Bulletin of Insectology ***60*** *(2): 383-384, 2007*
ISSN 1721-8861

Molecular identification of phytoplasmas infecting myrtle plantations in Sardinia (Italy)

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Abstract

Myrtus communis is a bushy species of the Mediterranean area that produce very popular liquor: Red mirto and White mirto. Preliminary studies in North Sardinia, resulted in the observation of a severe symptomatology associated with phytoplasma presence. These studies continued, and recently a total of 33 symptomatic plants belonging to different cultivars in two plantations were mapped and repeatedly tested using molecular methods. Phytoplasmas belonging to the 16SrI, 16SrII-F, 16SrIII, 16SrV-A, 16SrX-A, and 16SrXII-A subgroups were identified. Molecular assays on potential insect vectors showed that 16SrX-A+16SrI-B, 16SrXII-A and 16SrIII subgroups were present.

Key words: Phytoplasmas, witches' broom, PCR/RFLP analyses, detection, *Myrtus communis*.

Introduction

Myrtus communis L. is a bushy species covering thousands of hectares in Sardinia (Italy) from the coast to the interior of the island. The berries and the leafy biomass are used also in the production of very popular liquors such as Red and White Mirto, with around three million bottles sold per year in a growing market. Given the species importance for the region's economy, recent research increased knowledge of the domestication processes (Mulas, 2007) and many new cultivars were selected for their productive characteristics.

In preliminary research comparing different cultivar performances, severe stunting and witches' broom symptoms were observed and associated with phytoplasma presence (Garau *et al*., 2005). Studies were continued for three more years in two myrtle plantations of the north Sardinia where the disease was rapidly spreading. Symptomatic plants died in one or two years, and percentage of new infection was about 70% per year. Identification of phytoplasmas present in the plantations is important to devise strategies to reduce and/or prevent the disease from spreading.

Materials and methods

During a 3 year survey a total of 33 symptomatic myrtle plants, belonging to different cultivars, in two North Sardinia plantations, were mapped for symptom evolution and sampled three times to verify phytoplasma detectability and identity. Potential insect vectors were also identified and tested for phytoplasma presence.

The plant and insect nucleic acids were extracted following the procedure of Doyle and Doyle (1990), and amplified in direct PCR with universal primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), in nested PCR with F1/B6, and in a second nested PCR with R16F2n/R2 (Gundersen and Lee, 1996) then subjected to RFLP analyses as described (Duduk *et al*., 2004). To increase system sensitivity F1/B6 products were further amplified in nested PCR with R16(X)F1/R1 and/or R16(I)F1/R1 (Lee *et al*., 1994; 1995).

The R16F2/R2 amplicons from two myrtle plants showing RFLP profiles of phytoplasmas belonging to subgroups 16SrXII-A and 16SrX-A in mixed infections were sequenced after cleaning with the Concert Rapid PCR Purification System (Gibco, BRL), and cloning using the InsT/Aclone PCR Product Cloning Kit (Fermentas, MBI, Vilnius, Lithuania). Selection and screening of recombinant colonies were performed using universal primers M13f/r as described (Bertaccini *et al*., 2005). PCR assays with R16F2n/R2 primers and RFLP analyses with selected restriction enzymes identified 3 diverse recombinants 1-B2, 1-B4 and 1-B5. Sequencing of colonies 1-B2 and 1-B5 using primers M13f/r was performed. Sequences were aligned with those of phytoplasmas available in GenBank and percentage of homology was verified.

Results

The results of the molecular survey indicate that phytoplasma detection in symptomatic myrtle is not always consistent during different periods of the year and over the years, while symptoms are persistent and kill the plants in 1-2 vegetative seasons. Samples from only 18 out of the 33 symptomatic plants tested, resulted in amplification of phytoplasma-related sequences. RFLP identification showed presence of molecularly distinguishable phytoplasmas, in single or mixed infection, belonging to ribosomal groups or subgroups: 16SrI, 16SrII-F, 16SrIII, 16SrV-A, 16SrX-A, and 16SrXII-A.

Cloning and sequencing the samples with mixed infection by 16SrX-A and 16SrXII-A phytoplasmas, resulted in one clone (1-B4) showing the expected RFLP profiles for 16SrX-A phytoplasmas ('*Candidatus* Phytoplasma mali'-related). The other two cloned fragments (1-B2 and 1-B5) were sequenced since RFLP profiles were not identical to reference strains. After sequences alignment two 1,238 bp DNA fragments were obtained; blast searches showed that 1-B5 sequence shared 99% similarity with the majority of '*Ca.* P. mali' strains deposited in GenBank; 1-B2 sequence showed both 95% similarity with stolbur strains, mainly from grapevine i.e. from Spain (AJ964960), France (AF248959), and 95% with '*Ca.* P. mali' reference and related strains from apple and from rhododendron from diverse countries (Germany, Italy and Czech Republic). Virtual RFLP analyses of the latter sequence show that *Rsa*I restriction profile of cloned sequence was identical to one of the profiles present in the original amplicons (figure 1).

Molecular analyses on insects identified phytoplasmas in *Exitianus capicola* (Stål) and *Laodelphax striatellus* (Fallen) species that were similar to those detected in symptomatic myrtle plants, but was not detected in *Anaceratagallia ribauti* (Ossiannilsson) (table 1).

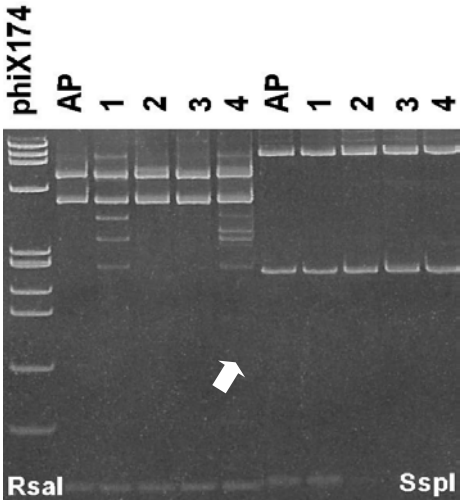

Figure 1. RFLP profiles of R16F2n/R2 amplicons from myrtle samples (n. 1 and 4 were cloned). Sample n. 4 (arrow) show the profile observed in cloned DNA sample 1-B2.

Table 1. Phytoplasmas detected in insect species collected from severely symptomatic myrtle cultivations.

Insect species	No. of insects with phytoplasma infection (no. of tested insects)	RFLP identification
E. capicola	2 (4)	16SrX+16SrI-B; XII-A
L. striatellus	2 (4)	16SrI+16SrXII-A; 16SrIII
A. ribauti	0 (1)	

Discussion

Molecular identification of phytoplasmas in myrtle plantations indicates that different phytoplasmas are present in symptomatic plants. Only 6 out of the 18 plants that tested positive during the survey showed the presence of mixed phytoplasma infection, and there are no indications about increased severity of the disease in relationship to the number/type of phytoplasma(s) identified. The prevalent phytoplasma detected is apple proliferation-related that was also identified in some weeds (Prota *et al*., unpublished) as well as in *E. capicola* (table 1) collected in infected plantations.

RFLP analyses and sequencing of apple proliferation strains confirm that '*Ca.* P. mali' in myrtle show high 16S rRNA similarity with the reference strain. The finding after cloning of a phytoplasma 16S rRNA sequence (1-B2) with a phylogenetic position between stolbur and apple proliferation phytoplasmas could indicate recombination between the two phytoplasmas. Further evidence is needed to confirm this event.

References

BERTACCINI A., FRÁNOVÁ J., BOTTI S., TABANELLI D., 2005.- Molecular characterization of phytoplasmas in lilies with fasciation in the Czech Republic.- *FEMS Microbiology Letters,* 249: 79-85.

DENG S., HIRUKI C., 1991.- Genetic relatedness between two non-culturable mycoplasmalike organisms revealed by nucleic acid hybridyzation and polymerase chain reaction.- *Phytopathology*, 81: 1475-1479.

DOYLE J. J., DOYLE J. L., 1990.- Isolation of plant DNA from fresh tissue.- *Focus,* 12: 13-15.

DUDUK B., BOTTI S., IVANOVIĆ M., KRSTIĆ B., DUKIĆ N., BERTACCINI A., 2004.- Identification of phytoplasmas associated with grapevine yellows in Serbia.- *Journal of Phytopathology*, 152: 575-579.

GARAU R., PROTA V. A., PALTRINIERI S., TOLU G., SECHI A., BERTACCINI A., 2005.- *Myrtus communis*: nuovo ospite di fitoplasmi in Sardegna.- *Petria*, 15 (1/2): 33-35.

GUNDERSEN D. E., LEE I.-M., 1996.- Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs.- *Phytopathologia mediterranea*, 35: 144-151.

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 MLO-infections in a single host plant.- *Phytopathology*, 83: 834-842.

LEE I.-M., BERTACCINI A., VIBIO M., GUNDERSEN D. E., 1995.- Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy.- *Phytopathology*, 85: 728-735.

MULAS M., 2007.- Orientamenti per la scelta delle cultivar di mirto, pp. 60-69. In: *Atti della quarta giornata di Studio sul mirto*.- Sassari, 8 maggio 2007.

SCHNEIDER B., SEEMÜLLER E., SMART C. D., KIRKPATRICK B. C., 1995.- Phylogenetic classification of plant-pathogenic mycoplasma-like organisms or phytoplasmas, pp. 369-380. In: *Molecular and diagnostic procedures in mycoplasmology, vol. 2* (RAZIN S., TULLY J. G., Eds).- Academic Press, New York, USA.

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