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FIRST REPORT OF 16SrII-D PHYLLODY PHYTOPLASMA AND ASSOCIATED INSECT VECTORS INFECTING MULTI-FLOWER INBRED LINES OF SUNFLOWER (*Helianthus annuus L.*) in FAISALABAD, PAKISTAN

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Multi-flower inbred lines of sunflower (*Helianthus annuus L.*) showing phyllody, virescence and big bud disease like symptoms were collected from experimental fields in Faisalabad, Pakistan. Light and electron microscopic observation confirmed the presence of phytoplasma in infected sunflower plants. DNA was extracted from infected samples for nested PCR using phytoplasma universal and specific primers based on 16Sr DNA sequence. The PCR detection, restriction fragment length polymorphism (RFLP) and nucleotide sequence (phylogeny) comparison of 16S rDNA showed the close association (>99-100% sequence similarity) of submitted accession number (MK421430.1) of sunflower phytoplasmas with peanut witches'-broom group (16SrII-D) available at NCBI. Transmission trials for disease transmission confirmed that the leaf hoppers, *Orosius argentatus* and *Circulifer tenellus*as were responsible to spread the sunflower phyllody diseases from symptomatic to asymptomatic sunflower plants. According to our knowledge, this is the first time identification of 16SrII-D subgroup phytoplasma and associated potential insect vectors for sunflower phytoplasma disease transmission. It is further suggested to screen existed germplasm of sunflower against phytoplasma and not to use susceptible cultivars/germplasm to develop new varieties. The developed varieties from existing susceptible germplasm will not be able to resist phytoplasma diseases. Moreover, the identified potential insect vectors of sunflower phyllody diseases should be controlled so that it does not spread to other agricultural crops.
Keywords: Sunflower (*Helianthus annuus L.*), phyllody phytoplasma16SrII-D, *C. tenellus*, *O. argentatus*, PCR and phylogeny.

INTRODUCTION

Sunflower (*Helianthus annuus L.*) is an important oil seed crop grown worldwide for oil, food and fodder purposes. Pakistan is a dynamic agricultural country but unfortunately 70% of the oil is imported from other countries. Soya bean and sunflower have a great potential for the increase of edible oil production in the country (Nasir, 2013). Although sunflower was introduced in Pakistan during 1960s but due to attack of insect pests, various diseases, low quality seeds and less market value, a declining trend in production was observed since 2010 (Basit *et al.*, 2016). Phytoplasma is a very destructive phytopathogen that changes the physiology and gene expression of attacked plant (Ahmad *et al.*, 2013; 2014) inducing multiple infections and symptoms in various agricultural and medicinal plants (Lee*et al.*, 2000; Ahmad *et al.*, 2017) The main symptoms observed are floral virescence, phyllody, development of abnormal floral organs, extreme internode shortening and proliferation, small leaf size and overall yellowing(Akhtar *et al.*, 2008; Sharif *et al.*, 2019; Malik *et al.*, 2020). Phloem sap-sucking insect vectors such as *Psyllidae*, *Cicadellidae*, and *Cixidae* are thought to transmit phytoplasmas between plants (Lee & Davis, 1992; Ahmad *et al.*, 2017). These are also transmitted through grafting or asexual propagation (Ahmad *et al.*, 2013; Sharif *et al.*, 2019) including storage tubers, cuttings, rhizomes & bulbs (Lee & Davis, 1992) and parasitic plants (*Cuscuta campestres*) (Salehi *et al.*, 2014; Ahmad *et al.*, 2017). Phytoplasma are restricted in the sieve tube element of infected plants and insect vectors where they reproduce successfully (Perilla-Henao and Casteel, 2016; Lee *et al.*, 2000; Hogenhout*et al.*, 2008). Significant yield losses due to phytoplasma diseases have been observed in ≥ 1000 species of different plant families (Lee *et al.*, 2000; Bertaccini and Duduk 2009; Hosseini *et al.*, 2017; Ahmad *et al.*, 2017). Based on 16S rRNA gene, different groups and subgroups of phytoplasma have been identified and characterized. Symptoms observation and staining of infected parts of plants and light microscopy observation has been defined as a simple and quick method for spotting phytoplasma diseases (Deeley*et al.*, 1979; Malik *et al.*, 2020) Advanced techniques involve fluorescent microscopy (Hibben*et al.*, 1986; Franova*et al.*, 2007), electron microscopy (TEM) (Sharif *et al.*, 2019) as well as molecular techniques (Pavlovic *et al.*, 2014; Ahmad *et al.*, 2017; Sharif *et al.*, 2019). Phytoplasma diseases associated with sunflower have been reported to cause significant economic losses all over the world (Hosseini *et al*., 2017). First time sunflower phyllody disease of phytoplasma was observed in Argentina during 2010–2011 (Mulpuri and Muddanuru, 2016; Guzmán *et al*., 2014). Sunflower phyllody diseases of Phytoplasma have also been reported from Iran (Hoseini *et al*., 2017; Salehi *et al*., 2015), Argentina (Guzmán *et al*., 2014), India (Mulpuri and Muddanuru, 2016) and Bulgaria (Avramov *et al*., 2016). In Pakistan, phyllody as well as other phytoplasma associated symptoms have been reported on different oilseeds, vegetables and medicinal plants (Akhtar *et al*., 2008, 2009ab; Ahmad *et al*., 2015abc; Ahmad *et al*., 2017; Sharif *et al*., 2019; Malik *et al*., 2020). Recently, along with some potential insect vectors, a few invasive lepidopterist insect species have also been identified and reported first time from Pakistan. Screening of existing varieties against phytoplasma and development of new varieties resistant to pest and disease is direly needed. The current study was conducted to observe the phytoplasma infection and occurrence of potential insect vectors on inbred lines of multi-flower sunflower which are used to develop sunflower varieties in Faisalabad.

MATERIALS AND METHODS

Plant and Insect samples collection: Leaf samples from multi-flower sunflower (*Helianthus annuus L.*) lines (Inbred lines) showing phyllody, virescence, witches' broom and big bud like disease symptoms were collected from Ayub Agriculture Research Institute (AARI), Faisalabad, Post Agricultural Research Station (PARS), University of Agriculture Faisalabad and Agronomy fields of the University of Agriculture Faisalabad (UAF) during August and November 2017-2019. Different leaf hoppers (Fig. 5) were also captured during field survey by using hand-held vacuum apparatus. The collected insects were cage-reared along with healthy and infected sunflower plants under laboratory conditions and some were stored at -20 °C for molecular testing by PCR assays for the phytoplasma presence or absence.

***Light and Electron Microscopic Observation*:** A 1–2 mm hand cut cross section of leaf midrib or stem portion from 25 healthy and 100 infected samples were collected during survey. Samples were treated with Dienes' stain (stock solution: 0.5 g methylene blue, 1.25 g azure II, 0.25 g sodium carbonate and 10 g maltose dissolved in 100 ml distilled H_2O) 0.2% v/v in distilled water, at 30 °C for 10 min (Deeley *et al*., 1979). Added one drop of xylol on objective lenz and observed under light microscope MCX100 Daffodil Micros Austria (microscope model) and observed at 40 X magnification. For SEM, a small piece of 1-2 mm length was cut with the help of fine razor blade from each infected and healthy plant sample. These pieces were fixed on microscopic slides with pH 7.4 and stored at 4°C for two days (Nienhaus *et al*., 1982). Then the cross sections of samples were made and stained with 0.2% Diene s' stain solution for 10 minutes at 30°C (Deeley *et al*., 1979). Washed excess stain with distilled water and then one drop of xylol was added to the slide with sample piece. The oil immersion lens of light microscope was used to focus the phloem sieve area of prepared samples.

***Disease transmission study*:** Leaf hoppers *O. argentatus, Ciculifer tenellus and Exitianus indicus* were used to transmit phytoplasma from infected sunflower plants to healthy after collection from fields and reared on healthy periwinkles under controlled laboratory conditions at 30 ± 5 °C and photoperiod (14:7). First, different groups of 25 adults of each leaf hoppers were tested for phytoplasma presence through PCR. Then, after confirmation of non-presence of phytoplasma, these were allowed to feed for 7 days on sunflower plants severely infected by phytoplasma for acquisition period. Then, a group of 25 (*O. argentatus)* and *50 (Ciculifer tenellus* and *Exitianus indicus)* insects were used for the transmission of sunflower phyllody disease from infected to healthy sunflowers. These insects were shifted into separate cages containing healthy plants for getting 3-5 days' latency period. After that, they were moved to healthy caged plants and monitored daily up to three months until the development of symptoms on transmitted plants. Upon the onset of phyllody symptoms, the samples were collected for molecular studies.

***Molecular Study*:** Genomic DNA was isolated from control and infected flowers of sunflower using recommended protocol (Ahmad *et al.*, 2013; Ahmad *et al*., 2017). Amplification of extracted DNA (0–20 ng) was performed through a simple PCR assay using P1/P7 primer pairs and amplicons were re-amplified in a pre-programmed thermal-cycler (PeqSTAR, Germany) by nested PCR assays using internal primers Fu5/Ru3 and/or R16F2n/R16R2 (Gundersen and Lee, 1996; Smart *et al*., 1996). The content mixed were 0.2 mM each dNTPs, 0.5 µl of each primer pair (20 pmol), 1 unit of DNA polymerase including buffer (10X Taq polymerase) in 0.5mL microfuge tubes to make final volume of 20 µl PCR reaction mixture. Pure PCR water and healthy sunflower samples were used as a negative control. Application of phytoplasmal DNA were carried out in programmable thermocycler. Following temperature conditions were maintained in thermo cycler: 1 min denaturation cycle at 94 °C, 2 min annealing cycle at 55 °C and 2 min extension cycle at 72 °C. The later cycle was extended to 10 min at 72 °C. For nested PCR, same as above thermal conditions were maintained in thermocycler except 2 min annealing step at 50 °C. Agarose gel electrophoresis was performed with amplified phytoplasmal DNA, followed by visualization of DNA band stained with Ethidium bromide under UV Trans illuminator. PCR amplicons of 16Sr RNA gene were purified and sequenced. Representative 16S rRNA gene sequences were deposited at GenBank and compared with other closely related phytoplasma sequences. Version 5 of MEGA6 software was used to construct a phylogenetic tree by the neighbor-joining method with 1,000 replications for each bootstrap value (Tamura *et al.*, 2011).

RESULTS AND DISCUSSIONS

***Symptoms observation and Microscopic study*:** Naturally infected and artificially inoculated sunflower plants exhibited distinctive symptoms like virescence, shoot proliferation, phyllody, reduced leaf size, infertile flowers, and seedless weak capsules. The most distinctive symptoms observed alteration of floral leaves into green leaf-like structures (phyllody), replacement of ovary by shoot like elongated structures, the calyx turns to polysepalous and petals become leaf-like structures (Fig. 1). Disease symptoms were developed initially on the upper part of the canopy and prevailed rapidly to the older leaves during flowering. Several sunflower plants exhibiting distinctive phyllody symptoms were harvested from the field and tested through direct PCR assays. Light microscopy of Dienes' staining section showed phytoplasma unit in the phloem region of sesame plant infected with phytoplasma. In contrast, no intense colour was observed in similar stained section of symptomless tissues (Fig. not shown here). Scanning electron microscopic observation of infected sunflower samples showed pleaomorphic bodies having diameter ranging from 200 to 600nm limited in phloem areas while healthy samples did not show any type of bodies (Fig. 2).

***Identification of sunflower diseases by Molecular Analysis*:** Sunflower plants bearing phyllody symptom were subjected to direct and nested PCR that produced positive DNA fragments of about 1.8 kbp and 1.25 kbp, respectively whereas negative DNA fragment resulted from control and healthy sunflower plants (Fig. 3).

Figure 1. Symptoms appeared on phytoplasma infected sunflower plant parts. (A) Healthy flower (B-F) Capitola having green color flowers, phylloid flowers appeared on all branches, capitola with disc florets and ligulae malformed in green color structures, big bud like structures with severe proliferation. Healthy plants with normal plant growth having flowers and pods formation but (C and G) infected plants showed severe abnormalities in vegetative and reproductive parts of plants.

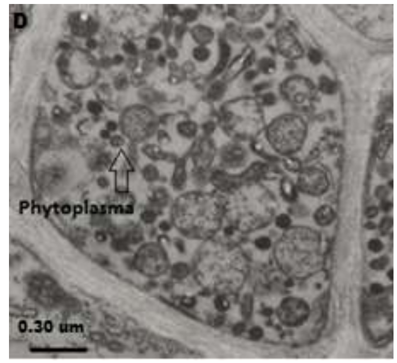

Figure 2. Scanning Electron microscopic observation of phloem cell of phytoplasma affected Sunflower plant showing phytoplasma bodies (bar = 0.30µm).

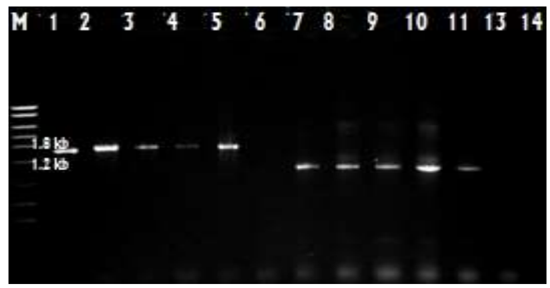

Figure 3. Nested PCR detection of sunflower associated phytoplasma by using universal primer primers P1/P7 (Lanes 1-5) followed by RI6F2n/R2 (Lanes 7-11). Lane 6 and 14- healthy samples; Lane 1-5- and 7-13 infected samples; Lane M- I kb DNA ladder (Invitrogen).

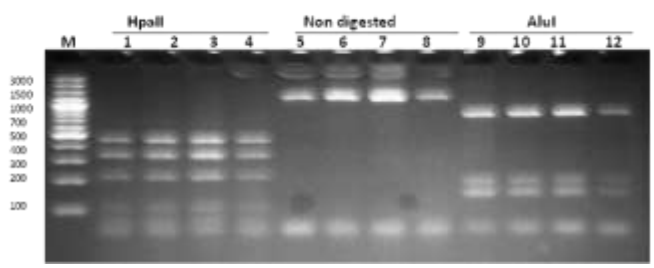

Figure 4. Restriction Fragment Length Polymorphism (RFLP) using restriction enzymes (*Hpa* II and *Alu*I);The wells (1-12) contain the RFLP and nested PCR products from sunflower samples digested with the *Hpa*II (1-3 wells), non-digested(5-7 wells) of PCR2 Product, *Alu*I (9-11 wells). The wells 4, 8 and 12 contain Nested PCR DNA samples obtained from phytoplasma infected sesame (16SrIID reference strain). Electrophoresis was conducted in 2 % agarose gel dyed with ethidium bromide (1 µg µL-1) in

the TAE 1X buffer. M: DNA ladder (100BP Invitrogen).

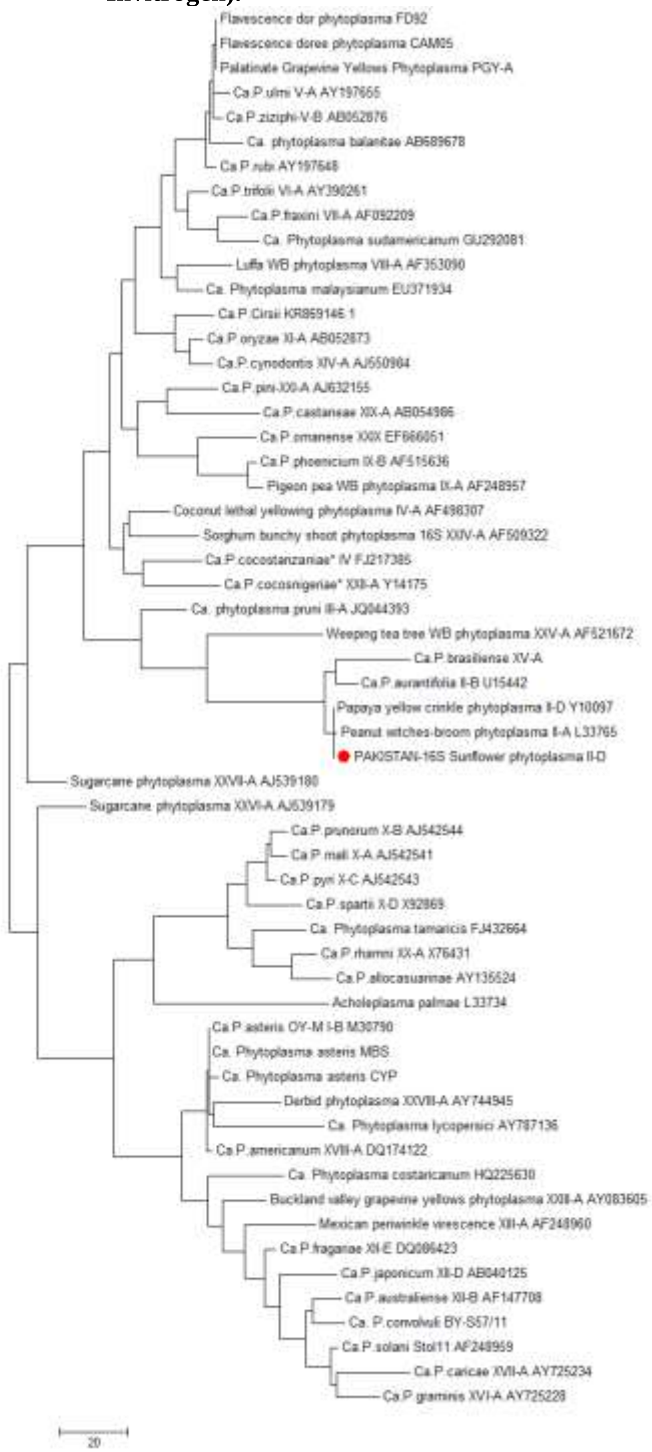

Figure 5. Phylogenetic tree through multiple alignment of nucleotide sequences of genes (16S rRNA) for sunflower phylody phytoplasma (MK421430.1) and GenBank available '*Candidatus* species' using MEGA6 software with the Neighbor-Joining method (Felsenstein, 1985; Saitu and Nei, 1987).

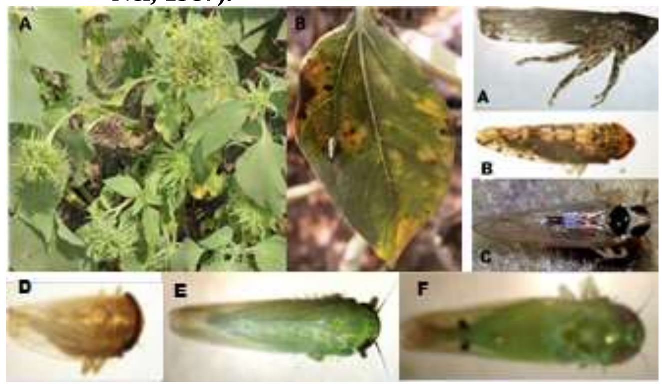

Figure 6. Leafhoppers detected by nested PCR and 16SrDNA sequencing. (A-B left) infected sunflower with *Circulifertenelus* feeding on leaves. (A) *Orosius orientalis (B) Orosius argentatus* (C) *Laodelphax striatellus* (D) *Exitianus indicus* (E) *Empoasca spp* (F) *Amrasca biguttula.*

After sequencing, desired amplified and sequenced PCR products of P1/P7 and F2/R2 were deposited in GenBank with accession numbers (MK421430.1) and compared with phytoplasma species available in the GenBank through BLAST search tool. Phylogenetic investigation for percentage homology was determined between the 16SrDNA sequences that showed that sunflower isolateexhibited >99-100% association with *Ca*. *P*. *australasia* strain of 16Sr II-D subgroup (acc.no Y10097). Further, digestion of the nested PCR products with *Hpa*II, *Alu*I (restriction enzymes) revealed the same restriction fragment length polymorphism (RFLP) pattern of 16S rDNA sequence as obtained with reference strain of *sesamum indicum* phyllody phytoplasma of 16SrII-D group (Fig. 3).

***PCR tests and Transmission Analysis*:** The potential insect vectors were captured from infected sunflower fields and used for PCR analysis and transmission tests. Table 2 shows the different species of insect vectors collected during sunflower sampling which were positive for phytoplasma presence when tested by PCR analysis. Except for white fly (*B. tabaci*) and aphids (*A. gossipy*), nearly all collected insect vectors has phytoplasma presence in their bodies (Table 2). Maximum detection of phytoplasma presence was observed in Orosius species (*O. orientalis* and *O. argentatus*) and *Circulifer tenellus* and minimum from*E. Indicus* collected from three different locations of Faisalabad. Among phytoplasma positive insects, three potential insect vectors (*O. argentatus, C.tenellus*and *E. indicus*) were used for transmission trials. *E. indicus* failed to transmit the disease but *O. argentatus* and *C.tenellus* were able to transmit sunflower phyllody from infected to healthy sunflower plants. Symptoms of sunflower phyllody appeared on plants within 25-55 days after

Table 1. Phytoplasmas associated sunflower disease: Country name and their identified Phytoplasma groups and subgroups.

Country	Identified Phytoplasma group and subgroup	References
1. Iran	16SrII-Z, 16SrII group,	Esmailzadeh Hoseini *et al*., 2017, Salehi *et al*., 2015
2. Argentina	16SrIII-J	Guzmán *et al*., 2014
3. India	16SrII-D	Mulpuri and Muddanuru, 2016
4. Bulgaria	16SrXII-A	Avramov *et al*., 2016
5. Pakistan	16SrII-D	This study

Table 2. PCR detection of phytoplasma from different insects captured from sunflower fields and surroundings during 2017-2019. Number of PCR positive insects/total number of tested insects from three locations

S. No.	Insect Species	Location 1 (no.) PCR +/Total AARI	Location 2 (no.) PCR +/Total UAF	Location 3 (no.) PCR +/Total PARS
1	*Orosius argentatus*	08/25	12/20	10/20
2	*Orosius orientalis*	10/30	08/25	05/15
3	*Exitianus indicus*	04/25	00/30	03/20
4	*Empoasca fabae*	10/30	09/25	03/10
5	*Bemesia tabaci*	00/25	00/20	00/10
6	*Amrasca biguttula.*	05/25	11/50	05/20
7	*Laudelphax striatellus*	11/50	08/30	05/15
8	*Aphis gossipy.*	00/50	00/25	00/20
9	*Circuler tenellus*	24/50	13/30	16/30

transmission of insect vector. PCR analysis showed positive DNA for symptomatic plants, while negative phytoplasmal DNA resulted from asymptomatic plants. Ten out of 25 sunflower plants were infected after transmission of leafhopper *O. argentatus* whereas 15 out of 25 sunflower plants were infected by the leafhopper *C. tenellus*. After 50 days of transmission, all plants exhibiting phyllody symptoms were PCR positive on nested PCR assays. The transmission studies with other phytoplasma positive leafhoppers particularly (*Empoasca* spp, *L. striatellus* and *Amrasca biguttula*) are under progress.

DISCUSSION

Sunflower is an important oilseed crop in Pakistan but because of unavailability of high yielding diseases and pest resistant varieties, low quality seeds and low market value is the main constraint of low yield in Pakistan. Diseases and pest attack also discourages farmers to grow oilseeds crops on wider area. The healthy germplasm which is used to develop resistant and high yielding varieties is the primary part in good quality seed production. Phytoplasma is an important disease that interferes with plant developmental, molecular and physiological process (Ahmad *et al.,* 2013, 2014; Yaseen *et al.,* 2020).

Current research was conducted to observe the susceptibility or resistant status of multi-flower sunflower germplasm (Inbred lines) being used to develop new sunflower varieties in Pakistan. Based on symptom observation as well as microscopic and molecular techniques, selected multi-flower inbred lines of sunflower at AARI and UAF were seen to be highly susceptible against phyllody phytoplasma diseases in Faisalabad, Pakistan. The amplicon of 16S rRNA genes of infected sunflower plant identified phytoplasmas strain as a member of 16Sr-II-D. The presence of phytoplasma further verified through light and electron microscope as well as insect transmission. Sunflower phyllody associated to phytoplasma subgroup16Sr-II-D have also been reported in chickpea (Akhtar *et al*., 2008, 2009b; Ahmad *et al.*, 2019), fenugreek (Malik *et al.*, 2020), parthenium, tomato, brassica, sesamum (Ahmad *et al*., 2015a, 2015b, 2015c), carrot, radish and onion (Sharif *et al*., 2019). However, the phytoplasmas detected in sunflower from different geographical areas (Table 1) are not alike and have been reported as a member of 16Sr-III group (X group) in Argentina (Guzmán *et al*., 2014), 16SrII and 16SrVI (Tazehkand *et al*., 2010), and 16SrII-D groups in Iran and India (Salehi *et al*., 2015; Mulpuri and Muddanuru, 2016). The 16SrII phytoplasmas belong to subgroup 16SrII-D have been reported in sunflower, pot marigold, white clover, alfalfa witches'-broom, tomato, chickpea, *Picris hieracioide*, sesame, solanaceous and cucurbit crops (Hosseini *et al*., 2013; Singh *et al*., 2012; Mitrovic *et al*., 2012; Alfaro-Fernández *et al*., 2012; Hosseini *et al*., 2011; Khan *et al*., 2002; Omar and Foissac, 2012). Among 15000 described species of Cicadellidae, 88 species are insect vector of phytoplasmas diseases in plants (Rojas-Martínez, 2009). Brown leafhopper *Orosious orientalis* (Hemiptera: Cicadellidae) transmits Phytoplasma Phyllody disease in different agriculture crops (Sertkaya *et al*., 2007; Nabi *et al*., 2015; Martini *et al*., 2018; Gogoi *et al*., 2019;

Salehi *et al*., 2016) all over the world. In Pakistan, *Orosious orientalis*is also a confirmed insect vector for oils seed crops as *sesamum indicum*, *brassica campestris* and vegetables (Akhtar *et al*., 2008, 2009ab; Ahmad *et al*., 2015abc; Ahmad *et al*., 2017; Ahmad *et al*., 2019; Sharif *et al*., 2019; Malik *et al.,* 2020). Other than sucking insect vectors, different lepidopterist species have also been identified and reported in Pakistan (Manzoor *et al.,* 2018, 2020; Ahmad *et al.,* 2020ab). In this study, *O. argentatus* and *C. tenellus* are responsible for spreading 16SrII-D phytoplasma from infected to healthy plants. *Orosius species* are the natural insect vectors of phytoplasma associated phyllody in sunflower. The *O. orientalis* has also been reported the insect vector of aster yellows (Tanne*et al*., 2001), alfalfa witches-broom (Salehi *et al*., 1995), garden beet witches'-broom (Mirzaie *et al*., 2007) and sesame phyllody associated phytoplasma (Ishihara, 1982). Moreover, *Orosius* species reported as the main insect vector of agricultural, horticultural and ornamental plants in Asia and Africa (Ishihara, 1982), Iran (Hosseini *et al*., 2007), Turkey (Sertkaya *et al*. 2007) as well as in Pakistan (Akhtar *et al.* 2009; Ahmad *et al*., 2015abc; Ahmad *et al*., 2017; Ahmad *et al*., 2019; Sharif *et al*., 2019). Other than other agricultural crops, *sesamum indicum* is the most affected phytoplasma associated (16SrII-D) oilseed crop in Pakistan.It is quite possible that sunflower associated phytoplasma is being transmitted from other crops to sunflower through potential insect vectors. To stop the spread of phytoplasma diseases to other major crops, it is essential to manage insect vectors as well as to develop resistant germplasm for the development of resistant varieties in Pakistan.

***Conclusion*:** This is a first case study and report of sunflower phyllody and its associated insect vector in Faisalabad, Pakistan. The detected phytoplasma in this investigation was closely related to '16SrII group' and sub-group "D". There is dire need to manage this hazardous disease and its causal agent. Development of insect and disease resistant cultivars is the most effective and long-term approach to control sunflower phyllody disease. Further, it is recommended to screen all existed germplasm and not to use susceptible multi-flower sunflower inbred lines for the development of new varieties because of their high susceptibility against phytoplasma. Field surveys are being conducted to investigate the symptoms of sunflower phyllody in several other important crop growing regions of Pakistan.

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