JEB®

Journal of Environmental Biology

p-ISSN: 0254-8704 • e-ISSN: 2394-0379 • CODEN: JEBIDP

Journal website : www.jeb.co.in ★ **E-mail :** editor@jeb.co.in

Triveni Enterprises (Educational Services Set-up)

Original Research

DOI : http://doi.org/10.22438/jeb/43/3/MRN-2002

Detection, characterization and management of brinjal little leaf disease in Assam

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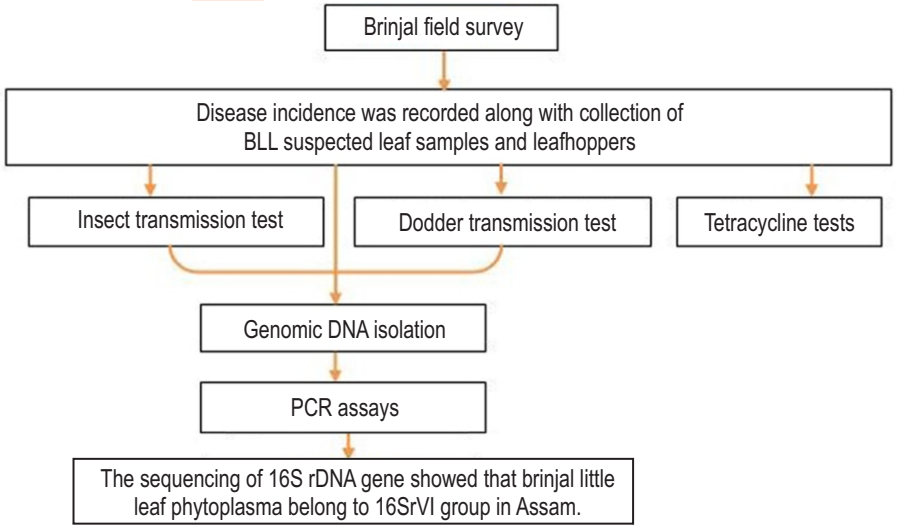
Received: 29.05.2021 *Revised*: 01.08.2021 *Accepted*: 12.11.2021

Abstract

Aim: To record the prevalence, detection, molecular and biological characterization of phytoplasma associated with brinjal little leaf disease in Assam and its management.

Methodology: Roving survey was conducted during the 2018-2020 brinjal growing seasons to record the disease incidence and symptoms associated with the disease. Insect and dodder transmission studies were carried out along with tetracycline tests. Further, molecular detection of all the samples collected during the survey and after dodder and insect transmission studies were done through PCR assays by using universal primers.

Results: The disease incidence varied from 3.62 to 23.63 per cent in surveyed districts. Transmission studies revealed that *Hishimonus phycitis* (Dist.) is acting as a vector of brinjal little leaf phytoplasma and found to be transmitted by dodder (*Cuscuta campestris*). The phylogenetic analysis indicated that the Brinjal little leaf phytoplasma understudy was associated with '*Candidatus* phytoplasma trifolii' and plants like *Datura stramonium* play a major role as potential alternate host of the phytoplasma. Further, studies on the effect of the antibiotic tetracycline hydrochloride in managing the disease elucidated that the antibiotic was effective only for temporary remission of symptoms.



Interpretation: This study reveals a broad picture about the incidence, phytoplasma associated with the disease, putative leafhopper vector transmitting the phytoplasma in the field, alternate host harbouring the phytoplasma, and effect of antibiotic on the disease suppression, which will be useful to develop effective management strategies.

Key words: Antibiotic, Brinjal little leaf disease, *Hishimonus phycitis*, Little leaf, Phytoplasma

How to cite : Dutta, D.S., M.K. Kalita and P.D. Nath: Detection, characterization and management of brinjal little leaf disease in Assam. *J. Environ. Biol.*, **43**, 460-467 (2022).

Introduction

Brinjal or eggplant (*Solanum melongena* L.) belongs to the family *Solanaceae*, is one of the most common, popular and widely grown vegetable crops of the world. In India, it is one of the principal vegetable crops grown throughout the country. India is the second-largest producer of brinjal in the world after China. In Assam, brinjal is grown in an area of about 17,760 ha with a production of 2,86,350 metric tonnes (Saxena *et al.,* 2018). Because of its high nutritive value, it serves as the main dietary component in India and is also known to possess medicinal properties in curing diabetic patients, asthma, cholera, bronchitis, diarrhoea and other complaints (Tomar and Kalda, 1998). Worldwide, brinjal is known to infect by many fungal, bacterial, and viral diseases and are the major constraints in its production and productivity. Besides these, little leaf disease caused by phytoplasma is one of the most important diseases causing considerable economic yield losses (Mitra, 1993). Brinjal little leaf disease was first reported in the central farm, Coimbatore by Thomas and Krishnaswamy (1939) and subsequently, several biological aspects of the disease have been described (Varma *et al.,* 1969; Mitra, 1993). The infected plants are characterized by reduced leaf size, shortened internodes and petiole, leaf cupping or curling, proliferation of auxiliary buds resulting in witches' broom symptoms, proliferation of shoots, phyllody, generalized stunting and yellowing, due to profound disturbance in the normal balance of growth regulators (Mazumdar and Das, 2020, Kumari, 2019 and Bertaccini, 2007).

Phytoplasma are mostly dependent on insect transmission for their spread and survival (Hogenhout *et al.,* 2008) and is also efficiently transmitted via dodder (Rao, 2021). Their vectors include diverse leafhoppers (Cicadellidae), planthoppers (Fulgoridae) and psyllids (Psyllidae). In 1969, the leafhopper *Hishimonus phycitis* Dist. was reported as the natural insect vector of brinjal little leaf phytoplasma (Bindra and Singh, 1969); It was later reported in several states in the country, namely New Delhi (Azadvar and Baranwal, 2012) Uttar Pradesh, Haryana, Maharashtra, Odisha and Bihar (Kumar *et al.,* 2017). Till date no vector transmission assay has been carried out in the North East region of India. Hence, this study was undertaken to identify the vector associated with little leaf phytoplasma in Assam. As this phytoplasma is also reported to be transmitted by dodder to tomato, potato, and tobacco (Mishra, 2004), therefore an attempt has been made in this study to determine the efficiency of dodder to transmit the phytoplasma from infected to healthy brinjal plant.

As phytoplasmas are not cultured on artificial medium and lack measurable phenotypic characters, their classification has been based primarily on molecular analysis of conserved gene sequences (Lee *et al.,* 1998). So far, six groups of phytoplasmas have been reported to cause brinjal little leaf disease globally *viz.,* 16SrI from Japan, Bangladesh and India (Okuda *et al.,* 1997; Kelly *et al.,* 2009; Kumar *et al.,* 2012), 16SrII-D from Egypt (Omar and Foissac, 2012), 16SrIII-J and 16SrIII-U from Brazil (Mello *et al.,* 2011), 16SrVI-A and D from Turkey and India (Sertkaya *et al.,* 2007; Azadvar and Baranwal, 2012: Kumar *et al.,* 2017), 16SrIX-C from Iran (Tohidi *et al.,* 2015) and 16SrXII-A from Russia (Ember *et al*., 2011). Whereas only two phytoplasma groups (16SrI and 16SrVI) were reported to be associated with brinjal little leaf disease in India (Azadvar and Baranwal, 2012; Kumar *et al.,* 2012). Even though phytoplasma diseases are of common occurrence, only a few of them have been properly studied in the North East region of the country; phylogenetic position of brinjal little leaf phytoplasma is not clear and no comprehensive molecular work has been done on the phytoplasma associated with this disease in Assam. Therefore, in the present study, systematic and regular studies were conducted to ascertain the prevalence of brinjal little leaf disease in different brinjal growing regions of Assam during 2018-2020 brinjal growing seasons.

Phytoplasma etiology of brinjal little leaf disease in India has been confirmed based on symptoms, electron microscopy, polymerase chain reaction and transmission assays (Varma *et al.,* 1975; Verma and Dubey, 1978; Shantha and Lakshmanan, 1984; Azadvar and Baranwal, 2012). Timely and correct identification of plant diseases is a critical factor in disease management (Rao, 2021). Limited attempt has been made to manage the phytoplasma diseases in India. Presently, there is no curative treatment for brinjal little leaf disease, however, the disease symptoms of phytoplasma infected plants can be remission temporarily by application of antibiotic tetracycline, as mycoplasmas are known to be sensitive to it, but penicillin treatments has no effect (Ishiie *et al.,* 1967; Davis *et al.,* 1968). Earlier, influence of treatment of different concentration of tetracyclines has been reported for remisson of symptoms and control of phytoplasma strains associated with brinjal little leaf in India (Verma and Dubey, 1978). There is scanty literature pertaining to the management of this disease in the North Eastern states of India, hence this research was undertaken to study the effects of tetracycline antibiotic in suppressing the disease symptoms. In India, recent evidence show that severity of brinjal little leaf disease is increasing at an alarming rate and causing serious economic losses every year. Brinjal little leaf suspected symptoms have been observed in Assam from time to time, but no systematic studies have been carried out to date. Therefore, the present investigation aimed for verification of brinjal little leaf phytoplasma disease incidence, characterization, transmission, insect vector identification and management.

Materials and Methods

Field survey and samples collection: Roving surveys were conducted in seven major brinjal growing districts of Assam during the period 2018-2020. A total of 133 leaf samples were collected from Biswanath (21), Darang (15), Golaghat (27), Jorhat (20), Nagaon (14), Sivasagar (26), and Sonitpur (10), respectively. Information regarding disease incidence and variety grown in the surveyed fields were also recorded. In these fields, leaf samples from plants expressing symptoms associated with brinjal little leaf disease were collected alongside with samples

from plants suspected to be healthy. While in transit, samples were stored in an ice-box and were later preserved in a deep freezer at -20°C for further use. The predominant leafhopper species feeding on brinjal crop in surveyed fields were also collected using a sweeping net in the morning and evening hours and stored at -20°C for further studies. The identification of leafhoppers was carried out in the Department of Entomology, Assam Agricultural University, Jorhat.

Molecular analysis of brinjal little leaf phytoplasma

DNA isolation from leaf samples and insects: Total genomic DNA from suspected healthy and symptomatic leaves samples collected from the surveyed locations was isolated by the CTAB protocol (Ahrens and Seemüller, 1992). On the other hand, the isolation of insect DNA was done following the protocol described by Marzachi *et al.* (1998) with some modifications. After isolation, the DNAs were stored at -20°C for further studies.

Detection by PCR assays: Conventional PCR was carried out to test the presence of phytoplasma in the DNAs isolated by using the universal primer pairs P1/P6 (Deng and Hiruki, 1991), followed by nested primer pairs R16F2n/R16R2 (Gundersen and Lee, 1996). The PCR reaction mixture consisted of 100 ng of DNA template (0.5µl), 5µl of 2x Emerald master mix (Takara ®), 0.5 µl each of forward and reverse primers (10pmol concentration) and 3.5 µl of sterile distilled water. For conventional PCR, the amplification conditions steps followed with denaturation (94°C for 45 sec), annealing (54.9°C for 2 min), and extension (72°C for 3 min) of 32 cycles. The final extension step was set for 10 min at 72°C.

As for the nested PCR, the PCR product of conventional PCR assay was diluted in a ratio of 1:15 with sterile water, and 0.5 µl was used as a template in nested PCR assay. However, similar amplification cycles and programs as earlier stated were used for nested PCR, except for the annealing temperature which was 54.8°C for 2 min. All the PCR amplifications were carried out in a thermal cycler (Gene Amp® PCR System 9700, Applied Biosystems, USA). The amplified PCR products were resolved on 1.5% agarose gel electrophoresis along with a 100bp ladder (Puregene) and were viewed in a gel documentation unit (Gel Doc™ EZ Imager, Bio-Rad, USA).

Transmission studies

Insect transmission test: Brinjal seedlings were grown in insect-free condition inside net house. Four different types of leafhoppers *viz., Amrasca biguttula biguttula* (Ishida), *Exitianus indicus* (Dist.), *Hishimonus phycitis* (Dist.) and *Nephotettix nigropictus* (Stal) were tested for their ability in vectoring the phytoplasma. The leafhoppers were allowed for seven days acquisition feeding on brinjal little leaf phytoplasma infected brinjal plants followed by inoculation feeding for seven days on thirty-five-day-old healthy brinjal plants, releasing five leafhoppers per plant. A set of five healthy brinjal plants were kept as uninoculated control. After the inoculation period was over, the leafhoppers were killed by spraying 0.2% imidacloprid and the killed leafhoppers were collected and stored in a deep freezer (-20°C) for further use. The inoculated brinjal plants were observed regularly for expression of symptoms. All symptomatic and asymptomatic inoculated plants and the uninoculated brinjal plants were subjected to PCR assay by using phytoplasma specific primers (P1/P6 and R16F2n/R16R2) to confirm the phytoplasma presence.

Dodder transmission: The dodder transmission studies were carried out as per the procedure of Marcone *et al.* (1997). Five brinjal plants were used for the transmission with dodder and a healthy brinjal plant was maintained as control. The dodder inoculated brinjal plants were observed regularly for expression of symptoms. All symptomatic and asymptomatic inoculated plants and the uninoculated brinjal plants were subjected to PCR assays by using phytoplasma specific primers (P1/P6 and R16F2n/R16R2) for confirmation of infection.

Effect of tetracycline sprays on brinjal little leaf disease: To evaluate the effect of antibiotic tetracycline hydrochloride on symptom remission and reappearance of brinjal little leaf disease, three different concentrations *viz.,* 300, 400 and 500 ppm of the antibiotic was sprayed on brinjal plants of the age 90-95 days showing typical symptoms of the disease. Each antibiotic concentration was applied on infected brinjal plants giving single and double sprays at seven days intervals. Five little leaf infected brinjal plants were maintained as control plants by spraying only water. The plants were observed regularly to record the duration for symptom remission and reappearance.

Results and Discussion

The brinjal little leaf disease was prevalent in all the areas under survey. During the survey, different phytoplasma associated symptoms were observed in brinjal fields. The major symptoms include; reduction in the size of leaves, yellowing, stunting and floral virescence. Variation in the disease incidence was observed according to the variety and location. The highest disease incidence was observed in the Sivasagar district (23.63%) which was followed by Golaghat (21.68%) and the lowest disease incidence was recorded in Darang (3.62%) district (Table1). In accordance with the present study, variation in disease incidence was also observed in different districts of Karnataka in the same brinjal varieties.

A wide range of symptoms are produced by phytoplasmas in the infected plants which may vary depending with the strain, host, time of infection, age of the plant and environmental conditions (Bertaccini and Lee, 2018). Typical symptoms of brinjal little leaf phytoplasma; stunted growth, yellowing, malformed leaves, little leaf, shortened internodes, virescence, phyllody and witches' broom were also reported from Kerala, West Bengal, New Delhi, Uttar Pradesh, Haryana, Maharashtra, Odisha, Chhattisgarh and Bihar (Yadav *et al.,*

Table 1: Incidence of brinjal little leaf disease in farmers' field of Assam during 2018-2020

Districts	Locations	GPS data	Varieties	Disease incidence (%)	
				Range	Mean
Biswanath	Bamgaon	26°44'03.3"N 93°09'25.3"E	Singhnath	7.84-16.36	12.91
	Komalia	26°41'46"N 93°06'13"E	Local cultivar		
	Monabari	26°45'22.6"N 93°17'19.8"E	Basudha		
Darang	Kharupetia	26°30'32"N 92°09'21"E	Pusa Hybrid-5	3.33-3.92	3.62
	Sialmari	26°30'18"N 92°07'23"E	Pusa Bhairav		
	Thekerabari	26°31'24"N 92°07'36"E	Arka Nidhi		
Golaghat	Hahchowa Gaon	26°42'39"N 93°56'27.3"E	Singhnath	21.56-21.81	21.68
	Mamoroni gaon	26°43'17.99"N 93°56'12.4"E	Kaveri 402		
Jorhat	ICR farm (AAU)	26°43'07.5"N 94°11'23.7"E	Surya	7.27-16.36	10.90
	Nam Deori Gaon	26°47'08"N 97°05'38"E	Kuchia		
	Upper Deori Gaon	26°49'03.6"N 94°07'28.3"E	Kaveri 402		
Nagaon	Rangagora	26°44'09.7"N 93°48'42.5"E	Singhnath	9.09-16.36	12.72
	Sibasthan	26°29'39"N 92°52'52"E	Bholenath		
Sibsagar	Dikhowmuk	26°53'35.2"N 94°26'13.4"E	Singhnath	21.81-25.45	23.63
	Dishangmukh	27°05'02.9"N 94°33'13.3"E	Basudha		
Sonitpur	Balipara	26°48'51"N 92°46'18"E	Krinti	5.45-5.88	5.66
	Napam	26°42'29.7"N 92°49'20.5"E	Pusa Hybrid-5		

2015, Majumdar and Das, 2020, Azadvar and Baranwal, 2012, Kumar *et al.,* 2017), suggesting the widespread nature of this disease in most states of India. Out of the four leafhopper species tested, only *H. phycitis* (Dist.) could transmit the phytoplasma from infected to healthy brinjal plants with a transmission efficiency of about 80%. First symptoms appeared at 30 to 35 days after inoculation of healthy plants. In the PCR analysis, band sizes of 1.5 kb (Fig. 1A) and 1.25 kb from amplified PCR products were obtained only from *H. phycitis* (Dist) through conventional and nested PCR, respectively.

No amplifications were obtained from other three types of leafhoppers. Similarly, all symptomatic brinjal plants inoculated with *H phycitis* (Dist.) were positive to brinjal little leaf phytoplasma (Fig. 1B). In India, brinjal little leaf transmission was demonstrated by *H. phycitis* earlier, but only on the basis of presence and identification of leafhoppers in infected fields and presence of phytoplasma in insects by PCR assays (Bindra and Singh, 1969; Azadvar and Baranwal, 2012). Later in 2015, *H. phycitis* was reported as a vector of brinjal little leaf phytoplasma (strain 16Sr VI-D) through transmission trials in Delhi. In Assam, no proper transmission assays were performed till date to confirm any leafhopper species as natural vector of brinjal little leaf disease. In the present study, *H. phycitis* was a confirmed natural vector of little leaf phytoplasma in Assam.

The leafhopper, *H. phycitis* was also reported as a vector of sesame phyllody in India (Un Nabi *et al.,* 2015), lime witches' broom in Iran (Salehi *et al.,* 2007) and brinjal little leaf disease in India (Azadvar and Baranwal, 2012 and Kumar *et al.,* 2017) which indicates its potentiality as a natural vector of phytoplasma (Rao and Kumar, 2017). The brinjal little leaf phytoplasma was successfully transmitted by dodder from infected to healthy brinjal plants with a transmission efficiency

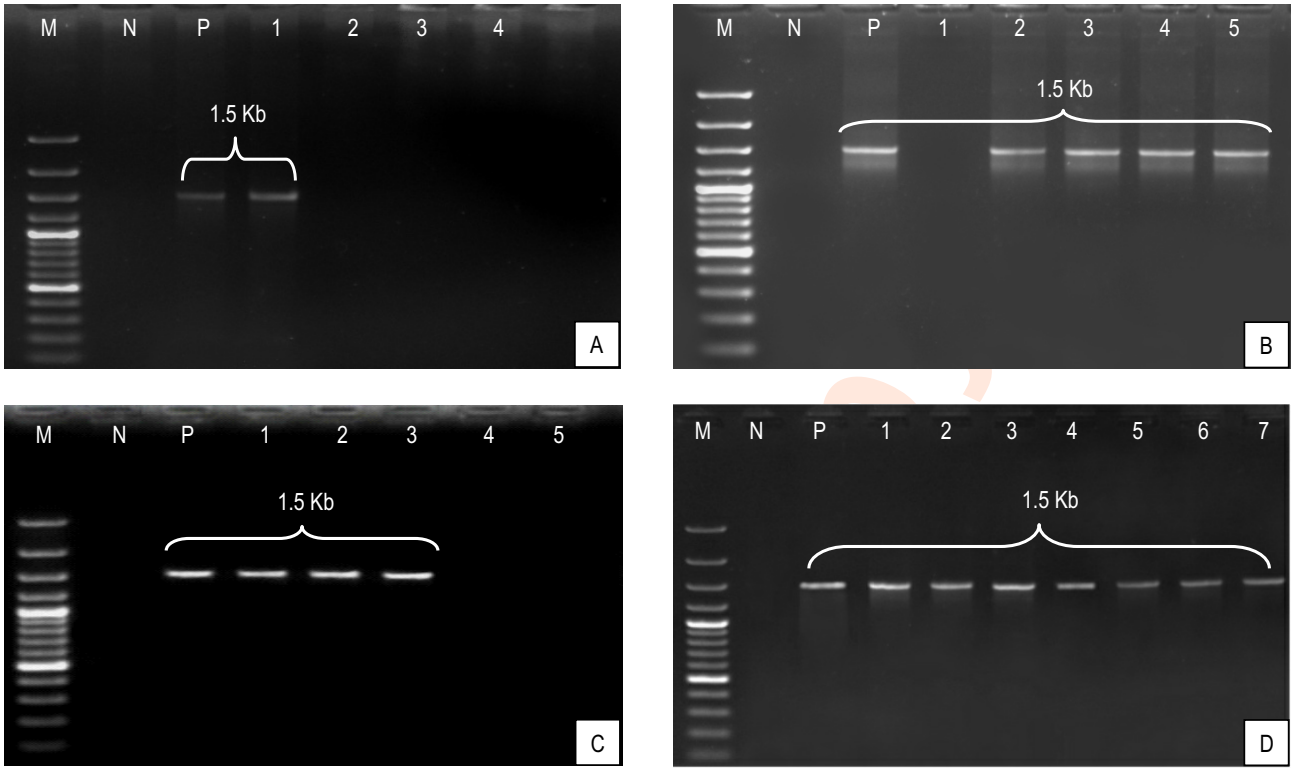


Fig.1: (A-D) Identification of phytoplasma by PCR in 1% agarose gel electrophoresis. M- 100bp DNA ladder, N- negative control, P- positive control, (A) lane 1- *Hishimonus phycitis* Dist., lane 2- *Exitianus indicus* (Dist.), lane 3- *Amrasca biguttula biguttula* (Ishida) and lane 4- *Nephotettix nigropictus* (Stal). (B) lane 1 to 5- *Hishimonus phycitis* Dist. inoculated brinjal plants, (C) lane 1 to 5- dodder inoculated brinjal plants, (D) lane 1 to 7- Brinjal little leaf infected samples from different districts of Assam.

of 60% and the dodder transmitted plants developed little leaf symptoms within 35-45 days after inoculation. All the symptomatic brinjal plants yielded visible amplified DNA band of 1.5 kb from first-round PCR with primer pair P1/P6 (Fig. 1C) and 1.25 kb from nested PCR with primer pair R16F2n/R16R2 confirming the presence of phytoplasma in them. Similar to our study, the transmission ability of dodder was also reported with a transmission efficiency of 40%, where the dodder transmitted plants developed little leaf symptoms within 30-35 days after inoculation. Dodder transmission is an effective way to study wide range of phytoplasmas as it might provide some insight into their pathogenic properties, such as their host range and cross infectivity (Kumari *et al.,* 2019).

In the present study all the symptomatic brinjal samples collected from the seven districts of Assam gave an expected amplicon size of about 1.5 Kb from direct PCR with universal phytoplasma primer pair P1/P6 (Fig. 1D). Also, nested-PCR assay with the primer pair R16F2n/R16R2 yielded DNA fragments of about 1.25 Kb in the same samples (data not shown). No amplification was obtained from DNA isolated from healthy and asymptomatic brinjal plants. The 16S rDNA sequences of four strains from Biswanath, Sonitpur, Jorhat and Nagaon generated in this study were submitted in NCBI GenBank having accession numbers MW261862, MW261864, MW261865 and MW261866, respectively. Comparison of the 1.5 Kb of P1/P6 primed sequence of 16S rDNA from little leaf disease symptomatic brinjal plants showed 99-100% identity with the existing accessions '*Candidatus* phytoplasma trifolii' (Acc. No. MN861370.1), brinjal little leaf phytoplasma (Acc. No. KX284703.1) and brinjal little leaf phytoplasma (Acc. No. KX284697.1). Phylogenetic analysis also supported the above results and all the brinjal little leaf strains clustered together with strains classified under the '*Candidatus* Phytoplasma trifolii' Clade (16SrVI) (Fig. 2).

The phytoplasma detected in *Datura stramonium* plant collected from Sonitpur district was also found to be associated with 16SrVI *Candidatus* Phytoplasma trifolii'. The brinjal little leaf disease has a history of more than 75 years (Rao *et al.,* 2021). Among the major biotic constraints in brinjal production, little leaf and phyllody disease is a serious disease capable of inflicting up to 34% yield loss (Abraham *et al.,* 1977). Till date no comprehensive genetic diversity study has been worked out on phytoplasma strains associated with brinjal little leaf disease in Assam. Hence, the results of this study, confirmed that 16SrVI group of phytoplasma is the most widespread infecting brinjal

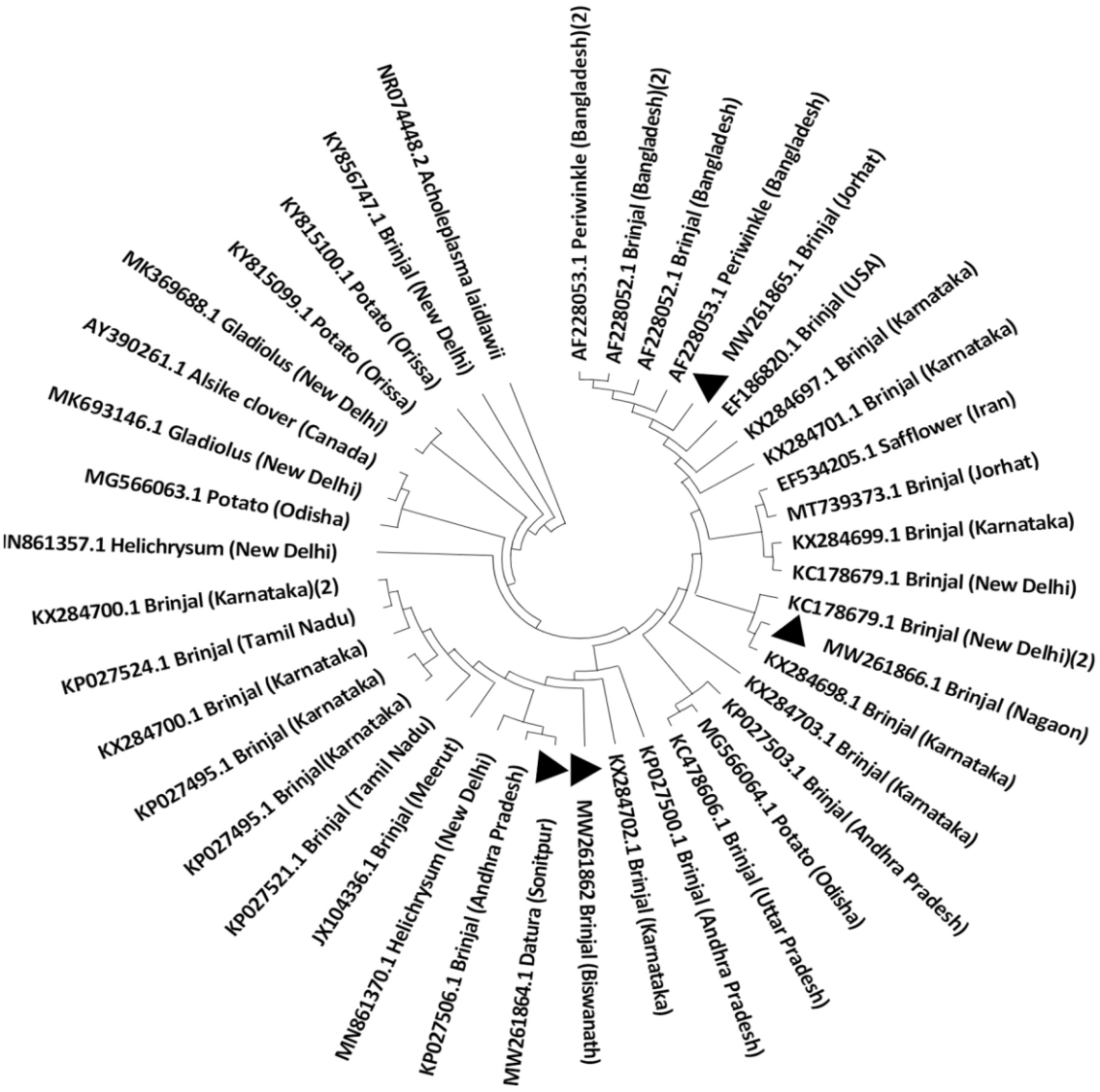


Fig. 2: Phylogenetic tree, based on 16S rDNA, showing the relationships among brinjal little leaf phytoplasma strains constructed by maximum likelihood method using Mega X software.

crops in Assam. This phytoplasma clade has been associated with diseases of large number of plants including alsike clover, pepper, tomato, alfalfa, Illinois elm and potato in different parts of the world. Similar to our study, in India it has been reported in *Datura inoxia* (Raj *et al.,* 2008) and *Datura stramonium* (Singh *et al.,* 2012) as an alternate host for brinjal little leaf phytoplasma. Hence, early detection of phytoplasma in weeds is very important to check their further spread to other commercial crops (Kumar *et al.,* 2017). The antibiotic tetracycline hydrochloride can successfully suppress the disease symptoms of brinjal little leaf disease at 400 and 500 ppm concentrations in both single and double sprays. Brinjal plants treated with 400 ppm concentration could suppress the symptoms for 5-8 days with a single spray and 9-11 days with two sprays. However, disease symptoms reappeared between 18-21 days and 21-24 days after the first spray in plants treated with single and double sprays, respectively. Plants treated with 500 ppm of antibiotic concentration can suppress the symptoms for up to 13-16 days and 19-24 days at one and two sprays, respectively.

The reappearance of disease symptoms was observed 24-27 days after the first spray in single spray and 30-35 days after the first spray in plants with double spray. Infected brinjal plants treated with 300 ppm concentration of antibiotic did not affect the symptom remission. The temporary suppressive effects of antibiotic spray on the disease agents were also reported earlier

(Bowyer and Atherton, 1971; Bindra *et al.,* 1972). Anjenayulu and Ramakrishnan (1972) also reported short-term remission in symptoms by tetracycline in plants infected with brinjal little leaf phytoplasma. As such, developing cultivars resistant to either phytoplasmas or insect vectors would be a long-lasting tool for controling phytoplasma associated diseases or an integrated approach may be the most viable and sustainable option to check the further spread of disease (Kumari *et al.,* 2019).

Little leaf of brinjal is a prevalent phytoplasma etiology disease causing severe economic losses in India. This study revealed that the severity of brinjal little leaf disease is growing rapidly in the brinjal growing tracts of Assam and almost all the brinjal varieties under cultivation are susceptible to disease. The sequencing of 16S rDNA gene showed that brinjal little leaf phytoplasma belong to 16SrVI clover proliferation group in Assam. This study also confirmed the association of brinjal little leaf phytoplasma with leafhopper *H. phycitis* suggesting its role as a vector of this phytoplasma.

In the present investigation, only 7 districts of the Assam were surveyed and there is need to cover larger areas in the state to gather more information on the presence of other phytoplasma groups, alternative host plant species and identification of resistant brinjal genotypes to formulate novel brinjal little leaf disease management strategies. Moreover, there is a need to continue the search for understanding the epidemiology, biology of the phytoplasmas and the vectors, and the relationship between the hosts, vector and pathogen to explore phytoplasma diseases.

Acknowledgments

The authors would like to thank Assam Agricultural University, Jorhat, Assam, for financial support to carry out this research work.

Add-on Information

Authors' contribution: D.S. Dutta: Contributed to the study conception, design, material preparation, data collection, analysis and draft of the manuscript; **M.K. Kalita and P.D. Nath:** Major and Co- Major Advisors to Dibya Sree Dutta. All authors read and approved the final manuscript.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: Not applicable.

Conflict of interest: The authors declare that there is no conflict of interest.

Data from other sources: Not applicable.

Consent to publish: All authors agree to publish the paper in *Journal of Environmental Biology.*

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