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Molecular Characterization of Phytoplasma Associated with Crops, Weeds and Forest Trees in Andhra Pradesh, India

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A B S T R A C T

Totally 32 weed hosts and crop plants were collected and the DNA was isolated from 32 weeds and crops showing phytoplasma disease symptoms and subjected to PCR amplification with phytoplasma specific primers R16F2n/R16R2. The result shows that R16F2n/R16R2 primer amplified 1250bp product in 19 weeds and other crop species. The notable contribution in the present study was identification of six new hosts for phytoplasma for the first time in the Andhra Pradesh. They are *Cleome gynendra, Solanum duclamara, Portulaca oleracea, Aerva lanata, Celosia argentea* and *Pergularia daemia* in Andhra Pradesh.

Introduction

Phytoplasma are wall less prokaryote bounded by a unit membrane and have cytoplasm, ribosome and nucleic acid and filamentous or polymorphic in shape, bodies ranging from 0.15-1.0 µm in diameter and 0.5-1.8 µm in length. Phytoplasma transmitted from plant to plant by leafhopper and plant hopper (Florence and Cameron, 1978). Phytoplasmas are generally present in phloem sieve tubes and in the salivary glands of insect vectors. While phytoplasmas are multiply in the phloem, little is known about its mechanism.

A common symptom resulting from phytoplasma infection is phyllody, a condition in which a plant produces leaf like structures instead of flowers, Leaf yellowing, one of the common symptoms associated with the presence of these organisms, phytoplasma infected plants may also show virescence, the development of green flowers due to the loss of pigment in the petal cells and many phytoplasma infected plants acquire a bushy or witches' broom appearance due to changes in their normal growth patterns. Different types of crops and weeds act as a collateral or alternate host for phytoplasma species needs

to be identified because they may provide reservoirs for disease organisms that infect crop species. In this study 32 crop species and weeds were collected from RARS farm, Tirupati and other places in Andhra Pradesh.

Materials and Methods

Phytoplasma infected weeds and other crop samples were collected from the fields of Regional Agricultural Research Station (RARS), Tirupati and different places of Chittoor and Anantapur district of Andhra Pradesh. The total DNA from phytoplasma infected plant samples and weed samples were extracted from leaves using CTAB method (Murray and Thomson, 1980). The total isolated DNA used as a template in first round PCR for amplification with P1/P7 primers (Deng and Hiruki. 1991; Smart *et al.*, 1996) followed by nested PCR was done using 2 µl of diluted standard PCR product with phytoplasma specific primers R16F2n/R16R2 (Gundersen and Lee.1996).

The first round PCR and nested PCR were carried out sequentially in a final volume of 25 µl reactions containing 2.5 µl of 10X PCR buffer, 2.0 µl (25 mM) MgCl2, 0.5 µl (10 mM each) dNTPs, 1.0 µl (10 µM) each primers, 0.2 µl Taq DNA polymerase (5 u/ µl), and 2 µl template DNA (50 ng/ µl). The DNA was amplified by an initial denaturation of 94°C for 4 min followed by 35 cycles of 94°C for 30 seconds denaturation, 56°C for 1 min primer annealing (55°C for 1 min for nested PCR), 72°C for 2 min primer extension and final extension at 72°C for 10 min.

The PCR products were analysed by electrophoresis in 1% (w/v) agarose gel. The DNA fragments in the gel were recorded using gel documentation system. The PCR amplified 1250bp DNA from gel slices was extracted using the ultra-clean gel kit as per the manufacturer's protocol.

Results and Discussion

Crop and non-crop species with phytoplasma species needs to be identified because they may provide reservoirs for disease organisms that infect crop species. In this study 32 crop species and weeds were collected from RARS farm, Tirupati and other places in Andhra Pradesh. The details of crops and weeds collected from different places, their symptoms and place of collection were given in Table 1.

Thirty two weeds and other crop species showing typical phytoplasma disease symptoms (Fig. 1-2) were collected from the fields of Regional Agricultural Research Station (RARS), Tirupati and Anantapur district. The DNA was isolated from 32 weeds and other crops showing phytoplasma symptoms by CTAB method as described earlier. The amount of DNA and purity of DNA (260/280 ratio) was measured in Nanodrop spectrophotometer. The concentration of DNA ranged from 81.17 ng/µl to 4094 ng/µl and 260/280 ratios ranged between 1.30 to 2.16. The extracted DNA gave good amplification of 16S rDNA gene in PCR when used at concentration of 100 ng/µl.

The isolated DNA from 32 weeds and other crop species was amplified in nested PCR with P1/P7 and R16F2n/R16R2 primers and obtained 1800 bp and 1250 bp product respectively. About 32 weeds and other crop species belonging to various families were tested by nested PCR using above two primers and results were furnished in Table 2.

PCR amplification with R16F2n/R16R2 primer resulted 1250 bp product in 19 weeds and other crop species. They are *Cleome gynandra, Solanum duclamara, Portulaca oleracea, Pergularia daemia, Aerva lanata, Celosia argentea, Parthenium hysterophorus, Tephrosia purpurea, Solanum melongena,*

Solanum lycopersicum, Sesamum indicum, Cleome viscosa, Croton bonplandianum, Saccharum officinarum, Citrullus lanatus, Capsicum annuum, Borreria hispida, Cassia auriculata, Arachis hypogaea.

Several workers amplified phytoplasmas rDNA sequence in PCR with universal primers using total DNA isolated from phytoplasma infected *Portulaca olaracea* (Savita *et al.*,2014), *Pergularia daemia* (Rangaswamy *et al.,* 2011), *Celosia argentea* (Samuitiene and Navalinskiene., 2006), *Parthenium hysterophorus* (Raj *et al.,* 2008), *Tephrosia purpurea* (Yadav *et al.,* 2014), *Solanum melongena* (Kumar *et al.,* 2012), *Solanum lycopersicum* (Singh *et al.,* 2012) *Sesamum indicum* (Khan *et al.,* 2007), *Cleome viscosa* (Li *et al.,* 2014), *Saccharum officinarum* (Guar *et al.,* 2008), *Capsicum annuum* (Khan *et al.,* 2005) and *Arachis hypogaea* (Li *et al.,* 2014).

To determine the 16S rRNA group of phytoplasma associated with certain selected weed species, the 1250 bp rDNA gene from *Cleome gynendra, Solanum duclamara, Portulaca oleracea, Aerva lanata, Celosia argentea* and *Pergularia daemia* were gel eluted from agarose gel and cloned into pTZ57R/T vector and transformed into competent *E. coli* cells (JM109) as described earlier.

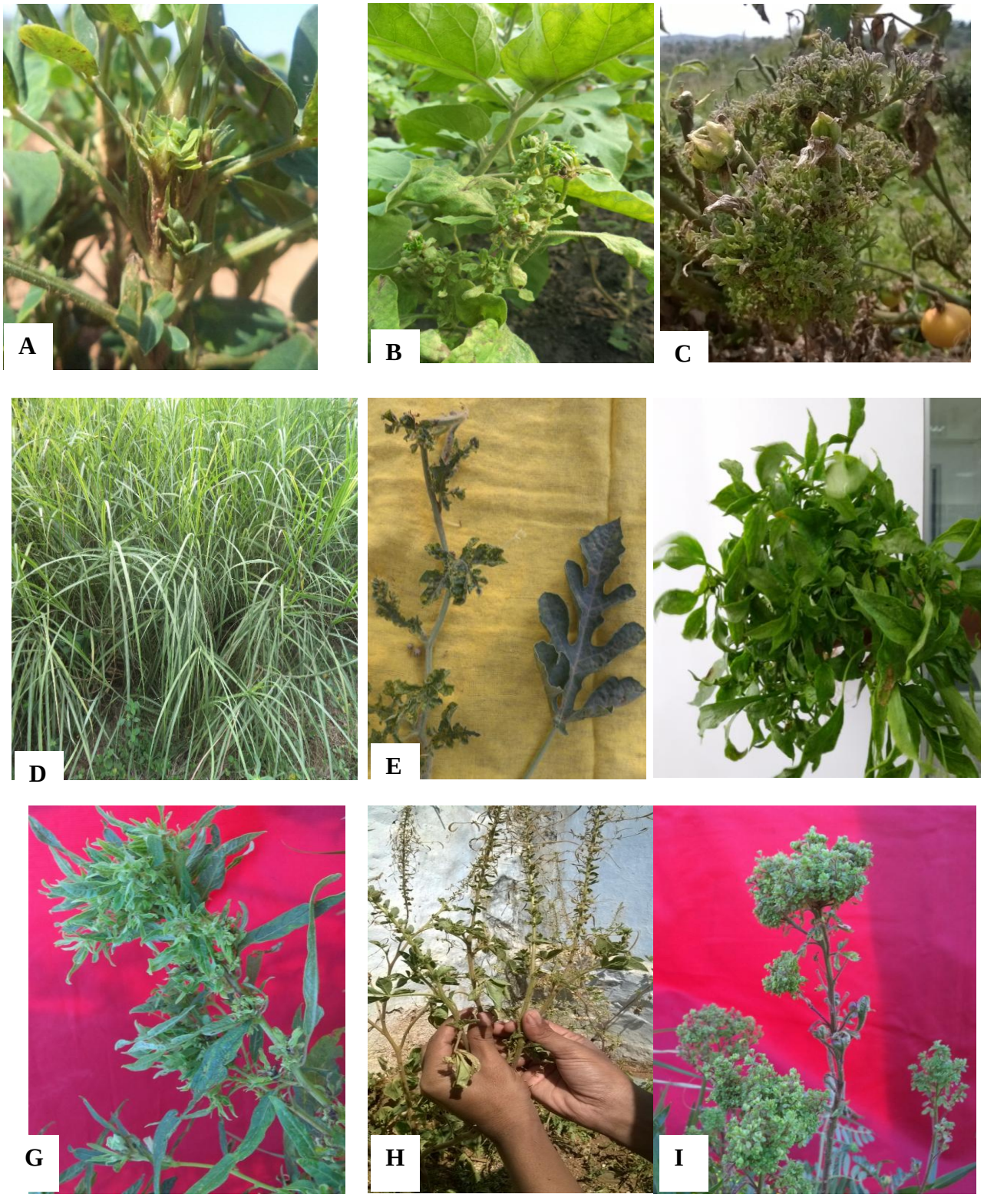
The recombinant clones containing 1250 bp 16S rDNA gene were identified by Colony PCR with R16F2n/R16R2 primers in above 6 weed species (Fig. 3a). The correct size of the insert was further confirmed by restriction digestion with *PstI* and *EcoRI* flanking cloning site in the vector. Restriction digestion with *PstI* and *EcoRI* gave two products of approximately 530 bp and 750 bp instead of single product of 1250 bp due to internal restriction site in rDNA sequence in above 6 weed species (Fig. 3b). Several workers cloned the rDNA gene of phytoplasmas infecting *Celosia argentea* (Samuitiene and Navalinskiene, 2006), *Portuluca oleracea* (Savita *et al.,* 2014) using universal primer R16F2n/R16R2 in nested PCR.

The clones having correct size of 1250 bp were sequenced using M13 primers and data was assembled using BioEdit software. The partial rDNA sequences of 6 weed species and brinjal infected with phytoplasma were aligned with 26 reference phytoplasma species downloaded from GenBank using BioEdit software to confirm the identity of phytoplasma species associated with 6 weed species and brinjal under study. Multiple sequence analysis of rDNA with reference phytoplasma species belonging to various rDNA groups and sub groups was carried out to confirm the identity. The similarity matrix for phytoplasma infecting weed species and brinjal with 26 other reference phytoplasma isolates were generated using BioEdit software.

The partial rDNA sequence analysis of phytoplasma infecting *Cleome gynendra, Pergularia daemia, Portuluca oleracea and Parthenium hysterophorus* share 99.4% identity with '*Ca*. P. australasiae' 16S rDNA II-D and papaya mosaic phytoplasma (Y10096). Phytoplasmas infecting *Solanum duclamara* and *Celosia argentea* share 100% rDNA sequence identity with '*Ca*. P. australasiae' 16S rDNA II-D and papaya mosaic phytoplasma (Y10096).

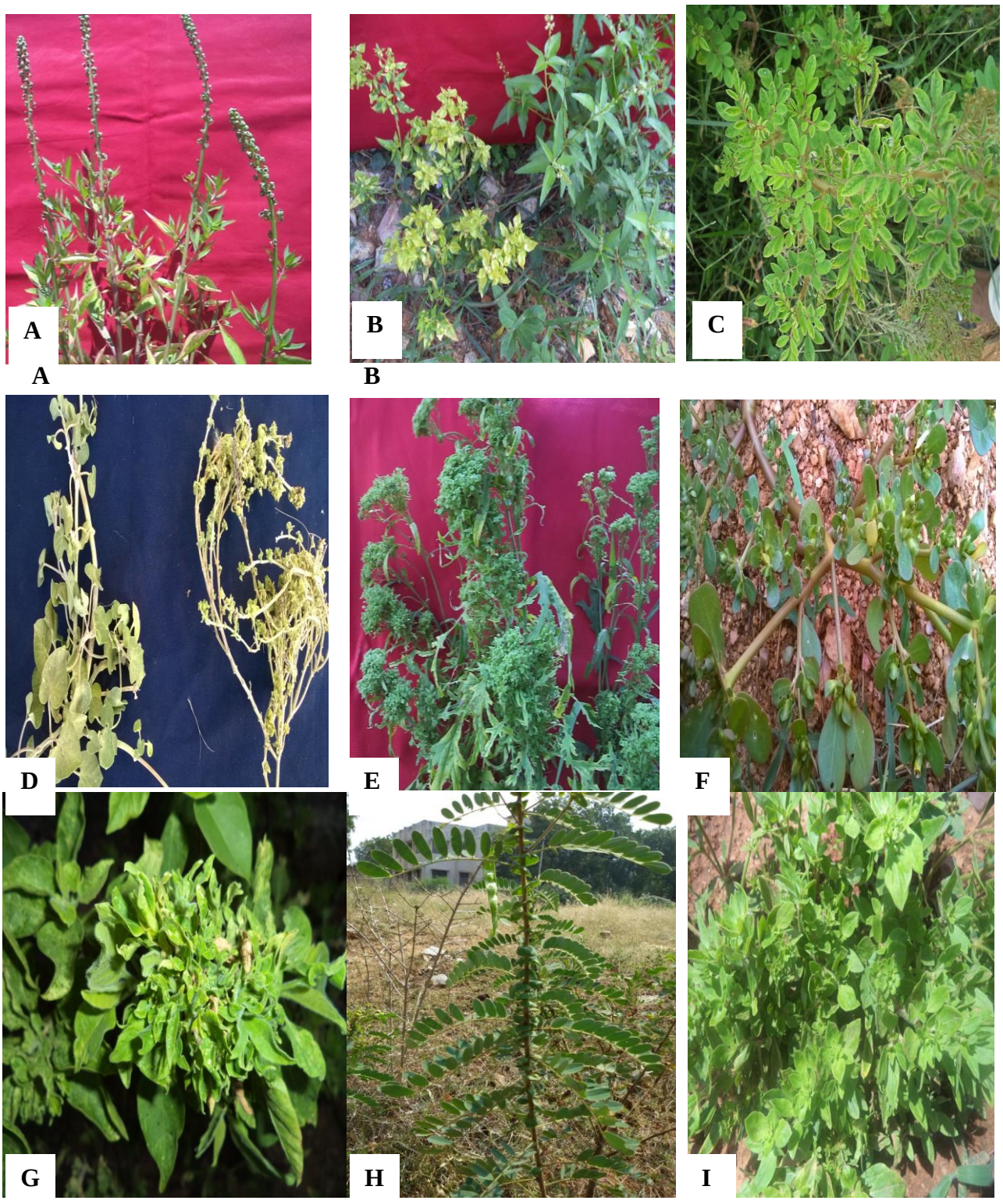
Phytoplasma associated with *Tephrosia purpurea* share 98% rDNA sequence identity with '*Ca. P. australasiae'* 16S rDNA II-D and papaya mosaic phytoplasma (Y10096). *Aerva lanata* infected with phytoplasma share maximum rDNA sequence identity of 95% with greengram phyllody (AB690305) from Myanmar and < 94% with several members of 16S rDNA II group.

Fig.1 Crops showing typical phytoplasma disease symptoms



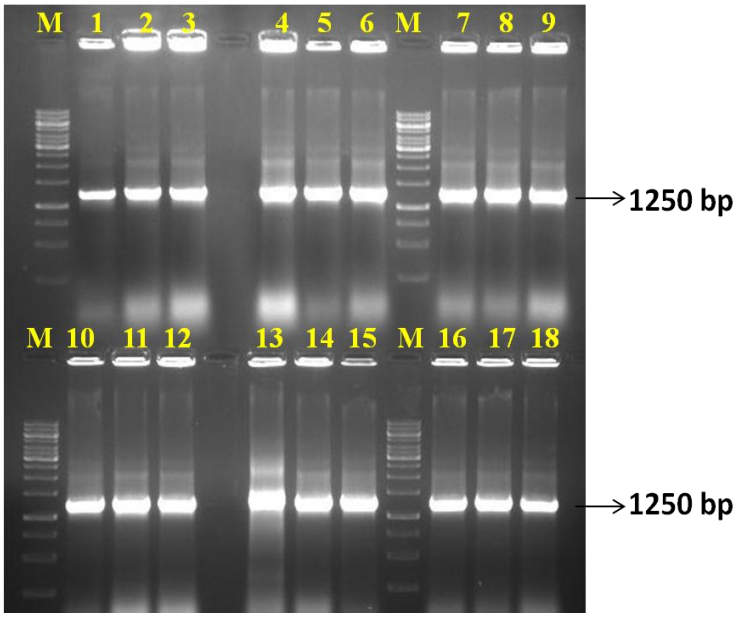
(A) *Arachis hypogaea* (B) *Solanum melongena* (C) *Solanum lycopersicum* (D) *Saccharam officinarum* (E) *Citrullus lanatus* (F) *Capsicum annuum* (G) *Sesamum indicum* (H) *Cleome gynendra* (I) *Cleome viscose*

Fig.2 Crops showing typical phytoplasma disease symptoms



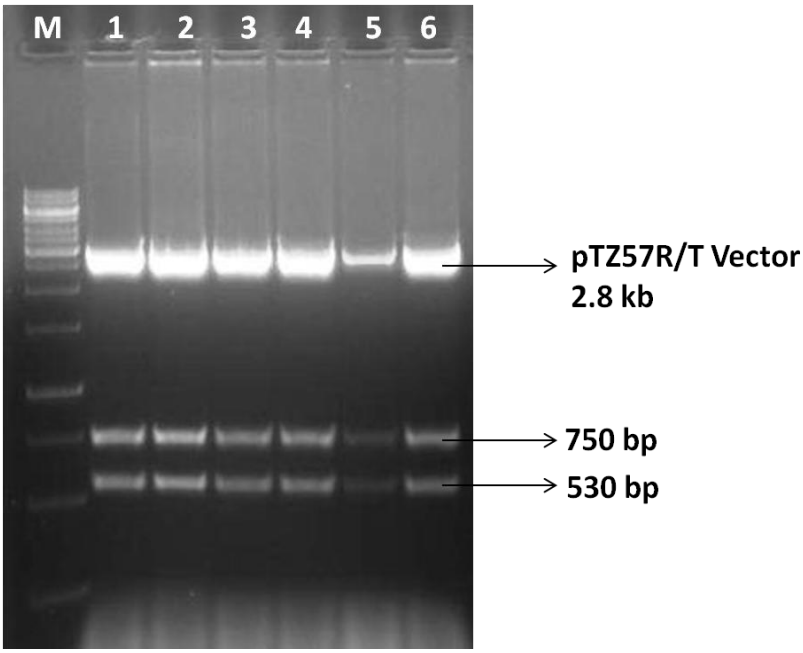
(A) *Celosia argentea* (B) *Croton bonplandianum* (C) *Tephrosia purpurea* (D) *Pergularia daemia* (E) *Parthenium hysterophorus* (F) *Portulaca oleracea* (G) *Solanum duclamara* (H) *Cassia auriculata* (I) *Borreria hispida*

Fig.3 (a) Confirmation of 16S rDNA clones of phytoplasma by Colony PCR using with R16 F2n/R16R2 primers



Lanes: M-1 Kb DNA ladder, Lanes: 1, 2, 3- *Cleome gynendra*, Lanes: 4, 5, 6- *Solanum duclamara*, Lanes: 7, 8, 9- *Portulaca olaracea*, Lanes: 10, 11, 12- *Aerva lanata*, Lanes: 13, 14, 15- *Celosia argentea*, Lanes: 16, 17, 18- *Pargularia daemia*.

Fig.3 (b) Confirmation of 16S rDNA recombinant clones by restriction digestion with *Pst1* and *Eco R1* in plants



Lanes: M-1 Kb DNA ladder, Lanes: 1-*Cleome gynendra*, Lanes: 2-*Solanum duclamara*, Lanes: 3-*Portulaca olaracea*, Lanes: *4-Aerva lanata*, Lanes: *5-Celosia argentea*, Lanes: *6-Pargularia daemia.*

Fig.4 Phylogenetic tree sharing the relationship of present isolates under study (marked) with other references isolates from the database

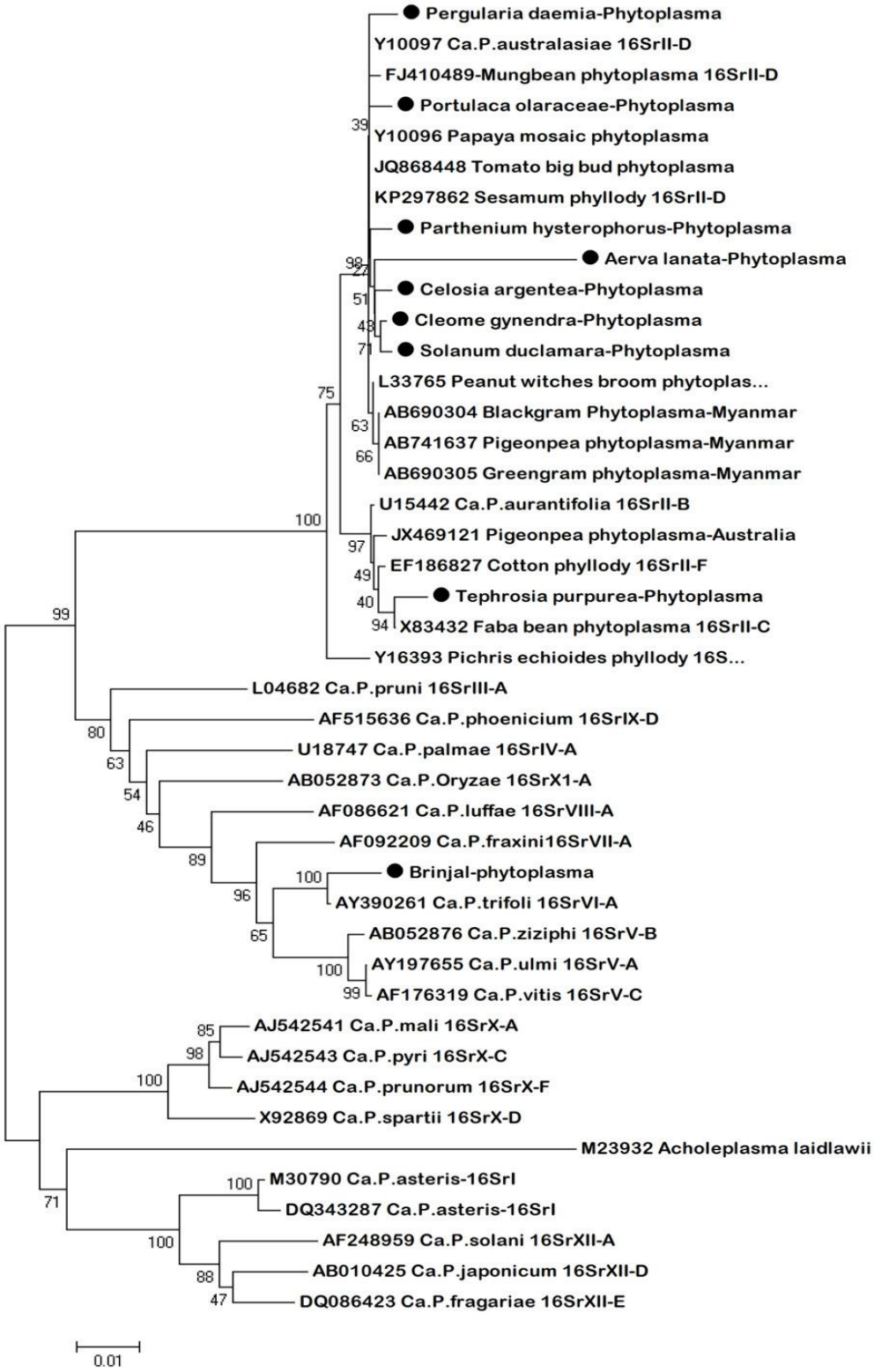


Table.1 List of economically important crop plants and weed hosts collected for detection of phytoplasmas, their families, symptoms and location

S. No	Name of the crops	Family	Symptoms	Place of collection
01	*Citrullus lanatus*	Cucurbitaceae	Little leaves yellowing	Tirupati
02	*Arachis hypogaea*	Fabaceae	Phyllody	Tirupati
03	*Capsicum annuum*	Solanaceae	Little leaves	Chittoor
04	*Saccharam officinarum*	Poaceae	Narrow leaves, stunted growth	Chandragiri
05	*Solanum melongena*	Solanaceae	Little leaves	Chandragiri
06	*Solanum lycopersicum*	Solanaceae	Little leaves	Dharmavaram
07	*Sesamum indicum*	Pedaliaceae	Phyllody	Tirupati
08	*Cynodon dactylon*	Poaceae	Leaf yellowing	Dharmavaram
09	*Parthenium hysterophorus*	Asteraceae	Phyllody, Virescence	Dharmavaram
10	*Cleome gynandra*	Brassicaseae	Phyllody	Dharmavaram
11	*Solanum duclamara*	Solanaceae	Little leaves	Dharmavaram
12	*Aerva lanata*	Amaranthaceae	Little leaves	Dharmavaram
13	*Portulaca oleracea*	Portulacaceae	Stunted growth, little leaves	Dharmavaram
14	*Pergularia daemia*	*Asclepiadaceae*	Little leaves	Dharmavaram
15	*Celosia argentea*	Amaranthaceae	Phyllody, Virescence	Dharmavaram
16	*Cleome viscosa*	Brassicaseae	Phyllody	Tirupati
17	*Croton bonplandianum*	Euphorbiaceae	Leaf yellowing, little leaves	Tirupati
18	*Borreria hispida*	Rubiaceae	Little leaves	Dharmavaram
19	*Acanthospermum hispidum*	Asteraceae	Leaf yellowing, little leaves	Tirupati
20	*Acalypha indica*	Euphorbiaceae	Leaf yellowing	Tirupati
21	*Tephrosia purpurea*	Fabaceae	Leaf yellowing, little leaves	Tirupati
22	*Ziziphus jujube*	Rhamnaceae	Leaf yellowing, little leaves	Dharmavaram
23	*Cassia auriculata*	Fabaceae	Little leaves	Tirupati
24	*Alternanthera pungens*	Amaranthaceae	Leaf yellowing, little leaves	Dharmavaram
25	*Achyranthes aspera*	Amaranthaceae	Stunted growth, little leaves	Dharmavaram
26	*Trichodesma indicum*	Boraginaceae	Little leaves	Dharmavaram
27	*Commelina benghalensis*	Commelinaceae	Little leaves	Tirupati
28	*Eclipta prostrata*	Asteraceae	Little leaves	Tirupati
29	*Phyllanthus madaraspatensis*	Phyllanthaceae	Little leaves	Dharmavaram
30	*Tribulus terrestris*	Zygophyllaceae	Leaf yellowing	Dharmavaram
31	*Amaranthus viridis*	Amaranthaceae	Stunted growth, little leaves	Dharmavaram
32	*Malvastrum coromandelianum*	Malvaceae	Leaf yellowing	Dharmavaram

Table.2 Detection of phytoplasma in crop plants and weed hosts by nested PCR with primers P1/P7 and R16F2n/R16R2 primers

S. No	Name of the other crop plants and weed hosts	primers	
		P1/P7	R16F2n/R16R2
01	*Citrullus lanatus*	+	+
02	*Arachis hypogaea*	+	+
03	*Capsicum annuum*	+	+
04	*Saccharam officinarum*	+	+
05	*Solanum melongena*	+	+
06	*Solanum lycopersicum*	+	+
07	*Sesamum indicum*	+	+
08	*Cynodon dactylon*	+	+
09	*Parthenium hysterophorus*	+	+
10	*Cleome gynendra*	+	+
11	*Solanum duclamara*	+	+
12	*Aerva lanata*	+	+
13	*Portulaca olaracea*	+	+
14	*Pergularia daemia*	+	+
15	*Celosia argentea*	+	+
16	*Cleome viscose*	+	+
17	*Croton bonplandianum*	+	+
18	*Borreria hispida*	+	+
19	*Acanthospermum hispidum*	-	-
20	*Acalypha indica*	-	-
21	*Tephrosia purpurea*	+	+
22	*Ziziphus jujube*	-	-
23	*Cassia auriculata*	+	+
24	*Alternanthera pungens*	-	-
25	*Achyranthes aspera*	-	-
26	*Trichodesma indicum*	-	-
27	*Commelina benghalensis*	-	-
28	*Eclipta prostrata*	-	-
29	*Phyllanthus madaraspatensis*	-	-
30	*Tribulus terrestris*	-	-
31	*Amaranthus viridis*	-	-
32	*Malvastrum coromandelianum*	-	-

The interesting results of present work is that phytoplasma infecting brinjal share maximum identity of 99% at rDNA sequence level with '*Ca*.P.trifolii' 16S rDNA VI-A group, 97% with '*Ca*.P.fraxini' 16S rDNA VIII-A and < 95% with all other reference isolates under analysis.

Phylogenetic and molecular evolutionary analysis were conducted using rDNA

sequences of phytoplasmas infecting 7 weed species and brinjal with 26 reference phytoplasma isolates by neighbour- joining method using Mega 4.0 software with *Acholeplasma laidlawii* as out group.

The data was bootstrapped 500 times and percentage values are given at the nodes of the tree (Fig. 4). The phytoplasma infecting *Pergularia daemia, Portuluca oleracea, Parthenium hysterophorus, Celosia argentea*, *Cleome gynendra* and *Solanum duclamara* formed unique cluster with '*Ca*. P. australasiae' 16S rDNA II-D and papaya mosaic phytoplasma (Y10096), tomato big bud phytoplasma (JQ868448), and sesame phyllody 16S rDNA II-D group. From the above results it is clear that phytoplasma infecting above six crops in Andhra Pradesh belongs to 16S rDNA II-D group.

The notable contribution in the present study was identification of six new hosts for phytoplasma for the first time in the India. They are *Cleome gynendra, Solanum duclamara, Portulaca olaracea, Aerva lanata, Celosia argentea* and *Pergularia daemia* in Andhra Pradesh.

In the present study association of phytoplasma was observed with *Parthenium hysterophorus.* Raj *et al.,* (2008) also reported phytoplasma in *Parthenium hysterophorus* in Bahraich and Gorakhpur districts of Uttar Pradesh, India.

Yadav *et al.,* (2014) reported '*Candidatus* Phytoplasma aurantifolia' associated with witches′ broom disease of *Tephrosia purpurea* in India. The present results are in agreement with above report.

Kumar *et al.,* (2012) reported little leaf disease of brinjal caused by '*Candidatus* Phytoplasma asteris' in the field of Bihar, India. The present study also confirmed little leaf disease of brinjal in Andhra Pradesh, but causal agent is '*Candidatus* Phytoplasma trifolii'. Samuitiene and Navalinskiene. (2006) reported phytoplasma disease on *Celosia argentea* in Lithuania.

Amplification by nested PCR and RFLP analysis shows that the plants were infected by the phytoplasma belonging to the 16S rI-M subgroup.

The present results also confirmed phytoplasma disease on *Celosia argentea* but causal agent comes under 16S rII group.

The objective of this study was to identify the weed host of phytoplasma infecting pulses in Andhra Pradesh. Because weeds are reservoirs of phytoplasma that infect crop and they play an important part in the emergence of epidemics affecting crops. Weeds may serve as reservoirs of phytoplasmas during the non-cropping season. In this study we have identified several weeds as host for phytoplasmas. Transmission studies from weeds to pulses and vice versa is also required to get information on exact role of them in spreading disease under field conditions.

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