Journal of Medicinal Plants Research Vol. 6(5), pp. 906-911, 9 February, 2012
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR11.1584
ISSN 1996-0875 ©2012 Academic Journals

*Full Length Research Paper*

# The first Stolbur Phytoplasma occurrence on two St. John's Worth species (*Hypericum perforatum* L. and *Hypericum barbatum* L.) in Serbia

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Accepted 22 December, 2011

**The symptoms indicating phytoplasma like leaf yellowing, reddening and early drying were observed on two St. John's worth species (*Hypericum perforatum* L. and *Hypericum barbatum* L.) on infected fields (Pancevo, Indjija and Stara Pazova) in Serbia in 2008. Electron microscopy examination of the ultra-thin sections revealed the presence of numerous polymorphic phytoplasma-like bodies in the phloem tissue of leaf midribs and petioles. The phytoplasma etiology was confirmed by polymerase chain reaction (PCR) using 3 sets of primers (P1/P7, P1/16S-Sr and R16F2n/R16R2). Restriction fragment length polymorphism (RFLP) analysis of amplification products of 1.2 kb (obtained with R16F2n/R2 primer pair) in 51 from 60 symptomatic plants, indicated the presence of 16SrXII-A phytoplasma subgroup from all three affected localities. Sequence of R16F2n/R2 amplicon for representative phytoplasma *H. perforatum* L. isolate Hp22 was deposited in the GeneBank with accession number JQ033928. This is the first report of the natural occurrence of Stolbur phytoplasma in two cultivated St. John's worth species in Serbia.**

**Key words:** *Hypericum perforatum*, *Hypericum barbatum*, phytoplasma diseases, 16SrXII-A subgroup.

## INTRODUCTION

In the last few decades, the trends in increased use of herb in alternative medicinal purposes, have led to increased cultivation of medicinal plants such as *Hypericum*. In these new crops, unique diseases and pest problems are emerging. Some of these are rare or unknown in the wild, and have emerged by the use of agricultural systems (Scheffer, 1997). *Hypericum* (Hypericaceae) is one of the plants used traditionally in medicine for over 2000 years (Patocka, 2003) and one of the best – selling herbs of the past decade (Grunwald, 1999). In order to preserve the natural population of St. John's worth and due to a huge demand, *Hypericum perforatum* has been cultivated on as a crop since 1997 in Serbia.

Phytoplasmas are associated with serious plants disseases. They are plant-pathogenic bacteria of the class Mollicutes, have no cell wall and inhabit plant phloem of numerous plants. Cell sizes of phytoplasmas are 0.1 to 0.8 µm in diameter and genome size are the smallest among bacteria (Hren et al., 2009). Current classification of the phytoplasmas is based on the nucleotide sequence and restriction fragment length polymorphism (RFLP) of the 16S rRNA gene. Polymerase chain reaction (PCR) amplification of 16S rDNA of the phytoplasmas has significantly contributed to the identification and characterization of unidentified phytoplasmas (Montano et al., 2001; Harrison et al., 2002).

Diverse phytoplasma infections have been found on various cultivated medicinal plants: *Galega oficinalis* L., *Digitalis lutea* L., *Hyssopus officinalis* L., *Parietaria*

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**Table 1.** Presence of Stolbur Phytoplasma detected by nested PCR in the samples of *H. perforatum* and *H. barbatum* with symptoms in 3 localities in Serbia during 2010.

| Locality | Symptomatic plants | | Asymptomatic plants | |
|---|---|---|---|---|
| | Samples | No. nested PCR positive/ analysed | Samples | No. nested PCR positive/ analysed |
| Pancevo | HpP* 1-10 | 10/10 | HpP 31-33 | 0/3 |
| | HbP 1-10 | 9/10 | HbP 31-33 | 0/3 |
| Stara Pazova | HpS 11-20 | 7/10 | HpS 34-36 | 0/3 |
| | HbS 11-20 | 8/10 | HbS 34-36 | 0/3 |
| Indjija | HpI 21-30 | 9/10 | HpI 37-39 | 0/3 |
| | HbI 21-30 | 8/10 | HbI 37-39 | 0/3 |

*Hp- *H.perforatum*; Hb- *H.barbatum*; P- Pancevo; S- Stara Pazova; I-Indjija.

*officinalis* L., *Tagetes patula* L., *Spartium junceum* L., *Vinca rosea* (Lee et al., 2000), *Marticaria perforata* (Khadair et al., 1999), *H. perforatum* L. (Bruni et al., 2005), *Valeriana officinalis* (Khadhair et al., 2008), *Echinacea purpurea* (Radisek et al., 2008, Pavlovic et al., 2011), *Plantago lanceolata* L. (Credi et al., 2006, Franova and Simkova, 2009) and *Plantago major* (Josic et al., in press).

During the observation of the St. John's worth plants in the locality Pancevo, at the end of May 2008, the phytoplasma like symptoms were noted: yellowing, followed by reddening of leaves and proliferation. The following year, all plants developed a yellow-red color on all green part of the plants: stem and leaves. The diseased tissue showed necrosis, drying and death before flowering. The percentage of affected plants expressed as the reduction in yield was over 70 in some fields. This very high percentage of diseased plants brought into the question the commercial viability of the production.

## MATERIALS AND METHODS

### Electron microscopy

Samples for the observation of phloematic areas by transmission electron microscopy were taken from the symptomatic plants during June 2009 and May 2010. Main veins and petioles from symptomatic and asymptomatic leaves were fixed in 5% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2) for at least for 2 days at 4°C and, subsequently, post-fixed in 2.0% osmium tetroxide in the same buffer. The specimens were dehydrated by an ethanol series. Ultra-thin sections were stained with uranyl acetate in 70% ethanol and examined by the transmission electron microscopy (TEM) (Hopkins et al., 1973).

### Disease symptoms and incidence

Samples of *H. perforatum* and *Hypericum barbatum* showing symptoms of phytoplasma infection were collected in May 2010 from three different localities in Serbia - Pancevo, Stara Pazova and Indjija (Table 1). The symptoms included leaf yellowing, premature leaf drying and, occasionally, desiccation of the whole plant.

Disease incidence, expressed as a percentage of the area with symptomatic plants, was calculated approximately in all three localities during 2008 to 2010.

### Phytoplasma detection

A total of 30 *H. perforatum* and *30 H. barbatum* symptomatic leaf samples were collected. Eighteen asymptomatic plants (3 plants per locality for both species) were also collected as control samples.

For detection of phytoplasmas, total DNA was extracted from the veins of symptomatic and asymptomatic leaves using the protocol of Angelini et al. (2001). Oligonucleotide universal primers P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) were used in the direct PCR assays to amplify a 1.8 kb fragment including the 16S rRNA gene, the 16S–23S spacer region and the 5' end of the 23S rRNA gene.

The second set of primers was P1/16S-Sr and amplicons of 1.5 kb were expected in infected plants. For nested PCR, the R16F2n/R16R2 primers (Gundersen and Lee, 1996) were used to amplify a 1.2 kb fragment of the 16S rRNA gene included in the 1.8 or 1.5 kb fragment. Amplifications were performed in 50 µl reaction mixture using Dream Taq Green master mix (Fermentas, Lithuania). PCR using primer pairs P1/P7 and P1/16S-Sr were performed for 35-cycles: denaturation at 95°C for 60 s (2 min for first cycle), annealing for 30 s at 56°C and primer extension for 90 s at 72°C. For the nested PCR the same conditions were applied, except that annealing was done at 57°C. The PCR products were separated on 1.2% agarose in TBE buffer, stained with ethidium bromide and visualized on UV transilluminator.

### Phytoplasma identification

For the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis, the samples that yielded amplicons with R16F2n/R16R2 primers were used for further characterization using *Alu*I and *Tru*I restriction enzymes (Fermentas, Lithuania) as recommended by the manufacturer. RFLP profiles were analyzed by electrophoresis trough 2.5% agarose with GeneRuler SM0331 marker (Fermentas, Lithuania) as the DNA marker size standard. The following phytoplasma isolates

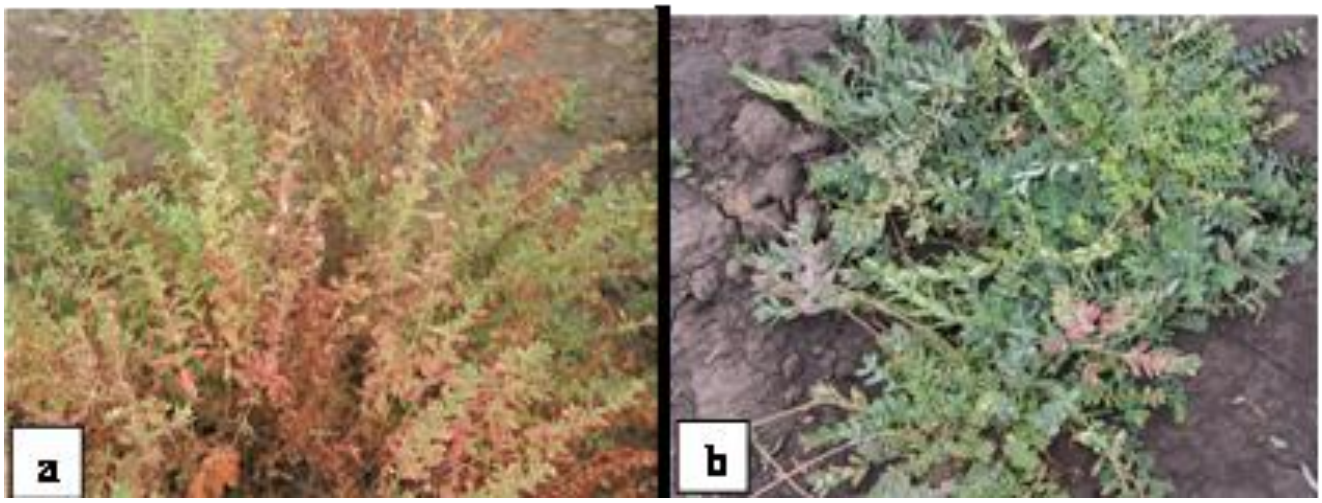


**Figure 1.** *H. perforatum* (a) and *H. barbatum* (b) plants infected with Stolbur phytoplasma.

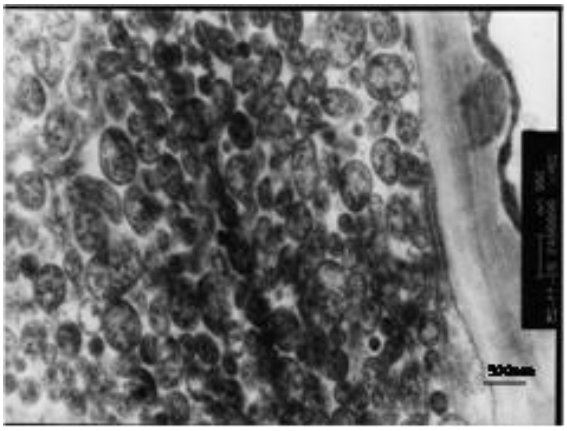

**Figure 2.** Ultra-thin cross-section of *H. perforatum* main vein from the symptomatic leaves (bar = 300 nm).

were used as references for PCR/RFLP analysis: STOL-phytoplasma DNA, grapevine FD-C and AY (kindly provided by M. Martini) phytoplasmas.

The PCR product of representative isolate Hp22 primed by R16F2n/R16R2 primers for partial 16S rRNA gene was subjected to the sequence analysis using facilities of IMGGI, Belgrade.

## RESULTS

### Disease symptoms and incidence

During the three years of observation (2008, 2009 and 2010) the first symptoms, consisting in diffuse yellowing and reddening of the leaves, proliferation and stunting, were observed from the beginning of May (Figure 1), increasing in severity with time. Symptomatic plants died within one or two months. The percentage of infected plants in first year of observation (2008) varied from 15 (Indjija and Stara Pazova) to 20% (Pancevo); in the second year from 40 (Indjija and Stara Pazova) to 70% (Pancevo), and in the third year of observation, this percentage in locality Indjija and Stara Pazova were higher than 70 while in Pancevo was over 85%.

### Electron microscopy

Thin section of the leaf main veins revealed the presence of typical phytoplasma-like bodies of 75 x 100 to 100 x 300 nm in diameter (Figure 2). They were observed in mature and immature phloem sieve tubes. In the control (healthy plants) there were no phytoplasma like bodies present.

### Phytoplasma detection

The PCR amplification from total DNA of symptomatic plants generated 1.8 kb (in 28 out of 40 samples) or 1.5 kb (in 17 out of 20 samples) DNA fragments of the 16S ribosomal DNA, when universal primers P1/P7 and P1/16S-Sr (Figure 3a) were used, respectively. The PCR amplification of a 1.2 kb (Figure 3b) fragments were obtained in 51 samples with R16F2n/R2 primers (Table 1). No PCR products were obtained from asymptomatic, healthy, control plants.

### Phytoplasma identification

All R16F2n/R2 amplified products gave identical RFLP patterns corresponding to the profile of the Stolbur phytoplasma (subgroup 16SrXII-A) using *Alu*I and *Tru*I restriction enzymes. RFLP patterns of representative isolates were shown in Figure 4. Partial 16S rRNA gene

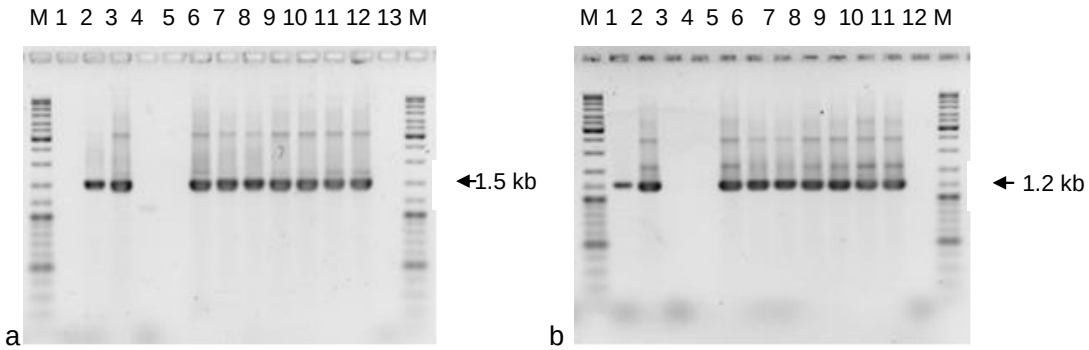


**Figure 3.** PCR amplification of 16S ribosomal DNA using (a) P1/16S-Sr primers: lane 1. HpI37; lane 2-7: HpI22; HpI24; HpI26; HpI27; HpI28; HpI30; lane 8-12: HbI21; HbI22; HbI23; HbI25; HbI27; HbI29; lane 13. HbI 38; (b) R16F2n/R2 primers: line 1-6. HpP1; HpP7; HpP33; HpS36; HpS14; HpS19; lane 7-12. HbP1; HbP4; HbP7; HbS11; HbS15; HbS36; M-Marker: GeneRuler DNA Ladder mix SM0331 (Fermentas, Lithuania).

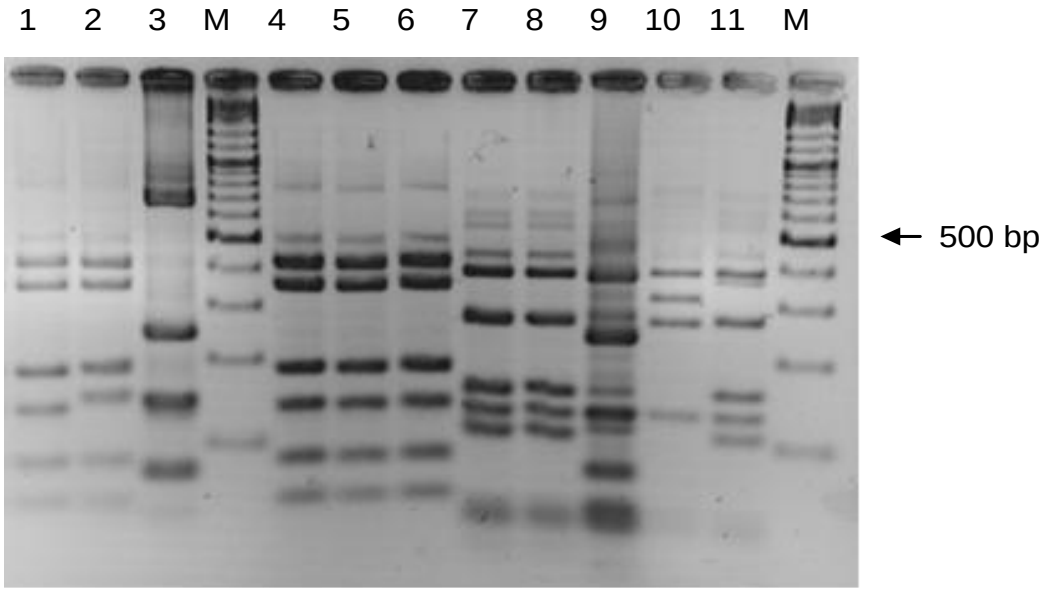


**Figure 4.** RFLP analysis of the 1.2-kb PCR product (R16F2n/ R2) digested by *Alu*I (lane 1-6): lane 1. control 16SrXII group (STOL); lane 2. control AY phytoplasma; lane 3. control FD-C phytoplasma; Marker; lane 1-3: HpS19; HpI22; HbP4; and *Tru*I (lane 7-11): lane 7-8: HpI22; HbP4; lane 9. control FD-C phytoplasma; lane 10. control AY phytoplasma; lane 11. control 16SrXII group (STOL); Marker: GeneRuler DNA Ladder mix SM0331 (Fermentas, Lithuania).

and the 16S–23S spacer region sequence of representative isolate Hp22 was deposited in the GenBank database under accession No. JQ033928.

## DISCUSSION

The ultra-thin transverse or longitudinal sections of the deseased tissues clearly show the presence of typical phytoplasma – like bodies of 75 to 300 nm in diameter, which corresponds to the known dimensions already cited in literature (Franova et al., 2004; Irti et al., 2008; Hren et al., 2009).

According to the results of the RFLP analysis, all symptomatic *H. perforatum* and *H. barbatum* plants showed the same pattern as STOL reference isolate. The analysis of partial 16S rRNA gene sequence (JQ033928) showed high level of similarities with 16S ribosomal RNA gene partial sequences of Bulgarian 'Rubus fruticosus' phytoplasma Stolbur-Rubus-Bg deposited by Bobev and De Jonghe (2011) (under accession number JF293091.1), Italian 'Bois noir'

phytoplasma strain CH-1 from periwinkle (HQ589193.1) deposited by Kube et al. (2010) and Russian Stolbur-Rus phytoplasma strain Rus93 (GU004375.1) deposited by Lee et al. (2010).

Phytoplasmas of the Stolbur group (subgroup 16SrXII-A) infect a wide range of vegetable crops. In Serbia, Stolbur is a long known pathogen of pepper plant (Martinovic and Bjegovic, 1950) and bindweed *Convolvulus arvensis* (Aleksic et al., 1969). The high incidence of Stolbur occurred in past decade, mostly infecting grapevine (Duduk et al., 2004; Kuzmanovic et al., 2008; Josic et al., 2010), corn (Duduk and Bertaccini, 2006), peach (Duduk et al., 2008) and weed species such as *Cirsium arvense* (Rancic et al., 2005). Recently, Stolbur disease was detected in important medicinal plants - purple coneflower (Pavlovic et al., 2011) and plantain (Josic et al., in press).

The presence of pathogens has a significant influence on the composition of the secondary metabolites in plants. A study of Bruni et al. (2005) revealed that *H. perforatum* plants infected with an ash yellows phytoplasma (ribosomal group 16SrVII) produced lower amounts of essential oil (0.11%) than healthy plants (0.75%), and that a higher sesquiterpene and aliphatics ratio was observed in the infected plants.

The chemical variation in plants affected by phytoplasmas may decrease their therapeutic efficacy and commercial value. The first detection of Stolbur phytoplasma in *H. perforatum* and *H. barbatum* plants in the three locations in Serbia (Pancevo, Stara Pazova and Indjija) indicates the need for further investigations in the way in which phytopatological conditions affect the quality of secondary metabolites in medicinal plants and commercial crops.

## ACKNOWLEDGEMENTS

We thank Dr Marta Martini (Università di Udine, Italy) for kindly providing the AY phytoplasma. This research was supported by the Ministry of Education and Science of the Repbulic of Serbia, through Projects TR-31018 and III46007.

### REFERENCES

Aleksic Z, Sutic D, Aleksic D (1969). Study of stolbur disease as a problem of pepper production in Yugoslavia. Zastita bilja, 105: 235-239.

Angelini E, Clair D, Borgo M, Bertaccini A, Boudon-Padieu E (2001). Flavescence golden in France and Italy - Occurence of closely related phytoplasma isolates and their near relationships to Palatinate grapevine yellows and an alder yellows phytoplasma. Vitis, 40: 79-86.

Bobev SG, de Jonghe KWA (2011). First Report of Stolbur Phytoplasma on Blackberry *(Rubus fruticosus*) in Bulgaria. http://www.ncbi.nlm.nih.gov/nucleotide/326378237?report=genbank&log$=nucltop&blast_rank=1&RID=C08YG814013.

Bruni R, Pellati F, Bellardi GM, Paltrinieri S, Bertaccini A, Bianchi A (2005). Herbal Drug Quality and Phytochemical Composition of *Hypericum perforatum* L. affected by Ash Yellows Phytoplasma Infection. J. Agric. Food Chem., 53: 964-968.

Credi R, Terlizzi F, Milanesi L, Bondavalli R, Cavallini G, Montermini A, Dradi D (2006). Wild host plants of stolbur phytoplasma and its vector, *Hyalesthes obsoletus*, at sites of grapevine bois noir occurrence in Emilia-Romagna, Italy. Extended abstracts, 15th Meeting of the International Council for the Study of Virus and Virus-like Diseases of the Grapevine, Stellenbosch, South Africa, pp. 182-183.

Deng S, Hiruki C (1991). Amplification of 16S rRNA genes from culturable and nonculturable Mollicutes, J. Microbiol. Methods, 14: 53-61.

Duduk B, Botti S, Ivanovic M, Krstic B, Dukic N, Bertaccini A (2004). Identification of Phytoplasmas Associated with Grapevine Yellows in Serbia. J. Phytopathol., 152: 575-579.

Duduk B, Bertaccini A (2006) Corn with symptoms of reddening: new host of stolbur phytoplasma. Plant Dis., 90: 1313-1319.

Duduk B, Ivanovic M, Paltrinieri S, Bertaccini A (2008). Phytoplasmas infecting fruit trees in Serbia. Proceedings of the XX International Symposium on virus an virus-like diseases of temperate fruits crops-fruit thee diseases, 781: 351-358.

Franova J, Paltrinieri S, Botti S, Simkova M, Bertaccini A (2004). Association of Phytoplasmas and Viruses with Malformed Clovers. Folia Microbiol., 49: 617-624.

Franova J, Simkova M (2009): Association of "*Candidatus* Phytoplasma asteris" with Yellowing and Phyllody of *Plantago lanceolata*. Folia Microbiol., 54(5): 469-472.

Grunwald J (1999). The world market for hypericum products. Nutraceuticals World, 5-6: 22-25.

Gundersen DE, Lee IM (1996). Ultrasensitive detection of phytoplasmas by nested-PCR assays using two universal primer pairs. Phytopathologia Mediterranea, 35: 144-151.

Harrison NA, Womack M, Carpio ML (2002). Detection and characterization of a lethal yellowing (16SrIV) group phytoplasma in Canary Island date palms affected by lethal decline in Texas. Plant Disease, 86: 676-681.

Hopkins DL, Mollenhauer HH, French WJ (1973). Occurrence of a Rickettsia-like Bacterium in the Xylem of Peach Trees with Phony Disease. Phytopathology, 63: 1422-1423.

Hren M, Nikolic P, Rotter A, Blejec A, Terrier N, Ravnikar M, Dermastia M, Grueden K (2009). Black wood, phytoplasma induces significant reprogramming of the leaf transcriptome in the field grown grapevine. BMG Genomics, 10: 460.

Irti M, Quaglino F, Maffi D, Casati P, Bianco A, Faoro F (2008). Solanum malocoxylon, a New Natural Host of Stolbur Phytoplasma. J. Phytopathol., 156: 11-14.

Josic D, Kuzmanovic S, Stojanovic S, Aleksic G, Pavlovic S, Starovic M (2010). Detection of XIIA Phytoplasma group on Cultivar Zupljanka in Zupa vineyard region by RFLP analysis of 16S rDNA sequences. Genetika, Belgrade, 42: 146-153.

Khadhair AH, McClay A, Hwang SF, Shan H (1999). Aster Yellows Phytoplasma Identified in Scentless Chamomile by Microscopical Examinations and Molecular Characterization. J. Phytopathol., 147: 149-154.

Khadhair AH, Hiruki C, Heyholos M (2008). Molecular Characterization of Aster Yellows Phytoplasma Associated with Valerian and Sowthistle Plants by PCR-RFLP Analyses. J. Phytopathol., 156: 326-331.

Kube M, Mitrovic J, Duduk B, Bertaccini A (2010). http://www.ncbi.nlm.nih.gov/nucleotide/315258455?report=genbank&log$=nucltop&blast_rank=2&RID=C08YG814013.

Kuzmanovic S, Martini M, Ermacora F, Ferrini F, Starovic M, Carraro L, Osler R, Tosic M (2008). Incidence and molecular characterization of *Flavescence doree* and *stolbur* phytoplasma in grapevine cultivars from different viticultural areas of Serbia. Vitis, 47: 105-111.

Lee IM, Davis RE, Gundersen-Rindal DE (2000). Phytoplasma: Phytopathogenic mollicutes. Annu. Rev. Microbiol., 54: 221-255.

Lee IM, Bottner-Parker KD, Zhao Y, Davis RE, Harrison NA (2010). Phylogenetic analysis and delineation of phytoplasmas based on the secY gene. Int. J. Syst. Evol. Microbiol., 60(12): 2887-2897.

Martinovic M, Bjegović P (1950). On somediseases and pests identified in the People's Republic of Serbia in 1949. Plant Protect., 2: 59-68.

Montano MG, Davis RE, Dally EL, Hogenhout S, Pimentel JP, Brioso PST (2001). Int. J. Syst. Evol. Microbiol., 51: 1109-1118.
Patocka J (2003). The chemistry, phyrmacology and toxicology of the biologically active constituents of the herb *Hypericum perforatum L*. Appl. Biomed., 1: 61-70.
Pavlovic S, Starovic M, Stojanovic S, Popovic T, Aleksic G, Drazic S, Josic D (2011). *Echinacea purpurea* – a host of 16SrXII-A phytoplasma group in Serbia. Phytopathogenic Mollicutes, 1(1): 35-39.
Radisek S, Ferant N, Jakse J, Javornik B (2008). Identification of a phytoplasma from the aster yellows group infecting purple coneflower (*Echinacea purpurea*) in Slovenia. New Dis. Reports, 18: 13.
Rancic D, Saltrineri S, Tosevski I, Petanovic R, Stevanovic B, Bertaccini A (2005). First report of multiple inflorescence disease of *Cirsium arvense* and its association with a 16SrIII-B subgroup phytoplasma in Serbia. Plant Pathol., 54: 561.
Scheffer RP (1997). The Nature of Disease in Plants; Cambridge University Press: Cambridge, UK.. p. 325.
Schneider B, Seemüller E, Smart CD, Kirkpatrick BC (1995). In Molecular and Diagnostic Procedures in Mycoplasmology (eds Razin S, Tully JG), Academic Press, San Diego, USA, 1995, 1: 369-380.