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Changes in the composition of volatile compounds of *Spartium junceum* induced by the phytoplasmal disease, Spartium witches'-broom

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Abstract
In southern Italy, *Spartium junceum* (Spanish broom) is severely affected by a phytoplasmal disease, Spartium witches'-broom (SpaWB). The volatile fractions extracted from flowers of healthy and diseased plants, examined by gas chromatography and gas chromatography–mass spectrometry, appeared to be quantitatively and qualitatively different. In both the healthy and the diseased plants, the main components were *n*-alkanes, which occurred at a rate of 55.2% and 38.8%, respectively. The level of aliphatic acids was considerably lower in flowers of the diseased plants than in those of the healthy plants (4.5% vs. 18.7%). Sesquiterpenes were detected only in the diseased plants. It is possible that the changes in the composition of secondary metabolites of diseased plants can be related to plant defense responses.

Abbreviations: AP, apple proliferation; EY, elm yellows; SpaWB, Spartium witches'-broom

Keywords: *Phytoplasmas, plant defense responses, sesquiterpenes,* Spartium junceum*, volatile fraction*

Introduction

Spartium junceum L. (Spanish broom) (Fabaceae) is a thornless shrub, up to 3 m tall, with deep golden-yellow flowers, that is common in Mediterranean areas (Pignatti 1982). This rapidly growing plant is highly adaptable to various environmental conditions (Bezic et al. 2003) and is of considerable importance due to its role in decreasing soil erosion. The plant is also used as an ornamental shrub and, mainly in the past, for fiber production. In addition, it has medicinal properties. The young herbaceous tips of flowering shoots, harvested in spring, are considered cardiotonic, emetic and purgative; the entire plant is claimed to be a strong diuretic, whereas the seeds have both properties (Gastaldo 1987). The flowers possess a potent anti-ulcerogenic activity (Yesilada et al. 1993; Yesilada & Takaishi 1999). *S. junceum* is also commonly called fragrant broom due to the fragrance of its flowers, containing a volatile fraction widely used in perfumery (Miraldi et al. 2004; Lawrence 2006).

In southern Italy, Spanish broom is severely affected by a lethal phytoplasmal disease, Spartium witches'-broom (SpaWB). The most characteristic symptoms of the disease are pronounced "witches'-brooms," shortened internodes, off-season growth and, ultimately, death of the plant (Figure 1). SpaWB is associated with two genetically different phytoplasmas that induce the same symptoms. These are (1) "*Candidatus* Phytoplasma spartii," a member of the apple proliferation (AP) phylogenetic group, and (2) a phytoplasma that belongs to the elm yellows (EY) phylogenetic group (Marcone et al. 1996, 2004; Seemüller et al. 1998). Most of the diseased plants are doubly infected with the two phytoplasmas, one of which is predominant and readily detectable by direct ("one-round") polymerase chain reaction (PCR) assays, whereas the other occurs at a very low titer and can be detected only by the highly sensitive nested PCR method (Marcone et al. 1996, 1998, 2004). In contrast to the progress made in the detection, differentiation

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Figure 1. Witches'-brooms on diseased *Spartium junceum* (Spanish broom) plants.

and phylogenetic classification of phytoplasmas, very little is known about the effects of phytoplasmal infections on the biochemical content of diseased plants. Phytoplasmas are wall-less, unculturable bacteria of the class Mollicutes that induce diseases in more than 1000 plant species worldwide. In diseased plants, they reside almost exclusively in the phloem sieve tube elements and are transmitted from plant to plant by phloem-feeding homopteran insects, mainly leafhoppers (Cicadellidae).

The aim of this work was to identify possible changes in the chemical composition of the volatile fraction from flowers of SpaWB-affected Spanish broom plants in comparison with healthy ones.

Material and methods

Plant material

Sampling of fully symptomatic and non-symptomatic *S. junceum* L. plants was carried out in one location near Salerno in the Campania region (southern Italy) in the spring of 2008 at the full flowering stage. Voucher specimens of healthy and diseased plants are deposited in the herbarium of the Medical Botany Chair at the state university of Salerno.

Phytoplasma reference strains

For comparison, DNA samples from Spanish broom plants infected by "*Candidatus* Phytoplasma spartii" and the EY-related phytoplasma, which were previously examined (Marcone et al. 1996), were included in the study. In addition, the phytoplasma reference strains AT of "*Candidatus* Phytoplasma mali," ULW of "*Candidatus* Phytoplasma ulmi" and ALY of the alder yellows agent, which were previously transmitted to *Catharanthus roseus* (L.) G. Don (periwinkle) and maintained in this experimental host by periodic grafting, were used (Marcone et al. 1996; Seemüller et al. 1998).

DNA extraction, PCR amplification and RFLP analysis

For DNA extraction, phloem preparations from 2- to 4-year-old stem portions of the healthy and the diseased Spanish broom plants were used. Phloem tissue was prepared as described by Ahrens and Seemüller (1994). Young shoots, including leaves, were taken from healthy and diseased periwinkles. DNA was isolated from approximately 1.0 g of fresh tissue using a phytoplasma enrichment procedure as described previously (Ahrens & Seemüller 1992).

PCR amplification ("one-round") was performed using either the universal phytoplasma primer pair P1/P7 or the primer pair fB1/rULWS, which specifically amplifies phytoplasmal ribosomal DNA (rDNA) from the EY agents (Marcone et al. 1996). In nested PCR assays, initial amplification was carried out with the primer pair P1/P7. The products obtained were then re-amplified with primer pairs fB1/rULWS or P1/rSP. The reverse primer rSP is specific for "*Candidatus* Phytoplasma spartii" (Marcone et al. 1996, 2004). PCR conditions, gel electrophoresis of PCR products and restriction fragment length polymorphism (RFLP) analysis of P1/P7 and fB1/rULWS amplicons were carried out as described in a previous work (Marcone et al. 1996, 1997, 1998).

Volatile fraction extraction

For volatile fraction extraction, flowers from the healthy and the diseased Spanish broom plants were used. Due to difficulties in obtaining flowers from a single diseased plant in sufficient amounts, pooled flowers taken from 15 plants that were phytoplasma-positive were employed. Also, flowers from a representative number of healthy plants were pooled. The volatile fraction was extracted from 50 g of fresh flowers by hydrodistillation for three hours and following the procedure described by the European Pharmacopoeia (Council of Europe 2004). The extraction procedure was repeated three times.

GC and GC–MS analyses

The volatile fractions were analysed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS). Analytical GC was carried out on a PerkinElmer Sigma 115 gas chromatograph fitted with an HP-5 MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The column temperature was initially kept at 40°C for 5 min, then gradually increased to 250°C at a rate of

2°C/min, held for 15 min and finally raised to 270°C at a rate of 10°C/min. Amounts of 1 µl were injected at 250°C, manually and in the splitless mode. Flame ionization detection was performed at 280°C. The analysis was also run by using a fused silica HP Innowax polyethylene glycol capillary column (50 m × 0.20 mm i.d., 0.20 µm film thickness). In both cases, helium was used as the carrier gas (1 ml/min). GC–MS analysis was performed on an Agilent 6850 Series II apparatus; fitted GC–MS analysis was performed using an Agilent 6850 Ser. A apparatus, equipped with a fused silica HP-5 capillary column (30 m × 0.25 mm i.d., 0.33 µm film thickness), linked on line with an Agilent Mass Selective Detector MSD 5973 (ionization voltage 70 eV, electron multiplier energy 2000 V). Gas chromatographic conditions were as given above; transfer line was kept at 295°C.

Most components were identified from their GC retention indices, either with those reported in the literature (Jennings & Shibamoto 1980; Davies 1990) or with those of authentic compounds purchased from Sigma-Aldrich Co. (Milan, Italy). The retention indices were determined in relation to a homologous series of *n*-alkanes (C_8–C_{24}) under the same operating conditions. Further identification was made by comparison of their MS spectra either with spectral data stored in NIST 02 and Wiley 275 libraries or with mass spectra from the literature (Jennings & Shibamoto 1980; Adams 2001) and our home-made library. The relative concentrations of the components were calculated based on GC peaks without using correction factors.

Results

PCR assays

By PCR assays using primers derived from rDNA sequences, phytoplasmal infections were detected in all symptomatic Spanish broom plants examined. Neither by direct ("one-round") nor by nested PCR assays was DNA amplified from template DNA isolated from any of the non-symptomatic plants. On the basis of primer specificity and RFLP analysis of PCR-amplified phytoplasmal rDNA, all 15 diseased plants examined proved to be primarily infected by the EY-related phytoplasma, whereas "*Candidatus* Phytoplasma spartii" occurred in low concentration and thus could be detected only through nested PCR. Examples of PCR amplification and RFLP analysis of PCR-amplified phytoplasmal rDNA from SpaWB-affected Spanish broom plants have been previously shown (Marcone et al. 1996, 1997, 1998) and are also shown in Figures 2 and 3.

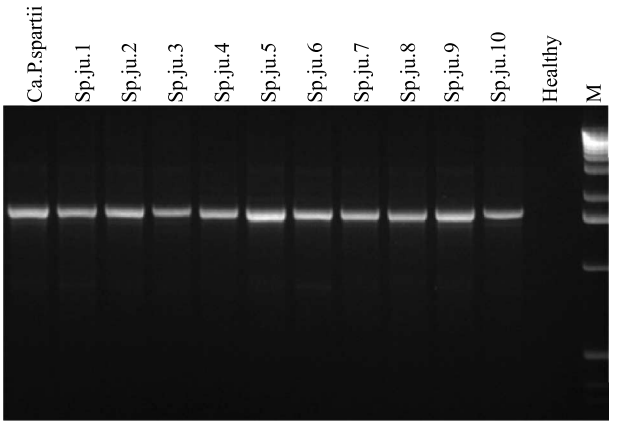

Figure 2. Detection of "*Candidatus* Phytoplasma spartii" by nested PCR assays using the universal phytoplasma primer pair P1/P7 followed by the primer pair P1/rSP.
Note: Ca. P. spartii, "*Candidatus* Phytoplasma spartii"; Sp.ju.1 through Sp.ju.10, samples from diseased Spanish broom plants; Healthy, healthy Spanish broom; M, 1-kb DNA ladder (BRL Life Technologies).

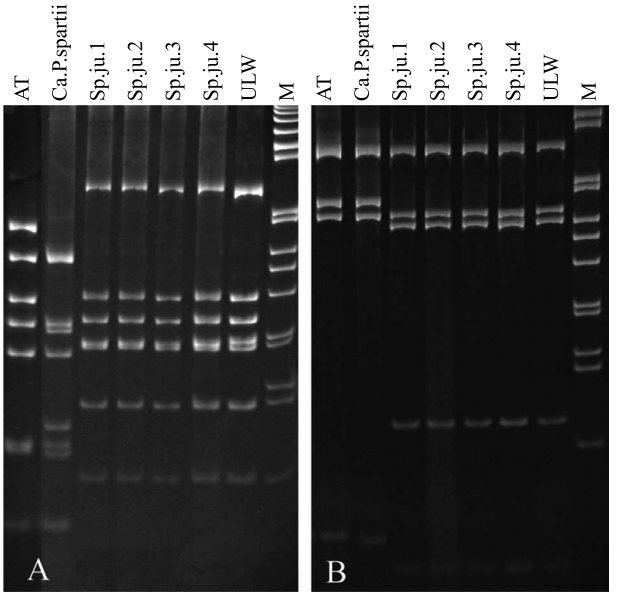

Figure 3. *Alu*I (A) and *Rsa*I (B) restriction profiles of phytoplasma ribosomal DNA amplified by PCR using the universal primer pair P1/P7.
Note: AT, strain of "*Candidatus* Phytoplasma mali"; Ca. P. spartii, "*Candidatus* Phytoplasma spartii"; Sp.ju.1 through Sp.ju.4, samples from diseased Spanish broom plants; ULW, strain of "*Candidatus* Phytoplasma ulmi"; M, 1-kb DNA ladder (BRL Life Technologies).

Analysis of volatile fraction

Flowers from the healthy and the diseased plants yielded a volatile fraction at a rate, calculated on a fresh weight basis, of 0.022 ± 0.017% and 0.015 ± 0.004%, respectively. Also, the qualitative profile of the oil extracted from the healthy and the diseases plants differed significantly. The composition of the

Table I. Volatile fraction composition of flowers from healthy and spartium witches'-broom-affected *S. junceum* plants.

K_i^a	K_i^b	Compound	Healthy (%)	Diseased (%)	Identification[c]
1234	1662	Pulegone	0.6 ± 0.1	ND	R_i, MS
1435	1650	γ-Elemene	ND	5.0 ± 0.3	R_i, MS
1563	2050	(*E*)-Nerolidol	ND	3.6 ± 0.5	R_i, MS
1567	2503	Dodecanoic acid	1.6 ± 0.1	ND	R_i, MS, Co-GC
1574	2069	Germacrene D-4 ol	ND	0.5	R_i, MS
1578	2008	Caryophyllene oxide	ND	1.1	R_i, MS, Co-GC
1640	2187	τ-Cadinol	ND	19.2 ± 0.4	R_i, MS
1686	2219	α-Bisabolol	ND	1.1 ± 0.1	R_i, MS
1758	2713	Tetradecanoic acid	7.7 ± 0.1	1.8 ± 0.1	R_i, MS, Co-GC
1959	2931	Hexadecanoic acid	6.9 ± 0.1	ND	R_i, MS, Co-GC
1983	2234	Hexadecanoic acid ethyl ester	1.6 ± 0.1	1.0 ± 0.1	R_i, MS, Co-GC
2100	2100	Heneicosane	0.8 ± 0.1	ND	R_i, MS
2163	2569	(*Z,Z,Z*)-9,12,15-Octadecatrienoic acid ethyl ester	ND	1.7 ± 0.2	R_i, MS, Co-GC
2187	2467	(*Z*)-9-Octadecenoic acid ethyl ester	0.9 ± 0.3	ND	R_i, MS, Co-GC
2200	2200	Docosane	0.6 ± 0.1	0.5 ± 0.2	R_i, MS, Co-GC
2300	2300	Tricosane	14.4 ± 0.7	5.5 ± 0.1	R_i, MS, Co-GC
2400	2400	Tetracosane	2.9. ± 0.5	1.9 ± 0.8	R_i, MS, Co-GC
2500	2500	Pentacosane	7.3 ± 0.6	3.1 ± 0.5	R_i, MS
2592		Hexacosene	0.3 ± 0.1	ND	R_i, MS
2600	2600	Hexacosane	2.5 ± 0.1	2.8 ± 0.7	R_i, MS
2700	2700	Heptacosane	4.6 ± 0.4	3.8 ± 0.5	R_i, MS
2800	2800	Octacosane	3.3 ± 0.6	3.2 ± 0.5	R_i, MS
2827	3063	Squalene	1.5 ± 0.2	1.5 ± 0.1	R_i, MS
2894		Nonacosene	0.4 ± 0.1	0.6 ± 0.4	R_i, MS
2900	2900	Nonacosane	4.4 ± 0.1	5.3 ± 0.5	R_i, MS
2985		Methyltriacontane	1.2 ± 0.3	ND	R_i, MS
3000	3000	Triacontane	4.5 ± 0.5	5.0 ± 0.4	R_i, MS
3100	3100	Hentriacontane	3.3 ± 0.4	2.8 ± 0.1	R_i, MS
3200	3200	Dotriacontane	2.7 ± 0.1	2.4 ± 0.2	R_i, MS
3300	3300	Tritriacontane	2.0 ± 0.6	2.5 ± 0.7	R_i, MS
3400	3400	Tetratriacontane	0.7 ± 0.1	ND	R_i, MS

[a]Retention index on HP-5 column.
[b]Retention index on HP Innowax column.
[c]R_i, retention index identical to bibliography; MS, identification based on the comparison of mass spectra; Co-GC, retention time identical to authentic compound.
Note: ND, not detected.

essential oils are reported in Table I, in which the components are listed in the order of elution on an HP-5 MS column (w/w). Twenty-four components, accounting for 76.7% of the volatile fraction composition, were identified in the healthy plants, whereas 23 components, accounting for 75.9% of the volatile fraction, were present in the diseased plants. In both the healthy and the diseased plants, the main components were *n*-alkanes, which occurred at a rate of 55.2% and 38.8%, respectively. Among them, tricosane (14.4%), pentacosane (7.3%) and heptacosane (4.6%) were the main components in the healthy plants, whereas tricosane (5.5%), nonacosane (5.3%) and triacontane (5.0%) were prevalent in the diseased plants. The amount of aliphatic acids was considerably lower in flowers of the diseased plants than in those of the healthy plants. In the healthy plants, such compounds amounted to 18.7% of the volatile fraction, with tetradecanoic acid (7.7%) and hexadecanoic acid (6.9%) present at the highest amounts. In the diseased plants, the aliphatic acid level was 4.5%. Monoterpenes were almost absent in the healthy plants except pulegone, which occurred at a rate of 0.6%. Conversely, approximately one-third of the volatile fraction of the diseased plants was represented by six sesquiterpenes (30.5%). Among them, five (25.5%) were oxygenated compounds. τ-Cadinol (19.2%) was the main component.

Discussion

Our data demonstrate that the volatile fraction yield was reduced in the diseased plants and that there was a marked increase in the amount of sesquiterpenes and a decrease in the amount of *n*-alkanes and aliphatic compounds. The results of our study largely agree with the findings of Bruni et al. (2005),

who found sesquiterpenes as defense compounds in ash yellows phytoplasma infections in *Hypericum perforatum*. Moreover, Miraldi et al. (2004) found that the main components of the volatile fraction extracted from flowers of apparently healthy *S. junceum* plants sampled in Tuscany, Italy, were hydrocarbons, which altogether accounted for about 48% of the total volatile fraction composition.

The presence of substantial amounts of sesquiterpenes in the volatile fraction of SpaWB-affected Spanish broom plants and their absence in the healthy plants may be related to the role of phytoplasmal infections in triggering plant defense responses. Plant antimicrobial compounds include a broad array of low-molecular-weight secondary metabolites known as phytoalexins. Among these, sesquiterpenes have been shown to be induced in several plants challanged by fungi, bacteria and abiotic and other biotic factors (Hammerschmidt 1999; Xu et al. 2004; Cardoza & Tumlinson 2006). The relationship between the pest or pathogen and the infected plant has been studied previously as well. Rajeswara Rao et al. (2004) reported that leaf-sucking insects affected the composition of the essential oil of *Cymbopogon winterianus* Jowitt and that the total amount of sesquiterpenes increased with the increase in the severity of the disease. The effects of *Fusarium oxysporum* var. *redolens* (Wollenweb.) on the yield and quality of oils of *Pelargonium* species have also been reported (Rajeswara Rao et al. 1999).

Thus, the present work can contribute to expanding our knowledge of the effects of phytoplasmal infections on the secondary metabolism of plants.

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