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MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF PHYTOPLASMAS ASSOCIATED WITH CARROT, CABBAGE AND ONION CROPS AND THEIR INSECT VECTORS IN PUNJAB, PAKISTAN

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The study was undertaken to observe the phytoplasma incidence in carrot (*Daucus carota*), cabbage (*Brassica oleracea* var. *capitata*) and onion (*Allium cepa*) plants during 2017 in Punjab, Pakistan. Phytoplasma induced symptoms such as yellowness, proliferation, phyllody and stunted growth were observed on studied plants. Moreover, pleomorphic phytoplasma bodies were seen in phloem cells of infected plant samples using electron microscopy. The presence of phytoplasma in infected plant samples was further confirmed by nested PCR amplification of 16SrDNA using universal primer pairs (P1/P7 and RI6F2n/R2). Amplicons of 1.8 Kbp and 1.2 Kbp were obtained in PCR, visualized on 1.5 % agarose gel electrophoresis. Restriction Fragment Length Polymorphism (RFLP) profiles and Sequencing proved that pattern of studied vegetables isolates is same with Sesamum phyllody reference strain of 16SrII-D subgroup. The phylogenetic analysis confirmed the 99-100% sequence homology to Peanut witches-broom phytoplasma strain of 16SrII-D subgroup. Various insect species were collected from vegetable fields of three above mentioned crops. Among those, *Empoasca* spp. *O. albicinctus*, *A. bigutula* and *Nervosa* spp. were resulted phytoplasma positive while some other species of unknown leafhoppers and aphids were negative. The PCR positive insect vectors could be involved in transmission of phytoplasma in vegetables. This is the first report of vegetable association with phytoplasma and their potential insect vectors.
Keywords: *D. carota*, *B. oleracea*, *A. cepa*, 16SrII-D phytoplasma, insect vectors, PCR, phylogeny.

INTRODUCTION

Phytoplasmas are bacterial plant pathogens and obligate parasites lacking cell walls. They are phloem-limited, uncultivable mostly causing diseases in numerous plants worldwide (Lee *et al*., 2000; IRPCM, 2004). Globally, phytoplasmas cause multiple diseases in several crop species including vegetables, fruits, cereals (Lee *et al.,* 2000). Different crops have been reported to be infected by many viruses, bacteria and fungal diseases but in addition nested PCR studies using particular phytoplasma primers (P1/P7 and R16F2n/R16R2) specified phytoplasma incidence in symptomatic plants (Ahmad *et al*., 2015). A varied of symptoms induced due to phytoplasma infection including leaf yellowing, little leaf, virescence, growth aberrations (proliferation, dwarfism), and more commonly flower abnormalities and alteration of gene expression are faced (McCoy *et al*., 1989; Del Serrone *et al.*, 2001; Ahmad *et al.*, 2013). Phytoplasma is also reported to change the plant physiology as DNA methylation was studied as a potential mechanism for ruling floral gene expression in tomato buds infected with stolbur phytoplasma (Ahmad *et al.*, 2013). In year 2007, phytoplasmas connected with various subgroups of the AY phytoplasma group (16SrI) were spotted to be linked with low prevalence of infections in carrots presenting leaves redness, qualitative reduction of tap roots and shoot proliferation (Duduk *et al*., 2007). Phytoplasmas are transferred among plants by phloem sap-sucking insect vectors of different families including *Psyllidae*, *Cicadellidae*, and *Cixidae*, in which they reproduce (Lee and Davis, 1992). Transovarial transmission of some phytoplasmas in insects has also been reported (Danielli *et al*., 1996; Alma *et al.*, 1997; Mitsuhashi *et al*., 2002). Transmission of phytoplasmas can also be conceded through grafting and vegetative propagation including cutting, storage tubers, rhizomes or bulbs (Lee and Davis, 1992). Different dodder species (*Cuscuta campestres*, *epilinum* and *trifolli*), the plant parasites, affecting various plants including tomatoes are also responsible for the transmission of phytoplasmas (Salehi *et al*., 2014). So far, various phytoplasma strains have been categorized into 28 groups using PCR with universal primers and RFLP examination

amplifying 16S rDNA sequences (Serrone *et al*., 2001; Wei *et al.*, 2007; Lee *et al.*, 2007). Recently various pathogens and insect vectors have been identified from Pakistan (Ahmad *et al*., 2018a,b). Keeping in view the economic importance of vegetable crop and the probability of quick dispersion of phytoplasmas in carrot and as well as other field crops, it is compulsory to inhibit huge infestation. The purpose of present study was to define phytoplasma populations and documentation of potential insect vector species on vegetables in Pakistan.

MATERIALS AND METHODS

The research presented here was undertaken during 2017 at Integrated Genomics Cellular, Developmental and Biotechnology Laboratory (IGCDBL) PARS Campus, University of Agriculture Faisalabad (Pakistan).

***Field surveys*:** A field surveillance of carrot, cabbage and onion crop was accompanied during 2017 in different zones of Punjab province and the areas involved in that survey were Faisalabad, Lodhran, Bahawalpur, DG Khan and Rahim Yar Khan. The activities performed during survey of mentioned areas were observations of infected plants, collection of infected leaves samples as well as capturing insect species from the field.

***Electron microscopy*:** Water agar-entrenched infected as well as healthy samples of carrot stem were preceded overnight in 5% of glutaraldehyde, pounded via 0.2 M Pipes buffer while post-fixed in 1% of osmium tetraoxide for the time period of 18 hrs at room temperature. Later, the samples were washed away through utilization of distilled water then treated via uranyl acetate (5%) for the period of 16-18 hrs and washed once more with distilled H2O. Furthermore, dehydration was done through absolute ethanol and entrenched in Spur resin at the temperature of 70°C for duration of 48 hrs. RMC MT 7000 ultra-micro- tome was employed to cut thick sections of 120 nm, and then picked on copper grids. Next, for staining of those sections uranyl acetate (5%) for time period of 30 min and lead citrate for time period of 10 min was applied. At the end, observations were made through application of JEOL JEM1010 transmission electron microscope functioning at 80 KV.

DNA extraction*:* Extraction of DNA from 0.5 g samples was carried out from field collected plants samples that were initially crushed with the help of mortar and pestle by CTAB extraction protocol as documented by Doyle and Doyle (1990; Ahmad *et al.*, 2014).

***PCR assays for phytoplasma in test plants*:** Each reaction mixture (50 ml) for PCR comprised of 1 µl of DNA, Taq polymerase (1.25 units), Taq buffer comprising 1.4 mM MgCl2, primers (0.4 µM) and dNTP (0.1 mM). For the first round PCR universal primer pair P1/P7 (Deng and Hiruki 1991; Kirkpatrick *et al.*, 1995) while in case of nested PCR primers pair RI6F2n/R2 (Gundersen and Lee, 1996) were used for phytoplasma detection. Conditions applied for PCR cycling were: 1 min denaturation at 95°C (2 min duration for first cycle), 1 min annealing at 55°C temperature and 1.5 min time for the process of extension at the temperature of 72°C for 35 cycles (9.5 min on final cycle). Carrot phytoplasma DNA product, collected from those plants showing phytoplasma associated symptoms and sterile dH2O (SDW) were used as positive and negative controls correspondingly. After the completion of each nested PCR investigation, PCR product of 2 µl were analyzed on 1% agarose gel and stained with ethidium bromide and then visualized under UV light using Gel documentation system.

***RFLP analysis of plants*:** Nested-PCR products of 5-8 µl (1.25 Kbp from 16S ribosomal-DNA) from three isolates of various carrot, cabbage and onion fields in Punjab were individually digested with *Hpa*II and *Alu*I (restriction enzymes) regarding manufacturer's guidelines at the temperature of 37°C overnight. Then, electrophoresis of digestion products was done through agarose gels (2%) electrophoresis and visualized staining with illuminating chemical "ethidium bromide" (1 µg μl^{-1}) in the TAE 1X buffer by ultraviolet Trans illumination under Gel Documentation System (SYNGENE, UK). The resulting patterns of restriction fragments length polymorphism (RFLP) were matched with those already searched and documented for 16S ribosomal-DNA of some another phytoplasmas (Lee *et al.*, 1998; Marcone *et al.*, 2000).

***Sequencing and phylogenetic analysis*:** Amplification of 16S ribosomal-DNA sequence through nested polymerase chain reaction (1.25 Kbp) of tested plants was purified through commercial kit and then sequencing was done by AbiPrism 3100 Genetic Analyzer apparatus (Applied Biosystems, USA). Data obtained through sequencing of plant samples was aligned & examined working with Lasergene v. 7.1 software package (DNASTAR, USA) and for homology phylogenetic studies were performed with MEGA6 software using a methodology designated as "408oil408bor joining method" (Tamura *et al.*, 2007). Numerous phytoplasma strains along with their accession numbers utilized for the purpose of phylogenetic tree construction are given bellow (Table 2).

RESULTS

***Symptomatology*:** Carrot associated infections can result various types of phytoplasma symptoms, but the main symptoms observed in carrot plants were phyllody, hairy roots, shoot proliferation, and yellowish and purplish leaves coloring. While, the symptoms spotted in cabbage diseased plants exhibited thicker leaves, protracted thick shoots and failure to heads formation. The phytoplasma triggering indications in onion crop from different districts of Punjab, Pakistan and those indications include phyllody and

virescence in onion inflorescence, axillary growth, yellowing and proliferation (Fig. 1)

***Electron microscopy*:** Infested carrot tissues exhibited characteristically pleomorphic bodies of phytoplasma in diameter range of about 200-600 nm that were restricted to the sieve elements but healthy samples were lacking such type of bodies (Fig. 2).

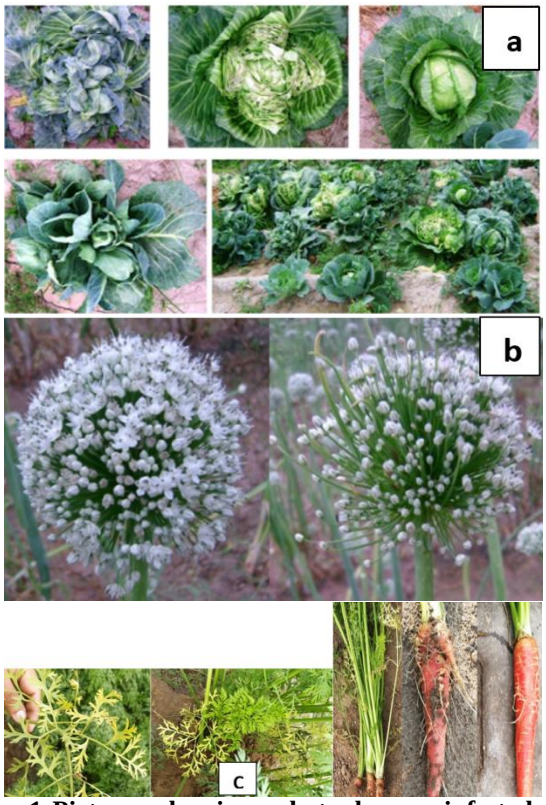

Figure 1. Picture showing phytoplasma infested and healthy plants: a= healthy cabbage plants on upper right side while middle and top left open headed plants are phytoplasma infested. b= inflorescence of infected onion plant on top right side exhibiting phyllody and virescence. c= infested carrot plants on left side with leaf yellowing and hairy roots while at extreme right side healthy carrot is placed.

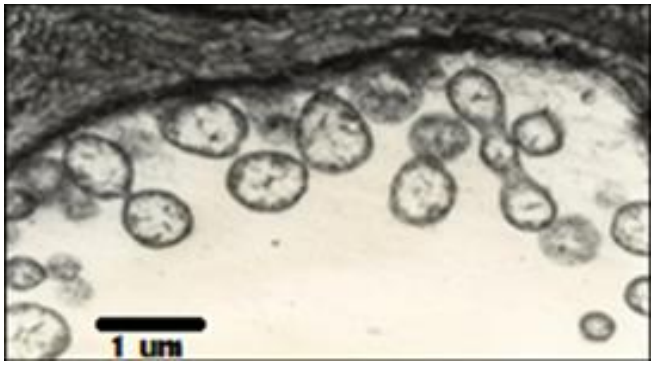

Figure 2. SEM picture exhibiting pleomorphic bodies observed through electron microscopy in carrot leaf midrib.

Table 1. Phytoplasma strains and their accession numbers for construction of phylogenic tree

Sr.	Strain/Groups/subgroups	Accession numbers
1	Jahrom (Iran) sesame phyllody phytoplasma	F607109
2	Chrysanthemum morifolium phyllody phytoplasma	Y693690
3	Catharanthus roseus phytoplasma II	U096500
4	Alfalfa witches broom phytoplasma strain AlfWB-S	Y365528
5	Helianthus annuus phyllody phytoplasma clone HAP1	T005455
6	Faba bean phyllody phytoplasma	P869129
7	Alfalfa phytoplasma (Sudan)	Y449416
8	Peanut witches-broom phytoplasma strain PnWB-Hn1	GU113148
9	Tomato big bud Iran	JF508510
10	*Ca.* P. *rhamni*	L33765
11	*C*a. P. pyri	AJ542543
12	A. laidlawii PG8A	NR076550

Table 2. PCR results of different insects (hoppers) and their population collected during field Surveillance.

Insects	Family	No. of collected insects	Nested-PCR results (+ve/-ve)
Empoasca spp	Cicadellidae	35	15/35 +ve
Nervosa spp		28	8/28+ve
Circulifer haematoceps	Cicadellidae	31	20/31 +ve
Stictocephala bisonia	Membracidae	17	-ve
Orosius albicinctus	Cicadellidae	22	20/22 +ve
Eufairmairia spp	Membracidae	16	-ve
Exitianus sp.	Cicadellidae	19	-ve
Muirodelphax arvensis	Delphacidae	11	not tested
Laodelphax striatellus	Delphacidae	13	not tested
Amrasca bigutula	Cicadellidae	10	4/10 +ve
Aphid spps.	Aphididae	15	-ve
Unidentified leafhoppers		39	-ve

Molecular Characterization:

***PCR and RFLP analysis*:** Extraction of DNA carried out from phytoplasma infested vegetable plant samples and insect species was successfully carried out and their amplification using the universal primer P1/P7 and RI6F2n/R2 indicated amplification of phytoplasma gene in PCR. Total plant samples exhibiting phytoplasma symptoms yielded the PCR product of 1.8 kbp (Figure 3). Characterization of PCR product was also undertaken through RFLP investigation. As a consequence RFLP summaries via *Alu*I and *Hpa*II

restriction enzymes were same for all DNA products (Figure 4). This pattern was consistent to profile of "sesame phyllody strain" which previously belongs to 16SrII-D subgroup. Multiple insect species (Hoppers) were collected from fields of variant above mentioned zones.

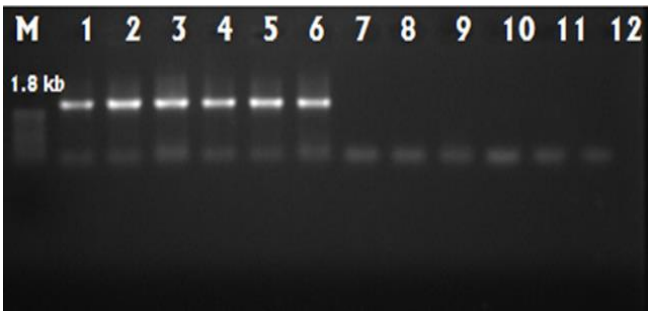

Figure 3. Nested PCR detection of vegetables associated phytoplasma by utilizing universal primer primers P1/P7 & RI6F2n/R2. Lane 1-2 Carrot diseased samples; Lane 3-4 Cabbage diseased samples; Lane 5-6 Onion diseased samples; Lane 7-12 are healthy samples of these vegetables correspondingly while Lane M- 1kb DNA ladder (Invitrogen).

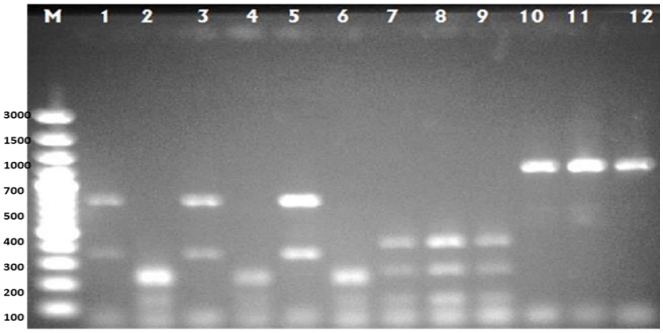

Figure 4. RFLP analysis with *Alu*I, *Hpa*II restriction enzymes; M, Molecular weight DNA Ladders (100 bp Invitrogen; Carrot and cabbage with sesame (References) samples digested with the *Alu*I (1, 3 and 5 wells), *Hpa*I (7, 8 and 9 wells) and PCR2 DNA (10-12 wells). While, well 2, 4, 6 contain undigested PCR product. Electrophoresis was conducted in 3 % agarose gel dyed with ethidium bromide (1 µg µL-1) in the TAE 1X buffer.

Those insect species were recognized as *Empoasca* spp, *Nervosa* spp. (white-winged planthopper), tree hoppers or horn tree hoppers (*Stictocephala bisonia*; *Eufairmairia* spp), *Muirodelphax arvensis*, *Circulifer haematoceps*, *Laodelphax striatellus*, *Exitiana* spp, *Orosius albicinctus*, *Aphids* and some other unidentified leafhoppers (Figure 5). Empoasca spp., Nervosa, Circulifer, Orosius and Emrasca species were positive for phytoplasma with numbers 15, 8, 20, 20 and 4 respectively whereas other insects were negative. PCR results of possible collected insect vectors are shown in Table 1.

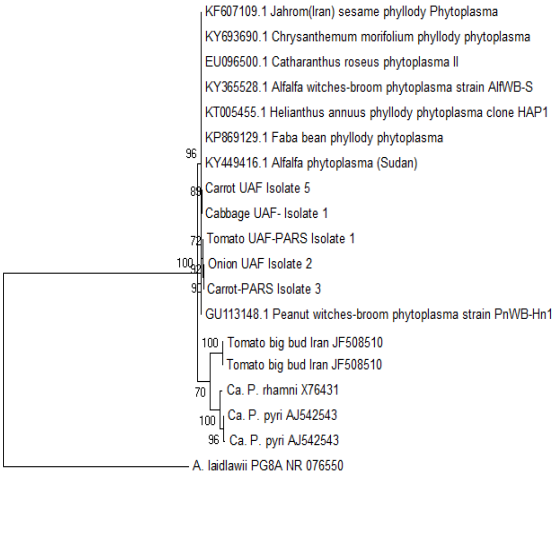

Figure 5. Construction of a phylogenetic tree through multiple alignments of nucleotide sequences of genes (16S rRNA) for isolates of carrot, cabbage and onion phytoplasma achieved from the GenBank database using MEGA6 software along with methodology designated as "neighbour joining method".

Table 3. Insect vector species detected for phytoplasma transmission in various crops.

Sr.	Insect vectors	Country	Crop	References
1	*Circulifer haematoceps* *Neoaliturus fenestratus*	Israel	Carrot	Weintraub *et al.* (2004)
2	*A. laevis ; A. ribauti* *A. venosa ; P. striatus* *P. confinis ; P. alienus*	Italy	Carrot	Drobnjakovic *et al.* (2010)
3	*Macrosteles fascifrons*	Canada	Carrot	Wally *et al.* (2004)
4	*Orosius albicinctus*	Iran	Carrot	Salehi *et al.* (2016)
5	*M. quadripunctulatus* *M. sexnotatus* *M. laevis*	Serbia	Carrot	Duduk *et al.* (2008)
6	*Macrosteles fascifrons* *M. quadrilineatus,* *Scaphytopius irroratus* *Ceratagallia abrupta*	USA	Cabbage	Brcak.,1979; Lee *et al*., 2001; Lee *et al*., 2003; Zhang *et al*., 2004
7	*M. striifrons*	Japan	Onion	Wei *et al*., 2004

***Sequencing and phylogenetic analysis*:** Sequencing of nested-PCR products achieved from utilization of RI6F2n/R2 were carried out and then compared between some other 16S-rDNA of groups and subgroups available in Genbank. The phylogenetic tree (Figure 6) constructed by NCBI available sequences (Table 2) proved that Pakistani isolates (Carrot UAF isolate, Carrot UAF-PARS isolate1, Onion UAF-PARS isolate 2, Cabbage UAF-PARS isolate3, Cabbage UAF isolate3) formed same cluster with 16Sr-II-D group of

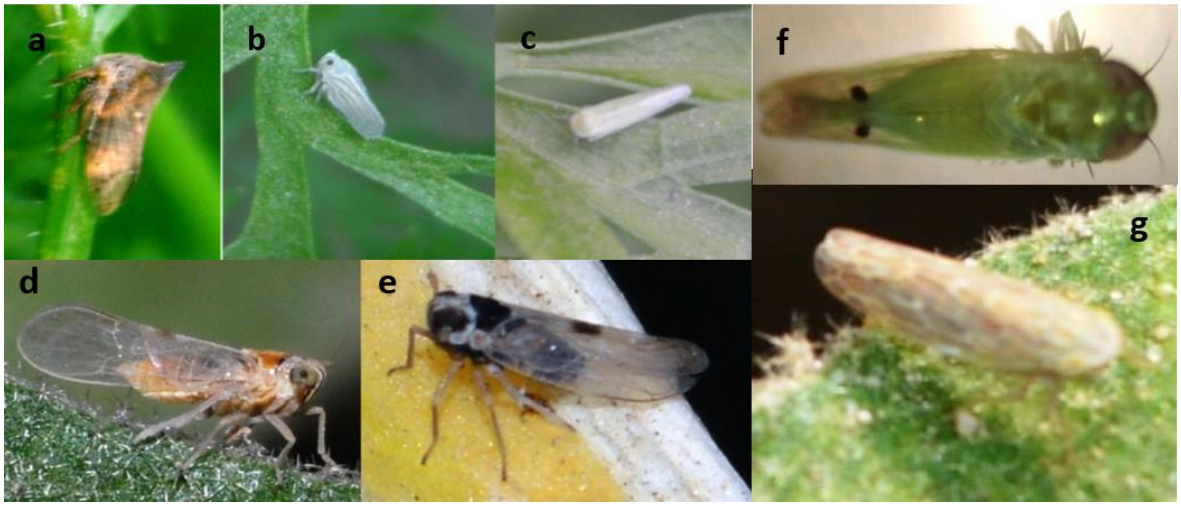

Figure 7. Plant-hopper species collected from vegetable fields of carrot, cabbage and onion crops: a, horn tree hopper; b, *Muirodelphax arvensis;* c, *Empoasca* spp; d-e, *Laodelphax striatellu; f, Amrasca bigutula; g, Orosius spp.*

phytoplasma showing 99-100% identity with Peanut witches-broom phytoplasma PnWB-Hn1 (Access no. GU113148).

DISCUSSION

The phytoplasma infection of carrot crop has been detected in Israel (Orenstein *et al.*, 1999; Weintraub *et al*., 2004), Canada (Wally *et al.*, 2004), Washington State (Lee *et al*., 2006), Serbia (Duduk *et al*., 2008), Italy (Drobnjakovic *et al.*, 2010), USA (Nisbet *et al.*, 2014), Saudi Arabia (Omar, 2014), Iran (Salehi *et al*., 2016) and Qassim region of Saudi Arabia (Omar *et al.*, 2017). While in Pakistan the spread of this syndrome is being reported for the very first time in 2017 in Pakistan. The key symptoms of phyllody disease associated with carrot plants in current study include phyllody, hairy adventitious roots, proliferation of shoots, and field outlook exhibiting yellowish and purplish leaves pattern. Same the phytoplasma symptoms are reported recently by Omar, (2017) in Saudi Arabia. Furthermore, Nisbet *et al.* (2014) stated the infected carrot symptoms that were said to report by carrot growers of Scotland and the symptoms reported by those growers were leaf curling, reddening and yellowing of leaves and occurrence of adventitious roots was also noted. On another hand Salehi *et al.* (2016) documented the related symptoms in carrot plants, the symptoms they stated were yellowing, reduced size leaves, yellowing, shoot proliferation from taproot, taproot stunting, phyllody, virescence, reddening of leaf and witches' broom. The symptoms spotted in cabbage diseased plants exhibited thicker leaves, extended thick shoots and termination of heads formation. Same the consequences of phytoplasma infection related indications were spotted in cabbage crop in Hungary by Fodor *et al*. (1999) while Ahmad *et al*. (2015) has also reported such kinds of phytoplasma triggering indications in onion crop from different districts of Punjab, Pakistan and those indications include phyllody and virescence in onion inflorescence, axillary growth, yellowing and proliferation. Based on main syndrome symptoms, existence of insect vector species, reaction with Dienes stain, direct inspection of sieve cells linked pleomorphic bodies and amplification of specific 16S rDNA fragment (1.25 kb), it was ratified that carrot plants infection is due to phytoplasma. Dienes staining exhibited frequently scattered areas in the phloem zone of the phytoplasma infected carrot plants (Salehi and Izadpanah, 1992).The phylogenetic study of our overall carrot isolates exhibited that they connect more closely together with Peanut witches-broom of 16SrII-D sub group. Omar *et al.* (2017) also documented 16SrII-D subgroup infecting carrot in Qassim region of Saudi Arabia. Similar phytoplasma (Papaya yellow crinkle phytoplasma) with 16SrII-D subgroup was also reported by Omar and Foissac (2012). Blast analysis, RFLP and phylogenetic investigation regarding partial sequence of 16Sr DNA and 16Sr RNA genes exhibited that phytoplasma connecting carrot witches-broom syndrome has maximum homology and close association with Peanut witches broom of 16SrII group (Salehi *et al.*, 2014; Salehi *et al.*, 2016). Peanut witches-broom phytoplasma strain (GU214176) was also spotted in Taiwan triggering the indications related to virescence in Peanut (Liu *et al.*, 2015). Such subgroups were also reported to infect papaya, Pale Purple Coneflower (Pearce *et al.*, 2011), and tomato plants (White *et al.*, 1998) in Australia but the strains have not been differentiated so far on the base of genetics. Hoppers have been acknowledged for many years as the vectors of many diseases. In current investigation we have collected variable hopper species from different districts, the species including *Empoasca* spp,

Nervosa spp, tree hoppers (*S. bisonia*; *Eufairmairia* spp), *M. arvensis, C. haematoceps, L. striatellus*, *Exitiana* spp, *O. albicinctus* and some other unidentified leafhoppers but couple of hopper species (*Empoasca* spp and *Nervosa* spp) ensued phytoplasma positive but some of them were phytoplasma negative and others could not tested that time to confirm their vector status that may be the vectors of such infections. Salehi *et al.* (2016) detected *O. albicinctus* as causative agent of peanut witches' broom associated phytoplasma group (16SrII) in carrot crop of Iran. Ahmad *et al*. (2017) also reported *O. albicinctus* responsible for phytoplasma infestation in Brassica campestris in Pakistan.Three hopper species including, *Macrosteles quadripunctulatus, M. laevis* and *M. sexnotatus* collected from the carrot field were resulted positive for the similar phytoplasmas recognized in the samples with phytoplasma infection (Duduk *et al*., 2008). On another hand *Empoasca decipiens* (Cicadellidae; Typhlocybinae) are the potential vectors for the phytoplasma transmission in tomato plants (Ahmed *et al*., 2014). Catindig *et al*. (1995) documented a planthopper specie (*Nisia nervosa*) as leaf sucking insect of rice crop while Kumar *et al*. (2015) reported this insect as potential putative vector instigating Weligama Coconut Leaf Wilt Syndrome in Sri Lanka. While the vector status of *C. haematoceps* for transmission of *spiroplasma citri* has also reported by Bretin *et al.* (2010). Additional experiments should be conducted to determine whether currently detected phytoplasma is transferred in nature by such insect vectors.

***Conclusion*:** The recent studies confirmed the spreading of phytoplasma associated diseases and insect vectors in vegetables. The Pakistani phytoplasma isolates triggering infestations are members of subgroup 16SrII-D clade connected with phytoplasma 16Sr-DNA RFLP classification. They have same partial sequences of 16SrDNA. The phytoplasma 16 Sr-II-D group surely is being transmitting from one crop to another or from wild reservoir to crop by means of insect vectors. Severe deformations were noticed causing carrot crop unremarkable and the deformities include symptoms like phyllody, hairy roots, proliferation, and field outlook exhibiting yellowish and purplish leaves pattern. The study also proposes the proper management of phytoplasma diseases and insect vectors. Furthermore, investigations on non-tested hopper species are mandatory to detect their vector status, accountability for the transmission of phytoplasma in the country and to define its plant and insect host range. Additionally, better genetic variation of isolates will be required to find out the geography and dynamics of its epidemics.

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