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Molecular characterization of phytoplasma strains associated with brinjal little leaf and screening of cultivated and wild relatives of eggplant cultivars for disease resistance

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Abstract Brinjal little leaf (BLL) is one of the most important and widespread disease of eggplant associated with a phytoplasma in India. It severely infects eggplant cultivation in India and causes serious economic losses. Severe incidence (8 to 30%) of the disease was recorded in three districts of Uttar Pradesh (Varanasi, Mirzapur and Jaunpur) state of India during 2015 and 2016. A total of 58 symptomatic BLL leaf samples were collected from the surveyed fields and processed for nested PCR assays using phytoplasma-specific primer pairs (P1/P7, R16F2n/R16R2). Pair wise sequence identity and phylogeny analysis of 16S rRNA gene sequences of BLL isolates in the study confirmed association of *Ca*. P. trifoli (16SrVI group) with the BLL symptomatic samples. Association of similar strain of phytoplasma was identified in *Hishimonus phycitis* collected in brinjal fields at all the locations, utilizing a similar set of primers pairs as described above. The population of *H. phycitis* was positively correlated with the incidence of BLL disease in the fields. The phytoplasma indexing of 55 eggplants varieties and 17 wild *Solanum* species through PCR assays revealed that one eggplant cultivated variety (Uttara) and 17 wild *Solanum* species were found immune, one resistant (Pusa Ankur) and 12 eggplant varieties/lines were found moderately resistant to BLL. However, all the 17 wild *Solanum* species were recorded free from phytoplasmas in PCR assays. Further, biochemical analysis of the resistant eggplant varieties showed higher peroxidase and polyphenol oxidase enzymatic activities along with the increased total phenols content. These resistant varieties identified in the present study can be utilized as pre-breeding materials to breed and develop eggplant resistance to BLL phytoplasma disease.

Key words Brinjal · Phytoplasma · Genetic diversity · Defense related enzymes · Wild *Solanum* varieties · Resistance

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Introduction

Eggplant (*Solanum melongena* L.) also known as brinjal is an important vegetable crop mainly grown in tropical and sub-tropical countries. It is prone to attack by several pathogens, among them brinjal little leaf (BLL), a phytoplasma associated disease is considered as the most important as it causes significant yield losses up to 40% (Mitra et al. 1993). BLL disease was reported first time in India by Thomas and Krishnaswamy (1939). So far, six groups and nine subgroups of phytoplasmas (16SrI-B, 16Sr II-D, 16Sr III-B, –J and –U, 16Sr VI-A, 16Sr VI-D, 16SrIX-C and 16SrXII-A) have been reported to be associated with BLL disease in different countries (Rao and Kumar 2017). Under epidemic condition yield loss up to 100% was reported in affected eggplants (Rao et al. 2018). This disease could be artificially transmitted from infected to healthy plants through the parasitic plant dodder (*Cuscuta* spp.) or through vegetative grafting. Several leafhopper species are reported as potential vectors for transmission of BLL phytoplasmas under natural conditions (Kumar et al. 2015). Besides, several weeds such as *Datura inoxia, D. stramonium, Cannabis sativa* subsp. *sativa, Portulaca oleracea* and *P. grandiflora* have also been reported as natural and putative alternative hosts of BLL associated phytoplasma strains in India. They play an important role in the natural spread of the BLL disease through leafhopper vectors (Rao and Kumar 2017).

Farmers in India are repeatedly using broad spectrum insecticides to manage pests and diseases in the eggplant crop which results in higher production costs, resistance to pesticides, harmful pesticide residues having adverse implications towards consumer health. Hence, there is an urgent need to find out alternative and safer methods including host plant resistance for the management of BLL disease. Identification of durable resistance sources through screening of available eggplant cultivars, lines, and wild species of *Solanum* against BLL disease is an effective prerequisite to develop a resistant variety. Little attempt has been made, so far, for identifying the resistance sources in eggplant for BLL. Das and Mitra (2000) screened eggplant varieties against the BLL disease and germplasms under natural field conditions and identified eight resistant and 14 moderately resistant lines to BLL. Chakrabarti and Choudhury (1975) were also screened eggplant genotypes against the BLL disease and identified two wild species of eggplant viz., *Solanum integrifolium* and *S. gilo* as resistant to BLL. In addition, they also developed S.212–1 (cross between *S. integrifolium* and *S. gilo*), which was graded as resistant to BLL. Afterwards, no attempts have been made towards screening of potential cultivated eggplant genotypes in India for the resistance potential against BLL.

In addition to phenotypic characteristics, the biochemical parameters also play a key role in the selection of resistance sources. Biochemical compounds like, phenols, polyphenol oxidase and peroxidase enzymes help in protecting the host plant against several biotic stresses (Jabeen et al. 2009; Kumar et al. 2010). Therefore, analyses of biochemical parameters are also important to exploit host plant resistance potential in eggplant varieties against insect vector and phytoplasma disease.

Thus, the aim of the present study was: (a) to characterize the phytoplasma strains associated with brinjal little leaf disease in three major eggplant growing districts of Uttar Pradesh; (b) to record the major leafhopper vector responsible for natural spread of BLL; (c) to screen popular cultivated eggplant varieties/genotypes and wild *Solanum* species to identify resistant sources against BLL disease; and (d) to study the effect of phytoplasma infection on biochemical properties in different eggplant genotypes.

Materials and Methods

Survey and collection of eggplant little leaf samples

A survey was conducted during the years 2015 and 2016 to record the incidence and severity of BLL disease and presence of leafhopper species in eggplant fields of three districts (Varanasi, Mirzapur and Jaunpur) of Uttar Pradesh state, India. The incidence of BLL disease (percentage of plants with little leaf symptoms) was recorded visually in each field (40 fields in Varanasi, 13 in Mirzapur and five in Jaunpur) by counting the number of symptomatic over asymptomatic plants by randomly selecting 10×10 m plots at each location during October 2015 to November 2016 between vegetative and reproductive stage of

the crop. Per cent Disease Index (PDI) was calculated by the following procedure.

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

Fifty-eight samples showing the typical phytoplasma-like symptoms (little leaf and bunchy top) were collected from Varanasi (40), Mirzapur (13) and Jaunpur (5) along with two asymptomatic plant samples from each location (Table 1, Fig. 1). The collected eggplant samples were kept in separate polythene bags after labelling and brought to Plant Pathology laboratory of ICAR-Indian Institute of Vegetable Research (IIVR), Varanasi for further processing through PCR assays.

Insect vector sampling, identification, and dynamics

During the field survey, different leafhopper species feeding on brinjal plants were collected using a sweeping net method during vegetative and reproductive stage of the crop. The collected insects were kept in plastic vials containing 70% ethanol and stored at 4 °C. They were identified at Division of Entomology, ICAR-Indian Agricultural Research Institute, New Delhi. The identified leafhoppers were analyzed for phytoplasma indexing (Table 1). To monitor the insect vector population, yellow sticky trap cards @ 1–2 traps per 50 m^2 (placed at East, West, South and North side of the field, 2 m inside the border row at a height of 40 cm near to the crop canopy) were fixed when the crop (eggplant cv. Punjab Barsati) was 4 weeks old at ICAR-Indian Institute of Vegetable Research, Varanasi (Fig. 3a). Leaf hopper population was counted at weekly intervals from September to March months during two consecutive years (2015 and 2016) and correlated with the incidence of BLL disease in the field (Un Nabi et al. 2015).

Phytoplasma source and maintenance

The BLL samples collected from the ICAR-IIVR experimental plots were maintained in the polyhouse under insect-proof cage through graft transmission on eggplant cv. Punjab Barsati (Acc. No. KC478607). This highly susceptible cultivar was used as standard check for screening resistant in different eggplant germplasms and the wild *Solanum* species (Fig. 2).

Molecular detection and characterization of phytoplasma in eggplant

DNA isolation and PCR assay

Total genomic DNA was isolated from 100 mg of leaf midrib tissue from 58 symptomatic eggplant samples (collected from different surveyed locations) using CTAB method. The DNA extracted from asymptomatic samples collected from healthy brinjal fields was used as negative control. Before PCR assays, the quality of the DNA was checked with Nanodrop. The DNA extracted from the susceptible standard check maintained at ICAR-IIVR was used as positive control in the study. Approximately, 50 ng/µl DNA was used in the PCR for the detection of phytoplasma using phytoplasma-specific 16S ribosomal DNA (rDNA) primers P1/P7 followed by R16F2n/R2 nested primer pairs (Deng and Hiruki 1991; Gundersen and Lee 1996). The samples were collected from fields at distances of 10 km and used for 16S rDNA gene sequencing as described by Ashwathappa et al. (2020). Similarly, the total genomic DNA extracted from leafhoppers (five each from different surveyed locations as well as from eggplant experimental plot at ICAR-IIVR) was used as template for PCR assays (De Barro and Driver 1997).

Pair wise sequence comparison of 16Sr RNA gene and phylogenetic analysis

The 16S rRNA gene sequences derived from the eggplant and insect samples were assembled and edited using ClustalX2 software. They were subjected to BLAST, NCBI to search for similar sequences in the database. Query sequences with close and similar matches to 16S rRNA gene sequences of the members of phytoplasma group/subgroup representative's available in the GenBank database were retrieved (Table 2). The sequence identity matrices for the BLL strains together with phytoplasma group/subgroup available in the database were generated using Bioedit Sequence Alignment Editor (version 5.0.9) and a phylogenetic tree was constructed by MEGA 7 software using the neighbor-joining method with

Table 1 Survey and incidence of little leaf disease of eggplant in different places of Uttar Pradesh state of India along with GenBank submission and phytoplasma identification in brinjal and insect vector

Place	BLL isolates	No. of filed surveyed	Stage of crop	Type of symptoms	Accession number	Group/ subgroup identified	Av.% disease incidence	No. Samples collected	
								Plant	insect
Varanasi	BLL-1	40	Pre and Post flowering stage	Pale green of young leaves, little leaf, Witch's broom	KC478607	16SrVI-D	8–30	40	11
	BLL-14				MW273752 MW273753	16SrVI-D			
	BLL-15				MW273754	16SrVI-D			
	BLL-16				MW273755	16SrVI-D			
	BLL-22				MW273756	16SrVI-D			
	BLL-23				MW273766	16SrVI-D			
	Hp1				MW273767	16SrVI-D			
	Hp2					16SrVI-A			
Mirzapur	BLL-24	15	Pre and Post flowering stage	Little leaf, Witch's broom	MW273757	16SrVI-D	15–30	13	6
	BLL-25				MW273758	16SrVI-D			
	BLL-26				MW273759	16SrVI-D			
	BLL-27				MW273760	16SrVI-A			
	BLL-28				MW273761	16SrVI-D			
	BLL-29				MW273762	16SrVI-D			
	Hp3				MW273768	16SrVI-D			
Janupur	BLL-30	5	Pre and Post flowering stage	Little leaf, Witch's broom	MW273763	16SrVI-A	10–15	5	2
	BLL-31				MW273764	16SrVI-A			
	BLL-32				MW273765	16SrVI-D			
	Hp4				MW273769	16SrVI-A			

1000 bootstrap replications (Kumar et al. 2016). The 16S rRNA gene sequence (Acc. No. U14905) of *Acholeplasma laidlawii* was used as outgroup to root the tree.

Virtual RFLP analysis

The 16S rRNA gene sequences of BLL strains corresponding to the R16F2n/R16R2 fragments were subjected to *in silico* RFLP analysis using the iPhyClassifier online tool and compared with published RFLP patterns of representative sequences of phytoplasma strains of 16SrVI-D (Ac. No. X83431) and 16SrVI-A (Ac. No. AY390261) subgroups (Zhao et al. 2009).

Screening of eggplant varieties and wild *Solanum* spp. against BLL disease

The seeds of 55 eggplant varieties and 17 wild *Solanum* spp. were obtained from the Crop Improvement Division, ICAR-Indian Institute of Vegetable Research (IIVR), Varanasi for screening against BLL disease. The seeds were sown in the raised seed bed (10 m × 5 m) with potting soil in an insect proof greenhouse at ICAR-IIVR. The seedlings were transplanted in an open field 5 weeks after sowing and also in plastic pots for natural and artificial screening by grafting technique, respectively.

Open field screening

A separate field experiment was conducted using 55 cultivated eggplant genotypes and 17 wild *Solanum* spp. at the research farm of ICAR- Indian Institute of Vegetable Research, Varanasi (82.52 E longitude; 68 25.10 N latitude), Uttar Pradesh, India for the two consecutive seasons during 2015 and 2016 in a randomized block design with three replications. The 100 seedlings of each eggplant genotypes and wild *Solanum* spp. accessions were transplanted and raised in plots of size 5 m × 5 m with a spacing of 75 × 75 cm by following the recommended standard agronomic practices. Based on preliminary studies, eggplant cultivar Punjab Barsati was used as a standard susceptible check for this experiment. The transplanted eggplant genotypes and wild *Solanum* spp. accessions were

Fig. 1 Eggplant displayed different types of symptoms under field conditions (**a**) witches' broom at Varanasi; (**b**) Bunchy top symptoms at Mirzapur; (**c**) little leaves turned into brown and fell down at Uttar Pradesh; (**d**) Upper leaves turn pale yellow in colour at Varanasi; (**e**) growing buds are converted into little leaf symptoms at Janupur; (**f**) eggplant showing the big bud symptoms at Varanasi

exposed to natural infestation of leafhoppers. The incidence of the BLL disease was recorded at weekly intervals after 5 weeks of transplanting till the flowering and fruiting stage of the crop. The mean percentage of disease incidence was calculated based on diseased over healthy plants from all the replications. Based on per cent disease incidence, the eggplant genotypes were classified into five categories: (i) immune (0%), (ii) resistant (0.1–10%), (iii) moderately resistant (10.1–20%), (iv) susceptible (20.1–50%), and (v) highly susceptible (> 50%) (Memane et al. 1987; Devi et al.

Fig. 2 Test egplant cv. Punjab barsati showing (**a**) pale yellowing of leaves and malformed buds, (**b**) Little leaf with witches' broom symptoms by graft transmission.

Table 2 List of the 16Sr DNA sequences of phytoplasmas employed in the phylogenetic analysis.

Phytoplasma	Accession	Subgroups	BLL1	BLL14	BLL15	BLL.16	BLL22	BLL.23	BLL24	BLL25	BLL26	BLL27	BLL28	BLL29	BLL30	BLL31	BLL32	Hp1	Hp2	Hp3	Hp4
Brinjal little leaf	16SrVI-D	KC478607	97.2	100	99.9	97.9	97.3	97.6	97.8	97.8	99.4	97.9	97.4	98.6	97.6	97.9	99.9	97.9	97.9	97.3	97.9
Brinjal little leaf	16SrVI-D	X83431	99.1	97.6	97.5	99.2	98.8	99.5	99.6	99.6	98.1	99.2	98.9	98.4	99.1	98.9	97.5	98.9	98.9	98.8	98.9
Brinjal little leaf	16SrVI-D	AF228052	99.2	97.6	97.6	99.4	99.0	99.6	99.8	99.8	98.2	99.4	99.1	98.6	99.2	99.1	97.6	99.1	99.1	99.0	99.1
Brinjal little leaf	16SrVI-D	KX689234	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Brinjal little leaf	16SrVI-D	KX689235	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Brinjal little leaf	16SrVI-D	KX689238	99.3	97.7	97.6	99.5	99.1	99.7	99.9	99.9	98.3	99.5	99.2	98.7	99.2	99.2	97.6	99.2	99.2	99.1	99.2
Brinjal little leaf	16SrVI-D	KX689239	99.3	97.7	97.6	99.5	99.1	99.7	99.9	99.9	98.3	99.5	99.2	98.7	99.2	99.2	97.6	99.2	99.2	99.1	99.2
Brinjal little leaf	16SrVI-D	KX689241	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Brinjal little leaf	16SrVI-D	KX689242	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Brinjal little leaf	16SrVI-D	KX689243	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Brinjal little leaf	16SrVI-D	KX689244	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Brinjal little leaf	16SrVI-D	KX689245	99.3	97.7	97.6	99.5	99.1	99.7	99.9	99.9	98.3	99.5	99.2	98.7	99.2	99.2	97.6	99.2	99.2	99.1	99.2
Brinjal little leaf	16SrVI-D	KX689246	99.3	97.7	97.6	99.5	99.1	99.7	99.9	99.9	98.3	99.5	99.2	98.7	99.2	99.2	97.6	99.2	99.2	99.1	99.2
Brinjal little leaf	16SrVI-D	KX689251	99.3	97.7	97.6	99.5	99.1	99.7	99.9	99.9	98.3	99.5	99.2	98.7	99.2	99.2	97.6	99.2	99.2	99.1	99.2
Brinjal little leaf	16SrVI-D	KX689252	99.3	97.7	97.6	99.5	99.1	99.7	99.9	99.9	98.3	99.5	99.2	98.7	99.2	99.2	97.6	99.2	99.2	99.1	99.2
Brinjal little leaf	16SrVI-D	KX689247	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Brinjal little leaf	16SrVI-D	KX689248	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Brinjal little leaf	16SrVI-D	KX689249	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2

Table 2 (continued)

Phytoplasma	Accession	Subgroups	BLL1	BLL14	BLL15	BLL.16	BLL22	BLL.23	BLL24	BLL25	BLL26	BLL27	BLL28	BLL29	BLL30	BLL31	BLL32	Hp1	Hp2	Hp3	Hp4
Brinjal little leaf	16SrVI-D	KX689250	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Brinjal little leaf	16SrVI-D	KX689237	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Cannabis sativa	16SrVI-D	KX689240	99.3	97.7	97.6	99.5	99.1	99.7	99.9	99.9	98.3	99.5	99.2	98.7	99.2	99.2	97.6	99.2	99.2	99.1	99.2
Portulaca oleracea' little leaf	16SrVI-D	KX689236	99.4	97.8	97.7	99.6	99.2	99.8	100	100	98.4	99.6	99.2	98.8	99.3	99.2	97.7	99.2	99.2	99.2	99.2
Fragaria multicipita	16VI-B	AF036354	95.0	93.6	93.5	95.6	94.8	95.4	95.6	95.6	94.1	95.6	94.8	94.5	95.4	95.0	93.5	95.0	95.0	94.8	95.0
Illinois Clover proliferation	16SrVI-C	AF409069	98.8	97.6	97.6	99.6	98.8	99.2	99.3	99.3	98.2	99.6	98.9	98.6	99.3	99.1	97.6	99.1	99.1	98.8	99.1
Centaurea solstitialis virescence	16SrVI-E	AY270156	98.8	97.2	97.2	99.0	98.6	99.2	99.4	99.4	97.8	99.0	98.7	98.2	98.8	98.7	97.2	98.7	98.7	98.6	98.7
Catharanthus phyllody	16SrVI-F	EF186819	98.4	97.2	97.2	99.3	98.4	98.8	98.9	98.9	97.8	99.3	98.5	98.2	99.2	98.7	97.2	98.7	98.7	98.4	98.7
Indian Portulaca little leaf	16SrVI-H	EF651786	97.7	96.7	96.6	98.4	97.8	98.1	98.3	98.3	97.2	98.4	97.9	97.6	98.1	98.0	96.6	98.0	98.0	97.8	98.0
Fragaria multicipita	16SrVI-G	AF190225	98.4	97.2	97.2	99.3	98.4	98.8	98.9	98.9	97.8	99.3	98.5	98.2	99.1	98.7	97.2	98.7	98.7	98.4	98.7
Passion fruit witches-broom	16SrVI-I	GU292081	96.5	95.5	95.4	97.2	96.6	97.0	97.1	97.1	96.0	97.2	96.7	96.4	97.2	96.8	95.4	96.8	96.8	96.6	96.8
Valeriana yellows	16SrI-M	AY102274	89.7	88.7	88.8	90.6	89.8	90.1	90.2	90.2	89.1	90.6	89.9	89.5	90.8	90.1	88.8	90.1	90.1	90.0	90.1
Ca.P. australasiae	16SrII-D	Y10097	89.7	88.6	88.7	90.5	89.7	90.0	90.1	90.1	88.9	90.5	89.8	89.3	90.7	90.0	88.7	90.0	90.0	89.9	90.0
Ca.P. pruni	16SrIII-A	L04682	93.3	92.4	92.5	94.3	93.4	93.7	93.9	93.9	92.8	94.3	93.5	93.2	94.4	93.8	92.5	93.8	93.8	93.6	93.8
Ca.P. palmae	16SrIV-A	U18747	94.6	93.6	93.7	95.6	94.7	95.0	95.2	95.2	94.0	95.6	94.8	94.4	95.7	95.1	93.7	95.1	95.1	94.8	95.1
Ca.P. ulmi	16SrV-A	AY197655	96.7	95.5	95.6	97.4	96.8	97.0	97.2	97.2	96.0	97.4	96.8	96.4	97.6	97.1	95.6	97.1	97.1	96.9	97.1
Ca.P. ziziphi	16SrV-B	AB052876	96.0	94.8	94.8	96.7	96.0	96.3	96.4	96.4	95.3	96.7	96.1	95.7	96.8	96.4	94.8	96.4	96.4	96.2	96.4
Ca.P. vitis	16SrV-C	AF176319	96.5	95.3	95.4	97.2	96.6	96.8	97.0	97.0	95.9	97.2	96.7	96.3	97.4	96.9	95.4	96.9	96.9	96.8	96.9
Ca.P. trifolii	16SrVI-A	AY390261	99.0	97.9	97.8	100	99.1	99.4	99.6	99.6	98.4	100	99.2	98.8	99.7	99.3	97.8	99.3	99.3	99.1	99.3
Ca.P. fraxini	16SrVII-A	AF092209	96.4	95.3	95.2	97.4	96.5	96.8	97.0	97.0	95.9	97.4	96.6	96.3	97.4	96.8	95.2	96.8	96.8	96.5	96.8
Ca.P. luffae	16SrVIII-A	AF086621	95.8	94.8	94.7	96.6	95.9	96.0	96.2	96.2	95.1	96.6	96.0	95.5	96.6	96.0	94.7	96.0	96.0	95.9	96.0
Ca.P. phoenicium	16SrIX-D	AF515636	93.2	92.4	92.4	94.0	93.2	93.6	93.7	93.7	92.6	94.0	93.3	93.0	94.1	93.6	92.4	93.6	93.6	93.4	93.6

Table 2 (continued)

Phytoplasma	Accession	Subgroups	BLL1	BLL14	BLL15	BLL.16	BLL22	BLL.23	BLL24	BLL25	BLL26	BLL27	BLL28	BLL29	BLL30	BLL31	BLL32	Hp1	Hp2	Hp3	Hp4
Ca.P. mali	16SrX-A	AJ542541	90.0	89.0	89.1	90.8	90.0	90.4	90.5	90.5	89.4	90.8	90.1	89.8	91.0	90.4	89.1	90.4	90.4	90.2	90.4
Ca. P. pyri	16SrX-C	AJ542543	90.1	89.2	89.3	91.0	90.2	90.5	90.7	90.7	89.6	91.0	90.3	90.0	91.2	90.6	89.3	90.6	90.6	90.4	90.6
Ca.P. spartii-].	X92869	X92869	89.1	88.3	88.4	90.0	89.2	89.6	89.6	89.6	88.6	90.0	89.2	89.0	90.1	89.6	88.4	89.6	89.6	89.3	89.6
Ca.P. prunorum	16SrX-F	AJ542544	90.0	89.0	89.1	90.8	90.0	90.4	90.5	90.5	89.4	90.8	90.1	89.8	91.0	90.4	89.1	90.4	90.4	90.2	90.4
Ca.P. oryzae	16SrXI-A	AB052873	94.0	93.2	93.2	95.0	94.1	94.4	94.6	94.6	93.5	95.0	94.2	93.9	95.1	945	93.2	94.5	94.5	94.3	94.5
Ca.P. solani	16SrXII-A	AF248959	88.7	87.6	87.7	89.5	88.8	89.1	89.2	89.2	88.0	89.5	88.8	88.4	89.7	89.1	87.7	89.1	89.1	88.9	89.1
Ca.P. australiense		L76865	88.5	87.2	87.3	89.0	88.3	89.0	89.0	89..0	87.6	89.0	88.4	88.0	89.2	88.6	87.3	88.6	88.6	88.5	88.6
Ca.P. japonicum	16SrXII-D	AB010425	89.4	88.4	88.4	90.3	89.5	89.8	89.9	89.9	88.8	90.3	89.6	89.2	90.5	89.8	88.4	89.8	89.8	89.6	89.8
Ca.P. fragariae	16SrXII-E	DQ086423	89.6	88.5	88.6	90.4	89.6	90.0	90.0	90.0	88.9	90.4	89.7	89.3	90.7	90.0	88.6	90.0	90.0	89.8	90.0
Mexican periwinkle Vir	16SrXIII-A	AF248960	89.4	88.4	88.4	90.2	89.5	89.8	89.9	89.9	88.8	90.2	89.6	89.2	90.4	89.8	88.4	89.8	89.8	89.6	89.8
Ca.P. cynodontis	16SrXIV	AJ550984	94.4	93.7	93.8	95.4	94.5	94.8	95.0	95.0	93.9	95.4	94.6	94.3	95.6	94.9	93.8	94.9	94.9	94.7	94.9
Ca.P. brasiliense	16SrXV	AF147708	89.6	88.4	88.5	90.4	89.7	89.8	90.0	90.0	88.9	90.4	89.7	89.3	90.6	89.9	88.5	89.9	89.9	89.8	89.9
Ca.P. graminis	16SrXVI	AY725228	86.1	85.1	85.2	86.9	86.2	86.5	86.6	86.6	85.5	86.9	86.3	85.9	87.1	86.5	85.2	86.5	86.5	86.3	86.5
Ca.P. caricae	16SrXVII	AY725234	85.1	84.1	84.2	85.9	85.2	85.5	85.6	85.6	84.5	85.9	85.3	84.9	86.1	85.5	84.2	85.5	85.5	85.3	85.5
Ca.P. americanum	16SrXVIII	DQ174122	89.7	88.6	88.7	90.4	89.7	90.1	90.2	90.2	89.0	90.4	89.8	89.4	90.7	90.0	88.7	90.0	90.0	89.9	90.0
Ca.P. castaneae	16SrXIX	AB054986	93.2	92.4	92.4	94.1	93.2	93.6	93.7	93.7	92.6	94.1	93.3	93.0	94.1	93.6	92.4	93.6	93.6	93.4	93.6
Ca.P. rhamni	16SrXX	X76431	90.0	89.2	89.2	90.9	90.1	90.5	90.6	90.6	89.6	90.9	90.2	90.0	91.1	90.5	89.2	90.5	90.5	90.3	90.5
Ca.P. pini	16SrXXI	AJ632155	94.0	93.2	93.2	94.9	94.0	94.4	94.5	94.5	93.4	94.9	94.1	93.8	95.1	94.4	93.2	94.4	94.4	94.2	94.4
Ca.P. cocosnigeriae	16Sr XXII-A	Y14175	92.8	92.0	92.0	93.8	92.9	93.2	93.4	93.4	92.3	93.8	93.0	92.7	94.0	93.3	92.0	93.3	93.3	93.1	93.3
Buckland Valley GY	16SrXXIII-A	AY083605	89.6	88.6	88.7	90.5	89.7	90.0	90.1	90.1	89.0	90.5	89.8	89.4	90.8	90.0	88.7	90.0	90.0	89.9	90.0
Sorghum bunchy shoot	16SrXXIV-A	AF509322	94.3	93.3	93.4	95.2	94.4	94.7	94.8	94.8	93.7	95.2	94.4	94.1	95.4	94.8	93.4	94.8	94.8	94.5	94.8
Weeping tea WB	16SrXXV-A	AF521672	89.9	88.8	88.9	90.5	90.0	90.2	90.3	90.3	89.2	90.5	90.0	89.6	90.8	90.2	88.9	90.2	90.2	90.1	90.2
Sugarcane yellows	16SrXXVI-A	AJ539179	92.3	91.3	91.4	93.2	92.4	92.7	92.8	92.8	91.7	93.2	92.4	92.1	93.4	92.8	91.4	92.8	92.8	92.5	92.8
Sugarcane yellows	16SrXXVII-A	AJ539180	92.7	91.7	91.8	93.6	92.8	93.1	93.2	93.2	92.1	93.6	92.8	92.5	93.9	93.2	91.8	93.2	93.2	92.9	93.2
Ca.P. omanense	16SrXXIX	EF666051	92.8	92.0	92.0	93.8	92.9	93.3	93.4	93.4	92.3	93.8	93.0	92.7	93.8	93.3	92.0	93.3	93.3	93.1	93.3
Ca.P. tamaricis	16SrXXX	FJ432664	89.6	88.8	88.8	90.5	89.7	90.1	90.2	90.2	89.1	90.5	89.8	89.5	90.7	90.1	88.8	90.1	90.1	89.9	90.1
Ca.P. cocostanzaniae.		X80117	94.4	93.4	93.5	95.3	94.4	94.8	94.9	94.9	93.8	95.3	94.5	94.2	95.5	94.8	93.5	94.8	94.8	94.6	94.8
Chinaberry yellows.		AF495882	89.2	88.3	88.4	90.0	89.3	89.6	89.7	89.7	88.7	90.0	89.4	89.1	90.3	89.6	88.4	89.6	89.6	89.5	89.6

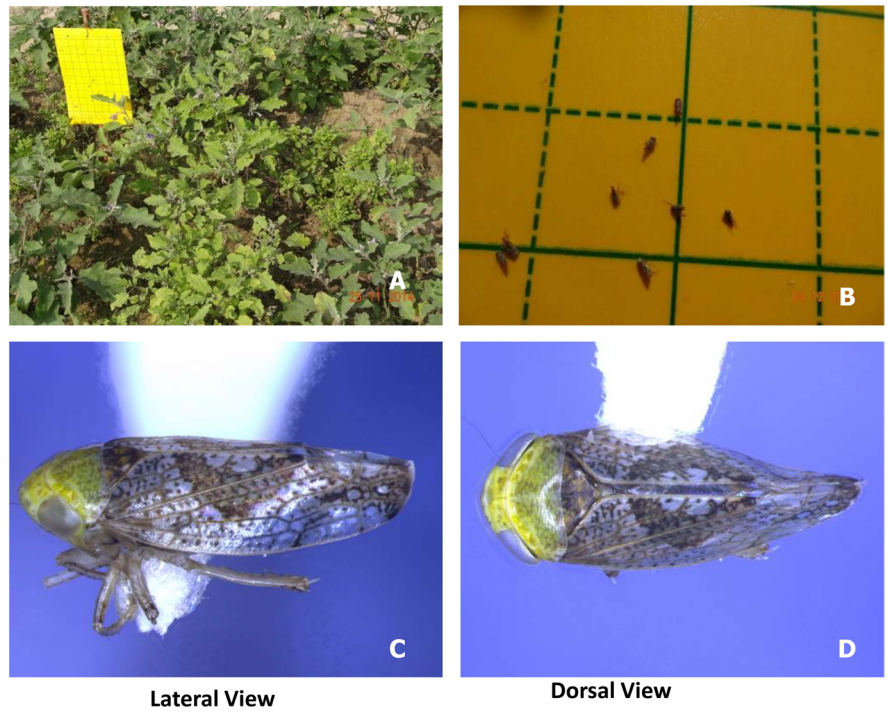


Fig. 3 (**a**) Installation of yellow sticky trap under field condition, (**b**) Insect vector (*Hishimonus phycitis*) on yellow sticky trap, (**c**) Lateral and (**d**) dorsal view of *Hishimonus phycitis*

1995). Further, the infected and healthy plant samples collected from the 55 eggplant genotypes and 17 wild *Solanum* spp. accessions were subjected to molecular DNA-based screening (PCR assay) using the primer pairs R16F2n/ R16R2 through nested PCR assay as described above.

Artificial Screening by grafting

The 55 accessions of eggplant genotypes and 17 wild *Solanum* spp. were also subjected to further screening through graft transmission technique (Venkataravanappa et al. 2018a). Ten seedlings from each of different accessions of eggplant genotypes were transplanted in plastic pot with dimension of 30 cm × 15 cm filled with a potting mixture. Grafting was made on the 35 days old eggplant as described earlier using scion from the phytoplasma positive eggplant maintained under polyhouse conditions (Venkataravanappa et al. 2018a). The grafted plants were maintained and observed for symptom expression up to 95 days after grafting followed by PCR indexing.

Study of biochemical parameters

The leaf samples of cultivated eggplant genotypes infected with phytoplasma were collected during peak incidence of the disease including susceptible cultivar (Punjab Barsati) after 45 days after planting for the estimation of defence enzyme activities such as peroxidase, polyphenol oxidase and total phenol content.

Peroxidase enzyme

Peroxidase enzyme activity was analyzed by following the method of Shannon et al. (1996). One gram of fresh leaf tissue from each cultivar was macerated with help of sterilized pestle and mortar by adding Tris-HCl buffer (pH 7.6). The extract was centrifuged for 30 min at 15000 × g at 4 °C. The chilled supernatant (0.1 ml) was mixed with 2.8 ml of reaction mixture containing 0.5% o-dianisidine dissolved in methanol, 0.28 ml sodium acetate buffer and 2.4 ml water. Hydrogen peroxide (30%) was added (0.1 ml) to reaction mixture to start the reaction. Changes in absorbance values were recorded at an interval of one minute at 430 nm up to 3 min.

Polyphenol oxidase enzyme

For estimation of polyphenol oxidase activity, one-gram fresh leaf tissue from each cultivar was macerated with phosphate buffer (pH 6.0) at 4 °C in a sterilized pestle and mortar. The extract was centrifuged at 15000 × g for 30 min at 4 °C. To the extract (0.5 ml), 2 ml phosphate buffer and 0.5 ml (0.01 M) catechol was added. Changes in absorbance values were recorded at 410 nm up to 3 min at an interval of one minute (Matto and Diamond 1963).

Total phenol content estimation

Total phenol content of the eggplant cultivars was estimated by grinding 1 g leaf sample with 10 ml of 95% ethanol in a pestle and mortar, subsequently boiling in a water bath for 30 min. Then, the extract was centrifuged for 20 min at 10000 rpm. Each sample was extracted three times and the supernatants were pooled followed by evaporation of ethanol at 80 °C in a hot water bath. The residue was dissolved in 50 ml of water, 3 ml of extract was taken and 0.5 ml of Folin-Ciocalteau reagent and 4 ml of Na_2CO_3 (20%) was added. The mixture was kept in boiling water bath for 1 min and cooled. The phenol content was estimated using a catechol standard by recording the absorbance at 650 nm.

Results

Survey, incidence, and symptomatology

Under natural conditions, the major symptoms recorded on eggplant varieties were pale green-coloured little leaf leaves, phyllody, big bud, witches' broom, and excessive axillary shoot proliferation (Fig.1a–f). The floral buds were malformed into leaf like structures and infected plants failed to set fruits. Infected plants could be easily identified from a distance because of their little leaf and bushy appearance. The incidence and severity of the BLL in various fields of Varanasi, Mirzapur and Janupur was recorded from 8 to 30%.

Leafhopper identification and population studies

The major leafhopper species collected from the eggplant fields at surveyed locations and experimental plots were identified as *Amrasca biguttula* (Ishida), *Empoasca prima* (Distant) and *Hishimonus phycitis* (Distant). Out of these, *H. phycitis,* was identified as predominant species present in brinjal fields at all the surveyed locations as well as at the experimental plots of ICAR-IIVR, Varanasi (Fig. 3b-d). The highest count of insect vector (*H. phycitis*) was recorded during second fortnight of September (29 insects/trap) and first fortnight of October (26 numbers/trap) (Table 1). The high populations of *H. phycitis* in experimental eggplant plots were found to be positively correlated with the incidence of BLL disease in later months. The percent little leaf disease in the experimental plots ranged from 10.21 to 52.48% (Table 1).

Detection of phytoplasma

Phytoplasma indexing in 58 symptomatic eggplant samples (collected from different surveyed locations) along with standard susceptible check as a positive control were confirmed through amplification of ~1.8 kb products in the first round PCR (P1/P7 primers) and ~1.2 kb products in nested PCR assays with primer pair R16F2n/R2, respectively (Table 2). No amplifications were noticed in any of the asymptomatic samples collected from different surveyed locations.

Three leafhopper species, *Amrasca biguttula*, *Empoasca prima* and *Hishimonus phycitis,* were counted as major dominant species in eggplant fields based on populations trapped during the 2 years. For phytoplasma indexing (19 samples of three species of leaf hopper collected from surveyed locations of 10 m × 10 m plot size at Varanasi, Mirzapur and Jaunpur and five samples from experimental plots at ICAR-IIVR) were subjected to PCR assay using primer pairs, P1/P7 and R16F2n/R2. Only four *H. phycitis* samples (two each from the experimental plots at Varanasi and Mirzapur) were detected positive for phytoplasma with the amplification product of 1.25 kb in nested PCR assay (data not shown). However, no phytoplasma amplification was achieved in any of other two leafhopper species (*A. biguttula*, *E. prima*) collected from Varanasi,

Mirzapur and Jaunpur districts of Uttar Pradesh in the present study.

Since most of the samples from similar places have 16S rRNA sequence identity of 100%, representative 15 BLL samples of which six were from Varanasi (BLL-1, BLL-14, BLL-15, BLL-16, BLL-22, BLL-23), six from Mirzapur (BLL-24, BLL-25, BLL-26, BLL-27, BLL-28, BLL-29), three from Jaunpur districts (BLL-30, BLL-31, BLL-32) and four leaf hopper samples of *H. phycitis* (Hp1 to Hp4) (two from the experimental plot and two from Varanasi and Mirzapur) (data not shown) were sequenced in both directions and the consensus sequences of the isolates were deposited in the NCBI database (Acc. Nos KC478607, MW273752-MW273769) (Table 1).

Pair wise comparison of 16S rRNA gene sequences of BLL and leafhopper phytoplasma strains

Pair wise comparison of 16S rRNA gene sequences from 15 eggplant phytoplasma isolates (BLL-1, BLL-14, BLL-15, BLL-16, BLL-22, BLL-23, BLL-24, BLL-25, BLL-26, BLL-27, BLL-28, BLL-29, BLL-30, BLL-31, BLL-32) and four leafhopper isolates (Hp-1 to Hp-4) with different phytoplasma group/subgroup representatives available in NCBI database showed maximum 16S rRNA gene sequence similarity (99.3 to 100%) of BLL and Hp isolates with *Ca*. P. trifoli (16SrVI group) related strains (Table 2).

Phylogenetic analysis of 16S rRNA gene sequences

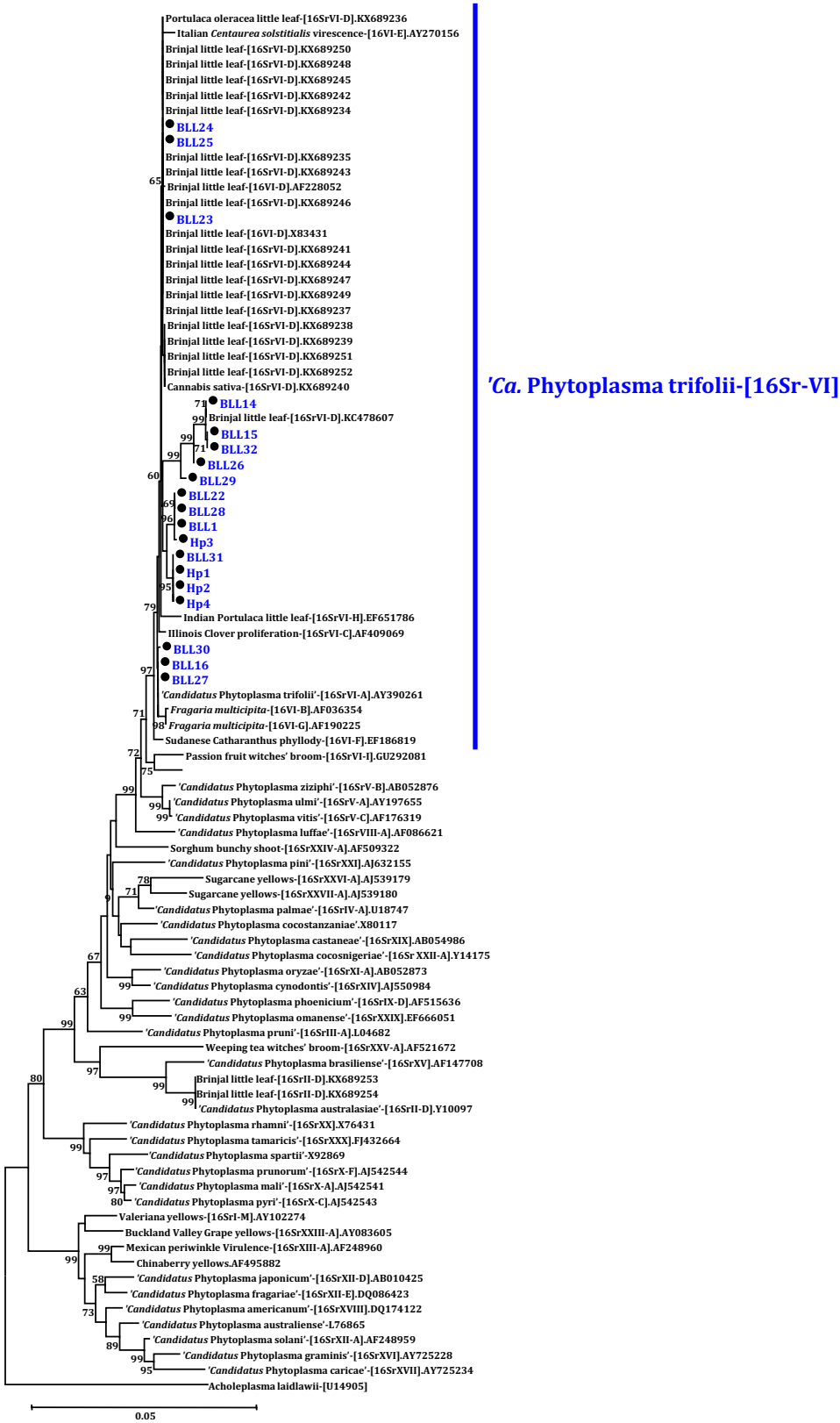
The phylogenetic tree was generated by comparing the 19 (15 eggplants and four from insect vector) 16S rRNA gene sequences of BLL phytoplasma and Hp isolates identified in the present study with other selected phytoplasma of different groups and subgroups retrieved from GenBank database. The phylogenetic tree analysis of 16SrDNA sequences of BLL and Hp phytoplasma isolates showed clustering of BLL and Hp isolates into two subgroups strains,16SrVI-D (KC478607) and 16SrVI-A (AY390261), which belonged to *Ca*. P. trifoli (16SrVI group) related strains (Kumar et al. 2015; Venkataravanappa et al. 2018a) (Fig. 4).

The restriction RFLP profiles of the three BLL phytoplasma isolates (BLL23, BLL24, and BLL25) showed 100% similarity to the restriction profiles of 16SrVI-D subgroup representative strain (Acc No. X83431) with a similarity coefficient of 1.00 and restriction profiles of the other three BLL isolates (BLL16, BLL27 and BLL30) were found similar with 16SrVI-A subgroup representative strain (Acc. No. AY390261) with a similarity coefficient of 1.00. However, the *Bfa*I endonuclease restriction profile of thirteen BLL isolates (BLL1, BLL14, BLL15, BLL22, BLL26, BLL28, BLL29, BLL31, BLL32, Hp1, Hp2, Hp3 and Hp4) of the present study was matched different from all previously reported 16SrVI subgroup strains with a similarity coefficient ranging from 0.86 to 0.97 (Fig. 5).

Screening of eggplant genotypes/lines and wild *Solanum* sp. against BLL disease

Open field screening

Responses of 55 eggplant genotypes and 17 wild *Solanum* species to phytoplasma infection was observed and their levels of resistance was recorded (Table 3). Out of these, only one cultivated eggplant variety Uttara and 17 wild *Solanum* species were found to be immune to the BLL disease, as no visual symptoms were observed during the entire course of study and were tested negative for phytoplasma DNA amplification in nested PCR assays (Fig. 6a-b). Whereas Pusa Ankur variety was found resistant with disease incidence of 6.5% and other 12 eggplant genotypes *viz*., KKM 01, Ajad Eggplant 4, CHBR 1, Rajendra Eggplant 9, BH 2, CHBR 2, Utkal Madhuri, Bhagyamati, Shobha, Arka Nidhi, CH 215 and Arka Keshav were recorded as moderately resistant with disease incidence ranging between 10.1–20%. The remaining 41 eggplant genotypes were graded as susceptible and highly susceptible to BLL disease (Table 3). Further, total genomic DNA extracted from all the 55 eggplant genotypes/lines and 17 wild *Solanum* species were subjected to PCR analysis using phytoplasma specific universal primers. Further, immunity in Uttara and 17 wild *Solanum* species was confirmed with no amplification of phytoplasma DNA in PCR indexing (Fig. 7a & b).


Portulaca oleracea little leaf-[16SrVI-D].KX689236
Italian Centaurea solstitialis virescence-[16VI-E].AY270156
Brinjal little leaf-[16SrVI-D].KX689250
Brinjal little leaf-[16SrVI-D].KX689248
Brinjal little leaf-[16SrVI-D].KX689245
Brinjal little leaf-[16SrVI-D].KX689242
Brinjal little leaf-[16SrVI-D].KX689234
BLL24
BLL25
Brinjal little leaf-[16SrVI-D].KX689235
Brinjal little leaf-[16SrVI-D].KX689243
Brinjal little leaf-[16VI-D].AF228052
Brinjal little leaf-[16SrVI-D].KX689246
BLL23
Brinjal little leaf-[16VI-D].X83431
Brinjal little leaf-[16SrVI-D].KX689241
Brinjal little leaf-[16SrVI-D].KX689244
Brinjal little leaf-[16SrVI-D].KX689247
Brinjal little leaf-[16SrVI-D].KX689249
Brinjal little leaf-[16SrVI-D].KX689237
Brinjal little leaf-[16SrVI-D].KX689238
Brinjal little leaf-[16SrVI-D].KX689239
Brinjal little leaf-[16SrVI-D].KX689251
Brinjal little leaf-[16SrVI-D].KX689252
Cannabis sativa-[16SrVI-D].KX689240
BLL14
Brinjal little leaf-[16SrVI-D].KC478607
BLL15
BLL32
BLL26
BLL29
BLL22
BLL28
BLL1
Hp3
BLL31
Hp1
Hp2
Hp4
Indian Portulaca little leaf-[16SrVI-H].EF651786
Illinois Clover proliferation-[16SrVI-C].AF409069
BLL30
BLL16
BLL27
'Candidatus Phytoplasma trifolii'-[16SrVI-A].AY390261
Fragaria multicipita-[16VI-B].AF036354
Fragaria multicipita-[16VI-G].AF190225
Sudanese Catharanthus phyllody-[16VI-F].EF186819
Passion fruit witches' broom-[16SrVI-I].GU292081
'Candidatus Phytoplasma ziziphi'-[16SrV-B].AB052876
'Candidatus Phytoplasma ulmi'-[16SrV-A].AY197655
'Candidatus Phytoplasma vitis'-[16SrV-C].AF176319
'Candidatus Phytoplasma luffae'-[16SrVIII-A].AF086621
Sorghum bunchy shoot-[16SrXXIV-A].AF509322
'Candidatus Phytoplasma pini'-[16SrXXI].AJ632155
Sugarcane yellows-[16SrXXVI-A].AJ539179
Sugarcane yellows-[16SrXXVII-A].AJ539180
'Candidatus Phytoplasma palmae'-[16SrIV-A].U18747
'Candidatus Phytoplasma cocostanzaniae'.X80117
'Candidatus Phytoplasma castaneae'-[16SrXIX].AB054986
'Candidatus Phytoplasma cocosnigeriae'-[16Sr XXII-A].Y14175
'Candidatus Phytoplasma oryzae'-[16SrXI-A].AB052873
'Candidatus Phytoplasma cynodontis'-[16SrXIV].AJ550984
'Candidatus Phytoplasma phoenicium'-[16SrIX-D].AF515636
'Candidatus Phytoplasma omanense'-[16SrXXIX].EF666051
'Candidatus Phytoplasma pruni'-[16SrIII-A].L04682
Weeping tea witches' broom-[16SrXXV-A].AF521672
'Candidatus Phytoplasma brasiliense'-[16SrXV].AF147708
Brinjal little leaf-[16SrII-D].KX689253
Brinjal little leaf-[16SrII-D].KX689254
'Candidatus Phytoplasma australasiae'-[16SrII-D].Y10097
'Candidatus Phytoplasma rhamni'-[16SrXX].X76431
'Candidatus Phytoplasma tamaricis'-[16SrXXX].FJ432664
'Candidatus Phytoplasma spartii'-X92869
'Candidatus Phytoplasma prunorum'-[16SrX-F].AJ542544
'Candidatus Phytoplasma mali'-[16SrX-A].AJ542541
'Candidatus Phytoplasma pyri'-[16SrX-C].AJ542543
Valeriana yellows-[16SrI-M].AY102274
Buckland Valley Grape yellows-[16SrXXIII-A].AY083605
Mexican periwinkle Virulence-[16SrXIII-A].AF248960
Chinaberry yellows.AF495882
'Candidatus Phytoplasma japonicum'-[16SrXII-D].AB010425
'Candidatus Phytoplasma fragariae'-[16SrXII-E].DQ086423
'Candidatus Phytoplasma americanum'-[16SrXVIII].DQ174122
'Candidatus Phytoplasma australiense'-L76865
'Candidatus Phytoplasma solani'-[16SrXII-A].AF248959
'Candidatus Phytoplasma graminis'-[16SrXVI].AY725228
'Candidatus Phytoplasma caricae'-[16SrXVII].AY725234
Acholeplasma laidlawii-[U14905]
0.05
'Ca. Phytoplasma trifolii-[16Sr-VI]

◂**Fig. 4** Phylogenetic tree based on sequences of 16SrRNA gene from little leaf phytoplasma and *Hishimonus phycitis* with other phytoplasma strains using Neighbor-joining algorithm. Horizontal distances are proportional to sequence distances; vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed and the bootstrap percent values more than 50 are numbered along branches.

Artificial screening by grafting

All the 55 cultivated eggplant genotypes and 17 wild *Solanum* species screened under field conditions were also used for artificially screening against BLL disease through grafting technique. Among them, 54 cultivated eggplant genotypes expressed the typical little leaf and phyllody symptoms after incubation of 20–25 days; whereas, the variety Uttara did not show any symptoms even after grafting for one month. Similarly, 17 wild *Solanum* species did not show any trace of phytoplasma symptoms after incubation of 20–25 days (Table 4). Further, total genomic DNA extracted from all the 72 eggplant genotypes and wild *Solanum* species were subjected to PCR analysis using phytoplasma universal primers. PCR amplicons of ~1.2 kb were detected in all genotypes of eggplant except DNA isolated from the variety Uttara and 17 wild *Solanum* species (Table 4).

Evaluation of eggplant varieties for defence enzyme activities

The eggplant genotypes were evaluated in two consecutive years under natural disease pressure, showed varying degree of susceptibility to BLL disease. Therefore, to know the status of defence enzymes activity in eggplant genotypes infected with BLL disease, the healthy and diseased leaf samples of 55 eggplant genotypes were collected during peak severity and incidence of the disease at 60 days after sowing from the field for the estimation of peroxidase, polyphenol oxidase activities and the total phenol content.

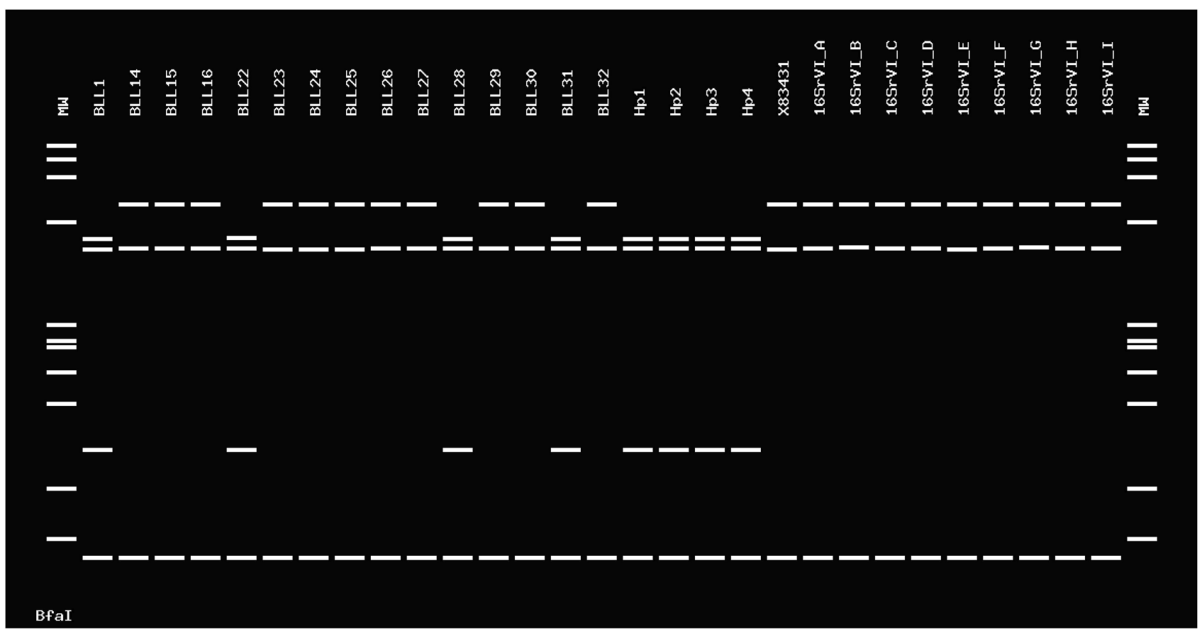


Fig. 5 Virtual RFLP patterns derived from *in silico* digestions, using *i*PhyClassifier for R16F2n/R16R2 fragment of 16S rRNA gene from strains of BLL and insect vector (BLL1, BLL22, BLL28, BLL31, Hp1, Hp2, Hp3 and Hp4). The virtual RFLP patterns strains of BLL (*Bfa*I) distinguish the strain from those in a number of subgroups in group 16SrVI. The restriction fragments were resolved through 3% virtual agarose gel. M: Molecular Ladder phiX174 DNA *Hae*III digest.

Table 3 Reaction of different eggplant varieties/lines and wild *Solanum* spp. to phytoplasma (Little leaf disease)

Variety/Line	Disease Incidence	Category
Uttara (1), *Solanum torvum*, *S. incanum*, *S. gilo* (ADM-117) *S. kashianum* (ADM-183), *S. laciniatum* (EC-790351), *S. sisymbifolium* (EC-790352), *S. aethiopicum* (EC-790353), *S. macrocarpum* (EC-790354), *S. anguivi* (EC-790358), *S. aethiopicum* (EC-790360), *S. aethiopicum* (EC-790361), *S. viarum* (EC-790363), *S. xathocarpum* (EC-790365), *S. sundatum* (EC-790349), *S. anguivi* (EC-790359), *S. aethiopicum* (EC-790357) (17)	0%	Immune
Pusa Ankur (1)	0.1–10%	Resistant
KKM 01, Ajad Eggplant 4, CHBR-1, Rajendra Eggplant-9, BH- 2, CHBR-2, Utkal Madhuri, Bhagyamati, Shobha, Arka Nidhi, CH 215, Arka Keshav (12)	10.1–20%	Moderately Resistant
Punjab Barasati, Kashi Prakash, IVBL-22, Ajad Eggplant 1, JB-8, JB-9, Arka Neelkanth, CO2, Arka Kranti, JB-69,IBH 3, DBL 24, Aruna, Uttakal Tarini, BR-14, Pusa Shymel, RS-356, Pusa Uphar, JB-6, KS-339, B. Devariya, ABSR-2, RCMBL-02, ADM-190, Pant Rituraj, RM Jaint, DBR 8, Punjab Sadabahar, SLW, Gulabi, S. pratibha, JB-67, JB-2, Co-11, Pusa Purple Long, RCMBL-04 (37)	20.1–50%	Susceptible
Azad eggplant-3, Azad eggplant-2, DBR-31, Jawahar Eggplant (4)	>50%	Highly Susceptible

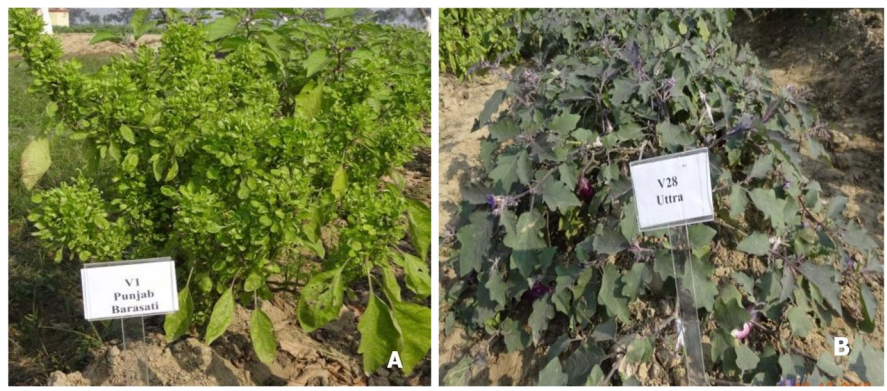


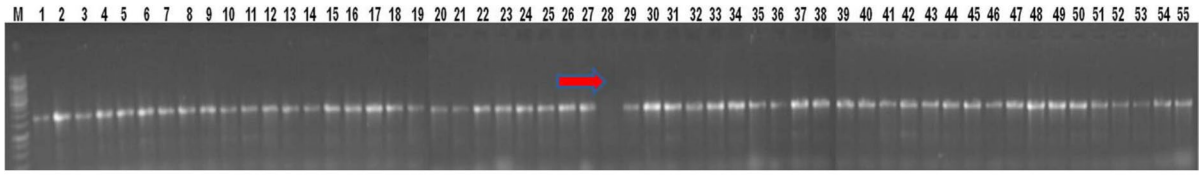
Fig. 6 Response of Uttara variety along with a susceptible check (Punjab barasati) to phytoplasma disease under field conditions (**a**) Punjab barasati, (**b**) Uttara variety

Peroxidase enzyme activity

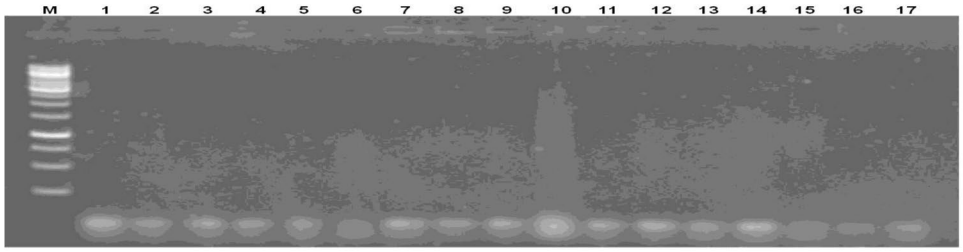
The peroxidase enzyme activity in terms of changes in absorbance (g/min) was recorded in range from 0.47–2.11 and 0.58–1.81 in healthy and BLL diseased eggplant genotypes, respectively. The highest activity was recorded in Uttara, an immune variety followed by PR-5, Shobha, ABSR-2, Pusa Barsati, KKM01, and Rajendra Brinjal-9. The lowest enzyme activity was observed in the highly susceptible genotypes JB6, Co11, Pusa purple long, Akra Nidhi and Pusa Uphar. Overall, the healthy samples of the eggplant varieties/lines recorded maximum peroxidase enzyme activity than the phytoplasma associated identified diseased varieties/lines (Fig. 8a).

Polyphenol oxidase enzyme activity

The polyphenol oxidase enzyme activity profile in terms of changes in absorbance (g/min) ranged from 0.50–0.94 and 0.41–0.83 in healthy and BLL infected eggplant genotypes. The genotypes with maximum polyphenol oxidase activity showed resistance reaction to phytoplasma infection. The eggplant genotypes such as Azad Kranti, SLW, Pusa Shymel, CO2, JB6, CHBR1 and KKM01 recorded highest enzyme activity along with the immune variety Uttara. The susceptible



(a) 55 brinjal varieties/lines



(b) 17 wild *Solanum* spp.

Fig. 7 PCR amplification of 16S rRNA gene of little leaf phytoplasma infecting (**a**) 55 eggplant varieties/lines and (**b**) 17 wild *Solanum* spp.

(**a**) Marker 1 kb, Lane **1 to 55**: Punjab Barasati, KKM 01, Kashi Prakash, IVBL-22, Ajad Brinjal-3, Ajad Brinjal-1, Ajad Brinjal-2, Ajad Brinjal-4, PR-5, CHBR 1, Rajendra Eggplant-9, Pusa Ankur, JB-9, JB-8, Arka neelkanth, Co-2, Arka kranti, Jb 69, IBH-3, BH 2, DBL-24, CHBR-2, Aruna, Uttakal Tarini, Br14, Pusa Shymel, RS 356, Uttra, Utkal Madhuri, Bhagyamati, Pusa Uphar, JB-6, KS-339, B. Devariya, ABSR-2, RCMBL-02, Shobha, Adm-190, Pant Rituraj, RM Jaint, DBR-8, DBR-31, Arka Nidhi, Jawahar Brinjal, CH-215, SLW, Punjab sadabahar, Gulabi, S. pratibha, JB- 67, Arka keshav, Co-11, JB-2, Pusa Purple Long, RCMBL 04–04,

(**b**) Marker 1 kb, Lane **1 to 17**: *Solanum torvum*, *S. incanum*, *S. gilo* (ADM-117) *S. kashianum* (ADM-183), *S. laciniatum* (EC 790351), *S. sisymbifolium* (EC 790352), *S. aethiopicum* (EC 790353), *S. macrocarpum* (EC 790354), *S. anguivi* (EC 790358), *S. aethiopicum* (EC 790360), *S. aethiopicum* (EC 790361), *S. viarum* (EC 790363), *S. xathocarpum* (EC 790365), *S. sundatum* (EC 790349), *S. anguivi* (EC 790359), *S. aethiopicum* (EC 790357)

eggplant genotypes such as Utkal Tarini, Aruna, BH2, Punjab Sadabahar and ABSR-2 were recorded with lowest enzyme activity (Fig. 8b).

Total phenol content in eggplant varieties

The total phenol content (mg/100 g) ranged from 133.72–785.12 and 349.2–567.21 in healthy and BLL infected eggplant genotypes, respectively. The moderately resistant varieties like Arka Nidhi, Rajendra Brinjal 9 and Azad Brinjal 4 recorded a higher phenol content. The lowest phenol content was observed in susceptible genotypes such as DBL24, DBR8, Pant Rituraj, CHBR2, JB2, RM_Giant, BH2, DBR31 and other susceptible varieties/lines (Fig. 8c).

Discussion

Phytoplasma diseases are major constraints for production of many economically important field crops (Rao et al. 2018). The diseases caused by phytoplasmas are constantly increasing over the years with an uncertain etiology and diverse geographic distribution (Bertaccini and Duduk, 2009; Rao et al. 2018). Accurate detection of phytoplasma is a prerequisite for the management of the disease. The nucleic acid-based diagnostic technique such as polymerase chain reaction is routinely used for authentic detection of phytoplasmas. In the present study, initial diagnosis was done based on symptoms appearance in different eggplant samples collected from three eggplant growing districts of Uttar Pradesh, India. This was subsequently confirmed by PCR and nested-PCR assays (Kumar et al. 2015; Rao and Kumar 2017; Venkataravanappa et al. 2018a).

Table 4 Artificial Screening (grafting) different eggplant varieties/lines against phytoplasma (Little leaf disease)

Sl. No	Eggplant varieties/lines	Grafting#	PCR*
1	KKM 01	+	+
2	BR 14	+	+
3	R M Jaint	+	+
4	Kashi Prakash	+	+
5	IVBL 22	+	+
6	SLW	+	+
7	Pant Ritura	+	+
8	CHBR 2	+	+
9	BH2	+	+
10	Azad eggplant 1	+	+
11	Arka kusumakar	+	+
12	JB 9	+	+
13	Utkal tarini	+	+
14	CO2	+	+
15	Punjab Sadabahar	+	+
16	Arka nidhi	+	+
17	Arka kranti	+	+
18	RCMBL- 04	+	+
19	KKM 01	+	+
20	Pusa shymel	+	+
21	Swarna pratibha	+	+
22	B. Devariya	+	+
23	Gulabi	+	+
24	Arka neelkanth	+	+
25	RCMBL −02	+	+
26	Azad Eggplant 4	+	+
27	DBL 24	+	+
28	Jawahar Eggplant	+	+
29	Azad eggplant 2	+	+
30	Azad eggplant 3	+	+
31	Rajendra eggplant 9	+	+
32	BH 2	+	+
33	CHBR 2	+	+
34	Uttkal Madhuri	+	+
35	Pusa Ankur	+	+
36	Punjab Barsathi	+	+
37	Uttara	–	–
38	DBR 31	+	+
39	Bhagyamati	+	+
40	Shoba	+	+
41	CH215	+	+
52	Arka Kesav	+	+
53	Pusa Purple Long	+	+
54	JB-6	+	+
55	KS 339	+	+
56	*Solanum torvum*	–	–
57	*S. incanum*	–	–
58	*S. gilo* (ADM-117)	–	–
59	*S. kashianum* (ADM-183)	–	–
60	*S. laciniatum* (EC-790351)	–	–
61	*S. sisymbifolium* (EC-790352)	–	–
62	*S. aethiopicum* (EC-790353)	–	–
63	*S. macrocarpum* (EC-790354)	–	–
64	*S. anguivi* (EC-790358)	–	–
65	*S. aethiopicum* (EC-790360)	–	–
66	*S. aethiopicum* (EC-790361)	–	–
67	*S. viarum* (EC-790363)	–	–
68	*S. xathocarpum* (EC-790365)	–	–
69	*S. sundatum* (EC-790349)	–	–
70	*S. anguivi* (EC-790359)	–	–
71	*S. aethiopicum* (EC-790357)	–	–

Total twenty five plants were used in each experiment for grafting

*Individual plant was subjected to PCR assay using phytoplasma specific primers

Phytoplasma specific primers designed based on conserved 16S rRNA gene are being used for detection of a wide range of phytoplasmas associated with different plants and insect vectors (Namba et al. 1993). However, nested-PCR assay is performed for preliminary amplification of universal primer pair followed by use of a second set of internal sized primer capable of detection of phytoplasmas in the infected tissues. It also enhances the sensitivity and specificity for detection of phytoplasmas present even in low titres in the plant tissue (Ashwathappa et al. 2019,2020; Hemmati et al. 2020).

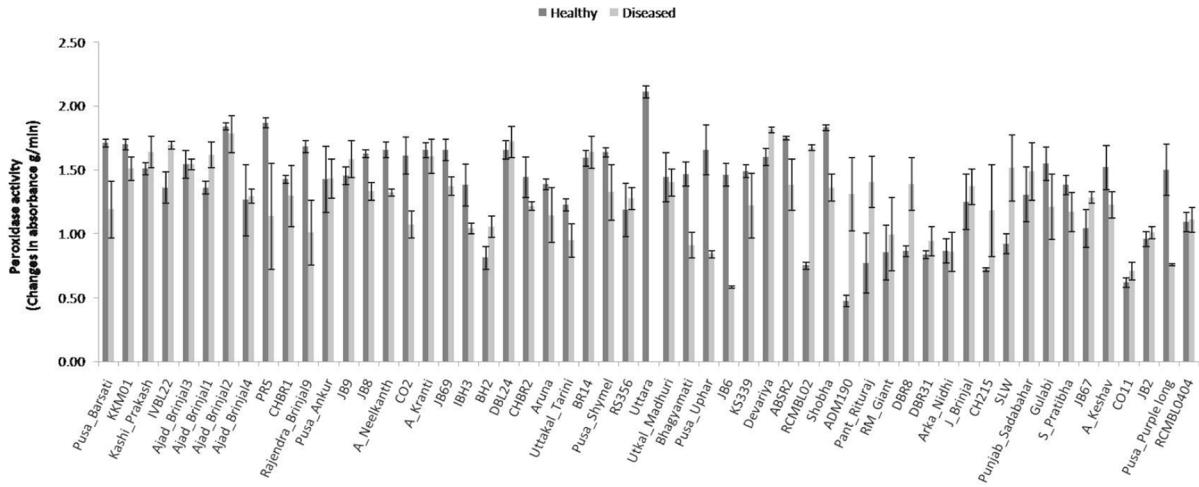
So far, phytoplasmas belonging to 16Sr II-D and 16Sr VI-D were reported to infect eggplant crop in India (Kumar et al. 2017; Rao 2021; Venkataravanappa et al. 2018a). In the present study, 16SrVI-A and 16SrVI-D subgroups have been identified and characterized in eggplant samples collected from three districts (Varanasi, Mirzapur and Jaunpur) of Uttar Pradesh, India based on 16S rRNA gene sequence comparison and restriction profiles in virtual RFLP analysis. Further the virtual RFLP analysis 16S rRNA gene of thirteen BLL isolates (BLL1, BLL14, BLL15, BLL22, BLL26, BLL28, BLL29, BLL31,

BLL32, Hp1, Hp2, Hp3 and Hp4) revealed that they could be tentatively classified into a new variant under 16SrVI phytoplasma group, because of different restriction pattern of *Bfa,* which needs further investigations with real RFLP analysis and other multilocus gene mapping. Available literature also suggests that 16SrVI group infects a wide range of plants including weeds, ornamentals, vegetables, fruits and trees in India (Rao 2021).

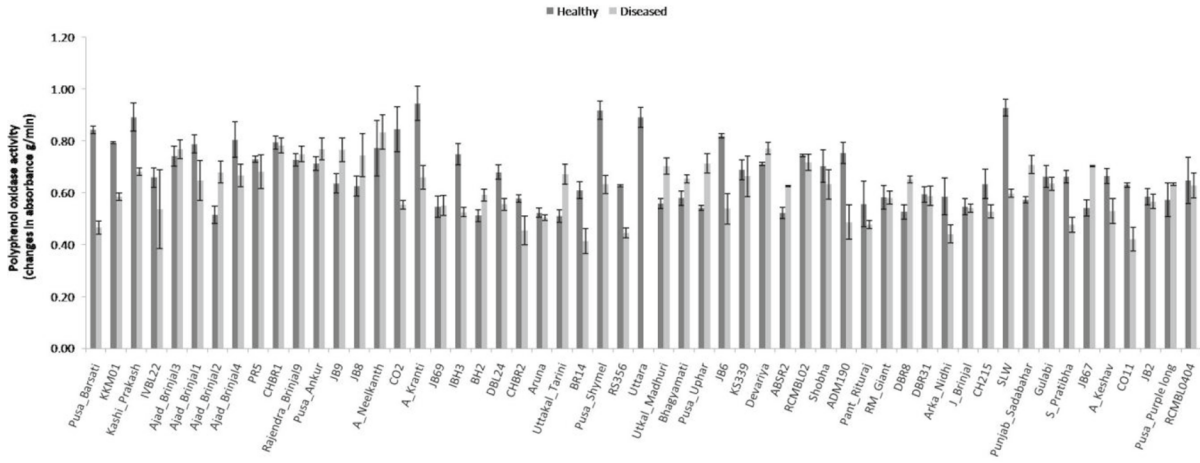
In the present study, the leaf hopper *H. phycitis* has been identified as a putative vector that transmits BLL disease under natural conditions. It was confirmed based on the presence of phytoplasma in leafhoppers collected from infected eggplant fields in PCR assays. The high population densities of vector, *H. phycitis* was recorded in eggplant fields from March to May and Sept to Nov during 2015 and 2016 followed by high incidence of the BLL disease in eggplant fields at Varanasi. The *H. phycitis* was reported as a natural and putative vector for BLL phytoplasma strain associated with 16SrVI-D subgroup in different states of India (Kumar et al. 2017; Dutta et al. 2020). Similarly, *H. phycitis* is also reported as natural vector of sesame phyllody in India (Un Nabi et al. 2015), lime witches' broom in Iran (Siampour et al. 2006; Salehi et al. 2007) and several phytoplasma strains associated with ornamentals in India (Rao 2021).

Different defense mechanisms function in host plants to tolerate/overcome different biotic/abiotic stresses. The mechanism of host plant resistance in response to biotic stress consists of a series of changes in biochemical events such as emergence of free radicals, damage of cellular biomolecules, and subsequently malfunctioning of immune system (Bendich 1996). The antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), ascorbate peroxidase (APX) play major roles in the detoxification of ROS and maintain adequate level of antioxidants in the cells (Baker and Orlandi, 1995). In the present study, the peroxidase (PO), polyphenol oxidase (PPO) enzyme activity and total phenols content in the eggplant was studied in BLL resistant and susceptible eggplant genotypes. An increased activity of defense enzymes such as PO and PPO were observed in resistant genotypes of eggplant vis-à-vis susceptible ones. The literature survey showed that, defense enzymes like PO and PPO play a major role in the defense mechanism of plants (Shivalingaiah and Umesha 2016). PPO carries out oxidation of phenols to quinones. The level of resistance to pathogens varies with different plants and varieties of same plant (Bingham et al. 2009). Enhanced PO and PPO enzyme activities confer resistance against pathogen infection (Melo et al. 2006). Similarly, the total phenol content and antioxidative enzyme levels were compared in tomato leaf curl Palampur virus in infected and healthy pumpkin plants revealed that, there was substantial increase in the total phenol content and antioxidative enzymes (SOD, GPX, APX and CAT) levels in leaves (72%) and fruits (300%) in response to virus infection of pumpkin plants (Namrata et al. 2013). The increased activity of polyphenol oxidase has been well documented in many plants subjected to various biotic and abiotic stresses (Kumar et al. 2010). Phenols also play an important role in plant defense, host pathogen interaction and disease development in infected plants (Jabeen et al. 2009; Kumar et al. 2010). The increased quantity of phenolics in phytoplasma-infected eggplants may also contribute towards the resistance against infection.

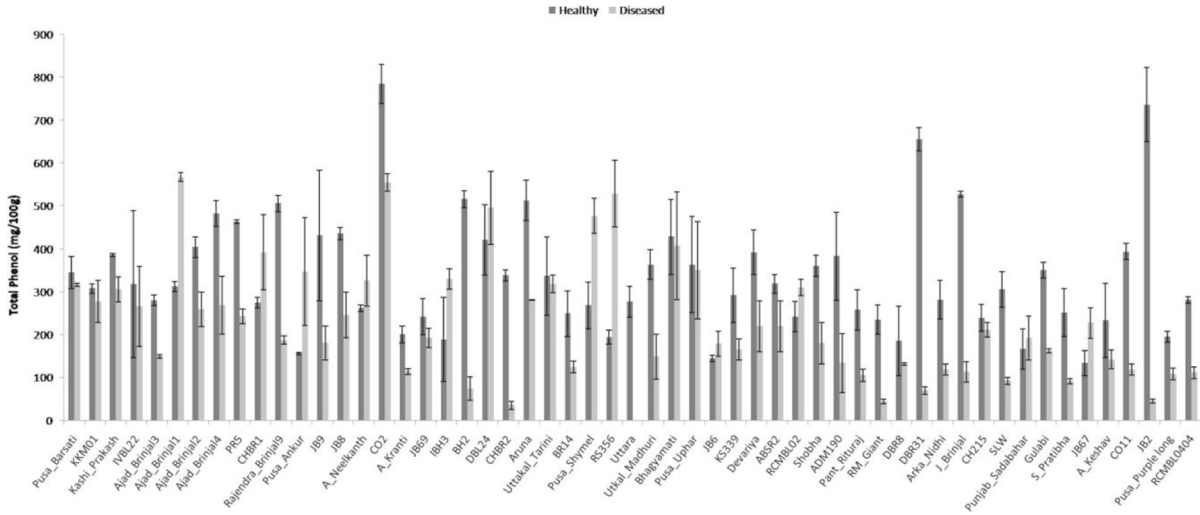
The major limitation in the production of eggplant is its susceptibility to different biotic stresses. Among them, little leaf, is an important disease of eggplant which is caused by phytoplasma and transmitted by leaf hoppers (Rao and Kumar 2017). At present, there is a lack of information about resistant sources and reliable screening techniques to identify resistance source in India for effective phytoplasma management. Also, there was often a confusion between resistance of the plant to the phytoplasma and resistance to the insect vector. In the present study, we have identified one immune variety (Uttara), 17 immune wild *Solanum* species, one resistant (Pusa Ankur) and 12 moderately resistant eggplant varieties against BLL phytoplasma. These resistant accessions identified in the present study could be used as pre-breeding materials to breed resistant cultivars and map BLL resistance genes in eggplant. We have also confirmed the presence of new tentative 16SrVI subgroup variant strains of phytoplasma associated with BLL disease in north eastern plain region (Varanasi, Mirzapur and Jaunpur) of India, which needs further investigation for finer classification and nomenclature of the



a **Peroxidase**



b ***Polyphenol oxidase***



c **Total phenol content**

◂**Fig. 8** (**a**) Peroxidase, (**b**) Polyphenoloxidase activity and (**c**) Phenol content in the 616 healthy and phytoplasma infested 55 eggplant varieties/lines

subgroups associated. Further studies on survey, detection, identification, and characterization of hitherto unknown phytoplasma strains are required to understand the clear picture of the diversity and geographical distribution phytoplasmas associated with BLL to develop a suitable and effective management strategies to manage the BLL incidence in India.

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Declarations

Compliance This article does not contain any studies with human or animal subjects performed by any of the authors.

Competing interests The authors declare that they have no conflict of interest in publishing this research work.

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