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Short Communication

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First Report of Phytoplasma Infection in Olive Trees (*Olea Europea* L.)

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With 3 figures

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Abstract

In the Sarca valley (Trento, north Italy) the presence of olive trees (*Olea Europea* L.) with yellow symptoms in a few branches was observed. Some leaves showed diffuse chlorotic mottling affecting almost all the blade, while others showed mottling with green and yellow areas; leaf deformation was also sometimes present. Phytoplasmas were only detected in olive trees with yellow leaves by polymerase chain reaction (PCR) amplification and subsequent restriction fragment length polymorphism (RFLP) analysis. The results obtained suggested a high similarity between this phytoplasma and the elm yellows phytoplasma. This is the first time that a phytoplasma has been reported in the genus *Olea*.

Zusammenfassung

Erster Bericht über eine Phytoplasma-Infektion bei Olivenbäumen (*Olea europaea* L.)

Im Sarca-Tal (Trento, Norditalien) wurden bei Olivenbäumen (*Olea europaea* L.) in einigen Ästen gelbe Symptome beobachtet. Mehrere Blätter Zeigten eine diffuse chlorotische Scheckung, die fast die ganzen Blattspreiten betraf; andere wiesen Scheckungen mit grünen und gelben Flächen auf, manchmal lagen auch Blattdeformationen vor. Durch Amplifikation mit Hilfe der Polymerase-Kettenreaktion (PCR) und anschließende Analyse der Restriktionsfragmentlängenpolymorphismen (RFLP) wurden Phytoplasmen nur in Olivenbäumen festgestellt, die gelbe Blätter aufwiesen. Die Ergebnisse legten eine starke Ähnlichkeit dieses Phytoplasmas mit dem für die Ulmenvergilbung verantwortlichen Phytoplasma nahe. Dies ist der erste Bericht über das Auftreten eines Phytoplasmas in der Gattung *Olea*.

Introduction

Despite the economic importance of olive trees (*Olea Europea* L.) in the Mediterranean countries, the studies of the infections associated with viruses and phytoplasmas (formerly called mycoplasma-like organisms) can be considered relatively recent. To our knowledge, natural infection by phytoplasmas has never been detected in olive trees. A disease called sickle-leaf disorder, characterized by sickle-shaped leaves with lateral distortion and chlorosis has been reported in California, Northern Italy, Portugal and Chile (Thomas, 1958). Sickle-leaf disorder was graft-transmitted to a few healthy olive plants, but sap transmission to 16 herbaceous hosts failed and no phytoplasmas or virus particles were observed by electron microscopy (Waterworth and Monroe, 1975). Moreover, symptoms resembling phytoplasma infections such as branch dwarfing with shortened internodes, leaf malformation, branch die-back, leptonecrosis are generally attributed to boron deficiency (Barba, 1993), which is quite diffuse, especially around the Garda lake (Sancassani and De Rossi, 1993).

The introduction of molecular techniques, particularly polymerase chain reaction (PCR) with primers based on the 16S rRNA gene provided rapid and sensitive means for detection of phytoplasmas in several herbaceous and woody hosts (Ahrens and Seemüller, 1992). Subsequently, restriction fragment length polymorphism (RFLP) analysis made it possible to group phytoplasmas in seven major phylogenetic clusters, including a few subgroups (Schneider et al., 1993). More recently, the analysis of the 16/23S spacer regions (SR) of more than 40 phytoplasma isolates revealed a highly variable nucleotide sequence. This region was studied to design strain-specific primers used as reverse PCR primers in combination with 16S rDNA primers. These combinations amplified phytoplasmas associated with elm yellows, ash yellows and pear decline (Kirkpatrick et al., 1994).

During autumn/winter 1994, trees with branches with yellow leaves were observed in several orchards of cultivars Frantoio and Leccino, located in the Sarca valley, close to the Garda lake. Some leaves showed diffuse chlorotic mottling affecting almost all the blade whereas other leaves showed mottling with green and yellow areas (Fig. 1). Sometimes malformations of the affected leaves, such as irregular margins occurred. In general, only a few branches of a tree showed these symptoms, but normally all the shoots on these branches had symptomatic leaves. By spring and early summer many of the symptomatic leaves had dropped and the younger leaves showed no symptoms. No other symptoms, such as growth of latent axillary buds, branch die-back or reduced growth, were observed. The incidence of trees with symptoms in the orchards observed was always less than 10%.

This paper reports the detection of phytoplasmas in olive trees using PCR with 'universal' and SR-specific primers and their identification by RFLP analysis of the amplified products.

Fig. 1 Shoot of olive tree showing diffuse yellowing and mottling

Materials and methods

DNA was extracted from 0.8 g of leaf petioles and midribs in late Winter and early Spring; five olive trees with yellow leaves, three with yellow leaves and accentuated malformations and five asymptomatic plants were chosen for DNA extraction according to Ahrens and Seemüller (1992). Japanese and European plum trees with leptonecrosis (PLN), elm trees with severe elm witches' broom symptoms — collected in the vicinity of the olive groves — and periwinkles affected with ash yellows phytoplasmas (kindly provided by Dr E. Seemüller) were included as positive controls. The PCR mixture contained 5 μl of DNA preparation (150 ng), 250 nM of each primer, 100 μM of four dNTPs and 1 unit of *Taq* polymerase (Boehringer Mannheim, Mannheim, Germany). The following primer pairs were chosen: fU5/rU3 which amplified a 880 bp DNA fragment from all phytoplasmas, fO1/rO1 which amplified a 1050 bp DNA fragment from all European fruit phytoplasmas (Ahrens et al., 1994), fBI/rULWS which amplified a 1630 bp DNA fragment from elm phytoplasmas and fBI/rASHYS which amplified a 1630 bp DNA fragment from ash phytoplasmas (Kirkpatrick et al., 1994). The primers chosen were based on the 16S rRNA gene (fBI, fO1, fU5, fAT, rO1, rU3) or on the spacer region (rPRUS, rULWS, rASHYS). The PCR parameters were as follows: denaturation 1 min at 95°C, annealing 1 min at 55°C (50°C with fBI/rULWS and fBI/rASHYS), extension 1.5 min at 72°C for 35 cycles in a DNA Thermal Cycler 480 (Perkin Elmer, Norwalk, CT, USA). The PCR amplification products were subjected to electrophoresis on 1% agarose gel in TBE buffer (45 mM Tris–borate, 1 mM EDTA, pH 8.0) and stained with ethidium bromide (1 μg/ml). To characterise the amplified DNAs, from each sample, 20 μl of the PCR reaction products was digested with 1 μl of *Alu*I, *Rsa*I and *Kpn*I restriction endonucleases (Boehringer Mannheim) at 37°C for 4 h and analysed on 10% polyacrylamide gels at 5 V/cm. Molecular weights were determined using the 1 kb ladder (BRL, Eggenstein, Germany).

Results and Discussion

The analysis of the PCR products obtained from olive trees with yellow leaves after 35 cycles revealed the consistent presence of two DNA fragments of approximately 880 bp and 1630 bp using primer pairs fU5/rU3 and fBI/rULWS (Fig. 2). No DNA amplification was observed with the other primer pairs or with DNA samples from olive trees with yellow leaves and accentuated malformations or from asymptomatic olive trees. Positive controls were amplified by appropriate primer pairs. *Alu*I digestion of amplified fragments with fU5/rU3 from affected olive trees produced an identical restriction profile — two fragments of 615 and 247 bp — similar to that obtained from elm and ash phytoplasmas. All isolates showed no *Kpn*I sites, as predicted (Schneider et al., 1993). RFLP analysis of PCR products, amplified with fBI/rULWS, provided better identification of the phytoplasmas recovered from olive trees. *Alu*I digestion gave six fragments (693, 254, 247, 232, 130 and 76 bp; Fig. 3), a pattern identical to that obtained from elm phytoplasmas (Schneider et al., 1993, G. Firrao pers. comm.); *Rsa*I digestion also produced a pattern of seven fragments (760, 425, 319, 73, 24, 20, 16, 4) as expected for elm phytoplasmas (Schneider et al., 1993). Conversely, the DNA fragments obtained from ash phytoplasmas, amplified with fBI/rASHYS and cut with the same endonucleases, were consistently different.

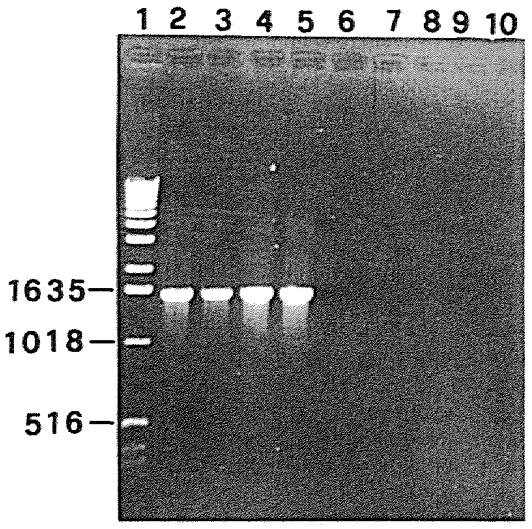

Fig. 2 Agarose gel electrophoresis of PCR products from DNA obtained from: elm trees with witches' broom (lane 2), olive trees with yellow leaves (lanes 3–5), olive trees with yellow leaves with accentuated malformations (lanes 6 and 7), periwinkles affected by ash yellows (lane 8), asymptomatic olive trees (lanes 9 and 10). Lane 1: 1 kb DNA ladder. Primer pair used was fBI/rULWS

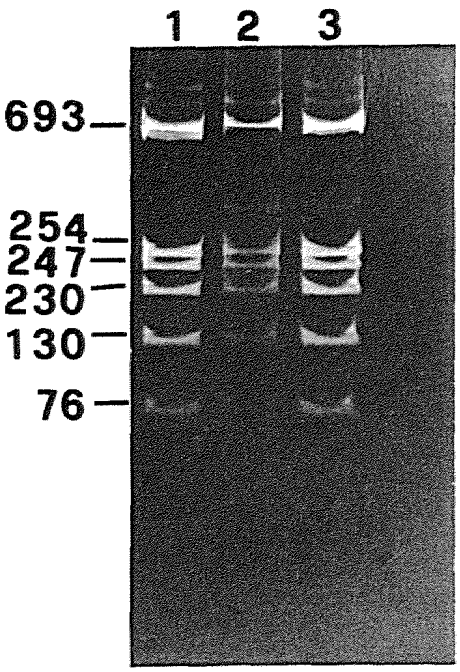

Fig. 3 Restriction profiles after *Alu*I digestion of the PCR products obtained from DNA obtained from DNA isolated from elm trees with witches' broom (lane 1) and olive trees with yellow leaves (lanes 2 and 3). Primer pair used was fBI/rULWS

This is the first time that the presence of phytoplasmas has been reported within the genus *Olea*. The constant detection of phytoplasmas using the PCR technique in olive trees with yellowing symptoms, but not in healthy ones, strongly suggests that the above-mentioned symptoms are associated with phytoplasmas. Moreover, all attempts to detect viruses by sap transmission, ELISA and electron microscopy gave negative results, indicating phytoplasma aetiology. It should be noted that phytoplasmas were not detected in olive trees with yellows associated with severe leaf deformations. However, symptoms such as sickle leaf and leaf malformation have been reported in Italy since 1953 and are generally attributed to insect injury, boron deficiency or genetical disorders (Barba, 1993). The data obtained by PCR amplification and RFLP analysis suggest that the phytoplasmas recovered from olive trees can be included in the IV cluster (the EY cluster) described by Schneider et al. (1993), which comprises elm yellows, elm witches' broom, rubus stunt and alder yellows phytoplasmas (Schneider et al., 1993; Maurer et al., 1993). Elm yellows is epidemically diffuse in North-east and central Italy where elm plantations are often in the vicinity of wild blackberry plants (*Rubus* sp.) with witches' broom symptoms (Conti et al., 1987). Elm yellows phytoplasmas have also been reported in Italy in yellows diseased grapevines around Vicenza (Bianco et al., 1994). To our knowledge the only phytoplasmas recovered to date in Oleaceae were ash yellows, found in several taxa of Fraxinus and in several taxa or Syringa affected with lilac witches' broom in North America (Griffiths et al., 1994). Ash yellows phytoplasmas, however, have never been reported in Europe and are closely related to EY phytoplasmas but belong to a different cluster (III) (Maurer et al., 1993; Schneider et al., 1993).

Further investigations are necessary to further characterize the phytoplasma examined and to determine the real incidence of this disease on olive production and its presence in other regions of Central and Southern Italy where olive trees are widespread and have a strong economic importance.

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