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First report of stolbur phytoplasmas in *Prunus avium* in Bulgaria

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Abstract

Since 2005 the National Plant Protection Service in Bulgaria has included a monitoring program for detection, among other quarantine pests and pathogens, of quarantine phytoplasmas. During surveys conducted under this program. symptoms similat to those reported for phytoplasma diseases were observed in cherry trees. Sampling and molecular analyses allow to verify presence of stolbur phytoplasmas in symptomatic plants of cherry and bindweed in the same orchards suggesting the association of the disease in cherry with phytoplasmas.

Key words: phytoplasmas, nested PCR, stolbur, molecular identification.

Introduction

Stolbur, grapevine yellows, apple proliferation, pear decline and European stone fruit yellows are reported to be associated with serious phytoplasma diseases of vegetables, grapevine and fruit trees in Bulgaria (Topchiiska and Sakalieva, 2001; 2002; EPPO, 2006; Sakalieva *et al*., 2007).

At the beginning of 2005, the National Plant Protection Service in Bulgaria (part of the Food safety agency) developed the monitoring program for quarantine pests on fruit trees and grapevine. The object of surveys was to verify the presence of quarantine phytoplasmas. The samples were tested at the Central Laboratory for Plant Quarantine and during these monitoring programs 'bois noir' infection in grapevine was associated with specific stolbur phytoplasma presence in cultivar Merlot (Avramov *et al*., 2008). The stolbur disease is very common in Bulgaria and it was detected and identified in tomatoes (Bertaccini *et al*., 1995), potatoes and other horticultural crops. During the monitoring a cherry disease possibly related to phytoplasma presence was observed in scattered plants and molecular analyses to verify this were carried out.

Materials and methods

Wilting, dying, and phloem necrosis were first observed in 2009 cherry (*Prunus avium* L.) plants located in South Eastern Bulgaria. Samples from symptomatic and asymptomatic cherry were collected in Stara Zagora region of Bulgaria together with bindweed (*Convolvulus arvense* L.) growing under the symptomatic trees and analyzed at the Central Laboratory for Plant Quarantine.

Total DNA was extracted from leaf midribs, secondary roots and scrape of phloem from small branches or trunk (cherry) and from roots and leaves (bindweed) using a CTAB method (Doyle and Doyle, 1990) and the Plant DNeasy mini kit (Qiagen GmbH, Hilden). Preliminary identification was carried out performing PCR assays with universal phytoplasma rDNA primers P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995), followed by nested PCR using the universal primers U3/U5 (Lorenz *et al*., 1995). Reference controls included DNA extracts from asymptomatic cherry, naturally infected tomato samples showing typical stolbur symptoms, naturally infected apple showing apple proliferation symptoms, and PEY (*Pichis echioides* yellows, subgroup 16SrIX-C) and CX (X disease from peach, subgroup 16SrIII-A) infected *Catharanthus roseus*. Tubes without DNA were used as negative controls.

RFLP analysis of nested PCR products was performed using restriction enzymes *Rsa*I (*Afa*I) and *Alu*I (Amersham Biosciences, USA). Further amplification were carried out using P1A/P7A (Lee *et al*., 2004; Martini, 2006) and R16F2/R2 (Lee *et al*., 1995) primers in nested PCR on P1/P7 amplicons under published conditions; the final amplicons were subjected to RFLP analyses with *Tru*I, *Tsp509*I, and *Mbo*II restriction enzymes (Fermentas Vilnius, Lithuania). Amplification and RFLP analyses of *tuf* gene (Langer and Maixner, 2004) were also performed on samples resulted infected by stolbur phytoplasmas using as reference strain stolbur from pepper from Serbia (STOL). Polyacrylamide 5% gels stained with ethidium bromide were employed to compare RFLP profiles to reference phytoplasmas for all obtained amplicons.

Results

Nested PCR results with general phytoplasma primers U5/U3, P1/P7A and R16F2/R2 provides positive results from both cherry and bindweed samples. In particular samples from roots and small branches of symptomatic cherry trees were positive as well as samples from

bindweed roots and leaves, negative results were obtained from negative controls employed (data not shown). RFLP analyses on both P1A/P7A and R16F2/R2 amplicons allow to identify phytoplasmas in cherry and bindweed as stolbur or 16SrXII-A phytoplasmas (Figure 1). RFLP analyses on tuf gene indicated that both cherry phytoplasmas could be assigned to tuf type-b as well as STOL reference strain and infected tomato from Bulgaria.

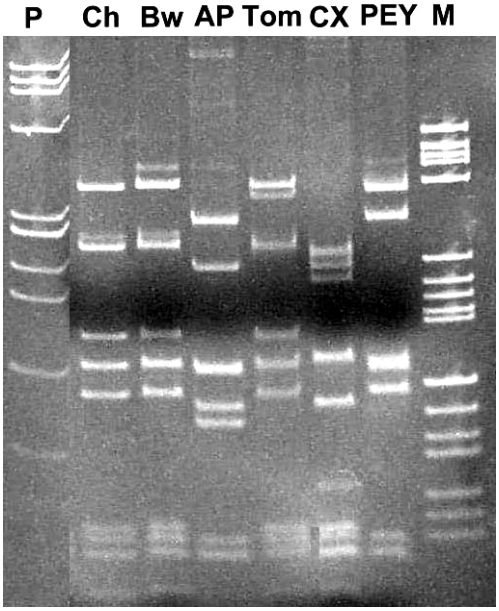

Figure 1. RFLP profiles of R16F2/R2 amplicons after *Tru*I digest. Acronyms: Ch, cherry: Bw, bindweed; AP, apple proliferation; Tom, tomato infected by stolbur; CX, X disease phytoplasmas from peach in periwinkle (Canada; 16SrIII-A); PEY, *Pichris echioides* yellows in periwinkle (Italy, 16SrIX-C).

Discussion

Stolbur phytoplasmas were identified in the past in fruit trees showing symptoms referred to as Moliére diseases, in these cases at least stolbur phytoplasmas were transmitted to periwinkle by dodder. Recently in Italy stolbur phytoplasmas were identified in cherry with lethal decline syndrome together with other phytoplasmas referable to ribosomal subgroup 16SrV-B ('*Candidatus* Phytoplasma ziziphae'), and 16SrIII-B (Paltrinieri *et al*., 2008). To our knowledge, this is the first report of stolbur infecting cherry in Bulgaria; more research should be carried out in order to verify the epidemiology of the disease as well as the insect vector role in its spreading.

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