Molecular detection of 16SrI-B and 16SrII-D subgroups of phytoplasma associated with flat stem and witches' broom disease of *Celosia argentea* L.

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Abstract Symptoms of stunting (shortening of internodes), twisting and flat stem (the fasciation of a stem), discoloration of petals, deformed flowers, and witches' broom were recorded on an ornamental plant, plumed cockscomb (*Celosia argentea* L., fam: Amaranthaceae). The survey conducted at Indian Agricultural Research Institute (IARI) campus, New Delhi and Karnal region, Haryana, India, during September 2014 to March 2015 revealed disease incidence of 40 and 10%, respectively. The *16S rRNA* gene sequence comparison and phylogenetic relationships of *Celosia* phytoplasma strains under study confirmed that they were associated with two different phytoplasma groups ('*Candidatus* Phytoplasma australasia' and '*Ca. P. asteris*'). Virtual RFLP analysis of *16S rRNA* gene sequences allowed further classification of the *Celosia* phytoplasma strains into the 16SrI-B and 16SrII-D subgroups. Notably, the detection of '*Ca. P. asteris*' phytoplasma was reported in seeds of *C. argentea* by nested PCR assays; however, no evidence of phytoplasma presence was detected in seedlings raised from these seeds. This observation is the first record of the association of 16SrI-B and 16SrII-D subgroups of phytoplasmas with flat stem and witches' broom disease of *C. argentea* anywhere from the world.

Keywords Plumed Cocks comb · Phytoplasma disease · *Ca. P. asteris* · *Ca. P. australasia*

Phytoplasmas are cell wall-less Mollicutes that colonize plant phloem and insects salivary glands. They are known to cause devastating losses in the important crops worldwide. They are transmitted by phloem-feeding insects (Bertaccini et al. 2014). The phytoplasmas are known to be associated with diseases in 65 different ornamentals plant species which belongs to 14 different groups (Rao et al. 2017) causing severe economic losses. *Celosia argentea* L., commonly known as plumed cockscomb or the silver cock's comb, is an herbaceous ornamental species of tropical origin, commercially used as cut flower in India (Varadharaj and Muniyappan 2017). So far, no reports are available on phytoplasma-associated diseases on cockscomb plants from India. Hence, in this study, the attempt was made to characterize the phytoplasma association with flat stem and witches' broom disease of *C. argentea* from Delhi and Karnal regions.

C. argentea plants showing symptoms of stunting (shortening of internodes), twisting and flat stem (fasciation of stem), discoloration of petals, deformed flowers, and witches' broom (Fig. 1) were collected from Indian Agricultural Research Institute (IARI) campus, New Delhi and Regional Research Station of Haryana Agricultural University campus, Karnal, Haryana from September 2014 to February 2015. Seeds from the symptomatic cocks' comb plants were collected at New Delhi to check the possibility of seed transmission of associated phytoplasmas. The DNA from the five symptomatic plant tissues and seeds were extracted (Ahrens and Seemuller 1992), and the phytoplasma *16S rRNA* gene was amplified using primers P1 (Deng and Hiruki 1991) and P7 (Schneider et al. 1995)

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Fig. 1 *Celosia* plants showing different symptoms. Flattened stem (a, b), Yellowing of leaves (c), Witches' broom (d, e), and discoloration of flower associated with phytoplasma diseases (f)

followed by nested PCR assay using primer pair R16F2n/R16R2 (Gundersen and Lee 1996). The DNA isolated from periwinkle infected with toria phyllody phytoplasma (16SrIX group, Pigeon pea witches' broom phytoplasma) maintained in the greenhouse used as positive control (Azadvar et al. 2009). The DNA extracted from asymptomatic cocks comb stem/flower tissues and seeds collected from symptomless cocks' comb plants used as negative controls. The PCR reactions were carried out in a Master cycler (Eppendorf, Germany) and the cycling protocol was followed as described by Rao et al. (2014). The amplicons of expected size of phytoplasma DNA products were eluted with Gel Elution Kit (Promega, USA) and sequenced directly as described earlier (Yadav et al. 2015).

The 1.25 kb of *16S rRNA* gene sequences derived from *Celosia* plant and seed phytoplasma isolates were subjected to in silico RFLP analysis using pDRAW32 software (<http://www.acaclone.com>) and compared with representative sequences of the phytoplasmas strains of 16SrI-B (M30790) and 16SrII-D (KF773149) subgroups. Seeds collected from the phytoplasma-positive *Celosia* plants sown in earthen pots and observed for the development of disease symptoms for the 60 days under glasshouse condition. The newly grown *Celosia* plants checked for phytoplasma presence using PCR assays described earlier.

Nested PCR assays with P1/P7 followed by R16F2n/R16R2 primer pairs amplified ~1.2 kb of *16S rRNA* gene in all the five symptomatic plant and seed samples from New Delhi along with the positive control. No DNA was amplified from the template DNA isolated from any of the plant tissues and seeds collected from asymptomatic plants. The obtained sequences were compared with nucleotide sequences in the GenBank database, using BLAST (version BLASTN 2.2.18) (NCBI, Bethesda, MD). These *16S rRNA* gene sequences and the reference *Ca.* phytoplasma strain sequences retrieved from GenBank were aligned using CLUSTALW and used to construct phylogeny tree using MEGA6 (Tamura et al. 2013).

The pairwise DNA sequence alignment of *16S rRNA* gene of *C. argentea* Karnal isolates (Karnal 1, Acc. no. KT306676; Karnal-2, Acc. no. KX671550) revealed 100% sequence identity with phytoplasma strains of sesame phyllody (KF429485), tomato big bud (KP027532), and faba bean phyllody (KP869129) belonged to '*Candidatus* Phytoplasma australasia' (16SrII group). However, the *16S rRNA* gene sequences of *C. argentea* plant (IARI-1, Acc. no. KX671548) and seed (IARI-2, Acc. no. KX671546) from New Delhi shared 99% sequence identity with sesame phyllody (KC920749), mulberry dwarf (KP662132), and periwinkle virescence (KP662136) members of

'*Candidatus Phytoplasma asteris*' (16SrI group). Phylogenetic analysis of 16S rRNA gene sequences of *C. argentea* phytoplasma strain from Karnal and New Delhi revealed their close relationships with 16SrII-D and 16SrI-B strains of '*Candidatus Phytoplasma australasia*' and '*Candidatus Phytoplasma asteris*,' respectively (Fig. 2).

Virtual RFLP analysis using 17 restriction enzymes further confirmed that the Karnal isolates (KT306676 and KX671550) produced RFLP profiles identical to *Tylophora indica* little leaf phytoplasma (KF773149) which belong to the 16SrII-D sub group (Fig. 3b). However, restriction profiles of the Delhi isolates (seed, KX671546, and stem, KX671548) produced RFLP profiles identical to the 16SrI-B sub group (*Oenothera* phytoplasma, M30790) (Fig. 3a).

No symptoms recorded up to 60 days of seed germination in seedlings grown from seeds collected from phytoplasma-associated cockscomb plants. To check phytoplasma transmission through seeds, DNA was extracted from the germinated seedlings; PCR assays were

performed, but no amplification was found with universal primer pairs of phytoplasma either in the first round (P1/P7) or nested PCR (R16F2n/R16R2) assays (data not shown).

Earlier reports are available for identification of phytoplasmas of 16SrI group on *Celosia* from Lithuania (Samutiene and Navalinskiene 2006), Iran (Aldaghi and Bertaccini 2015), and 16SrII-A subgroup from China (Chen et al. 2016) and 16SrVI group from Iran (Babaie et al. 2007). However, phytoplasma strains of 16SrI-M and 16SrIII groups were reported on other species of *Celosia* (*C. plumose* and *C. cristata*) from Israel (Tanne et al. 2000). Till date, no reports are available for phytoplasma association with any species of *Celosia* in India. Hence, assignment of 16SrII-D and 16SrI-B subgroups phytoplasma associated with flat stem and witches' broom disease of *C. argentea* in the present study is the first report from the world.

Fig. 2 Phylogenetic relationship between the *Celosia argentea* flat stem phytoplasma India isolates and reference identified phytoplasma strains based on 16S rRNA gene sequences

Fig. 3 Virtual RFLP patterns from digestion of R16F2n/R16R2 generated 16S rRNA gene fragments of phytoplasmas strains infecting sesame plants in India and the phytoplasma reference strains with 17 restriction enzymes (*AluI*, *BamHI*, *BfaI*, *BstUI*, *DraI*, *EcoRI*, *HaeIII*, *HhaI*, *HinfI*, *HpaI*, *HpaII*, *KpnI*, *MboI* (*Sau3AI*), *MseI*, *RsaI*, *SspI*, and *TaqI*) using PDRAW software. Φ X174: fragment size

The transmission of phytoplasma through seeds were reported previously in alfalfa (*Medicago sativa*) (Khan et al. 2002), stolbur phytoplasma in pea, tomato, corn, and winter oil seed rape (Zwolinska et al. 2010; Botti and Bertaccini 2006; Calari et al. 2011). However, the seed transmission of phytoplasma is a controversial issue considering the poor connection of embryo with the mother plant (Bertaccini et al. 2014). Recent studies have highlighted that the late infections in herbaceous crops allow the natural seed production with chances of transmission of the pathogens through the seeds (Olivier and Galka 2008). In our study, we detected phytoplasma in seeds collected from symptomatic *Celosia* plants, but not in seedlings grown from these seeds inferring that *Celosia* phytoplasma

standard Φ X174 *HaeIII* digested. The patterns were compared with the reference strain *Oenothera* (16SrI-B, M30790) with Delhi isolate (KX671546 and KX671548) (a) and reference strain *Mollicutes* sp. (associated with papaya mosaic disease) little leaf yellowing phytoplasma (16SrII-D, Y10096) with Karnal isolate (KT306676 and KX671550) (b)

was not truly transmitted via seeds and needs further elaborate study to establish the phytoplasma seed transmission hypothesis.

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Compliance with ethical standards

Conflict of interest No potential conflict of interest was reported by the authors.

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