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Candidatus Phytoplasma Australasia Associated with Alfalfa Witches' Broom: Symptomatology, Quantitative Loss, Qualitative Loss and Molecular Characterization

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ABSTRACT

Background: Alfalfa, *Medicago sativa* L. is the most important and widely grown leguminous fodder crop in temperate and tropical regions of the world. The production of alfalfa crop is limited by several biotic stresses, among which witches' broom disease (AWB) was reported to cause significant economic losses.

Methods: The phytoplasma infected alfalfa plants were collected from a central research farm, ICAR-IGFRI, Jhansi, U.P. Qualitative parameters such as crude protein content, acid detergent fibre and neutral detergent fibre were estimated in diseased and healthy plants. Phytoplasma universal primer (P1/P7) and nested primer (R16mF2/R16mR1) were used for the molecular characterization of AWB infected plants and phytoplasma infected, *Parthenium hysterophorus*.

Result: The incidence of AWB disease ranged from 8-10%. The quantitative analysis of disease plants showed reduced plant height (-35%), fresh weight (-46.89%) and dry weight (-50.08%) compared to healthy plants. The diseased plant recorded low crude protein content (-21.38%) and higher dry matter content (+0.68%), acid detergent fibre (+33.72%) and neutral detergent fibre (+13.06%). The association of phytoplasma in diseased alfalfa and parthenium samples was confirmed by using P1/P7 and R16mF2/R16mR1 primer pair and Blastn analysis shared 99.6-100% similarity with '*Candidatus* Phytoplasma australasia' belongs to the 16Sr group II-D.

Key words: Alfalfa, *Ca*. Phytoplasma australasia, Crude protein, Witches' broom.

INTRODUCTION

The alfalfa (*Medicago sativa* L.), a leguminous plant vernacularly known as lucerne, is the most important and widely cultivated fodder crop in temperate and tropical regions worldwide (Khan *et al.* 2001). The crop is often referred to as the queen of fodder crops because of its higher nutritional quality (crude protein (≈16-25%) with higher digestible vitamins and minerals), biomass, seed yield, persistency under dry conditions with its ability to fix atmosphere nitrogen (Ruckle *et al.* 2017; Kumar *et al.* 2021). Several biotic stresses limit the production of alfalfa crop. Among which, fungal pathogens like *Verticillium*, *Phytophthora*, *Aphanomyces* and phytoplasma are the most important pathogens responsible for significant yield loss in alfalfa (Alhudaib, 2009). However, little leaf or witches' broom disease associated with phytoplasma is reported to cause significant economic losses in several countries (Gopurenko *et al.* 2016).

Alfalfa witches' broom (AWB) or lucerne little leaf disease caused by seven distinct phytoplasma groups (16Sr I, II, VI, VII, IX, XII) has been observed worldwide (Gopurenko *et al.* 2016). The important phytoplasma group reported on alfalfa includes '*Candidatus* Phytoplasma asteris' from Bolivia (Jones *et al.* 2005); '*Ca*. Phytoplasma aurantifolia' from Iraq (Al-Kuwaiti *et al.* 2019); '*Ca*. Phytoplasma australasia' fromTurkey (Ayvaci *et al.* 2020); '*Ca*. Phytoplasma trifolii' reported from Canada (Khadhair *et al.* 1997); '*Ca*. Phytoplasma fraxini' from Argentina (Conci *et al.* 2005); '*Ca*. Phytoplasma phoenicium' from Italy (Marcone *et al.* 1997) and '*Ca*. Phytoplasma solani' from Serbia (Starovic *et al.* 2012).

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In India, the little leaf disease of alfalfa was first identified in Karnataka and documented the association of phytoplasma based on the symptoms (Suryanarayana *et al.* 1996). However, the study did not establish whether the symptoms observed were due to phytoplasma infection. Further, the effect of phytoplasma infection on leaf quality was substantial. Hence, the effort was made to quantify the qualitative and quantitative loss caused by AWB and to confirm the phytoplasma association, molecular characterization was carried out in the present study.

MATERIALS AND METHODS

Sample collection and disease incidence

The infected leaf samples showing witches' broom and little leaf symptoms were collected from the alfalfa field located in central research farm and technology demonstration block, ICAR-Indian Grassland and Fodder Research Institute, Jhansi (IGFRI-1-25° 5′ N 78° 55′ E and IGFRI-2-25° 30′ N 78° 3′ E), Uttar Pradesh, India during *Kharif* (July-December 2020). The per cent disease incidence was recorded during the sample collection. In addition, the phytoplasma infected weed samples *i.e*., *Parthenium hysterophorus* adjacent to the alfalfa fields collected to detect the presence of phytoplasma. Leaf samples from asymptomatic healthy plants were used as a negative control.

Estimation of quantitative and qualitative loss in the infected plant samples

To determine the quantitative loss of biomass in the infected alfalfa, whole infected and healthy alfalfa plants were collected and recorded plant height (n=25), leaf size (n=25), fresh weight and dry weight (n=10). The important fodder qualitative parameters such as crude protein content (% CP: Van Soest *et al.* 1991), dry matter content (% DM: AOAC, 1990), acid detergent fibre (% ADF: Van Soest *et al.* 1991) and neutral detergent fibre (% NDF: Van Soest *et al.* 1991) of infected and healthy plant samples were analyzed. The loss or gain in biomass and nutrients of alfalfa owing to disease was estimated by comparing it with healthy plants.

PCR amplification and sequencing

The total genomic DNA was extracted from leaves of five symptomatic and one asymptomatic alfalfa and parthenium plant using the CTAB method described by Doyle and Doyle (1990). The DNA from a known phytoplasma infected sample (sesamum) and DNA from asymptomatic plants were used as a positive and negative control, respectively. The detection of phytoplasma was based on PCR amplification of 1.8 and 1.2 kb fragments by using universal primer pair P1/P7 (Deng and Hiruki, 1991; Balol *et al.* 2021) and nested PCR was conducted using universal primers R16F2n/R16R2, respectively (Gundersen and Lee, 1996). The polymerase chain reaction (PCR) was carried out in a 25 µl reaction mixture and amplification was performed in a Thermal cycler as described by Venkataravanappa *et al.* (2017). Amplified PCR products were separated electrophoretically on 1.2% agarose gel using a capillary electrophoresis unit. Four confirmed positive (including one sample of parthenium) samples containing phytoplasma DNA were sequenced.

Phylogeny and virtual RFLP analysis

The nucleotide sequences of the 16Sr RNA gene of alfalfa and parthenium phytoplasma were edited, aligned and assembled using CLC Genomics Workbench 12.0 and sequences were deposited in NCBI GenBank. The reliable reference sequences were retrieved from NCBI GenBank and aligned using the ClustalW program available in MEGA X software. A phylogenetic tree was constructed by MEGA X software using Neighbour-Joining [NJ] method adopting 1000 bootstrap replications. All characters were run unordered and of equal weight and gaps were treated as missing data. Further, R16F2n/R16R2 sequence fragments were subjected to virtual RFLP analysis with the iPhyClassifier software (Zhao *et al.* 2009).

RESULTS AND DISCUSSION

Disease symptoms and incidence

The distinctive symptoms incited by phytoplasma include stunted growth, witches' brooms (Fig 1), leaf yellowing followed by proliferation of shoots with short intermodal length and severe reduction in leaf size with short petioles (Fig 2A). Later floral parts were converted into leaf-like structures leads to sterility in the plants (Fig 2B). Jones *et al.* (2005) observed witches' broom and little leaf symptoms in phytoplasma infected alfalfa plants at Bolivia. Numerous genetically distant 16Sr RNA phytoplasma groups have been reported as the causal agents of diseases leading to different symptoms in alfalfa, including "yellows" and "witches' broom."

The average disease incidence ranged from 8-10% in the infected fields. However, the disease incidence reported by Hosseini *et al.* (2015) was substantially greater than the current study, which revealed disease incidence ranging from 17.8 to 70% in different provinces of Iran. The parthenium plants infected with phytoplasma in the vicinity of an alfalfa field had identical symptoms such as short proliferation with short internodal followed by floral deformation (Fig 2C). Yadav *et al.* (2015) reported the phytoplasma infecting *Parthenium hysterophorus* shares 99.3% rDNA sequence similarity with '*Ca*. P. aurantifolia' 16S rDNA II-D.

Quantitative and qualitative losses due to AWB disease

Quantitative parameters includes plant height, fresh weight, dry weight and leaf size were measured. Phytoplasma infected alfalfa plant recorded lower plant height (38.60 cm ≈ -35%), fresh weight (40.36 g ≈-46.89%), dry weight (9.07g ≈ -50.08%) and mean leaf size (0.74 × 0.39 cm) as compared to healthy plants (59.80 cm, 76.00 g, 18.17 g and 2.29 × 1.1 cm, respectively). Qualitative parameters *viz*., crude protein content in the infected plant was reduced by 21.38%, but

DM, ADF and NDF were increased by 0.68%, 33.72% and 13.06%, respectively (Table 1). Similar observations were made by Saxena *et al.* (2002), who reported that alfalfa weevil reduces the green matter yield up to 1.7 t/ha and crude protein content by 0.3 t/ha. The crude protein and hemicelluloses content was reduced by 16.43% and 40.00%, but ADF and NDF were increased by 25% in powdery mildew infected cowpea.

Fig 1: Alfalfa plant infected by phytoplasma: Healthy plant (Left), Alfalfa showing witches broom symptoms (Right).

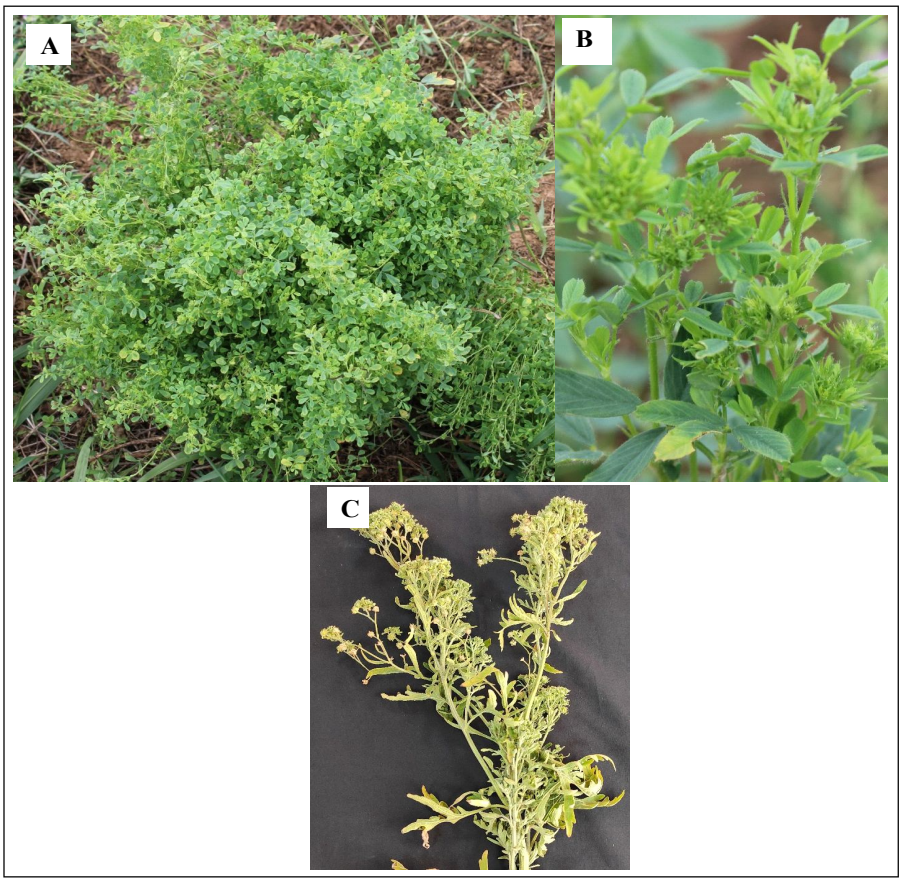

Fig 2: Phytoplasma infected plant showing little leaf symptoms in alfalfa (A), floral proliferation in alfalfa (B), reduced leaf size and floral deformation in *Parthenium hysterophorus* (C).

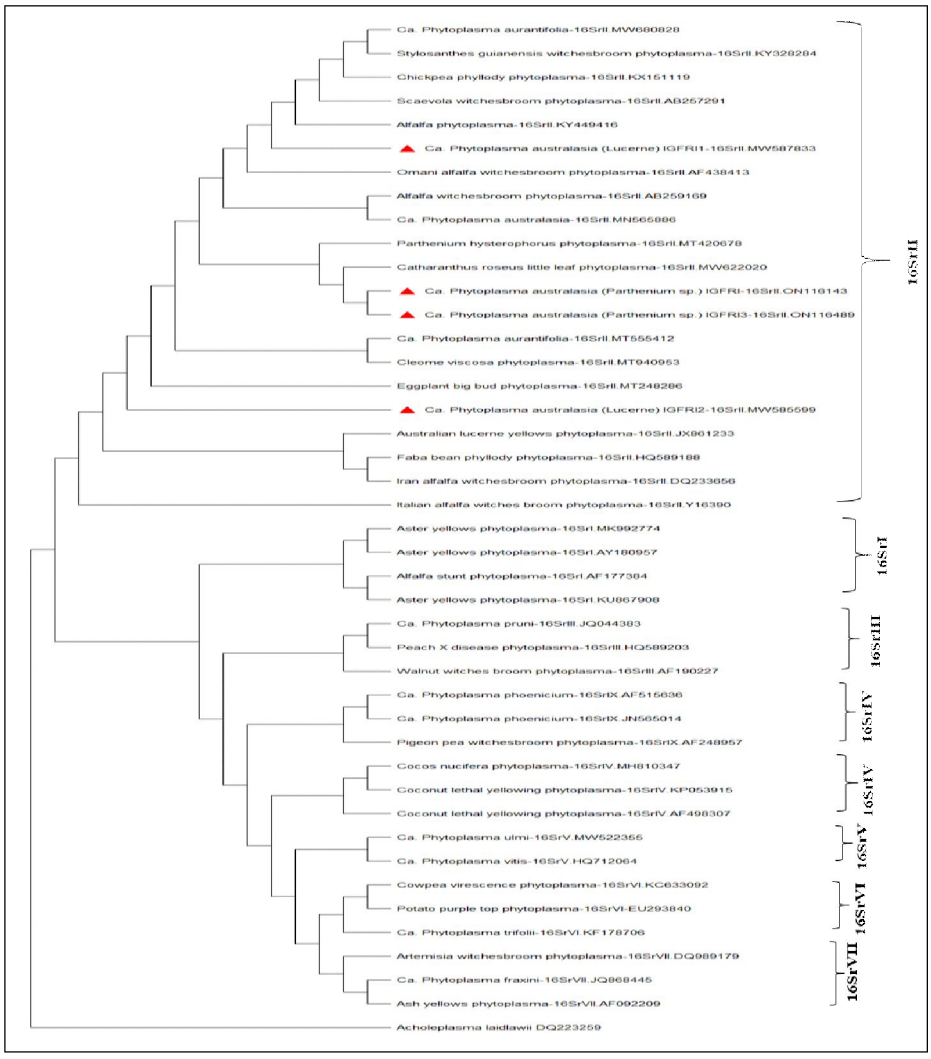

Fig 3: Phylogenetic tree constructed based on nucleotide sequences of 16S rRNA gene of alfalfa phytoplasma with other phytoplasma retrieved from NCBI database using Neighbor-joining algorithm. Horizontal distances are proportional to sequence distances, vertical distances are arbitrary. The trees are unrooted. A bootstrap analysis with 1000 replicates was performed and bootstrap percentage values more than 50 are numbered along the branches.

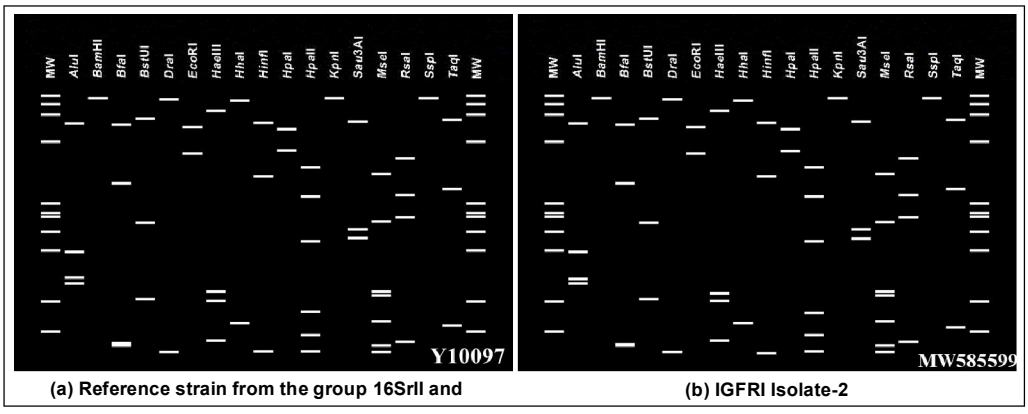

Fig 4: *In-silico* digestion using iPhyClassifier for the R16Fn2/R16nR2 fragment of the 16S rRNA genesequence of the alfalfa phytoplasma (IGFRI-2) (GenBank accession number MW585599) in comparison with reference strain (Y10097) 16SrII-D. MW used as a marker.

Table 1: Quantitative and qualitative analysis of phytoplasma infected and healthy alfalfa plants.

Parameters	Infected plants	Healthy plants	Per cent gain or loss as compared to healthy plant
Plant height (cm)*	38.60	59.80	-35.45
Fresh weight (g)*	40.36	76.00	-46.89
Dry weight (g)*	9.07	18.17	-50.08
Leaf size (L×B) (cm)**	0.74×0.39	2.29×1.1	-
Crude protein (%)	12.98	16.51	-21.38
Dry matter (%)	92.15	92.78	+0.68
Acid detergent fiber (%)	40.58	34.89	+33.72
Neutral detergent fiber (%)	52.62	46.54	+13.06

*=Mean of 25 plants, **=Mean of 25 leaves.

Table 2: List of gene sequences of Phytoplasma isolates used for comparison of AWB disease.

Name	Phytoplasma group	Location	GenBank accession numbers
Aster yellows phytoplasma	16SrI	Hungary	MK992774
Aster yellows phytoplasma	16SrI	USA	AY180957
Aster yellows phytoplasma	16SrI	USA	KU867908
Alfalfa stunt phytoplasma	16SrI	USA	AF177384
Scaevola witches' broom phytoplasma	16SrII	Oman	AB257291
Alfalfa phytoplasma	16SrII	Sudan	KY449416
Alfalfa witches' broom phytoplasma	16SrII	Alfalfa	AB259169
Candidatus phytoplasma aurantifolia	16SrII	Taiwan	MW680828
Stylosanthes guianensis witches' broom phytoplasma	16SrII	China	KY328284
Italian alfalfa witches' broom phytoplasma	16SrII	Italy	Y16390
Ca. Phytoplasma australasia	16SrII	Iran	MN565886
Australian lucerne yellows phytoplasma	16SrII	Australia	JX861233
Faba bean phyllody phytoplasma	16SrII	Sudan	HQ589188
Iran alfalfa witches' broom phytoplasma	16SrII	Iran	DQ233656
Omani alfalfa witches' broom phytoplasma	16SrII	Oman	AF438413
Chickpea phyllody phytoplasma	16SrII	India	KX151119
Ca. Phytoplasma aurantifolia	16SrII	India	MT555412
Parthenium hysterophorus phytoplasma	16SrII	India	MT420678
Eggplant big bud phytoplasma	16SrII	India	MT248286
Catharanthus roseus little leaf phytoplasma	16SrII	India	MW622020
Cleome viscosa' phytoplasma	16SrII	India	MT940953
Ca. Phytoplasma pruni	16SrIII	USA	JQ044383
Walnut witches' broom phytoplasma	16SrIII	USA	AF190227
Peach X-disease phytoplasma	16SrIII	Germany	HQ589203
Coconut lethal yellowing phytoplasma	16SrIV	Jamaica	AF498307
Cocos nucifera phytoplasma	16SrIV	Mexico	MH810347
Coconut lethal yellowing phytoplasma	16SrIV	France	KP053915
Ca. Phytoplasma ulmi	16SrV	South Korea	MW522355
Ca. Phytoplasma vitis	16SrV	Croatia	HQ712064
Ca. Phytoplasma trifolii	16SrVI	USA	KF178706
Cowpea virescence phytoplasma	16SrVI	Iran	KC633092
Potato purple top phytoplasma YN-6	16SrVI	China	EU293840
Ca. Phytoplasma fraxini	16SrVII	Denmark	JQ868445
Ash yellows phytoplasma	16SrVII	USA	AF092209
Artemisia witches' broom phytoplasma	16SrVII	Argentina	DQ989179
Ca. Phytoplasma phoenicium	16SrIX	France	AF515636
Ca. Phytoplasma phoenicium	16SrIX	Iran	JN565014
Pigeon pea witches' broom phytoplasma	16SrIX	USA	AF248957
Ca. Phytoplasma australasia IGFRI-1	16SrII	India	MW587833
Ca. Phytoplasma australasia IGFRI-2	16SrII	India	MW585599
Ca. Phytoplasma australasia IGFRI-3	16SrII	India	ON116489
Ca. Phytoplasma australasia IGFRI	16SrII	India	ON116143

Molecular detection of AWB disease

Five symptomatic alfalfa samples and one parthenium sample suspected for phytoplasma infection yielded expected amplicon sizes of 1.8 kb and 1.2 kb for P1/P7 and R16F2n/R16R2 primer pair, respectively, which confirms the presence of phytoplasma in the infected samples. The 16S rRNA gene PCR products of alfalfa (MW 587833 and MW 585599) and parthenium (ON116143 and ON116489) phytoplasma were sequenced, analyzed and deposited in the NCBI GenBank. Sequence similarity search via Blastn showed that the phytoplasma associated with alfalfa witches' broom shared 99.6 to 100% with '*Ca*. phytoplasma australasia' belongs to 16SrII phytoplasma group *viz*. Scaevola witches'-broom phytoplasma (AB257291), Alfalfa witches'-broom phytoplasma (KY449416, AB259169), all above sequences belong to '*Ca*. P. australasia' (16SrII group). It confirms that, *Parthenium hysterophorus* acts as a reservoir of phytoplasma and helps in survival during the off-season. *Ca*. Phytoplasma aurantifolia survives in *P. hysterophorus* and spread through insect vector, *Orosius albicinctus* and causes witches' broom disease of *P. hysterophorus* (Yadav *et al.* 2015).

Phylogeny and virtual RFLP analysis

The phylogenetic tree was constructed based on the 16S rRNA sequences of alfalfa phytoplasma (IGFRI1 and IGFRI2) and parthenium (IGFRI and IGFRI3) phytoplasma with the corresponding region 16S rRNA sequences of different groups of phytoplasma retrieved from NCBI database (Table 2). Based on the 16S rDNA gene sequence, the present study showed that phytoplasma associated with AWB clustered with '*Ca*. P. australasia' related strains belong to 16Sr II group (Fig 3). Similarly, '*Ca*. phytoplasma australasia' was identified as the causal agent of alfalfa witches' broom disease in Turkey (Ayvaci *et al.* 2020; Khan *et al.* 2001).

In-silico RFLP analysis of the F2nR2 fragment of 16S rDNA sequence of alfalfa phytoplasma using iPhyClassifier indicated that the virtual RFLP pattern derived from the query of the F2nR2 fragment of 16S rRNA sequence of alfalfa phytoplasma was identical (similarity coefficient 1.00) to the reference pattern of 16Sr group II and subgroup D (Y10097). Therefore, based on a similar restriction profile, the alfalfa phytoplasma strains in the present study were classified under the 16SrII-D sub-group (Fig 4). Similar observations were made by Ayvaci *et al.* (2020) alfalfa witches' broom disease caused by *Ca*. phytoplasma australasia belong to 16Sr group II and sub group D.

CONCLUSION

The present study provides molecular evidence that '*Ca*. *P. australasia*' belonging to 16Sr group II and subgroup D responsible for AWB disease in India. The results of quantitative and qualitative properties provide basic knowledge regarding loss caused by the disease. The symptomatology, detection and virtual restriction analysis lead to a better understanding of the pathogen and its host. It also provides a way to develop a holistic approach for diagnosis and designing novel disease management strategies.

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Declaration of competing interest

All authors declare that they have no conflict of interest.

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