First Report of Carrot Infection with Phytoplasmas in Slovenia

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Abstract—In Slovenia, in years 2016 and 2017, carrot plants were sampled for ‘Candidatus Liberibacter solanacearum’ presence verification. All samples were negative for this pathogen. Because some samples displayed symptoms reminiscent of phytoplasma diseases they were also tested for phytoplasma infection. An association of diseased plants with phytoplasmas was confirmed with the universal quantitative PCR and (ii) the ribosomal groups of 16SrI and 16SrXII were identified by specific quantitative PCRs. Sequence analysis of tuf, secY and stamp gene fragments indicated that the 16SrXII phytoplasmas in carrot samples belong to the ‘Candidatus Phytoplasma solani’. This research is the first report of carrot infection in phytoplasmas in Slovenia.

Key words—aster yellows, ‘Candidatus Phytoplasma solani’, carrot, Slovenia.

I. INTRODUCTION

In carrot plants infected with phytoplasma symptoms of leaf reddening, purpling and yellowing, formation of chlorotic adventitious shoots, proliferation and reduction of size and quality of roots have been observed in Israel (Orenstein et al. 1999), Lithuania (Valiunas et al. 2001), Canada (Wally et al. 2004), Serbia (Duduk et al. 2008), India (Arocha et al. 2009), the UK (Nisbet et al. 2014), Iran (Vali-Sichani et al. 2014) and Saudi Arabia (Omar 2017). Further analyses of samples from symptomatic carrot plants have shown their association with different phytoplasma subgroups of 16SrI, 16SrII or 16SrXII groups. This research is the first report of phytoplasmas associated with carrot plants in Slovenia.

II. MATERIAL AND METHODS

Samples, DNA extraction and ‘Candidatus Liberibacter solanacearum’ testing

During the official survey for ‘Candidatus Liberibacter solanacearum’ of the Administration of the Republic of Slovenia for Food Safety, Veterinary Sector and Plant Protection, 14 symptomatic samples and one asymptomatic sample of carrot (Daucus carota subsp. sativus) were collected in 2016 and in 2017 in 12 locations in Slovenia (Table 1). DNA was extracted from different parts of carrot samples using a kit (QuickPick Plant DNA kit; Bio-Nobile, Finland) and a purification system (KingFisher mL; Thermo Scientific, USA) as described in Pirc et al. (2009); the samples were then tested by Li et al. (2009) quantitative PCR for the presence of ‘Ca. L. solanacearum’.

Phytoplasma testing

Since ‘Ca. L. solanacearum’ had not been detected in the samples and the symptoms could indicate phytoplasma infection, the samples were further tested by quantitative PCR assays with phytoplasma universal- (Christensen et al. 2004), “stolbur” (16SrXII) group specific- (Hren et al., 2007) and aster yellows (AY; 16SrI) group specific primers and probes (Angelini et al. 2007; Nikolić et al. 2009). The qPCR was performed on an ABI PRISM® 7900HT Fast Detection System (Applied Biosystems), as described by Mehle et al. (2013). The final reaction volume of 10 µl contained 2 µl sample DNA, 1× TaqMan® Universal PCR Master Mix (Applied Biosystems), 300 nM (forward primer of phytoplasma universal assay)/ 900 nM (all except forward primer of phytoplasma universal assay) primers, plus 100 nM (phytoplasma universal and AY specific assays)/ 250 nM (16SrXII specific assay) probe. All type of quality controls for PCR based diagnostics that are described by Dermastia et al. (2017) have been included. A sample was considered positive if it produced an exponential amplification curve distinguishable from negative controls. If no exponential amplification curve was produced, a sample was considered negative.

Molecular characterisation of 16SrXII strains

The 16SrXII-positive samples were further investigated. Nested PCR procedures were performed with primers fTUF1/ rTUF1 and fTUFAY/ rTUFAY (tuf gene; Schneider et al. 1997), with POSecF1/ POSecR1 and POSecF3/ POSecR3 (secY gene; Fialová et al. 2009) as well as with StampF/ StampR0 and StampF/ StampR1 (stamp gene; Fabre et al. 2011). All the PCR assays were performed in a PCR cycler (PCR System 9700 Gene Amp) in 50 µl final reaction volumes that contained: 2 µl 10-fold diluted DNA sample; 1× High Fidelity buffer (Invitrogen); 2 mM MgSO4 (Invitrogen); 200 µM dNTPs (Applied Biosystems); 0.02 U/µl Platinum Taq DNA Polymerase High Fidelity (Invitrogen); and 0.2 µM (secY, stamp)/ 0.4 µM (tuf) of each primer. The PCR conditions for the initial denaturation were 2 min (secY, stamp)/ 3 min (tuf) at 94°C, which was followed by 35 cycles of denaturation for 15 s (secY, stamp)/ 30 s (tuf) at 94°C, annealing for 30 s at 54°C (secY)/ 56°C (stamp)/ 45°C (tuf), and extension for 1 min at 68°C. The final extension was for
7 min at 68°C (tuf). Nested PCR was carried out using 2 µl of the PCR amplification product. The nested PCR conditions were as for PCR except the annealing temperature (secY: 62°C, stamp: 52°C, tuf: 53°C). The nested PCR products were separated on 1% agarose gels stained with ethidium bromide, observed under UV light, and purified later (MiniElute PCR purification kits, Qiagen).

The forward and reverse sequencing reactions for the nested PCR products were performed by Macrogen Europe using the Sanger method. The partial amplified sequences of tuf, secY and stamp were compared with sequences from the GenBank database, using the BLAST algorithms (http://www.ncbi.nlm.nih.gov/blast). DNA sequence alignments of the Slovenian strains were carried out by creating contiguities using the ContigExpress software (Vector NTI). TUFAY fragments were also analysed by restriction fragment length polymorphism (RFLP) with HpaII (New England Biolabs) according to the manufacturer’s instructions. HpaII restriction products were separated by electrophoresis on 2.5 % (W/v) polyacrylamide gels, stained with ethidium bromide and visualized on a UV transilluminator. RFLP patterns were compared with RFLP patterns identified for ‘Candidatus Phytoplasma solani’ by Aryan et al. (2014): two carrot samples [D356/17 and D369/16 (subsample: part between the root and stem)] showed a tuf-type b pattern, while the remaining sample (D509/16) revealed a tuf-type a pattern. To confirm these results sequencing of the TUFAY fragments was performed. This allowed a discrimination of three tuf sequence types, which were all identical to sequences already described for different ‘Ca. P. solani’ strains: tuf-type a (D509/16), tuf-type b1 (D356/17) and tuf-type b2 (D369/16) (Table 2).

Nested PCR products of secY and stamp were also directly sequenced by the Sanger method. The obtained sequences of the 781-bp segment of secY and the 472- (D369/16), 511- (D356/17), 534-bp segments (D509/16) of stamp were compared. All 16SrXII-positive samples had the same secY genotype. The secY genotype has been found identical to several different ‘Ca. P. solani’ reported strains (Table 2). More variability was observed for the sequence of the stamp gene. The stamp sequences of D369/16 and D356/17 differed in only one nucleotide, while the stamp sequence of D509/16 differed from D369/16 and D356/17 stamp sequences in 22 and 23 nucleotides, respectively. However, stamp genotypes of all three carrot samples were identical to stamp genotypes of some previously described ‘Ca. P. solani’ strains (Table 2).
et al. 2008).

from the study on the carrot phytoplasma disease in Serbia (Duduk et al., 2012). Phytoplasmas have been previously confirmed to be responsible for the carrot phytoplasma diseases in other countries (Duduk et al., 2012; Nisbet et al., 2014; Vali-Sichani et al., 2014). Phytoplasmas from different groups cannot be differentiated by specific symptomatology. Such results are consistent with the findings from the study on the carrot phytoplasma disease in Serbia (Duduk et al., 2008).

### Table 1: Carrot samples analysed by qPCRs for the presence of phytoplasmas

<table>
<thead>
<tr>
<th>Sample ID (ID/year of sampling)</th>
<th>Geographical origin (part of Slovenia)</th>
<th>Symptoms</th>
<th>Material for phytoplasma detection</th>
<th>Results of phytoplasma testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>D337/16-1</td>
<td>Stojnči (NE)</td>
<td>leaf reddening</td>
<td>roots part between the root and stem</td>
<td>pos* (16SrI)</td>
</tr>
<tr>
<td>D363/16</td>
<td>Dol pri Ljubljani (central)</td>
<td>leaf reddening</td>
<td>roots part between the root and stem</td>
<td>pos (16SrI)</td>
</tr>
<tr>
<td>D310/17</td>
<td>Moste pri Komendi (central)</td>
<td>leaf reddening</td>
<td>roots part between the root and stem</td>
<td>pos (16SrI)</td>
</tr>
<tr>
<td>D322/17</td>
<td>Brunsice (SE)</td>
<td>leaf reddening, presence of adventitious roots</td>
<td>roots part between the root and stem</td>
<td>pos (16SrI)</td>
</tr>
<tr>
<td>D357/17</td>
<td>Formin (NE)</td>
<td>reddening of some leaves, presence of adventitious roots</td>
<td>roots part between the root and stem</td>
<td>pos (16SrI)</td>
</tr>
<tr>
<td>D369/16</td>
<td>Dolgavas (NE)</td>
<td>leaf reddening</td>
<td>roots part between the root and stem</td>
<td>pos (16SrI)</td>
</tr>
<tr>
<td>D309/16</td>
<td>Grbovje pri Preklopi (SE)</td>
<td>leaf reddening, decay of plants</td>
<td>roots part between the root and stem</td>
<td>pos (16SrI)</td>
</tr>
<tr>
<td>D356/17</td>
<td>Stojnči (NE)</td>
<td>weak reddening of some leaves, presence of adventitious roots</td>
<td>roots part between the root and stem</td>
<td>neg</td>
</tr>
<tr>
<td>D337/16-2</td>
<td>Stojnči (NE)</td>
<td>asymptomatic</td>
<td>roots part between the root and stem</td>
<td>neg</td>
</tr>
<tr>
<td>D370/16</td>
<td>Zgornji Dupešek (N)</td>
<td>weak leaf bronzing</td>
<td>roots part between the root and stem</td>
<td>neg</td>
</tr>
<tr>
<td>D290/17</td>
<td>Zalog pri Cerkljah (central)</td>
<td>weak leaf bronzing</td>
<td>roots</td>
<td>neg</td>
</tr>
<tr>
<td>D291/17</td>
<td>Spodnji Brnik (central)</td>
<td>weak leaf yellowing</td>
<td>roots</td>
<td>neg</td>
</tr>
</tbody>
</table>

*pos – the presence of phytoplasma was confirmed by universal phytoplasma qPCR; neg – the presence of phytoplasma was not confirmed by universal qPCR; AY – positive signal with two qPCRs specific for ‘Ca. P. aseris’; 16SrXII – positive signal with 16SrXII group specific qPCR.

*a weak signal was observed using phytoplasma universal qPCR.

### Table 2: Genotypes of 16SrXII positive carrot samples. ‘Ca. P. solani’ strains from GenBank database that have identical nucleotide sequence are also listed.

<table>
<thead>
<tr>
<th>Sample ID (ID/year of sampling)</th>
<th>Tuf type</th>
<th>References: Acc. No. (country, host)</th>
<th>SecY type</th>
<th>References: Acc. No. (country, host)</th>
<th>Stamp type</th>
<th>References: Acc. No. (country, host)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D369/2016 b2</td>
<td></td>
<td>KJ469709 (Austria, Hyalethes obsoletus), LT899729 (Azerbaijan, Vitis vinifera)</td>
<td>secY-Slo-carrot 1</td>
<td>FM163376 (France, H. obsoletus), KP635228 (Switzerland, Reptalus panzeri), KU374896 (Bosnia and Herzegovina, Zea mays), JQ977710 (Germany, Convolvulus arvensis), LT941330 (France, Salvia sclarea), FO393427 (Serbia, Nicotiana sp.)</td>
<td>stamp-Slo-carrot 1</td>
<td>KP877603 (Serbia, H. obsoletus), KP877604 (Serbia, Reptalus quinquecostatus), KC703022 (Serbia, Vitis sp.)</td>
</tr>
<tr>
<td>D509/2016 a</td>
<td></td>
<td>KJ469707 (Austria, H. obsoletus), GU220558 (Italy, V. vinifera)</td>
<td>secY-Slo-carrot 1</td>
<td>FO393427 (Serbia, Nicotiana sp.)</td>
<td>stamp-Slo-carrot 2</td>
<td>FO393427 (Serbia, Nicotiana sp.)</td>
</tr>
<tr>
<td>D356/2017 b1</td>
<td></td>
<td>KJ469708 (Austria, H. obsoletus), LT899728 (Azerbaijan, V. vinifera), GU220562 (Italy, V. vinifera), KF907507 (Bulgaria, Zea mays), EU552455 (Czech Republic, Trifolium pratense), LA6370 (France, Lycopersicon esculentum), FO393427 (Serbia, Nicotiana sp.)</td>
<td>secY-Slo-carrot 1</td>
<td>FO393427 (Serbia, Nicotiana sp.)</td>
<td>stamp-Slo-carrot 3</td>
<td>KP739853 (Bosnia and Herzegovina, V. vinifera)</td>
</tr>
</tbody>
</table>

### IV. DISCUSSION

The results obtained in this work revealed the presence in symptomatic carrot, of two different phytoplasmas: aster yellows (group 16SrI) and ‘Ca. P. solani’ (group 16SrXII). Both phytoplasmas have been previously confirmed to be responsible for the carrot phytoplasma diseases in other countries (Duduk et al., 2008; Nisbet et al., 2014; Vali-Sichani et al., 2014). Phytoplasmas from different groups cannot be differentiated by specific symptomatology. Such results are consistent with the findings from the study on the carrot phytoplasma disease in Serbia (Duduk et al., 2008).

Aster yellows group phytoplasmas reported here for the first time in carrots in Slovenia, they were previously described in Echinacea purpurea, as first report of this phytoplasma in the country (Radišek et al. 2009). It has also been detected in a sample of Euscelis incisus (sample from 2003; NE Slovenia), Trifolium sp. (sample from 2012; SW Slovenia) and Malus domestica (sample in 2016; NE Slovenia), and in three samples of Vitis vinifera (two samples from 2014 and in one sample from 2016; all from SE Slovenia) (unpublished data).
‘Ca. P. solani’ is widespread in all wine-growing regions in Slovenia (Mehle et al. 2011). Phytoplasmas belonging to the “stolbur” group has been detected also in several samples of Convolvulus arvensis, in different known and putative vectors and in Lycopersicon esculentum plants (Mehle et al. 2011). In 2016, it has been detected also in Urtica dioica (unpublished data). However, this is the first report of ‘Ca. P. solani’ in carrots in Slovenia. Further research is necessary to get more insight into epidemiology of phytoplasmas that affects carrot plants in Slovenia e.g. incidence of the diseases, identification of relevant vectors and the infection of surrounding plants. In addition, the significance of similarities among strains found in carrots and in other hosts plants are yet to be clarified.

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