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Occurrence of phytoplasmas in leafhoppers and cultivated grapevines in Canada

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ABSTRACT

Following the recent detection of bois noir, X-disease and aster yellow (AY) in Canadian vineyards, a survey was conducted in 2006–2008 in vineyards located in Ontario (ON), British Columbia (BC) and Quebec (QC). PCR tests were used to detect phytoplasmas in grapevine cultivars and leafhopper species, and identify phytoplasma strains. The overall detection of phytoplasmas was <1% in BC, and ranged from 2.4% to 8.3% in ON and QC, respectively, with significant variability between years, cultivars and locations. A large proportion of phytoplasma-infected grapevines were symptomless and the cultivars merlot and pinot gris were the only two cultivars in which no phytoplasma were detected. Aster yellow phytoplasma strains belonging to the subgroups 16SrI-A, -B, and -C were detected in grapevines, with 16SrI-A being the most frequently found. Phytoplasma DNA was detected in 37 leafhopper species, out of which 11 are known vectors. Most leafhopper species in which phytoplasma DNA were detected are grass feeders, with the AY vector *Macrosteles quadrilineatus* constituting a strong potential candidate for AY transmission in ON and QC vineyards. Five new sequences of phytoplasma were found in grapevines, and 11 in leafhoppers: they were registered in GenBank. The possibility of phytoplasma transmission between leafhoppers and grapevines is discussed.

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1. Introduction

Phytoplasmas are non-culturable prokaryotic microorganisms belonging to the mollicute class. Phytoplasmas have been classified based on molecular analysis of the highly conserved 16Sr genes, as well as other genes (Weintraub and Jones, 2010; Lee et al., 2010).

Phytoplasmas are transmitted by phloem-feeding insects, most of them belonging to the Auchenorrhyncha, such as leafhoppers (Weintraub and Beanland, 2006; Olivier et al., 2012). Many insects can acquire phytoplasmas and be considered as "carriers" (i.e., determined phytoplasma-positives by molecular methods). However, few species can be competent "vectors", i.e., acquire and successfully transmit phytoplasmas to a plant. Vectoring ability is the result of a successful specific tritrophic interaction between the phytoplasma, the insect vector and the host plant (Weintraub and Beanland, 2006; Hogenhout et al., 2008; Weintraub and Jones, 2010).

Grapevine yellows (GYs), caused by phytoplasmas, are economically important diseases that have been detected in Europe, North Africa, Australia, USA and Chile (Weintraub and Jones, 2010; Steffek et al., 2007). Typical symptoms of GYs include leaf chlorosis and downwards rolling, flower abortion or berry withering, die-back of shoot tips, uneven or total lack of lignification of canes, and reduced vitality (Weintraub and Jones, 2010), as well as lower wine quality (Steffek et al., 2007).

Until recently, little was known about phytoplasmas associated with vineyards in Canada. The closest identified GY was the North America grapevine yellow (NAGY), associated with the aster yellow (AY) group (16SrI-I) and X-disease group (SrI-III) and detected in the states of Virginia and New York (Pearson et al., 1985; Beanland et al., 2006). However, in 2006, bois noir (BN), a disease associated with a phytoplasma belonging to the stolbur group (16SrXII) was detected for the first time in vineyards from Ontario (ON) and British Columbia (BC) (Rott et al., 2007), and X-disease was

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identified in grapes from ON (Johnson, personal communication). In 2007, phytoplasmas belonging to the AY group were detected in symptomatic and symptomless grapevines in BC, ON and Quebec (QC) (Olivier et al., 2009).

In USA, several species of leafhoppers were identified as potential vectors of NAGY in vineyards of Virginia (Beanland et al., 2006) and New York (Maixner et al., 1993). Several leafhopper species, such as *Macrosteles quadrilineatus*, *Amplicephalus inimicus* or *Exitianus exitiosus*, known or suspected vectors of phytoplasmas (Beanland et al., 2006; Weintraub and Beanland, 2006) have been collected in Canadian vineyards (Bostanian et al., 2003; Olivier et al., 2009; Saguez et al., 2014).

Planthoppers can also vector phytoplasma belonging to the stolbur and coconut lethal yellowings phytoplasma (Weintraub and Beanland, 2006). However, coconut lethal yellowing phytoplasma has not been detected in North America and BN, the only stolbur phytoplasma detected in Canada in 2006, has been eradicated and is now considered absent from Canada, under IPPC standards (NAPPO, 2007). Therefore, only leafhopper species were sampled and analysed in this project.

Essential steps to design science-based programmes to address phytoplasma issues in vineyards are to (i) determine the biodiversity of leafhoppers associated with vineyards, and (ii) identify the phytoplasma strains associated with grapevines and their potential insect vectors. Between 2006 and 2008, we addressed these two steps in commercial Canadian vineyards. The first step, i.e., the study of the biodiversity of leafhoppers, has been treated in Saguez et al. (2014). The second step is hereafter presented, with two main objectives: (1) to detect phytoplasmas in grapevine cultivars and leafhopper species and (2) to identify, using PCR tests, phytoplasma strains present in symptomatic and symptomless grapevines and in leafhoppers.

2. Materials and methods

In 2006, 2007 and 2008, insects and plants were collected in 37 commercial vineyards of BC (throughout the Okanagan Valley, from Vernon in the north to Osoyoos in the south, and in the Similkameen Valley near Cawston and Keremeos), and in 51 sites located in 47 commercial vineyards of ON (Vineland, Beamsville, Niagara-on-the-Lake, St. Catharines and Stoney Creek). In 2007 and 2008, collections were done in five vineyards of QC (Saint-Jacques-le-Mineur, Saint-Rémi, Dunham and Saint-Armand) (GPS coordinates listed in Table A1, Appendix A).

Vineyards in BC, ON and QC were visited throughout the growing season from May until October. Every year, BC and ON vineyards were visited at least once a month. In QC, the same vineyards were visited weekly from July to October in 2007 and 2008. Before our study, no GY phytoplasma was previously detected or identified in the surveyed vineyards. However, in few cases, some grapevines had symptomatic appearance.

In each vineyard, five independent rows (one cultivar/row) in which symptomatic grapevines, i.e., plants exhibiting characteristic symptoms (e.g., yellowing, leaf rolling), were selected. Grapevine leaves were collected in the same rows throughout the study. At each sampling time, 50 leaves were collected per row from 10 individual grapevines, i.e., five leaves/grapevine randomly collected at different levels of the canopy. If the number of symptomatic leaves was <50 in the five selected rows, symptomless leaves that presented damage due to leafhopper feeding (e.g., punctures, hopperburn) were randomly collected in the same five selected rows. Leaves were placed in plastic bags, labelled per cultivar, and stored in a cooler for transportation to the laboratory.

In BC and ON, leaves were individually cut in small pieces of about 1 cm^2 placed in 2.0 ml Eppendorf tubes, each containing leaves collected on one grapevine. In QC, two leaf discs (diameter 1.8 cm) were punched from each leaf (out of five leaves/grapevine). The 100 leaf discs from a same row were pooled in a single 50 ml Corning® tubes (Corning Inc., Corning, NY). For all discs or leaf pieces, care was taken to include main veins and part of the petioles. They were freeze-dried and kept at −20 °C until analysis.

Leafhoppers were collected as described in Saguez et al. (2014). Briefly, they were collected in the same rows as grapevine leaves using tapping or sweeping methods. Insects were kept in ethanol 70% until identification. All collected specimens were identified using morphological characteristics and male genitalia. Species were keyed as mentioned in Saguez et al. (2014).

2.1. Detection and identification of phytoplasmas

Each grapevine sample was composed of leaves collected in a same row. For BC and ON, 2–4 leaf pieces were taken from each Eppendorf tubes and pooled per row. For QC, 10 discs were randomly taken in each Corning® tube. This procedure was repeated three times. The total number of analysed samples for BC, ON and QC was 905, 3788, and 990, respectively.

After identification, leafhopper specimens belonging to species known to be phytoplasma vectors or potential vectors (Weintraub and Beanland, 2006; Olivier et al., 2009) were individually assayed. Leafhoppers belonging to *Erythroneura* sp., *Empoasca* sp. and *Edwardsiana rosae* were tested in groups of 10 specimens, and *Graminella nigrifrons* in groups of three specimens. Other species were individually tested. Each group, pooled in a 2.0 ml Eppendorf tube, constituted a sample. DNA extraction and purification was performed using the method described by Daire et al. (1997).

For grapevines, negative and positive controls for PCR analysis consisted respectively of DNA extracts of uninfected grapevine (cv. chardonnay, provided as in vitro plants from Canadian Food and Inspection Agency-Sydney, BC), and AY-infected periwinkle grown in cages containing AY-infected *M. quadrilineatus*. For leafhoppers, negative and positive controls consisted respectively of DNA extracts from uninfected, and AY-infected *M. quadrilineatus* reared on barley and periwinkles. All plants were grown in greenhouses and insects reared in separated growth chambers (16 h day/8 h night, 23 °C, 63% R.H.) at AAFC-Saskatoon, SK.

All DNA extracts were amplified according to the methods described by Olivier et al. (2010), using the universal phytoplasma-specific primer pair P1/P7 (Deng and Hiruki, 1991; Schneider et al., 1995) for a first amplification, followed by primer pair R16R2/R16F2 (Lee et al., 1993) for a second amplification. The amplifications were performed in 20 μl mixtures containing 2 μl of 10× Invitrogen buffer (Invitrogen Canada Inc., Burlington, ON, Canada), 200 μM dNTP, 5 μM of each primer and, for the first amplification, 4 μl of undiluted DNA with 5 mM $MgCl_2$ and, for the second amplification, 2 μl of diluted PCR product (1/200) of the first amplification with 2.5 mM $MgCl_2$. Denaturation was performed at 92 °C for 1 min 30 s, followed by 30 cycles of 45 s at 92 °C, 45 s at 57 °C and 1 min 45 s at 72 °C, for the first amplification and at 92 °C for 1 min 15 s, followed by 35 cycles of 30 s at 92 °C, 30 s at 67.5 °C and 1 min 15 s at 72 °C for the second amplification. PCR products were separated on 1.9% agarose electrophoresis gels followed by staining in ethidium bromide. Phytoplasma DNA was revealed by the presence of a 1200 bp DNA fragments when observed under a UV transilluminator set at 365 nm (AIML 26, Alpha Innotech Corp., San Leandro, CA, USA).

1200 bp DNA fragments of phytoplasma DNA contained in leaves and leafhoppers were extracted from the agarose gel and purified using a Qia-Quick Gel Extraction Kit (Qiagen Canada, Mississauga, ON, Canada) (Fig. A1, Appendix A). Sequencing was performed at the Plant Biotechnology Institute of the National

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