MOLECULAR DETECTION AND CHARACTERIZATION OF PHYTOPLASMA INFECTING BRINJAL (*Solanum melongena* L.)

by

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DECLARATION

I hereby declare that this thesis entitled '**Molecular detection and characterization of phytoplasma infecting Brinjal (*Solanum melongena* L.)**' is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, associate ship, fellowship or other similar title of any University or Society.

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microlitre
ºC	Degree Celsius
APS	Ammonium Persulphate
BAP	Bezyl amino purine
BLL	Brinjal Little Leaf
bp	Basepair
BSA	Bovine serum albumin
Ca. P	*Candidatus* Phytoplasma
CD	Critical difference
CLL	Catharanthus Little Leaf
cm	Centimeter
DAI	Days after inoculation
EDTA	Ethylene diamine tetra acetic acid
et al.	And other co workers
Fig.	Figures
g	Gram
GA_3	Gibberellic acid
hr	Hour
IAA	Indole acetic acid
i.e.	That is
kb	Kilobase
kDa	Kilodalton
M	Molar

mg	Milligram
min	Minute
mM	Milli molar
ml	Millilitre
MS medium	Murashige Skoog medium
N	Normal
NAA	Naphthalene acetic acid
ng	Nanogram
nm	Nanometre
PAL	Phenyl alanine ammonialyase
PCR	Polymerase Chain Reaction
PO	Peroxidase
PPO	Polyphenol oxidase
pM	Picomolar
PR proteins	Pathogenesis Related Proteins
PVP	Poly vinyl pyrrolidone
REP	Roodan Eggplant Phyllody
Rm	Relative mobility
rpm	Revolutions per minute
SDS-PAGE	Sodium do-decyl sulphate polyacrylamide gel electrophoresis
sp	Species
TEMED	Tetramethyl ethylene diamine

Introduction

1. INTRODUCTION

Brinjal or egg plant (*Solanum melongena* L.) is an important solanaceous vegetable crop cultivated all over the world, especially in the tropics and subtropics. It is of much importance in the warm areas of Far East, being grown extensively in India, Bangladesh, Pakistan, China and the Philippines. In India, it is one of the most common, indigenous and principal vegetable crops grown throughout the country except higher altitudes. The name brinjal is popular in India and it is also called aubergine (French word) in Europe. It is a perennial but grown commercially as an annual crop. About 90% of brinjal production comes from five countries. China is the top producer (58% of world output), India ranks second (25%), followed by Iran, Egypt and Turkey. More than 4,000,000 acres (1,600,000 ha) were devoted to the cultivation of eggplant in the world (FAO, 2012).

Brinjal production is affected by several diseases, of which little leaf is an emerging problem in Kerala. Brinjal Little Leaf (BLL) is a very serious disease of brinjal causing considerable economic losses and brings about drastic morphological changes in the infected plants (Varma *et al*., 1969; Mitra, 1993). In India, the symptoms of little leaf disease were recorded in brinjal (Mitra, 1988; Schneider *et al*., (1995). The infected plants were characterized by stunting, excessive branching, phyllody of flowers and reduction of leaf size. The disease was reported to be transmitted through the leafhopper vector *Hishimonas phycitis* (Bindra and Singh., 1969) and by grafting. The association of mycoplasma like bodies was demonstrated in little leaf of brinjal (Varma *et al.,* 1969).

Numerous yellows-type diseases of plants were believed to be caused by viruses considering their infective spreading, symptomatology, and transmission by insects (Kunkel, 1926). The first demonstration that the etiological agents of these diseases could be wall-less prokaryotes rather than viruses caught the field of plant pathology by surprise (Doi *et al.*, 1967). Phytoplasmas are specialized prokaryotes that are obligate parasites of plant phloem tissue coming under the class *Mollicutes* and named mycoplasma-like organisms or MLOs. They are characterized by lack of cell wall, pleiomorphic or filamentous shape, normally with a diameter less than 1 μm, and very small genomes. The enigmatic status of MLOs amongst the prokaryotes was resolved with molecular technologies and led to the new trivial name of "phytoplasma", and eventually to the designation of a new taxon named '*Candidatus* phytoplasma' (IRPCM, 2004). The preliminary evidence that the phytoplasmas can be grown in or on cell free laboratory media has been reported by Bertaccini *et al*., (2010) and Contaldo *et al*., (2013). Phytoplasma diseases have been reported worldwide infecting a broad range of crops, causing considerable economic losses.

Taking into consideration, the above aspects and also the importance of phytoplasma in the cultivation of brinjal in our state, this study has been undertaken to investigate the symptomatology, transmission, host pathogen interaction, molecular detection and characterization of phytoplasma infecting brinjal.

Review of Literature

2. REVIEW OF LITERATURE

Brinjal is one of the most commonly grown vegetable crops of the country. Among the diseases, Brinjal little leaf (BLL) is becoming a serious problem during the cultivation as the crop suffers heavy yield loss. The disease was first reported by Thomas and Krishnaswamy (1939) and they reported *Hishimonas phycitis* and *Empoasca devastans* as the vectors. Like other similar diseases, BLL was considered to be due to virus. Varma *et al*. (1969) demonstrated typical MLBs in the phloem cells of roots and stems of diseased plants. The size of the MLBs ranged from 40 to 300 nm and a majority of these were about 250 nm. Further evidence for mycoplasmal etiology of BLL was obtained by the susceptibility of the causal agent to tetracycline antibiotics, which considerably delayed the appearance of symptoms in plants (Anjaneyulu and Ramakrishnan, 1969). Raychaudhuri *et al*. (1970) reported that similar bodies were also present in the leaf parenchyma cells. Among the several tetracyclines tried for their efficacy against the BLL under field and glasshouse condition, dimethylchlortetracycline hydrochloride was found to be most effective when applied by spraying (Raychaudhuri *et al.*, 1970; Varma *et al.*, 1975). Varma *et al.* (1975) detected Mycoplasma Like bodies (MLBs) in phloem cells of affected eggplant roots and remission of symptoms was noticed on treatment with achromycin, aureomycin, ledermycin and terramycin, but not with garramycin and pencillin. Mitra *et al*. (1976) studied the effects of BLL on the respiration and the enzymatic activities of brinjal at a particular stage of infection. Several biological aspects of the disease have been studied by Mitra, (1993). Singh *et al*. (2015) provided the first report of mixed infection of phytoplasma and bigomoviruse in eggplant from India.

Phytoplasmas were discovered in ultrathin sections of plant phloem tissue in aster and other plants with "yellows" symptoms opening phytoplasmology history and named MLOs due to the physical resemblance and the temporary disappearance of phytoplasma was observed when treated with tetracycline (Doi *et al*., 1967; Ishiie *et al*., 1967). Several studies showed uneven phytoplasma distribution in the host plant (Seemüller *et al.*, 1984), and a seasonal fluctuation of the pathogen population in woody hosts. Generally, levels were low in roots (sink organ) and moderate in stems. The highest concenration was found in source organs (mature leaves), sometimes ≈40 times higher concenration than in roots. In sink leaves, phytoplasma concentration was low or below detection. The highest concentration of phytoplasma in source region gives a direct indication of faster multiplication of phytoplasma. Christensen *et al.* (2004) reported that phytoplasmas were able to move within plants through the phloem from source to sink through sieve tube elements. Weintraub and Beanland, (2006) identified that these microorganisms were transmitted in a persistent manner by insects belonging to the families Cicadellidae, Cixidae, Psyllidae, Delphacidae and Derbidae.

2.1 SYMPTOMATOLOGY

A common symptom caused by phytoplasma infection is phyllody, the production of leaf like structures in place of flowers. Moreno *et al.* (1985) described and compared several yellows diseases on *Catharanthus roseus* in Spain. Lee *et al.* (2000) reported that the phytoplasma infected plants also suffered from virescence i.e. the development of green flowers due to the loss of pigment in the petal cells. Sterility of the flowers was also described in the infected plants. Many phytoplasma infected plants exhibited a bushy or witches' broom appearance due to changes in normal growth patterns caused by the infection. Lee *et al*. (2000) also found that most of the infected plants showed the proliferation of axillary (side) shoots and decreased size of internodes. He also reported that the symptoms of diseased plants varied with

the phytoplasma, host plants, stage of the disease, age of the plants at the time of infection and environmental conditions. Bertamini and Nedunchezhian (2001) observed that the infected plants had yellowing which was caused by the breakdown of chlorophyll and carotenoids, the biosynthesis of which was also inhibited.

Evidence suggested that the phytoplasma deregulated a gene involved in flower formation (Pracros *et al.*, 2006). Other symptoms, such as the yellowing of leaves, were thought to be caused by the presence of phytoplasma in the phloem, which affected its function, and changed the transport of carbohydrates. photosynthesis, especially photosystem II, was also inhibited in many phytoplasma infected plants. Plants infected by phytoplasma exhibited an array of symptoms that suggested profound disturbances in the normal balance of growth regulators. Bertaccini (2007) studied the symptoms associated with phytoplasma infection and observed virescence/phyllody (development of green leaf like structures instead of flowers), sterility of flowers, proliferation of axillary (side) buds resulting in witches' broom appearance, abnormal internodes elongation and generalized stunting. Omar *et al.* (2008) observed little leaves, shortened internodes, virescence and witches' broom symptoms associated with infected catharanthus plants grown in Egypt.

McGibbon (1924) first described the occurrence of sesame phyllody in Burma. The disease was reported in India, Iran, Iraq, Israel, Burma, Sudan, Nigeria, Tanzania, Pakistan, Ethiopia, Thailand, Turkey, Uganda and Mexico. The pathogen was transmitted by the leaf hopper *Orosius albicinctus* (Akhtar *et al*., 2009). In India, the disease was transmitted by *Orosius orientalis* and the associated phytoplasma in Thailand belonged to peanut witches' broom group (16Sr II) (Schneider *et al*., 1995). The affected plants showed pale green and bushy growth due to excessive stunting and axillary proliferation. The abnormal green sterile structures were developed in the place of flowers along with yellowing.

In India, the symptoms of little leaf disease were recorded in brinjal (Mitra, 1988; Schneider *et al.*, 1995). According to Mitra (1988), the infected plants were

characterized by stunting, excessive branching phyllody of flowers and reduction of leaf size. He also reported that in early infection, no fruiting occurred and in late infection, fruits formed were deformed and the seeds were shrivelled. Omar and Foissac (2012) reported severe symptoms such as malformed leaves, virescence (excessive greening of floral tissues) and phyllody (leaf-like structure) in naturally infected egg plants. They also reported symptoms associated with phytoplasma infection in tomato and squash.

2.2 TRANSMISSION STUDIES

Tohidi *et al*. (2015) reported that the agent of Roodan (province of Iran) Eggplant Phyllody (REP) was transmitted from phyllody affected eggplant to healthy egg plant and tomato by grafting and to catharanthus via dodder inoculation that induced phytoplasma-type symptoms.

2.2.1 Graft transmission

Coleman (1923) first demonstrated the graft transmissibility of sandal spike disease to healthy trees. Kaminska and Korbin (1999) attempted to transmit the phytoplasma from naturally infected lily and experimentally infected Alstroemerias to catharanthus by top grafting and resulted in stunted growth, dull yellowing and malformation of leaves in 4-6 weeks. Lee *et al*. (2000) reported that phytoplasma could be transmitted through grafts and could not be transmitted mechanically by inoculation with phytoplasma containing sap. Torres *et al*. (2004) observed little leaf symptoms in graft inoculated catharanthus plants and the disease progressed towards the young vegetative organs along with virescence and phyllody. Salehi *et al*. (2009) observed the transmission of safflower phyllody phytoplasma in Iran from diseased to healthy safflower by grafting, which was verified by nested PCR. Nejat *et al*. (2010) also reported the transmission of phytoplasma associated with the proliferation of catharanthus in Malaysia by grafting.

In brinjal, out of different methods tested, grafting was found to be the most efficient one and a period of 7-9 days were required for the infected scion to remain united with the healthy stock for the optimum inoculation (Chakrabarti and Choudhury, 1975).

2.2.2 Dodder transmission

Deng and Hiruki, (1991b) demonstrated the dodder transmission from diseased alsike clover to catharanthus by means of *Cuscuta subinclusa*. Ghosh *et al.* (1999) used dodder (*Cuscuta reflexa* Roxb.) to transmit the causal agent of witches' broom disease (WBD) from citrus to catharanthus plants and the disease was succesfully transmitted from infected acid lime to catharanthus plants and vice-versa by dodder. Kaminska and Korbin (1999) observed that catharanthus plants that were bridged by *Cuscuta odorata* from the diseased lilies and Alstroemerias resulted in stunted growth, dull yellowing and malformation of leaves. Marcone *et al.* (1999) reported the dodder transmission of pear decline, European stone fruit yellows, rubus stunt, *Picris echioides* yellows and cotton phyllody phytoplasmas to catharanthus. They also reported that the transmission had association with both the type of phytoplasmas and C*uscuta* sp. Salehi *et al.* (2009) reported the transmission of safflower phyllody phytoplasma from diseased to healthy safflower and catharanthus by dodder (*Cuscuta campestris*) and verified by nested PCR. Toria Phyllody (TP) phytoplasma was successfully transmitted from toria to toria and to catharanthus by dodder transmission (Azadvar *et al*., 2011).

2.3 MAINTENANCE OF PHYTOPLASMA

Micropropagation of phytoplasma diseased plant tissue was used by different researchers for the maintenance of phytoplasma in their original host plants. McCoy (1978) reported the maintenance of lethal yellowing phytoplasma in shoot cultures. Wang *et al*. (1994) identified that the concentration of paulownia witches' broom phytoplasma in tissue culture was significantly higher than that of plants grown under

greenhouse conditions. Jarausch *et al*. (1996) reported the micropropagation of *Malus pumila* for the maintenance of apple proliferation (AP) phytoplasma for more than ten years. Kaminska *et al.* (2000) performed the detection of phytoplasma in tissue culture plants of gladiolus grown under various conditions and the best detection was obtained in MS media containing kinetin and NAA in presence of light. Phytoplasma was also detected in MS medium with 0.5 mg l^{-1} BAP+0.2 mg l^{-1} NAA. The PCR amplification indicated that during two years of tissue culture at standard conditions, on MS medium with 1mg l^{-1} BAP, phytoplasma could be detected in rose cultivars Sacha and Jazz (Kaminska *et al*., 2005). Shekari *et al*. (2011) studied the maintenance of phytoplasma, which caused witches' broom disease of small-fruited lime (WBDL) in Murashige and Tucker medium (MT medium) and confirmed by PCR using the primers P1/P7 followed by P3/P7. Maintenance of phytoplasma strains in tissue culture using MS solid medium supplemented with 0.12 mg l^{-1} BAP was standardized in all strains transmitted to catharanthus and also in naturally infected host plant species (Bertaccini *et al.*, 2013).

In brinjal, the highest amount of shoots was reported on MS medium containing 2 mg l^{-1} BAP and 0.5 mg l^{-1} Kinetin and further shoot elongation was obtained on MS medium with GA_3 at 1.5 mg l^{-1} and rooting was done in MS medium containing 3 mg l^{-1} IBA (Shivaraj and Rao, 2011). Ray *et al.* (2011) reported that the highest amount of callus growth and maximum number of plant regeneration through callus in brinjal was produced on MS medium containing 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA. Robinson and Saranya (2013) found that the maximum efficiency of multiple shooting in brinjal was observed in MS medium supplemented with 0 0.2 mg l^{-1} BAP, 0.6 mg l^{-1} NAA and 0.4 mg l^{-1} IAA.

2.4 BIOCHEMICAL ANALYSIS

Plant diseases produce radical metabolic changes in the host tissues and the little leaf of brinjal is no exception (Diener, 1963).

2.4.1 Carbohydrates

In pear plants with pear decline, accumulation of sugar and starch was reported in leaves (Catlin *et al.*, 1975). Phytoplasma infection led to a significant increase of starch in source leaves and a decrease in sink leaves and roots than the healthy plants. The similar effect was reported in fruit trees and woody plants (Kartte and Seemuller, 1991), which showed that the phytoplasma infection led to the accumulation of carbohydrates in mature leaves and decreased starch in the roots. Magat (1993) reported that reduction in protein increased the C/N ratio, which resulted in higher carbohydrate content. Lepka *et al.* (1999) studied the effects of phytoplasma infection on the concentration and translocation of carbohydrate in tobacco and catharanthus and found that the levels of sucrose and reducing sugars were higher in infected plants than healthy ones. The ultra structural observations of starch accumulation in chloroplasts were associated with severe disorganization of thylakoids and a reduction in chlorophyll content. Higher carbohydrate content was reported in papaya leaves infected with phytoplasma (Guthrie *et al*., 2001). Maust *et al.* (2003) studied the accumulation of carbohydrates in coconut palms affected by lethal yellowing and considered as a secondary effect of infection by phytoplasma and explained through an inhibition of phloem transport. The accumulation of photosynthetic products in chloroplast caused the inhibition of photosynthesis and reduction in the supply of sugars from source leaves to roots. According to Junqueira *et al.* (2004), the amount of reducing sugar was higher in corn plants infected by maize bushy stunt phytoplasma.

The reduction in carbohydrate content was also noticed in some phytoplasma infected plants. The BLL disease decreased the reducing and non-reducing sugars,

starch and total carbohydrate in the resistant wild species and increased the same in the susceptible cultivar (Chakrabarti and Choudhury, 1975). Investigations on the biology of leaf hopper, *Hishimonus phycitis* in the healthy and little leaf-infected egg plants showed that there was distinct preference for the diseased leaves, which possessed higher amounts of moisture, total carbohydrates, sugars and organic acids than the healthy ones (Srinivasan and Chelliah, 1980). Mitra (1988) reported that the carbohydrate content was reduced in brinjal plants as a result of little leaf infection.

2.4.2 Chlorophyll

Srinivasan and Chelliah (1980) observed marked reduction in the total chlorophyll content in phytoplasma infected egg plants. Mitra (1988) found that the reduction in photosynthetic activity in phytoplasma infected egg plants was 30% in case of mildly infected plants and 91% in case of severely infected plants.

Variations in carbohydrate metabolism in plants affected by phytoplasmas were associated with a marked reduction of total chlorophyll content due to the decrease of both Chl a and Chl b in leaves. Krapp *et al.* (1993) reported that the loss of chlorophyll was followed by a general sugar mediated repression of genes involved in photosynthesis. The reduction in the chlorophyll content was also reported in root (wilt) affected coconut palms (Koshy, 1999). One of the main effects of phytoplasma infection was decreased plant productivity caused by the inhibition of photosynthesis in phytoplasma infected grapevine, which could be the direct effect of infection on photosynthetic electron transport and enzymatic activities (Bertamini and Nedunchezhian, 2001). Jagoueix-Eveillard *et al*. (2001) demonstrated that stolbur phytoplasma induced a down regulation of genes involved in photosynthesis. Bertamini *et al.* (2002a) suggested that phytoplasma had a role in the inhibition of chlorophyll biosynthesis in the host leaves. Bertamini *et al.* (2002b) identified that the reduction in chlorophyll was due to the enhanced chlorophyllase activity in the infected leaves. Bertamini *et al*. (2003) analysed the effects of phytoplasma infection on pigments, chlorophyll-protein complex and photosynthetic activities in

field grown apple leaves, where the total chlorophyll content was drastically reduced to 52% in severely infected leaves of apple with a markedly lower Chl a/b ratio. Junqueira *et al.* (2004) reported that maize bushy stunt phytoplasma resulted in significant reduction in chlorophyll content in infected maize plants. Significant reduction of Chl was reported in lime plants infected by *Candidates* Phytoplasma aurantifoliae (*Ca*. Phytoplasma aurantifoliae) along with early leaf chlorosis (Zafari *et al*., 2012).

The decline of photosynthesis may be due to phytoplasma infection on photosynthetic electron transport and enzymatic activities. Water stress also caused reduction in chlorophyll content in plants (Mukherjee and Kumar, 2005). Otitoju and Onwurah (2010) identified that the amount of chlorophyll in leaf tissues was affected by the environmental stresses and nutrient availability.

2.4.3 Protein

Usually plants infected by pathogens showed a high protein content, which could be due to both the activation of host defence mechanisms and the pathogen attack mechanisms. Among the proteins produced in the host plants, there were pathogenesis related proteins (PR proteins), including peroxidases. Zhong and Shen (2004) found that six soluble proteins, accumulated specifically in *Chrysanthemum coronarium* infected with phytoplasmas, belonging to the 16SrI and 16SrIII groups and the proteins shared high sequence similarities with the N-terminal amino acid sequence of thaumatin-like or osmotin-like proteins, which were PR5 protein group members.

Contradictory results were obtained for different plants with different phytoplasmas. The reduction in leaf protein content was reported in lethal yellowing affected coconut palms (Leon *et al.*, 1996). The similar results were obtained in phytoplasma infected corn plants and in tomato plants affected by stolbur phytoplasma (Favali *et al.*, 2001). Bertamini *et al.* (2002a) reported the reduction of

protein content in apple trees with apple proliferation. Bertamini *et al.* (2002b) reported the same results in bois noir affected grapevine. The grapevine affected by flavescence doree also showed the same results (Musetti *et al.*, 2007). But the maize plants infected by maize bushy stunt phytoplasma exhibited an increased amount of protein content (Junqueira *et al*., 2004).

Friedrich and Huffaker (1980) reported that early senescence induced by *Ca.* Phytoplasma aurantifoliae enhanced the activity of RUBPC-specific protease. Eichelmann and Laisk (1999) reported that a part of RUBPC played the role of storage protein. Bertamini *et al*. (2003) reported that the total protein reduction in phytoplasma infected leaves was probably due to the decrease in the synthesis of ribulose-1, 5-biphosphate carboxylase (RUBPC), the major soluble protein of the leaf.

2.4.4 Phenol

Nicholson and Hammerschmidt (1992) reported that the phenolic compounds were formed in response to the ingress of pathogens and their appearance was considered as part of an active defense mechanism. Agrios (1997) observed that certain common phenolic substances were toxic to the pathogens and accumulated in plants after the infection, especially in resistant varieties. Different phenolic substances were found to appear concurrently in the same diseased tissue, which showed a synergistic activity against the pathogens.

Mitra and Majumdar (1977) reported that out of four varieties of brinjal tested, total phenol content was decreased throughout the disease development, as a result of infection by little leaf phytoplasma in three brinjal varieties. Marked reduction in the phenolic content was also reported in brinjal plants with little leaf disease (Srinivasan and Chelliah, 1980).

Musetti *et al*. (2000) determined the total phenol content in phytoplasma infected apples and plums. Junqueira *et al.* (2004) observed the accumulation of

phenolics in phytoplasma infected *Zea maize*, where the presence of phytoplasma triggered an increase in phenolic componds. Choi *et al.* (2004) reported the same result in phytoplasma infected *Catharanthus roseus* leaf. He revealed that the metabolites related to the biosynthesis of phenyl propanoids were present in higher amounts in phytoplasma infected plants, along with relatively higher abundance of chlorogenic acid and polyphenols in the infected leaves. Rusjan *et al*. (2012) observed statistically significant differences in total phenolic content in the berry skins of chardonnay grape variety between uninfected and phytoplasma symptomatic berries at all analysed phenological stages but at harvest stage the total phenolic content drastically reduced in phytoplasma symptomatic berries.

2.4.5 Defence Related Enzymes

Dasgupta (1988) reported that the defence related enzymes could act as an important factor in the induction of resistance. Nurnberger *et al*. (2004) reported that the constitutive defenses of plants included structural barriers, such as the plant cell wall as well as inhibitory compounds including phenolics.

The activity of peroxidase, a defence related enzyme could be related to the resistance level of host plants (Leherer, 1969). Mitra (1988) studied the activity of PO in brinjal affected by little leaf phytoplasma and found that the activity was reduced as a result of infection. But enhanced activity has been reported in many plants affected by phytoplasma. Rangaswamy (1995) confirmed the enhanced activity of peroxidase in sandal spike affected trees than the healthy control trees. Mehlhorn *et al.* (1996) reported that peroxidases are enzymes with numerous functions in plant cell, including response to pathogens. The significant enhancement of peroxidase activity was reported in apple trees infected with apple tree phytoplasma (Musetti *et al.*, 2005). He also reported low peroxidase activity during natural recovery from *Ca*. Phytoplasma prunorum infection in apricot trees. Junqueira *et al.* (2011) reported increased peroxidase activity in corn plants infected by maize bushy stunt phytoplasma. He found that the presence of phytoplasma

caused an average increase in enzyme activity around 89% in the susceptible hybrid and 54% in the resistant one. The maximum peroxidase activity for both hybrids was observed at 40-50 days and thereafter the activity was decreased.

Lax and Cary (1995) demonstrated the role of Polyphenol oxidase (PPO), a copper containing enzyme with molecular oxygen as co-substrate in phenol metabolism and in defense mechanisms against pathogens. Mitra and Majumdar (1977) tested four varieties of brinjal and observed that the PPO activity was decreased at all stages of disease development, as a result of infection by little leaf phytoplasma in three brinjal varieties. The reduced PPO activity was also reported in BLL as a result of phytoplasma infection (Mitra, 1988). Zafari *et al*. (2012) reported increased activities of PO and PPO in the lime plants infected by *Ca*. Phytoplasma aurantifoliae than the healthy control.

The activity of phenylalanine ammonia-lyase (PAL), an important enzyme in phenyl propanoid pathway in plants having the role in plant development and pathogen defense rapidly changed under the influence of various factors, such as pathogen attack and treatment with elicitors (Dixon and Lamb, 1990). Romanazzi *et al*. (2007) reported that grapevines, both affected by bois noir and recovered from the disease, showed an upregulation of PAL, as compared to the healthy plants. The activities of PO and PAL could be rapidly enhanced under the influence of elicitors or pathogen attack. Patui *et al.* (2013) reported that the activity of PAL was increased in apple trees affected by apple proliferation phytoplasma.

2.4.6 SDS-PAGE (Sodium do-decyl sulphate polyacrylamide gel electrophoresis)

Favali *et al.* (2001) reported the alterations in several proteins in many plant-phytoplasma systems. SDS-PAGE analysis in lime plants infected by *Ca*. Phytoplasma aurantifoliae revealed that the protein patterns in infected and control leaves were not identical and the differences were both quantitative and qualitative. The presence of two extra proteins of molecular weights 15 and 26 kDa was reported

from the infected leaves, which suggested their possible role in response to phytoplasma infection (Zafari *et al.*, 2012). Proteomic analysis was found to be an important tool to gain insight into the plant host responses to stresses. Prete *et al.* (2011) investigated the effects of Pear Decline (PD) phytoplasma on the pear protein profile by conducting SDS-PAGE on leaf proteins from infected and healthy plants, which showed differentially expressed protein bands.

2.4.7 Isozyme Analysis

Isozymes were found to be the most efficient and inexpensive tool for the study of genetic variation in tree species as compared to other molecular markers or morphological characteristics (Soltis and Soltis, 1989). Variation in their structure provided reliable information on the variability of the genome (Hoelzel, 1991). Asiedu (1992) also reported that isozyme analysis was a powerful tool for the estimation of genetic variability in identifying cultivars and germplasm accessions. Isozymes were identified as multiple molecular forms of enzyme proteins as well as primary gene products. Isozymes appeared on electrophoretic gels as multiple bands and genetic interpretations could be made from the banding profile.

The presence of different isoforms of antioxidative enzymes in healthy control and phytoplasma infected leaves was determined in lime plants with *Ca*. Phytoplasma aurantifoliae by native PAGE (Zafari *et al.*, 2012). The analysis revealed four peroxidase isoforms in the healthy and phytoplasma infected plants.

2.5 MOLECULAR DETECTION AND CHARACTERIZATION

2.5.1 Molecular Detection by PCR

Detection and identification of phytoplasma are necessary for the accurate disease diagnosis. Sensitive methods need to be implemented in order to monitor the presence and spread of the phytoplasma infections. Smart *et al.* (1996) suggested that polymerase chain reaction (PCR) was the most versatile tool for detecting

phytoplasmas in the host plants and their vectors. Lee *et al*. (2000) reported that molecular diagnostic techniques introduced during the last two decades were more accurate and reliable than biological criteria long used for phytoplasma identification.

In the 1990's, following the first cloning of phytoplasma DNA (Kirkpatrick *et al*., 1987), nucleic acid-based probes (randomly cloned DNA or its complementary RNA) were widely applied in different assays to detect and differentiate phytoplasmas in plants and vectors (Lee and Davis, 1988; Harrison *et al.*, 1992). In the same year, probes based on cloned phytoplasma-specific chromosomal and extrachromosomal DNAs provided the first evidences of genetic differences in the phytoplasma DNA among the strains derived from different plant hosts or from different geographical locations (Lee *et al.*, 1992). Schaff *et al*. (1992) developed the genomic sequence-specific oligonucleotides for diagnostic purposes. PCR assays using primers based on cloned DNA fragments (nonribosomal DNAs), specific to a given phytoplasma, provided sensitive as well as specific means for phytoplasma detection.

PCR assays using universal primers were useful for preliminary identification of phytoplasma diseases. Ahrens and Seemüller (1992) designed several universal and many phytoplasma group specific primers for routine detection of phytoplasmas on 16S ribosomal gene. Rajan and Clark (1995) identified that immuno-capture PCR assay, in which the phytoplasma of interest was selectively captured by specific antibody adsorbed on microtiter plates, and the phytoplasma DNA, was released and amplified using specific or universal primers, could be an alternative method to increase sensitivity of detection. Baric and Dalla-Via (2004) suggested the real time PCR as a candidate for replacing standard PCR in routine testing as it had high sensitivity and direct reading of the results which reduced the risk of amplicon contamination and the need for a gel-based post PCR analysis.

Christensen *et al.* (2004) reported variation in the amount of phytoplasma DNA between individual plants propagated from one infected parent plant.

Bertaccini (2007) reported that the amount of phytoplasma DNA was lower than 1% of the total DNA extracted from the tissue. Different protocols were reported for total DNA extraction for the detection of the phytoplasma, with the main goal as to concentrate phytoplasma DNA while reducing enzyme-inhibitory plant phenolic and polysaccharide molecules, which is obtained by including a phytoplasma enrichment step.

Nested-PCR assay, designed to increase both sensitivity and specificity, was used for the amplification of phytoplasma DNA from samples having unusually low titers or inhibitors that might interfere with the PCR efficacy (Gundersen *et al*., 1994). Nested-PCR was performed by the preliminary amplification using a universal primer pair followed by a second amplification using a second primer pair. Lee *et al*. (1995) suggested that nested-PCR with a universal primer pair followed by a group specific primer pair, could detect phytoplasmas present in mixed infection. The advantage of nested-PCR was that if the wrong fragment was amplified in the direct PCR, then the amplification of that wrong fragment during the second PCR reaction could be avoided.

The detection of phytoplasma employed at least two primer pairs to test a sample e.g. P1/P7 (Deng and Hiruki, 1991a) and R16F2n/R16R2 (Gundersen and Lee, 1996). Genes encoding 16Sr DNA were highly conserved across the phytoplasma clade and served as a primary molecular tool for phytoplasma identification, genotyping, taxonomic assignment and group/subgroup classification by RFLP analyses (Lee *et al*., 1998). Heinrich *et al*. (2001) reported that dimmers or unspecific bands, formed by some of the primers had sequence homology in the 16S-spacer region to chloroplasts and plastids, which could increase the risk of false positives. The design of primers based on various conserved sequences such as 16S rRNA gene, ribosomal protein gene operon, *tuf* and *SecY* genes was the major breakthrough in identification, classification and the detection of a wide array of

phytoplasmas associated with plants and insects. (Gundersen *et al*., 1996; Martini *et al*., 2007).

Schneider *et al*. (1993) observed that PCR coupled with RFLP analysis allowed the accurate identification of different strains and species of phytoplasma. Lee *et al.* (1998) reported that differentiation of putative phytoplasmas was routinely carried out on 16S rRNA gene that must be accomplished through Restriction Fragment Length Polymorphism (RFLP) analysis of PCR amplified DNA sequences using a number of endonuclease restriction enzymes. As the RFLP patterns characteristics of each phytoplasmas were conserved, unknown phytoplasmas could be identified by comparing the patterns of the unknown with the available RFLP patterns for known phytoplasmas without co-analyses of all reference representative phytoplasmas (Wei *et al*., 2007; Cai *et al*., 2008).

The presence of phytoplasmas in symptomatic plant samples could be detected by a PCR procedure using the universal phytoplasma primer pair P1/P7 in direct PCR followed by the primer pair R16F2n/R16R2 in nested PCR (Lee *et al*., 2004). Kaminska *et al.* (2005) performed the PCR amplification using P1/P7 followed by R16F2n/R16R2 which resulted in ~1.8 kb and 1.24 kb respectively in phytoplasma-affected micropropagated rose cultivars Sacha and Jazz in MS medium with 1mg l^{-1} BAP. Delic *et al.* (2007) reported that the PCR products were usually visualized on 1% agarose gel prepared in 1X TAE buffer, stained with ethidium bromide. Salehi *et al*. (2009) demonstrated the presence of phytoplasma in safflower phyllody by the nested PCR assay employing primer pair P1/P7 followed by R16F2n/R16R2 that generated products of 1.8 and 1.2 kb respectively. The first report of 16SrXIV-A phytoplasma association with little leaf disease of *Ranunculus sceleratus* in the world was resulted from the nested PCR with the universal primers P1/P7 followed by R16F2n/R16R2 and further confirmed by phylogenetic analysis and computer simulated RFLP patterns (Singh *et al*. 2013).

Azadvar and Baranwal (2012) obtained the PCR amplification products of 1.8 kb in direct PCR with P1/P7 and nested PCR with P1/P7 followed by R16F2n/R16R2 resulted in 1.2 kb amplification in all little leaf affected brinjal samples and the vectors, *Hishimonas phycitis*. Phytoplasma infection was demonstrated by nested PCR with P1/P7 followed by R16F2n/R16R2 in Roodan (province of Iran) egg plant phyllody (REP) and blast search, phylogenetic virtual RFLP analyses and nucleotide homology per cent revealed that REP associated phytoplasma is classified in 16SrIX-C group (Tohidi *et al*., 2015).

2.5.2 Sequence Analysis

Phytoplasmas of 16SrVI group were responsible for BLL in India (Schneider *et al*., 1995). Siddique *et al*. (2001) reported that the two strains of phytoplasma in Bangladesh, which caused little leaf disease in brinjal and catharanthus were closely related and were identical to the strain of brinjal little leaf phytoplasma in India. The phytoplasmas in 16SrVI group were taxonomically described as '*Ca*. Phytoplasma trifoli' (Hiruki and Wang, 2004). Azadvar and Baranwal, (2012) reported that BLL disease was associated with phytoplasma of the 16SrVI clover proliferation (CP) phytoplasma group.

Materials and Methods

3. MATERIALS AND METHODS

3.1 SYMPTOMATOLOGY

Brinjal plants with little leaf and phyllody symptoms were collected from the crop museum, College of Agriculture, Vellayani. The phytoplasma culture was maintained by the repeated grafting of infected scion onto healthy root stocks. The culture of phytoplasma infecting catharanthus was obtained from Coimbatore and maintained by the same method for further studies.

3.2 TRANSMISSION

3.2.1 Graft Transmission

The infected shoots having the symptoms of phytoplasma were selected as the source or scion. The base of the scion was trimmed into a wedge shaped structure of approximately 4 cm and then it was inserted into the cleft of about 4 to 5 cm made on the healthy plant which was selected as the root stock. The graft was tied firmly using a high density polythene strip and covered using a polypropylene cover to keep the graft moist. The grafted plants were kept for the expression of symptoms on the new sprouts.

3.2.2 Dodder Transmission

Cuscuta sp. was selected for conducting dodder transmission. The dodder plants were established on the phytoplasma infected plants. The growing ends from the established dodder plants were twined into the young growing shoots of the healthy test plants. The dodder plants so established were allowed to grow as a bridge between the infected and healthy test plants. The plants were kept for the development of systemic symptoms, shown by the new sprouts of the test plants.

3.3 MAINTENANCE OF PHYTOPLASMA

3.3.1 *In vitro* Maintenance using Tissue Culture

In vitro maintenance of Phytoplasma culture was done using tissue culture. The infected shoots obtained from the diseased plants were used as explants and inoculated on Murashige and Skoog medium supplemented with 0.2 mg l^{-1} BAP, 0.6 mg l^{-1} NAA and 0.4 mg l^{-1} IAA for multiple shoot regeneration (Robinson and Saranya (2013).

3.3.1.1 *Culture Media*

The nutrient medium developed by Murashige and Skoog (1962) was used for culturing the explants (APPENDIX VII). The stock solutions of macro, micro, minor nutrients, iron and vitamins were prepared by dissolving adequate quantities of each chemical. Also the stock solutions of the growth regulators BAP, NAA and IAA were prepared at the required concentrations. After adding appropriate quantities of the stock solutions, the pH of the medium was adjusted to 5.8 using 1 *N* NaOH/HCl and the volume was made up using double distilled water. Agar was added at the rate of 8 g l^{-1} and the required quantities of sucrose (3%w/v) and Myoinositol were also added. The medium was heated for the proper mixing of agar and it was dispensed to the culture tubes. The test tubes were plugged with non absorbent cotton. The medium was sterilized by autoclaving and allowed to cool to room temperature.

3.3.1.2 *Preparation of the Sterile Tissue*

The infected younger shoots collected from the diseased plants were thoroughly washed under tap water and then soaked in 2% laboline solution for two minutes. Then the explants were washed with sterile distilled water three times. Then these were transferred to 0.1% Bavistin solution for 20 min and washed with three changes of sterile distilled water. Then the explants were transferred to 0.1%

mercuric chloride for 1-2 min and again washed with three to four changes of sterile distilled water.

3.3.1.3 *Inoculation of the Explants*

The surface sterilized explants were inoculated into the sterile media after it was cut into the required form, as shoot tips using sterile needle and forceps. Then the culture tubes were kept for the regeneration of plantlets.

3.3.2 *In vivo* Maintenance in Plants

The *in vivo* maintenance of phytoplasma culture was done by grafting. The infected shoots were grafted on the healthy plants and kept for the expression of systemic symptoms.

3.4 HOST PATHOGEN INTERACTION

Physiological changes in brinjal and catharanthus due to the infection by phytoplasma were studied by the biochemical analysis of healthy and infected plants. The analysis was done at different intervals of ten, twenty, thirty and sixty days after the graft inoculation and compared with that of the healthy plant.

The analysis was conducted to estimate the changes in total carbohydrates, chlorophyll, phenol and protein. The analysis of defence related enzymes such as peroxidase, poly phenol oxidase and phenyl alanine ammonialyase was also carried out. Protein profile and isozyme analysis of healthy and diseased plants were also conducted.

3.4.1 Estimation of Total Carbohydrate

The total carbohydrate content of healthy and phytoplasma infected plant samples at various intervals after graft transmission was estimated by anthrone method (Hedge and Hofreiter, 1962). The 100 mg each of healthy and diseased leaf samples were hydrolyzed with 5 ml of 2.5 *N* HCL in boiling water bath for three hr.

The hydrolyzate was neutralized with solid sodium carbonate until the effervescence ceased and the volume was made up to 100 ml. Then it was centrifuged at 5000 rpm for 15 min. From the supernatant, 0.5 ml aliquot was taken and made up to one ml by adding distilled water. To this, 4 ml anthrone reagent (Appendix I) was added and heated for eight min in a boiling water bath. This was cooled rapidly and absorbance was measured at 630 nm in a spectrophotometer (Systronics UV- VIS Spectrophotometer 118). The amount of carbohydrate present was calculated from the standard graph obtained using glucose and expressed as milligrams of glucose equivalent per gram of leaf tissue on fresh weight basis.

3.4.2 Estimation of Chlorophyll

Chlorophyll estimation was done by the method described by Arnon (1949). One gram leaf samples of healthy and diseased plants were homogenized with 20 ml of 80 per cent acetone. The homogenate was centrifuged at 5000 rpm for five minutes and the supernatant was transferred to a 100 ml volumetric flask. The above procedure was continued till the residue became colourless. The final volume in volumetric flask was made up to 100 ml with 80% acetone. Absorbance at 645 and 663 nm was read in a spectrophotometer against the solvent (80% acetone) as blank. The chlorophyll content was expressed as milligrams chlorophyll per gram leaf tissue on fresh weight basis (mg g^{-1}). The chlorophyll content can be calculated as

Chlorophyll a = $[12.7 (A_{663}) – 2.69 (A_{645})] V /1000 W$

Chlorophyll b = $[22.9 (A_{645}) – 4.68 (A_{663}) V /1000 W$

Total chlorophyll = $[20.2 (A_{645}) + 8.02 (A_{663})] V /1000 W$

Where, A = absorbance value at a specific wavelength.

V = final volume of chlorophyll extract in 80 % acetone.

W = fresh weight of tissue extracted.

3.4.3 Estimation of Protein

As per the procedure described by Bradford (1976), the total soluble protein content was estimated. One gram each of leaf samples were homogenized in 10 ml, 0.1 *M* sodium acetate buffer (pH 4.7) (Appendix II) and centrifuged at 5000 g for 15 minutes at 4^{o} C. The supernatant was saved for the estimation of soluble protein. The reaction mixture consisted of 0.5 ml enzyme extract, 0.5 ml distilled water and 5 ml of diluted (5 times) dye solution (Appendix II). The absorbance was read at 595 nm in a spectrophotometer against the reagent blank. Bovine serum albumin (BSA) was used as the protein standard and the protein content was expressed as milligram albumin equivalent of soluble protein per gram of leaf tissue on fresh weight basis.

3.4.4 Estimation of Phenol

The phenol content was estimated as per the procedure described by Bray and Thorpe (1954). One gram each of the leaf samples were homogenized in 10 ml of 80 per cent ethanol. The homogenate was centrifuged at 10, 000 rpm for 20 minutes. The supernatant was saved and residue was extracted with five times the volume of 80 per cent ethanol and centrifuged as above. The supernatant was saved and evaporated to dryness in a boiling water bath. The residue was dissolved in 5 ml distilled water. An aliquot of 0.2 ml was pipetted out and made up to 3 ml with distilled water. Folin- Ciocalteau reagent (0.5 ml) was added and 2 ml of 20 per cent sodium carbonate solution was added to each tube after three minutes. This was mixed thoroughly and kept in boiling water for one minute. The reaction mixture was cooled and absorbance was measured at 650 nm against a reagent blank. Standard curve was prepared using different concentrations of catechol and expressed in catechol equivalents as mg g^{-1} leaf tissue on fresh weight basis.

3.4.5 Estimation of Defense Related Enzymes

3.4.5.1 *Estimation Of Peroxidase (PO)*

Peroxidase activity was estimated using a spectrophotometric method described by Srivastava (1987). Leaf samples of 1 g each were homogenized in 5 ml of 0.1 *M* sodium phosphate buffer (pH 6.5) (Appendix III) to which a pinch of polyvinyl pyrrolidone (PVP) was added. The homogenization was done at 4^0C using a pre- chilled pestle and mortar. The homogenate was filtered through a muslin cloth and centrifuged at 5000 rpm for 15 minutes at 4^0C and the supernatant was used as the enzyme extract for the estimation of PO activity.

The reaction mixture consisting of 1 ml of 0.05 *M* pyrogallol and 50 µl of enzyme extract was taken in both reference and sample cuvettes, mixed and kept in a spectrophotometer (Systronics UV- VIS spectrophotometer 118) and the reading was adjusted to zero at 420 nm. To initiate the reaction, one ml of 1% hydrogen peroxide (H_2O_2) was added to the sample cuvettes and the changes in the absorbance were recorded at 30 seconds interval upto 180 seconds. The PO activity was expressed as changes in absorbance min^{-1} g^{-1} fresh weight of tissue.

3.4.5.2 *Estimation of Polyphenol Oxidase (PPO)*

Polyphenol oxidase activity was determined as per the procedure given by Mayer *et al*. (1965). The enzyme extract was prepared as per the procedure given for the estimation of peroxidase.

The reaction mixture contained one ml of 0.1 *M* sodium phosphate buffer (pH 6.5) (Appendix III) and 50 µl of enzyme extract. The reaction was initiated after adding one ml of 0.01 *M* catechol. The observations were recorded in a spectrophotometer (Systronics UV-VIS spectrophotometer 118). The change in absorbance was recorded at 495nm at 30 seconds interval upto 180 seconds.

PPO activity was expressed as change in the absorbance of the reaction mixture per minute per gram on fresh weight basis.

3.4.5.3 *Estimation of Phenylalanine Ammonia-Lyase (PAL)*

PAL activity was assayed spectrophotometrically by assaying the rate of conversion of L- phenylalanine to trans- cinnamic acid at 290 nm as described by Dickerson *et al*. (1984). The enzyme extract was prepared by homogenizing one gram leaf sample in 5 ml of 0.1 *M* borate buffer (pH 8.8) (Appendix III) containing a pinch of PVP using chilled mortar and pestle. The homogenate was centrifuged at 10000 rpm for 10 minutes at 4^0C. The supernatant was used for the assay of PAL activity. The reaction mixture contained 3 ml of 0.1 *M* sodium borate buffer (pH 8.8), 0.2 ml enzyme extract and 0.1 ml of 12 *mM* L-phenyl alanine prepared in the same buffer. The blank contained 3 ml of 0.1 *M* sodium borate buffer (pH 8.8) and 0.1 ml of 12 *mM* L-phenyl alanine. The reaction mixture and blank were incubated at 40^0C for 30 minutes and reaction was stopped by adding 0.2 ml of 3*N* hydrochloric acid. The absorbance was read at 290 nm in a spectrophotometer (Systronics UV-VIS spectrophotometer 118). PAL activity was expressed as micrograms of cinnamic acid produced per minute per gram on fresh weight basis.

3.4.6 Electrophoretic Analysis of Proteins: SDS- PAGE

Electrophoretic separation of soluble protein of brinjal leaves was done based on the procedure described by Laemelli (1970).

Preparation of Sample

One gram each of healthy and diseased leaf samples were homogenized in 1.5 ml of cold denaturing solution (Appendix IV) at 4°C. The homogenate was centrifuged at 5000 rpm for 15 min. The supernatant was mixed with chilled acetone in the ratio 1:2 and the protein was allowed to precipitate by keeping the mixture at 4°C for 30 minutes. Then it was centrifuged at 5000 rpm for 15 minutes at 4°C. The

supernatant was removed and the pellet was resuspended in 50 µl of denaturing solution (Appendix IV) and vortexed. The homogenate was centrifuged at 5000 rpm for 15 minutes. Then the supernatant was mixed with 10 µl of SDS sample buffer and kept in a boiling water bath for 3 minutes. The samples were used for PAGE analysis.

The protein concentration in each sample was adjusted to strength of 100 µg of protein following Bradford method. Standard was prepared using known molecular weight marker (Protein molecular weight marker, GeNei Cat No. 623110275001730) 10 µl added with 10 µl sample buffer.

Reagents

a) Acrylamide stock (30 %)

Acrylamide	–	29.2 g
Bis-acrylamide	–	0.8 g
Double distilled water	–	100.0 ml

b) Separating (resolving) gel buffer stock (1.5 *M* Tris-HCl, pH 8.8)

Tris base (18.15 g) was dissolved in approximately 50 ml of double distilled water. The pH was adjusted to 8.8 with 6 *N* HCl and made up the volume to 100 ml with double distilled water and stored at 4°C.

c) Stacking gel buffer stock (0.5 *M* Tris-HCl pH 6.8)

Tris base (6.0 g) was dissolved in approximately 60 ml of double distilled water and adjusted the pH to 6.8 with 6 *N* HCl and the volume was made upto 100 ml with double distilled water and stored at 4°C.

d) Polymerising agents

Ammonium persulphate (APS) 10 per cent was prepared freshly before use.

TEMED – Fresh from refrigerator

e) Electrode buffer pH 8.3

Tris base	- 6.0 g
Glycine	- 28.8 g
SDS	- 2.0 g
Double distilled water	- 2 litre

f) Sample buffer

Double distilled water	- 2.6 ml
0.5 *M* Tris HCl pH 6.8	- 1.0 ml
2-mecaptoethanol	- 0.8 ml
Glycerol	- 1.6 ml
SDS 20 % (w/v)	- 1.6 ml
0.5 % Bromophenol blue	- 0.4 ml

g) Staining solution

Comassie brilliant blue R 250	- 0.1 g
Methanol	- 40 ml
Glacial acetic acid	- 10 ml
Double distilled water	- 50 ml

h) Destaining solution

As above without Coomassie brilliant blue R 250

Procedure

Separating gel should be casted first followed by stacking gel by mixing the various solutions as shown below.

a) Preparation of separating gel (12%)

Double distilled water	- 13.4 ml
1.5*M* Tris HCl, pH 8.8	- 10 ml
SDS 10 %	- 0.4 ml
Acrylamide stock	- 16 ml

The above solution was mixed well and degassed for two to three minutes and then the following polymerizing agents were added immediately.

Freshly prepared 10% Ammonium persulphate (APS)	- 0.20 ml
Tetra methyl ethylenediamine (TEMED)	- 0.02 ml

The separating gel was mixed well and poured immediately between the glass plates and a layer of water was added above the polymerizing solution to make the polymerization process faster.

b) Preparation of stacking gel (4%)

Double distilled water	- 6.1 ml
0.5*M* Tris HCl, pH 6.8	- 2.5 ml
SDS 10 %	- 0.2 ml
Acrylamide stock	- 1.3 ml

The solution was mixed well, degassed and the following agents were added.

APS 10 %	- 0.05 ml
TEMED	- 0.01 ml

The water layered over the separating gel was removed and washed with a little amount of electrode buffer and then the stacking gel was poured over the polymerized separating gel, after keeping the comb in position.

After polymerization, the comb was removed and the samples were loaded into the wells. Standard with known molecular weight is also loaded into one of the wells. The electrophoresis was performed at 100 V till the dye reached the separating gel. Then the voltage was increased to 200 V and continued till the dye reached the bottom of the gel. Immediately after electrophoresis, the gel was removed from the glass plates and incubated in the staining solution for overnight with uniform shaking which was then transferred to the destaining solution. The protein appeared as bands in the gel was photographed after placing on a transilluminator (Appligene Model White / UV TMW-20).

3.4.7 Electrophoretic analysis of isozyme

The present work was carried out to understand the enzyme alterations in healthy and phytoplasma infected plant leaves.

The electrophoresis of protein extracts from plant tissues using different kinds of support media and buffer systems (Appendix V) allows the separation of multiple forms of enzymes (isozymes) on the basis of charge and molecular size. Discontinuous anionic polyacrylamide gel electrophoresis was conducted under non-dissociating conditions as described by Wagih and Coutts (1982) with slight modification.

3.4.7.1 *Peroxidase Isozyme Analysis*

Soluble and ionically bound enzymes were extracted by grinding the samples under chilled condition in 50 *mM* Tris-Cl (pH 7.6) in the ratio of 1:1 w/v. The homogenate was centrifuged at 15,000 rpm for 10 min at 4°C. The supernatant was used for isozyme analysis. The protein content was adjusted in each sample to the strength of 100 µg of protein following Bradford method. Proteins extracted by 50

mM Tris (pH 7.6) were separated by gel electrophoresis in 7.5 per cent gel. The gel was prepared using the stock solution prepared for protein gel electrophoresis without SDS (native gel). Triton X-100 (2%) was added in place of SDS. The gel was incubated in 0.6 *M* sodium acetate buffer (pH 5.4) containing 0.5 percent O–dianisidine HCl for 30 minutes at room temperature. The gel was transferred to 0.1 *M* hydrogen peroxide until visible bands were developed.

Reagents

a) Separating gel (7.5 %)

Tris chloride buffer stock solution (pH 8.9)	- 5 ml
Resolving gel acrylamide solution	- 10 ml
Distilled water	- 25 ml
APS	- 300 µl
Triton X-100	- 2%

b) Stacking gel (4%)

Tris chloride buffer stock (pH 6.7)	- 2.5 ml
Resolving gel acrylamide solution	- 3.1 ml
Distilled water	- 14.1 ml
APS	- 300 µl
Triton X-100	- 2%

3.5 MOLECULAR DETECTION

3.5.1 Isolation of genomic DNA

The genomic DNA was isolated using modified CTAB method (Lodhi *et al.* 1994) as well as by using DNeasy plant mini kit (QIAGEN: Cat. No. 69104) as per the manufacturer's protocol.

3.5.1.1 *Extraction of DNA by modified CTAB method*

Reagents used for DNA extraction

1. CTAB buffer

CTAB	: 2%
Tris HCl	: 100 *mM*
EDTA	: 20 *mM*
Nacl	: 1.4 *M*
β-Mercaptoethanol	: 1%

2. TE buffer

Tris HCl	: 10 *mM*
EDTA	: 1 *mM*
pH	: 8.0

3. TAE buffer/ TBE buffer

TAE buffer (50x, 100ml)

Tris	: 24.2 g
Glacial acetic acid	: 5.71 ml
EDTA	: 1.861 g
pH	: 8.0

TBE buffer (5x, 1000ml)

Tris base	: 54 g
Boric acid	: 27.5 g
EDTA, 0.5*M*	: 20 ml

4. Chloroform : Isoamyl alcohol (24:1)
5. Isopropanol
6. 70% Ethanol

Protocol

1. One gram of fresh leaf tissue with the symptom was taken in a sterile mortar, add liquid nitrogen to frozen the samples and powdered using sterile pestle along with 0.1g of PVP.
2. The powdered samples were transferred into 1 ml CTAB isolation buffer and mixed well by vortexing.
3. The tubes were incubated in a water bath at 65°C for 1hr with occasional gentle swirling.
4. The samples were then centrifuged at 8000 rpm for 5 min.
7. Collected the supernatant, add equal volume of Chloroform: Isoamyl alcohol (24:1) and mixed well.
5. Centrifuged at 13,000 rpm for 15 minutes (This produced two phases, an upper aqueous phase which contains the DNA, and a lower chloroform phase that contains some degraded proteins, lipids, and many secondary compounds. The interface between these two phases contains most of the "junk"- cell debris, many degraded proteins etc.).
6. The aqueous phase was collected and equal volume of ice cold isopropanol was added to precipitate the DNA. These were mixed by inverting the tubes.
7. The tubes were incubated at -20°C for 1 hr or 4°C overnight.
8. The mixtures were centrifuged at 13, 000 rpm for 15 min.
9. The pellet was washed by using 70% ethanol and centrifuged at 13, 000 rpm for 15 min.
10. The supernatant was discarded and the pellet was dried for 10-15 min.
11. The DNA was resuspended in TAE buffer (APPENDIX VI).

3.5.2 Confirmation of genomic DNA

The presence of genomic DNA was confirmed by the horizontal mini submarine gel electrophoresis unit (Hoefer, Sl No. 30036384) using 0.8% agarose gel made of 1x TAE buffer and ethidium bromide (0.5µg/ml). The DNA samples were mixed with the gel loading dye and added to the wells prepared. The electrophoresis was run till the loading dye reached about 3/4th of the gel. After the completion of electrophoresis, the gel was visualized with the help of gel documentation unit (BIO RAD Molecular Imager® Gel Doc™ XR+).

3.5.3 Nested PCR

Neasted PCR was carried out in a thermal cycler (BIO RAD T100™)by the preliminary amplification using the universal primer pair P1/P7 followed by a second amplification by R16F2n/R16R2, which amplifies a secondary target within the first run product, so that it reduced the contamination of products due to the amplification of unexpected primer binding sites. The relative positions of the two primers are represented in Fig 1. PCR was performed in 15 µl reaction mixture consisting of the following:

Preliminary PCR using P1/P7

10x Taq buffer (with MgCl2)	: 1.5 µl
10mM dNTP	: 0.6 µl
Taq polymerase (1unit/ µl)	: 1 µl
Forward primer P1 (10 *pM*)	: 0.5 µl
Reverse primer P7 (10 *pM*)	: 0.5 µl
Template DNA	: 2 µl

Sterile distilled water	: 8.9 µl

Secondary PCR using R16F2n/R16R2

10x Taq buffer (with MgCl2)	: 1.5 µl
10mM dNTP	: 0.6 µl
Taq polymerase (1unit/µl)	: 1 µl
Forward primer R16F2n (10 *pM*)	: 0.5 µl
Reverse primer R16R2 (10 *pM*)	: 0.5 µl
1° PCR product	: 1 µl
Sterile distilled water	: 9.9 µl

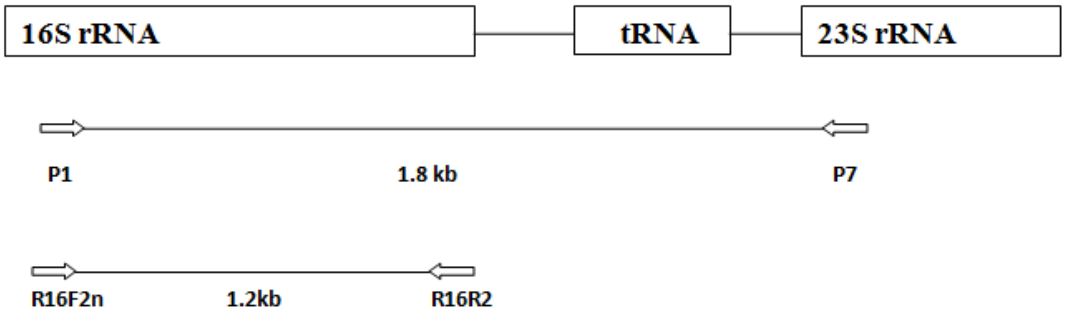


Fig .1 Diagrammatic representation of the 16S-23S rRNA operon, showing the relative positions of universal primers that have been used in the present study (indicated below the arrows) and the sizes of the expected amplicons (not drawn to scale).

Table 1. Primers used in nested PCR amplification

Primer	Primer sequence	Reference
P1(Forward primer)	5' AAGAGTTTGATCCTGGCTCAGGATT 3'	(Deng and Hiruki, 1991a; Smart *et al*, 1996; Schneider et al, 1995)
P7 (Reverse primer)	5' CGTCCTTCATCGGCTCTT 3'	
R16F2n (Forward primer)	5' GAAACGACTGCTAAGACTGG 3'	(Gundersen and Lee, 1996)
R16R2 (Reverse primer)	5' TGACGGGCGGTGTGTACAAACCCCG 3'	

All the PCR operations were performed in a Thermal cycler (BIO RAD T100^{TM}). The PCR conditions are represented in Table 2. The PCR products were analyzed by using horizontal electrophoresis unit. Agarose gel (1%) was prepared with ethidium bromide (0.5µg/ml) and TAE/TBE buffer (running buffer). A marker (1Kb DNA ladder: 2µl) was mixed with 1µl of 6x dye and 3µl of sterile water and load to the well. The PCR products each of 5µl was mixed with 2µl of the dye and load to the appropriate wells. After the dye has reached 3/4th of the gel, it was visualized in the gel documentation system (BIO RAD Molecular Imager® Gel Doc^{TM} XR+) and the amplification was analyzed. DNA fragments of 1.2 kb amplified from the nested PCR analysis were sequenced.

3.5.4 Sequencing using BigDye Terminator v3.1

Sequencing reaction was done at Rajiv Gandhi Centre for Biotechnology institute, Thiruvananthapuram in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems , USA) following manufactures protocol.

Table 2. PCR Conditions followed in nested PCR.

PCR Primer combination	Process	Temperature (°C)	Time	No. of cycles
P1/P7	Initial denaturation	94	5 min	35
	Denaturation	94	45 sec	
	Annealing	63	1 min	
	Extension	72	2 min	
	Final extension	72	10 min	
R16F2n/R16R2	Initial denaturation	94	3 min	35
	Denaturation	94	30 sec	
	Annealing	56	1 min	
	Extension	72	1min 30sec	
	Final extension	72	10 min	

The PCR mix consisted of the following components:

PCR Product	-	10-20 ng
Primer	-	3.2 *pM* (either Forward or Reverse)
Sequencing Mix	-	0.28 µl
5x Reaction buffer	-	1.86 µl
Sterile distilled water	-	make up to 10µl

Primers Used

R16F2n : 5’ GAAACGACTGCTAAGACTGG 3’

R16R2 : 5’ TGACGGGCGGTGTGTACAAACCCCG 3’

The sequencing PCR temperature profile consisted of a 1st cycle at 96ºC for 2 minutes followed by 30 cycles at 96ºC for 30 sec, 50ºC for 40 sec and 60ºC for 4 minutes for all the primers.

3.5.5 Post Sequencing PCR Clean up

1. Make master mix I of 10µl milli Q and 2 µl 125*mM* EDTA per reaction
2. Add 12µl of master mix I to each reaction containing 10µl of reaction contents and are properly mixed.
3. Make master mix II of 2 µl of 3*M* sodium acetate pH 4.6 and 50 µl of ethanol per reaction.
4. Add 52 µl of master mix II to each reaction.
5. Contents are mixed by inverting.
6. Incubate at room temperature for 30 minutes
7. Spin at 14,000 rpm for 30 minutes
8. Decant the supernatant and add 100 µl of 70% ethanol. Again spin at 14,000

rpm for 20 minutes.

9. Decant the supernatant and repeat 70% ethanol wash
10. Decant the supernatant and air dry the pellet.

The cleaned up air dried product was sequenced in ABI 3500 DNA Analyzer (Applied Biosystems).

3.5.6 Quality Analysis of Sequence

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.1 (Drummond *et al*., 2010).

3.5.7 Phylogenetic analysis

The partial 16S rDNA sequences of both brinjal (APPENDIX VIII) and catharanthus (APPENDIX IX) isolates were blasted at the NCBI website and phylogenetic relationship was studied by the comparison with certain phytoplasma isolates obtained from Genbank. The percentages of sequence homology with other phytoplasma isolates were also recorded. The phylogenetic tree was constructed using MEGA6 software with 1000 bootstrap replications. *Acholeplasma palmae* was used as the outgroup to root the tree.

Results

4. RESULTS

4.1 SYMPTOMATOLOGY

The graft inoculated brinjal plants showed characteristic little, narrow, soft, glabrous and smooth leaves produced as clusters and later showed the symptoms of yellowing. Newly formed leaves were much shorter and the petioles were shortened so that the leaves appeared to be sticking to the stem. The proliferation of axillary shoots along with shortened internodes and numerous clustered small leaves gave the plant a stunted bushy appearance. The typical symptom of phyllody, the production of leaf like structures in place of flowers was observed. The phylloid flowers were erect, green and mostly sterile. Fruits were rarely observed in the infected plants. Similar symptoms were also recorded from phytoplasma infected catharanthus plants (Plates 1-11).

4.2 TRANSMISSION

4.2.1 Graft Transmission

Graft transmission was carried out by wedge grafting of phytoplasma infected scion onto a healthy root stock and observed for the symptom development. The initial symptoms were noticed in the new sprouts of the rootstock 7-10 days after the graft transmission (Plates 12-13). The graft transmission was found to be 100% successful in both brinjal and catharanthus (Table 3).

4.2.2 Dodder Transmission

Cuscuta sp. was established between the phytoplasma infected plants and the healthy test plants and kept for the symptom expression in the healthy test plants (Plate 14-15). The percentage transmission of 10% was observed in brinjal and 20% in catharanthus (Table 3).

Plate. 1.a Little leaf symptom in mature twig of brinjal.

Plate. 1.b. Little leaf symptom in young twig of brinjal.

Plate. 2. Clustering of leaves in brinjal

Plate. 3.a Floral symptom: Loss of colour of petal in brinjal

Plate. 3.b Floral symptom: Erect and green phylloid flowers in brinjal

Plate. 4. Stunted and bushy growth in brinjal

Plate 5. Yellowing in brinjal

Plate. 6.a. Little leaf symptom in catharanthus.

Plate. 6.b. Little leaf and stunted growth in catharanthus.

Plate. 7. Yellowing in catharanthus.

Plate. 8.a Floral symptoms: Comparison between normal and infected flowers of pink variety of catharanthus

Plate. 8.b Comparison between normal and infected flowers of white variety of catharanthus

Plate. 8.c Changing colour of petal in catharanthus

Plate. 8.d Colour degradation of petals during early stage

Plate. 8.e Degradation of colour in advanced stage

Symptoms collected from the field

Plate. 9. Phyllody in bittergourd

Plate. 10. Phyllody in ashgourd

Plate.11. Phyllody in sesame

Table 3. Percentage of transmission obtained in brinjal and catharanthus after graft and dodder transmissions.

CROP	GRAFT TRANSMISSION		DODDER TRANSMISSION	
	No. of Plants inoculated	No. of Plants developed symptom	No. of Plants inoculated	No. of Plants developed symptom
Brinjal	10	10	10	1
	10	10	10	1
Catharanthus	10	10	10	2
	10	10	10	2
Percentage Transmission	100% in both brinjal and catharanthus		10% in brinjal & 20% in catharanthus	

4.3 MAINTENANCE OF PHYTOPLASMA

Tissue culture technique was used for the *in vitro* maintenance of phytoplasma culture (Plate 16). The surface sterilized phytoplasma infected shoots were cultured on MS medium supplemented with 0.2mg/l BAP, 0.6mg/l NAA and 0.4mg/l IAA and regenerated into plantlets (Robinson and Saranya (2013). The presence of phytoplasma was detected in the *in vitro* grown tissue culture plants of brinjal by the nested PCR with P1/P7 followed by R16F2n/R16R2 (Plates 19-20).

Plate. 12.a Graft inoculation in brinjal

Plate. 12.b Symptom expression on new sprouts after graft inoculation in brinjal

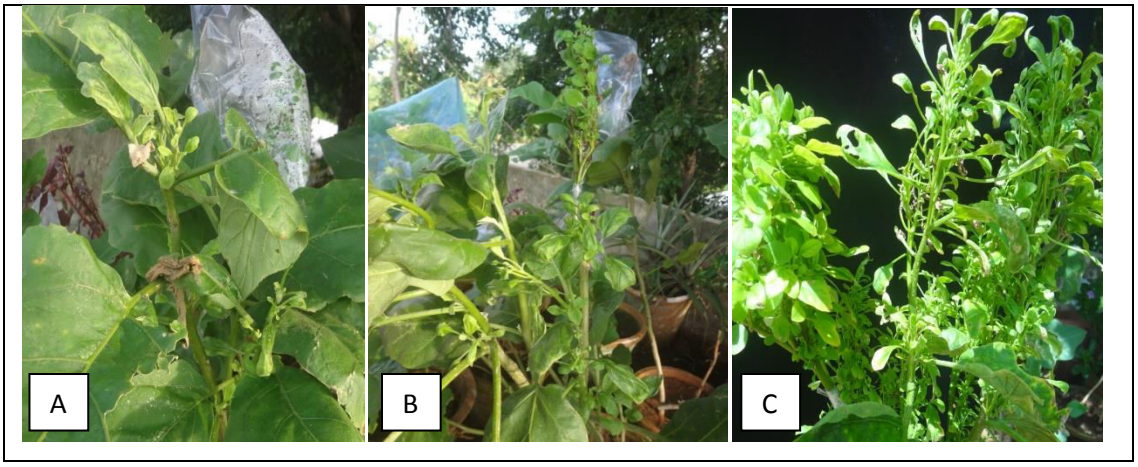


Plate. 12.c Different stages after graft inoculation in brinjal
A: 20DAI B: 30DAI C: 60DAI
(DAI: Days After Inoculation)

Plate. 13. Graft inoculation in catharanthus.

Plate. 14. Dodder transmission in brinjal

Plate. 15. Dodder transmission in catharanthus

Plate. 16.a Tissue culture plant developed from shoot tip culture of brinjal

Plate. 16.b Multiple shoot development in brinjal

Plate. 16.c Tissue culture plant with root growth in brinjal

The direct PCR with P1/P7 gave the amplicons of size ~1.8 kb and the nested PCR with P1/P7 followed by R16F2n/R16R2 produced the amplicons of size ~1.2 kb.

4.4 HOST PATHOGEN INTERACTION

Physiological changes in brinjal and catharanthus plants infected by phytoplasma were studied at different stages i.e. at 10^{th}, 20^{th}, 30^{th} and 60^{th} days after inoculation and compared with the healthy samples.

4.4.1 Estimation of Total Carbohydrate

The total carbohydrate content of healthy and diseased plant samples at different days after inoculation was studied and the results are presented in Table 4. The results in brinjal revealed that immediately after the inoculation, the carbohydrate content was significantly increased from 5.5 mg g^{-1} and reached the maximum content of 7 mg g^{-1} at 10 DAI, which was decreased at 20 DAI and found to be on par with that of healthy samples. Thereafter the carbohydrate content was significantly reduced with the least content of 2.3 mg g^{-1} at 60 DAI.

The results of carbohydrate estimation in healthy and inoculated samples of catharanthus plants are represented in the Table 4, which indicated an increased level of carbohydrate content in the inoculated plants than the healthy samples. The least carbohydrate content was 6 mg g^{-1}, estimated in healthy samples. The carbohydrate content was found to be significantly increased at 10 DAI, which was decreased and found to be on par with the content at 20 DAI. Thereafter, even though the carbohydrate content was increased, the contents were found to be on par, with the peak value of 8.42 mg g^{-1}at 60 DAI.

Table 4. Changes in total carbohydrate content of brinjal and catharanthus leaves in response to phytoplasma inoculation (mg g^{-1} fresh weight of tissue).

Treatments	*Change in carbohydrate content	Per cent increase (+) or decrease (-) over healthy	*Change in carbohydrate content	Per cent increase (+) or decrease (-) over healthy
	Brinjal		Catharanthus	
Healthy	5.5[b]	-	6.00[c]	-
10 DAI	7.0[a]	+27	7.25[b]	+21
20 DAI	6.0[b]	+9	7.08[b]	+18
30 DAI	4.0[c]	-27	8.02[a]	+34
60 DAI	2.3[d]	-58	8.42[a]	+40
CD Value (0.05) (Between treatments)	0.720		0.518	

*Mean of three replications

4.4.2 Estimation of Chlorophyll

The samples were estimated for chlorophyll a, chlorophyll b and total chlorophyll at different days after inoculation and compared with the healthy samples. The contents of chlorophyll a, chlorophyll b and total chlorophyll were found to be higher in healthy samples as compared to the inoculated samples. In brinjal with little leaf disease, the chlorophyll a content was significantly reduced towards later stages of inoculation except at 20 and 30 DAI, at which the contents of chlorophyll a were found to be on par and it is not reflected in the total chlorophyll content (represented in Table 5). The results revealed that chlorophyll b and the total chlorophyll content were found to be significantly reduced towards later stages of infection. The maximum content of total chlorophyll was 1.097 mg g^{-1}, noticed in the healthy samples and the least content was 0.373 mg g^{-1}in the inoculated samples at 60 DAI.

The similar effect was noticed in case of catharanthus with little leaf disease (Table 6). The content of chlorophyll b in healthy and inoculated samples at 10 DAI were found to be on par and thereafter the content was significantly reduced. The maximum chlorophyll content was 1.547 mg g^{-1}, obtained in the healthy samples and the least content was 0.933 mg g^{-1}in the inoculated samples at 60 DAI. The phytoplasma infection caused a significant reduction in the chlorophyll a and total chlorophyll content throughout the period of observation as compared to the healthy control plants.

4.4.3. Estimation of Protein

The total soluble protein content was estimated as per the procedure described by Bradford (1976) and the results are presented in the table 7. The results indicated that the infected samples had lower protein content, which is found to be decreasing at subsequent stages of infection than the healthy samples. The protein content in brinjal was significantly reduced throughout the period of observation, with the

Table 5. Changes in chlorophyll content of brinjal leaves in response to phytoplasma inoculation, mg g^{-1}

Treatments	*Changes in Chlorophyll content (mg g^{-1} fresh weight)		
	Chlorophyll a	Chlorophyll b	Total chlorophyll
Healthy	0.790[a]	0.307[a]	1.097[a]
10 DAI	0.560[b]	0.193[b]	0.753[b]
20 DAI	0.507[c]	0.110[c]	0.617[c]
30 DAI	0.483 [c]	0.080[d]	0.563[d]
60 DAI	0.340[d]	0.033[e]	0.373[e]
CD (0.05)	0.036	0.029	0.052

*Mean of three replications

Table 6. Changes in chlorophyll content of catharanthus leaves in response to phytoplasma inoculation, mg g^{-1}

Treatments	*Changes in Chlorophyll content (mg g^{-1} fresh weight)		
	Chlorophyll a	Chlorophyll b	Total chlorophyll
Healthy	1.087[a]	0.460[a]	1.547[a]
10 DAI	0.987[b]	0.433[a]	1.420[b]
20 DAI	0.927[c]	0.387[b]	1.313[c]
30 DAI	0.830[d]	0.310[c]	1.140[d]
60 DAI	0.687[e]	0.247[d]	0.933[e]
CD(0.05)	0.037	0.032	0.056

*Mean of three replications

Table 7. Changes in total soluble protein content of brinjal and catharanthus leaves in response to phytoplasma inoculation, (mg g^{-1} fresh weight of tissue).

Treatments	*Change in soluble protein content	Per cent increase (+) or decrease (-) over healthy	*Change in soluble protein content	Per cent increase (+) or decrease (-) over healthy
	Brinjal		Catharanthus	
Healthy	1.320[a]	-	0.760[a]	-
10 DAI	1.143[b]	-13	0.720[b]	-5
20 DAI	0.957[c]	-27.5	0.703[b]	-7.5
30 DAI	0.860[d]	-35	0.630[c]	-17
60 DAI	0.713[e]	-46	0.530[d]	-30
CD(0.05)	0.038		0.038	

*Mean of three replications

maximum protein content of 1.320 mg $g^{-1}$for the healthy samples and the least content of 0.713 mg g^{-1} at 60 DAI. The similar trend was also noticed in case of catharanthus except the fact that the protein content at 10 DAI was found to be on par with the protein content at 20 DAI. The highest protein content in catharanthus was 0.760 mg g^{-1} in healthy samples and the lowest content was 0.530 mg g^{-1} at 60 DAI.

4.4.4. Estimation of Phenol

The phenol content was estimated in healthy and diseased samples and the results are given in the Table.8. The study was undertaken to understand the effect of phytoplasma on the phenol content of the samples and the results indicated significant difference between the treatments. In brinjal and catharanthus affected by little leaf phytoplasma, the phenol content exhibited the same trend with significant reduction throughout the period of inoculation. The highest phenol content in brinjal was 11.25 mg g^{-1} in healthy samples and the lowest content was 8.02 mg g^{-1} in the inoculated samples at 60 DAI. The maximum phenol content in catharanthus was 12.75 mg g^{-1} in the healthy samples, which significantly reduced to 8.88 mg g^{-1} at 60 DAI.

4.4.5. Defence Related Enzymes

4.4.5.1. *Estimation of Peroxidase (PO)*

Peroxidase (PO) activity in healthy and inoculated samples of brinjal and catharanthus at different DAI was estimated and presented in the table 9. The results indicated a significant increase in PO activity in inoculated samples at all stages of infection in brinjal. Thus the activity of peroxidase was induced in brinjal as a result of infection by phytoplasma. The highest activity was obtained for brinjal samples at 60 DAI (7.207 $min^{-1}g^{-1}$) and that of healthy sample was the least (2.1 $min^{-1}g^{-1}$).

Table 8. Changes in phenol content of brinjal and catharanthus leaves in response to phytoplasma inoculation, (mg g^{-1} fresh weight of tissue).

Treatments	*Change in soluble phenol content	Per cent increase (+) or decrease (-) over healthy	*Change in soluble phenol content	Per cent increase (+) or decrease (-) over healthy
	Brinjal		Catharanthus	
Healthy	11.25[a]	-	12.75[a]	-
10 DAI	10.00[b]	-11	10.25[b]	-19.6
20 DAI	9.50[c]	-15.5	10.00[c]	-21.57
30 DAI	8.75[d]	-22	9.25[d]	-27.45
60 DAI	8.02[e]	-29	8.88[e]	-30.35
CD(0.05)	0.285		0.206	

*Mean of three replications

In case of catharanthus, the PO activity was enhanced and found to be significantly higher in the inoculated samples with the maximum activity of 5.107 $min^{-1}g^{-1}$ at 30 DAI and the activity was declined to 3.9 $min^{-1}g^{-1}$ at 60 DAI. The PO activity in brinjal and catharanthus exhibited similar trend up to 30 DAI. Immediately after the inoculation i.e. at 10 DAI, a threefold higher activity was observed in catharanthus than the healthy samples. The highest peroxidase activity in catharanthus was 5.107 at 30 DAI and the least activity was observed in the healthy samples (1.503 $min^{-1}g^{-1}$).

4.4.5.2. *Estimation of Polyphenol Oxidase (PPO)*

PPO activity in healthy and graft inoculated samples of brinjal and catharanthus at different DAI was studied (Table 10). The results revealed that the activity of PPO followed a reverse trend compared to the activity of PO. PPO activity was reduced in the inoculated plants of brinjal and catharanthus in response to phytoplasma inoculation, compared to the control plants. In brinjal, the activity was significantly reduced throughout the successive stages of inoculation. The PPO activity in brinjal was highest for the healthy samples (7.383 $min^{-1}g^{-1}$) and the least activity of 3.143 $min^{-1}g^{-1}$ was observed at 60 DAI. In catharanthus, the activity in healthy and inoculated samples at 10 DAI was observed to be on par and the activity at 20 and 30 DAI was also found to be on par. The maximum activity was 3.167 $min^{-1}g^{-1}$ in healthy control plants, which reduced to 0.810 $min^{-1}g^{-1}$ at 60 DAI.

4.4.5.3. *Estimation of Phenylalanine Ammonia-Lyase (PAL)*

The activity of PAL in healthy and inoculated samples was estimated (Table 11). The results revealed that the PAL activity was following similar trend with significant difference in both brinjal and catharanthus. The activity was significantly reduced in the inoculated plant samples compared to the healthy samples (25 µg $g^{-1}min^{-1}$) and reached the least value of activity at 20 DAI (12.87 µg $g^{-1}min^{-1}$) and

Table 9. Changes in peroxidase activity of brinjal and catharanthus leaves in response to phytoplasma inoculation (changes in absorbance $min^{-1}g^{-1}$ fresh weight).

Treatments	*Peroxidase activity	Per cent increase (+) or decrease (-) over healthy	*Peroxidase activity	Per cent increase (+) or decrease (-) over healthy
	Brinjal		Catharanthus	
Healthy	2.100[e]	-	1.503[e]	-
10 DAI	4.900[d]	+133	4.500[c]	+199
20 DAI	5.204[c]	+148	4.910[b]	+227
30 DAI	6.020[b]	+187	5.107[a]	+240
60 DAI	7.207[a]	+243	3.900[d]	+159
CD (0.05)	0.026		0.029	

*Mean of three replications

Table 10. Changes in Polyphenol oxidase activity of brinjal and catharanthus leaves in response to phytoplasma inoculation (changes in absorbance $min^{-1}g^{-1}$ fresh weight).

Treatments	*Polyphenol oxidase activity	Per cent increase (+) or decrease (-) over healthy	*Polyphenol oxidase activity	Per cent increase (+) or decrease (-) over healthy
	Brinjal		Catharanthus	
Healthy	7.383[a]	-	3.167[a]	-
10 DAI	6.440[b]	-13	2.900[a]	-8.4
20 DAI	5.600[c]	-24	2.097[b]	-34
30 DAI	4.767[d]	-35	1.797[b]	-43
60 DAI	3.143[e]	-57	0.810[c]	-74
CD (0.05)	0.610		0.444	

*Mean of three replications

Table 11. Changes in Phenylalanine ammonia-lyase activity of brinjal and catharanthus leaves in response to phytoplasma infection (changes in absorbance µg $g^{-1}min^{-1}$fresh weight).

Treatments	*Phenylalanine ammonia-lyase activity	Per cent increase (+) or decrease (-) over healthy	*Phenylalanine ammonia-lyase activity	Per cent increase (+) or decrease (-) over healthy
	Brinjal		Catharanthus	
Healthy	25.0[b]	-	18.22[a]	-
10 DAI	15.4[d]	-38	15.07[c]	-17
20 DAI	12.87[e]	-48.5	12.56[e]	-31
30 DAI	22.16[c]	-11	14.58[d]	-20
60 DAI	28.31[a]	+13	16.43[b]	-10
CD(0.05)	0.557		0.328	

*Mean of three replications

thereafter significantly enhanced and attained a peak activity of 28.31 µg $g^{-1}min^{-1}$at 60 DAI in brinjal. Even though catharanthus samples exhibited the same trend, with enhanced activity after 20 DAI, the activity was not enhanced up to that of healthy samples. The maximum PAL activity in catharanthus was 18.22 µg $g^{-1}min^{-1}$in healthy control plants and the least activity was 12.56 µg $g^{-1}min^{-1}$at 20 DAI. So the maximum PAL activity was attained at 60 DAI in brinjal while in catharanthus, the healthy samples exhibited the highest activity.

4.4.6. Electrophoretic Analysis of Proteins Using SDS-PAGE

Proteins were extracted from healthy and phytoplasma graft inoculated samples of brinjal and resolved by SDS- PAGE. The protein profile of healthy and inoculated samples at different intervals was analyzed and compared. The analysis revealed the presence of two extra protein bands in the infected samples with molecular weights of 29 kDa and 43 kDa, the sizes of which were estimated by the comparison with low range protein molecular weight markers (PMWM) loaded along with the samples visible on the Coomassie brilliant blue stained SDS polyacrylamide gels (Plate. 17). The extra novel proteins which were absent in the corresponding healthy sample are assumed to be induced in the graft inoculated plants as a result of the plant-phytoplasma interaction.

4.4.7. Electrophoretic Analysis of Isozyme.

Native polyacrylamide gel electrophoresis was carried out to find out the presence of isoforms of enzymes and their intensity in both healthy and phytoplasma infected plants. Isozyme pattern analysis of peroxidase revealed two isoperoxidase bands in the inoculated plants, one of which was absent in the healthy samples (Plate. 18). The Relative mobility (Rm) values of the samples are presented in the Table. 12. The intensity of bands was found to be increased as the days after inoculation increased.

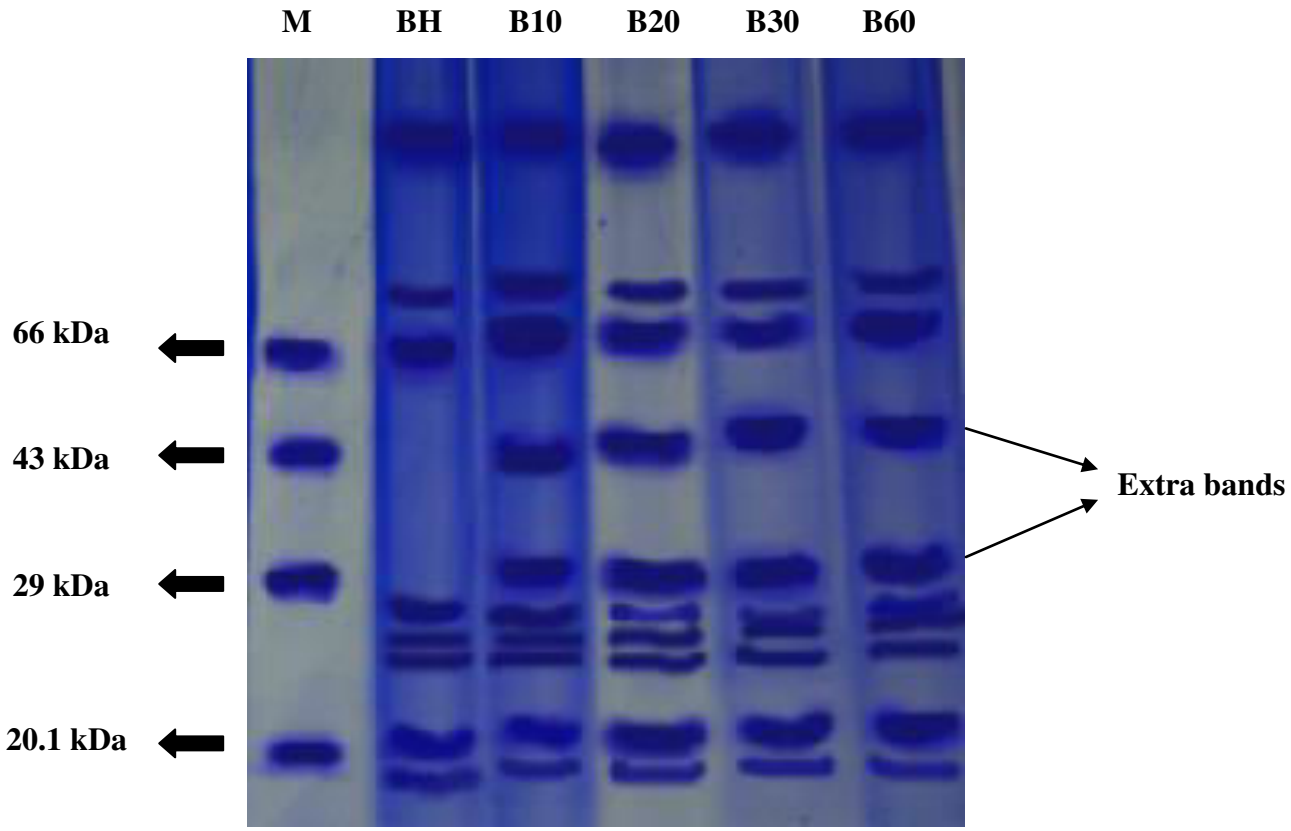


Plate. 17. Electrophoretic analysis of proteins: SDS-PAGE

M: Protein marker

BH: Healthy brinjal

B10: Brinjal at 10 DAI

B20: Brinjal at 20 DAI

B30: Brinjal at 30 DAI

B60: Brinjal at 60 DAI

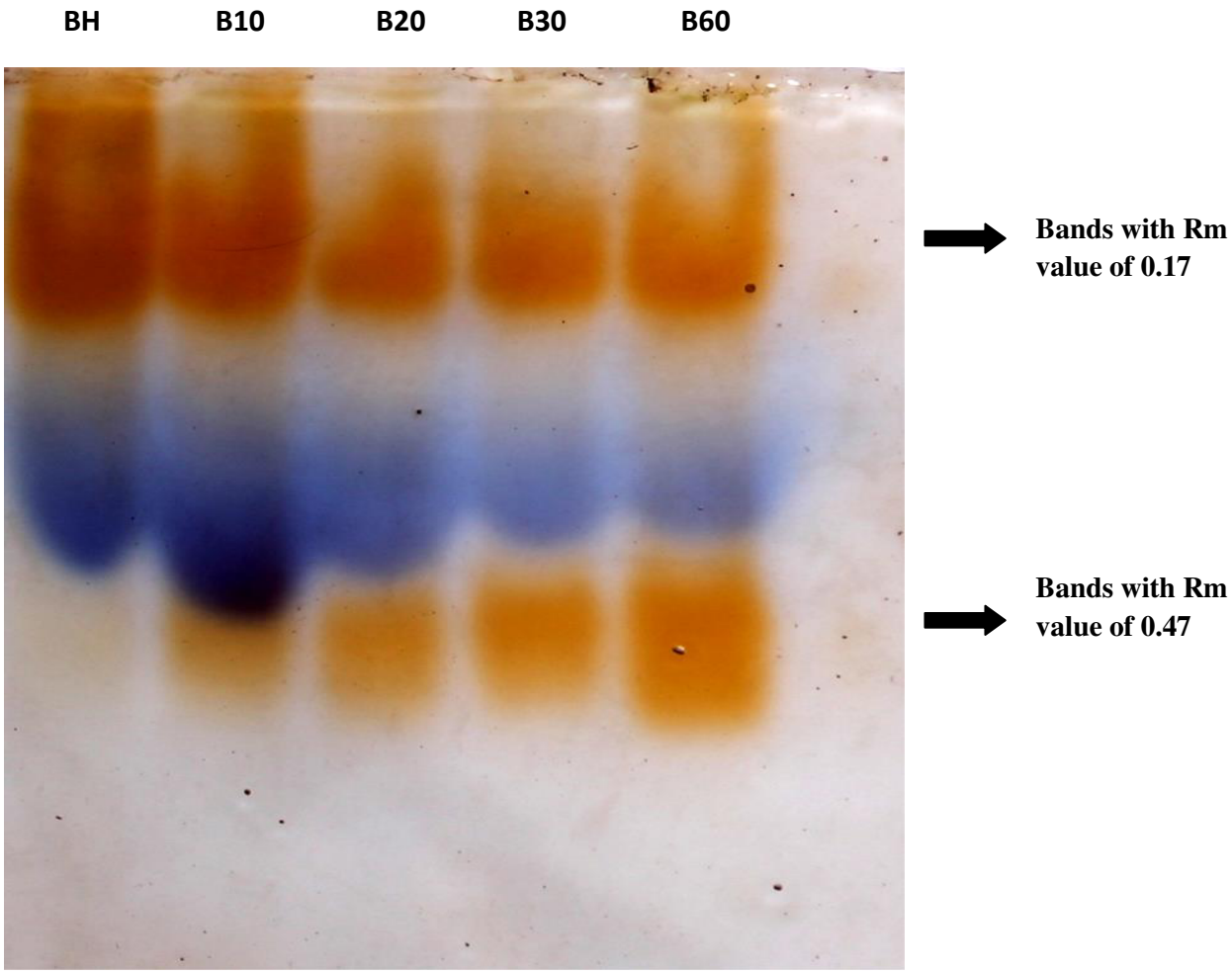


Plate. 18 . Electrophoretic analysis of isozyme.

BH: Healthy brinjal

B10: Brinjal at 10 DAI

B20: Brinjal at 20 DAI

B30: Brinjal at 30 DAI

B60: Brinjal at 60 DAI

The analysis revealed two isoperoxidase bands in the inoculated plants with Relative mobility (Rm) values, 0.17 and 0.47. The isoperoxidase band with Rm value of 0.47 was absent in the healthy sample.

Table 12. Rm values of isoperoxidase bands in healthy and infected brinjal samples.

Iso POs	Healthy control	Inoculated samples
Iso PO1	0.17	0.17
Iso PO2	-	0.47

4.5 MOLECULAR DETECTION AND CHARACTERIZATION

4.5.1 PCR Amplification and Sequence Analysis

Phytoplasma DNA was amplified by direct as well as nested PCR using the DNA extracted from samples of phytoplasma infected brinjal, tissue culture plants of brinjal and catharanthus. A 1.8 kb fragment corresponding to the entire 16S rDNA gene along with the 16S-23S spacer region and the 5'end of the 23S rDNA was obtained in the direct PCR using the universal phytoplasma primers P1/P7 (Plate 19). Further, nested PCR with universal internal primer pairs R16F2n/R16R2 amplified the fragment of size 1.2 kb corresponding to the partial 16S rDNA gene from all the symptomatic samples (Plate 20).

The PCR products obtained after the nested PCR were sequenced and analysed. The two isolates causing little leaf disease in brinjal and catharanthus were found to be closely related. The comparative analysis was conducted and the percentage homology was identified between the isolate under study and other

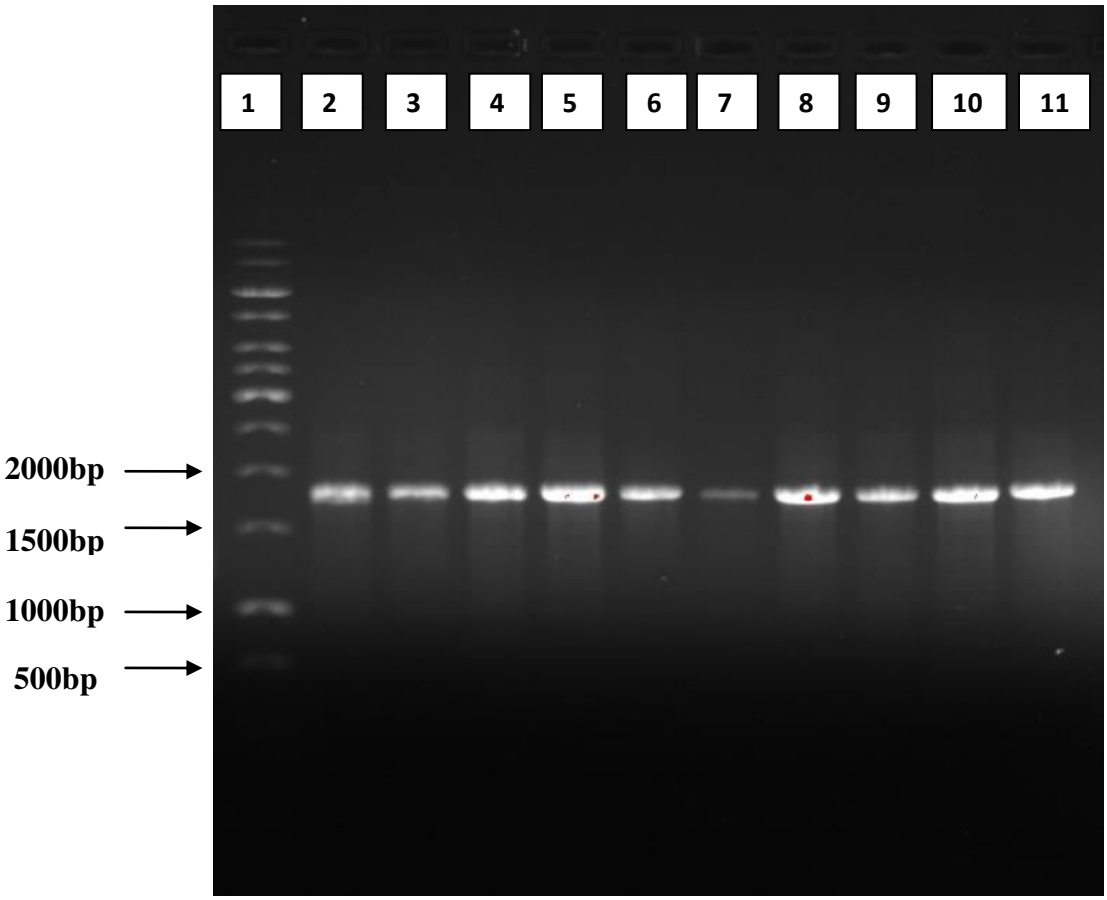


Plate. 19. Amplified products of little leaf phytoplasma from brinjal and catharanthus by P1/P7 primers.

Product size: 1.8 kb

Lane1 : 1kb DNA Ladder

Lane 2-4 : Graft inoculated brinjal

Lane 5-8 : Tissue culture brinjal

Lane 9-11 : Graft inoculated catharanthus

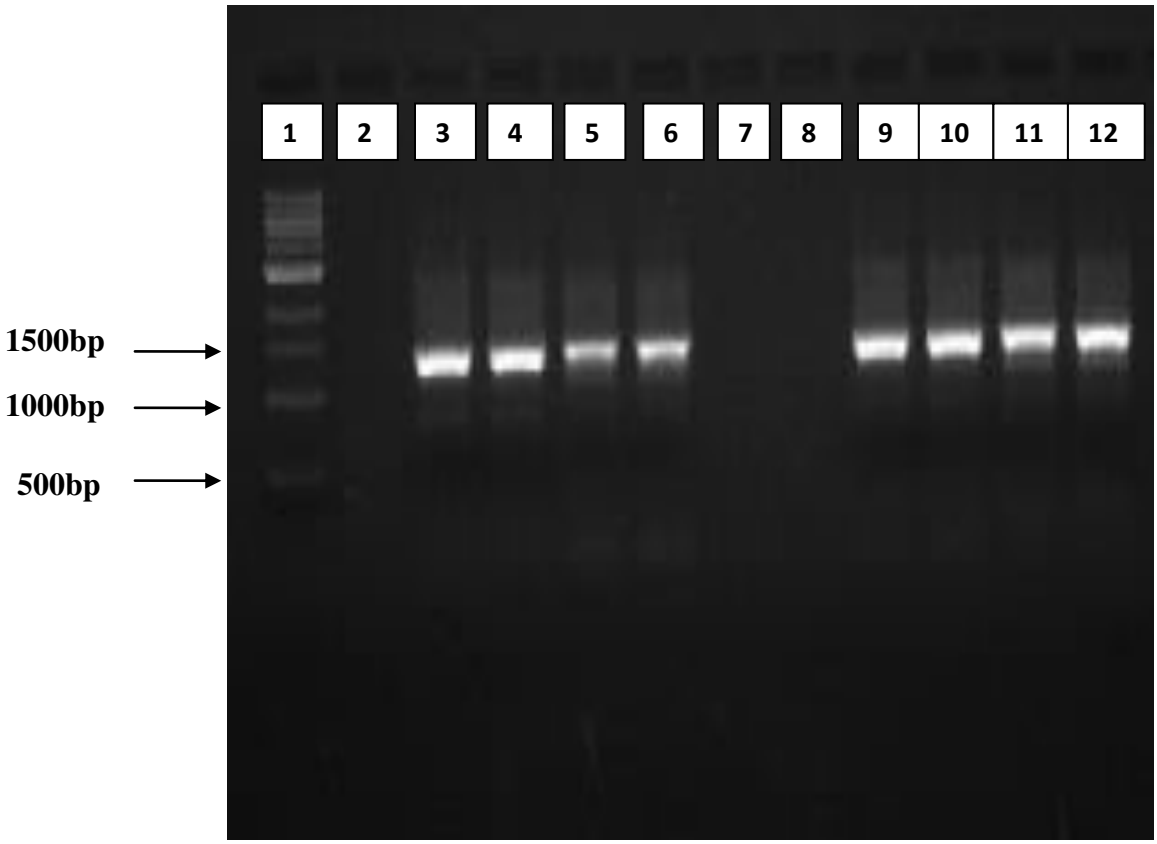


Plate. 20. Amplified products of little leaf phytoplasma from brinjal and catharanthus by nested PCR (P1/P7 nested with R16F2n/R16R2 primers).

Product size: 1.25 kb

Lane 1 : 1 Kb DNA ladder

Lane 3,4 : Grafted brinjal

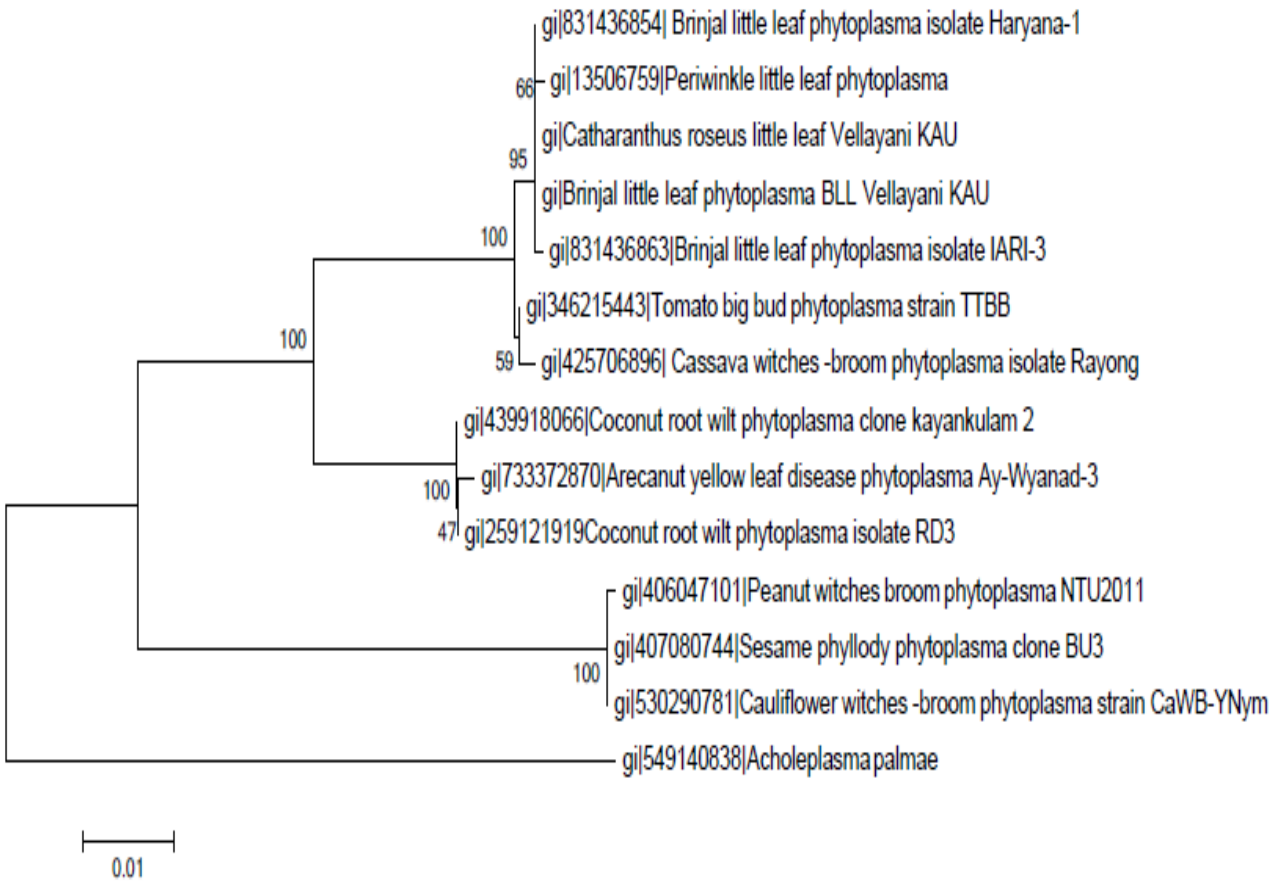
Lane 5,6 : Tissue culture brinjal

Lane 9-12 : Grafted catharanthus

Table. 13. Percent sequence identity between the nucleotide sequences of the BLL and CLL isolates with selected phytoplasmas.

Sequences used for comparison	Sequence identity (%)
gi|733372870|Arecanut yellow leaf disease phytoplasma Ay Wyanad 3	95.6
gi|439918066|Coconut root (wilt) phytoplasma clone Kayankulam_2	95.6
gi|259121919|Coconut root (wilt) phytoplasma isolate RD3	95.6
gi|346215443|Tomato big bud phytoplasma strain TTBB	99.7
gi|425706896| Cassava witches broom phytoplasma isolate Rayong	99.5
gi|407080744|Sesame phyllody phytoplasma clone BU3	90.5
gi|406047101| Peanut witches broom phytoplasma NTU2011	90.4
gi|530290781|Cauliflower witches broom phytoplasma strain CaWB YNym	90.5
gi|549140838|*Acholeplasma palmae* (outgroup)	88.2
gi|831436863|Brinjal little leaf phytoplasma isolate IARI 3	100
gi|831436854| Brinjal little leaf phytoplasma isolate Haryana 1	100
gi|13506759|Periwinkle little leaf phytoplasma	99.9

Fig. 2. Phylogenetic tree comparing the partial 16S rDNA sequences of the BLL and CLL phytoplasmas with selected phytoplasmas from GenBank.



phytoplasma isolates (Table. 13). The phylogenetic tree was constructed by comparing the partial 16S rDNA sequences of isolates under study with other phytoplasma isolates (Fig 2). The 16S rDNA sequences of Brinjal little leaf phytoplasma (BLL) and Catharanthus little leaf (CLL) phytoplasma shared 99.7% similarity with that of '*Candidatus* Phytoplasma trifolii ' reference strain (GenBank accession: AY390261). Thus the phytoplasmas causing little leaf in brinjal and catharanthus were identified as '*Ca.* Phytoplasma trifolii'-related strains. The blast analysis revealed that the BLL isolate of vellayani and CLL isolate from Coimbatore had 100% sequence homology with the BLL isolates from IARI and Haryana. The two isolates showed 99.8% sequence identity with potato witches' broom and potato purple top phytoplasmas. Also the isolates under study had 99.7% homology with tomato big bud phytoplasma. The phytoplasma associated with sesame phyllody was found to be distantly related to the phytoplasmas under study with the sequence homology of only 90.5% and came under different cluster.

Discussion

5. DISCUSSION

Brinjal or eggplant (*Solanum melongena* L.) is an important and widely consumed indigenous vegetable crop of India grown round the year. Among the various constraints, Brinjal little leaf (BLL) is becoming an emerging disease which is capable of causing even 100% loss. The disease is caused by phytoplasma, belonging to the class mollicutes, under which wall less prokaryotic organisms are grouped. Phytoplasma is a cosmopolitan group of plant pathogen and has been reported worldwide infecting a broad range of crops and considered as an emerging pathogen of the present time and hence considered to be one of the most economically important plant pathogens as viruses. Taking into consideration the importance of phytoplasma in the cultivation of vegetable crops, the present study was undertaken for obtaining information on the symptomatology, transmission, molecular detection and characterization of the organism.

5.1 SYMPTOMATOLOGY

The characteristic symptoms of the disease under study were marked reduction in the size of leaves and the phyllody of flowers. The newly formed leaves became progressively smaller. The petioles were much shortened, so that the leaves appeared to be appressed to the stem and became narrow, soft, glabrous, and yellow. The internodes were shortened. The axillary buds were stimulated to sprout, and they grew into short branches with very small leaves. The plants developed rosette appearance. Phyllody, the conversion of floral parts into green vegetative like structures was another typical symptom associated with the disease. The phylloid flowers were green, erect and mostly sterile, so that in severe cases, the affected plants did not bear any fruit. In catharanthus also, the similar symptoms were noticed.

The symptoms were found to be similar to those caused by phytoplasma reported earlier. Moreno *et al.* (1985) described and compared several yellows diseases on catharanthus in Spain. Mitra (1988) reported that the infected plants were characterized by stunting, excessive branching, reduction of leaf size and phyllody of flowers. He also reported that in early infection, no fruiting occured and in late infection, fruits were formed, but they were deformed and the seeds are shrivelled. Omar and Foissac (2012) reported severe symptoms such as malformed leaves, virescence (excessive greening of floral tissues) and phyllody (leaf-like structure) in naturally infected egg plants. The symptoms associated with REP in brinjal were foliar chlorosis, stem and flower proliferation, virescence, phyllody, shortened internodes, stunting and reduced fruit size (Tohidi *et al.*, 2015). The results of the present study were in conformity with the previous reports.

Plants infected by phytoplasma exhibited a wide range of specific and non-specific symptoms. The symptoms of diseased plants varied with the phytoplasma, host plants, stage of the disease, age of the plants at the time of infection and environmental conditions (Lee *et al.*, 2000). Jagoueix-Eveillard *et al*. (2001) demonstrated that some *mollicutes* could repress the genes involved in sugar transport, such as transketolase. Inhibition of these genes might also be responsible for the repression of genes involved in photosynthesis and this gene deregulation could explain the yellows symptoms induced in host plants. Bertaccini (2007) reported many symptoms associated with phytoplasma infection such as virescence/phyllody (development of green leaf like structures instead of flowers), sterility of flowers, proliferation of axillary (side) buds resulting in a witches' broom appearance and generalized stunting. Omar *et al.* (2008) observed little leaves, shortened internodes, virescence and witches' broom symptoms associated with infected catharanthus plants in Egypt. Omar and Foissac (2012) studied the symptoms such as stunting, yellowing or purplish leaves, intense proliferation of lateral buds, formation of hypertrophic calixes, virescence, inhibition of anther and ovary formation and fusion of petals, which led to big bud abnormalities etc.

associated with phytoplasma infected tomato plants. Also the plant apices generally lacked leaves and the youngest leaves were very small, thick and distorted.

5.2 TRANSMISSION STUDIES

In the present investigation, two methods of transmission were studied. The findings were in line with the earlier reports. Tohidi *et al.* (2015) reported the transmission of phytoplasma associated with Roodan Eggplant Phyllody (REP) from phyllody affected eggplant to healthy eggplant and tomato by grafting and to catharanthus via dodder.

5.2.1 Graft transmission

In the present study, the phytoplasma causing little leaf was efficiently transmitted through grafting and the percentage transmission was 100% in both brinjal and catharanthus. The successful wedge grafting resulted in the expression of initial systemic symptoms in healthy root stock 7-10 days after inoculation. In brinjal, out of different methods tested, grafting was found to be the most efficient one and a period of 7-9 days were required for the infected scion to remain united with the healthy root stock for the optimum inoculation (Chakrabarti and Choudhury, 1975).

Successful graft transmission was reported by different researchers. The results of the present study are in accordance with the findings of Kaminska and Korbin (1999), Torres *et al*. (2004) etc. Salehi *et al*. (2009) reported the transmission of phytoplasma associated with safflower phyllody from diseased to healthy safflower by grafting in Iran. Nejat *et al.* (2010) also reported the transmission of phytoplasma associated with the proliferation of catharanthus by grafting

5.2.2 Dodder transmission

In the present study, dodder transmission was carried out using *Cuscuta* sp. and the percentage transmission in brinjal was 10% and that of catharanthus was

20%. Successful dodder transmission was reported earlier by different researchers. Ghosh *et al.* (1999) reported the dodder transmission of phytoplasma associated with WBD from citrus to catharanthus. Marcone *et al.* (1999) reported the transmission of phytoplasma via dodder in case of pear decline, European stone fruit yellows, rubus stunt, *Picris echioides* yellows and cotton phyllody. Salehi *et al.* (2009) reported the dodder transmission of safflower phyllody. Azadvar *et al*. (2011) reported the successful dodder transmission of phytoplasma causing Toria Phyllody (TP). Here, *Cuscuta* sp, the plant parasite formed an infection bridge between the infected and healtthy plants and phytoplasma could move through the dodder into the healthy plants.

5.3 MAINTENANCE OF PHYTOPLASMA

The present investigation was conducted to check the maintenance of phytoplasma in the tissue culture plants obtained by culturing the phytoplasma infected explants. The multiple shoots were obtained on MS medium supplemented with 0.2 mg l^{-1} BAP, 0.6 mg l^{-1} NAA and 0.4 mg l^{-1} IAA. Root induction was observed in the basal MS media. The presence of phytoplasma was detected in the tissue culture plants by using nested PCR. The direct PCR with P1/P7 produced the amplicons of size ~1.8 kb and the nested PCR with P1/P7 followed by R16F2n/R16R2 produced the amplicons of size ~1.2 kb. Thus the maintenance of phytoplasma is possible through tissue culture technique. The maintenance and detection of phytoplasma in tissue culture of different plants grown under various conditions were studied by different researchers. Kaminska *et al*. (2000) studied the phytoplasma detection in tissue culture of Gladiolus plants grown under various conditions and the best detection was obtained in MS media containing kinetin and NAA in presence of light. Phytoplasma was also detected in MS medium containing 0.5 mg l^{-1} BAP+0.2 mg l^{-1} NAA. Kaminska *et al.* (2005) reported the phytoplasma detection in tissue culture of rose on MS medium with 1mg l^{-1} BAP. The phytoplasma was maintained in tissue culture plants of catharanthus using MS solid

medium supplemented with 0.12mg l^{-1} BAP (Bertaccini *et al.*, 2013). Thus the results of the present study were in conformity with earlier reports.

5.4 BIOCHEMICAL ANALYSIS

Physiological changes in brinjal and catharanthus plants infected by phytoplasma were studied in the present investigation with respect to the content of total carbohydrate, chlorophyll, total phenol and protein at ten, twenty, thirty and sixty days after graft inoculation of healthy plants with infected scion taken from the diseased plants and compared with the healthy samples. Diener (1963) reported that plant diseases bring about radical metabolic changes in the host tissues and the little leaf of brinjal was no exception. Xianling *et al*. (2009) showed that the infection with phytoplasma was generally associated with increased carbohydrate, starch content and decreased photosynthetic rate, carboxylation efficiency and pigment content of leaves. The data derived from the physiological and biochemical analyses could be supported by the findings of Prete *et al.* (2011).

The total carbohydrate content in brinjal was significantly increased at 10 DAI, which was found to be reduced in the inoculated plants especially during the later stages of infection (Fig. 3). The results in brinjal are in agreement with the earlier reports. The BLL disease decreased the reducing and non-reducing sugars, starch and total carbohydrate in the resistant wild species and increased the same in the susceptible cultivar (Chakrabarti and Choudhury, 1975). Mitra (1988) reported that the little leaf infection caused reduction of carbohydrate content in brinjal. In the present investigation, the reduced carbohydrate content may be due to some resistance mechanisms employed by the host plants.

In catharanthus, the estimation showed an increased level of carbohydrate content in the inoculated plants than the healthy samples (Fig. 3). The maximum content of carbohydrate was obtained at 60 DAI and the least content in the healthy samples. Several studies have shown similar results related to the carbohydrate

content in phytoplasma infected plants. Phytoplasma infection led to a significant increase of starch in source leaves and a decrease in sink leaves and roots than the healthy plants. In pear plants with pear decline, accumulation of sugar and starch was reported in leaves (Catlin *et al.*, 1975). These data supported those obtained from fruit trees and woody plants (Kartte and Seemuller 1991), which showed that phytoplasma infection led to the accumulation of carbohydrates in mature leaves and decreased starch in the roots. The ultra structural observations of starch accumulation in chloroplasts were associated with a severe disorganization of thylakoids and a reduction in chlorophyll content. Lepka *et al*. (1999) observed significant increase of starch in source leaves and a decrease in sink leaves and roots than healthy plants. He also reported some variations depending on the virulence of the phytoplasma isolate and on the host-phytoplasma association. Guthrie *et al.* (2001) reported higher carbohydrate content in papaya leaves infected with phytoplasma. The accumulation of carbohydrates, reported in coconut palms affected by lethal yellowing was considered as a secondary effect of infection and explained through an inhibition of phloem transport (Maust *et al*., 2003). Junqueira *et al.* (2004) reported higher amount of reducing sugar in maize bushy stunt phytoplasma affected corn plants. The increased accumulation of carbohydrate content was considered as a secondary effect of infection and can be explained through the disruption of phloem transport. As a consequence, photosynthetic product accumulation in chloroplasts inhibits photosynthesis and reduces the supply of sugars from source leaves to roots.

The total chlorophyll, chlorophyll a and chlorophyll b were significantly reduced in phytoplasma inoculated brinjal (Fig. 4) and catharanthus plants (Fig. 5). Similar to virus diseases, phytoplasma also caused the reduction or degradation of chlorophyll in the host plants and showed the symptoms of yellowing. The findings of the present investigation are in agreement with that of other reports. Srinivasan and Chelliah (1980) reported marked reduction in total chlorophyll content in little leaf-infected eggplants. Mitra (1988) reported that reduction in photosynthetic

activity due to little leaf infection in brinjal was 30% in case of mildly infected plants and 91% in case of severely infected plants. Significant reduction of Chl was reported in root (wilt) affected coconut palms (Koshy, 1999), in phytoplasma affected grapevine (Bertamini and Nedunchezhian, 2001), in phytoplasma affected field grown apple leaves (Bertamini *et al*., 2003), in maize plants infected by maize bushy stunt phytoplasma (Junqueira *et al.*, 2004) and in lime plants infected by *Ca. P. aurantifoliae* (Zafari *et al.*, 2012).

Carbohydrate metabolism and chlorophyll content are correlated. The loss of chlorophyll was accompanied by a general sugar mediated repression of genes involved in photosynthesis (Krapp *et al*., 1993). Changes in the photosynthetic activity were connected with several physiological parameters, such as stomatal conductance. Leon *et al*. (1996) reported that the stomatal conductance was decreased progressively as the disease developed in coconut palms affected with lethal yellowing (LY). One of the main effects of phytoplasma infection was a decrease in the plant productivity caused by the inhibition of photosynthesis, which might be due to the direct effect of infection on photosynthetic electron transport and enzymatic activities (Bertamini and Nedunchezhian, 2001). Bertamini *et al.* (2002a) reported that phytoplasmas had a role in the inhibition of chlorophyll biosynthesis in the host leaves. The reduction was probably the result of enhanced chlorophyllase activity in the infected leaves (Bertamini *et al.* 2002b). Zafari *et al.* (2012) reported that, in addition to the decrease in Chl a and Chl b, the Chl a/b ratio was also lower in infected leaves, indicating that Chl a, which is characteristic of photosynthetic activity decreased more than Chl b.

The protein content of phytoplasma infected plants in the present study was found to be decreased significantly towards the later stages of infection in brinjal (Fig. 6). The highest protein content was estimated in brinjal healthy samples and that of inoculated samples at 60 DAI was found to be the least. In catharanthus also

Fig. 3 Changes in total carbohydrate content of brinjal and Catharanthus leaves in response to phytoplasma inoculation

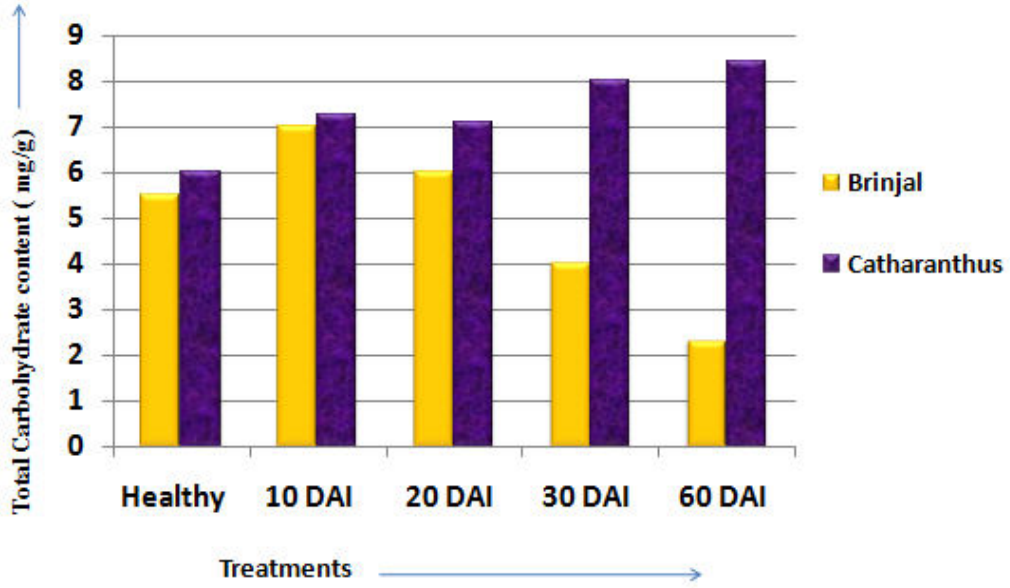


Fig. 4 Changes in chlorophyll content of brinjal leaves in response to phytoplasma inoculation (mg g^{-1})

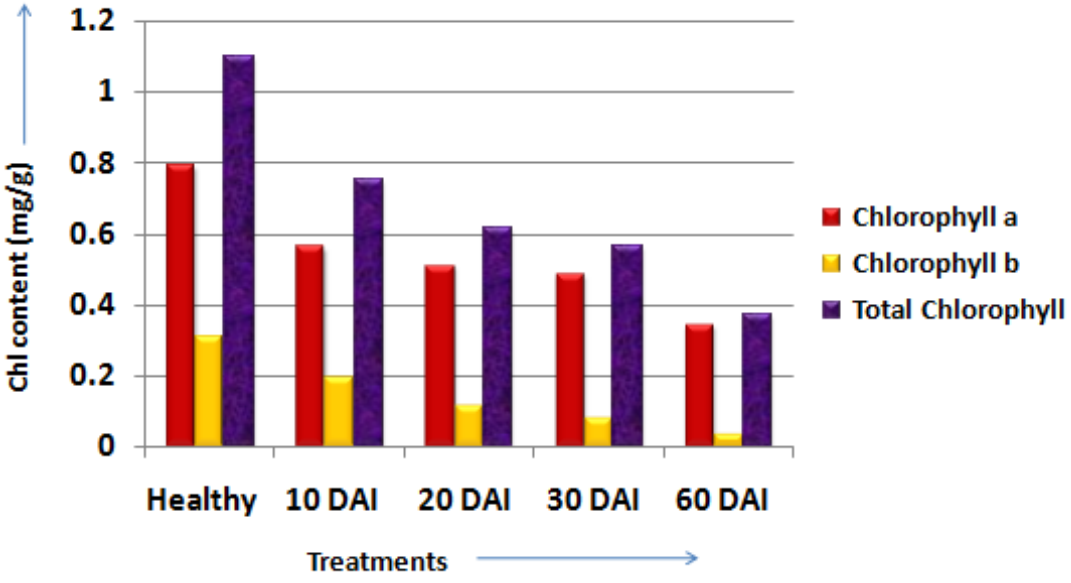


Fig. 5 Changes in chlorophyll content of Catharanthus leaves in response to phytoplasma inoculation

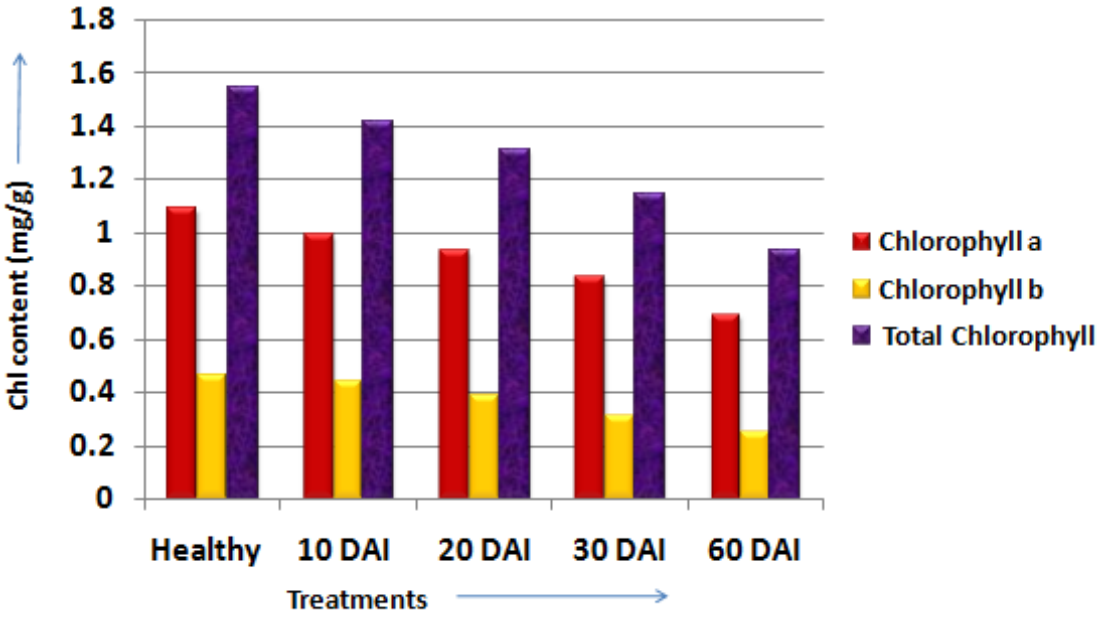
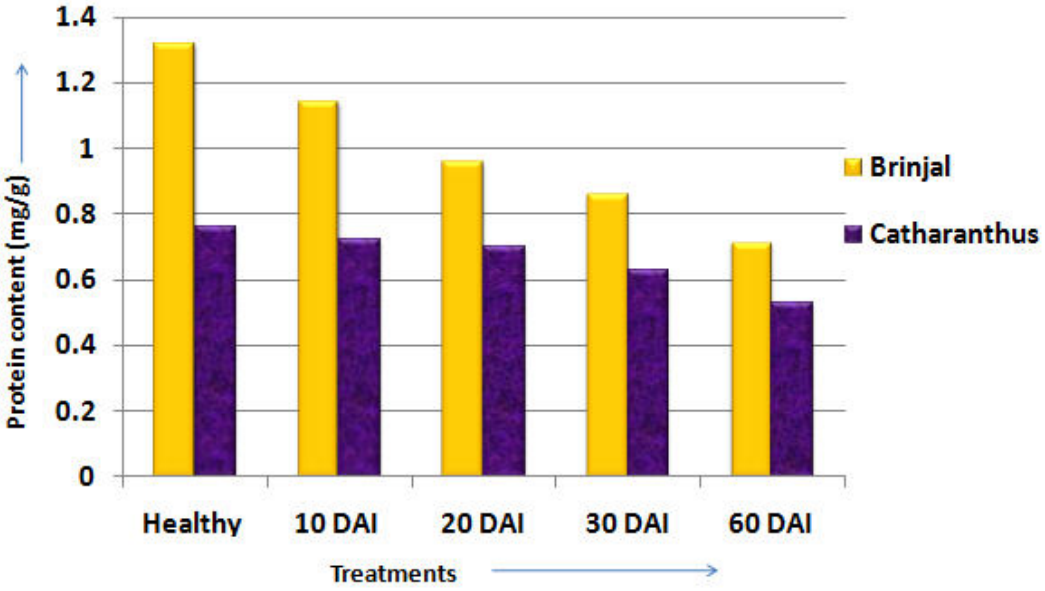


Fig. 6 Changes in protein content of brinjal and Catharanthus leaves in response to phytoplasma inoculation



similar effect was noticed. Usually plants infected by pathogens show high protein content. Contradictory results were obtained for different plants with different phytoplasmas. The reduction of proteins was reported in many plants infected by phytoplasma such as lethal yellowing affected coconut palms (Leon *et al.*, 1996), tomato plants affected by stolbur phytoplasma (Favali *et al*., 2001), apple plants affected by apple proliferation (Bertamini *et al*., 2002a) and grapevine affected by bois noir (Bertamini *et al*., 2002b). A possible explanation for the reduction could be due to the fact that extremely susceptible plants were used in these experiments or degradation of some proteins. It has been hypothesized that the total protein reduction in phytoplasma infected leaves might have been due to the decrease in the synthesis of ribulose-1, 5-biphosphate carboxylase (RUBPC), the major soluble protein of the leaf (Bertamini *et al*. 2003).

The present study revealed a significant reduction in phenol content in the inoculated plants as a result of phytoplasma infection in both brinjal and catharanthus (Fig. 7). In brinjal, the highest phenol content was reported in healthy sample and the inoculated samples have significantly lower phenol contents with the least value at 60 DAI. The same trend was also reported in catharanthus. Agrios (1997) reported the accumulation of phenolic compounds in pathogenic situations. But reduction in the phenolic compounds was also observed due to the infection by phytoplasma. The results of the present study are in line with the earlier reports. Mitra and Majumdar (1977) reported that out of four varieties of brinjal, total phenol content was decreased throughout the disease development in three brinjal varieties as a result of infection by little leaf phytoplasma. In brinjal affected by little leaf disease, marked reduction in phenolic content was reported (Srinivasan and Chelliah, 1980). These earlier studies support the present study that certain plant phytoplasma interaction resulted in the reduction of phenol content, which may be due to the changes in host metabolism caused by the phytoplasma infection or the susceptibility of the host plants.

The results of the present investigation indicated a significant increase in peroxidase (PO) activity of inoculated samples at all stages of infection in brinjal. The highest activity was obtained for brinjal sample at 60 DAI and the activity for the healthy sample was the least. In case of catharanthus, the activity was highest at 30 DAI and the least activity was shown by the healthy sample as in case of brinjal (Fig. 8). In the present study, the plants may have some degree of resistance, which could result in high PO activity in the infected plants as reported by Leherer (1969). But reduced peroxidase activity was reported in brinjal affected with little leaf phytoplasma (Mitra, 1988), which may be due to the high level of susceptibility of plants used in their experiment. The results of the present study are in conformity with the findings of other plant-phytoplasma systems of many researchers. The significant enhancement of peroxidase activity was reported in apple trees infected with apple proliferation phytoplasma (Musetti *et al*, 2005). Junqueira *et al.* (2011) reported increased activity of peroxidase in the inoculated plants of both resistant and susceptible corn hybrids, which was an evidence of changes in the host metabolism caused by the phytoplasma. According to them, PO had no role in the resistance response of the corn plants because the symptoms appeared at the same time in both resistant and susceptible hybrids. Zafari *et al*. (2012) found that the activity of peroxidase was higher in phytoplasma infected leaves of lime than the healthy control.

The activity of PPO was reduced in phytoplasma infected samples in both brinjal and catharanthus (Fig. 9). The activity of PAL was following similar trend with significant difference in both brinjal and catharanthus (Fig. 10). The similar results were reported earlier. In brinjal, out of four varieties tested, the PPO activity was decreased at all stages of the disease development in three brinjal varieties as a result of infection by little leaf phytoplasma (Mitra and Majumdar, 1977). Mitra (1988) observed that the PPO activity was reduced in BLL affected plants as a result of phytoplasma infection. The reduction in PPO activity could be due to the

Fig. 7 Changes in Phenol content of Brinjal and Catharanthus leaves in response to phytoplasma inoculation(mg g^{-1})

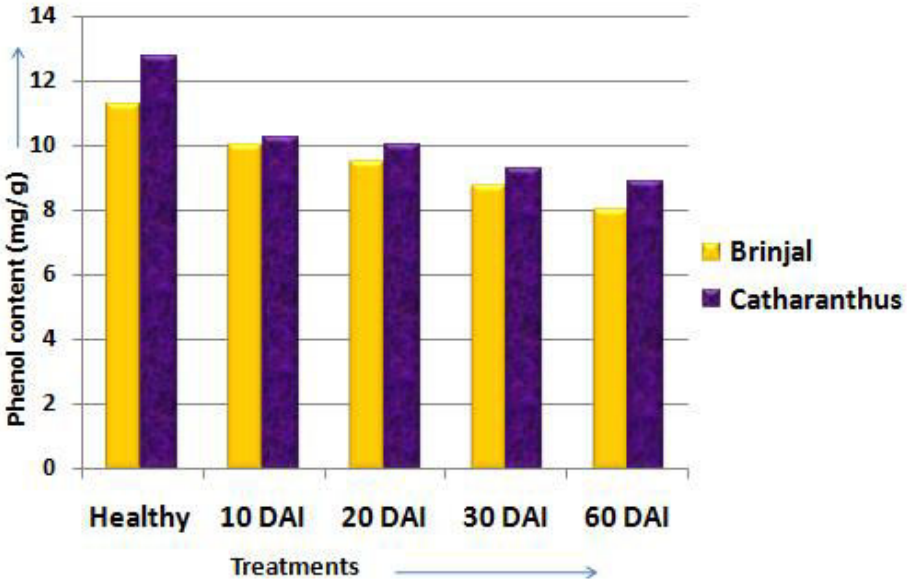


Fig. 8 Changes in peroxidase activity of Brinjal and Catharanthus leaves in response to phytoplasma inoculation

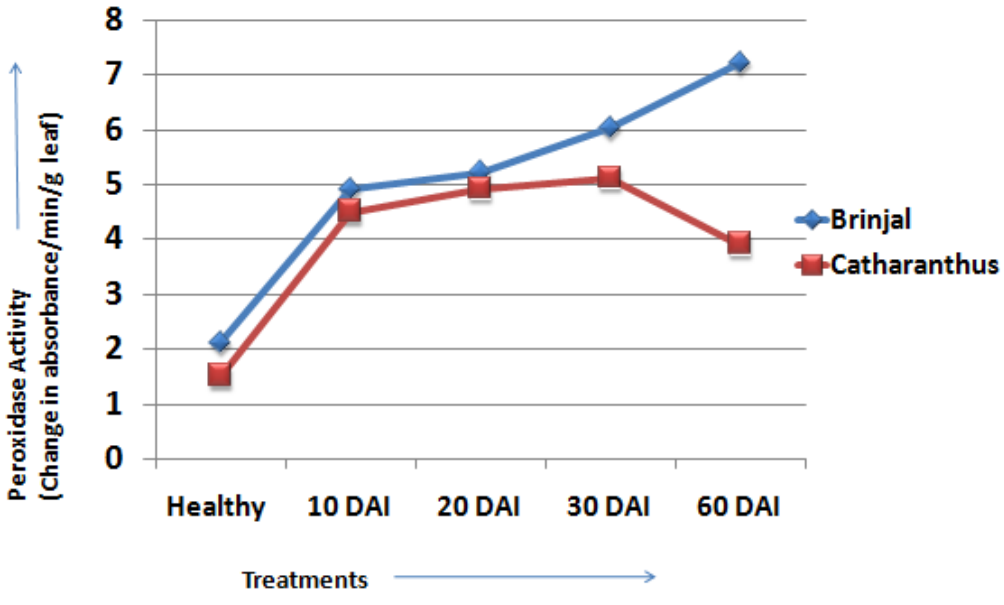


Fig. 9 Changes in Polyphenol Oxidase (PPO) activity of Brinjal and Catharanthus leaves in response to phytoplasma inoculation

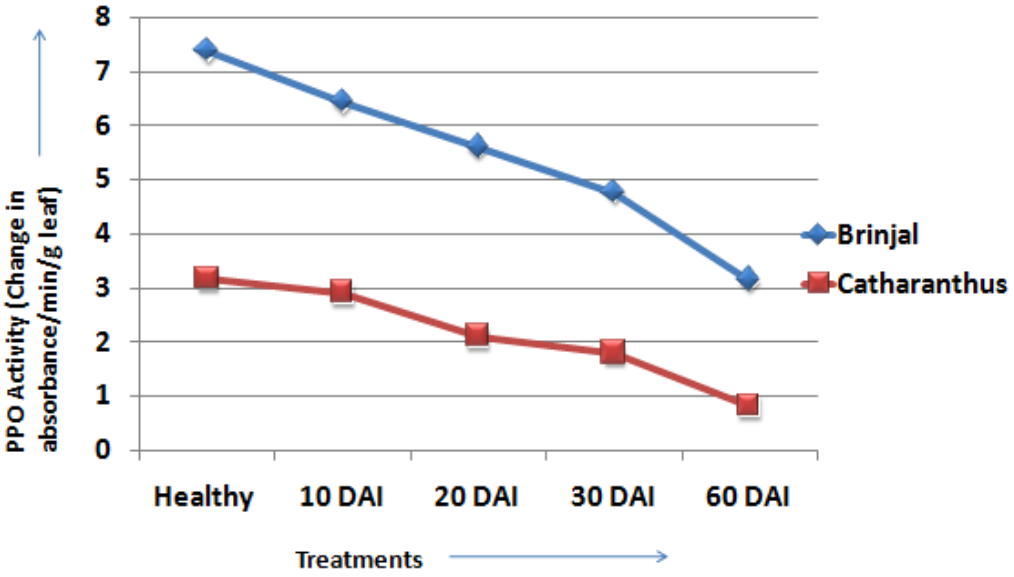
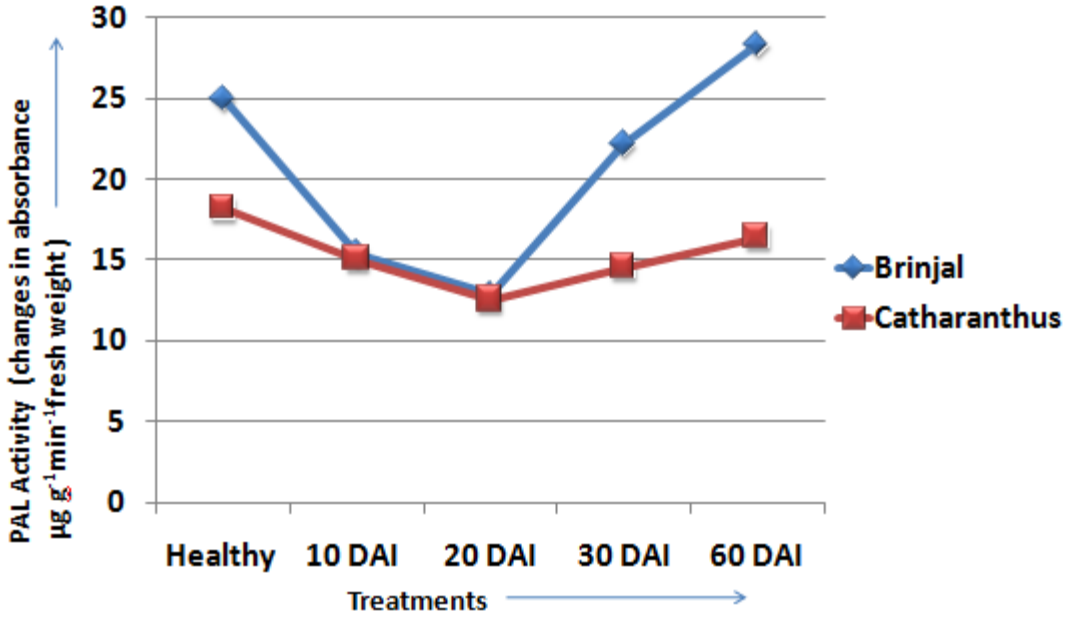


Fig . 10 Changes in Phenylalanine ammonia-lyase (PAL) activity of Brinjal and Catharanthus leaves in response to phytoplasma inoculation



susceptibility of the host plant. The activity of PAL was enhanced after 20 days of inoculation in the present study, which may be due to some host resistance reaction. The activity of PAL rapidly changed under the influence of various factors, such as pathogen attack and treatment with elicitors (Dixon and Lamb, 1990). Patui *et al.* (2013) reported that the activity of PAL was increased in apple trees affected by apple proliferation phytoplasma.

The protein profile analysis in the present study revealed the presence of two extra protein bands in the infected brinjal samples with molecular weights of 29 kDa and 43 kDa, which were absent in the corresponding healthy samples. The extra novel proteins are assumed to be induced in the inoculated plants as a result of the plant-phytoplasma interaction. The protein profile analysis was carried out earlier in some plant phytoplasma systems. Favali *et al.* (2001) reported the alterations of several proteins in other plant-phytoplasma systems. Moreover, the decrease in intensity of some protein bands under phytoplasma infection can also be attributed to the decrease in protein synthesis, accelerated proteolysis, decrease in the availability of amino acids and denaturation of enzyme involved in protein synthesis. Prete *et al.* (2011) investigated the effects of Pear Decline (PD) phytoplasma on the pear protein profile by conducting SDS-PAGE on leaf proteins from infected and healthy plants, which showed differentially expressed protein bands. They identified 18 proteins and all of them, except two were involved in diverse processes including photosynthesis, carbohydrate metabolism and metabolite transport. Proteomic analysis showed that the expression of many proteins changed during phytoplasma infection. These changes could alter many physiological and biochemical processes and resulted in diverse and severe symptoms in the infected plants. Zafari *et al.* (2012) performed SDS-PAGE to study the protein profile in lime plants infected by *Ca*. Phytoplasma aurantifoliae and the analysis revealed that the protein patterns in infected and control leaves were not identical and the differences were both quantitative and qualitative. They showed the presence of two extra proteins of

molecular weights 15 and 26 kDa from the infected leaves, which suggested their possible role in response to phytoplasma infection.

Electrophoretic analysis of isozyme was carried out by using native polyacrylamide gel electrophoresis to study the enzyme alteration in healthy and phytoplasma infected plants. In the present study, the isozyme pattern analysis of peroxidase revealed two isoperoxidase bands in all phytoplasma inoculated plants. The Relative Mobility (Rm) values of the bands were 0.17 and 0.47. The isoperoxidase band with Rm value of 0.47 was absent in the healthy sample. The intensity of bands was found to be increased towards the later stages of inoculation and the highest intensity was obtained at 60 DAI. The estimation of peroxidase enzyme also revealed the highest activity at 60 DAI. The isozyme analysis was performed earlier in lime plants infected by phytoplasma. Zafari *et al*. (2012) reported the presence of different isoforms of antioxidative enzymes in healthy and phytoplasma infected leaves of lime plants with *Ca.* Phytoplasma aurantifoliae by native PAGE. Four PO isoforms were detected in healthy control and infected leaves.

5.5 MOLECULAR DETECTION AND CHARACTERIZATION

Molecular detection was performed by using nested PCR. The direct PCR in brinjal with P1/P7 primer pair resulted in the amplification of DNA fragment with size of ~1.8 kb and the nested PCR using P1/P7 followed by R16F2n/R16R2 resulted in the product of ~1.2 kb. The same result was obtained in tissue culture plants of brinjal and catharanthus plants. The results of the present study were in line with the previous reports. Azadvar and Baranwal (2012) performed the molecular detection and they obtained PCR products of ~1.8 kb in direct PCR with phytoplasma universal primer pair P1/P7 in brinjal samples and the vectors. Also, the nested PCR assays with the primer pair P1/P7 followed by the phytoplasma universal primer pair R16F2n/R16R2 gave DNA fragment of ~1.2 kb in all the samples. The same results

were obtained earlier in different plant-phytoplasma systems by using the same primer combinations.

The highly conserved 16S rDNA gene sequence was used as the primary molecular tool for classification of phytoplasmas. Genes encoding 16Sr DNA were highly conserved across the phytoplasma clade and served as a primary molecular tool for phytoplasma identification, genotyping, taxonomic assignment and group/subgroup classification by RFLP analyses (Lee *et al*., 1998). In the present investigation, the conserved 16S rDNA gene sequence was targeted. The products obtained by the nested PCR were sequenced and blasted at the NCBI website. The two phytoplasma isolates were found to be closely related. Phylogenetic relationship was studied by the comparison with certain phytoplasma isolates obtained from Genbank. The comparative study revealed that BLL isolate of vellayani and CLL isolate from Coimbatore had the highest sequence homology of 100% with the BLL isolates from IARI and Haryana. The two isolates showed 99.8% sequence identity with potato witches' broom and potato purple top phytoplasmas and 99.7% homology with tomato big bud phytoplasma. The phytoplasma associated with sesame phyllody was found to be distantly related and came under different cluster. The phytoplasmas responsible for coconut root (wilt) and arecanut yellow leaf disease were also distantly related, but closer than the sesame phyllody phytoplasma. The 16S rDNA sequences of Brinjal little leaf phytoplasma (BLL) and Catharanthus little leaf (CLL) phytoplasma were blasted and analysed. The analysis revealed that the two isolates were closely related and they shared 99.7% similarity with that of '*Candidatus* Phytoplasma trifolii' reference strain (GenBank accession: AY390261). Thus the two phytoplasma isolates were identified as the related strains of '*Candidatus* Phytoplasma trifolii'. The results of the present study were in line with earlier reports.

Schneider *et al*. (1995) reported that the phytoplasmas of 16SrVI group were responsible for BLL in India. Siddique *et al*. (2001) reported that the two strains of phytoplasma, which caused little leaf disease in brinjal and catharanthus in Bangladesh were closely related and they were found to be identical to the strain of brinjal little leaf phytoplasma in India. The phytoplasmas in 16SrVI group were taxonomically described as '*Ca.* Phytoplasma trifoli' (Hiruki and Wang, 2004). Azadvar and Baranwal, (2012) reported that BLL disease was associated with phytoplasma of the 16SrVI clover proliferation (CP) phytoplasma group. They reported that the comparison of 1249 bp (1.249 kb) of R16F2n/R16R2 primed sequence of 16S rDNA of BLL phytoplasma isolate to other phytoplasma sequences showed 99% similarity to different strains of BLL, catharanthus etc. and all the isolates belonged to 16SrVI phytoplasma group.

Summary

6. SUMMARY

Brinjal little leaf, caused by phytoplasma is an emerging disease in Kerala capable of causing losses up to 100%. The study entitled "Molecular detection and characterization of phytoplasma infecting Brinjal (*Solanum melongena* L.) was conducted at the Department of Plant Pathology, College of Agriculture, Vellayani during 2013-2015 . Brinjal little leaf, collected from the Crop museum, College of Agriculture, Vellayani and catharanthus little leaf obtained from Coimbatore were maintained by repeated grafting for the comparative study.

The characteristic symptoms developed after graft transmission were the production of small, narrow, smooth leaves with yellowing. The typical phyllody symptoms were also observed, i.e. the production of leaf like structures in place of flowers. The phylloid flowers were mostly sterile. Proliferation of axillary shoots, shortened internodes, production of numerous little leaves etc. gave the plants bushy or rosette appearance. Newly formed leaves were much shorter and the petioles were shortened so that the leaves appeared to be sticking to the stem.

The phytoplasma transmission studies were conducted and the percentage of transmission was recorded. The wedge graft transmission was found to be 100% successful in both brinjal and catharanthus. The dodder transmission, conducted by using *Cuscuta* sp. was found to be 10% successful in brinjal and 20% successful in catharanthus. The dodder plants could act as a bridge between the infected and healthy control plants to cause the transmission.

The maintenance of phytoplasma was done in both *in vivo* and *in vitro* conditions. The *in vitro* maintenance was done using tissue culture technique by culturing the tissue of infected explants on MS media supplemented with 0.2 mg l^{-1} BAP, 0.6 mg l^{-1} NAA and 0.4 mg l^{-1} IAA. The *in vivo* maintenance was done by using grafting.

The pathophysiological studies of healthy and diseased plants revealed that the content of chlorophyll, phenol and protein were reduced in the inoculated plants as a result of phytoplasma infection. The chlorophyll a, chlorophyll b and the total chlorophyll were reduced in all the inoculated samples of both brinjal and catharanthus. The carbohydrate content in brinjal was significantly increased immediately after the inoculation and thereafter decreased, but increased in the inoculated catharanthus samples. The activity of peroxidase was enhanced while that of polyphenol oxidase was reduced in the inoculated plants. The activity of phenyl alanineammonialyase was reduced immediately after the inoculation, but enhanced at 30 and 60 days after inoculation (DAI).

Protein profile of healthy and phytoplasma infected samples of brinjal was analysed using SDS-PAGE. The analysis revealed the presence of two extra novel proteins with approximate molecular weight of 29 kDa and 43 kDa, which were absent in the corresponding healthy samples. Native PAGE was carried out for the isozyme analysis of peroxidase. The analysis revealed two isoforms in the inoculated plants with Relative mobility (Rm) values of 0.17 and 0.47, but a single isoperoxidase band in healthy plants with Rm value of 0.17. The isoperoxidase band with Rm value of 0.47 was absent in the healthy sample.

Molecular diagnosis using nested PCR was performed for the detection of the conserved 16SrDNA region of phytoplasma. The direct PCR with the phytoplasma universal primer pair P1/P7 revealed the products of size ~1.8 kb while the nested PCR with P1/P7 followed by R16F2n/R16R2 amplified the fragment of size 1.2 kb.

The phytoplasma was detected in graft inoculated brinjal, catharanthus and the tissue culture plants obtained by culturing the phytoplasma infected brinjal. The fragment of size 1.8 kb was the region corresponding to the entire 16S rDNA gene along with the 16S-23S spacer region and the 5'end of the 23S rDNA while the fragment obtained after the nested PCR with size 1.2 kb was corresponding to the partial 16S rDNA gene. The products of nested PCR were sequenced and analysed.

The partial 16S rDNA sequences of both Brinjal little leaf phytoplasma (BLL) and Catharanthus little leaf (CLL) phytoplasma isolates were blasted at the NCBI website and phylogenetic relationship was studied by conducting a comparative study with phytoplasma isolates obtained from Genbank. The blast analysis of both the isolates with the existing data base from NCBI revealed a 100% homology with brinjal little leaf phytoplasma isolates of Haryana and IARI. They shared 99.8% sequence identity with potato witches' broom and potato purple top phytoplasmas and 99.8% homology with tomato big bud phytoplasmas. The 16S rDNA sequences of both brinjal and catharanthus isolates shared 99.7% sequence similarity with that of *Candidatus* Phytoplasma trifolii' reference strain (GenBank accession: AY390261).

Brinjal little leaf is an emerging disease in Kerala, causing considerable economic losses. The disease produced characteristic symptoms including little leaf and phyllody. The phytoplasma was readily transmitted by wedge grafting of infected scion onto healthy rootstock. Tissue culture was found to be a good option for the *in vitro* maintenance of phytoplasma. The physiological and biochemical analyses revealed the expression of differential metabolisms in host plants as a result of infection by phytoplasma. The molecular diagnosis by nested PCR was found to be the most important detection method in case of phytoplasma, for which the highly conserved regions are commonly targeted. The sequence analysis revealed that the two phytoplasma isolates studied were closely related and identified to be related to '*Ca*. Phytoplasma trifolii'.

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Appendices

APPENDIX – I

Anthrone reagent

Anthrone reagent is made by dissolving 200 mg of anthrone in 100 ml ice cold 95% concentrated sulphuric acid.

APPENDIX – II

0.1 M Sodium acetate buffer (pH 4.7)

Stock solutions

A: 0.2 M solution of acetic acid (11.55 ml in 1000 ml)

B: 0.2 M solution of sodium acetate (16.4 g of $C_2H_3O_2$ Na or 27.2 g of $C_2H_3O_2$ Na. $3H_2O$ in 1000 ml).

22.7 ml of A is mixed with 27 ml of B, diluted to a total of 100 ml.

Preparation of stock dye solution for estimation of protein

100 mg of Coomassie brilliant blue G-250 was dissolved in 50 ml of 95 per cent ethanol and 100 ml of concentrated orthophosphoric acid was added. The volume was made upto 200 ml with water and kept at 4°C. The working dye was freshly prepared by diluting the stock solution to five times with water.

APPENDIX III

Buffers for enzyme analysis

0.1 M Sodium phosphate buffer (pH 6.5)

Stock solutions

A. 0.2 M solution of mono basic sodium phosphate (27.8 g in 1000 ml)

B. 0.2 M solution of di basic sodium phosphate (53.65 g NA_2HPO_4 .7 H_2O in 1000 ml)

68.5 ml of A mixed with 31.5 ml of B diluted to a total of 200 ml.

0.1 M Borate buffer (pH 8.8)

Stock solutions

A: 0.2 M solution boric acid (12.4 g in 1000 ml)

B: 0.05 M solution of borax (19.05 g in 1000 ml)

50 ml of A is mixed with 30 ml of B, diluted to a total of 200 ml.

APPENDIX IV

Protein denaturing solution

10 M urea	- 80 ml
1 M NaH_2PO_4. $2H_2O$ (pH 8)	- 5 ml
1 M Tris (pH 8)	- 1 ml
5 M sodium chloride	- 2 ml

Makeup volume to 100 ml by adding 12 ml of distilled water.

APPENDIX V

Stock solutions for isozyme analysis

1. Tris-glycine electrode buffer stock solution, pH 8.3

   Tris - 6.0 g

   Glycine - 28.8 g

   Distilled water - 1000 ml

2. Electrode buffer

   Dilute the Tris- glycine electrodebuffer stock solution with distilled water in a 1: 9 ratio.

3. Tris- chloride buffer stock solution, pH 8.9

   HCl, 1N - 48.00 ml

   Tris - 36.6 g

   TEMED - 0.23 ml

   Distilled water -100.00 ml

4. Tris- chloride buffer stock, pH 6.7

   HCl, 1N - 48.00 ml

   Tris - 5.98 g

   TEMED - 0.46 ml

   Distilled water - 100.00 ml

5. Resolving gel acrylamide stock solution

   Acrylamide - 28.00 g

   Bis-acrylamide - 0.74 g

   Double distilled water - 100.0 ml

   Store in dark bottle at 4^0C for upto 2 weeks.

6. Ammonium persulphate solution

   Ammonium persulphate - 0.1 g

   Dissolve in 1 ml distilled water. Prepared freshly before use

7. Bromophenol blue solution

   Bromophenol - 25 mg

   Make upto to 10 ml with Tris- chloride buffer solution, pH 6.7

8. Resolving gel solution (for one 1.5 mm gel, 7.5 %)

   Tris- chloride buffer Solution, pH 8.9 - 5 ml

   Resolving gel acrylamide solution - 10 ml

   Distilled water - 25 ml

   Ammonium persulphate solution - 300 µl

9. Stacking gel solution (for one 1.5 mm gel, 4%)

   Tris- chloride buffer stock Solution, pH 6.7 - 2.5 ml

   Resolving gel acrylamide solution - 10 ml

   Distilled water - 25 ml

   Ammonium persulphate solution - 300 µl

APPENDIX VI

Buffers for PCR reaction

50x TAE buffer (Tris-Acetate-EDTA) (pH 8.0)

Tris base -242.00 g

Acetic acid – 57.1 ml

0.5M EDTA-100 ml

Add distilled water to a final volume of 1 litre.

Sample loading buffer (6x)

0.25 % bromophenol blue and 40 % (w/v) sucrose in water

APPENDIX VII

Stock solutions for MS basal medium

Sl. No.	Constituents	Quantity of chemical (mg l^{-1})	Amount required, g	Quantity required for 1 litre media
1.	Stock solution I (250 ml 40 X)			
	$MgSO_4$. 7 H_2O	370	3.7	
	KH_2PO_4	170	1.7	25 ml
	KNO_3	1900	19	
	NH_4NO_3	1650	16.5	
2.	Stock solution II (100 ml, 200 X)			
	$CaCl_2$. 2 H_2O	440	8.8	5 ml
3.	Stock solution III (100 ml, 1000 X)			
	H_3BO_3	6.2	0.62	
	$MnSO_4$.4 H_2O	22.3	1.69	
	$ZnSO_4$. $2H_2O$	8.6	0.86	1 ml
	Na_2MoO_4. $2H_2O$	0.25	0.025	
	KI	0.83	0.083	

4.	*Stock solution IV (100 ml, 200 X) $FeSO_4$. 7 H_2O Na_2 EDTA. 2 H_2O	 27.8 37.3	 0.556 0.745	5 ml
5.	Stock solution V (100 ml 2000 X) $CuSO_4$. 5 H_2O $CoCl_2$. 6 H_2O	 0.025 0.025	 0.0125 0.0125	0.5 ml
6.	Stock solution VI (100 ml 1000 X) Thiamine HCl Pyridoxin HCl Nicotinic acid Glycine Myoinositol- 100mg l^{-1} Sucrose - 30 g l^{-1} Agar - 7 g l^{-1}	 0.1 0.5 0.5 2	 0.01 0.05 0.05 0.2	1 ml

*Dissolve $FeSO_4$. 7 H_2O and Na_2 EDTA. $2H_2O$ separately in 45 ml distilled water by heating and constant stirring. Mix the two solutions and adjust the pH to 5.5 and add distilled water to make up the final volume to 100 ml.

All the prescribed stock solutions, chemicals and hormones were taken in a beaker and the volume was made up to 1 litre. The agar was dissolved in the medium by melting before autoclaving.

APPENDIX VIII

Partial 16S rDNA sequence of Brinjal little leaf (BLL) phytoplasma.

GAAGGTTACTAAGACTGGATAGGAAACAAAAAGGCATCTTTTTGTTTT
TAAAAGACCTTCTTACGAAGGTATGCTTAAAGAGGGGCTTGCGCCACA
TTAGTTAGTTGGTAGAGTAAAAGCCTACCAAGACGATGATGTGTAGCT
GGACTGAGAGGTTGAACAGCCACATTGGGACTGAGACACGGCCCAAA
CTCCTACGGGAGGCAGCAGTAGGGAATTTTCGGCAATGGAGGAAACT
CTGACCGAGCAACGCCGCGTGAACGATGAAGTATTTCGGTATGTAAAG
TTCTTTTATTGAAGAAGAAAAAGTAGTGGAAAAACTATATTGACGTTA
TTCAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAAGA
CATAGGGGGCGAGCGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTA
GGCTGTTAGATAAGTCTATAATTTAATTTCAGTGCTTAACGCTGTCTTG
TTATAGAAACTGTCTTGACTAGAGTGAGATAGAGGCAAGCGGAATTCC
ATGTGTAGCGGTAAAATGTGTAAATATATGGAGGAACACCAGAAGCG
TAGGCGGCTTGCTGGGTCTTTACTGACGCTGAGGCACGAAAGCGTGGG
TAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TACTAAGTGTCGGGGTAAAACTCGGTACTGAAGTTAACACATTAAGTA
CTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGAC
GGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACAC
GAAAAATCTTACCAGGTCTTGACATACTCTGCAAAGCTATAGAAATAT
AGTGGAGGTTATCAGGGATACAGGTGGTGCATGGTTGTCGTCAGTTCG
TGTCGTGAGATGTTAGGTTAAGTCCTAAAACGAACGCAACCCTTGTCG
TTAATTGCCAGCACATAATGGTGGGGACTTTAACGAGACTGCCAATTA
AACATTGGAGGAAGGTGAGGATTACGTCAAATCATCATGCCCCTTATG
ATCTGGGCTACAAACGTGATACAATGGCTGTGACAAAGAGTAGCTGA
AACGCGAGTTTTTAGCCAATCTCAAAAAAGCAGTCTCAGTTCGGATTG
AAGTCTGTAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAA
TCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCG
TCA

APPENDIX IX

Partial 16S rDNA sequence of catharanthus little leaf (CLL) phytoplasma.

GAAGGTTACTAAGACTGGATAGGAAACAAAAAGGCATCTTTTTGTTTT
TAAAAGACCTTCTTACGAAGGTATGCTTAAAGAGGGGCTTGCGCCACA
TTAGTTAGTTGGTAGAGTAAAAGCCTACCAAGACGATGATGTGTAGCT
GGACTGAGAGGTTGAACAGCCACATTGGGACTGAGACACGGCCCAAA
CTCCTACGGGAGGCAGCAGTAGGGAATTTTCGGCAATGGAGGAAACT
CTGACCGAGCAACGCCGCGTGAACGATGAAGTATTTCGGTATGTAAAG
TTCTTTTATTGAAGAAGAAAAAGTAGTGGAAAAACTATATTGACGTTA
TTCAATGAATAAGCCCCGGCTAACTATGTGCCAGCAGCCGCGGTAAGA
CATAGGGGGCGAGCGTTATCCGGAATTATTGGGCGTAAAGGGTGCGTA
GGCTGTTAGATAAGTCTATAATTTAATTTCAGTGCTTAACGCTGTCTTG
TTATAGAAACTGTCTTGACTAGAGTGAGATAGAGGCAAGCGGAATTCC
ATGTGTAGCGGTAAAATGTGTAAATATATGGAGGAACACCAGAAGCG
TAGGCGGCTTGCTGGGTCTTTACTGACGCTGAGGCACGAAAGCGTGGG
TAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TACTAAGTGTCGGGGTAAAACTCGGTACTGAAGTTAACACATTAAGTA
CTCCGCCTGAGTAGTACGTACGCAAGTATGAAACTTAAAGGAATTGAC
GGGACTCCGCACAAGCGGTGGATCATGTTGTTTAATTCGAAGATACAC
GAAAAATCTTACCAGGTCTTGACATACTCTGCAAAGCTATAGAAATAT
AGTGGAGGTTATCAGGGATACAGGTGGTGCATGGTTGTCGTCAGTTCG
TGTCGTGAGATGTTAGGTTAAGTCCTAAAACGAACGCAACCCTTGTCG
TTAATTGCCAGCACATAATGGTGGGGACTTTAACGAGACTGCCAATTA
AACATTGGAGGAAGGTGAGGATTACGTCAAATCATCATGCCCCTTATG
ATCTGGGCTACAAACGTGATACAATGGCTGTGACAAAGAGTAGCTGA
AACGCGAGTTTTTAGCCAATCTCAAAAAAGCAGTCTCAGTTCGGATTG
AAGTCTGTAACTCGACTTCATGAAGTTGGAATCGCTAGTAATCGCGAA
TCAGCATGTCGCGGTGAATACGTTCTCGGGGTTTGTACACACCGCCCG
TCA

Abstract

MOLECULAR DETECTION AND CHARACTERIZATION OF PHYTOPLASMA INFECTING BRINJAL (*Solanum melongena* L.)

by

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ABSTRACT

The study entitled “Molecular detection and characterization of phytoplasma infecting Brinjal (*Solanum melongena* L.) was conducted at the Department of Plant Pathology, College of Agriculture, Vellayani, with the objectives to study the symptom development, transmission, molecular detection and characterization of phytoplasma infecting brinjal and its relationship with phytoplasma diseases of other crop plants. Brinjal little leaf (BLL), collected from the Crop museum, College of Agriculture, Vellayani and catharanthus little leaf (CLL) obtained from Coimbatore were maintained for further studies.

Symptomatology revealed the characteristic little, narrow, soft, glabrous and smooth leaves produced as clusters along with yellowing, proliferation of axillary shoots, shortened internodes, stunted bushy or rosette appearance and phyllody, the conversion of floral parts into leaf like structures.

The graft transmission was found to be 100% successful while the percentage transmission by dodder was only 10% in brinjal and 20% in catharanthus. Phytoplasma was maintained *in vivo* in plants by grafting and *in vitro* by culturing the infected explants on MS media supplemented with 0.2 mg l^{-1} BAP, 0.6 mg l^{-1} NAA and 0.4 mg l^{-1} IAA.

Biochemical analysis of healthy and diseased plants revealed that the contents of protein, phenol and chlorophyll were reduced in the inoculated plants as a result of phytoplasma infection. Carbohydrate content in brinjal increased immediately after inoculation and then decreased. The activity of peroxidase (PO) was enhanced in the inoculated plants while that of polyphenol oxidase (PPO) was reduced. The activity of phenyl alanineammonialyase (PAL) was reduced immediately after the inoculation, but enhanced at 30 and 60 days after inoculation (DAI).

The electrophoretic analysis of proteins using SDS-PAGE revealed the presence of two extra protein bands in the infected samples with molecular weights of 29 kDa (Kilo Dalton) and 43 kDa. The isozyme pattern analysis of peroxidase using native PAGE revealed two isoperoxidase bands in the inoculated plants with Relative mobility (Rm) values, 0.17 and 0.47, but a single band in healthy plants with Rm value of 0.17.

Molecular detection was done using nested PCR. PCR products of ~1.8 kb (Kilo base) were obtained in direct PCR with phytoplasma universal primer pair P1/P7 and the nested PCR with P1/P7 followed by R16F2n/R16R2 amplified the fragment of size 1.2 kb. The presence of phytoplasma in tissue culture plants was also confirmed using nested PCR.

Comparative nucleotide sequence analysis of brinjal and catharanthus isolates with the existing data base from NCBI revealed a 100% homology with brinjal little leaf phytoplasma isolates from Haryana and IARI and 99% homology with potato witches' broom, potato purple top, tomato big bud phytoplasma etc.

The 16S rDNA sequences of BLL and CLL phytoplasma shared 99.7% similarity with that of '*Candidatus* Phytoplasma trifolii (*Ca*. Phytoplasma trifolii)'. Thus the two phytoplasma isolates were identified as the related strains of '*Ca.* Phytoplasma trifolii'.