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Phytoplasma infected plants in Austrian forests: role as a reservoir?

Eduviges G. BORROTO FERNÁNDEZ[1], Alberto CALARI[2], Veronika HANZER[1], Hermann KATINGER[1], Assunta BERTACCINI[2], Margit LAIMER[1]
[1]*Plant Biotechnology Unit, IAM, Dept. Biotechnology, BOKU, Vienna, Austria*
[2]*Dipartimento di Scienze e Tecnologie Agroambientali, Patologia vegetale, University of Bologna, Bologna, Italy*

Abstract

Reports on phytoplasma diseases in plant wild species are rare. Particularly interesting is the case in an Austrian forest, in the region Rosalia, Lower Austria, where a high number of plants, including *Euonymus europaea*, *Sorbus aucuparia*, *Fraxinus excelsior*, *Fagus sylvatica*, *Betula alba*, *Sambucus nigra*, *Pyrus* sp. and *Picea abies,* appeared with typical symptoms attributed normally to phytoplasma infection. Plants of *Rubus ideaus*, *Rubus fruticosus* and *Vaccinium myrtillus*, which represent small fruit species, showed clear symptoms. In preliminary test to verify phytoplasma presence in *V. myrtillus* the presence of phytoplasmas belonging to ribosomal group 16SrVI were identified after nested PCR on 16S ribosomal gene and restriction digestion with the appropriate enzymes. These plants were introduced *in vitro* to assure the conservation of the phytoplasma isolate for further analysis. Since in this area only a few home gardens are present in the neighbourhood of the forest land, the way of introduction of the phytoplasmas remains obscure.

Key words: Vaccinium witches' broom, forest plants, phytoplasmas, molecular detection, micropropagation.

Introduction

Phytoplasma diseases in wild plant species have been described sporadically, but in the majority of cases are overlooked or attributed to other causes, e.g. herbicide treatments. Disease development and the inoculum build-up in woody species however deserve particular attention, since overlooked foci of infection may develop disproportionate consequences.

Similarly as in studies on the genetic diversity of wild relatives of domesticated species also the migration of pathogens from and into the forest might represent a more complex pattern than the simple assumption that the diseases originate from the natural flora. Detection and identification of phytoplasmas is necessary for accurate disease diagnosis in both host systems, i.e. in plants, where phytoplasmas are localized in phloem cells, and in insect vectors. The choice of a particular method will vary according to the intention/goal of research, e.g. the determination of the degree of infection of an area, the distribution of different strains. Of extraordinary value is therefore *in vitro* reference material, which provides control samples independently of the season and prolongs the availability of strains, and has been attempted by several laboratories (Bertaccini *et al*., 1992; 2000; Jarausch *et al*., 1996; Laimer, 2003).

In an Austrian forest, in the region Rosalia, Lower Austria, a high number of plants appeared with typical symptoms attributed normally to phytoplasma infection, including *Euonymus europaea*, *Sorbus aucuparia*, *Fraxinus excelsior*, *Fagus sylvatica*, *Betula alba*, *Sambucus nigra*, *Pyrus* sp., and *Picea abies*. Plants of *Rubus ideaus*, *Rubus fruticosus* and *Vaccinium myrtillus*, (figure 1) which represent small fruit species, showed also clear symptoms.

To verify phytoplasma presence molecular analyses were carried out.

Materials and methods

Symptomatic plant samples from the above described species were collected in the forest and preparation of midribs, or bark scrapings was carried out immediately from fresh material, before freezing 1 g samples in liquid nitrogen. For wild European blueberry (*Vaccinium myrtillus* L.) a plant exhibiting symptoms of shoot proliferation and small leaf was potted in the greenhouse (figure 1A) to serve as donor material for tissue culture experiments. Tissue cultures were established according to standard procedures (Steniczka *et al*., 2006).

DNA was extracted from plant samples using either the phenol/chloroform method (Prince *et al*., 1993) or with the commercial kit DNeasy-Qiagen according to the manufacturer's instructions.

To verify phytoplasma association with the described symptoms, molecular identification by PCR/RFLP analyses was carried out using general primers located in the 16S rDNA or at the beginning of spacer regions

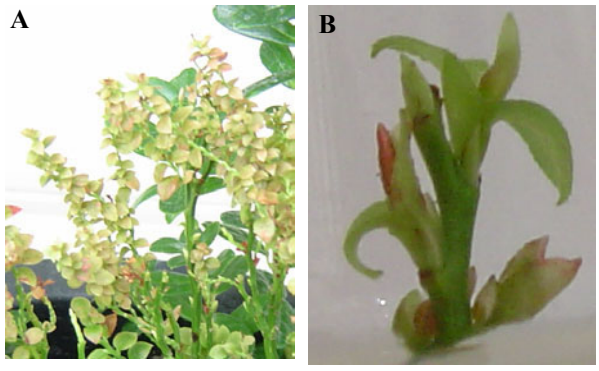

Figure 1. *Vaccinium myrtillus* with typical symptoms due to phytoplasma infection. A) *in vivo* B) *in vitro*. (In colour at www.bulletinofinsectology.org).

of the phytoplasma genome. Nested PCR experiments were carried out using in direct PCR R16mF2/mR2 (Gundersen and Lee, 1996) and PA2F/PA2R followed by R16F2/R2 (Lee *et al*., 1995) or NPA2-F/NPA2-R respectively. The latter primer combination was designed to amplify in direct PCR a product of 1,187 bp between nucleotide 482 and nucleotide 1,669. Nested NPA2F/R primers amplifies a product from nucleotide 1,182 to nucleotide 1,667 (Heinrich *et al*., 2001). A second nested PCR amplification on R16F2/R2 amplicons was also carried out using primers R16(I)F1/R1 (Lee *et al*., 1995).

RFLP analysis of PCR-amplified rDNA was employed using frequently cutting restriction endonucleases, such as *Taq*I, *Tru*I, *Tai*I, and *Tsp509*I (Fermentas, Vilnius, Lithuania).

The PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to manufacture's instructions. The extracted DNA was sequenced by VBC-BIOTECH Service GmbH and analyzed with DNASTAR (DNAstar software package).

Results and discussion

Using PA2R/PA2F followed by NPA2-F/NPA2-R in nested PCR assays, a product of 489 bp was amplified from DNA of blueberry samples from a plant with witches' broom symptoms, indicating that the plant was phytoplasma infected.

Nested PCR on P1/P7 amplicons with PA2R/PA2F primers produce the expected length DNA fragments only from symptomatic blueberry samples. Using RFLP analyses with *Tru*I and *Tsp509*I it was possible to preliminary identify the phytoplasmas as belonging to 16SrVI ribosomal group (clover proliferation, *sensu* Heinrich *et al*., 2001; A. Calari *et al*., unpublished).

Sequence analyses confirmed the presence of a phytoplasma of the 16SrVI group in *V. myrtillus*, which so far was only reported to contain phytoplasmas belonging to the 16SrIII group (Paltrinieri *et al*., 2000). The infected plant was successfully micropropagated (figure 1B).

Phytoplasma presence was also detected after a second nested PCR amplifications with primers R16(I)F1/R1 on R16F2/R2 amplicons in symptomatic samples from *R. fruticosus*, *R. ideaus* and *Fagus* spp.. RFLP characterization allow to verify that *R. fruticosus* was infected by phytoplasmas belonging to ribosomal group 16SrI-B while in *R. idaeus* and *Fagus* samples phytoplasmas belonging to ribosomal subgroup 16SrXII-A were identified. Aspecific amplification was obtained with some of the other samples, molecular tests to further verify phytoplasma presence in these samples are in progress to evaluate the role of these phytoplasmas as reservoirs for epidemic outbreaks.

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Corresponding author: Margit LAIMER (e-mail: m.laimer@iam.boku.ac.at), Plant Biotechnology Unit, IAM, Dept. Biotechnology, BOKU, Vienna, Austria.