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SHORT COMMUNICATION

MOLECULAR IDENTIFICATION OF PHYTOPLASMAS FROM DILL (*ANETHUM GRAVEOLENS* L.; *UMBELLIFERAE*)

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SUMMARY

Dills (*Anethum graveolens* L.) grown from seeds in a small area of the Italian Riviera showed symptoms including stalk discoloration, flower virescence and phyllody. The affected plants were tested for the presence of phytoplasmas. DNAs were PCR-amplified in reactions with ribosomal universal or group-specific primer pairs and analysed by restriction digestions with *Bfa*I or *Mse*I enzyme. Phytoplasmas, identified as members of 16SrI-B, I-C, V-A and XII-A subgroups were present in 18 out of 22 samples (81.8%). Of these, 9 (50% of the infected) carried a single phytoplasma [3 subgroup 16SrI-B (16.6%), 1 16SrI-C (5.5%), and 5 16SrV-A (27.7%)]. Seven samples (38.8%) carried two different phytoplasmas (6 members of subgroups V-A and XII-A; 1 member of subgroups I-C and V-A). Two samples carried phytoplasmas of three different subgroups in different mixes (I-C, V-A and XII-A; I-B,I-C and XII-A). There was no clear correlation between a given symptom and the presence of one or more phytoplasmas. This is the first report from Europe of phytoplasmas in dill plants.

Key words: dill, phytoplasma, PCR detection, RFLP analysis.

Within the class *Mollicutes*, the monophyletic clade of the phytoplasmas includes 14 16S rDNA RFLP groups, subdivided into 32 subgroups (Lee *et al.*, 1998), that encompass the phytoplasmas associated with hundreds of diseases distributed worldwide in several plant species (Lee *et al.*, 2000). Symptomatic plants may exhibit generalised stunting, unseasonal yellowing or reddening, abnormal proliferation of axillary buds resulting in witches'-broom appearance, decline and dieback, flower virescence and/or phyllody. Different phytoplasmas may cause identical symptoms in certain plant species, whereas closely related ones can induce different syndromes in the same host (Lee *et al.*, 2000).

In the agronomically important region of the Italian Riviera, a number of plant diseases, involving ornamental and horticultural crops, wild shrubs and cultivated trees, have been associated with several phytoplasmas, mainly of the subgroups I-B and XII-A, and less frequently I-C (Bertaccini *et al.*, 1990, 1992; Minucci *et al.*, 1994, 1995; Vibio *et al.*, 1994, 1995; Conti *et al.*, 1997; Marzachì *et al.*, 1999, 2000; Alma *et al.*, 2000). Representatives of subgroups V-C and V-E have been identified from diseased grapevines and stunted blackberry, respectively (Bertaccini *et al.*, 1995; Lee *et al.*, 1995b; Osti *et al.*, 2000; Davis and Dally, 2001).

The cultivation of aromatic dill (*Anethum graveolens* L.), grown from seeds in open fields in Spring and Autumn or, during Winter, under protected conditions, has been recently introduced to the region and maintained mainly for export. During March-April 2000 and 2001 we have observed, in both types of cultivation, the appearance of stunted plants showing whitening or reddening of the stalks, flower virescence, phyllody and witches'-broom, with almost complete loss of production. Over time, the incidence of such syndrome has considerably increased, affecting over 20% of the crops.

DNAs were extracted as described (Marzachì *et al.*, 1999) and amplified in assays driven by the universal primers fP1/rP7 (Schneider *et al.*, 1995). Irrespective of the results of direct amplifications, reaction mixtures were diluted 1:40 and subjected to nested PCRs separately by universal primers R16F2/R2 (Lee *et al.*, 1993) and one of the following group-specific primer pairs: R16(I)F1/R1, R16(III)F2/R1, R16(V)F1/R1 (Lee *et al.*, 1994) or R16(X)F1/R1 (Lee *et al.*, 1995a), according to conditions detailed in the original papers. Products were analysed in 1% (w/v) agarose gels and visualised by UV light as described (Marzachì *et al.*, 1999). Amplified fragments were restricted with appropriate enzymes following manufacturer's instructions and fractionated in 5% (w/v) polyacrylamide gels as described (Marzachì *et al.*, 1999). Phytoplasma reference isolates [acronyms and subgroup of affiliation (*sensu* Lee *et al.*,

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1998) in parenthesis], maintained and propagated in periwinkles [*Catharanthus roseus* (L.) G. Don], were: American aster yellows (AAY; I-A), European aster yellows (EAY; I-B), clover phyllody (CPh; I-C), peach yellow leaf roll (III-A), Vaccinium witches'-broom (III-B), American elm yellows (EY-1; V-A), Serbian stolbur strain from pepper (Stol; XII-A). DNAs from apple proliferation and pear decline phytoplasmas (X-A and X-C, respectively), jujube (*Zizyphus jujuba* Miller), blackberry (*Rubus fruticosus* L.) and grapevine (*Vitis vinifera* L.), naturally infected with jujube witches's-broom (JWB; V-B), rubus stunt (RuS; V-E) and Flavescence dorée (FD; V-C) phytoplasmas, respectively (Pasquini *et al.*, 2000), were also used.

No correlation between a distinctive symptom and a given phytoplasma could be determined from the results of PCR and RFLP analyses of samples listed in Table 1. Eighteen out of 22 samples (81.8%) did respond positively in nested assays with universal and groups I- and V-specific primer pairs. Failure to amplify phytoplasma DNA in 9% of the symptomatic samples could have been due to titres below detection limits or to the reduced size (1.5 g of leaf veins) of the analysed samples. Nine samples (50%) yielded phytoplasma-specific amplicons with both these primer pairs, thus indicating multiple infections with phytoplasmas of different groups (Table 1). Only the reference isolates, not the samples, yielded the expected phytoplasma-specific fragments using groups 16SrIII- or X-specific primer pairs (not shown).

*Bfa*I-digested amplicons obtained from dill DNA by nested PCR using group V-specific primer pair appeared indistinguishable among them and from those of V-A or V-B subgroups reference isolates, but differed from those of subgroup V-E (examples in Fig. 1). As subgroup V-B members have been reported only in China from jujube (*Z. jujuba*) and Chinese cherry (*Prunus pseudocerasus* Lindl.) (Zhu *et al.*, 1998), it is conceivable that these phytoplasmas belonged to subgroup V-A. Group V phytoplasmas have been detected in diseased tomatoes (*Lycopersicum esculentum* Mill) from central Italy (Del Serrone *et al.*, 2001) and *Celtis australis* L. (Bertaccini *et al.*, 1996). In Italy group V-A phytoplasmas have been detected in *Ulmus* spp. (Griffiths *et al.*, 1999) and *Eucaliptus* spp. (Camele *et al.*, 1999).

*Mse*I-RFLP profiles of 16S rDNA sequences amplified by nested PCR with group I-specific primer pair indicated that the majority of the positive samples were carrying phytoplasmas indistinguishable from the reference stolbur isolate (XII-A, Fig. 2 S and samples 9 and 6), whereas samples 3, 16 and 17 (Table 1) yielded restriction patterns identical to that of AAY and EAY phytoplasma (I-A and I-B, Fig. 2, A and E, respectively) and sample 21 had the same appearance as CPh phytoplasma (I-C, Fig. 2C).

Table 1. Nested PCR detection of phytoplasmas in symptomatic dill extracts collected during the years 2000 (samples 1-5) and 2001 (samples 6-22). Assignment to taxonomic subgroups (Lee *et al.*, 1998) has been attained by RFLP analysis of group-specific nested amplicons (see Figs 1 and 2).

Sample	Nested (universal)	Nested (I)	Nested (V)	RFLP
1	–	–	–	–
2	+	–	+	V-A
3	+	+	-	I-B
4	+	–	+	V-A
5	–	–	–	–
6	+	+	+	V-A, XII-A
7	+	+	+	V-A, XII-A
8	+	+	–	V-A, XII-A
9	+	+	+	V-A, XII-A
10	+	+	+	I-C, V-A, XII-A
11	+	+	+	V-A, XII-A
12	+	–	+	V-A
13	+	+	+	I-C, V-A
14	+	+	+	V-A, XII-A
15	+	–	+	V-A
16	+	+	–	I-B
17	+	+	–	I-B
18	+	+	–	I-B, I-C, XII-A
19	+	–	+	V-A
20	–	–	–	–
21	+	+	–	I-C
22	–	–	–	–

However, subgroup I-A members appear to be restricted to North America (Seemüller *et al.*, 1998; Lee *et al.*, 1998, 2000), and therefore it is likely that

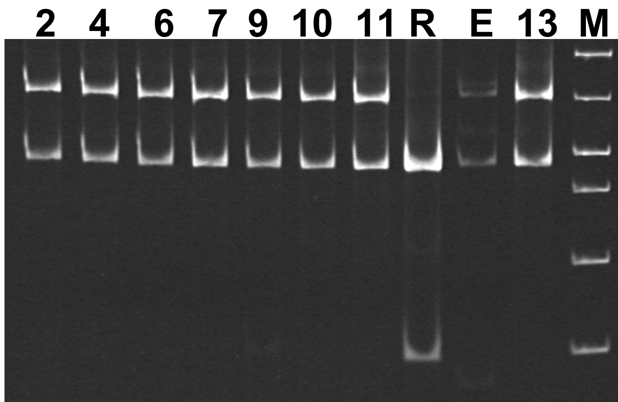

Fig. 1. Polyacrylamide gel electrophoresis of *Bfa*I-restricted 16S rDNA amplicons obtained from infected dills and reference phytoplasma isolates in nested PCRs by R16(V)F1/R1 primer pair. Numbers are the same as those of Table 1. M is the 1 Kb plus DNA size marker (Gibco-BRL); R and E: rubus stunt (V-E) and American elm yellows (V-A) phytoplasmas, respectively.

infected dills were in fact harbouring subgroup I-B phytoplasmas. Multiple infection by members of different subgroups was suggested by the complex RFLP patterns of samples 10 and 18 (Fig. 2). As mentioned, in the Italian Riviera, group I phytoplasmas appear to be the commonest in various crops and wild plants (Bertaccini *et al.*, 1990, 1992; Conti *et al.*, 1997; Marzachì *et al.*, 1999). This may well be due to the fact that they are efficiently vectored by several species of polyphagous leafhoppers (Guglielmone *et al.*, 1994; Bosco *et al.*, 1997; Alma *et al.*, 2000) and hence it is no surprise that new crops may become their targets.

Samples 6 and 9 showed identical RFLP patterns, indistinguishable from that of the reference stolbur isolate (Fig. 2), in line with previous reports (Bertaccini *et al.*, 1995; Marzachì *et al.*, 2000). Stolbur phytoplasmas have been identified in various hosts in the Italian Riviera (Bertaccini *et al.*, 1995; Marzachì *et al.*, 1999, 2000), while different stolbur isolates have been described from field bindweed (*Convolvulus arvensis* L.) from southern Italy and south western Germany (Schneider *et al.*, 1997). Two ciixid hoppers (*Hyalesthes obsoletus* Signoret and *Pentastiridius beieri* Wagner) had been demonstrated as vectors of stolbur (Fos *et al.,* 1992; Gatineau *et al.*, 2001). However, stolbur appears to actively spread within vineyards in the absence of *H. obsoletus* in Spain (Batlle *et al.*, 2000).

Yield losses in dill crops have been in the past attributed mainly to fungal attacks (*Fusarium* sp.; Frauenstein, 1968), but also, in the former German Democratic Republic and Italy, to sporadic alfamo-, cucumo- and poty-virus infections (Wolf, 1972; Bellardi *et al.*, 1998). An outbreak of aster yellows phytoplasmas (I-A and I-B) in dills, among other vegetable crops and biennial weeds, has recently been reported from south western Texas (Lee *et al.*, 2001).

We here report that dills can be affected, in single, double or triple infection, by phytoplasmas belonging to subgroups I-B, I-C, V-A and XII-A. Although one or more phytoplasmas could not be linked to any given syndrome, they appear to be rather common in the area surveyed and frequently associated with the dill crop. The increasing number of symptomatic plants over successive years indicates that dills, as newly introduced crop, are susceptible to phytoplasma infection.

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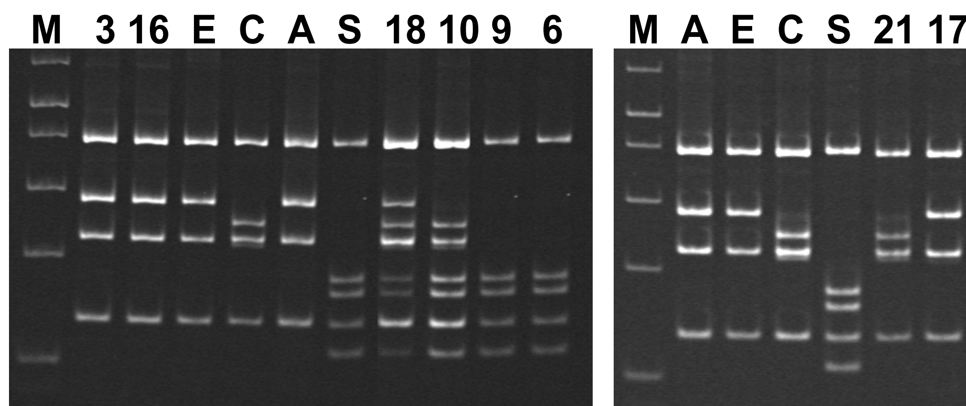

Fig. 2. Polyacrylamide gel electrophoresis of *Mse*I-restricted 16S rDNA amplicons obtained from dills and reference phytoplasma isolates in nested PCRs by R16(I)F1/R1 primer pair. Numbers are the same as those of Table 1. M is the 1 Kb plus DNA size marker (Gibco-BRL); A, E, C, and S are American and European aster yellows, clover phyllody and stolbur phytoplasmas, respectively.

REFERENCES

Alma A., Marzachì C., d'Aquilio M., Bosco D., 2000. Cyclamen (*Cyclamen persicum* L.): a dead-end host species for 16SrI-B and -IC subgroup phytoplasmas. *Annals of Applied Biology* **136**: 173-178.

Batlle A., Martínez M.A., Laviña A., 2000. Occurrence, distribution and epidemiology of grapevine yellows in Spain. *European Journal of Plant Pathology* **106**: 811-816.

Bellardi M.G., Rubies-Autonell C., Lugaresi C., 1998. Il deperimento dell'aneto. *L'informatore Agrario* **28**: 70-72.

Bertaccini A., Davis R.E., Hammond R.W., Vibio M., Bellardi M.G., Lee I.M., 1992. Sensitive detection of mycoplasma-like organisms in field-collected and *in vitro* propagated plants of *Brassica*, *Hydrangea* and *Chrysanthemum* by polymerase chain reaction. *Annals of Applied Biology* **121**: 593-599.

Bertaccini A., Davis R.E., Lee I.M., 1990. Distinction among mycoplasmalike organisms (MLOs) in *Gladiolus*, *Ranunculus*, *Brassica* and *Hydrangea* through detection with non-radioactive cloned DNA probes. *Phytopathologia Mediterranea* **29**: 107-113.

Bertaccini A., Mittempergher L., Vibio M., 1996. Identification of phytoplasmas associated with a decline of European hackberry (*Celtis australis*). *Annals of Applied Biology* **128**: 245-253.

Bertaccini A., Vibio M., Stefani E., 1995. Detection and molecular characterization of phytoplasmas infecting grapevine in Liguria (Italy). *Phytopathologia Mediterranea* **34**: 137-141.

Bosco D., Minucci C., Boccardo G., Conti M., 1997. Differential acquisition of chrysanthemum yellows phytoplasma by three leafhopper species. *Entomologia Experimentalis et Applicata* **83**: 219-224.

Camele I., Bertaccini A., Martini M., Rana G.L., 1999. Identificazione molecolare di fitoplasmi in piante di eucalipto e mirto con scopazzi. *Petria* **9**: 259-268.

Conti M., Minucci C., Territo V., Boccardo G., 1997. Epidemiology of grapevine dieback disease in Liguria, northern Italy. *Proceedings of the 12th Meeting -ICVG, Lisbon* 1997*,* 61-62.

Davis R.E., Dally E., 2001. Revised subgroup classification of group 16SrV phytoplasmas and placement of Flavescence Dorée - associated phytoplasmas in two distinct subgroups. *Plant Disease* **85**: 790-797.

Del Serrone P., Marzachì C., Bragaloni M., Galeffi P., 2001. Phytoplasma infection of tomato in central Italy. *Phytopathologia Mediterranea* **40**: 137-142.

Fos A., Danet J.L., Zreik L., Garnier M., Bové M., 1992. Use of monoclonal antibody to detect the stolbur mycoplasma-like organism in plants and insects and to identify a vector in France. *Plant Disease* **76**: 1092-1096.

Frauenstein K., 1968. Beobachtungen zum Auftreten von Krankheiten an Arznei- und Gewürzpflanzen aus der Familie der Umbelliferen in den Jahren 1964-1966. *Die Pharmazie* **23**: 78-82.

Gatineau F., Larrue J., Clair D., Lorton F., Richard-Molar M., Boudon-Padieu E., 2001. A new natural planthopper vector of stolbur phytoplasma in the genus *Pentastridius* (Hemiptera: Cixiidae). *European Journal of Plant Pathology* **107**: 263-271.

Griffiths H.M., Sinclair W.A., Boudon-Padieu E., Daire Xlee I.M., Sfalanga A., Bertaccini A., 1999. Phytoplasmas associated with elm yellows: molecular variability and differentiation from related organisms. *Plant Disease* **83**: 1101-1104.

Guglielmone L., Bozzano G., Conti A., Conti M., 1994. Piante ospiti e trasmissione di un micoplasma isolato da lattuga in Liguria. *Informatore Fitopatologico* **44**: 55-57.

Lee I.M., Bertaccini A., Vibio M., Gundersen D.E., 1995a. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology* **85**: 728-735.

Lee I.M., Bertaccini A., Vibio M., Gundersen D.E., Davis R.E., Mittempergher L., Conti M., Gennari F., 1995b. Detection and characterisation of phytoplasmas associated with disease in *Ulmus* and *Rubus* in northern and central Italy. *Phytopathologia Mediterranea* **34**: 174-183.

Lee I.M., Dane R.A., Black M.C., Troxclair N., 2001. Epidemiology of aster yellows diseases in various vegetable crops in Texas. *Phytopathology* **91**: S54.

Lee I.M., Davis R.E., Gundersen-Rindal D.E., 2000. Phytoplasma: phytopathogenic Mollicutes. *Annual Review of Microbiology* **54**: 221-255.

Lee I.M., Gundersen D.E., Hammond R.W., Davis R.E., 1994. Use of mycoplasmalike organism (MLO) group-specific oligonucleotide primers for nested-PCR assays to detect mixed-infections in a single host plant. *Phytopathology* **84**: 559-566.

Lee I.M., Gundersen-Rindal D.E., Davis R.E., Bartoszyk I.M., 1998. Revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *International Journal of Systematic Bacteriology* **48**: 1153-1169.

Lee I.M., Hammond R.W., Davis R.E., Gundersen D.E., 1993. Universal amplification and analysis of pathogen 16S rDNA for classification and identification of mycoplasma-like organisms. *Phytopathology* **83**: 834-842.

Marzachì C., Alma A., d'Aquilio M., Minuto G., Boccardo G., 1999. Detection and identification of phytoplasmas infecting cultivated and wild plants in Liguria (Italian Riviera). *Journal of Plant Pathology* **81**: 127-136.

Marzachì C., Veratti A., d'Aquilio M., Vischi A., Conti M., Boccardo G., 2000. Molecular hybridization and PCR amplification of non-ribosomal DNA to detect and differenti-

ate stolbur phytoplasma isolates from Italy. *Journal of Plant Pathology* **82**: 201-212.

Minucci C., Milne R.G., Conti M., Luisoni E., Boccardo G., 1994. A disease of lettuce in northern Italy caused by mycoplasmalike organisms related to European aster yellows. *Rivista di Patologia Vegetale* **4**: 69-79.

Minucci C., Ramasso E., Dellavalle G., Lisa V., Masenga V., Boccardo G., 1995. Caratterizzazione di un organismo simile a micoplasmi in zucchino in Liguria. *Informatore Fitopatologico* **45**: 61-64.

Osti M., Triolo E., Lucchi A., Santini L., 2000. La flavescenza dorata nelle Cinque Terre. *L'informatore Agrario* **56**: 89-91.

Pasquini G., Marzachì C., Poggi Pollini C., Faggioli F., Ragozzino A., Bissani R., Vischi, A., Barba M., Giunchedi L., Boccardo G., 2000. Molecular identification of phytoplasmas from olive trees in Italy. *Journal of Plant Pathology* **82**: 213-219.

Schneider B., Marcone C., Kampmann M., Ragozzino A., Lederer W., Cousin M.T., Seemüller E., 1997. Characterization and classification of phytoplasmas from wild and cultivated plants by RFLP and sequence analysis of ribosomal DNA. *European Journal of Plant Pathology* **103**: 675-686.

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Schneider B., Seemüller E., Smart C.D., Kirkpatrick B.C., 1995. Phylogenetic classification of plant pathogenic mycoplasma-like organisms or phytoplasmas. In: Razin S., Tully J.G. (eds.). Molecular and diagnostic procedures in mycoplasmology, Vol. I, pp. 369-380. Academic Press, San Diego.

Seemüller E., Marcone C., Lauer U., Ragozzino A., Göschl M., 1998. Current status of molecular classification of the phytoplasmas. *Journal of Plant Pathology* **80**: 3-26.

Vibio M., Bertaccini A., Lee I.M., Davis R.E., Minucci C., Milne R.G., 1994. Etiology of lettuce yellows in Italy: genetic characterization of associated phytoplasmalike organisms. *Phytopathologia Mediterranea* **33**: 179-186.

Vibio M., Bertaccini A., Schilirò E., Rapetti S., 1995. Caratterizzazione molecolare di fitoplasmi associati a virescenza in anemone. *Informatore Fitopatologico* **45**: 60-62.

Wolf P., 1972. Viruskrankheiten des Dills (*Anethum graveolens* L.). *Acta Phytopathologica Academiae Scientiarum Hungariae* **7**: 209-211.

Zhu S.F., Hadidi A., Lee I.M., Gundersen D.E., Zhang C.L., 1998. Characterization of the phytoplasma associated with cherry lethal yellows and jujube witches'-broom diseases in China. *Acta Horticulturae* **472**: 701-714.