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Full Length Research Paper

Cultivated and wild plantain (*Plantago major*) as a host of Stolbur phytoplasma in Serbia

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The wild plantain (*Plantago major*) is an important medicinal plant. Symptoms suggestive of phytoplasma diseases were observed in infected plantain plants in Serbia. A new disease on *P. major* has symptoms of reduced leaf size, leaf reddening and crinkling, and occasionally rolling of flowers and early drying up. This disease was found first on the plantain plantation in Pancevo locality, but later has been found in some other localities in Serbia (Kovin, Vrdnik). Restriction fragment length polymorphism (RFLP) analysis of amplification products of 1.2 kb, obtained in nested PCR with R16F2n/R16R2 primer pair after amplification with P1/16S-Sr primers, in 24 from 26 symptomatic plants indicated the presence of phytoplasma from the 16SrXII-A subgroup. Plantain plants collected from all three affected localities in Serbia were determined to be hosts of this phytoplasma. This is the first report of the natural occurrence of Stolbur phytoplasma in cultivated and wild *P. major* in Serbia.

Key words: *Plantago major*, phytoplasma diseases, 16SrXII-A subgroup, wild plantain.

INTRODUCTION

The leaves of *Plantago major* are used almost worldwide as a diuretic and astringent, and to treat wounds, insect stings, sunburn, skin diseases, eye irritation and inflammation of mouth and throat. In modern phytotherapy they are used to alleviate irritation in catarrh of the upper respiratory tract.

Phytoplasmas, the noncultivable, phloem-limited bacterial pathogens, cause many serious diseases of woody and herbaceous plants worldwide (McCoy et al., 1989). Despite their monophyletic origin, widely divergent phytoplasma lineages have evolved in adaptation to specific ecological niches. Phytoplasma are associated with plant diseases in several hundred plant species, including many important food, vegetable, and fruit crops, ornamental plants, and timber and shade trees (Jones, 2002). The list of diseases caused by phytoplasmas continues to grow (Bertaccini and Duduk, 2009).

There are a small number of papers about phytoplasma disease on the plantain. *P. major* was determined to be hosts of the phytoplasma from the aster yellows (AY) group in Hawaii (Borth et al., 2006), and *Plantago lanceolata* in Czech Republic (Franova and Simkova, 2009). Credi et al. (2006) found Stolbur phytoplasma at the different wild host plant among *P. lanceolata*. Alhudaib et al. (2009) reported sequences from the phytoplasma detected in lime *Citrus aurantifolia* (L.), and weed species *Chenopodium morale* L., *P. lanceolata* L., *Convolvulus arvensis*, showed 98 to 99% of identity with phytoplasmas from group 16SrII, Candidatus Phytoplasma aurantifolia.

Infected plantain plants in Serbia were detected during 2008 and 2009 in the plantation of medicinal plants in locality Pancevo. Symptomatic wild plants appeared in 2010 near Kovin and Vrdnik spa. We observed all collected symptomatic plants to determine type of phytoplasma as causing agents of disease of plantain (*P. major*) and compared results with those obtained for healthy plants.

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Figure 1. *Plantago major* plant showing phytoplasma like symptoms.

MATERIALS AND METHODS

Sample collection

Plantains (*P. major*) expressing typical symptoms of phytoplasma diseases, were first detected in a commercial field in Pancevo during 2008 after which the development of the symptoms was monitored. The first symptoms appeared in May, and the number of symptomatic plants later increased. Symptomatic and asymptomatic plants were collected at June in 2008 (Pc1-9) and 2009 (Pc10-19) and then were tested for phytoplasma infection. Symptomatic and asymptomatic plants in Vrdnik spa (Vd20-28) and near Kovin (Ko29-38) were collected at the same time during 2010.

DNA manipulation for phytoplasma detection

DNA isolation

Plantains leaf midribs (0.7 g) from 38 plants on all three localities were collected and total DNA was extracted with hexadecyltrimethil-ammonium bromide (CTAB) protocol (Daire et al., 1997) with modification described by Angelini et al. (2001). DNA extracted from asymptomatic plants was used as negative control.

DNA amplification

For direct amplification with P1/16S-Sr primers (Deng and Hiruki, 1991; Lee et al., 2004) the following conditions were used: 30 cycles of denaturation at 95°C for 40 s (2 min for first cycle), annealing for 30 s at 55°C and primer extension for 80 s at 72°C. The second PCR with R16F2n/R16R2 primers was carried out: for 35 cycles denaturation at 94°C for 1 min (3 min for first cycle), annealing for 1 min at 58°C and primer extension at 72°C for 2 min. DNA from healthy plants and without DNA templates were included in all cases as negative controls, and grapevine STOL-phytoplasma DNA, grapevine FD-C and AY were used as positive control.

RFLP analysis

The 1.2 kb PCR products (primers R16F2n/R16R2) of plantain symptomatic plants that represented the different localities were individually digested with the restriction enzymes *Alu*I and *Tru*I (Fermentas, Lithuania) according to respective instructions of the manufacturer. Restriction fragments were resolved in 2.5% agarose in TBE, stained with ethidium bromide and visualized by UV light. The restriction DNA patterns from plantain plants were compared with the RFLP pattern of the grapevine STOL phytoplasma strain.

RESULTS AND DISCUSSION

Symptoms

Typical phytoplasma symptoms were observed in *P. major* plants grown in fields in Pancevo, and in wild population of plantain around Kovin and Vrdnik spa. Symptoms first appeared in May, and the number of symptomatic plants later increased. The affected plants showed reddening, purpling, reduction of leaves size, and crinkling, rolling of flowers and early drying (Figure 1). On Pancevo plantation the percent of infected plants increased from 8.5 (in 2008) to 35% during 2009 and 2010. Yield on the infected fields decreased by two thirds compared with yield from fields with asymptomatic plants. Also, symptomatic plantains were lacking seed production. Symptoms observed in plantains in the present survey are similar to those described for phytoplasma infection of *P. lanceolata* (Franova and Simkova, 2009), on *P. major* (Borth et al., 2006), and other various host species (Bertaccini, 2007; Franova et al., 2009; Bertaccini et al., 2011).

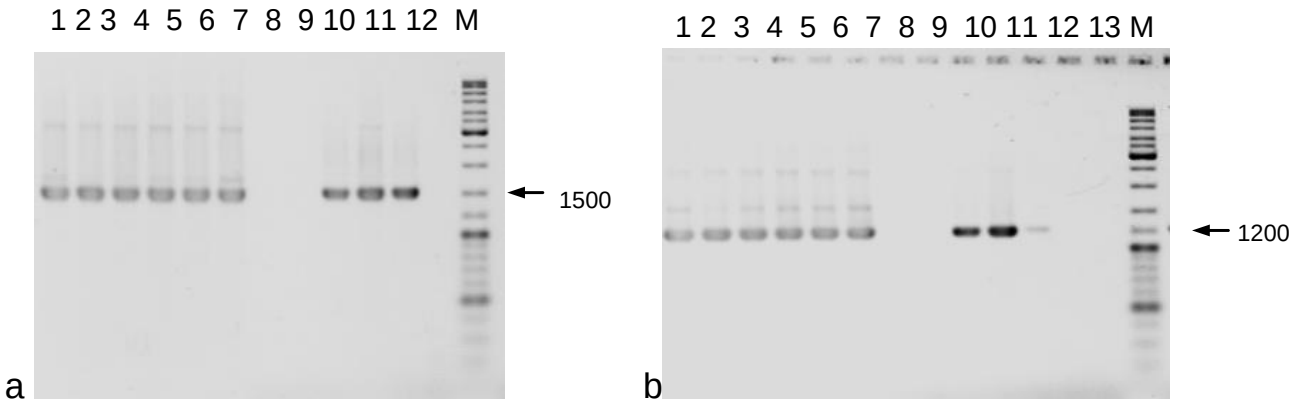

Figure 2. Nested PCR amplification of 16S ribosomal DNA using (a) P1/16S-Sr primers: lane 1-12. plantain samples Pc1, Pc2, Pc15, Pc16, Vd 22, Vd 23, Vd 25, Vd 26, Ko31, Ko32, Ko35, Ko36; lane 14. Marker; and (b) R16F2n/R2 primers: line 1-13: samples Pc1, Pc2, Pc15, Pc16, Vd 22, Vd 23, Vd 25, Vd 26, Ko31, Ko32, Ko35, Ko36, Ko 38; lane 14. Marker: GeneRuler DNA Ladder mix SM0331 (Fermentas, Lithuania).

Phytoplasma detection

PCR amplification from symptomatic plantain total DNA generated 1.5 kb (Figure 2a) and 1.2 kb (Figure 2b) DNA fragments of the 16S ribosomal DNA and 16S–23S spacer region when P1/16S-Sr primers and R16F2n/R16R2 were used, respectively. No PCR products were obtained from asymptomatic plants (Table 1).

RFLP analysis of the 16S rDNA 1.2 kb PCR product (R16F2n/R2) of 24 out of 26 investigated phytoplasma isolates showed identical *Alu*I (Figure 3a) and *Tru*I (Figure 3b) pattern as the reference STOL phytoplasma from grapevine and were different from the AY and FD-C reference strains. Obtained patterns were in agreement with revised classification scheme of phytoplasmas based on RFLP analyses of 16S rRNA (Lee et al., 1998).

The presence of affected plants in the plantation and presence of affected wild plants in/ near plantation or fields may be an important factor in the spread of the disease, as they provide a reservoir for the phytoplasmas and the insect vectors, which leads to increasing disease incidence (Mirzaie et al., 2007).

In our investigation, the first symptoms of disease (reddening, crinkling and leaf size reducing) were detected in plantain plantation (Pancevo) during 2008. No wild plantain with the same symptoms collected during 2008 and 2009 in Serbia. Only few symptomatic plants in wild population near vineyards were collected in 2010 near Kovin (7) and Vrdnik spa (6). All symptomatic plantains from plantation near Pancevo and 11 wild plantain tested positive for Solbur phytoplasma, even collected from distinct localities. This appearance of infected plants in wild population of plantain may be connected with spreading of disease from vineyard near these localities, which is in agreement with data reported by Credi et al. (2006). These authors investigated wild plant species, with or without symptoms, grown near and in different vineyards located in Italian Emilia-Romagna region. They found Stolbur phytoplasma in 48.1% of a total of 162 non-crop native plant samples (20 species belonging to 15 families) and among them one infected out of six investigated *P. lanceolata* plants. Berger et al. (2009) tested various species (41) of herbaceous plants from 21 families collected in BN-affected vineyards during three years (2006 to 2008) and positive results obtained for seven species belonging to six families, but reported absence of Stolbur infection (in the 13 investigated plants) on Plantaginaceae family (*P. lanceolata, P. major* and *P. media*). Our results showed 11 infected *P. major* plants and additionally 2 symptomatic plants without positive PCR results, probably caused by low phytoplasma concentration in tested plants at sampling time.

Few reports suggested infected plantain as weed species near affected field of cultivated plants, however affected with phytoplasma from other phytoplasma groups. *P. major* and five weed species (*Amaranth* sp., *Eclipta prostrata*, *Emilia sonchifolia*, *Myriophyllum aquaticum* and *Sonchus oleraceus*) collected from the vicinity of affected watercress (*Nasturtium officinale*) farms in Hawaii, were determined to be hosts of phytoplasma belong to the 16SrI-B phytoplasma group (Borth et al., 2006). Natural occurrence of the same phytoplasma subgroup belonging to aster yellows group reported Franova and Simkova (2009) for long plantain (*P. lanceolata* L.) in Bohemia, Czech Republic, showing similar symptoms as plantain in our investigation. Alhudaib et al. (2009) reported *P. lanceolata* as weeds found coexisting with the lime decline (LD)-affected lime trees which had symptoms of yellowing and stunting. The 16S rDNA sequence of the phytoplasmas identified in this plant species was 99.9% identical with that of the phytoplasma identified in LD-affected lime (16SrII phytoplasma).

Table 1. PCR detection of phytoplasma using P1/16S-Sr and R16F2n/R2 primers on symptomatic and asymptomatic *Plantago major*.

Locality and year of isolation	Symptomatic plants sample	P1/16S-Sr 1.5 kb product	R16F2n/R2 1.2 kb product	Asymptomatic plants sample	1.5 kb and/or 1.2 kb products
Pancevo 2008	Pc1- Pc6	4/6	6/6	Pc7-Pc9	0/3
Pancevo 2009	Pc10- Pc16	6/7	7/7	Pc17- Pc19	0/3
Vrdnik 2010	Vd20-Vd25	4/6	5/6	Vd26, Vd28	0/3
Kovin 2010	Ko29- Ko35	6/7	6/7	Ko36-Ko38	0/3
Total	26	20	24	12	0

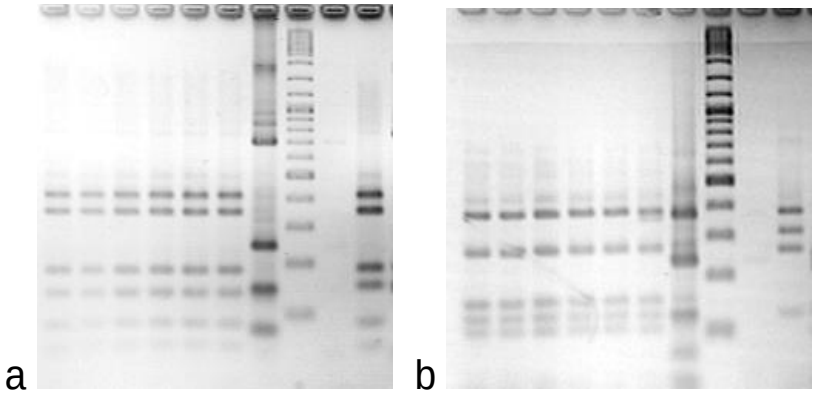

Figure 3. RFLP analysis of the 16S rDNA 1.2-kb PCR product (R16F2n/ R2) digested by a) *Alu*I: line 1 to 5. plantain samples Pc1, Pc15, Vd 22, Ko31, Ko32; lane 6. control 16SrXII group (Stolbur); lane 7. control FD-C; lane 8. Marker; lane 10. control AY phytoplasma. b) *Tru*1: lane 1 to 5. samples Pc1, Pc15, Vd 22, Ko31, Ko32; lane 6. control 16SrXII group (Stolbur); lane 7. control FD-C; lane 8. Marker; lane 10. control AY phytoplasma. Marker: GeneRuler DNA Ladder mix SM0331 (Fermentas, Lithuania).

Infected *P. lanceolata* and other infected weed species (*C. morale L., C. arvensis*) were considered as alternative phytoplasma reservoirs for the 16SrII phytoplasma associated with LD in Easter region of Saudi Arabia.

Analysis of symptomatic plantains in three different locations in Serbia- on cultivated field in Pancevo and wild population near Kovin and Vrdnik, showed the presence of Stolbur phytoplasma infection and tendency to increase Stolbur disease during 2010. So far, this is the first report of 16SrXII-A phytoplasma subgroup presence in *P. major* in Serbia.

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