SUMMARY

Safflower plants with phyllody symptoms were observed in Fars and Yazd provinces of Iran. Affected plants showed floral virescence, phyllody and proliferation, proliferation of axillary buds along the stem and little leaf symptoms. The causal agent (SP) was transmitted from diseased to healthy safflower by grafting and from safflower to safflower and periwinkle by dodder (*Cuscuta campestris*). The presence of phytoplasmas in diseased plants was shown by direct and nested polymerase chain reaction assays using phytoplasma-specific primer pairs P1/P7 and R16F2n/R2. Restriction fragment length polymorphism (RFLP) analysis of nested R16F2n/R2 primed PCR product (1.2 kb) classified SP phytoplasma in the clover proliferation phytoplasma group (16SrVI). Sequence homology, phylogenetic and putative restriction site analyses of 16S rRNA gene also identified SP phytoplasma as a member of 16SrVI group. On the basis of molecular analyses, SP phytoplasma was most closely related to brinjal little leaf and periwinkle little leaf phytoplasmas, members of subgroup 16SrVI-C.

Key words: safflower, phyllody, phytoplasma transmission, RFLP, sequencing.

INTRODUCTION

Safflower (*Carthamus tinctorius* L.) has been grown for many years in Iran, which is one of the centers of safflower culture in the old world (Knowles, 1969). In recent years, due to increasing demand for nutritional oil and for a drought and salt tolerant crop, safflower has received a great deal of attention. Several diseases affect safflower, including phytoplasmal phyllody (Kolte, 1985), which was previously reported only from Israel (Klein, 1970). A phytoplasma belonging to 16SrI-B group was found associated with this disease (Schneider et al., 1993; Lee et al., 2004) and the leafhopper *Neoalioturus fenestratus* was reported as its vector in Israel (Raccab and Klein, 1982; Weintraub and Bealand, 2006). During a survey carried out in 2003, safflower phyllody (SP) was observed in safflower fields in Zaraghan, Firouzabad and Darab (Fars province, southern Iran) and in Sarchahan (Yazd province, central Iran).

The objective of the present study was the identification of the agent associated with SP and its molecular characterizations. A preliminary report was recently published (Salehi et al., 2008).

MATERIALS AND METHODS

Source of the disease. A safflower plant with typical phyllody symptoms was selected in Zaraghan (30 km north of Shiraz, Fars province), transferred to a greenhouse and used as the source of the SP agent for transmission studies. The phytoplasma isolate was also used for cloning and sequencing. Other samples were collected from diseased and symptomless safflower plants in commercial fields in Zaraghan and Sarchahan and used for PCR assays. The disease agent was maintained in safflower and periwinkle by grafting.

Graft inoculation. For side-veenner grafting, 8-week old safflower plants were used as rootstock and small axillary shoots from a naturally infected safflower plant were used as scion. The graft unions were wrapped with parafilm and the plants were covered with plastic bags for two weeks to maintain humidity. Infection of graft-inoculated plants was verified by nested PCR.

Dodder transmission. Seeds of dodder (*Cuscuta campestris* Yunk.) were germinated on moist paper and transferred to a healthy sugar beet plant. After 3 weeks, connection was established between dodder strands from healthy sugar beet and a phyllody-affected safflower plant. Four weeks later, newly developed dodder strands from phyllody-affected safflowers were connected to healthy safflower and periwinkle plants. Connections were maintained for 4 weeks, after which test
plants were freed of dodder strands and kept in the greenhouse. Controls were exposed to dodder grown on healthy sugar beet. Infection of dodder-inoculated plants was verified by nested PCR.

**DNA extraction and PCR.** Samples were from rootlets of a symptomless control and eight phyllody-affected safflower plants taken from Zarghan and Sarchahan. Total DNA was extracted according to Zhang et al. (1998) with minor modifications, as reported by Abou-Jawdah et al. (2002) and Salehi et al. (2006). Positive controls consisted of total nucleic acid extracted from cabbage infected by an Iranian isolate of cabbage yellows (ICY) phytoplasma, belonging to 16SrVI-A subgroup (Salehi et al., 2007).

The universal phytoplasma primer pair P1/P7 (Schneider et al., 1995) was used in direct PCR to amplify a 1800 bp fragment of ribosomal operon consisting of the 16S rRNA gene, 16S-23S intergenic spacer region (ISR) and a portion of 5' region of 23S rRNA gene. In nested PCR a 1:40 dilution of PCR products amplified by P1/P7 primer pair was used as a template for a second round of PCR with primer pair R16F2n/R2 (Gundersen and Lee, 1996).

PCR was performed in 50 μl final reaction volumes containing 100 ng total DNA, 0.4 μM of each primer, 0.2 mM of dNTP mix, and 1.25 U Taq DNA polymerase (Cinagen, Iran), in 10x PCR buffer. The mixture was overlaid with 30 μl of mineral oil and subjected to initial denaturation step at 94ºC for 2 min followed by 35 cycles of 1 min at 94ºC, 2 min at 55ºC and 3 min at 72ºC. Final extension step was at 72ºC for 10 min. Ten μl of each PCR product were analyzed by electrophoresis in a 1% agarose gel in 1x TBE buffer. DNA bands were stained with ethidium bromide and visualized with a UV transilluminator.

**Restriction fragment length polymorphism (RFLP) analysis of 16S rDNA sequence.** The partial 16S rDNA sequence (1.2 kbp) amplified by nested PCR using primer pair R16F2n/R2 were analyzed by endonuclease digestion. ICY phytoplasma was used for comparison. Eight microliters of each PCR product were digested with the following enzymes, AluI, HaeIII, HhaI, HinfI, HpaII, MseI, Rsal, Sau3AI and TaqI, according to the manufacturer’s instructions. The restriction products were resolved in a 1% agarose gel in 1x TBE buffer. DNA bands were stained with ethidium bromide and visualized with a UV transilluminator.

**Cloning and sequencing of PCR products.** A 1800 bp PCR product of SP phytoplasma rDNA was ligated onto pTZ57R/T vector and cloned into Escherichia coli DH5-α cells using InstAclone™ PCR Product Cloning Kit (Fermentas, Lithuania) according to manufacturer’s instructions. Presence of the correct insert was confirmed by restriction endonuclease analysis using EcoRI and PstI enzymes. Plasmid DNA from cultures of recombinant colonies was purified using High Pure Isolation Kit (Roche, Germany). Sequencing was performed by SEQLAB (Germany) on both strands. M13 forward and reverse primers were used as sequencing primers. Internal primers were designed and used by the sequencing firm (SEQLAB). The whole length of 16S rDNA was sequenced and used for further analyses. Nucleotide BLAST search was performed to determine the closest phytoplasma relatives of the SP strain.

**Sequence homology, putative restriction site and phylogenetic analyses.** Nearly full length 16S rDNA sequences of 18 phytoplasmas including SP were aligned using Clustal X 1.81 (Thompson et al., 1994). Phylogenetic tree was constructed using the neighbor joining (NJ) plot option of Clustal X. Acholeplasma laidlawii (GenBank accession No. M23932) was used as outgroup to root the tree. Bootstrapping was performed 1000 times to estimate the stability and support for the branches. The 16S rDNA sequence homology between strains was evaluated after alignments were generated by using homology matrix distance option of DNA-MAN program version 4.02 (Lynon Corporation, Canada). Putative restriction site maps of 16S rDNA of SP and similar phytoplasma sequences belonging to 16SrVI group were generated using MapDraw option of DNASTAR (USA) program.

**RESULTS**

**Symptoms and infection rate.** Characteristic symptoms of the disease included phyllody, virescence, proliferation and sterility in the flowers, proliferation of axillary buds along the stems, leaf size reduction and stunting (Fig. 1). Infection rates of up to 9.8 % were
observed in safflower fields of Firouzabad. Lower rates were found in Darab, Zarghan and Sarchahan.

**Graft and dodder transmission.** Four out of 6 safflower plants successfully grafted with scions from diseased plants became infected and symptoms developed 45 to 70 days after inoculation. Two of 5 safflower and 3 of 6 periwinkle plants parasitized by dodder from infected safflower developed disease symptoms. The duration of the latent period in dodder-inoculated plants ranged from 10 to 13 weeks. The major symptoms shown by experimentally infected periwinkle plants were small leaves, virescence, phyllody, yellowing and stunting (Fig. 2). Infection of symptomatic graft- and dodder-inoculated plants was verified by nested PCR.

**PCR amplification.** DNA fragments of approximately 1800 bp were amplified with universal primer pair P1/P7 by direct PCR from total nucleic acid samples extracted from eight phyllody-affected safflower plants and a yellows-affected cabbage plant, used as a positive control. No amplification was observed in DNA samples from symptomless plants (data not shown). Nested PCR with primer pair P1/P7 followed by primer pair R16F2n/R2 yielded fragments of approximately 1.2 kbp from all eight symptomatic safflower plants and positive control, but none from healthy safflower or water control (Fig. 3).

**RFLP analysis.** The ribosomal DNA fragment amplified by nested PCR (1.2 kb) from SP phytoplasma and ICY positive control were separately digested with endonucleases AluI, HaeIII, HhaI, HinfI, HpaII, MseI, Rsal, Sau3AI and TaqI (Fig. 4). Based on comparison with reference strains (Lee et al., 1998; Wang et al., 1998; Davis and Dally, 2001; Wang and Hiruki, 2001; Jacobs et al., 2003; Hiruki and Wang, 2004) the SP phytoplasma pattern was similar to members of CP (16SrVI) phytoplasma group. However, SP strain was distinguishable from ICY (16SrVI-A) strain by two HhaI digestion sites (Fig. 4).

**Sequence homology, putative restriction site and phylogenetic analyses.** BLAST search showed that the 16S rDNA sequence of SP phytoplasma (GenBank accession No. DQ88948) shared the highest homology with phytoplasma sequences belonging to members of the CP (16SrVI) group. Phylogenetic analysis of 16S rDNA sequences of 18 phytoplasma strains and A. laidlawii yielded a tree (Fig. 5) whose branching order was in general agreement with previous findings (Seemüller et al., 1998; Davis and Dally, 2001). The tree clearly showed that the SP phytoplasma clustered with Ca. Phytoplasma trifolii and its relatives (CP group) being closely related to brinjal little leaf (BLL) phytoplasma. The determination of percentage homology between 16S rDNA sequences (Table 1) showed that among selected members of 16SrVI group, SP phytoplasma had maximum homology (99.7%) with BLL and periwinkle little leaf (PLL) phytoplasmas (16SrVI-C) and minimum homology with ICY phytoplasma (16SrVI-A).
**Fig. 4.** Restriction fragment length polymorphism (RFLP) profiles of 16S rDNA amplified by nested PCR using P1/P7 followed by R16F2n/R2 primer pairs from safflower phyllody phytoplasma (A) and Iranian cabbage yellows phytoplasma (B). DNA products were digested with *Taq*I, *Hin*II, *Sau*3AI, *Rsa*I, *Alu*I, *Hha*I, *Mse*I, *Hpa*II and *Hae*III and separated through a 2.5% agarose gel. Lane M, DNA ladder.

**Fig. 5.** Phylogenetic tree of full length 16S rDNA sequences of 18 phytoplasmas and *Acholeplasma laidlawii* as outgroup. The tree was constructed using NJ plot option of Clustal X program. Bootstrap values are from 1000 bootstrap repetitions. GenBank accession numbers are in parentheses.
homology (98.9%) with *Fragaria multipicta* (FM) phytoplasma (16SrVI-B). Homology of the SP phytoplasma with CP and potato witches'-broom (PWB) phytoplasmas (16SrVI-A members) was 99.3% and 99.1%, respectively, but was only 89.4% with oenothera aster yellows (OAY) (*Ca. Phytoplasma asteris*) (accession No. M30790), a non-16SrVI related phytoplasma.

Restriction maps of 16S rRNA gene showed that SP and BLL share identical restriction sites and are distinguishable from CP strain by the absence of the *HhaI* site at a position between 1000 to 1200 bp and the presence of an *MseI* site between 1 to 200 bp, and from FM strain by the absence of a *HhaI* site between 1000 to 1200 bp (Fig. 6).

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*Table 1.* Pairwise homology (%) among safflower phyllody phytoplasma and other reference phytoplasmas as determined by analysis of full length 16S rDNA sequences.

'BLL, Brinjal little leaf (X83431); CP, Clover proliferation (*Ca. Phytoplasma trifolii*) (AY390261); FM, *Fragaria multipicta* (AF500818); ICY, Iranian cabbage yellows (EF592606); OAY, Oenothera aster yellows (*Ca. Phytoplasma asteris*) (M30790); PLL, periwinkle little leaf (AF228053); PWB, Potato witches’-broom (AY300818); SP, Safflower phyllody (AY500818).

Fig. 6. Analysis of putative restriction sites of nearly full length 16S rDNA sequences of safflower phyllody phytoplasma with selected phytoplasms related to clover proliferation group. Maps were generated using the MapDraw option of DNASTAR program. Phytoplasma strain abbreviations and related sequence accession Nos. are in parentheses: BLL, Brinjal little leaf (X83431); CP, Clover proliferation (*Candidatus Phytoplasma trifolii*) (AY390261); FM, *Fragaria multipicta* (AF500818); SP, Safflower phyllody (AY500818).
DISCUSSION

Based on disease symptoms, graft and dodder transmission and positive PCR reaction with universal phytoplasma primers, the Iranian SP agent proved to have a phytoplasmal nature. RFLP analysis of PCR products amplified by the primer pair R1F2n/R2, sequence homology, putative restriction site and phylogenetic analyses of 16S rDNA indicated that SP is closely related to members of clover proliferation, 16SrVI group. Results of these analyses suggest classification of SP in the 16SrVI-C ribosomal subgroup, distinct from CP, PWB and ICY (16SrVI-A) (Hiruki and Wang, 2004; Salehi et al., 2007), FM (16SrVI-B) (Hiruki and Wang, 2004), and very close to BLL and PLL (16SrVI-C) (Siddique et al., 2001; Hiruki and Wang, 2004) strains. Previous investigations using various endonucleases, showed that phytoplasma members of the clover proliferation subgroup could be differentiated from each other by AflA, HpaI and MseI enzymes (Lee et al., 1998; Wang et al., 1998; Wang and Hiruki, 2001; Hiruki and Wang, 2004), in agreement with the results of RFLP analyses (Figs 4 and 6) of our study.

According to restriction-site analysis of PCR-amplified 16S rDNA (Schneider et al., 1993) and phylogenetic analysis of 16S rDNA (Lee et al., 2004), the safflower phyllody phytoplasma from Israel is caused by an agent belonging to 16SrI-Bs subgroup, vectored by Neoaliturus fenestratus (Raccah and Klein, 1982; Weintraub and Bealond, 2006), a leafhopper found also in safflower field in Zargarh, Iran. However, the ability of N. fenestratus to transmit the Iranian SP agent is yet to be tested. To our knowledge safflower is reported for the first time as a host of CP phytoplasma group.

REFERENCES


