SHORT COMMUNICATION

SURVEY OF “CANDIDATUS LIBERIBACTER SOLANACEARUM” IN CARROT CROPS AFFECTED BY THE PSYLLID TRIOZA APICALIS (HEMIPTERA: TRIOZIDAE) IN NORWAY

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SUMMARY

The carrot psyllid Trioza apicalis Förster (Hemiptera: Triozidae) is a serious insect pest of carrot (Daucus carota L.) in northern Europe, where it can cause up to 100% crop loss. Although it was long believed that T. apicalis causes damage to carrot by injection of toxins into the plant, it was recently established that this psyllid is a vector of the new bacterium “Candidatus Liberibacter solanacearum”, which severely damages carrot crops. This bacterium is also known to severely affect solanaceous crops and is the causal agent of zebra chip, a new and economically important disease of potato in North and Central America and New Zealand. Symptoms in psyllid-affected carrots include leaf curling, yellowish and purplish discoloration of the leaves, stunted growth of both leaves and roots, and proliferation of secondary roots. These plant symptoms resemble those caused by leafhopper-transmitted phytoplasmas and Spiroplasma citri in carrots. Using PCR assays, a survey of “Ca. L. solanacearum” in carrot crops in Norway determined that the bacterium is widespread in several counties in southeastern and eastern Norway, where most of the carrot crops are grown. Liberibacter infection rate ranged from 33.3 to 100% in carrot plants and from 21.2 to 56.3% in T. apicalis. No phytoplasmas or sproplasmas were detected in carrot or psyllid samples by PCR. Information from this research will help carrot producers reduce damage caused by “Ca. L. solanacearum” to carrot crops by vigorously monitoring and controlling T. apicalis, its insect vector.

Key words: carrot psyllid, liberibacter, phytoplasmas, sproplasmas, Trioza apicalis

The carrot psyllid Trioza apicalis Förster (Hemiptera: Triozidae) is a serious pest of carrot (Daucus carota L.) in northern Europe, particularly in Finland, Sweden, and Norway, where it can cause up to 100% crop loss (Markkula et al., 1976; Rygg, 1977; Burckhardt, 1986; Nehlin et al., 1994; Kristoffersen and Anderbrant, 2007; Nissinen et al., 2007; Meadow, 2010; Munyaneza, 2010). This insect is also known as an occasional pest in central Europe, including Switzerland (Burckhardt and Freuler, 2000). Symptoms in psyllid-affected carrots include leaf curling, yellowish and purplish discoloration of the leaves, stunted growth of both leaves and roots, and proliferation of secondary roots (Markkula et al., 1976; Rygg, 1977; Nissinen et al., 2007; Munyaneza et al., 2010a, 2010b). Feeding by T. apicalis significantly reduces carrot yield, but also lowers quality of the carrot as sucrose concentration in damaged roots is significantly decreased and concentrations of some phenolic compounds are increased (Nissinen et al., 2012; Seljåsen et al., 2013). These changes in metabolites result in production of carrots that are tough and lack crispness, in addition to having negative taste characteristics, including bitterness and astringency (Seljåsen et al., 2013). These plant symptoms resemble those caused by leafhopper-transmitted phytoplasmas and Spiroplasma citri in carrots (Font et al., 1999; Lee et al., 2006; Duduk et al., 2008; Cebrián et al., 2010).

It was recently discovered that “Candidatus Liberibacter solanacearum” (syn. “Ca. Liberibacter psyllaurous”), a new bacterium associated with economically important diseases of potato and other solanaceous crops in the United States, Mexico, Central America, and New Zealand is also associated with both T. apicalis and carrot crops affected by this psyllid in Finland (Munyaneza et al., 2010a, 2010b, 2011; Nissinen et al., 2013), Sweden (Munyaneza et al., 2012a), and Norway (Munyaneza et al., 2012b). This liberibacter species is the causal agent of zebra chip disease of potato and is transmitted to solanaceous species by the potato/tomato psyllid, Bactericera cockerelli (Šulc) (Munyaneza et al., 2007; Munyaneza, 2012). In addition, “Ca. L. solanacearum” has recently been reported on carrot and celery crops in Spain and
Carrot psyllids were collected with sweep nets and sticky traps from 10 commercial carrot fields from July to September of 2011 and 2012 in the municipalities of Rygge, Østfold county and Larvik, Vestfold county in southeastern Norway from late July to mid-September of 2011 and 2012 (Table 1, Fig. 1). Eighty to 90% of each of the sampled fields in Østfold, Vestfold, and Hedmark were covered with insect nets to exclude *T. apicalis*; thus, the samples were obtained from parts of the field that were not covered. The rate of symptomatic plants (leaf curling and/or discoloration) in each field ranged from 10 to 100%. Leaf and root samples were shipped to the USDA-ARS facility in Wapato (WA, USA) for testing. A total of 254 plant samples (Table 1) were collected and tested for *Ca. L. solanacearum*. The samples were also tested for phytoplasmas and spiroplasmas.

Carrot plants were collected from 30 commercial and research fields in nine municipalities of Østfold, Akershus, Vestfold, Oppland and Hedmark counties in southeastern Norway, and in Rogaland county in southwestern Norway from late July to mid-September of 2011 and 2012 (Table 1, Fig. 1). Eighty to 90% of each of the sampled fields in Østfold, Vestfold, and Hedmark were covered with insect nets to exclude *T. apicalis*; thus, the samples were obtained from parts of the field that were not covered. The rate of symptomatic plants (leaf curling and/or discoloration) in each field ranged from 10 to 100%. Leaf and root samples were shipped to the USDA-ARS facility in Wapato (WA, USA) for testing. A total of 254 plant samples (Table 1) were collected and tested for *Ca. L. solanacearum*. The samples were also tested for phytoplasmas and spiroplasmas.

Total DNA was also extracted from carrot plant material using a modified version of the CTAB buffer extraction method of Pastrik and Maiss (2000). DNA was extracted from petiole and root tissues from field-collected carrot plants using the procedure described below. Five hundred mg of carrot tissue (petioles or roots) was macerated in 1 ml of extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA, 500 mM NaCl, 10 mM mercaptoethanol) using BioReba sample bags and a ‘Homex’ apparatus (Bioreba, Switzerland). Carrot tissue macerate (300 µl) was mixed with 80 µl lysozyme [50 mg/ml in 10 mM Tris-HCl, pH 8.0 (Sigma-Aldrich, USA)] and incubated for 30 min at 37°C. After incubation, 500 µl CTAB buffer (2% CTAB, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0, 0.2% mercaptoethanol) was added to the homogenate and the sample was incubated for 30 min at 65°C. The sample was then placed at room temperature for 3 min before the addition of 500 µl of ice cold chloroform. Samples were vortexed to mix, then centrifuged at 21,000 g for 10 min. The aqueous layer was then transferred to a new microfuge tube and a 0.6 vol. of isopropanol was added and the tube was placed on ice for 20 min to precipitate DNA. DNA was recovered by centrifugation as above. The pellet was washed with ice cold 75% ethanol and centrifuged 21,000 g for 2 min. After removal of ethanol, the pellet was air dried then re-suspended in 100 µl sterile water.

Insect and plant DNA sample extracts were tested for the presence of “*Ca. L. solanacearum*” by PCR. To determine whether to use conventional or quantitative real-time PCR, a total of 89 psyllid and 144 carrot plant samples were tested by both methods. Thirty seven out the 89 psyllid samples and 53 out of the 144 carrot samples tested positive for “*Ca. L. solanacearum*” with each of the PCR methods. Given the equal sensitivity of the two PCR methods, all the remaining insect and plant samples were tested by conventional PCR only.

Real-time PCR was performed with primers and probe targeting the outer membrane protein of “*Ca. L. solanacearum*” as described by Crosslin *et al.* (2011) and Sengoda *et al.* (2013). Briefly, the testing was carried out in 25 µl reaction with 12.5 µl TaqMan Universal PCR Master Mix (Applied Biosystems, USA), 2.5 µl of each primer (9 µM), 2.5 µl of labeled probe (2.5 µM), 4 µl sterile water, and 1.0 µl of either psyllids or plants genomic DNA. PCR reactions were amplified on Chromo4 (Bio-Rad, USA) with the following cycling conditions: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 sec and 60°C for 60 sec. The threshold cycle (Ct) values were exported using Opticon Monitor 3.1 (Bio-Rad, USA) with cut-off value of 37.

During the present study, conventional PCR used primer pair OA2/OI2c (Jagoueix *et al.*, 1996; Liefting *et al.*, 2008, 2009a), which amplifies DNA sequences from the 16S rRNA gene of “*Ca. L. solanacearum*”. This primer pair is expected to produce 1168 bp-amplicon (Jagoueix *et al.*, 1996; Liefting *et al.*, 2008, 2009a; Munyaneza *et al.*, 2010a,
2010b). Negative and positive controls were included in all PCR assays. Amplifications were performed in 25 µl reactions with Green Go Taq Polymerase (Promega, USA) according to the manufacturer’s instructions. For each reaction, 10 pmol of each primer, and 1 µl of DNA extract was added, and incubated with the following conditions: initial denaturation for 3 min at 94°C, then amplification for 20 sec at 94°C, 20 sec at 65°C, 1 min at 72°C for 39 cycles, followed by a final 5 min incubation at 72°C. PCR products were separated on 1.5% agarose gels containing ethidium bromide for visualization.

For TA cloning, four carrot amplicons were randomly selected and excised using clean razor blades and ethidium bromide removed using GenElute Minus EtBr Spin columns (Sigma-Aldrich, USA). The purified PCR products were cloned using the TOPO TA cloning kit (Invitrogen, USA) with TOP 10 Escherichia coli chemically competent cells. Plasmid DNA was extracted from selected colonies using the QIAprep spin mini prep kit (Qiagen, USA) and the DNA clones were sequenced at MC Laboratories (MCLab, USA). Three clones from each of the four selected amplicons were sequenced. A consensus sequence was obtained from alignment of the 12 sequences from the clones and submitted to the GenBank database.

Similarly, the insect and plant DNA samples were tested for the presence of phytoplasmas and spiroplasmas by PCR. For phytoplasma amplification, nested PCR was used. The first round PCR was performed with universal primer pair P1/P7 followed by the second round PCR

Table 1. “Candidatus Liberibacter solanacearum” (Lso) infection rate in carrot crops in southeastern Norway. Conventional PCR was used as detection method.

<table>
<thead>
<tr>
<th>County</th>
<th>Municipality</th>
<th>2011</th>
<th>2012</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Lso-infected plants/symptomatic plants</td>
<td>Lso-infected plants/asymptomatic plants</td>
<td>Lso-infected plants/symptomatic plants</td>
</tr>
<tr>
<td>Østfold</td>
<td>Rygge</td>
<td>16/17</td>
<td>2/13</td>
</tr>
<tr>
<td>Akershus</td>
<td>Ås</td>
<td>1/3</td>
<td>0/4</td>
</tr>
<tr>
<td>Vestfold</td>
<td>Larvik</td>
<td>6/10</td>
<td>0/14</td>
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<td>Nøtterøy</td>
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<tr>
<td></td>
<td>Oppland</td>
<td>Østre Toten</td>
<td>12/16</td>
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<tr>
<td>Hedmark</td>
<td>Hamar</td>
<td>8/11</td>
<td>0/7</td>
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<tr>
<td></td>
<td>Stange</td>
<td>2/6</td>
<td>0/5</td>
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<tr>
<td>Rogaland</td>
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<td>Klepp</td>
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</table>

Table 2. “Candidatus Liberibacter solanacearum” (Lso) infection rate in Trioza apicalis in southeastern Norway. Conventional PCR was used as detection method.

<table>
<thead>
<tr>
<th>County</th>
<th>Municipality</th>
<th>2011</th>
<th>2012</th>
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<tbody>
<tr>
<td></td>
<td>Lso-infected psyllids/total number of psyllids tested</td>
<td>---</td>
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</tr>
<tr>
<td>Østfold</td>
<td>Rygge</td>
<td>18/32</td>
<td>32/150</td>
</tr>
<tr>
<td>Vestfold</td>
<td>Larvik</td>
<td>11/52</td>
<td>4/11</td>
</tr>
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</table>

Figure 1. Carrot plant and psyllid sampling sites in carrot-growing regions of southern Norway.
Currently, the only means to manage diseases associated with “Ca. L. solanacearum” is to focus on effective control of its psyllid vectors (Munyaneza, 2012). Thus, documenting occurrence of this bacterium and *T. apicalis* in carrot crops in affected regions is essential. Results of the present survey show that “Ca. L. solanacearum” is widespread in carrot growing regions of Norway, including Østfold, Akershus, Vestfold, Oppland, and Hedmark counties in the southeastern parts of the country, where about 80% of carrots are produced. The estimated “Ca. L. solanacearum” infection rate in carrot crops and psyllids was high, with infection rate ranging from 33.3 to 100% in carrot plant samples and from 21.2 to 56.3% in the insects. Therefore, there is a need to vigorously monitor and control *T. apicalis* in these carrot-growing regions of Norway to minimize damage caused by “Ca. L. solanacearum” to carrot crops in the country.

The carrot psyllid is very difficult to control using currently registered insecticides in Norway because of their poor efficacy (Meadow, 2010). The only effective method to control *T. apicalis* is the use of crop covers (Meadow, 2010), which is not economically sustainable. However, monitoring *T. apicalis* is a crucial tool to provide carrot producers with information on the timing of migration from overwintering hosts and field colonization by the psyllid in order to take appropriate psyllid control measures. Furthermore, it is recommended that similar surveys of “Ca. L. solanacearum” in carrot crops and psyllids be undertaken in neighbouring countries to better develop regional management strategies for this devastating plant pathogen and its insect vectors.

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