwide (McCoy et al. 1989), associated with over 600 plant diseases (Bertaccini et al. 2014). Many key fruit and vegetable crops are affected. Stone fruit trees are also vulnerable to numerous phytoplasma strains belonging to 14 distinct *Ca*. Phytoplasma species.

In Europe, apple proliferation, pear decline, and European Stone Fruit Yellows (ESFY) cause significant quality and yield losses in temperate fruit crops (Seemüller and Schneider 2004). ESFY alone kills about 5 percent of trees annually in major *Prunus*-growing regions.

In the United States, *Candidatus* Phytoplasma pruni causes X-disease in cherries and peaches, historically a major constraint on peach production (Stoddard et al. 1951; Douglas 1986; Davis et al. 2013). In North America, the X-disease resulted in removal of thousands of cherry trees from more than 100 cherry orchards during an epidemic in California (Uyemoto and Moratorio 1998). A recent outbreak of X-disease in Washington State led to the destruction of thousands of cherry trees, with sweet cherry production declining by up to 17.6% from 2018 to 2020 (Wright et al. 2021).

British Columbia, a key sweet cherry production area in Canada, experienced significant economic losses due to X-disease introduced from neighbouring Washington State (Urbez-Torres et al. 2024).

In the Middle East, phytoplasma infections impact over 164 plant species across various crops (Waller et al. 1978; Bové et al. 1988).

In China and India, *Ca.* P. ziziphi has caused serious outbreaks in jujube, cherry, and peach (Zhu et al. 1998, Thakur et al. 1998). Phytoplasma associated cherry lethal decline caused a severe outbreak in the orchards of Sichuan in Chinese province. Infected trees died within 3-4 years and did not produce fruits (Zhu et al. 1998). In India's Rajgarh valley, phytoplasma incidence in peaches reached up to 70% in more than 80 peach orchards.

2.3 Geographical Distribution

The United States has faced extensive outbreaks of X-disease across stone fruits like peach, nectarine, plum, and cherries, with *Ca.* P. pruni (16SrIII-A subgroup) as the primary agent (Davis et al. 2015). Disease is most destructive among all phytoplasma disease of temperate fruits, known by numerous names such as western X, little cherry, western X red leaf, wilt and decline, peach yellow leaf roll and smaller bitter cherry (Gilmer and Blodgett 1976; Dougas 1986).

In Europe, *Ca.* P. prunorum is responsible for European Stone Fruit Yellows (ESFY) outbreaks in France, Poland, and Hungary, where symptoms such as leaf yellowing and plant decline have devastated orchards (Bernhard et al. 1977).

In South America, Chile reported *Ca.* P. solani (16SrXII-A) infecting peaches and a 16SrIII-J strain affecting cherries (Firrao et al. 2013; Quiroga et al. 2015).

In the Middle East (Iran, Oman, Lebanon, Israel), 14 ribosomal phytoplasma groups threaten diverse crops (Hemmati et al. 2021), including severe infections in *Prunus* species. Several phytoplasma strains belonging to various ribosomal groups 16SrI-B, 16SrII (subgroup II-B, II-C), 16SrVI (subgroup VI-A and VI-D), 16SrIX (IX-B, IX-C, IX-D), 16SrX-F, and 16SrXII-A were reported to cause severe economic losses in *Prunus* species including plum, peach, sweet cherry, sour cherry, almond and apricot (Abou-Jawdah et al. 2003; Zirak et al. 2009 and Salehi et al. 2019).

In China, outbreaks of Sweet Cherry Virescence (SCV) and other diseases linked to *Ca.* P. ziziphi and *Ca.* P. asteris-related strains have been reported (Gao et al. 2011; Wang et al. 2014). Also, two sweet cherry phytoplasma diseases had been reported from two Provinces Yantai and Taian in China. Disease infection in Yantai Province was found to be associated with *Ca*. P. asteris related strain (Gao et al. 2011), on the other hand, the diseases infected sweet cherry trees in Taian Province was found to be linked to infect with *Ca*. P. ziziphi (Wang et al. 2014). In both the Provinces affected cherry trees exhibited floral symptoms including virescence and witches' broom

In India, phytoplasma infections have been confirmed in 129 plant species including vegetables, fruits, ornamental plants, oil crops, weeds, trees and palms across 17 states, belonging to 10 different ribosomal groups (Rao et al. 2017). Surveys were conducted in

cherry orchards by Shreenath et al. (2022) at the ICAR–Central Institute of Temperate Horticulture (CITH), Srinagar. The primary cherry cultivars observed were Bigarreau Nepoleon (BN), Bigarreau Noir Grossa (BNG), CITH-Cherry-9, Stella and Sunburst. Disease incidence ranged from 7.5% to 10% in cultivars Stella and Sunburst, while incidence in BN, BNG, and CITH-Cherry-*9* varied between 5.8% and 25%. BLAST analysis of the 16S rRNA gene sequences revealed that the phytoplasma strains infecting BN, BNG, and CITH-Cherry-9 belonged to the 16SrVI-D subgroup ('*Ca.* Phytoplasma trifolii'), whereas the strains infecting Stella and Sunburst belonged to the 16SrI-B subgroup ('*Ca.* Phytoplasma asteris') (Shreenath et al. 2022). Both phytoplasma groups have previously been reported infecting several economically important crops in India (Rao 2021).

Globally, six phytoplasma groups have been associated with cherry trees: 16SrI, 16SrII, 16SrIII, 16SrV, 16SrX, and 16SrXII (Shreenath et al. 2022), as detailed in Table 1.

Table 1: Phytoplasma strains associated with cherry trees around the world

Sr. No.	Ribosomal group/ Subgroup	*Candidatus* species	Geographic origin	References
1.	16SrI-B	'*Ca.* P. asteris'	Poland Czech Republic Turkey Iran Italy	Cieślińska and Smolarek (2015, 2019) Navratil et al. (2001) Caglayan et al. (2013) Zirak et al. (2021) Paltrinieri et al. (2001)
2.	16SrII-B	'*Ca.* P. aurantifolia'	Iran	Zirak et al. (2010)
3.	16SrIII-A 16SrIII-B	'*Ca.* P. pruni' NA	USA Italy	Uyemoto and Kirkpatrick (2011) Paltrinieri et al. (2008)
4.	16SrV-B	'*Ca.* P. ziziphi'	China Italy	Wang et al. 2018 Paltrinieri et al. (2008)
5.	16SrX-A	'*Ca.* P. mali'	Czech Republic Slovenia	Navratil et al. (2001) Mehle et al. (2007)
6.	16SrX-B	'*Ca.* P. prunorum'	Czech Republic Poland Italy	Czech Republic Navratil et al. (2001); Fialova et al. (2004); Ludvikova et al. (2011) Cieślińska and Smolarek (2019) Paltrinieri et al. (2001)
7.	16SrX-C	'*Ca.* P. pyri'	Poland Italy	Cieślińska and Smolarek (2015) Paltrinieri et al. (2001)
8.	16SrXII-A	'*Ca.* P. solani'	Bulgaria China Italy	Avramov et al. (2011) Li et al. (1997) Paltrinieri et al. (2001, 2008)

2.4 Symptomatology

Symptomatology plays a critical role in diagnosing phytoplasma involvement in plant diseases. Infection often leads to a profound imbalance of plant growth regulators, altering chlorophyll content, sugars, and secondary metabolites, which in turn disrupts the host's source-sink relationship (Xue et al. 2018; Tan et al. 2015). Interestingly, certain phytoplasma strains can induce traits that make infected plants desirable as ornamentals, such as strains affecting poinsettia (Bertaccini 1996).

Phytoplasma infections manifest in a variety of symptoms: stunting, virescence, shortened internodes, big bud, little leaf, witches' broom, phyllody, floral malformations, and vascular discoloration (Kumari et al. 2019). Yellows-type diseases are characterized by gradual yellowing or reddening of leaves, reduced leaf size, shoot proliferation, witches' broom formation, eventual dieback and death (Agrios 2024).

In cherries, symptoms include early reddening of foliage, dull leaf coloration in subsequent years, resetting of leaves on terminal branches and plant death. Zheng-Nan et al. (2014) reported that yellowing starts at minor veins and spreading to the entire leaf, accompanied by leaf size reduction, curling, necrosis and premature fruit drop.

In North America, X-disease (Little Cherry Disease) causes small, misshapen fruits with poor colour and flavour due to lower sugar contents. Symptoms typically begin on a few branches but can progress to the whole tree after several years. After multiple year of infection, tree is characterized by reduced fruit yield and dieback of limbs or even whole tree (Harper et al. 2020).

In China, sweet cherry virescence (SCV) disease causes floral virescence, shoot proliferation, and eventual branch death. Floral virescence was the earliest and most diagnostic symptom. The vegetative symptoms included development of densely clustered twigs with considerably shortened internodes called witches'-broom growth and abnormally small leave. Leaf necrosis and wilting occurred several weeks to a few months after the first appearance of floral symptoms, leading to eventual death of the affected branches or the entire plant. Due to the prominence of the floral symptom, the multi-location/year disease is referred to as sweet cherry virescence (SCV) disease (Wang et al. 2018).

Czech surveys during 2014–2015 documented symptoms such as shoot proliferation, chlorotic spots, premature reddening and leaf fall, and stunted growth in sweet and sour cherry cultivars. In sour cherry symptoms exhibited by commercial cv. Ujfehertoi furtop and cv. Fanal include bunches of bloom with smaller leaves growing at the apical part, shortened internodes and stunted growth of trees. Also, smaller fruit size and pale fruit colour was observed on cv. Morela pozdni and cv. Morellenfeuer (Franova et al. 2018).

In India, symptoms like leaf roll, flat branches, and witches' broom were observed in Srinagar cherry cultivars, caused by *Ca.* P. asteris and *Ca.* P. trifolii (Shreenath et al. 2022). Similarly, in Himachal Pradesh, peach trees exhibited yellowing, leaf rolling, and red spotting, leading to shriveled fruits and premature fruit drop. Visible symptoms of the disease started appearing after 7–8 weeks of budbreak in the form of chlorosis of leaves with typical longitudinal upward rolling and occasional red spotting. Later, the foliage turned pale to yellow-red in colour. DNA sequencing confirmed the presence of *Ca.* P. ziziphi (Khan et al. 2013).

2.5 Particle Morphology

Phytoplasmas are wall-less, pleomorphic, non-helical prokaryotes classified within the class *Mollicutes*. They are the smallest bacteria, ranging from 200 to 1000 nm. Due to their obligate parasitism in phloem sieve elements, in vitro cultivation attempts have failed (Christensen et al. 2004). Their genomes (530–1350 kb, low GC content) lack essential genes for vital metabolic pathways, indicating reductive evolution and complete host dependence (Marcone et al. 1999).

2.6 Transmission

Phytoplasma is phloem residing pathogen and is mainly transmitted by hemipteran insects. Leafhopper belonging to family Cicadellidae and plant hoppers of family Fulgoridae of order Hemiptera, indicate those species which mainly feed on leaves and sieve tube elements of plant species. Currently about 200 leafhopper species were recorded as confirmed or potential vectors of phytoplasma (Weintraub et al. 2019). The first record of experimental transmission of pathogen by leafhopper, *Reciladorsalis* was in Japan in 1883 and was again confirmed in 1893 (Fukusi 1969). Vectors transmit phytoplasma in persistent, propagative manner, which means that once vector acquire pathogen in its body it can actively multiply or increase its number. After acquiring the pathogen, a latent period is

required before vector can transmit the phytoplasma. During this period phytoplasma move through midgut epithelium to haemolymph followed by their multiplication and colonization in insect body. Once infected, vectors are usually infective for life. Vectors carrying phytoplasma in their salivary glands can inject it directly into the sieve tube elements of host while feeding. Nevertheless, various factors of vector influence the transmission efficiency, such as life stage, gender, flight behaviour and source plant. With regard to life stage, it has been reported that nymphs are more efficient in acquiring phytoplasma than adults (Chiykowski and Sinha 1982).

Stolbur group of phytoplasma affecting various vegetables and fruit crops like tomato, pepper (Avinent and Lla´cer 1995), avocado (Lavin˜a et al. 2002), apple, peach and pear (Martinez-Culebras et al. 2000) was mainly transmitted by *Hyalesthes obsoletus* (Fos et al. 1992), other species of planthopper identified as vector of stolbur group are *Pentastiridius beieri* in sugar beet (Gatineau et al. 2001), *Reptalus quinquecostatus* and *R. panzer* in vineyards (Palermo et al. 2004; Trivellone et al. 2005). Leafhopper species such as *Macrosteles laevis*, *Aphrodes bicinctus* and *Euscelis plebejus* have been identified as vectors of stolbur in experimental conditions (Valenta et al. 1961) or as carriers of the phytoplasma in the field (Fos et al. 1992).

Leafhoppers are the only known vectors reported to be responsible for transmitting X-disease in North America. Seven leafhoppers are known to transmit X-disease phytoplasma: *Colladonus montanus*, *Fiebriella florii*, *Scaphytopius acutus*, *Paraphlepsius irroratus*, *C. reductus, E. variegatus* and *C. geminatus* (Jensen 1969; Purcell and Elkinton 1980). The two most common leafhoppers in Washington are *C. reductus, C. geminatus*. In California, *C. geminatus* and *C. reductus* were also found to be the most common vectors of X-disease phytoplasma (Uyemoto et al. 1997).

Transmission studies conducted by Tiwari et al. (2016), found that three leafhopper species *Cofana immaculate* (Cicadellidae: Hemiptera), *Exitianus indicus* (Cicadellidae: Hemiptera) and *Maiestas portica* (Cicadellidae: Hemiptera) were the potential vectors of Sugarcane grassy stunt phytoplasma (SCGS) caused by *Ca*. P. oryzae (16SrXI group). The identification of new vectors of SCGS phytoplasma suggested that these leafhopper species may be responsible for secondary spread of SCGS phytoplasma (Tiwari et al. 2016). In the transmission study conducted by Sajad un Nabi et al. (2015), *Hishimonus*

phycitis was found to be positively associated with the transmission of sesame phyllody caused by *Ca* .P. asteris sub-group I-B (Sajad un Nabi et al. 2015).

2.7 Host Range

Phytoplasmas have been associated with diseases in several hundred plant species spanning over 98 families. Based on sequence data available in the GenBank database, phytoplasma diseases have been reported in more than 100 countries worldwide. Various stone fruits have been identified as natural hosts of the X-disease phytoplasma. Besides peach and chokecherry, other *Prunus* species reported to be susceptible include cherry (*Prunus avium* and *P. cerasus*), Japanese plum (*P. salicina*), almond (*P. dulcis*), apricot (*P. armeniaca*), nectarine (*P. persica* var. *nectarina*), Chinese bushcherry (*P. japonica*), Bessey cherry (*P. besseyi*), wild American plum (*P. americana*), wildgoose plum (*P. munsoniana*), and European plum (*P. domestica*) (Davis et al. 2013; Douglas 1986; Stoddard et al. 1951).

In India, six fruit crops including peach (Ahlawat and Chenulu 1981; Thakur et al. 1998; Singh et al. 2014), papaya (Rao et al. 2011; Verma et al. 2012), citrus Ghosh et al. 2017), jujube (Khan et al. 2013), lychee (Rao et al. 2017) and sapota (Madhupriya et al. 2016) are reported as the hosts of phytoplasma belonging to 16SrI, 16SrII, 16SrV, 16SrVI, 16SrXI and 16 SrXIV groups, respectively.

Between 2019 and 2021, Shreenath et al. confirmed the presence of phytoplasma in weeds and other host plants growing around peach, plum, and apricot orchards in Jammu and Kashmir, Himachal Pradesh, and Uttarakhand. Amplification of a 1.2 kb fragment corresponding to the R16F2n/R2 region revealed phytoplasma infection in seven weed species: *Toona sinensis*, *Cannabis sativa*, *Celosia argentea*, *Datura stramonium*, *Catharanthus roseus*, *Phyllanthus niruri*, and *Ageratum conyzoides*. Symptomatic observations included severe witches' broom in *C. sativa* and *T. sinensis*, flat stem in *C. argentea*, little leaf and witches' broom in *D. stramonium*, and little leaf and yellowing in *C. roseus* (Rajouri, Jammu & Kashmir). Witches' broom in *T. sinensis* and leaf rolling and chlorosis in *P. niruri* were noted in Rajgarh, Himachal Pradesh, while severe leaf yellowing in *A. conyzoides* was recorded from Pantnagar, Uttarakhand (Shreenath et al. 2022).

2.8 Detection

Detecting phytoplasmas is challenging due to their low concentration and uneven distribution in the sieve tubes of woody plants. Since no direct field control measures are

available, management primarily relies on infection prevention and early detection, making early diagnosis critical for disease control. Initially, detection was based on the observation of characteristic symptoms and pleomorphic bodies via electron microscopy. Biological characterization, based on symptomatology, was traditionally used but proved laborious, time-consuming, and nonspecific.

The development of phytoplasma-specific molecular probes and PCR assays has significantly advanced diagnostic capabilities. In the 1980s, serological diagnostic techniques using monoclonal and polyclonal antisera were developed to detect phytoplasmas in both plants and insect vectors (Chen et al. 1993; Sinha and Benhamou 1983; Boudon-Padieu et al. 1989). By the 1990s, the use of molecular probes, PCR (Schneider et al. 1993), nested-PCR (Lee et al. 1994; Lee et al.1995) and Restriction Fragment Length Polymorphism (RFLP) analysis facilitated broad phytoplasma detection (Lee et al. 1998). Deng and Hiruki (1991) first reported the PCR amplification of the phytoplasma 16S rRNA gene, leading to widespread adoption of 16S rRNA-targeted universal and group-specific primers for molecular detection (Gundersen and Lee 1996; Schneider et al. 1995).

2.8.1 Fluorescent microscopy

The 4',6-diamidino-2-phenylindole (DAPI) staining method was developed for the rapid and accurate localization of phytoplasmas in phloem sieve tubes of twigs, leaves, petioles, and roots. DAPI binds strongly to the AT-rich regions of phytoplasma DNA, staining infected phloem blue (Deeley et al. 1979; Eriksson 1993). Infected tissues exhibit bright phytoplasma-like bodies under fluorescence microscopy, whereas healthy tissues remain unstained. Due to its speed and cost-effectiveness, DAPI staining is widely used across various plant species (Thomas and Balasundar 1998; Arismendi et al. 2010).

Garcia-Chapa et al. (2003), detected Pear Decline (PD) phytoplasma in Germany, using 4',6-diamidino-2-phenylindole (DAPI) staining and found the high concentration of phytoplasma during summers. Furthermore, *Candidatus* Phytoplasma prunorum was detected in Japanese plum cv. 'Ozark Premier' inoculated by grafting the infected budwood by DAPI based fluorescence microscopy (Carror et al. 1998). In India, fluorescent based studies conducted by Thakur et al. (1998) and Thakur and Handa (2004) revealed the presence of fluorescent bodies in phloem sections prepared from roots, twigs and petiole sections of diseased peach tree.

2.8.2 Electron microscopy

Electron microscopy is precise and highly effective technique used for detection of phytoplasma in the plant cells. As early as 1967 phytoplasmas were detected in plant tissue using Transmission Electron Microscopy (TEM), the pathogen was seen in the phloem of plants showing dwarfing, "'witches' broom" and yellowing-type disease symptoms (Doi 1967). Use of transmission and scanning electron microscopy had greatly increased the knowledge about pathogen morphology, developmental stages, intracellular location and movement in plants and insect vectors. Electron microscopy has shown phytoplasmas to be pleiomorphic, having been observed in many shapes and sizes between 0.1 and 1 μm, this ability to modify their morphology enables phytoplasmas to pass through phloem sieve plates and plasmodesmata of the cell walls in plant vascular systems (Waters and Hunt 1980; Devonshire 2013).

El-Banna et al. (2015) investigated the association of phytoplasma with citrus witches' broom disease by molecular and electron microscopy. Ultrathin sections prepared from mandarin, orange leaf mid rib and albedo layer from orange fruit representing witches broom symptoms were investigated through transmission electron microscopy at different magnifications. The examination revealed the presence of numerous phytoplasma units in the sieve elements of the infected orange tissues. Furthermore, Musetti et al. (2002) used immune-electron microscopy technique to label *Chrysanthemum leuchanthemum* phytoplasma in infected leaf tissues of *C. leuchanthemum* L. and *Catharanthus roseus* L. plants. Use specific monoclonal antibodies at different dilutions and secondary anti-mouse antibody conjugated with colloidal gold particles of different sizes demonstrated the precise localization and identification of phytoplasmas in thin sections from infected tissues.

2.8.3 PCR based detection

Identification of phytoplasma is mainly based amplification of 16S rRNA gene sequence. PCR-based techniques offer highly specific and sensitive methods for phytoplasma detection. The conserved region ensures the ideal target for studying bacterial evolution. The presence of highly conserved domains that interact with ribosomal proteins, as well as its presence in every prokaryotic cell, are the two most important characteristics. Several conventional and nested PCR protocols have been developed for universal or group-specific detection (Torres et al. 2005; Yvon et al. 2009; Aladaghi et al. 2009). Universal and group-

specific primers are used in various combinations for direct, nested, and semi-nested PCR assays (Bertaccini et al. 2019).

Gundersen and Lee (1996) developed new universal primers (R16mF2/R1) and a modified pair (R16F2n/R2) for nested PCR assays targeting the 16S rRNA gene. These primers amplify 16S rDNA sequences from all known phytoplasma groups without amplifying DNA from healthy plants. Direct PCR with R16mF2/R1 enabled sensitive phytoplasma detection in woody hosts, while nested-PCR with R16F2n/R2 significantly enhanced sensitivity, allowing detection in both plants and insect vectors.

In Poland, Cieślińska and Morgaś (2011) used PCR and RFLP to detect and identify phytoplasmas in 435 stone fruit trees across 39 orchards. Phytoplasmas were detected in 29 trees (6.7% infection rate), corresponding to three apple proliferation group subgroups: *'Candidatus* Phytoplasma prunorum' (16SrX-B), *'Ca.* Phytoplama mali' (16SrX-A), and *'Ca.* Phytoplasma pyri*'* (16SrX-C).

In China, Gao et al. (2011) observed cherry virescence symptoms in Shandong Province and identified a phytoplasma (ChV-YT) associated with these symptoms. Molecular detection using nested-PCR revealed 98.9–99.4% sequence similarity with aster yellows group (16SrI) phytoplasmas, particularly *Ca.* Phytoplasma asteris (16SrI-B).

Shreenath et al. (2022) characterized phytoplasma strains infecting peach, apricot, and plum orchards in the northwestern himalayan region. Using nested-PCR with universal primers (P1/P7 and R16F2n/R2) and specific primers targeting secA, secY, and tuf genes, they confirmed the presence of '*Ca*. Phytoplasma asteris' (16SrI-B), '*Ca*. Phytoplasma australasia' (16SrII-D), and '*Ca*. Phytoplasma ziziphi' (16SrV-B) in stone fruits. In another study, Shreenath et al. (2022b) reported the detection of phytoplasmas in sweet cherry cultivars in Srinagar, Jammu and Kashmir. Using specific primers for the 16S rRNA, rp, secA, secY, and tuf genes, they confirmed the presence of *Ca*. Phytoplasma asteris and *Ca*. Phytoplasma trifolii strains (16SrI-B and 16SrVI-D).

Globally, various phytoplasma groups have been associated with cherry diseases: 16SrIII causes Western X-disease in America; 16SrX and 16SrI are linked with cherry decline and moliere's disease in Europe; and 16SrI, 16SrVII, and 16SrV cause lethal yellows and cherry virescence in China. The widespread detection of phytoplasmas in cherry

plantations and other plant species expands the pathogen's host range and aids its propagation, making early detection using fluorescence microscopy and molecular techniques essential for ensuring the production of healthy, high-quality fruits.

Chapter – 3

MATERIALS AND METHODS

The present studies with title "Investigations on a phytoplasma disease of cherry in Himachal Pradesh" was conducted in department of Plant Pathology, Dr YS Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh. The detail of materials used and methods followed during the present study are being explained under following sections:

3.1 To survey cherry growing regions of Shimla district for recording the incidence of disease.
3.2 To characterize the associated phytoplasma on the basis of fluorescent microscopy and PCR based molecular techniques.
3.3 To screen the available germplasm of cherry for source of resistance against phytoplasma.

3.1 SURVEY OF CHERRY ORCHARDS AT VARIOUS LOCATIONS IN SHIMLA DISTRICT OF HIMACHAL PRADESH

Surveys were conducted in various cherry growing areas of Shimla district of Himachal Pradesh during different growing seasons of crop starting from early spring to late autumn in the year 2023 and 2024. Table 2 represents different locations selected to conduct survey. Each location was critically screened visually to distinguish phytoplasma infected trees. Based on the visual symptoms, per cent disease incidence was calculated by counting the number of infected trees showing symptoms out of the total trees observed in an orchard. Table 3 represents the various details collected during the survey

The per cent disease incidence was calculated by using the formula given by McKinney (1923).

$$Per\ cent\ disease\ incidence = \frac{Number\ of\ diseased\ plants\ observed}{\text{Total number of plants observed}} \times 100$$

3.1.1 Marking of symptomatic trees and sample collection

During surveys, plants were selected based of the visual symptoms in different orchard at different locations. Trees were identified with mild to severe symptoms and all symptomatic and some asymptomatic trees were subjected to sampling. Samples included leaves, twigs and branches were collected from different branches of trees showing peculiar

symptoms of phytoplasma infection. The collected samples were placed in plastic sample bags labelled with date of collection, location, symptoms and brought to laboratory in ice box. Trees showing the characteristic symptoms of phytoplasma infection were marked for critically observing the symptom development in different growing seasons. Apart from the symptomatic trees samples were also collected from the healthy trees. In collected samples were placed at 4ºC for short term storage and at -80ºC in the laboratory for long term storage for further analysis.

Table 2: Survey of cherry growing areas of Shimla district of Himachal Pradesh

Sr. No.	District	Block	Orchard Location/Village	GPS Coordinates	
				Latitude	Longitude
1.	Shimla	Narkanda	Baghi	31° 14’ 22.1676’’ N	77° 34’ 6.0204’’ E
2.			Matlu	31° 14’ 0.4056’’ N	77° 33’ 59.0184’’ E
3.			Ratnari	31° 13’ 17.9616’’ N	77° 31’ 40.35’’ E
4.			Kungal	31° 12’ 12.4236’’ N	77° 30’ 58.3704’’
5.			Kotgarh	31° 18’ 45.0468’’ N	77° 28’ 32.3796’’ E
6.			Thanadhar	31° 19’ 24.6072’’ N	77° 29’ 29.5548’’ E
7.			Kainthla Mod	31° 13’ 26.256’’ N	77° 26’ 5.028’’ E
8.		Kumarsain	Kumarsain	31° 19’ 9.6816’’ N	77° 26’ 39.7716’’ E
9.			Kandyali	31° 13’ 26.256’’ N	77° 26’ 5.028’’ E
10.			Bhutti	31° 19’ 50.3112’’ N	77° 31’ 27.0732’’ E
11.		Rohru	Kdadrala	31° 14’ 45.3192’’ N	77° 38’ 17.8224’’ E
12.			Mashobra	31° 9' 13.1616" N	77° 12' 46.2744" E

3.2.1 TO CHARACTERIZE THE ASSOCIATED PHYTOPLASMA ON THE BASIS OF FLUORESCENT MICROSCOPY AND PCR BASED MOLECULAR TECHNIQUES

3.2.1 Histological detection of phytoplasma infection in cherry plants by using Fluorescence Microscopy

Fluorescence microscopy by using the 4’,6-diamidino-2-phenylindole (DAPI) stain was used to confirm the presence of phytoplasma in symptomatic leaves by observing under fluorescence microscope (EVOS FL Cell Imaging System, Carlsbad, California, USA) following the protocol given below (Andrade and Arismendi 2013):

- Infected leaf samples were stored at 4ºC were used for DAPI staining.
- Midrib of leaf sample was cut with a sharp blade up to a length of 1cm and placed in sterile distilled water to reduce dehydration of sample.
- The samples were immersed in 5 per cent glutaraldehyde solution and were fixed for 20 minutes.

Table 3: Various details collected during the survey

Sr. No.	Crop	Date of collection	Location	Variety	Age of plant	Source of planting material	Rootstock	Symptom	Incidence (%)	Farmers Name & Contact details
1.										
2.										
3.										
4.										
5.										

- The samples from the glutaraldehyde solution were removed, wash with 0.1 M phosphate buffer pH 6.9 for 5 min and transferred to fresh 0.1 M phosphate buffer pH 6.9.
- By using a sharp blade, samples were further cut into 1cm long midrib samples into thin transverse sections.
- Finally, sections were placed into 1X working solution of DAPI stain for 25 min.
- After 25 minutes of staining, the sections were put in sterile distilled water to remove excess dye and immediately mounted on a slide and covered with coverslip.
- Sections were finally observed under fluorescence microscope with a range of objective lenses (10X and 40X).

The composition of various buffers used in protocol is given Appendix-I

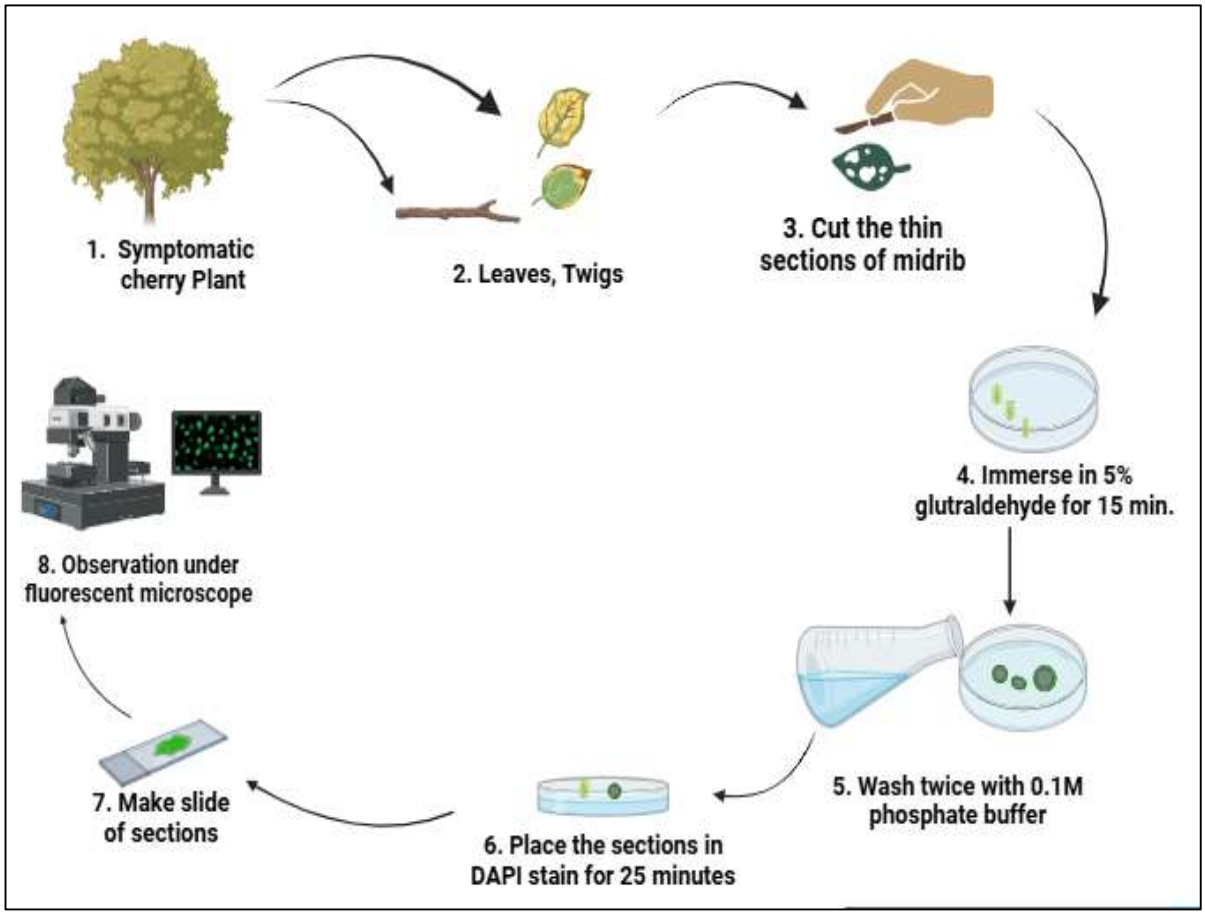

Fig. 1: Schematic diagram of fluorescent microscopy using DAPI staining (4,6-diamidino-2-phenylindole) for detection of phytoplasma in cherry samples

3.2.2 Biological detection of phytoplasma

Biological detection of phytoplasma was made by carrying out the graft transmission method (tongue grafting). One year old healthy seedlings (colt) were procured from Regional

Horticulture Research and Training Station, Mashobra, Shimla (HP) in the month of February, 2024. The seedlings were then bench grafted by using the scion wood of infected plants which are marked during the surveys conducted in the year 2023. For tongue grafting, a gradual cut is made on the rootstock at an angle of 15 to 30 degrees. The length of this cut is 4 to 5 times the diameter of stock. The second cut is made about one third of the way down the top of the first cut and should be made such that it run parallel to the length of stock. Similar cuts were made on scion wood of similar diameter. This formed a tongue on both scion and stock. Woods were then pushed together so that the two tongues get interlocked. The graft unions were finally sealed to prevent desiccation by using the grating tape or polyethene. Grafted seedlings were finally replanted at Krishi Vigyan Kendra, Rohru and at Dr. YS Parmar University of Horticulture and Forestry Nauni, Solan. Grafted plants were observed for the whole season for the development of symptoms. The transmission of phytoplasma from infected scion wood to healthy stocks were also confirmed by using PCR based detection techniques.

3.2.3 PCR based molecular detection and characterization

In order to further confirm and characterize the phytoplasma infection in cherry plants, PCR based detection techniques like normal PCR and nested PCR were performed on total DNA extracted from leaves of symptomatic and asymptomatic cherry plants.

DNA isolation

The isolation of total nucleic acid was made from leaf midribs of symptomatic and asymptomatic trees samples by using Cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle (1990) with some modifications. The composition of various buffers used in protocol is given Appendix-II.

- Fresh phloem tissue (300-500mg) samples taken from leaf midrib and petioles collected from symptomatic and asymptomatic trees were ground into fine powder in liquid nitrogen using pre chilled mortar and pestle. The homogenised product was immediately transferred to autoclaved eppendorf tubes.
- For cell lysis, 1ml pre heated of CTAB extraction buffer at 65ºC was added into each tubes containing homogenised samples and incubated at 65ºC for one hour in water bath with 3-4 intermittent shaking to optimize the enzyme activity in CTAB.

- After one hour of incubation at 65ºC tubes were cooled down to room temperature.
- Once the homogenate attained room temperature, 2/3rd volume of chloroform: isoamyl alcohol (24:1 *v/v*) was added to each tube and content was mixed gently by giving hand inversions for 10 to 15 minutes. Tubes were then centrifuged at 10,000 rpm for 10 minutes at room temperature.
- After centrifugation two phases were formed in each tube aqueous phase and plant debris. Aqueous phase containing DNA was carefully transferred to new 2 ml eppendorf tube without disturbing the interphase.
- 2/3rd volume of ice cold isopropyl alcohol was added to precipitate the DNA and tubes were kept at -20ºC overnight.
- After incubation, tubes were taken out next day and centrifuged at 12,000 rpm for 15 minutes.
- Supernatant was discarded and pellet was washed with 70 percent ethanol twice and again centrifuged at 10,000 rpm for 5 minutes.
- Supernatant was again discarded and pellet was air dried completely to evaporate ethanol and re-suspend the DNA pellet in 100-150 µl of 1x TE (Tris-EDTA) buffer.
- After complete dissolution of DNA pellet in TE buffer it was treated with RNase (1 µl /100 µl) and incubated in pre warmed water bath at 37ºC for 45 minutes and finally stored at -20ºC for further use.

DNA Quantification

The concentration of extracted DNA samples were measured spectrophotometrically at 260nm (A_{260}) absorbance using bio spectrophotometer (eppendorf Biospectrometer® basic, Corston, United Kingdom). Purity of DNA from protein and polysaccharides contamination was assessed by estimating the absorbance ratio at A_{260}/A_{280} and A_{260}/A_{230} respectively. Optical density was recorded at 260 nm wavelength against 1x TE buffer as control. The final concentration was calculated using the formula:

$$Total\ quantity\ of\ DNA\ (\frac{ng}{\mu l}) = \frac{O.D.at\ 260\ \times 50x\ dilution\ factor}{1000}$$

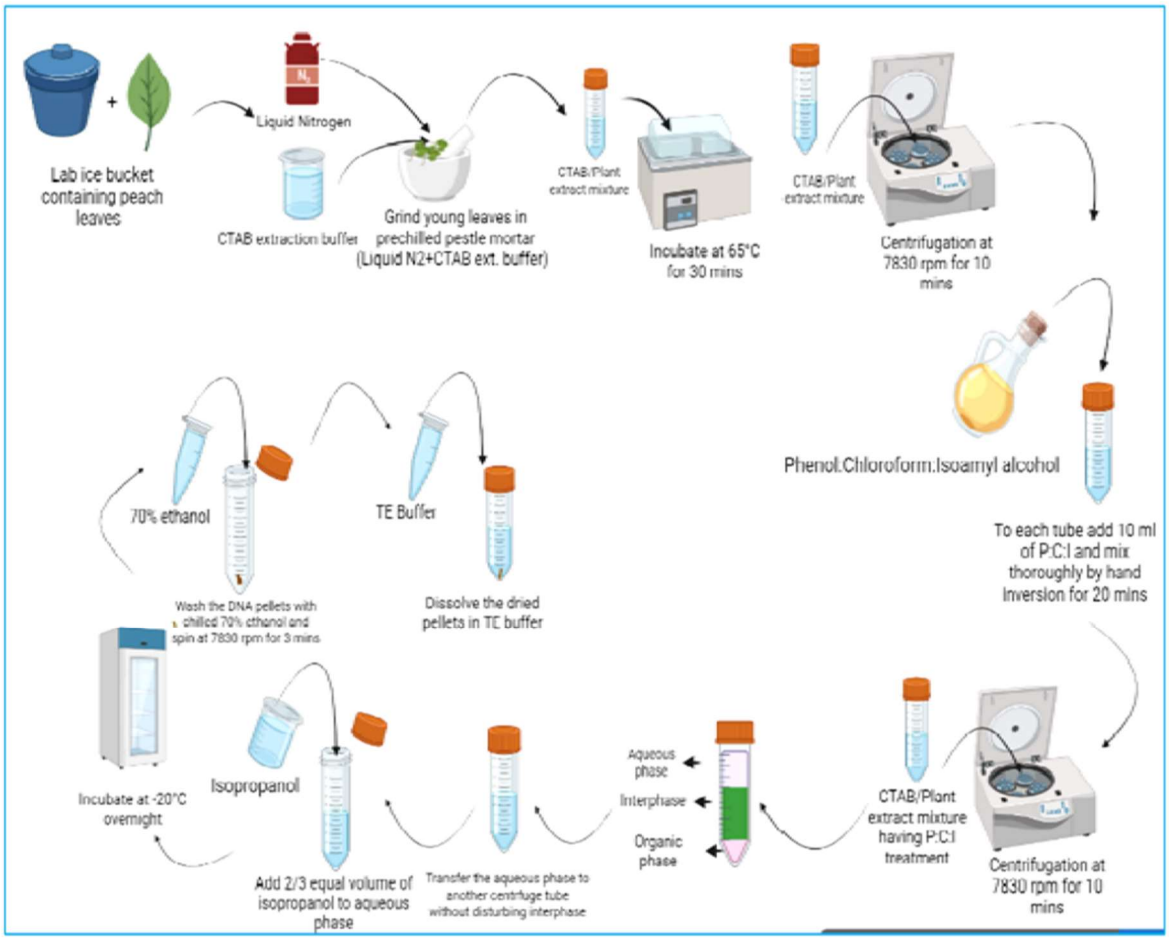

Fig. 2: Schematic diagram of the DNA extraction by CTAB method from plant parts

Agarose gel electrophoresis

DNA samples extracted were observed on 1 per cent agarose gel. To make 1 per cent agarose gel add 1 gm of agarose powder in 100 ml of 1x TAE buffer. The mixture was boiled till agarose dissolved completey to form a clear solution. After cooling the solution to 40-50ºC, 2µl of ethidium bromide (EdBr) was added. The molten gel was finally poured to casting tray with comb settled for well formation. Gel was then kept undisturbed at room temperature to solidify. Once solidified, gel was transferred to tank containing tank buffer (1xTAE) and combs were removed. DNA samples were then loaded in desired sequence along with 6x loading dye in a proportion of 5µl DNA sample and 2µl of 6x loading dye. The samples were run at 75 volts for 45 minutes in gel electrophoresis unit (Cleaver Scientific). Final results were observed by placing the gel in documentation system (BIO-RAD Molecular Imager® Gel Doc™ XR+ Imaging System). Similar procedure was also followed

for observing the PCR amplification results. Chemical reagents used in gel electrophoresis are mentioned in Appendix-III

Nucleic acid amplification

Different sets of universal and specific primer pairs designed for phytoplasma detection were used to amplify 16S rRNA gene in simple and nested PCR. The universal primer pair P1 and P7 were used for the amplification of a 1.8 kbp product of 16S rRNA gene, the spacer region between 16S and 23S rRNA and 5'- end of 23S rRNA gene of phytoplasma genome. After completion of the first cycle with primer pair P1 and P7, PCR products of first cycle were used as template in nested PCR with primer pair R16F2n and $R16FR_2$ after 1:40 dilution. Similarly, the second set of primers were used to amplify the 16S rRNA gene fragment of 1187bp with primer PA2F and PA2R and nested PCR with primer NPA2F and NPA2R after 1:20 dilution of first cycle product. The details of various primer sequences used are given in Table 4.

Table 4: Sequences of universal phytoplasma primers used

Target region	Primer	Primer sequence from 5' to 3' direction	Product size	Reference
16S-23S rRNA	P1	AAGAGTTTGATCCTGGCTCAGGATT	1784 bp	Schneider et al. 1995
	P7	CGTCCTTCATCGGVTCTT		
	R16F2n	GAAACGACTGCTAAGACTGG	1239bp	Lee et al. 1995
	R16FR2	TGACGGGCGGTGTGTACAAACCCCG		
	PA2F	GCCCCGGCTAACTATGTGCA	1187bp	Zirak et al. 2021
	PA2R	TTGGTGGGCCTAAATGGACTC		
	NPA2F	ATGACCTGGGCTACAAACGTGA	485bp	
	NPA2R	GGTGGGCCTAAATGGACTCG		

PCR Reaction mixture

PCR mixture was prepared by using 10x PCR buffer containing 25mM $MgCl_2$, 10mM dNTP mix, 10pM forward and reverse primer each, Taq DNA polymerase (3U/µl), 100ng/µl template DNA and finally adjusted to 25µl by addition of nuclease free water (Table 5). The direct PCR amplification for conserved region using the primer pairs given in Table 4 was carried out in thermocycler (applied biosystems by Thermo Fisher Scientific ProFlex PCR Systems, Massachusetts, United States). The conditions for various primers used for PCR amplification are given in Table 7 to 10. The amplified PCR products were separated by gel electrophoresis on 1 per cent agarose gel stained with ethidium bromide (EdBr) and run at 75 Volt for 45 minutes. The results were visualized using Gel Documentation system.

Procedure for nested PCR

To enhance the sensitivity of PCR, to universal primer pair R16F2n/R16FR2 and NPA2F/NPA2R were used for nested PCR to amplify the 16S-23S rRNA gene. The composition of various components used are listed in Table 6 and the conditions for primers are presented in Table 7 to 10. The final 25µl volume in each PCR tube contain the 10x PCR buffer containing 25mM $MgCl_2$, 10mM dNTP mix, 10pM forward and reverse primer each, Taq DNA polymerase (3U/µl) and template of first PCR cycle product diluted in 1:40 ratio and put for amplification in a thermocycler.

Table 5: Components of PCR reaction mixture used in first PCR cycle

Component	Stock Concentration	Reaction volume in µl	Working concentration
PCR reaction buffer	10x	2.5	1x
MgCl2	25mM	1.0	1mM
dNTP mix	10mM	1.5	2.5mM
Forward primer	100pmol	1.0	10pmol
Reverse primer	100pmol	1.0	10pmol
Taq DNA polymerase	3U/µl	0.5	0.03U/µl
Template		2.0	100ng/µl
Nuclease free water		15.5	-
Total		25.0	

Table 6: **Components of nested PCR reaction mixture used for nested-PCR**

Component	Stock Concentration	Reaction volume	Working concentration
PCR reaction buffer	10x	2.5	1x
MgCl2	25mM	1.0	1mM
dNTP mix	10mM	1.5	2.5mM
Forward primer	100pmol	1.0	10pmol
Reverse primer	100pmol	1.0	10pmol
Taq DNA polymerase	3U/µl	0.5	0.03U/µl
Template		2µl (product of first cycle PCR diluted at 1:40, 1:20)	100ng/µl
Nuclease free water		15.5	-
Total		25.0	

Agarose gel electrophoresis of the PCR products

After completion of second cycle the amplified nested PCR products, 5µl each with 6x gel loading buffer was loaded in 1 per cent agarose gel along with 1kbp or 100bp DNA

marker for size comparison of amplified product. The electrophoresis unit was run at 75V for 45 minutes. After completion results were visualized by using gel documentation system (BIO-RAD Molecular Imager® Gel Doc^{TM} XR+ Imaging System).

Table 7: PCR conditions for primer pair P1/P7

Steps	Temperature (ºC)	Duration (minutes)	No. of cycles
Initial denaturation	95	5	1
Denaturation	95	1	35
Annealing	53	1	
Extension	72	2	
Final extension	72	10	1

Table 8: PCR conditions for primer pair R16F2n/R16FR2

Steps	Temperature (ºC)	Duration (minutes)	No. of cycles
Initial denaturation	94	2	1
Denaturation	94	1	35
Annealing	50	1	
Extension	72	3	
Final extension	72	10	1

Table 9: PCR conditions for primer pair PA2F/PA2R

Steps	Temperature (ºC)	Duration	No. of cycles
Initial denaturation	94	5 min	1
Denaturation	94	30 sec.	35
Annealing	60	45 sec.	
Extension	72	60 sec.	
Final extension	72	10 min	1

Table 10: PCR conditions for primer pair NPA2F/NPA2R

Steps	Temperature (ºC)	Duration	No. of cycles
Initial denaturation	90	5 min	1
Denaturation	90	30 sec.	35
Annealing	60	30 sec.	
Extension	72	45 sec.	
Final extension	72	10 min	1

Elution of DNA

The PCR products were separated on 1 percent agarose gel to observe the presence of specific bands. The specific bands were excised carefully on a UV-transilluminator and was cut by using sterile blade and collected in a 1.5 ml sterile micro-centrifuge tube. The gel

elution was done by using HiYield^{TM}Gel/ PCRDNA Mini kit (Real Biotech). With some minor modifications, the elution was carried out in accordance with the manufacturer's protocol.

- 300mg of cut gel slice was transferred into a microcentrifuge tube and 500µl of DF buffer was added to the tube and mixed by vortexing.
- The tubes were incubated at 55ºC till the gel pieces were completely dissolved. During incubation 2-3 inversions were given.
- A DF column was placed into a collection tube and 800ul of sample mixture was then transferred into DF column. The setup was allowed to rest for a minute and then centrifuged at 13000 rpm for 30 seconds.
- The flow-through was discarded and the DF column was placed back in the collection tube.

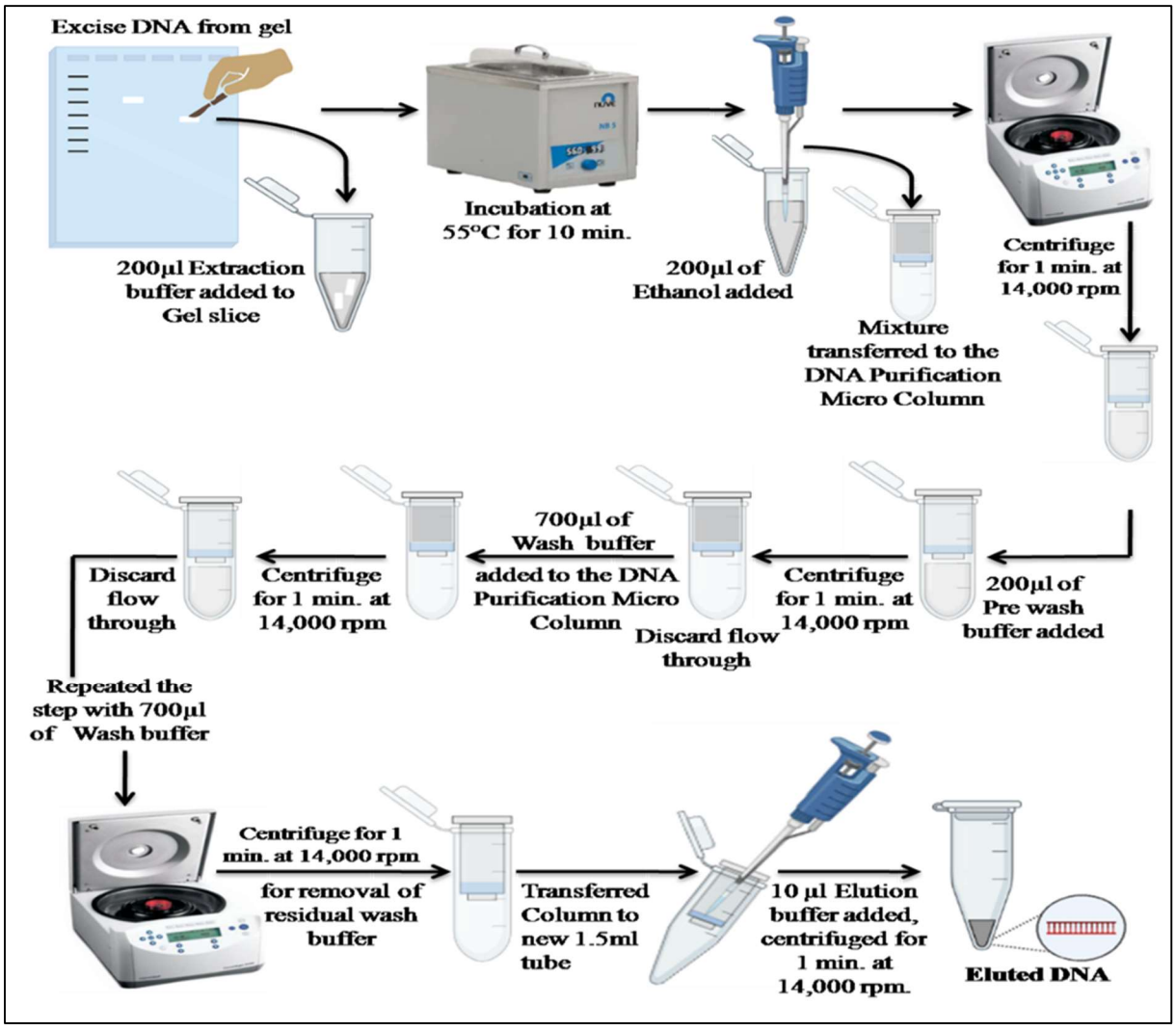

Fig. 3: Schematic diagram of elution of DNA bands for sequencing

- 600µl of wash buffer was added to the DF column and centrifuged at 13000 rpm for 30 seconds. The flow-through was discarded and DF column was again placed into collection tube.
- After drying the DF column by centrifuging it for 2 minutes at 13000 rpm, dried columns were transferred into new collection tube.
- 20-50µl elution buffer was added to the centre of column matrix and allowed to stand for 2 minutes until the elution buffer was absorbed by matrix. Finally the mixture was centrifuged for 2 minutes at full speed to elute purified DNA and stored at -20ºC till further use. Again 2µl of eluted DNA was checked on 1 per cent agarose gel to rule out any possibility of contamination.

Sequencing and submission of sequences to NCBI

The purified PCR products were sent to Eurofins Genomics India Pvt. Ltd., Bangalore for sequencing. The amplicons were sequenced in forward and reverse direction. The sequences obtained were subjected to local pairwise alignment using NCBI-BLASTn tool (https://www.blast.ncbi.nlm.nih.gov/BLAST). The chromatograms of the sequenced samples were analysed for the Q value and nucleotide positions with a Q value of more than 30 was considered to be standard. The sequences obtained from the chromatograms were aligned and assembled with the aid of reference sequence from NCBI GenBank using BioEdit (version 7.2.5) software (Hall 1999) and the nucleotide positions with Q values lesser than the standard were edited based on the aligned sequences. The edited sequences of the representative strains were deposited to GeneBank data library to get the accession number.

Computer analysis of sequencing data

The sequences received were analyzed with the help of tools like

i. BLAST (Basic Local Alignment Search Tool): BLAST is statistically driven method used for comparison of amino acid sequences of different proteins ot nucleotides sequences of nucleic acid. BLAST search compare the query sequence with database of sequences, and thereby identifies database of sequences sharing resemblance with the query sequence above certain threshold (https://www.blast.ncbi.nlm.nih.gov/BLAST).

ii. ClusterW: this is a basic multiple alignment algorithm consisting of three main stages:

1. All pairs of sequences are aligned separately in order to calculate a distance matrix giving the divergence of each pair of sequence.
2. A guide tree is calculated from the distance matrix.
3. The sequences are progressively aligned according to the branching order in a guiding tree (www.ddbj.nig.ac.jp).

iii. MEGA 11(Molecular Evolutionary Genetics Analysis) for constructing phylogenetic trees.

Phylogenetic analysis

The reference sequences of various representative strains of phytoplasma groups and subgroups were obtained from GeneBank. The reference sequence and test sequence were aligned using ClusterW (Thomson et al. 1994). Phylogenetic tree was constructed using MEGA 11 software. Bootstrap analysis was also used to place confidence interval on phylogenies. In this case a sufficient number of 1000 replications performed bootstrap analysis and bootstrap value of 75 minimum was considered well supported for the respective branch. *Acholeplasma laidlawii* was employed to root the tree of 16S rRNA gene employed in the study.

3.3 To screen the available germplasm of cherry for source of resistance against phytoplasma

Screening of cherry germplasm available at government farms and private orchards were conducted to ascertain the sanitary status of cultivars and rootstocks against the causal phytoplasma. Based on the visual symptoms produced by the plants, screening was done by using one to five disease severity category scale (Table 11) (Guo et al. 1996).

Table 11: Disease severity scale

Rating	Disease Severity
1	Leaves discoloured and most shoots and leaves very stunted, very low vigour.
2	Leaves discoloured and most shoots and leaves stunted, low vigour.
3	Leaves discoloured and most shoots and leaves moderately stunted, moderate vigour.
4	Few shoots and leaves with symptoms, high vigour.
5	No disease symptoms, high vigour.

All trees rated 3 or below were considered to be infected by phytoplasma disease. A rating of 4 or 5 indicates little or no effect of disease on tree. Also, DNA-based technique like nested-PCR was employed by using phytoplasma-specific universal primer pair P1/P7 followed by R16F2n/R16R2 that amplify 1200bp fragment of 16S RNA gene sequence of phytoplasma. Controls used were healthy or asymptomatic plants. If the typical bands were observed in gel electrophoresis from PCR product, the sample were considered positive.

Chapter – 4

RESULTS AND DISCUSSION

The present study was undertaken to assess the prevalence and percent incidence of phytoplasma disease affecting the cherry plants in Himachal Pradesh along with molecular identification, characterization and screening of cherry cultivars for resistance. Regular surveys were conducted across major cherry-growing regions to document disease incidence and observe symptoms variation in infected plants. The associated phytoplasma was subsequently characterized using molecular techniques. In addition, biological indexing and host range studies were performed to evaluate the transmission potential of the phytoplasma through grafting and to identify possible alternate host in the region. The findings of this investigation are organized under the following heads:

4.1 Occurrence, distribution and percent disease incidence of phytoplasma disease in cherry growing areas of Himachal Pradesh

4.2 Symptomatology

4.3 Detection

- 4.3.1 Fluorescence Microscopy
- 4.3.2 Biological Assay
- 4.3.3 Nucleic acid based molecular detection

4.4 Potential reservoir hosts of cherry phytoplasma

4.5 Screening

4.1 OCCURRENCE, DISTRIBUTION AND PERCENT DISEASE INCIDENCE OF PHYTOPLASMA DISEASE IN CHERRY GROWING AREAS OF HIMACHAL PRADESH

Surveys were conducted across various cherry growing regions of Himachal Pradesh during 2023-2024, encompassing approximately 100 cherry orchards in Shimla district (Plate 1). The observations were recorded from March to October during the growing season of 2023 and 2024. The percentage of disease incidence, calculated based on the symptoms observed during survey is presented in Table 12.

The data in Table 12 highlights the prevalence of phytoplasma infection in cherry trees across the surveyed locations. Disease incidence across different sites within three

blocks of Shimla district ranged from 0 to 38.8 percent. In Narkanda block, orchards around Baghi town exhibited the highest disease incidence, ranging from 15 to 38.8 percent, with an average incidence of 26.17 percent (Plate 2a). The Matlu location, situated near Baghi town, recorded the second-highest at 21.23 percent (Plate 2b). Conversely, locations such as Kumarsain, Kotgarh and Thanadhar within Narkanda block reported no incidence of disease. Similarly, surveys conducted in Rohru and Mashobra blocks revealed 0 percent disease incidence, as all trees observed were healthy and asymptomatic (Plate 3).

The findings from this investigation indicate that Baghi town of Narkanda block was most severely affected area, with maximum average phytoplasma incidence of 26.17 percent. Various cherry cultivars were grown across the surveyed regions, including Van, Black Heart, Early Liver, Stella, Merchant, Bing, Lapin, Durone Nero, Regina Glory, Decan, Sunburst and Red Heart. Predominant symptoms observed in infected plants included delayed bud break following winters, reduced leaf size, lighter foliage coloration, leaf rolling, uneven fruit ripening, partial infection of branches, reddening and tattering of leaves in the late season.

Global studies on the prevalence of phytoplasma disease on stone fruits reveal significant variation in disease incidence. In North America, X-disease phytoplasma was first identified in cherry trees in Washington State in 1946, with a low initial incidence of approximately 1 percent. However, the disease incidence has increased dramatically in recent years. Between 2015 and 2020, outbreak of X-disease and little cherry disease in Washington and Oregon led the removal of over 2,38,856 cherry trees, equivalent to 974 acres of orchards, as well as 33,082 peach, nectarine, plum and apricot trees across 81 acres. Yakima country of Washington was the most affected, with 33.2 percent of cherry blocks removed due to infection, followed by 16.8 percent in Chelan and 7.7 percent in Wasco country of Oregon (Molnar et al. 2021).

Chen et al. (2022) observed the symptoms such as the development of small branches with few leaves and branches dieback in Cherry blossom trees (*Cerus serrula*), it was found that more than 17 percent plants infected with phytoplasma was later confirmed as *Candidatus* Phytoplasma asteris (Chen et al. 2022). Similarly, a study conducted in 2013 reported sweet cherry trees exhibiting symptoms like floral virescence, wilting of infected branches and eventually tree death. All nine samples collected from symptomatic trees tested

positive for phytoplasma by nested-PCR with sequencing confirming the association of '*Candidatus* Phytoplasma ziziphi' with sweet cherry virescence disease (Wang et al. 2014).

Other studies from Europe have reported phytoplasma groups contributing to cherry plantation, including aster yellows group (16SrI), X-disease group (16SrIII) and apple proliferation group (16SrX) (Paltrinieri et al. 2006). A major factor in the spread of phytoplasma within limited regions is the use and distribution of phytoplasma infected planting material among farmers.

4.2 Symptomatology

Regular surveys of commercial cherry orchards in Shimla district of Himachal Pradesh were conducted to monitor symptom development year-round from 2023 to 2024. The observed orchards include trees ranging from 3 to 15 years old across a variety of commonly cultivated cherry cultivars.

Observations were made throughout different growing seasons, from bud break in spring to the onset of dormancy in early winters. Tracking the same infected trees over two growing seasons revealed a distinct spatial distribution pattern of phytoplasma within cherry trees, which changed over time. Initial infections were characterized by low titer with mild and scattered symptoms. Overtime, pathogen load increased, leading to more severe symptoms.

Early-stage symptoms, typically observed in May and June, included leaf yellowing, little leaf symptoms, leaf reddening, leaf tattering and inward leaf rolling (Plate 4 and 5). Symptoms were initially confined to a few branches with unaffected branches remaining healthy and bearing normal foliage and fruits. Severely infected branches exhibited poor flowering and poor fruit set. Additionally, phloem necrosis was observed in longitudinal sections (Plate 5d).

By August and September, infected trees became easily distinguishable from the healthy ones due to widespread yellow and red discoloration of foliage (Plate 2a and 2b). As the season progressed, infection spreads to additional branches, resulting in leaf discoloration, stunted shoot growth, a gradual decline in tree vigor and eventual tree death. Major scaffold branches dieback typically led to tree death within 3-4 years of symptom onset, while the

younger trees often succumbed within 1-2 years (Plate 6a and 6b). Symptomatic trees exhibited significantly reduced growth and further decline in subsequent growing season.

In India, previous reports of *Candidatus* Phytoplasma species affecting cherry plants were from Jammu and Kashmir. Shreenath et al. (2022) documented symptoms such as leaf rolling, swollen buds, bud proliferation, malformation symptoms and flat branches with swollen nodes. Similarly, a study by Zirak et al. (2021) in East Azerbaijan province of Iran, reported common phytoplasma symptoms in stone fruits including witches's broom, little leaf, leaf rolling leaf scorching, dieback, decline, yellowing, reddening and rosetting with leaf reddening and rosetting being the most prevalent symptoms observed in sweet cherry trees.

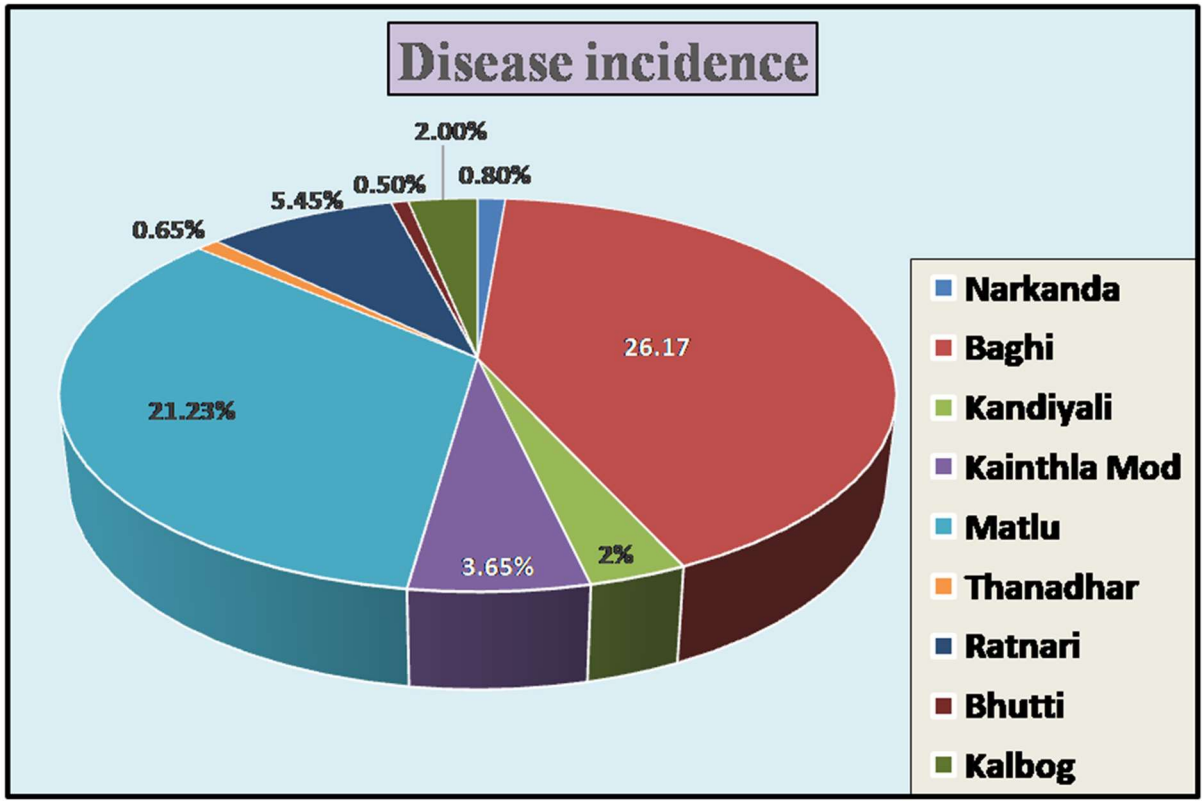

Fig.4: Comparative disease incidence in Shimla district

Furthermore, Franova et al. (2018) reported visual inspection of sweet cherry and sour cherry trees in germplasm collection and old commercial orchards in Czech Republic with symptoms such as bunches of small leaves on old branches, defoliation, decline, shoot proliferation and decline, chlorotic spots, premature leaf yellowing, reddening, and over all tree dwarfing. Trees often exhibited premature leaf fall, particularly during late summer, accompanied by extensive foliage discoloration.

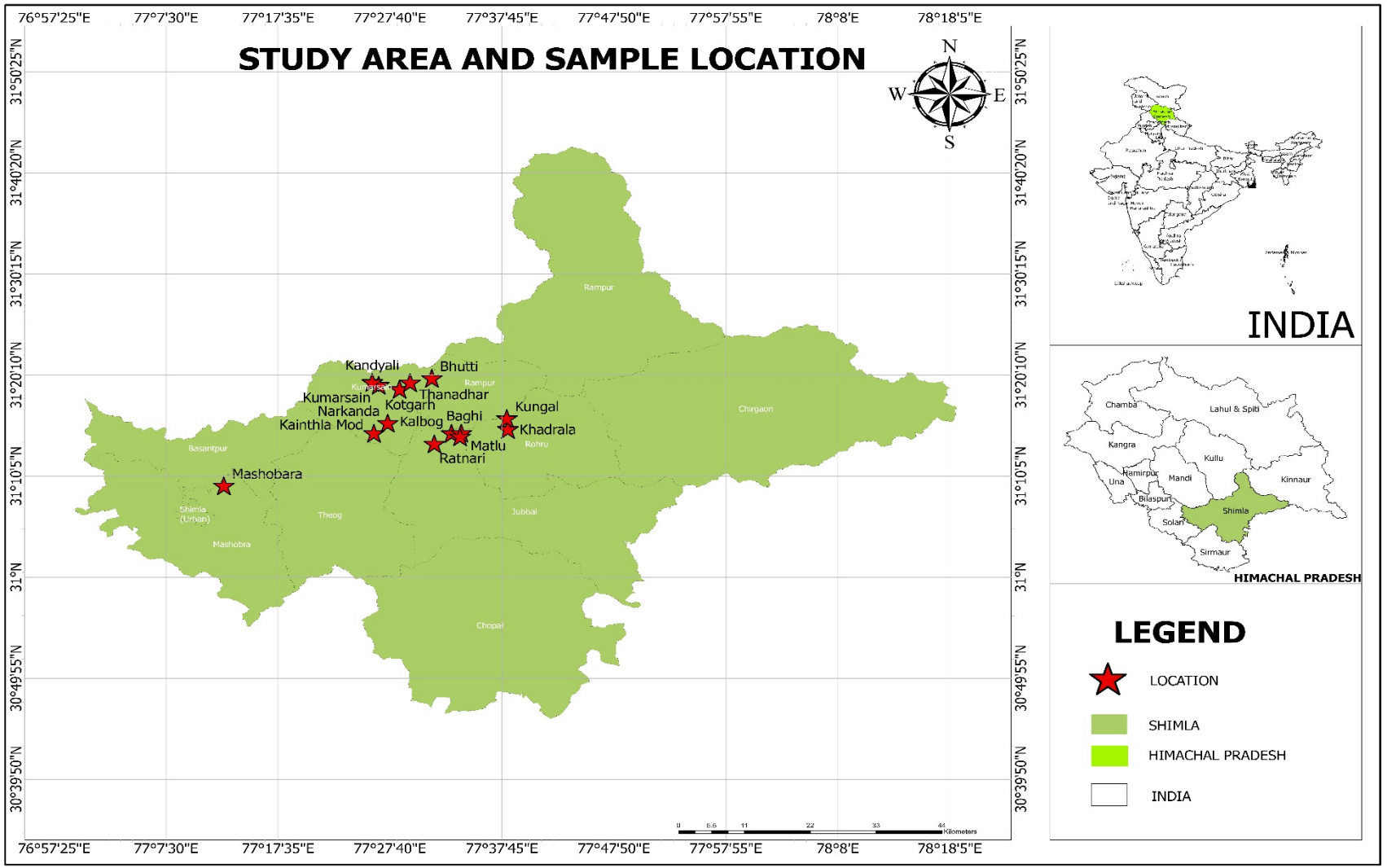

Plate 1: Map of district Shimla showing various locations surveyed

Plate 2a: Severely infected cherry orchard at Baghi

Plate 2b: Severely infected cherry orchard at Matlu near Baghi

Plate 3: Healthy cherry orchards at Kotgarh, Kumarsain and PCDO Khadrala

Table 12: Incidence of phytoplasma disease in different cherry orchards of Shimla district

Block	Location	Cultivar/Variety	Number of trees		Source of planting material	Percent disease incidence	Approximate age of orchard (years)	Characteristic symptom
			Total trees observed	Symptomatic				
Narkanda	**Narkanda**							
	Orchard 1	Black Heart, Stella, Durone Nero	45	0	Colt, Paja	0	15	-
	Orchard 2	Van, Durone Nero	60	2	Colt, Paja	3.3	10	LR,LT,C
	Orchard 3	Early Liver, Van	25	0	Gisela	0	3	-
	Orchard 4	Stella, Merchant	50	0	Colt	0	5	-
	Mean					**0.8**		
	Kainthla Mod							
	Orchard 1	Durone Nero, Black Heart, Van	100	6	Paja	6	15	QD,C
	Orchard 2	Black Heart, Early Liver	52	2	Paja, Colt	3.8	5	LR
	Orchard 3	Durone Nero, Black Heart	30	0	Paja	0	8-10	-
	Orchard 4	Stellla, Durone Nero	125	6	Colt	4.8	12	LR
	Mean					**3.65**		
	Kandiyali							
	Orchard 1	Early Liver, Van	50	2	Colt	4	5-6	LR,LT
	Orchard 2	Stella, Merchant	35	0	Colt	0	4	-
	Mean					**2**		
	Baghi							
	Orchard 1	Black heart, early liver, Duro Neuro, Van	600	130	Colt, Paja	22.5	14-15	C,LT,QD,LR
	Orchard 2	Early Liver, Merchant, Durone Nero	200	56	Colt, Gisela	26.5	5	C,LR,QD
	Orchard 3	Stella, Early Liver, Van	340	120	Paja	35.2	8-10	RLS,LR,LT
	Orchard 4	Van, Stella, Bing, Sam	250	97	Colt	38.8	5-8	PN,QD,RLS,C
	Orchard 5	Durone Nero, Black Heart, Stella, Van	725	148	Paja, Colt	20.4	11-12	LR,LT
	Orchard 6	Black Heart, Durone Nero,	500	75	Paja, Colt	15	15	PN,QD

		Van						
	Orchard 7	Stella, Merchant, Van	150	42	Paja, Colt	28	10-12	C, RLS, PN
	Orchard 8	Merchant, Durone Nero	200	36	Colt, Paja, Gisela	18	>20	QD,PN
	Orchard 9	Merchant, Van	280	86	Colt	30.7	10	LR,LT
	Orchard 10	Early Liver, Black Heart, Van	240	64	Paja, Colt	26.6	>20	LR,IR
	Mean					**26.17**		
	Ratnari (Sundernager, Chilkari, Gandhinagar)							
	Orchard 1	Van, Merchant	58	4	Colt	6.8	8-10	C
	Orchard 2	Black Heart, Van	70	3	Paja, Colt	4.2	10-15	LR
	Orchard 3	Stella, Van	40	0	Colt	0	7-8	-
	Orchard 4	Stella, Van	110	3	Colt, Paja	2.7	12	C, RLS
	Orchard 5	Stella, Van	30	0	Colt,	0	5-6	-
	Orchard 6	Merchant, Stella	50	6	Paja, Colt	12	10-12	LR,LT
	Orchard 7	Merchant, Van	80	10	Colt	12.5	12	LT
	Mean					**5.45**		
	Kalbog							
	Orchard 1	Early Liver, Stella	25	1	Colt	4	4	LR, C
	Orchard 2	Van, Stella	30	0	Colt	0	2	-
	Mean					**2**		
	Matlu							
	Orchard 1	Early Liver, Stella, Durone Nero	350	85	Colt	24.2	15	C, LR, LT, QD
	Orchard 2	Stella, Durone Nero	148	28	Colt	18.9	12	LR, LT
	Orchard 3	Merchant, Stella	300	62	Colt, Paja	20.6	10-12	QD, PN
	Mean					**21.23**		
	Bhutti							
	Orchard 1	Black Heart, Early Liver	350	7	Colt, Paja	1.7	12	LR
	Orchard 2	Van, Bing	200	0	Colt	0	8-10	-
	Orchard 3	Black Heart, Van	150	0	Colt, Paja	0	10	-
	Mean					**0.50**		
	Kumarsain							
	Orchard 1	Early liver, Van	100	0	Colt	0	6	-
	Orchard 2	Early Liver, Black Heart, Van	150	0	Colt, Gisela	0	4-5	-

	Orchard 3	Van, Early Liver	120	0	Colt	0	10	-
	Orchard 4	Black Heart, Stella	80	0	Colt	0	12	-
	Mean					**0**		
	Thanadhar							
	Orchard 1	Stella	50	0	Colt, Paja	0	18	-
	Orchard 2	Early liver, Stella	25	0	Gisela	0	8	-
	Orchard 3	Black Heart, Van	150	0	Colt, Paja	0	10-12	-
	Orchard 4	Merchant, Stella	200	0	Colt, Paja	0	15	-
	Mean					**0**		
	Kotgarh							
	Orchard 1	Stella, Early Liver	60	0	Colt, Paja	0	15	-
	Orchard 2	Black Heart, Early Liver	50	0	Colt, Paja	0	10	-
	Orchard 3	Black Heart, Van	50	0	Colt	0	4-5	-
	Orchard 4	Early Liver, Stella	70	0	Colt	0	10	-
	Mean					**0**		
Rohru	**Khadrala**							
	Orchard1 PCRO Khadrala	Glory, Ragina, Lapin, Benten, Chalan, Coral Champagne	150	0	Gisela-5, Gisela-6, Gisela-12 Mazard, Mahalab, Seedling	0	5 to 15	-
	Orchard 2	Ragina, Merchant	100	0	Gisela-5, Colt	0	12	-
	Mean					**0**		
Mashobra	**Mashobra**							
	Orchard 1	Durone Nero-II, Durone Nero-III, Sun Burst, Merchant, Bing, Sam, Decan, Red Heart, Triamph Domain, Van, Glory	150	0	Gisela-5, Gisela-6, Colt	0	6-8	-
	Mean					**0**		

LR- Leaf Reddening, LT- Leaf Tattering, C-Chlorosis, QD-Quick Decline, RLS-Reduced Leaf Size, LT-Leaf Thickening, PN-Phloem Necrosis, IR- Inward Rolling

Amaral-Mello et al. (2006) pointed out that phytoplasmas may alter the balance of hormones in the host plant, eventually inducing distortions of growth. They also stated that phytoplasmas produce certain proteins, e.g. glucanases and hemolysin-like proteins, which can act as virulence factors. In addition, phytoplasmas import numerous metabolites from the host plant, which eventually could change the physiological equilibrium of the host.

On the other hand, Pracros et al. (2007) indicated that phytoplasma multiplication in the phloem sieve tubes, results in deregulation of floral meristem gene expression, recorded that expression of genes controlling the maintenance of the shoot apical meristem and the floral organ identify were ap3, ag, Ify and regulated resulting in deformations and distortion of infected plants, as the lateral shoot growth is stimulated by the absence of the apical dominance resulting in witches –broom symptoms .

4.3 Detection

4.3.1 Fluorescent Microscopy

Samples collected from symptomatic trees were analyzed for the presence of phytoplasma using fluorescence microscopy. Leaves exhibited prominent symptoms were carefully transported to laboratory in clean polyethene bags placed in an ice box. Fluorescence microscopy, performed with DAPI staining, revealed strong fluorescence in the phloem cells of symptomatic leaves, while healthy leaf samples showed no presence of the bright, phytoplasma-like fluorescent spots within phloem sieve tubes (Plate 7). The observation of fluorescence in in sieve tubes of symptomatic leaves clearly indicates an association between phytoplasma and the infected cherry plants. Furthermore, the likelihood of phytoplasma detection by fluorescence microscopy increases from summers through early winters, peaking in late summers as the phytoplasma titer in sieve tube elements progressively rises with advancement of season.

Multiple studies on the use of fluorescence microscopy for phytoplasma detection have demonstrated that DAPI stain preferentially binds to the (A+T)-rich regions of phytoplasma DNA. To enhance specificity, phloem tissue, where the pathogen resides were selected for examination. Moreover, mature sieve elements are devoid of organelles, meaning that healthy mature sieve tubes do not exhibit fluorescence. Das and Mitra (2004) used fluorescence microscopy to detected brinjal little leaf phytoplasma, observing intense bluish fluorescent spots irregulary distributed within the tissues. Phytoplasma presence was

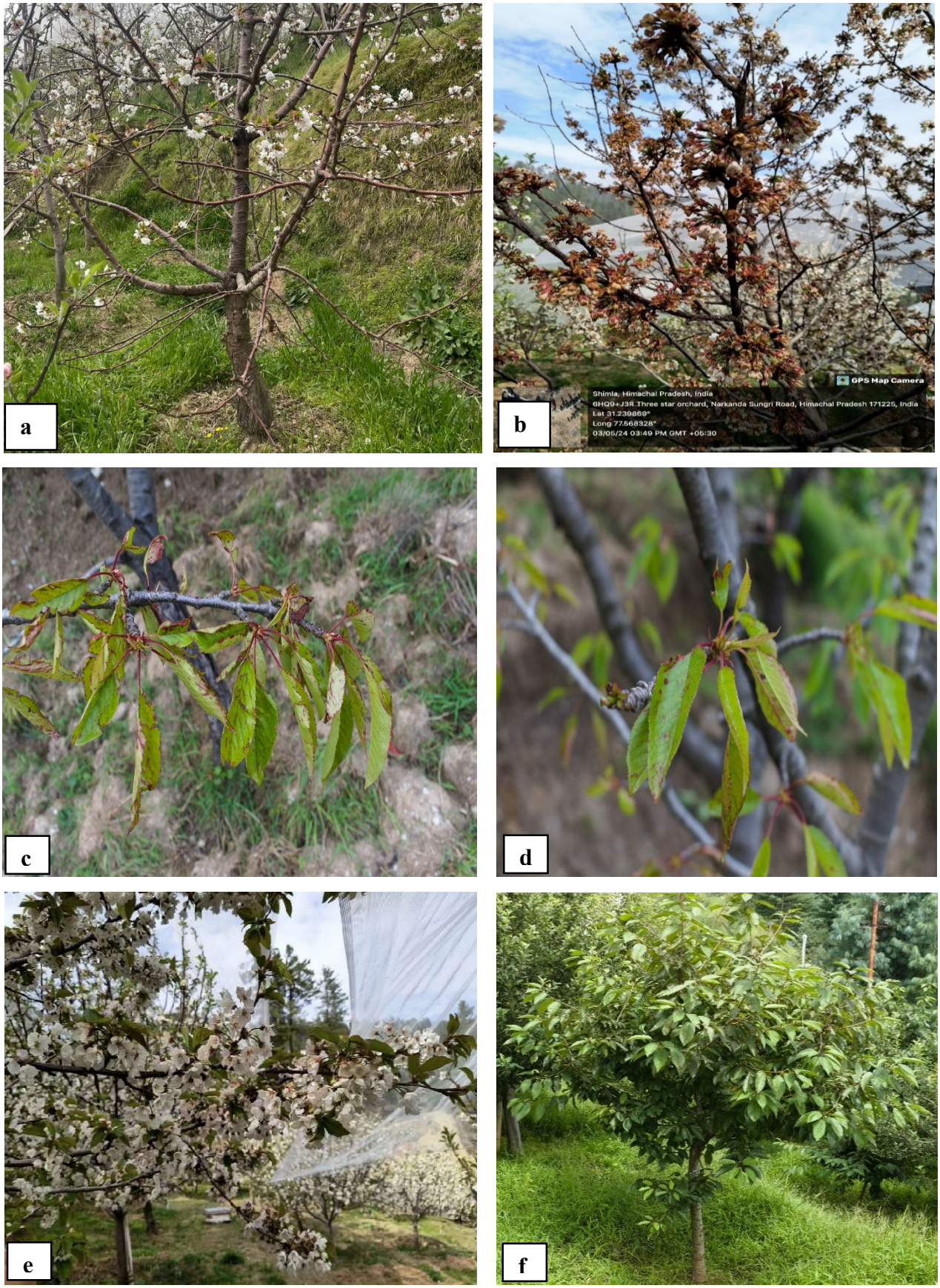

Plate 4: Symptoms of phytoplasma infection on cherry plants: a) Sparce and delayed flowering, b) dried flowers on infected branch, c) d) leaf chlorosis and reduced leaf size, e) normal flowering on healthy branches and f) healthy tree

Plate 5: Symptoms of phytoplasma infection on cherry leaves and twigs : a) Reddening and tattering of leaves, b) inward leaf rolling, c) healthy leaves, d) necrosis in phloem tissue e) healthy twig with no necrosis

Plate 6a: Quick decline and premature death of severely infected tree

Plate 6b: Progression of symptoms over time on infected cherry tree a) Partial infection on cherry tree (August 2023), b) decline of infected tree (July 2024), c) dead tree (September 2024),

confirmed in all six samples five weeks post-graft inoculation with infected plant material. Similar results were observed by Thakur et al. (1998) in peach trees from the northwestern region of India.

Ledere and Seemuller (1992) further validated the presence of mycoplasma in five *Prunus* species including peach, apricot, almond, Japanese plum and flowering cherry showing foliar symptoms like stunting and decline. Moderate to high concentration of phytoplasma was detected in rootstock and scion of flowering cherry. Additionally, scion wood from trees showing sparse foliage and reduced vigor were grafted on various *P. avium* rootstock (F12/1, G1, G3 and G5). DAPI staining phytoplasma presence in most parts of all rootstocks. In 1999, Jarausch et al. confirmed the presence of European Stone Fruit Yellows (ESFY) in *P. domestica* during spring and summers, 2 to 3 years after graft inoculation using DAPI staining.

4.3.2 Biological detection

Biological assay for detection of phytoplasma in cherry plants were conducted by grafting infected and healthy scion wood, obtained from previously marked trees, onto one-year-old rootstocks (Colt) at KVK Rohru and in department's net house. Grafted plants were regularly monitored for symptom development throughout the growing season. A total of 40grafts were performed at both the locations (Plate 8a). Over time, some grafted plants exhibited reduced growth and delayed graft failure (Plate 8b). Further detection was carried out using PCR-based techniques to identify phytoplasma presence in grafted plants during late summers (August-September) of the same year. Out of 30 successful grafts, 20 percent (6 plants) tested positive for phytoplasma, with bands detected at 1200 bp using universal primers. This confirmed the presence of phytoplasma in graft-inoculated plants. However, no typical phytoplasma symptoms appeared during 2024 growing season, aside from slow growth in positive tested grafts. This lack of symptom development may be attributed to low phytoplasma titer in the grafted plants, which are expected to increase in subsequent years. Despite the absence of visible symptoms, pathogen presence was confirmed in several plants via PCR-based detection technique, such as nested-PCR, which is highly sensitive.

The results align with the findings of Yavuz et al. (2011) and Caglayan et al. (2014) who reported graft transmission of *Candidatus* Phytoplasma pruni in wild apricot and B29 rootstock, as well as *Candidatus* Phytoplasma pyri in pear. In their studies, 50 grafts of each cultivars were made and kept in screen house for symptom observation. The only symptoms

observed in inoculated plants were dwarfing and weak shoot growth compared to healthy control. Nested-PCR assay confirmed 6 percent transmission rate for *Candidatus* Phytoplasma pyri and 18 percent transmission rate for *Candidatus* Phytoplasma pruni one year after graft inoculation. Similar results were reported in Japanese plum and apricot varieties. Five out of ten apricot varieties and all the Japanese plum plants grafted with ESFY-infected planting material tested positive for ESFY via PCR. Symptoms in apricot included little leaves and dying young branches, while in Japanese plum, only one plant exhibited witches' broom symptoms (Pastore et al. 2001).

4.3.3 Nucleic acid based molecular detection of cherry phytoplasma

4.3.3.1 DNA isolation

Leaf samples from symptomatic cherry trees, collected during 2023 and 2024, were transported to laboratory and initially subjecting to DAPI for primary detection. These samples were then used for DNA isolation, which was further used for PCR-based detection. DNA was extracted from both infected and healthy control leaves, sampled across the spring, summers and autumn season, following the procedure outlined in Figure 2 of the Material and Methods section. The DNA yield and quality were quantified by Biospectrometer (Eppendorf, Germany), with the results presented in Table 13. Additonally, DNA was isolated from various parts of the symptomatic trees, including the leaf lamina, leaf midribs, petiole, flowers, fruits, pedicle and woody stem. Plate 9a illustrates the isolated DNA from plant samples, visualized through electrophoresis on 1 percent agarose gel.

Table 13. DNA quantification by using Biospectrometer

Sample no.	Conct. (ng/µl or µg/ml)	$A_{260/280}$
1.	106.5	1.82
2.	113.8	1.80
3.	96.7	1.90
4.	110.2	1.89
5.	98.5	1.82
6.	103.5	2.10

4.3.3.2 PCR amplification of phytoplasma DNA with universal primers

To confirm the presence of phytoplasma in symptomatic plants, DNA from symptomatic and healthy plants were isolated and subjected to nested PCR assays. The assays utilized two sets of universal primer pairs: P1/P7 followed by R16F2n/R16R2 and PA1F/PA2R followed by NPA2F/NPA2R, targeting the 16S-23S rRNA region of the

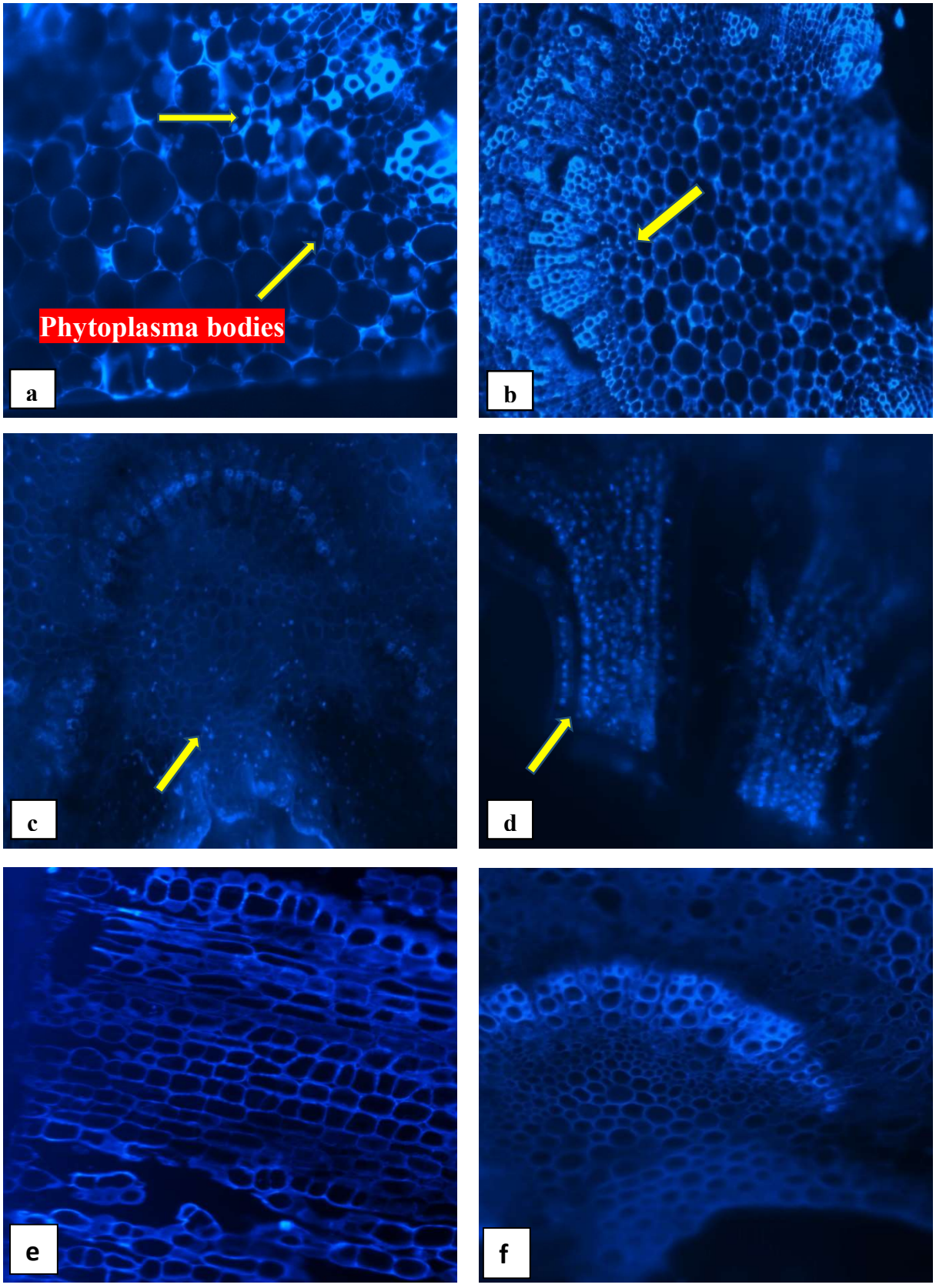

Plate 7: Fluorescent micrograph of cross-section of a), b), c), d) infected cherry leaf midrib e) and f) healthy leaf midrib

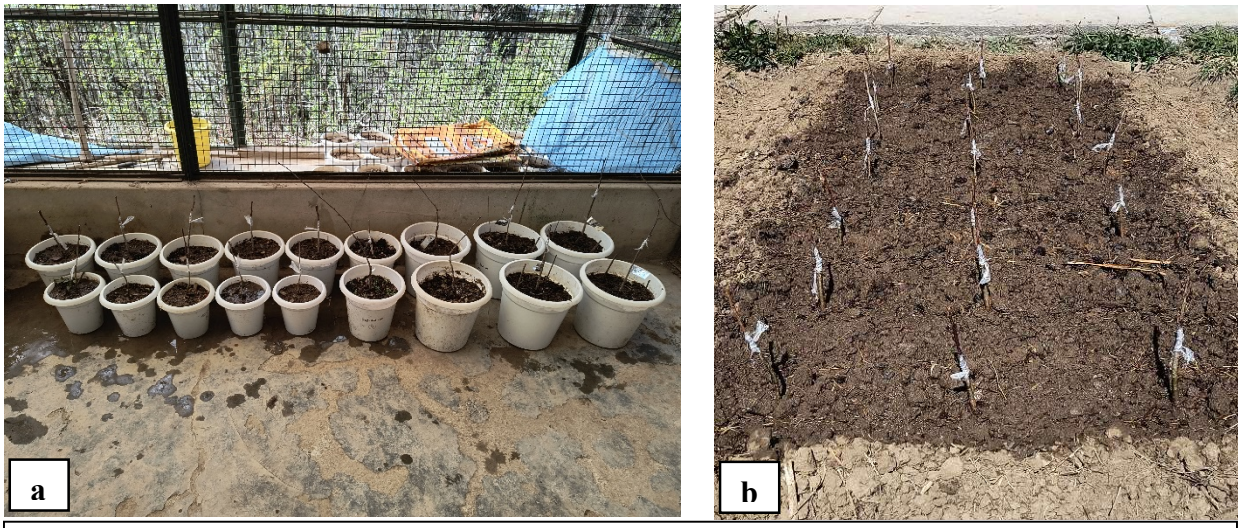

Plate 8a: Grafted budwood from infected cherry plants a) maintained in screen house at the university main campus b) KVK Rohru

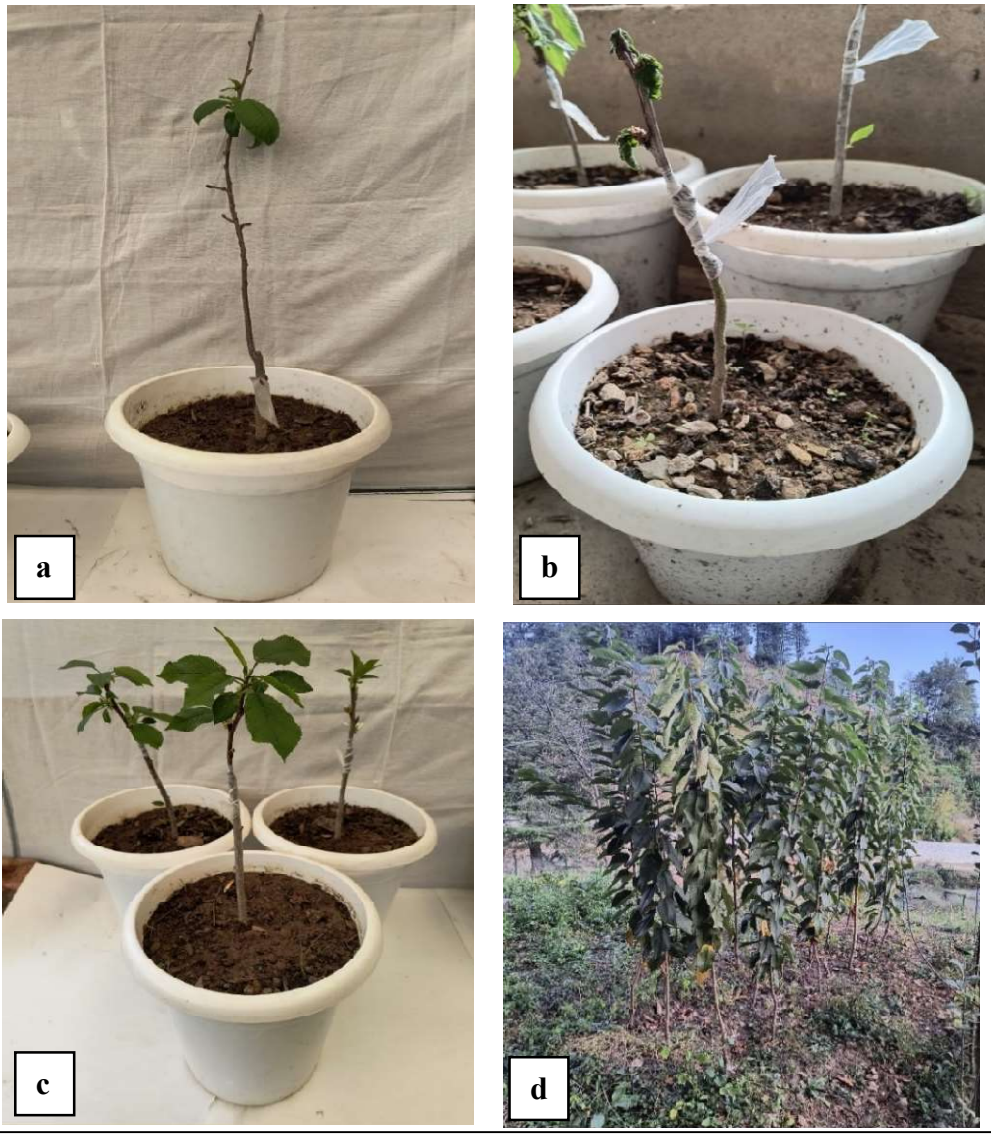

Plate 8b: Grafted plants showing symptoms of phytoplasma infection a) reduced growth b) delayed graft failure c) and d) Healthy plants maintained in screen house at the university main campus and KVK Rohru

phytoplasma genome. The first cycle of PCR, using the universal primers (P1/P7 and PA2F/PA2R), generated amplified products which served as templates for the second PCR cycle, utilizing primers R16F2n/R16R2 and NPA2F/NPA2R, respectively. Reaction mixture were prepared as described in Table 4 and 5 and gradient PCR was conducted at various annealing temperatures (46ºC, 48ºC, 50ºC, 52ºC, 54ºC, 56ºC, 58ºC and 60ºC). Additional components, including the dNTP mix (10mM) at concentration of 1.5, 2.0 and 2.5µl and $MgCl_2$ at 1, 1.5 and 2mM, were tested to optimize PCR amplification. The sharpest bands were observed at an annealing temperature of 54ºC and 50ºC for the first set of primers and 60ºC for second set of primers. Optimal concentration of dNTP (1.5µl) and MgCl2 (1mM) were determined for final PCR amplification, as detailed in Table 6, 7, 8 and 9 for respective primers. After standardizing the PCR conditions, DNA samples from other plant parts were tested.

Following PCR amplification, the samples were visualized on 1.2 percent agarose gel via electrophoresis. The first set of primer produced 1200 base pair amplicon (lane 1-6), while the second set amplified a 485 bp fragment specific to diseased plant samples (lane 1-7) with no amplification observed in asymptomatic leaf DNA (lane 8). The amplified products of both the primer sets were compared with a 1,000 and 100 bp marker ladder (lane M) (Genei^{TM}, Bangalore) respectively (Plate 9b and 10a). Among the plant parts tested, leaf midribs and petiole showed clear bands, while other parts such as leaf lamina, flower, fruits, pedicle and woody stem showed faint or no bands (Plate 10b). These findings suggest that phytoplasma titer is highest in vascular tissues during late summer, while other plant parts have either a low titer or no detecteble phytoplasma.

Similar detection methods were used by Henrich et al. 2001 to efficiently detect phytoplasma in infected trees, using PA2F/R and NPA2F/R primers to reduce detection time and minimise the risk of false positive results. The set of primers can detect 19 different phytoplasma strains from various phylogenetic groups. Zirak et al. (2021) also used these primer for detection and characterizing phytoplasma in stone fruits in Iran, concluding that PA2F/R and NPA2F/R primer were the most effective for detection of phytoplasma with a 485bp amplicon found in 37 percent of symptomatic trees. In contrast, the P1/P7 and R16F2n/R16R2 primer pair failed to amplify phytoplasma DNA from many symptomatic tree. Nested-PCR utilizing two universal primer pairs, bypasses the inhibitory effects present in DNA and enhance the specificity of target DNA detection. It also increases sensitivity, especially for detecting low-titer phytoplasma in perennial tree crops, where the pathogen is

unevenly distributed and present in low concentration. Thus, the low titer in woody host and the presence of inhibitors justify the need for nested-PCR to improve detection specificity and sensitivity.

The present study also showed that during the early stage of disease, no phytoplasma was detected even in symptomatic plants. However, as the season progressed and symptom severity increased, PCR detection technique became much more efficient. Phytoplasm titer varied significantly throughout the growing season, from very low in April to substantial increase by September. This pattern is consistent with findings by Jarausch et al. (1999) in ESFY and similar studies by Wright et al. (2022) which observed uneven and scattered symptom development in the early stage. Observations made were in line with the findings of Wright et al. who observed scattered and non-uniform symptom development in the early stages. Using real-time quantitative PCR (qPCR), they demonstrated a correlation between phytoplasma titer and symptom severity showing that the infection progresses in stages, initially spreading basipetally, accumulating in roots and later populating aerial parts. As titer increases, symptom severity follows.

4.3.3.3 Sequencing

To confirm and perform phylogenetic analysis of the phytoplasma associated with with cherry disease, the specific bands obtained from PCR were excised from agarose gel and subjected to sequencing. The PCR products were sequenced using Sanger sequencing at M/S Eurofins IT Pvt. Ltd., Bangalore India. The resulting nucleotide sequences of the 16S-23S rRNA region were analyzed and submitted to NCBI GeneBank, yielding accession numbers PQ145172 and PV248702. BLAST analysis revealed that these sequences shared high homology with *Candidatus* Phytoplasma ziziphi-related strains, with 98.86 percent identity for PV248702 and 86 percent for PQ145172.

4.3.3.4 Phylogenetic Analysis

Phylogenetic analysis was conducted using Mega 11 software. A phylogenetic tree was constructed using partial 16S rDNA sequences of the submitted sequences (Accession No. PQ145172 and PV248702) and other *Candidatus* Phytoplasma strains deposited in NCBI, using Neighor-Joining method. Bootstrap analysis with 1000 replicates was performed to assess the robustness of internal branches. *Acholeplasma laidlawii* (Accession no. NZ LZGE01000019) was used as outgroup. The phylogenetic tree shown in Plate 11a was

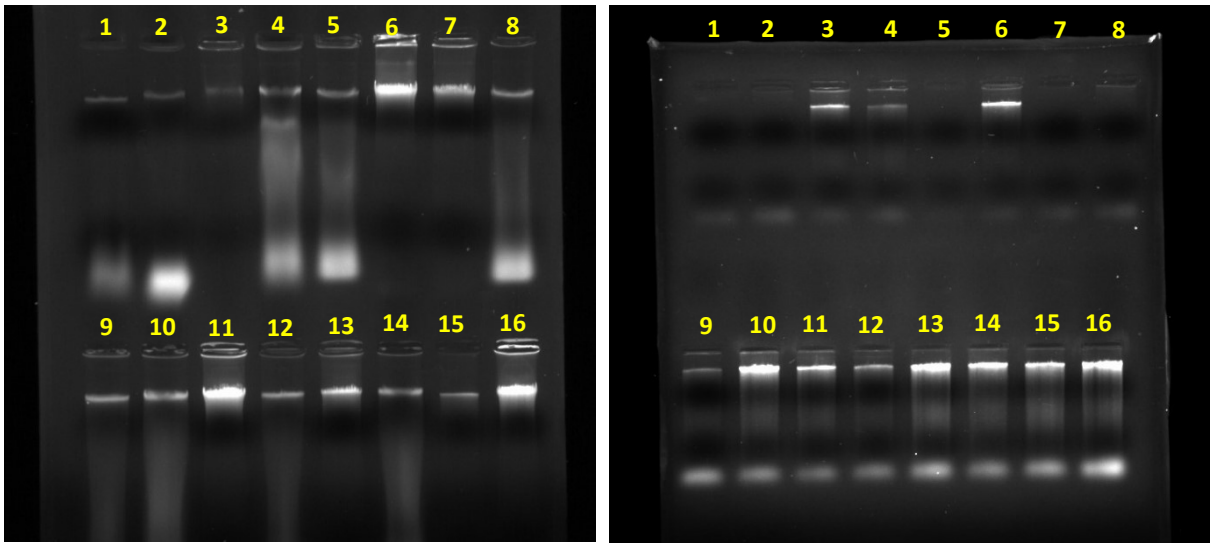

Plate 9a: Isolated DNA from healthy and infected cherry plants during different growing seasons

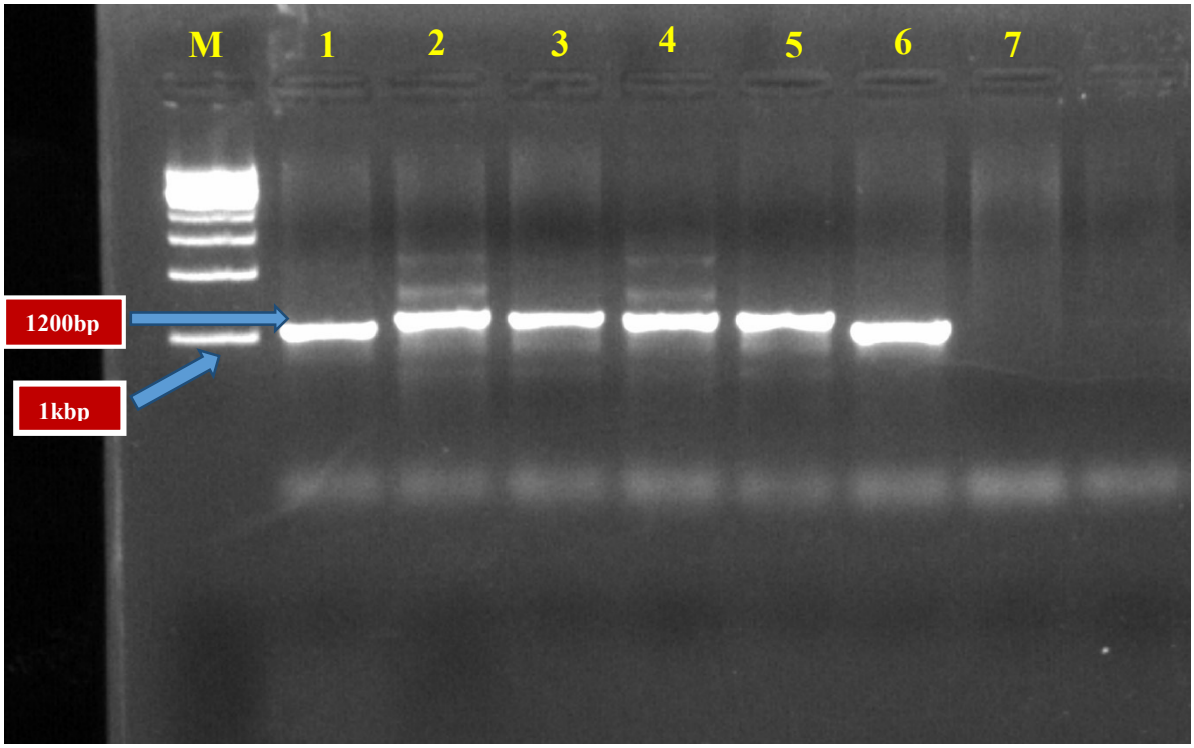

Plate 9b: Amplified bands of PCR product on agarose gel using primer pair P1/P7 and R16F2n/R16R2 showing bands at ~1200bp. Lane M-1kbp marker, lane 1-6 diseased samples , lane 7- Healthy control

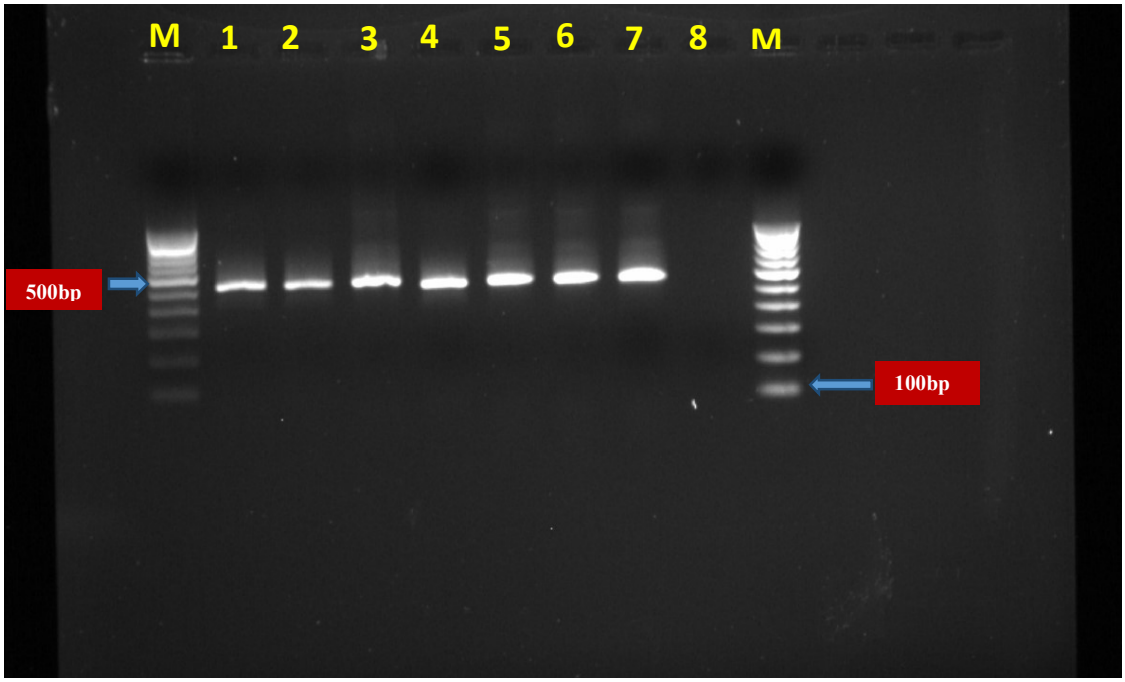

Plate 10a: Amplified bands of PCR product on agarose gel using primer pair PA2F/PA2R and NPA2F/NPA2Rshowing bands at ~485 bp. Lane M-100 bp marker, lane 1-7 diseased samples, lane 8- Healthy control

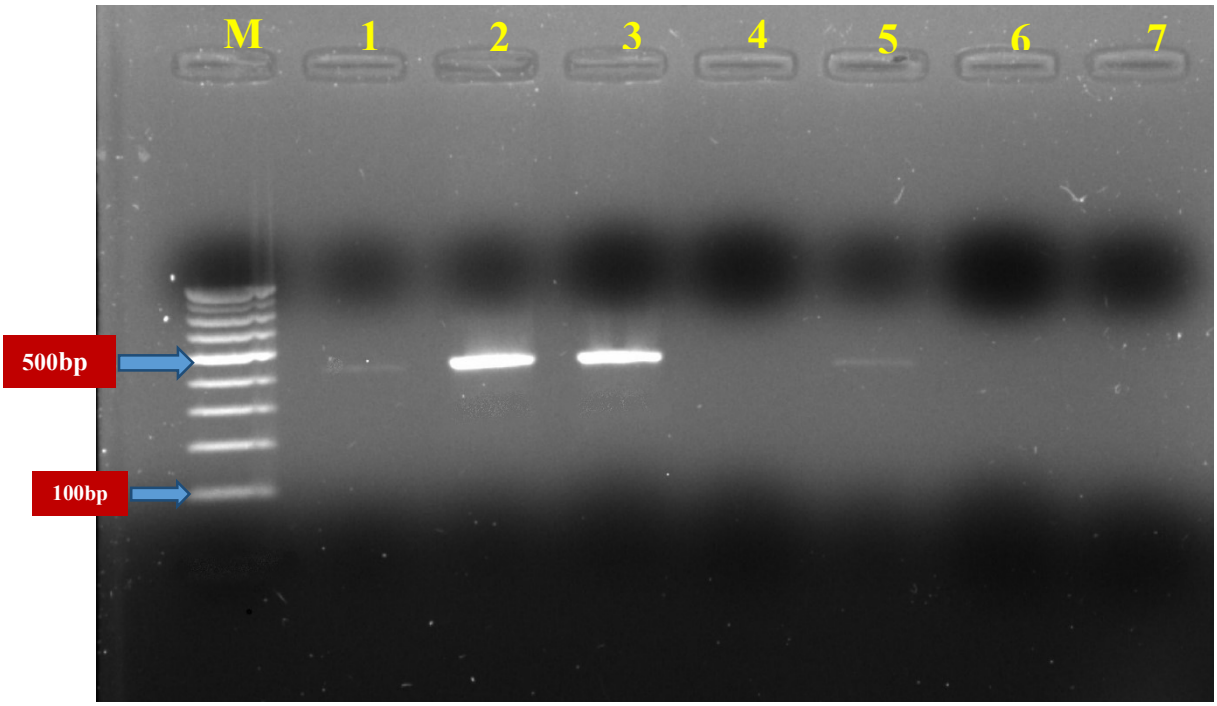

Plate 10b: Amplified PCR products from different plant parts: lane M-100bp marker, lane 1-Leaves, lane 2- Midrib, lane 3-Petiole, lane 4-Flower, lane 5-Bark, lane 6- Fruits, lane 7- pedicle

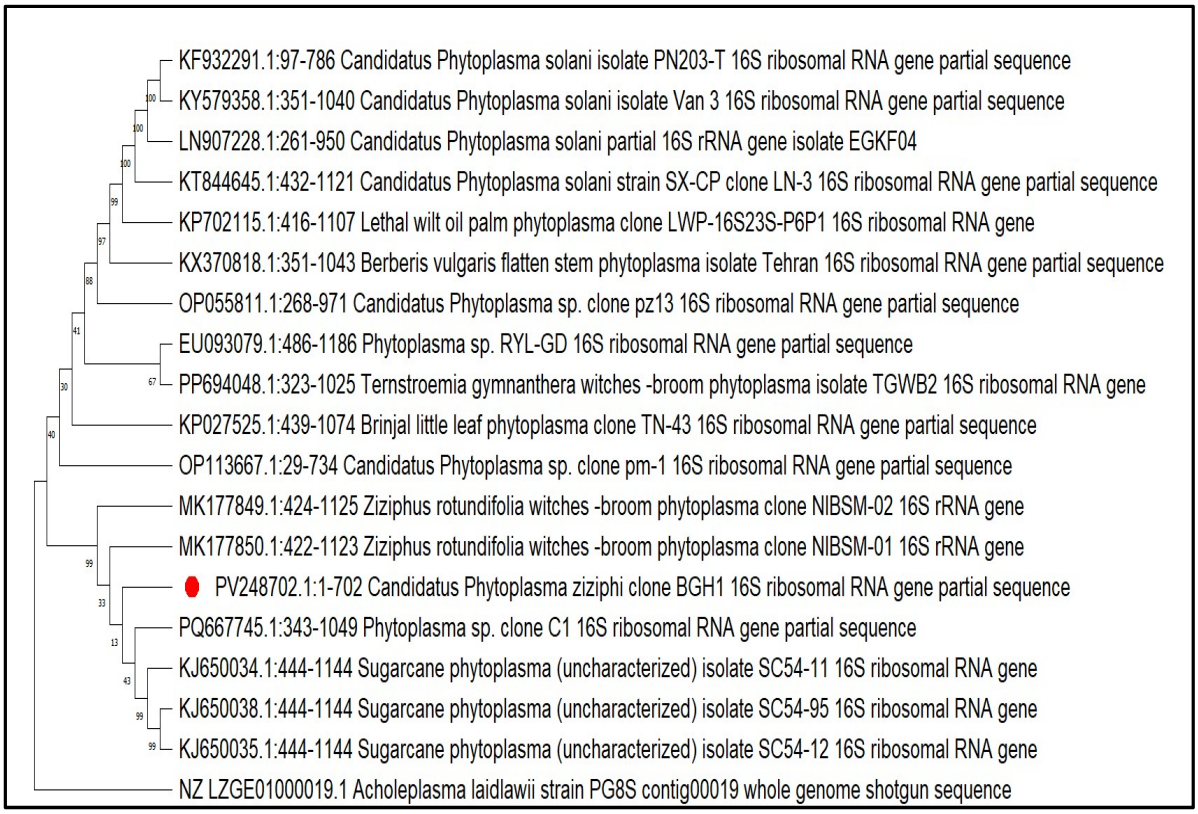

Plate 11a: Phylogenetic tree of *Ca.*phytoplasma strain Acc. No. PV248702 with other NCBI strains

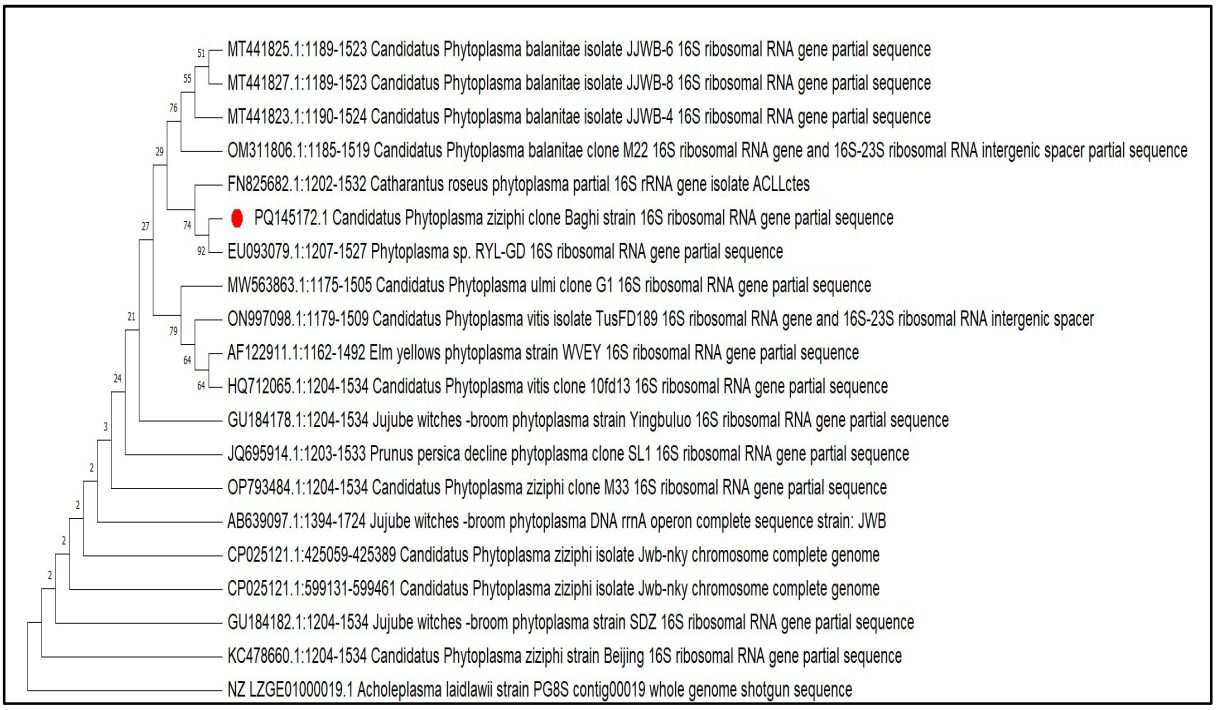

Plate 11b: Phylogenetic tree of *Ca.*phytoplasma strain Acc. No. PQ145172 with other NCBI strains

constructed using the sequence of accession no. PV248702 and compared with 15 to 20 other *Candidatus* Phytoplasma sequences from NCBI 98.86 percent similarity with *Candidatus* Phytoplasma ziziphi causing witches' broom disease in *Ziziphus rotundifolia* (GeneBank Accession no. MK177850). Similarly, Plate 11b shows the phylogenetic tree for accession no. PQ145172, which clustered with *Candidatus* Phytoplasma ziziphi.

4.4 Potential reservoir hosts of cherry phytoplasma

In 2024, a series of surveys were conducted around cherry orchards to investigate the presence of potential reservoir host in the surrounding area. Visual observations were made to assess symptoms on nearby plant species, including *Cannabis sativa, Ageratum conyzoides, Urtica dioica* (Nettle)*, Datura stromonium, Fern* spp. and *Rubus* spp. Leaf and stem samples were collected for further analysis in the laboratory to confirm the presence of phytoplasma. During the survey, critical symptoms such as leaf yellowing, witches' broom, little leaf, dwarfing and dense shoot proliferation were observed in plants species near cherry orchards (Plate 12a).

Phytoplasma detection in symptomatic plant was initially performed using DAPI staining. The results showed distinct blue fluorescence in the phloem sieve tubes of symptomatic plants while no fluorescence was present in some other species. This fluorescence confirmed the presence of phytoplasma infection in *Urtica dioica* (Nettle) and *Fern* spp. only. However, no fluorescence was detected in *Cannabis sativa, Ageratum conyzoides, Datura stromonium, Rubus* spp. and some *Fern* spp. indicating an absence of phytoplasma infection in these plants.

The positive DAPI test samples were further subjected to molecular analysis where DNA was isolated and nested-PCR assay was performed using the same PCR conditions and chemical concentrations applied to cherry samples. Both *Urtica dioica* (Nettle) and *Fern* spp., which had shown positive results in DAPI test, also displayed the presence of DNA bands at 485 bp in nested-PCR amplification using primer PA2F/PA2R and NPA2F/NPA2R. Lane 1 represents the PCR amplification from nettle and lane 2 from fern, with lane M containing 100bp DNA marker (Plate 12b).

The current findings are consistent with the research conducted by Khan et al. (2013) which identified a *Cadidatus* Phytoplasma ziziphi related strain belonging to elm yellows group (16SrV-B), as the causative agent of peach yellow leaf roll disease in peach trees. Similarly, phytoplasma-like symptoms were observed in *Ziziphus jujube* and *Z. numularia*

plants in Utter Pradesh. BLAST comparision of the 16S rDNA sequences from both plant species revealed 98 percent similarity with elm yellow group (16SrV) with accession no. AB052857 and AB052877 (Khan et al 2008). This same group has also been reported infecting the other plant species including *Tamarindus indica* and *Rosa damascena* in India (Kirdat et al. 2019; Saeed et al. 2016). The presence of elm yellows group phytoplasma in various plant species suggests that *Ca.* Phytoplasma ziziphi have a broad host range and can persist year-around in plant surrounding cherry orchards. The present study also indicates the potential role of nettle and fern species as reservoir hosts for *Candidatus* Phytoplsma ziziphi.

4.5 Screening

A significant variation in infection severity was observed among the trees ranging from severe symptoms to no visual sign of disease. More than 50 percent of the trees surveyed were classified as diseased based on disease rating scale with rating of 4 or lower, while the remaining trees with a rating 5 were considered healthy (Table 14). PCR testing of symptomatic samples revealed that most were infected with phytoplasma, as evidenced by a 1200 bp band in gel electrophoresis. Nearly all symptomatic samples tested positive for phytoplasma using nested-PCR, while the samples from asymptomatic trees were negative.

Table 14: Detection made based on disease severity categories

Disease Symptoms	Disease Rating	No. of samples	Nested-PCR
Leaves discoloured and most shoots and leaves very stunted, very low vigour	1 (D)	30	+
Leaves discoloured and most shoots and leaves stunted, low vigour.	2 (D)	45	+
Leaves discoloured and most shoots and leaves moderately stunted, moderate vigour.	3 (D)	55	+
Few shoots and leaves with symptoms, high vigour.	4 (D)	85	+
No disease and high vigour	5 (H)	170	-
	Total	**330**	

D= Diseased, H= Healthy

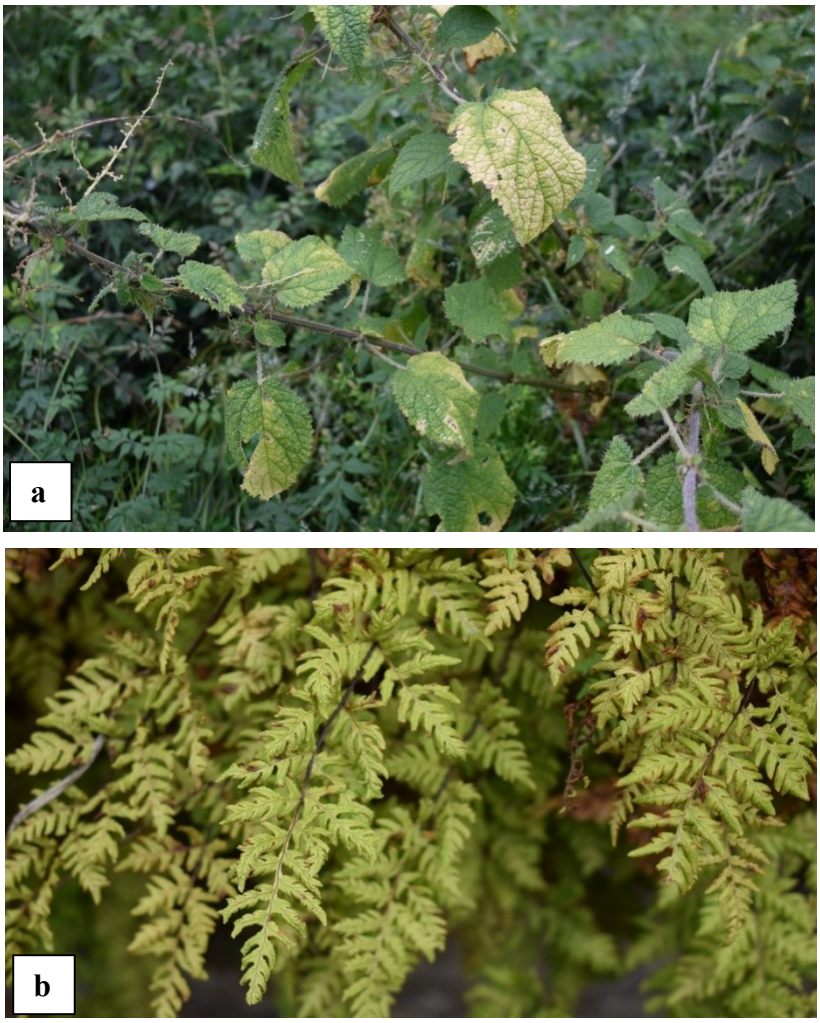

Plate 12a: Potential reservoir hosts of phytoplasma a) *Urticadioica (*Nettle) and b) *Fern* sp.

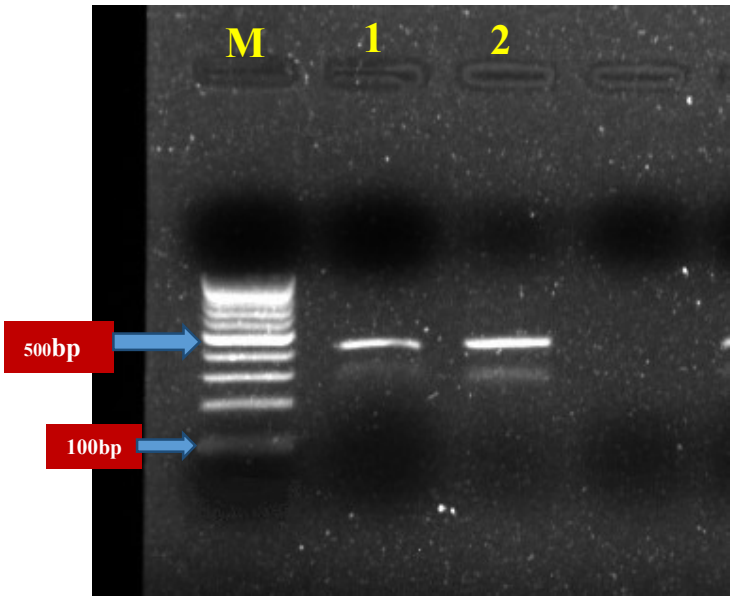

Plate 12b: Amplified bands of PCR product on agarose gel using primer pair PA2F/PA2R and NPA2F/NPA2R at ~485 bp in weeds. Lane M- 100bp ladder, lane 1- Nettle, lane 2-Fern sp.

The screening also included various cultivars grown by farmers, based on symptom development. Among the cultivars Van, Stella, Merchant, Early liver, Black Heart and Durone Nero characteristic symptoms such as leaf reddening, inward rolling, tattering, reduced size of leaves, phloem necrosis, quick decline and ultimately death of tree were observed. The Black Heart cultivar exhibited the highest percent incidence at 34.44 percent, followed by Van at 32.50 percent (Table 15). In contrast, no symptoms were observed in cherry cultivars Glory, Regina, Lapin, Benten, Chalen and Bing which were primarily grown at the Regional Horticulture Research and Training Station (RHRTS), Mashobra and Progeny Cum Demonstration Orchard (PCDO), Khadrala. The investigation revealed that only the cultivars Van, Stella, Merchant, Early liver, Black Heart and Durone Nero grown by farmers of Baghi area were susceptible to phytoplasma infection, whereas the cultivars at RHRTS, Mashobra and PCDO, Khadrala exhibited some resistance to disease.

Table 15: Screening of cherry cultivars for resistance against phytoplasma disease

Sr. no.	Cultivars	Total no. of trees		No. of orchards taken for observations	Percent disease incidence	Characteristic symptoms
		Total trees surveyed	Infected			
1.	Van	80	26	18	32.5	LR, LT,C
2.	Merchant	105	10	20	9.52	LR
3.	Stella	118	12	12	10.16	C,LR
4.	Black Heart	90	31	9	34.44	RLS, LR,IR
5.	Early Liver	68	20	7	29.41	QD, PN
6.	Durone nero	70	22	15	31.42	LR, IR, C
7.	Glory	25	0	4	0.00	-
8.	Ragina	30	0	4	0.00	-
9.	Lapin	25	0	2	0.00	-
10	Benten	24	0	1	0.00	-
11.	Chalen	15	0	1	0.00	-
12.	Bing	20	0	2	0.00	-

LR- Leaf reddening, LT- Leaf tattering, C- Chlorosis, RLS- Reduced leaf size, IR- Inward leaf rolling, QD- Quick Decline and PN- Phloem necrosis

Due to the lack of effective measure to control the phytoplasma disease in perennial trees, developing resistant cultivars is considered a preferred management strategy. Screening of germplasm is commonly employed to identify the source of resistance to pathogen in various breeding programs. Symptom-based detection, along with more sensitive and specific

techniques like nested PCR can also be utilized to screen for phytoplasma infection in other species. A similar approach was undertaken by Guo et al. (1998) who screened chokecherry trees for X-disease phytoplasma infection. In their study, 1,792 chokecherry trees were initially screened by observing characteristic disease symptoms, followed by monoclonal antibody assay and nested-PCR. The combination of these three techniques proved to be an effective and efficient method for examining a large number of planting material.

Chapter – 5

SUMMARY AND CONCLUSION

The present study, entitled *"Investigations on a Phytoplasma Disease of Cherry in Himachal Pradesh,"* was undertaken with the primary objectives of documenting the prevalence of phytoplasma-associated disease in cherry orchards of district Shimla, standardizing diagnostic techniques for accurate detection, and conducting molecular characterization of the associated phytoplasma.

Extensive field surveys were conducted during the years 2023 and 2024 across more than 100 cherry orchards in Shimla district, which is the leading cherry-producing region in Himachal Pradesh, contributing over 90 percent of the state's total production. Surveys were concentrated in major cherry-growing areas including Narkanda, Baghi, Ratnari, Kotgarh, Kumarsain, Mashobra and Khadrala. The disease prevalence was predominantly confined to Baghi and its surrounding localities, with the highest disease incidence recorded at Baghi (26.17 percent), followed by Matlu (21.33 percent).

In contrast, no disease incidence was reported from several locations such as Kumarsain, Kotgarh and Thanadhar within the Narkanda block. Similarly, surveys in the Rohru and Mashobra blocks revealed no symptomatic trees, suggesting a geographically restricted disease distribution. These findings indicate that Baghi, situated in the Narkanda block, represents the most severely affected area concerning phytoplasma disease in cherry trees.

Initial symptoms typically appeared in May–June, including leaf yellowing, little leaf syndrome, reddening, leaf tattering, and inward rolling of leaves. Severely infected branches exhibited poor flowering and diminished fruit set. By late summer (August–September), affected trees were visually distinguishable due to prominent foliar discoloration in yellow and red hues. As the season progressed, the infection spread to adjoining branches, resulting in stunted shoots, reduced foliage density and a marked decline in tree vigor. Over time, the disease advanced slowly but was ultimately lethal, with complete tree mortality occurring within 1 to 4 years following symptom onset.

The presence of phytoplasma in symptomatic trees was confirmed using fluorescence microscopy and biological assays. Bright fluorescent bodies were observed in sieve tube

elements of affected tissues, indicating phytoplasma infection. Grafting of infected scion wood onto Colt rootstocks further substantiated phytoplasma transmission, with both microscopy and polymerase chain reaction (PCR)-based techniques yielding positive results.

To achieve more definitive identification, nested-PCR was employed using universal primer sets targeting the 16S–23S rRNA intergenic spacer region. This approach demonstrated high sensitivity and specificity, particularly when phytoplasma titers were low or unevenly distributed. Tissue-specific analysis indicated that the leaf midrib and petiole sections harboured the highest concentrations of phytoplasma, especially during the late summer and pre-dormancy period when the pathogen titer in woody hosts peaks.

Sequencing and phylogenetic analysis of PCR amplicons revealed a high degree of genetic similarity (up to 98.86 percent) with *Candidatus* Phytoplasma ziziphi, placing the pathogen within elm yellows group (16SrV). These results confirmed the identity of the phytoplasma associated with diseased cherry plants in the region.

During the survey, several weed species including *Urtica dioica* (common nettle) and various *Fern* spp. exhibited symptoms such as yellowing, witches' broom, dwarfing, and dense shoot proliferation. These species, commonly growing in proximity to cherry orchards, were suspected as potential reservoir hosts. Subsequent confirmation via fluorescence microscopy and nested-PCR using the same universal primers validated the presence of phytoplasma in these plant species.

Screening of various cherry cultivars revealed differential susceptibility to phytoplasma infection. Cultivars such as 'Black Heart' and 'Van' were found to be highly susceptible, showing prominent symptoms and disease progression. Conversely, cultivars including 'Regina,' 'Glory' and 'Lapin' displayed resistance and remained symptom-free throughout the observation period. Notably, these resistant cultivars were primarily located in research and demonstration orchards rather than in commercial farms, highlighting a gap in the widespread adoption of tolerant germplasm.

This investigation establishes the presence of a localized but significant phytoplasma disease in cherry orchards of Himachal Pradesh, with *Candidatus* Phytoplasma ziziphi identified as the primary pathogen. The disease exhibits a chronic, progressive and ultimately fatal course in infected trees, underscoring the need for early detection and comprehensive disease management.

Among the diagnostic techniques evaluated, nested-PCR emerged as the most reliable method for pathogen detection due to its high sensitivity, particularly during the low-titer stages of infection. The identification of nettle and fern species as potential alternative hosts emphasizes the importance of integrated orchard management, including the monitoring and management of surrounding vegetation.

Furthermore, the identification of resistant cultivars offers a promising avenue for long-term disease control. Given that cherry is a vegetatively propagated crop, there is a high risk of pathogen transmission through infected planting material. Therefore, it is imperative to implement a phytoplasma-indexing program for existing nurseries and to establish new facilities capable of producing certified, disease-free planting stock.

To mitigate the spread and economic impact of phytoplasma disease on cherry cultivation in Himachal Pradesh, emphasis should be placed on the adoption of molecular diagnostic tools for early detection; elimination of reservoir host plants near orchards. Cultivation of resistant cherry cultivars and establishment and enforcement of certified planting material standards. These strategies, when integrated into the existing cherry production framework, will contribute significantly to sustaining cherry orchard health and productivity in the region.

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APPENDIX-I

REAGENTS AND BUFFERS USED IN DAPI STAINING

0.1M PHOSPHATE BUFFER: Add 5.8g NaH_2PO4 to 1000 ml of double distilled (dd) water. Mix for 10 minutes. Add 15.5g of Na_2HPO4 to 1000 ml of ddH2O and stir for 10 minutes and adjust the pH of solution to 6.9 by adding NAOH or HCl solution.

GLUTARALDEHYDE (5 PERCENT SOLUTION): Add 20 ml of 25 per cent glutaraldehyde to 80 ml of 0.1 M phosphate buffer to make final volume to 100ml.

DAPI STAIN (4',6-DIAMIDINO-2-PHENYLINDOLE): Add 1µl of DAPI dye to 1 ml phosphate buffer and store at 4ºC.

APPENDIX-II

COMMON REAGENTS AND BUFFERS USED IN DNA ISOLATION PROTOCOL

STOCK CHEMICALS

- 1M Tris- HCl
- 0.5M Na_2EDTA
- 5M NaCl
- 10 per cent CTAB
- β-mercaptoethanol
- Chloroform:Isoamyl alcohol (24:1 v/v)
- Isopropanol
- Polyvinylpyrrolidone (PVP)
- 70 per cent ethanol
- 1x TE buffer

DNA EXTRACTION BUFFER (DEB) - 100 ML

Sr. No.	Chemicals	Volume per 100 ml
1.	1M Tris- HCl	10 ml
2.	0.5M Na_2EDTA	4 ml
3.	5M NaCl	30 ml
4.	10 per cent cTAB	20 ml
5.	β-mercaptoethanol	0.2 ml
6.	PVP	1g
7.	ddH_2O	36 ml

APPENDIX-III

REAGENTS AND BUFFER USED FOR GEL ELECTROPHORESIS

Component	Constituents	Quantity
50X TAE	Tris Base	242g
	Glacial acetic acid	57.1ml
	0.5M EDTA (pH 8.0)	100ml
	Distilled water added to make volume up to 1 liter	
Loading Dye 1%	Bromophenol Blue	200µl
	Glycerol	200 µl
	10% SDS	60 µl
	0.5M EDTA	50 µl
	10X TAE	60 µl
	Distilled Water	30 µl

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ABSTRACT

Cherry (*Prunus avium* L.) is a fleshy drupe and is an important fruit grown in temperate regions belonging to family Rosaceae. In India, Jammu and Kashmir, Himachal Pradesh and Uttarakhand mainly contribute in the cherry production. However, during the recent surveys conducted in cherry orchards, the plants were found to be severely affected by a disease resulting in reddening of leaves, wilting of branches over time, which ultimately lead to the death of whole plant and is causing severe economic losses to the growers. Extensive surveys were conducted during the year 2023 and 2024 in more than 100 cherry orchards of district Shimla of Himachal Pradesh. Various symptoms observed during the surveys included leaf yellowing, little leaf symptoms, leaf reddening, leaf tattering and inward leaf rolling during the initial stages of infection on cherry trees. With time widespread yellowing and red discoloration of foliage occur on infected plants. The maximum disease incidence recorded was 26.17 percent in orchards of Baghi region followed by 21.23 percent at Matlu location. Conversely, locations such as Kumarsain, Kotgarh, Thanadhar, Rohru and Mashobra reported no incidence of disease. For histological detection DAPI based fluorescence microscopy was used which revealed the presence strong fluorescence in the phloem cells of symptomatic leaves, while healthy leaf samples showed no such fluorescent spots. Biological indexing carried out by tongue grafting of infected scion wood on colt rootstock resulted in the transmission of phytoplasma. PCR results showed that the 20 percent of the grafted plants were tested positive for the presence of phytoplasma. To further confirm the association of pathogen with disease production in cherry tree, nested-PCR using universal primers targeting the 16S-23S rRNA region was used. Also, the plant parts such as leaf midrib and petiole portion were subjected to detection also showed the highest concentration of phytoplasma in vascular tissues during the late summers. Sequencing and phylogenetic analysis revealed high genetic similarity of phytoplasma associated with cherry trees (up to 98.86%) with *Candidatus* Phytoplasma ziziphi, linking the pathogen to the elm yellows group (16SrV). Surveys also identified *Urtica dioica* (nettle) and Fern spp. showing symptoms such as leaf yellowing, witches' broom, little leaf, dwarfing and dense shoot proliferation indicating a possible role in disease perpetuation and the potential reservoir hosts. Presence of phytoplasma in symptomatic plant species was also confirmed by fluorescence microscopy and nested-PCR using the same set of universal primer pairs. Screening of cherry cultivars showed differential susceptibility, with Black Heart and Van being the most vulnerable, while cultivars like Regina, Glory and Lapin displayed resistance and remained symptom-free. These resistant cultivars were primarily located at research and demonstration stations rather than on commercial farms.

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Education Qualification:

Certificate/Degree	Year	University/Board	Marks (%)	Division
10th	2012	DAV Public School, Nahan (HP) (CBSE)	92	First
12th	2014	AVN Sr. Sec. School, Nahan (HP) (HPBOSE)	88	First
B.Sc. (Horticulture)	2019	Dr. YS Parmar University of Horticulture and Forestry, Nauni, Solan, (HP)	84.50	First
M.Sc. (Agriculture) Plant Pathology	2021	Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur (MP)	78.80	First

Title of Thesis in M.Sc. : Studies on the anthracnose of chilli and its management

Scholarship/Stipend/Fellowship, any other financial assistance received during the study period :

- University Merit Scholarship during B.Sc.
- National Talent Scholarship (NTS)-ICAR during M.Sc.
- University Stipend during Ph.D.

Publication :

Popular Article : 3
Research Paper: 2
Book Chapter: 1

(Aman Chauhan)