Identification of a 16SrII-E Phytoplasma in *Calendula arvensis*, *Solanum nigrum*, and *Chenopodium* spp.

G. Tolu, Dipartimento di Protezione delle Piante-sezione di Patologia vegetale, Università degli Studi, via De Nicola 1, 07100 Sassari, Italy; S. Botti, DiSTA, Patologia vegetale, Alma Mater Studiorum, Università di Bologna, viale Fanin, 42, 40127 Bologna, Italy; R. Garau, V. A. Prota, A. Sechi, and U. Prota, Dipartimento di Protezione delle Piante-sezione di Patologia vegetale, Università degli Studi, via De Nicola 1, 07100 Sassari, Italy; and A. Bertaccini, DiSTA, Patologia vegetale, Alma Mater Studiorum, Università di Bologna, viale Fanin, 42, 40127 Bologna, Italy

ABSTRACT

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Epidemiological surveys were performed in Northern Sardinia (Italy) in a 10-year-old vineyard affected by “Bois noir” disease. Samples collected between May and October 2003 from chlorotic and stunted weeds belonging to 14 different taxonomic groups were indexed molecularly for detection of phytoplasmas. Nested polymerase chain reaction (PCR) assays using primers specific for the phytoplasma 16SrDNA gene showed three of six *Calendula arvensis*, one of two *Solanum nigrum*, and one of seven *Chenopodium* spp. assayed positive. Restriction fragment length polymorphism analyses and sequencing of amplified 16SrDNA fragments identified a putative phytoplasma in the ribosomal subgroup 16SrII-E. Further characterization of the *rps3* gene, coding a ribosomal protein, confirmed the identification. However, the weeds and leafhopper species collected in the vineyard tested negative by PCR assays for the Stolbur phytoplasma, the causal agent of “Bois noir”. This is the first report of a phytoplasma of the 16SrII-E subgroup infecting *C. arvensis*, *S. nigrum*, and *Chenopodium* spp.

Phytoplasmas, prokaryotes with phloematic habitats, are found throughout the world on wild and cultivated plants (25); they belong to the class *Mollicutes* and are taxonomically distinguished by molecular characteristics, especially on 16S ribosomal genes, established principally by polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and phylogenetic comparisons (17). Phytoplasma infections cause symptoms on the plant which are often non-specific and not associated with a particular phytoplasma taxonomic group. Frequent symptoms caused by phytoplasmas are yellowing, discoloration, witches'-broom, dwarfing, virescence, and phyllody. In nature, phytoplasmas are transmitted by leafhoppers (Auchenorrhyncha). These insects may have an occasional or a permanent and essential trophic relationship with their hosts. *Scaphoideus titanus* Ball, for example, is an essential ampelophaga, i.e., it feeds only on grape, whereas *Euscelis lineolatus* Brullè, *Laodelphax striatellus* Fallén, and others are well known to be dependent on herba-

ceous species and may only occasionally be ampelophaga.

On *Vitis vinifera*, the phytoplasmas cause a syndrome called “yellows”, found in Europe and in grape-growing areas outside Europe. Yellows is mainly characterized by yellow leaves, yellow perivascular bands, leaf curling, and lack of cane lignification. These symptoms are found in both “Flavescence dorée” and “Bois noir” diseases. In “Flavescence dorée”, the symptoms are caused by phytoplasmas that belong to the 16SrV group (Elm yellows), transmitted by the cicadellid *S. titanus*. In “Bois noir” disease, symptoms are caused by phytoplasmas of the 16SrXII-A subgroup transmitted by the cixid *Hyalesthes obsoletus* Signoret. Although this phytoplasma has frequently been found in the herbaceous flora of vineyards and in various species of insects (15,16,20), the epidemiological relationship between the host and the vector is not completely clarified.

Grapevine yellows is widespread in different viticultural areas of Sardinia (Italy). Based on recent studies, symptomatic plants are infected by the Stolbur phytoplasma belonging to the 16SrXII-A subgroup, inducing the “Bois noir” disease on grapevines (6). The leafhopper *Goniathus guttulineris* (Kirschbaum) is a natural host of this phytoplasma; however, its role as a vector has not yet been established (5). Although the presence of this phytoplasma in weeds and in different leafhopper species has been repeatedly

reported, there are cases in which the epidemiology of grapevine yellows and its relationship with possible vectors has not been established. In Sardinia, for example, outbreaks of “Bois noir” have occurred in an apparent absence of the vector *H. obsoletus*. Thus, a study was undertaken to assess the presence of Stolbur phytoplasmas in weeds and insects in a vineyard affected by “Bois noir”.

MATERIALS AND METHODS

Field survey. Weed samples were collected from May to October 2003 among the most common species growing within a Chardonnay vineyard of northern Sardinia, and analyzed for phytoplasma presence using molecular techniques. Seventy-eight samples were tested: *Lolium* spp. (6 plants), *Convolvulus arvensis* L. (7), *Chenopodium* spp. (7), *Trifolium repens* L. (6), *Malva silvestris* L. (5), *Calendula arvensis* L. (6), *Erigeron canadensis* L. (6), *Helminthia echinoides* Gaertn. (5), *Plantago lanceolata* L. (5), *Amaranthus* spp. (6), *Solanum nigrum* L. (2), *Bromus sterilis* L. (6), *Avena fatua* L. (6), and *Hordeum murinum* Huds. (5). Most plants showed apical yellowing, while *C. arvensis* plants were stunted.

Preliminary studies to determine the presence of Auchenorrhyncha on the weeds of the vineyard were carried out monthly from May to August 2003. Standard entomological nets were used to collect the samples; the insects were collected from 150 m of the vineyard in all, and from four rows. The captured insects were classified, preserved in a -20°C freezer for a week, and then examined molecularly. The tests were carried out as described below on 14 samples, in batches ranging from 5 to 15 individuals, according to the size of the species.

PCR and RFLP on 16SrDNA and *rps3* gene sequences. Nucleic acid extraction for PCR analyses was performed on weed and insect samples using the Doyle and Doyle system (4). The DNA was stored in TE at -20°C. A direct PCR was carried out with universal primers R16F2/R2 (9). A touch-down procedure was used, consisting of heating at 95°C for 5 min, at 58°C for 1.5 min, followed by a one degree decrease with eight 1.5-min cycles to 50°C, and a final extension step

Corresponding author: A. Bertaccini
E-mail: Bertaccini_a@biblio.cib.unibo.it

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Table 1. Phytoplasma 16SrDNA sequences retrieved from GenBank, including AJ744767 – *Calendula* stunt from Sardinia – and employed for phylogenetic analysis

Accession no.	Phytoplasma strain	Abbreviation	Origin	16SrRNA group ^a
AF438413	Alfalfa witches'-broom	OmanAlfWB	Oman	16SrII-D
AF248958	Apple proliferation	AP	Italy	16SrX-A
AF189215	Ash yellows	ASHY	New York	16SrVII-A
AF222063	Aster yellows	AY	Maryland	16SrI-B
AF248961	Bermuda grass white leaf	BGWL	Thailand	16SrIV-A
AF228052	Brinjal little leaf	BLL	India	n.a.
AF200718	Cactus	Cactus	Mexico	n.a.
AJ744767	<i>Calendula</i> stunting	<i>Calendula</i> stunt	Italy	16SrII-E
AF515637	" <i>Candidatus</i> Phytoplasma phoenicium"	CP	Lebanon	16SrIX
AF495882	Chinaberry yellows	CbY	Bolivia	16SrXIII
AF028813	Chinese pigeon pea witches'-broom	CPPW	Taiwan	16SrII
AF173558	Clover yellow edge	CYE	Canada	16SrII-B
AY101386	Epilobium phyllody	EP	Estonia	16SrI
AF411592	Erigeron witches'-broom	EWB	Brazil	16SrVII
AY029540	European stone fruit yellows from apricot	ESFY	Austria	16SrX-B
X83432	Faba bean phyllody	FBP	Sudan	16SrII-C
AB026155	Gerbera phyllody	GP	Japan	16SrII-C
Y16390	Italian alfalfa witches'-broom	ItalAWB	Italy	n.a.
AF305240	Jujube witches'-broom	JJWB	China	16SrV-B
Y18053	Knautia phyllody	KP	Italy	n.a.
AF515638	Lactuca serriola phyllody	LsP	Lebanon	16SrIX
U15442	Lime witches'-broom	LWB	Oman	16SrII-B
AF248956	Loofah witches'-broom	LfWB	Taiwan	16SrVIII-A
AF248960	Mexican periwinkle virescence	MPV	Mexico	16SrXIII-A
Y10097	Papaya yellow crinkle	PapayaYC	Australia	n.a.
AF228053	Periwinkle little leaf	PerLL	Bangladesh	n.a.
Y15866	Phytoplasma sp. Strain Vill	ViLL	Australia	n.a.
Y16393	<i>Picris echioides</i> phyllody	PEP	Italy	16SrII-E
Y16389	<i>Picris echioides</i> yellows	PEY	Italy	16SrIX
AJ310849	Pine phytoplasma	Pine	Germany	n.a.
U89378	Rape phyllody	RP	Czech Rep.	n.a.
Y16395	Rubus stunt	RuS	Italy	16SrV-E
AJ289192	Stilosantes little leaf	StiLL	Australia	n.a.
AF248959	Stolbur	STOL	Serbia	16SrXII-A
AJ243044	Strawberry green petals	AustrSG	Australia	16SrXII-B
AF056095	Sugarcane yellows	SCY	South Africa	n.a.
AF222064	Tomato big bud	TomBB	Arkansas	16SrI-B
AF521672	Weeping tea tree witches'-broom	WttWB	Australia	n.a.

^a n.a. = group not available.

Fig. 1. Polyacrylamide gel showing restriction fragment length polymorphism patterns of 16SrDNA amplified with primer pair R16(I)F1/R1 from weed samples of *Chenopodium* spp. (Ch), *Solanum nigrum* (SN), and *Calendula arvensis* (L4, L7, and L8) and selected reference phytoplasma strains digested with restriction enzymes *TruI* and *Tsp509I*. CLP, cleome phyllody (16SrII-A); LWB, Lime witches'-broom (16SrII-B); CrP, crotalaria phyllody (16SrII-C); TBB, tomato big bud from Australia (16SrII-D); PEP, *Picris echioides* phyllody (16SrII-E); CoP, cotton phyllody (16SrII-F). P, ΦX174, marker *HaeIII* digested; fragment sizes in base pairs from top to bottom: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

at 72°C for 1.2 min. Then PCR continued for 25 cycles at 95°C for 1 min, 50°C for 1.30 min, and 72°C for 1.2 min with a final step at 72°C for 5 min. Amplification was done with a Thermocycler (Hybaid, Middlesex United Kingdom, EU) in 50-μl reactions containing 1× PCR buffer, 2 mM MgCl₂, 200 μM of each dNTP (Invitrogen, Carlsbad, CA, USA), R16F2/R2 100 nM, and 1 U of Red *Taq* (Sigma-Aldrich, St. Louis, MO, USA).

Two microliters of diluted (1:50) PCR products from the first amplification were employed as templates for nested PCR using the primer pair R16(I)F1/R1 (18). Thirty-five PCR cycles were run with the following parameters: 1 min (3 min for the first cycle) at 94°C, 1 min at 50°C, and 75 s (5 min in final cycle) at 72°C. The 50-μl reaction mix contained 1× PCR buffer, 2 mM MgCl₂, 200 μM of each dNTP, 1 μM R16(I)F1/R1, and 1 U of Red *Taq*. Amplified DNA was separated by electrophoresis in 1.2% agarose gels stained with ethidium bromide; the bands were visualized with a Gel-Doc 2000 Documentation System (Bio-Rad Laboratories, Milan, Italy, EU).

Nucleic acid from the positive samples and from the reference samples in periwinkle (*Catharanthus roseus*): CLP, cleome phyllody (16SrII-A); CrP, crotalaria phyllody (16SrII-C); TBB, tomato big bud from Australia (16SrII-D); PEP, *Picris echioides* phyllody (16SrII-E); CoP, cotton phyllody (16SrII-F), as well as from lime witches'-broom phytoplasma (16SrII-B) obtained from lime (*Citrus aurantiaca*) (12), which represented different ribosomal subgroups in ribosomal group 16SrII (12), was amplified again using primers P1/P7 (3,26) followed by nested PCR as described above with the primer pair R16F2/R2. The amplicons were obtained in a total volume of 25 μl PCR mixture containing 2.5 μl of 10× PCR buffer, 200 μM of each dNTP, 0.4 μM of each primer, and 0.625 U of *Taq* polymerase (Polymed, Florence, Italy, EU). Samples lacking DNA were run as negative controls. After amplification, 5-μl aliquots from each sample were separated by electrophoresis in a 2% agarose gel, stained with ethidium bromide, and visualized using Gel-Doc.

Two hundred to 300 ng of R16(I)F1/R1 amplicons were then subjected to RFLP analysis using *TruI* and *Tsp509I* (Fermentas, Vilnius, Lithuania, EU) following the manufacturer's instructions. Further RFLP analyses were carried out on R16F2/R2 nested PCR products with *TruI*, *Tsp509I*, and *DdeI* (Fermentas). The restriction patterns were compared after electrophoresis in a 5% polyacrylamide gel and staining of the products with ethidium bromide.

Under similar conditions, further amplifications for molecular characterization of phytoplasmas were performed on the S3 ribosomal protein of the *C. arvensis* L4 sample using semi-nested PCR primers

Fig 2. Polyacrylamide gel showing restriction fragment length polymorphism patterns of reference phytoplasma strains and strains from weed samples amplified with phytoplasma-specific primers R16F2/R2 and digested with restriction enzymes *Tsp509I* and *DdeI*. CLP, cleome phyllody (16SrII-A); LWB, Lime witches'-broom (16SrII-B); CrP, crotalaria phyllody (16SrII-C); TBB, tomato big bud from Australia (16SrII-D); PEP, *Pichris echinoides* phyllody (16SrII-E); CoP, cotton phyllody (16SrII-F). P, Φ X174, marker *HaeIII* digested; fragment sizes in base pairs from top to bottom: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Fig. 3. Polyacrylamide gels showing the restriction fragment length polymorphism of reference phytoplasma samples and sample L4 amplified with primers rp(II)F1/rp(I)R1A followed by primers rp(II)F2/rp(I)R1A in seminested polymerase chain reaction (PCR) and digested with restriction enzymes *Tsp509I* and *AluI*. CLP, cleome phyllody (16SrII-A); LWB, Lime witches'-broom (16SrII-B); CrP, crotalaria phyllody (16SrII-C); TBB, tomato big bud from Australia (16SrII-D); PEP, *Pichris echinoides* phyllody (16SrII-E); CoP, cotton phyllody (16SrII-F). P, Φ X174, marker *HaeIII* digested; fragment sizes in base pairs from top to bottom: 1,353, 1,078, 872, 603, 310, 281, 271, 234, 194, 118, and 72.

Table 2. Analysis of sequence similarities among 16SrRNA gene sequences from *Calendula arvensis* sample L4 (Calendula stunt) and nine phytoplasmas, the most of them belonging to 16SrII group

Phytoplasma ^a	16SrRNA subgroup		1	2	3	4	5	6	7	8	9	10
Calendula stunt	II-E	1	100	...	...	...	...	...	...	...	...	...
PEP	n.a. ^b	2	99.38	100	...	...	...	...	...	...	...	...
ItalAWB	n.a.	3	98.24	98.87	100	...	...	...	...	...	...	...
PapayaYC	II	4	97.98	98.61	98.87	100	...	...	...	...	...	...
CPPW	II	5	97.80	98.44	98.70	99.82	100	...	...	...	...	...
GP	n.a.	6	97.63	98.27	98.53	99.65	99.48	100	...	...	...	...
LWB	II-B	7	97.54	98.18	98.10	98.53	98.36	98.18	100	...	...	...
FBP	II-C	8	97.45	98.10	98.18	98.44	98.27	98.10	98.81	100	...	...
Cactus	n.a.	9	97.36	97.43	97.70	98.10	98.09	97.73	98.58	99.30	100	...
OmanAlfWB	II-D	10	97.02	97.67	97.93	99.05	98.88	98.70	97.32	97.50	97.35	100

^a Phytoplasma abbreviations are explained in Table 1.

^b n.a. = group not available.

specific for ribosomal group 16SrII: rp(II)F1/rp(I)R1A followed by primers rp(II)F2/rp(I)R1A (22). Amplified products were then digested with *Tsp509I* and *AluI* (Fermentas), and compared with reference phytoplasma strains from subgroups of the 16SrII ribosomal group.

Sequencing and phylogenetic analysis.

Further amplifications to obtain DNA for sequencing were performed on the L4 sample from *C. arvensis* using the P1/P7 primer pair; the product from this reaction was amplified by nested PCR using the primers R16F2/R2. The conditions for direct and nested PCR were as follows: 95°C for 2 min, 96°C for 30 s, 50°C for 30 s, 72°C for 1.3 min, and 72°C for 10 min for the last step, 35 cycles for the first PCR and 40 cycles for nested PCR. The 50- μ l reaction mix contained 1 $\times$ PCR buffer, 2 mM MgCl₂, 200 μ M each of the four dNTPs, 1 U of Red *Taq*, 350 nM of P1, 500 nM of P7 for the direct PCR, and 500 nM of the R16F2/R2 primer pair for the nested PCR.

R16F2/R2 amplified products (1,256 bp) were purified using a Qiagen PCR Purification Kit (Qiagen GmbH, Hilden, Germany, EU), and both strands were sequenced using R16F2 and R16R2 primers with an ABI Prism 377 Genetic Analyzer and the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction kit with AmpliTaq®DNA Polymerase (Perkin Elmer-Applied Biosystem, El Cajon, CA, USA). The gene sequence (about 1,164 bp) obtained in the Centro Ricerche Interdipartimentale Biotecnologie Innovative (CRIBI, Padua, Italy, EU) was deposited in the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) under accession no. AJ744767.

Partial sequences of the 16S ribosomal gene from 32 representative strains of the genus "*Candidatus* Phytoplasma" (10) (Table 1), along with the above-described sequence from *C. arvensis* sample L4 (Calendula stunt), were aligned using the Clustal algorithm with Bioedit v.6.0.7. software (Tom Hall and Isis Pharmaceutical, Inc., Raleigh, NC, USA). Cladistic analyses were performed with Treecon

version 1.3 B (Y. van de Peer, Konstanz, Germany, EU). A phylogenetic tree was constructed using the neighbor-joining criterion and visualized by Treecon. *Acholeplasma palmae*, a culturable *Mollicutes*, was designed as the out-group taxon to root the tree; bootstrap analysis was performed and replicated 1,000 times. To estimate the genetic relationships with the other phytoplasmas, considered as belonging to the same ribosomal group,

the partial 16SrDNA sequence from sample *Calendula* stunt (L4) was compared with those of the following phytoplasmas: Oman Alfalfa witches'-broom, Chinese pigeon pea witches'-broom, Faba bean phyllody, Gerbera phyllody, Italian alfalfa witches'-broom, Lime witches'-broom, Papaya yellow crinkle, and *Pichris* echinoides phyllody. The sequences were aligned using the BLAST engine for local alignment (version Blast N 2.2.10)

(NCBI), and percent identity was determined.

RESULTS

All weed samples produced no product in direct PCR with R16F2/R2 primers. The only samples yielding positive results after nested PCR with R16(I)F1/R1 primers were those collected in July. In particular, positive samples were: one of seven *Chenopodium*, three of six *C. arvensis*, and

Fig. 4. Phylogenetic tree constructed by parsimony analysis of 16SrRNA and spacer gene sequences from 38 phytoplasma taxa from GenBank. Numbers on branches of tree indicate bootstrap values. *Acholeplasma palmae* was employed as the out-group taxon. Abbreviations are given in Table 1.

one of two *S. nigrum*. Based on RFLP analyses on R16(I)F1/R1 amplicons (Fig. 1), phytoplasmas from these weeds were assigned to the 16SrII ribosomal group (17). Further confirmation of these results was obtained after RFLP analysis on R16F2/R2 amplicons from the positive samples that produced banding patterns identical to each other and to those observed in the *Picris echioides* phyllody (PEP) phytoplasma control (Fig. 2). Moreover, sample *Calendula* stunt L4 showed an identical profile with the PEP phytoplasma after RFLP analyses of the S3 ribosomal protein gene (Fig. 3).

Comparison of the sequence obtained from the *C. arvensis* L4 sample showed higher and more significant homology (about 98%) with some phytoplasmas: 99.38% identity with phytoplasma strain PEP from Southern Italy, 98.24% with Italian alfalfa witches'-broom (AlfWB), and 97.98% with Papaya yellow crinkle (PapayaYC) from New Zealand (Table 2). Phylogenetic analysis confirmed that the *Calendula* stunt phytoplasma was closely related to *Picris echioides* phyllody (PEP) (Fig. 4) and to all members of the 16SrII group.

The insects identified as most common in the vineyard were: *Agallia ribauti* Osianilsson with 10 adults captured in June, *Empoasca vitis* Göthe with 42 adults captured in August, *Euscelis lineolatus* Brullé with 57 adults and neanids captured in May, *Laodelphax striatellus* Fallén with 65 adults captured in June, and *Psammotettix alienus* Dahlbom with 20 adults captured in June. The tests carried out on samples of *A. ribauti*, *E. lineolatus*, *L. striatellus*, and *P. alienus* did not detect the Stolbur or any other 16SrII phytoplasmas. *E. vitis* samples were not analyzed.

DISCUSSION

In diseases caused by viruses or phytoplasmas, epidemiological studies are important, especially when there is a lack of such studies in a specific geographical area. The presence of unknown hosts and vectors in the epidemic process make it difficult to define the epidemiological framework. Phytoplasma infections in wild species, which act as intermediate hosts, have been reported (2,23,24), and natural weed hosts specific for phytoplasma strains of subgroup 16SrXII-A have been described (20). We did not find Stolbur phytoplasmas in the wild species in the vineyard sampled in this study. This could indicate that secondary weed hosts were not present, but research was not exhaustive enough to confirm this hypothesis. By contrast, the identification of *G. guttulinervis* infected with 16SrXII-A in a vineyard insect population (5) leaves open the question of how the "Bois noir" disease is spread. No trophic relationships were found between this leafhopper and the plant species present in the vineyard sampled in this study.

Although epidemiological surveys done in a Chardonnay vineyard affected by "Bois noir" failed to detect 16SrXII-A phytoplasmas in weeds, three weed species, *C. arvensis*, *S. nigrum*, and *Chenopodium* spp., appear to be new hosts of 16SrII-E subgroup phytoplasmas. Our results confirm the suitability of R16F2/R2 primers for phytoplasma detection and the use of the group-specific primer pairs R16(I)F1/R1 for detection of 16SrII ribosomal group phytoplasmas (17). This is the first evidence that the R16(I)F1/R1 primers, in addition to amplifying phytoplasmas of ribosomal groups 16SrI and 16SrXII, can amplify phytoplasmas in all subgroups described within ribosomal group 16SrII (12) (Fig. 1). The molecular characterization of the gene coding the S3 ribosomal protein confirmed the identification of *Calendula* stunt phytoplasma, as well as indicated the possibility of using this gene to differentiate members of the 16SrII phytoplasma subgroup (22).

The *Calendula* stunt phytoplasma was 99.38% similar to the PEP strain described in Southern Italy in *Picris echioides* based on the sequence of the 16S gene (21). Finding genetically indistinguishable phytoplasmas in Sardinia indicates a wider distribution of 16SrII phytoplasmas, which until now have been reported in weeds and herbaceous crops in areas of Southern Europe (21,27), Africa (14,23), New Zealand (29), Asia (24,25,30), Australia (2,8,28), and America (19). In Oman, they were found in herbaceous and woody plants (1,7,11) associated with severe losses of production; the leafhoppers *Astragallia avicula* Ribaut and *Empoasca* spp. were also described as potential carriers of the phytoplasma group 16SrII (13). In Mali, *Orosius cellulosus* Lindberg was found to play an active role in transmitting 16SrII phytoplasma-associated phyllody from cotton plants and spontaneous mallows (*Sida cordifolia* and *S. rhombifolia*) (14).

Further investigations to verify the presence of leafhopper populations in the sampled vineyard showed a significant occurrence of *A. ribauti* and *E. vitis* during June and July 2003 (unpublished results); however, tests on both species for the presence of phytoplasmas gave negative results.

Soil management through no tillage, where possible, has gained increasing attention in viticulture. Implementation of this practice should be carefully evaluated in the environments where phytoplasma infections are spread, especially in the presence of alternative plant hosts and of possible arthropod vectors of phytoplasmas.

LITERATURE CITED

1. Al-Sakeiti, M. A., Al-Subhi, A. M., Al-Saady, N. A., and Deadman, M. L. 2005. First report of witches'-broom disease of Sesame (*Sesamum indicum*) in Oman. *Plant Dis.* 89:530.

2. Davis, R. I., Schneider, B., and Gibb, K. S. 1997. Detection and differentiation of phytoplasmas in Australia. *Aust. J. Agric. Res.* 48:535-544.
3. Deng, S., and Hiruki, C. 1991. Genetic relatedness between two nonculturable mycoplasma-like organisms revealed by nucleic acid hybridization and polymerase chain reaction. *Phytopathology* 81:1475-1479.
4. Doyle, J. J., and Doyle, J. L. 1990. Isolation of plants DNA from fresh tissue. *Focus* 12:3-15.
5. Garau, R., Sechi, A., Tolu, G., Protta, V. A., Lentini, A., and Protta, U. 2004. *Goniognathus guttulinervis* (Kirschbaum) new natural host of the Stolbur subgroup 16SrXII-A phytoplasma in Sardinia. *J. Plant Pathol.* 86(2):177.
6. Garau, R., Tolu, G., Protta, V. A., Sechi, A., Mungianu, M. P. M., and Protta, U. 2002. Osservazioni sul "Bois noir" della vite in Sardegna. *Petria* 12(3):445-446.
7. Garnier, M., Zreik, L., and Bové, J. M. 1991. Witches'-broom, a lethal mycoplasma disease of lime trees in the Sultanate of Oman and the United Arab Emirates. *Plant Dis.* 75:546-551.
8. Gibb, K. S., Constable, F. E., Moran, J. R., and Padovan, A. C. 1999. Phytoplasmas in Australian grapevines – detection, differentiation and associated diseases. *Vitis* 38(3):107-114.
9. Gundersen, D. E., Lee, I.-M., Schaff, D. A., Harrison, N. A., Chang, C. J., Davis, R. E., and Kingsbury, D. T. 1996. Genomic diversity among phytoplasma strains in 16S rRNA Group I (Aster Yellows and related phytoplasmas) and III (X-Disease and related phytoplasmas). *Int. J. Syst. Bacteriol.* 46:64-75.
10. IRPCM Phytoplasma/Spiroplasma Working Team – Phytoplasma taxonomy group. 2004. Description of the genus '*Candidatus* Phytoplasma', a taxon for the wall-less non-helical prokaryotes that colonize plant phloem and insects. *Int. J. Syst. Evol. Microbiol.* 54:1243-1255.
11. Khan, A. J., Azam, K. M., Deadman, M. L., Al-Subhi, A. M., and Jones, P. 2001. First report of alfalfa witches' broom disease in Oman caused by a phytoplasma of the 16Sr II group. *Plant Dis.* 85:1287.
12. Khan, A. J., Botti, S., Al-Subhi, A. M., Gundersen-Rindal, D. E., and Bertaccini, A. F. 2002. Molecular identification of a new phytoplasma associated with alfalfa witches' broom in Oman. *Phytopathology* 92:1038-1047.
13. Khan, A. J., Botti, S., Al-Subhi, A. M., Zaidi, M. A., Altosaar, I., Alma, A., and Bertaccini, A. 2003. Molecular characterization of the 16S-rRNA gene of phytoplasmas detected in two leafhopper species associated with alfalfa plants infected with witches' broom in Oman. *Phytopathol. Mediterr.* 42:257-267.
14. Laboucheix, J., Offeren, A. L., and van Desmids, M. 1973. Étude de la transmission par *Orosius cellulosus* (Lindberg) (Homopt., Cicadellidae) de la virescence florale du cotonnier et de *Sida* sp. *Cotton Fibre Trop.* 28(4):461-471.
15. Langer, M., Darimont, H., and Maixner, M. 2003. Characterization of isolates of Vergilbungskrankheit-phytoplasma by RFLP-analysis and their association with grapevine herbaceous host plants and vectors. Pages 66-67 in: ICVG Conf., 14th, Locorotondo, (Bari) Italy; 2003 Sept. 12-17.
16. Langer, M., and Maixner, M. 2004. Molecular characterization of grapevine yellows associated phytoplasmas of the Stolbur-group based on RFLP-analysis of non-ribosomal DNA. *Vitis* 43:191-200.
17. Lee, I.-M., Gundersen, D. E., Davis, R. E., and Bartoszyk, I. M. 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int. J. Syst. Bacteriol.* 48:1153-1169.
18. Lee, I.-M., Gundersen, D. E., Hammond, R.

- W., and Davis, R. E. 1994. Use of mycoplasma-like organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-MLO infections in a single host plant. *Phytopathology* 84:559-566.
19. Leyva-López, N. E., Ochoa-Sánchez, J. C., Leal-Klevezas, D. S., and Martínez-Soriano, J. P. 2002. Multiple phytoplasmas associated with potato diseases in Mexico. *Can. J. Microbiol.* 48(2):1062-1068.
20. Maixner, M., Ahrens, U., and Seemüller, E. 1995. Detection of the German grapevine yellows (Vergilbungskrankheit) MLO in grapevine, alternative hosts and a vector by a specific PCR procedure. *Eur. J. Plant Pathol.* 101:241-250.
21. Marcone, C., Ragozzino, A., and Seemüller, E. 1997. Detection and identification of phytoplasmas in yellows-diseased weeds in Italy. *Plant Pathol.* 46:530-537.
22. Martini, M. 2004. Ribosomal protein gene-based phylogeny: A basis for phytoplasma classification. Ph.D. thesis. University of Udine, Italy.
23. Saeed, E., Sarindu, N., Davies, D. L., Clark, M. F., Roux, J., and Cousin, M. T. 1994. Use of polyclonal antibodies to identify mycoplasma-like organisms (MLOs) from Sudan and from Thailand. *J. Phytopathol.* 142:345-349.
24. Schneider, B., Cousin, M. T., Klinkong, S., and Seemüller, E. 1995. Taxonomic relatedness and phylogenetic positions of phytoplasmas associated with diseases of faba bean, sun hemp, sesame, soybean and eggplant. *Z. Pflanzenschutz* 102:225-232.
25. Schneider, B., Marcone, C., Kampmann, M., Ragozzino, A., Lederer, W., Cousin, M. T., and Seemüller, E. 1997. Characterization and classification of phytoplasmas from wild and cultivated plants by RFLP and sequence analysis of ribosomal DNA. *Eur. J. Plant Pathol.* 103:675-686.
26. Schneider, B., Seemüller, E., Smart, C. D., and Kirkpatrick, B. C. 1995. Phylogenetic classification of plants pathogenic mycoplasma-like organisms or phytoplasmas. Pages 369-380 in: *Molecular and Diagnostic Procedures in Mycoplasmaology*. Vol. 2. S. Razin and J. G. Tully, eds. Academic Press, New York.
27. Tessitori, M., Masenga, V., and Marzachi, C. 2005. First report of a phytoplasma associated with abnormal proliferation of cladodes in cactus pear (*Opuntia ficus indica*) in Italy. *Plant Dis. Rep.* 54:851-853.
28. Trau-Nguyon, L. T. T., Persley, D. M., and Gibb, K. S. 2003. First report of phytoplasma disease in capsicum, celery and chicory in Queensland, Australia. *Aust. Plant Pathol.* 32(4):559-560.
29. Waite, D. T., Blackall, L. L., Scott, P. T., and Walsh, K. B. 1998. Phylogenetic positions of phytoplasmas associated with dieback, yellow, crinkle and mosaic diseases of papaya, and their proposed inclusion in '*Candidatus Phytoplasma australasia*'. *Int. J. Syst. Bacteriol.* 48:941-951.
30. Zreick, L., Carle, P., Bové, J. M., and Garnier, M. 1995. Characterization of the mycoplasma-like organism associated with witches'-broom disease of lime and proposition of a '*Candidatus Phytoplasma aurantifolia*'. *Int. J. Syst. Bacteriol.* 45:449-453.