


Phytoplasma Infection could Affect Chemical Composition of *Artemisia sieberi*

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Artemisia sieberi showing symptoms resembling those caused by phytoplasma were observed in Geno Mountain, Hormozgan Province, Iran, and were examined for phytoplasma presence by PCR assays. In addition, the essential oils hydrodistilled from the aerial parts of phytoplasma-infected and healthy plants have been analyzed and compared by GC and GC/MS. Phylogenetic and virtual RFLP analysis of the 16S rRNA gene sequences revealed that the phytoplasma associated with *A. sieberi* witches' broom (AsWB) was a strain of 'Candidatus Phytoplasma aurantifolia'. The presence of the disease, however, induced a further enrichment (from 4.9 to 45.2%, a relative increase of 90%) of the entire monoterpene class as compared to the abundance in healthy samples. Conversely, a matching decrease in monoterpenoid (from 48.7 to 2%, a relative decrease of 90.2%) was observed in the infected plants. Besides the first report of phytoplasma infection of *A. sieberi*, the changes of its essential oils are reported.

Keywords : dermaneh, essential oils, GC-MS, medicinal plants, phytoplasma disease

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Aromatic and medicinal plants are important sources of secondary metabolites, which have a wide range of applications in control of plant and human diseases, cosmetics, as well as in the pharmaceutical industry (Pandey and Tripathy, 2011). In the plant kingdom, family Asteraceae is endowed with essential oil-yielding plants, and among these plants, the genus *Artemisia* occupies top position for its bio-prospection (Bora and Sharma, 2011). *Artemisia* genus includes 34 species that are found wild all over the Iran. *Artemisia sieberi* (*Artemisia herba alba*) known as 'Dermaneh' in Iran is a prominent perennial dwarf grey woolly shrub that grows in open fields, road sides and waste ground of the Irano-Turanian steppes of Spain, North Africa and the Middle East, Sinai, Jordan, Syria, Iraq, Iran and Afghanistan with 50-150 cm in height (Pandey and Singh, 2017). *Artemisia* species are widely used in folk and traditional medicine for treatment of colds, coughing, intestinal disturbances, intestinal worms and wound healing in human and livestock (Mahboubi et al., 2015). The *Artemisia* genus contains more than 160 individual components, such as flavonoids, flavones, luteolin, apigenin, santonin, sesquiterpene lactones and bicyclic monoterpene glycosides, davanone and related compounds, cyclic sesquiterpenes and artemisinin (Mahboubi et al., 2015).

Phytoplasmas are cell-wall less bacterial pathogens, which inhabit the plant phloem and insects. Their hosts include crops, fruit trees, ornamentals and weeds (Bertaccini et al., 2014). Phytoplasma-infected plants showed many typical symptoms, such as witches'-broom, flower virescence, phyllody, bushy top, and so on. Up to now, most of the identified phytoplasmas have been classified into 34 groups and more than 40 subgroups according to the RFLP patterns of 16S rDNA gene (Bertaccini et al., 2014).

Phytoplasma diseases of medicinal and aromatic plants affect plant species belonging to over 70 families, mostly to Apiaceae and Asteraceae. They differ considerably in geographic distribution and size of the various taxonomic



Fig. 1. Symptoms of witches' broom and little leaf (A) in comparison with healthy *Artemisia sieberi* (B).

groups and subgroups of the associated phytoplasmas (Marcone et al., 2016).

It is known that the synthesis of essential oils can be influenced by diseases, stress factors, or elicitors (Bruni et al., 2005). For example, it has been demonstrated that phytoplasma infection results in increasing components like sesquiterpenes, alkaloids and esters (Favali et al., 2004; Mayer et al., 2008; Rid et al., 2016). However, the reduction in some specific components like flavonoids and phenolic compounds had been also reported (Bellardi et al., 2009; Bruni et al., 2005; Marcone et al., 2016). In May 2018, typical symptoms of phytoplasma disease, including witches' broom and little leaf were observed in *A. sieberi* grown in Geno Mountain of Bandar Abbas, Hormozgan province, Iran. Accordingly, they were suspected of phytoplasma infection. So, this study was carried out to characterize phytoplasma associated with this species and if infection by phytoplasma can change the composition of essential oils of *A. sieberi*.

Samples were collected from five symptomatic plants and three asymptomatic plants in May 2018 from Geno Mountain, Hormozgan province, Iran (N27°38'92"; E56°16'55") (Fig. 1). Total DNA was extracted from symptomatic and asymptomatic *A. sieberi* plants by cetyltrimethylammonium bromide (CTAB) extraction procedure

described by Sahu et al. (2012) with some modifications. Genomic DNA was used as the template for PCR analysis. 16S rDNA fragment was amplified the using phytoplasma universal primer P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995), then with nested PCR primer pair R16F2n/R16R2 using the prior PCR amplicons (1:30 dilution) as the template (Gundersen and Lee, 1996). Leaf tissue infected with "*Candidatus* Phytoplasma trifolii" (a member of 16SrVI group) was used as a positive control (Hemmati et al., 2018). The 16S rDNA fragment was cloned into pMD™ 18-T cloning vector (TaKaRa, Dalian, China) and inserted into *Escherichia coli* strain DH5a competent cells (TaKaRa). Three positive transformants were selected and sent to MacroGen Sequencing Service (Republic of Korea) for sequencing. Sequences received in this study were assembled and aligned using software: DNASTAR and ClustalX. The sequences from infected samples were identical, and three consensus sequences were deposited into the GenBank database (GenBank accession MK299844-6). Phylogenetic analyses were conducted by neighbor joining (NJ) method using MEGA 6.0 software (Tamura et al., 2013) with 10,000 replicates for bootstrap analysis. *Acholeplasma laidlawii* was used as out-group to root the tree. The sequences of 16S rDNA of different phytoplasma groups used in comprehensive phylogenetic analyses were

downloaded from GenBank database.

Aerial parts of *A. sieberi* (5 symptomatic and 5 asymptomatic) were collected at full flowering stage. The collected plant was dried naturally on laboratory benches at room temperature (23-27°C) for 5 days until it was crisp dry. Essential oil was extracted by a hydrodistillation method

using a Clevenger-type apparatus for 4 h. Analysis the oils was carried out using gas chromatography GC-mass spectrometry (GC-MS). The GC-MS were conducted on an HP 6890 GC system coupled with 5973 network mass selective detectors with a capillary column of HP-5MS (30 m × 0.25 mm, film thickness 0.25 μm). The oven temperature

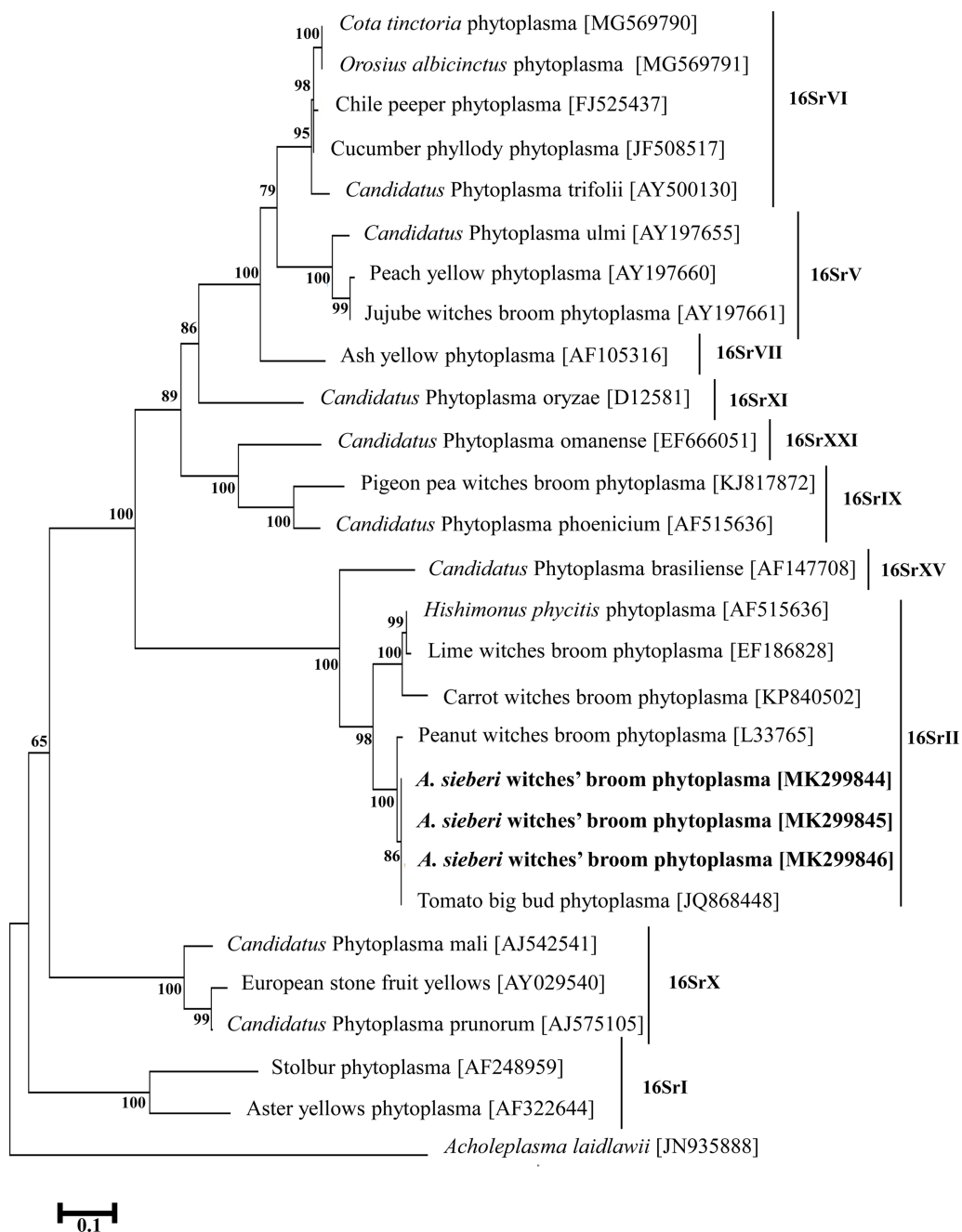


Fig. 2. Phylogenetic tree of partial 16S rDNA gene sequence from *Artemisia sieberi* witches' broom phytoplasma isolates (marked in bold) and selected phytoplasma reference sequences. GenBank accession numbers are shown in brackets, and 16Sr groups are annotated to the right. *Acholeplasma laidlawii* was used as outgroup to root the tree. The tree was constructed by the neighbor-joining method using MEGA 6 software. The bar indicates the number of nucleotides substitution per site. Bootstrap values are shown at nodes with greater than 50% support.

program was initiated at 60°C, held for 1 min, and then raised to 245°C at a rate of 3°C/min held isothermally for 10 min. Helium was used as the carrier gas at a flow rate of 1.5 ml/min. The detector and injector temperatures were 250 and 230°C, respectively. The sample was injected by splitting and the split ratio was 1:100. The quadruple mass spectrometer in electron impact ionization mode at 70 eV and an interface temperature of 250°C was scanned over the 40-500 m/z. Retention indices were calculated for all components using a homologous series of alkanes injected in conditions equal to those of the samples and by computer search using the libraries of Wiley275.L and Wiley7n.1, as well as by comparisons of their fragmentation pattern in mass spectra with published ones in the literatures (Adams, 2007).

The expected sizes of 1.8 and 1.25 kb of 16S rDNA were amplified from all symptomatic plants and the positive control, respectively, but not from the asymptomatic plants. Consequently, the plants were confirmed to be infected by a phytoplasma. The pathogen associated with *A. sieberi* witches' broom was designated as *A. sieberi* Witches' broom (AsWB) phytoplasma. Compared against the NCBI database using the BLASTn tool, the derived sequence was 99% identical with *Zinnia elegans* phyllody phytoplasma (KY501142), Alfalfa witches' broom phytoplasma (KT634120), *Cosmos bipinnatus* phyllody phytoplasma (MF186858), all of which were '*Ca. P. aurantifolia*'-related strains. Therefore, we concluded that the AsWB phytoplasma was a '*Ca. P. aurantifolia*'-related strain. A phylogenetic tree constructed was in accordance with the outcome of BLAST analysis and the sequence from present study was clustered in group 16SrII (Fig. 2). This result was further confirmed by the analysis using the *iPhyClassifier* online tool (Zhao et al., 2009) (<http://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi>) where it was determined that the *A. sieberi* phytoplasma was related to 16SrII group, subgroup D.

After steam distillation, the aerial parts of healthy and phytoplasma-infected *A. sieberi* plants produced a pale oil at a yield of 0.75 and 0.11%, respectively. A quantitative increase in excess of 60% was induced in phytoplasma-infected plants (Table 1). A total of 15 and 16 components were characterized, accounting for 97.1 and 95.65% of the essential oil in infected and healthy *A. sieberi*, respectively.

The main components were Camphor, α -Thujone, β -Thujone and 1,8-Cineole. The presence of the disease, however, induced a further enrichment (from 4.9 to 45.2%, a relative increase of 90%) of the entire monoterpene class as compared to the abundance in healthy samples. Conversely, a matching decrease in monoterpenoid (from 48.7

Table 1. Quantitative analysis of essential oil of 5 healthy and 5 phytoplasma-infected *A. sieberi* plants

Compound	Infected plant	Healthy	RI
Santolina triene	0.0	0.2 ± 0.01	930
α -Pinene	0.1 ± 0.02	0.1 ± 0.02	948
Camphene	4.0 ± 0.1	2.3 ± 0.2	960
Sabinene	0.2 ± 0.02	0.0	985
α -Terpinene	0.0	0.2 ± 0.02	1032
Cymene<o>	1 ± 0.02	1.4 ± 0.07	1037
Limonene	0.1 ± 0.08	0	1040
1,8-Cineole	2.0 ± 0.5	48.7 ± 1.6	1044
Alcohol Santolina	1.1 ± 0.1	0.0	1054
γ -Terpinene	0	0.3 ± 0.02	1071
Alcohol Artemisia	0.8 ± 0.01	0	1095
Terpinolene	0	0.2 ± 0.03	1100
α -Thujone	35.4 ± 2.45	2.6 ± 0.9	1116
β -Thujone	6.1 ± 0.91	0.1 ± 0.02	1130
Camphor	42.9 ± 3.4	32.7 ± 1.83	1158
Borneol	2.2 ± 0.09	0.95 ± 0.01	1179
Terpinen-4-ol	0.8 ± 0.02	3.1 ± 0.03	1189
Myrtenal	0.0	1.1 ± 0.01	1208
Pulegone	0.0	0.2 ± 0.01	1218
Carvone	0.2 ± 0.02	0.6 ± 0.01	1259
Bornyl acetate	0.2 ± 0.02	1 ± 0.03	1302

to 2%, a relative decrease of 90.2%) was observed in the infected plants (Table 1). Myrtenal was detected in the one healthy sample (1.1%), and it was absent from the oil obtained from diseased plants.

Based on symptoms and positive PCR reaction with universal phytoplasma primers, the AsWB is associated with a strain of "*Candidatus* Phytoplasma aurantifolia". In Iran, group 16SrII phytoplasmas have been identified in association with many diseases such as witches' broom disease of lime (WBDL), cabbage yellow, clover little leaf, alfalfa witches' broom, tomato witches' broom, sunflower phyllody, cucumber and squash phyllody, carrot witches' broom, parsley witches' broom, pomegranate little leaf, bell pepper big bud and elegant zinnia phyllody (Hemmati and Nikooei, 2017). Although the association between phytoplasmas and *A. sieberi* has not been reported yet, the association between other *Artemisia* species and phytoplasmas has been previously studied. For example, Meneguzzi et al. (2008) indicated that *A. annua* was infected by Ash yellows group in Argentina. In addition, Mori et al. (2015) reported that 16SXII-A was associated with *A. vulgaris* in Italy. Recently, the association between stolbur group (16SXII-A) and *A. scoparia* was reported in China (Yu et al., 2016). To the best of our knowledge, this is the first report of association of 16SII-D phytoplasma with *A. sie-*

beri in Iran and may be in the world. Further studies are required to find the insect vector of this phytoplasma.

The effect of phytoplasma infection on chemical composition of *A. sieberi* was studied. The component which increased drastically in infected plants was thujone. Thujone is a ketone and a monoterpene that occurs naturally in two diastereomeric (epimeric) forms: α -thujone and β -thujone. It has a menthol odor (Perry et al., 1999). Thujone acts on GABA as an antagonist (opposite to the effects of alcohol) and as a component of several essential oils, is also used in perfumery. There are controversial findings on the effects of phytoplasma infection on chemical composition of medicinal plants. Some authors indicated that phytoplasma infection could increase specific components like sesquiterpenes, esters and alkaloids (Favali et al., 2004; Mayer et al., 2008; Rid et al., 2016). However, the decrease in a main component flavonoids and phenolic compounds had been also reported (Marcone et al., 2016). According to our results, phytoplasma disease could thus become an issue in defining the market value for cultivation product of *A. sieberi* cultivated plants. So, we could reinforce a hypothesis that artificial infection could yield a high amount of thujone which can be used in pesticides and perfumery. However, the phytopathological status of *A. sieberi* propagation material must be carefully controlled and such controls should be included in the guidelines for good agricultural practice.

In addition, camphor which had the highest value among all component, was increased in infected plants. Camphor is believed to be toxic and is thus sometimes used as a repellent to insects, antimicrobial substance and used for mummification. Many researchers demonstrated that the insecticidal activity of *A. sieberi* is due to camphor. For example, Negahban et al. (2006) conducted a research and reported that the insecticidal activity of *A. sieberi* is related to camphor compared with other component. Like the above hypothesis, to reach high amount of this component, we can artificially infect the plants and get more such component to be used in pesticides.

Myrtenal was detected in the one healthy sample (0.92%), and it was absent from the oil obtained from diseased plants. Such behavior seems to fit with the consequences of drought stress in essential oil bearing plants and could be related with phloem necrosis typically caused by phytoplasmas (Mohamed et al., 2002).

In conclusion, plant secondary metabolites concentration in essential oils can be up- or downregulated in phytoplasma infected plants (Favali et al., 2004). Because the medicinal properties of *A. sieberi* are derived from secondary metabolites, whether the bioactive substance is also affected by phytoplasma infection needs further investiga-

tion.

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