Pestic. Phytomed. (Belgrade), 21 (2006) 107-111
Pestic. fitomed. (Beograd), 21 (2006) 107-111

UDK: 632.38:582.998.16 {497.11}
Scientific Paper * Naučni rad

Stolbur Phytoplasmas Infecting Chrysanthemum Plants in Serbia

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SUMMARY

During a survey on ornamental crops growing in open field, chrysanthemum plants showing proliferation, virescence and stunting were observed. To verify possible phytoplasma association with described symptoms, polymerase chain reaction (PCR) followed by restriction fragment length polymorphism (RFLP) analyses on phytoplasma 16Sr DNA were carried out. Phytoplasmas belonging to 16SrXII-A ribosomal subgroup (stolbur) were identified in all samples tested from symptomatic plants. This first report of 16SrXII-A stolbur phytoplasmas in chrysanthemum with flower proliferation and stunting symptoms allow to distinguish this from already known chrysanthemum yellows associated with phytoplasmas belonging to aster yellows 16SrI-B ribosomal group, '*Candidatus* Phytoplasma asteris'.

Keywords: Chrysanthemum; *Chrysanthemum indicum hybridum*; Stolbur phyto plasma

INTRODUCTION

Chrysanthemum (*Chrysantemum indicum hybridum*), originated from China and Japan, is grown in our country as ornamental plant under green houses and in open fields.

During a survey on ornamental crops, in an open field in Vinča locality, Belgrade, about 1% of chrysanthemum plants showing flower proliferation, virescence and stunting were observed (Fig. 1 and 2). The symptoms resembled those already described in *Chrysanthemum frutescens* and *C. carinatum* in Italy, and associated with phytoplasmas belonging to 16SrI-B subgroup, aster yellows '*Candidatus* Phytoplasma asteris' (Bertaccini et al., 1990; Lee et al., 2004).

Phytoplasmas are prokaryotes with phloematic habitats found throughout the world on wild and cultivated plants. These organisms cause symptoms often referred as yellowing, discoloration, witches'-broom, dwarfing, virescence, and phyllody. In nature they are transmitted by leafhoppers Auchenorrhyncha that may have occasional or permanent trophic relationship with their hosts.

Aim of this study was to verify phytoplasma presence and molecular identity of detected phytoplasmas in chrysanthemum plants with flower proliferation, virescence, and stunting symptoms.

Fig 1. Virescence of yellow flower and abnormal flower proliferation
Sl. 1. Simptomi virescencije i proliferacije cvetova

Fig 2. Lack of flowers and yellow flower malformations in Chrysanthemum inflorescence
Sl. 2. Simptomi deformacije cvetova

MATERIAL AND METHODS

Samples were collected from symptomatic as well as from asymptomatic chrysanthemum plants (as negative control) during November 2005.

Total nucleic acids were extracted separately from flowers showing proliferation and virescence and from leaf midribs following the protocol described by Prince et al. (1993), dissolved in the TE buffer, and maintained at 4°C. Before performing polymerase chain reaction (PCR) assays, nucleic acids were quantified and diluted in sterile distilled water to the final concentration of 20 ng/μL.

For phytoplasma detection, nested PCR with two pairs of universal phytoplasma primer amplifying 16S ribosomal DNA were used. Direct PCR reaction with P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) primer pair was followed by nested PCR amplification with F1/B6 (Davis and Lee, 1993; Padovan et al., 1995) primer pair. For molecular identification, samples positive in nested PCR with F1/B6 primer pair were subjected to a second nested PCR with R16F2/R2 (Lee et al., 1995) primer pair. Each 25 μL PCR reaction mix contained 20 ng template DNA, 2.5 μl 10X PCR buffer, 0.8 U *Taq* polymerase (Polymed, Italy, EU), 0.2 mM dNTPs, 1.5 mM $MgCl_2$ and 0.4 mM each primer. Samples lacking DNA were employed as negative controls. In nested PCR reactions, 1 μL of amplicon from direct PCR diluted 1: 30 in sterile distilled water, was used as template. Thirty-five PCR cycles were performed under the following conditions: 1 min (2 min for the first cycle) for denaturation step at 94°C, 2 min for annealing at 50°C, and 3 min (10 min for the last cycle) for primer extension at 72°C. Six μL of PCR products were separated in 1% agarose gel, stained with ethidium bromide and visualized with UV transilluminator.

Identification of detected phytoplasmas was done using restriction fragment length polymorphism (RFLP) analyses with *TruI* and *Tsp*509I (New England Biolabs, Beverly, MA, USA) restriction enzymes on R16F2/R2 amplified fragments. Visualization of RFLP products was performed in a 5% polyacrylamide gel, stained with ethidium bromide under UV transilluminator.

Reference phytoplasma strains. Phytoplasma reference strains maintained in collection in periwinkle [*Catharanthus roseus* (G.) Don.] (Bertaccini, 2003) and employed for phytoplasma identification were: aster yellows from apricot (AY-A, ribosomal subgroup 16SrI-F), faba bean phyllody (FBPSA, ribosomal subgroup 16SrII-C), Green Valley X disease (GVX, ribosomal subgroup 16SrIII-A), *Pichris echioides* yellows (PEY, ribosomal group 16SrIX), apple proliferation (AP-

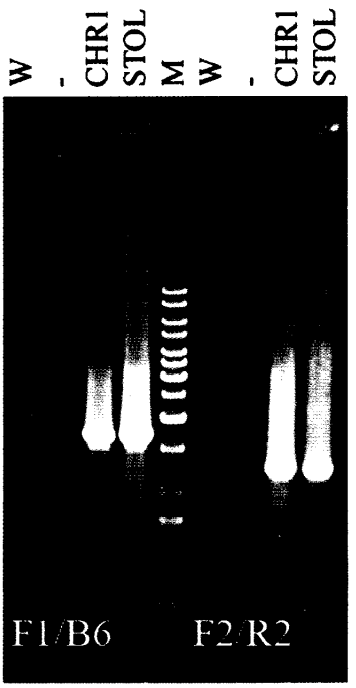

Fig. 3. Visualization of the amplicons obtained with F1/B6 primer pair (about 1.7 kb) and obtained with R16F2/R2 (F2/R2) primer pair (about 1.2 kb) in 1% agarose gel. W: water negative control, -: sample of asymptomatic chrysanthemum as negative control, CHR1: sample of symptomatic chrysanthemum, STOL: stolbur phytoplasma, positive control, M: 1 kb DNA ladder marker, with fragment sizes in bp from top to bottom: 10000; 8000; 6000; 5000; 4000; 3500; 3000; 2500; 2000; 1500; 1000; 750; 500 and 250.

Sl. 3. Vizualizacija amplikona, dobijenih korišćenjem F1/B6 para prajmera (veličine oko 1,7 kb) i amplikona, dobijenih korišćenjem R16F2/R2 (F2/R2) para prajmera (veličine oko 1,2 kb) u 1% agaroznom gelu. chrysanthemum: W: vodena negativna kontrola, -: uzorak zdrave hrizanteme kao negativna kontrola, CHR1: uzorak simptomatične hrizanteme, STOL: stolbur fitoplazma pozitivna kontrola, M: 1 kb, marker sa veličinama fragmenata u bp od gornjeg ka donjem: 10000; 8000; 6000; 5000; 4000; 3500; 3000; 2500; 2000; 1500; 1000; 750; 500 i 250.

15, ribosomal subgroup 16rX-A), and stolbur from pepper from Serbia (STOL, ribosomal subgroup 16SrXII-A) representing some of ribosomal phytoplasma groups described in literature (Lee et al., 1998a; Lee et al., 1998b).

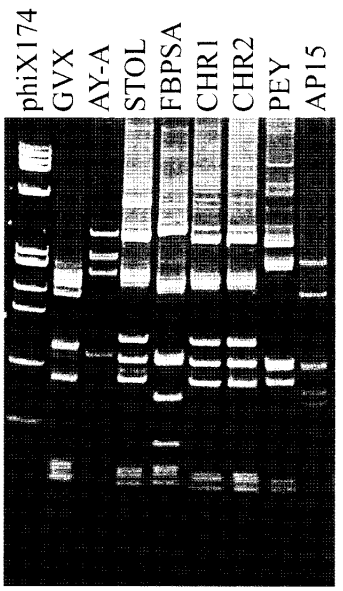

Fig. 4. Polyacrylamide gel 5% showing the *Tru*I RFLP patterns of phytoplasma 16SrDNA amplified with R16F2/R2 primer pair from two chrysanthemum samples and from phytoplasma reference strains in periwinkle. Sample abbreviations: GVX, Green Valley X disease; AY-A, aster yellows from apricot; STOL, stolbur; FBPSA, faba bean phyllody; CHR1, CHR2, samples of symptomatic chrysanthemum; PEY, *Pichris echioides* yellows; AP15, apple proliferation; phiX174, marker phiX174 *Hae*III digested; fragment sizes in base pairs from top to bottom: 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 and 72.

Sl. 4. *Tru*I restrikcioni profili 16SrDNA regiona fitoplazmi dobijenog korišćenjem R16F2/R2 para prajmera sa dva uzorka hrizanteme i referentnih izolata fitoplazmi održavanih u biljci *Catharanthus roseus*, vizualizirani u 5% poliakrilamidnom gelu. Akronimi uzoraka: GVX, Green Valley X disease; AY-A, aster yellows sa kajsije; STOL, stolbur; FBPSA, faba bean phyllody; CHR1, CHR2, uzorci simptomatičnih hrizantema; PEY, *Pichris echioides* yellows; AP15, apple proliferation; phiX174, marker phiX174 *Hae*III digestirani; veličine fragmenata u bp od gornjeg ka donjem: 1353; 1078; 872; 603; 310; 281; 271; 234; 194; 118 i 72.

RESULTS

Only positive controls were amplified in direct PCR with P1/P7 primer pairs (data not shown). Nested PCR reactions with F1/B6 primer pair resulted in expected length fragment amplification (about 1700 bp) (Fig. 3) from all the symptomatic chrysanthemum samples tested; no amplification was obtained from asymptomatic samples.

For molecular identification, positive samples after nested PCR with R16F2/R2 primer pair (Fig. 3), were subjected to RFLP analyses with *Tru*I and *Tsp*509I restriction enzymes. Restriction profiles from amplicons of all chrysanthemum samples were identical to each other and referable to the one of reference strain STOL, belonging to 16SrXII-A ribosomal subgroup (Fig. 4).

DISCUSSION

Chrysanthemum yellows is a disease of chrysanthemum known in Europe since long time. In the past a similar disease observed in Liguria (Italy) was associated with phytoplasmas belonging to 16SrI-B ribosomal group, aster yellows, 'Ca. Phytoplasma asteris', by using dot-hybridization assays and PCR amplification (Bertaccini et al., 1990; Bertaccini et al., 1992; Lee et al., 1998a; Lee et al., 2004). 'Ca. Phytoplasma asteris' associated with previously described Chrysanthemum yellows is transmitted by *Euscelidius variegatus, Euscelis incisus, Macrosteles quadripunctulatus* and *M. strifrons* (Alma et al., 2001; Palermo et al., 2001).

The presence of phytoplasmas in symptomatic samples of chrysanthemum from Serbia was indicated by the nested PCR positive results on 16SrDNA with F1/B6 primer pair. The phytoplasma detected in all examined symptomatic samples of chrysanthemum were identified as belonging to stolbur, 16SrXII-A ribosomal subgroup, on the basis of RFLP analyses of 16Sr DNA. Therefore they were different from those reported and associated with similar symptoms in Italy; this is not an uncommon feature for phytoplasma associated diseases in both herbaceous and woody hosts (Lee et al., 1995; Padovan et al., 1995; Bertaccini et al., 1999).

On the other hand the presence of stolbur phytoplasma associated diseases was for the first time reported in Serbia in 1949 on pepper

(Martinović and Bjegović, 1950). Since then stolbur is always sporadically present and periodically affects production of Solanaceae plants, especially in dry years. Previously, stolbur phytoplasma in Serbia was reported in *Convolvulus arvensis* (Aleksić et al., 1969); later on by using molecular identification tools it was recently identified in grapevine and peach plants (Duduk et al., 2004; Duduk et al., 2005). The most relevant vector of stolbur in Serbia was determined as *Hyalestes obsoletus* (Aleksić et al., 1967; Aleksić et al., 1969; Šutić et al., 1983). Although this phytoplasma-associated disease was not severely affecting chrysanthemum cultivation in 2005, more severe appearance of the disease can be expected, especially in open fields and in years with appropriate conditions for the insect vector *H. obsoletus* multiplication, due to increasing production of ornamentals in Serbia during last years.

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Stolbur fitoplazme na hrizantemi u Srbiji

REZIME

Tokom pregleda otvorenog polja sa ukrasnim biljakama na lokalitetu Vinča, Beograd, primećene su biljke hrizanteme sa simptomima fitoplazmoza. Primećeni simptomi su obuhvatali proliferaciju cvetova, virescenciju i kržljavost. Radi provere moguće zaraze fitoplazmama, simptomatične biljke hrizanteme analizirane su molekularnim tehnikama, umetnutom lančanom reakcijom polimeraze (nested PCR) praćenom analizom polimorfizma dužine restrikcionih fragmenata (RFLP) na 16Sr DNA. U svim testiranim uzorcima uzetim sa simtomatičnih biljaka hrizanteme identifikovane su fitoplazme iz 16SrXII-A ribozomalne podgrupe. Prvi nalaz 16SrXII-A stolbur fitoplazmi u biljkama hrizanteme sa simptomima proliferacije cvetova, virescencije i kržljavosti razlikuje ovu fitoplazmozu od već poznate „chrysanthemum yellows" koja je povezana sa fitoplazmama koje pripadaju aster yellows 16SrI-B ribozomalnoj podgrupi, '*Candidatus* Phytoplasma asteris'.

Ključne reči: Hrizantema; *Chrysanthemum indicum hybridum*; stolbur fitoplazma