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Transmission of 16SrIII-J phytoplasmas by the leafhoppers *Paratanus exitiousus* and *Bergallia valdiviana*

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Summary. Two of the most common leafhoppers present in Chile are the Cicadellidae *Paratanus exitiosus* and *Bergallia valdiviana*. They commonly occur in vineyards of central Chile, including some vineyards infected by phytoplasmas. The present study demonstrates that *P. exitiosus* and *B. valdiviana* can transmit 16SrIII-J phytoplasmas to grapevine and periwinkle plants. This provides improved understanding of the 16SrIII-J phytoplasma epidemiology in Chilean vineyards.

Keywords. Auchenorrhyncha, transmission trials, nested-PCR, *tuf* gene, 16S rRNA gene, RFLP.

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INTRODUCTION

In Chile, grapevine yellows (GY) is associated with phytoplasmas belonging to diverse ribosomal subgroups, including 16SrI-B and 16SrI-C ('*Candidatus* Phytoplasma asteris'-related), 16SrIII-J ('*Ca*. P. pruni'-related), 16SrV-A ('*Ca*. P. ulmi'), 16SrVII-A ('*Ca*. P. fraxini'), 16SrXII-A ('*Ca*. P. solani' or "stolbur") (Gajardo *et al*., 2009; Fiore *et al*., 2015). However, the phytoplasmas in the 16SrIII-J group were prevalent in the vineyards of the central zone of the country. This phytoplasma has been reported to infect various crops and spontaneous plant species, and a draft sequence of the genome of a Chilean strain was obtained (Gonzalez *et al*., 2011; Zamorano and Fiore, 2016; Quiroga *et al*., 2017a).

Phytoplasma dissemination in the field occurs with the use of infected plant materials and by insect vector transmission. The insects generally feed on weeds and only occasionally on grapevine, allowing the transmission of phytoplasmas to this species. In order to determine which insects are involved in transmission of the 16SrIII-J group-phytoplasma in Chile, an epidemiological study was carried out in symptomatic phytoplasma-infected vineyards. During dedicated surveys, several leafhoppers species (Hemiptera, Auchenorryncha, Cicadellidae) were found to be positive for the 16SrIII-J phytoplasma (N. Fiore, unpublished data), and among them, two of the most common insect species were *Paratanus exitiousus* (Beamer) and *Bergallia valdiviana* Berg 1881. This paper reports transmission of 16SrIII-J phytoplasma to periwinkle (*Catharanthus roseus* L.) and grapevine plants by both leafhoppers.

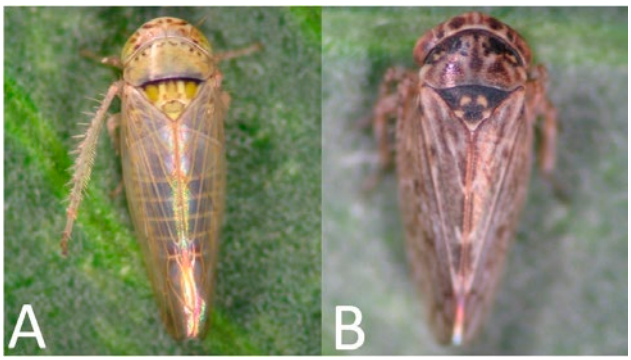

Figure 1. The two insect species used in transmission trials: A) *Paratanus exitiosus*; B) *Bergallia valdiviana.*

MATERIALS AND METHODS

In 2011 (September–December) and 2012 (January–May) surveys for *P. exitiosus* presence were carried out in three selected 16SrIII-J phytoplasma infected Chilean vineyards, two in the Metropolitana region (Buin and Pirque), and one in the Valparaiso region (Casablanca). The capture of *B. valdiviana* adults was carried out in 2012 (October–December) and 2013 (January–June) in a vineyard of cv. Pinot noir infected with 16SrIII-J phytoplasmas, in the Valparaiso region (Casablanca). *Paratanus exitiosus* and *B. valdiviana* (Figure 1) were captured using an entomological sweeping net and 150 sweepings directed to weeds present inside the vineyards. At the end of each capture, the adults of both insects were separated by species and divided in two batches. All the individuals belonging to one species were released into two entomological cages to let them feed on three grapevine plants of cv. Cabernet Sauvignon (first cage), and three periwinkle plants (second cage). The number of individuals released each month in each cage is reported in Tables 1 and 2.

Periwinkle and grapevine plants used in the transmission trials were from seeds, and tested by nested PCR (described below) to verify the absence of phytoplasmas before the transmission trials. Eighty-one periwinkle and 81 grapevine plants were used for the trials with *P. exitiosus,* while the phytoplasma transmission by *B. valdiviana* was performed to 27 periwinkle and 21 grapevine plants. All the plants were kept in a conditioned

Table 1. Numbers of *Paratanus exitiosus* individuals captured in three vineyards. Insects were released each month in cages to feed on periwinkle and grapevine plants for phytoplasma transmission trials.

Month	Number of individuals used in transmission trials to grapevine plants			Number of individuals used in transmission trials to periwinkle plants		
	Vineyard			Vineyard		
	Buin	Pirque	Casablanca	Buin	Pirque	Casablanca
September 2011	33	6**	2	32	7	2
October 2011	25**	17	20	24	18	20
November 2011	34	8	22	33**	7	22
December 2011	36	18**	9	35**	17	8**
January 2012	33	20	21	32	20	20
February 2012	31	14	30	30	14	30
March 2012	34	10	24	33	10	23
April 2012	18	5	21**	17	4	20
May 2012	33**	3	23	32	3	22

** Successful transmission trials for phytoplasma 16SrIII-J.

Table 2. Numbers of *Bergallia valdiviana* individuals captured in the Casablanca vineyard. Insects were released each month in cages to feed on periwinkle and grapevine plants for phytoplasma transmission trials.

Month	Number of individuals used in transmission trials to grapevine plants	Number of individuals used in transmission trials to periwinkle plants
October 2012	0	4
November 2012	0	16
December 2012	17	22
January 2013	20**	25
February 2013	28**	33
March 2013	24	30
April 2013	26	21**
May 2013	26	19
June 2013	0	23

** Successful transmission trials for phytoplasma 16SrIII-J.

incubator at 25ºC with 16 h of photoperiod. Each feeding period lasted until the death of all insects in the cages (four to six leafhoppers per test).

The cages were inspected on a daily basis to collect the dead insects and store them in 70% ethanol for further analyses. The cages without insects were then moved into a screen house for symptom onset. Phytoplasma detection in plants was carried out every three months. All insects from each cage were also tested in order to verify the phytoplasma presence.

Total nucleic acids were extracted from plants using a chloroform/phenol method (Prince *et al*., 1993), while insect nucleic acid was extracted with a CTAB method (Angelini *et al*., 2001). Nucleic acid was dissolved in Tris-EDTA pH 8.0 buffer and maintained at 4°C. PCR amplification was carried out using 20 ng μL^{-1} of nucleic acid. Direct and nested PCR on the *tuf* gene were performed according to the protocol of Makarova *et al*. (2012). Further direct PCR with primer pair P1/P7 (Deng and Hiruki, 1991; Schneider *et al*., 1995) and nested PCR with R16F2n/R2 primers on the *16S rRNA* gene (Gundersen and Lee, 1996), were performed as described by Schaff *et al*. (1992). Amplicons from nested PCRs for both genes were purified using the EZNA gel extraction kit (OMEGA Bio-tek). DNA fragments were ligated into the pGEMT-Easy Cloning Kit (Promega). Putative recombinant clones were analyzed by colony PCR, and selected fragments were sequenced in both directions by Macrogen USA Corp. The sequences were then aligned with those of classified strains deposited in GenBank using BLAST engine for local alignment (version Blast N 2.2.12). The phytoplasma identification was carried out using *in silico* restriction fragment length polymorphism (RFLP) analysis with *Hha*I, *BstU*I, and *Rsa*I restriction enzymes, in the *i*PhyClassifier online tool (https://plantpathology.ba.ars.usda.gov/cgi-bin/resource/iphyclassifier.cgi) (Zhao *et al*., 2009).

RESULTS

Leafhopper surveys

In the vineyard located in Buin, the *P. exitiosus* capture rate remained constant during all months, except for decreases in October 2011 and April 2012 (Table 1). In the Pirque vineyard, the greatest numbers of individuals were collected in October and December 2011, and January 2012. In the Casablanca vineyard, *P. exitiosus* was less abundant in September 2011, remained constant during the other months and increased in February 2012. These results indicated that in the surveyed areas *P. exitiosus* tended to be more abundant during summer.

Adult individuals of *B. valdiviana* were captured in both years in greatest abundance through the summer and remained constant in autumn (Table 2). This insect was of greatest abundance in the coastal valleys, where Casablanca is located. For this reason, only the insects captured in Casablanca vineyards were used for the transmission trials.

Phytoplasma identification

Phytoplasma 16SrIII-J was detected in some grapevine and periwinkle plants used in the transmission trials, and in *P. exitiosus* and *B. valdiviana* specimens that were fed on these plants. Sequences of cloned PCR fragments from both amplified genes were the same from the positive plants and insects in the *tuf* gene sequence (438 bp). On the other hand, sequence identity of the *16S rRNA* gene clones was 99.9 to 100% (1,230 bp) in the same samples. Two sequences of the *tuf* gene were deposited in GenBank under the accession numbers (Acc. No.) MH743135, MH743136, and two of the *16S rRNA* gene and were deposited under Acc. Nos MH743137, MH743138. For the *16S rRNA* gene, the nucleotide similarity percentages of the detected phytoplasmas showed a close correlation (99.8%) with those of the strain Ch10 (GenBank Acc. No, AF147706), corresponding to the chayote witches' broom phytoplasma (16SrIII-J) from Brazil. For the *tuf* gene, the greatest nucleotide identity was 100%, with the strain Hort72, belonging to a 16SrIII-J phytoplasma from sugarbeet from Chile (GenBank Acc. No. KM658259). The 16S rRNA amplicons were also subjected to *in silico* RFLP

analysis that assigned the detected phytoplasmas to the ribosomal subgroup 16SrIII-J (Figure 2).

Transmission trials with Paratanus exitiosus *to grapevine plants*

Paratanus exitiosus survived for 3 to 4 days on the grapevine plants. In the twelve months post-transmission trials, the phytoplasma presence was detected by nested PCR with primers for *tuf* and *16S rRNA* genes in five out of the 81 grapevine plants. Two plants (V47 and V78A) were from the transmission trials carried out with insects captured in Buin in two different months (respectively October 2011 and May 2012). Two plants (V43 and V61) were infected from insects captured in Pirque, during, respectively, September and December 2011. The plant V76B was infected by individuals of *P. exitiosus* captured in Casablanca, during April 2012 (Table 1). The transmission rates were 7.5% for the insects captured in Buin and Pirque, and 3.7% for those from Casablanca. At 24 months after the transmission trials were set up, two out of five grapevine plants infected with 16SrIII-J were asymptomatic (V43 and V61), while the other three plants showed short internodes, and leaves with downward curling and deformation (Figure 3).

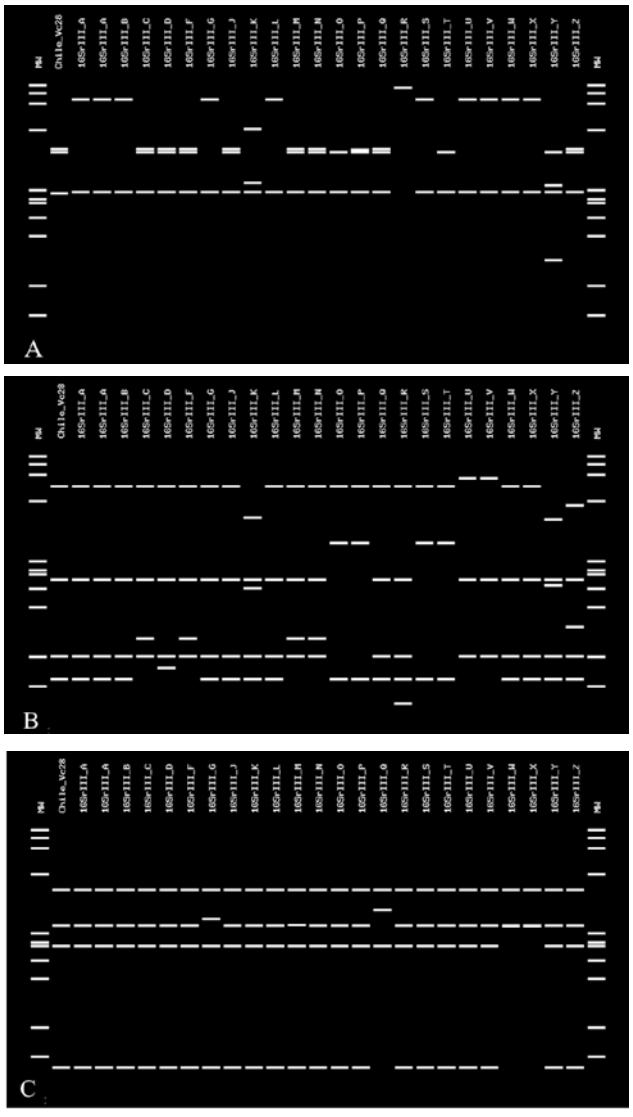

Figure 2. Virtual RFLP patterns obtained from the restriction with *Hha*I (A); *BstU*I (B) and *Rsa*I (C), of a 1,200 bp *16S rRNA* gene fragment obtained from sample VC28 (phytoplasma detected in a periwinkle plant used in transmission trials with *Paratanus exitiosus*). The phytoplasmas transmitted are classified in subgroup 16SrIII-J. The strains representative of the diverse subgroups are those enclosed in the *i*PhyClassifier (Zhao *et al*., 2009).

Transmission trials with Paratanus exitiosus *to periwinkle plants*

Paratanus exitiosus survived for 4 to 5 days on periwinkle plants. Three months after the transmission trials, in three out of the 81 periwinkle plants, the phytoplasma presence was detected by nested PCR with the primers for *tuf* and *16S rRNA* genes (Table 1). Two of the positive plants (VC28C and VC31C) were from the transmission trials carried out with insects captured in the Buin vineyard in two different months (November and December 2011, respectively). The third plant (VC33A) was infected by individuals captured in the Casablanca vineyard during December 2011. The three periwinkle plants infected with 16SrIII-J phytoplasmas showed symptoms of virescence, phyllody and witches' broom five months after the start of the transmission trials (Figure 4).

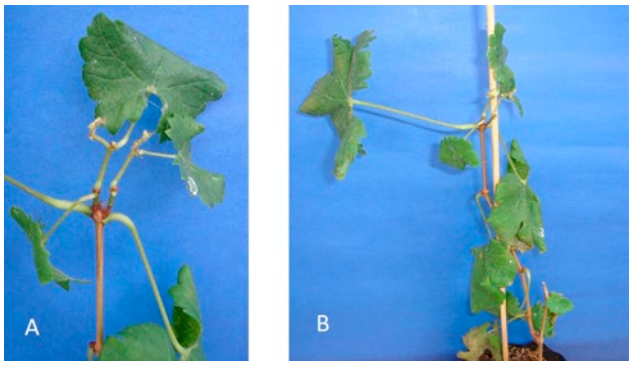

Figure 3. Symptoms associated with phytoplasma presence in grapevine after *Paratanus exitiosus* transmission. A) V43, short internodes; B) V76, leaves with downward rolling, deformations and necrosis.

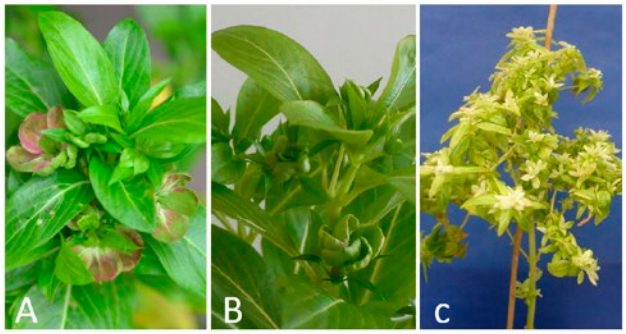

Figure 4. Symptoms associated with phytoplasma presence in periwinkle after *Paratanus exitiosus* transmission. A) VC28C, flowers with virescence; B) VC31C, flowers with virescence and phyllody; C) VC33A, plant with witches' broom, small and yellow leaves.

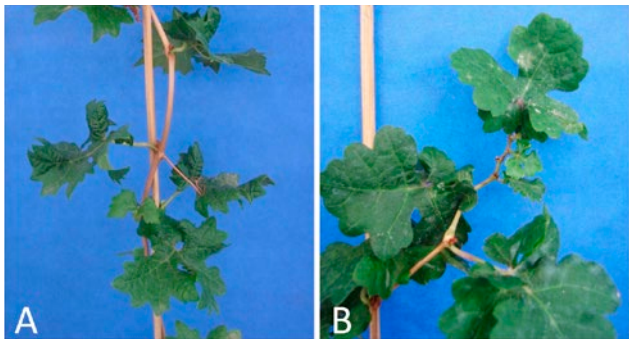

Figure 5. Symptoms associated with phytoplasma presence in grapevine after *Bergallia valdiviana* transmission. Short internodes, and leaves with downward rolling and deformation: A) VC85A; B) V86C.

Transmission trials with Bergallia valdiviana *to grapevine plants*

Bergallia valdiviana survived for 4 to 5 days on grapevine plants. At 24 months post-transmission trials, two of the 21 plants were positive for phytoplasma presence in nested PCR tests using primers for *tuf* and *16S rRNA* genes (Table 2). The positive grapevine plants (V85A and V86C) corresponded to the transmission trials carried out with insects captured in two different months (January 2013 for V85A and February 2013 for V86C). The transmission rate was 9.5%. The two grapevines infected with 16SrIII-J were symptomatic, with short internodes, and leaves with downward rolling and deformation (Figure 5).

Figure 6. Symptoms in periwinkle after *Bergallia valdiviana* transmission; A), B) VC60B, leaf deformation and severe yellowing; C) VC62B, flowers showing virescence.

Transmission trials with Bergallia valdiviana *to periwinkle plants*

Bergallia valdiviana survived for 6 to 7 days on periwinkle plants. Two out of the 27 plants were positive for phytoplasma presence, as determined with nested PCR using primers for *tuf* and *16S rRNA* genes (Table 2) 12 months after the transmission trials. These plants (VC 60B and V62B) corresponded to the transmission trials performed with insects captured in April 2013. The transmission rate was 3.7%. The 16SrIII-J infected periwinkle plants showed symptoms of virescence, leaf deformation and severe yellowing, 15 months after the transmission trials (Figure 6).

DISCUSSION

P. exitiosus and *B. valdiviana* transmitted the phytoplasma 16SrIII-J to grapevine and periwinkle plants. Both insects live on weeds and only occasionally feed on grapevine or other crops. *P. exitiosus* transmitted the phytoplasmas at a higher rate to grapevine than to periwinkle plants, while *B. valdiviana* had the same percentage of transmission to grapevine and periwinkle. Survival times of both insects on the grapevine plants was less than on periwinkle plants. The detection of 16SrIII-J phytoplasmas and the appearance of symptoms in grapevine occurred later than in the periwinkle plants. In the plants used for the transmission assays with *B. valdiviana*, phytoplasma detection occurred later in comparison with *P. exitiosus* assays and, as expected, the symptoms took longer to appear. This could indicate that the amount of inoculum of the pathogen in the *B. valdiviana* individuals used for transmission trials was less than in *P. exitiosus*, or that its transmission efficien-

cy was less. The main differences from the transmission trials were that higher transmission resulted from the *P. exitiosus* captured in the spring and summer periods, while *B. valdiviana* was only able to transmit the phytoplasma when collected at the end of summer and autumn. This vector difference could play a fundamental role in maintaining the phytoplasma population in host weeds during the periods of grapevine vegetative recess.

The phytoplasma 16SrIII-J and its newly identified insect vectors are widely distributed in Chile, infecting different weed species and crops of agronomic importance (Castro *et al*., 2000; Hepp and Vargas, 2002; González *et al*., 2010; 2011; Longone *et al*., 2011). Taking the observed *P. exitiosus* and *B. valdiviana* phytoplasma-transmission rates into account, if environmental conditions are favorable, there is high possibility that grapevine yellows outbreaks, associated with the presence of 16SrIII-J phytoplasmas, will occur in the central zone of Chile. Recent studies have indicated that climate change could modify the habitat of these species of insects, and increase their reproduction rates in the central zone of Chile (Quiroga *et al*., 2017b).

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