



Evaluation of rust pathogenicity (*Phragmidium violaceum*) as a biological control agent for the invasive plant *Rubus ulmifolius* on Robinson Crusoe Island, Chile

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Received: 7 May 2018 / Accepted: 21 January 2019
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Abstract

Rubus ulmifolius is a highly invasive species that negatively affects biodiversity, especially on Robinson Crusoe Island (RCI), one of the two global mini-hotspots of vascular plants. The rust *Phragmidium violaceum* has been used as a biological control agent for some *Rubus* species in mainland Chile, New Zealand and Australia. We aim to quantify *P. violaceum* pathogenicity over *R. ulmifolius* on RCI, under both field and laboratory conditions, to determine its potential as a biological control. In addition, in order to broaden our knowledge about the pathogenicity of *P. violaceum* from RCI, it was also tested on a highly susceptible *Rubus* species: *R. cf. constrictus*. We sampled four forest sites on RCI, measuring abiotic, micro-site, and vegetation variables that could be related with infection. In the laboratory, we inoculated *P. violaceum* into *R. ulmifolius* seedlings from RCI and *R. cf. constrictus* seedlings from mainland Chile. We found a low infection incidence in the field, where only 62 of 182 plots presented infected individuals. Applying a fitted generalized linear model, we predicted ten times more infection of *P. violaceum* on *R. ulmifolius* when located in southern aspect slopes and shallow soils (< 30 cm). In the laboratory we determined that rust from RCI infected *R. ulmifolius* individuals from the island in low density, and did not infect *R. cf. constrictus*; this low infection rate reinforces our field results. The low infection on *R. cf. constrictus* may mean that *P. violaceum* from RCI could originate from very impoverished genetic material. We recommend testing different rust genetic origins, mainly from the Old World, to increase their genetic diversity.

Keywords Blackberry · Juan Fernández islands · Restoration · Control invasive species · *Rubus constrictus*

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Introduction

Rubus ulmifolius Schott is a cosmopolitan Rosaceae species native to Europe and North Africa. This taxa has been reported as invasive in several countries, such as: Argentina, Australia, New Zealand, United States, Chile, and some islands of the Azores archipelago (i.e., Terceira, Graciosa and Pico) (Thorp and Lynch 2000; Mazzolari et al. 2011; Dirnböck et al. 2003; Smith-Ramírez et al. 2017). The damage of invasive plant species on the communities they invade has been well documented, and is considered a serious threat to biodiversity worldwide (Knops et al. 1999; Hejda et al. 2009; Vilà et al. 2011).

R. ulmifolius is found in most agricultural and forest landscapes of southern-central Chile (33°–43°S, Matthei et al. 1995), probably confused in some areas with *R. praecox*, recently described in Chile by Rejmánek (2015). By the first half of the 1970s about 5% of the agricultural land area in

mainland Chile was infested by *R. ulmifolius*, *R. cf. constrictus*, and probably *R. praecox* (Oehrens and González 1974). In 1927, the botanist Walterio Looser reported *R. ulmifolius* on Robinson Crusoe Island (RCI), Chile, for the first time (Looser 1927). This climbing shrub rapidly became highly invasive on the island. Currently, it is estimated that about 18% of the island is covered by shrubland composed of *R. ulmifolius*, strongly associated with another invasive exotic species, *Aristotelia chilensis* (Díaz 2013; Greimler et al. 2002). The forest area of RCI, constituted mainly by endemic species, is considered one of the two mini global hotspots, after Galápagos (Arroyo 1999), and the top conservation priority worldwide by the Durrell Conservation Trust (Saunders et al. 2011). Furthermore, it has been reported as being the fourth island with most forest displaced by invasive species worldwide, after Tahiti, Moorea and Pico Island (Smith-Ramírez et al. 2017).

On RCI, *R. ulmifolius* causes severe damage to the forest ecosystem (Vargas et al. 2013). Due an effective propagule dispersal strategy through gravity, wind, birds and asexual reproduction (Smith-Ramírez et al. 2013; Mora and Smith-Ramírez 2016), *R. ulmifolius* can establish itself almost anywhere, even climbing into the canopy of large trees (>10 m), showing unusually high secondary growth in diameter (up to 15 cm). It hinders the main regeneration strategies of the endemic forest taxa, which can result in local extinction for some plant species (Vargas et al. 2013), also affecting the habitat of endemic land bird species (Hahn et al. 2011).

Phragmidium violaceum (Schulz) Winter is a leaf-rust fungus indigenous to Europe and Asia (Tykhonenko 2007) that currently has a global distribution (Morin et al. 2013). This rust is an obligate biotrophic pathogen, characterized by high aggressiveness and strong specificity (Bruzzese and Hasan 1986; Morin et al. 2006, 2011, 2013). Consequently, it has been used as a biological control for some species of the *Rubus* genus in Australia, New Zealand and Chile (Oehrens and González 1974; Morin et al. 2011). However, the pathogenicity on different species of *Rubus* has been shown to vary (Evans et al. 2005, 2011; Bruckart III et al. 2017). This rust does not eradicate the host, but decreases its aggressiveness, which is the effect required on RCI, where invasive species also have a positive effect in terms of preventing soil erosion (Castillo and Smith-Ramírez 2018).

In 1973, *P. violaceum* from Germany was tested and deliberately released in mainland Chile near Valdivia (39° 48'S, 71° 14'W) to control two *Rubus* species (Oehrens and González 1974). After liberation, *P. violaceum* isolates were transported and released in several other locations in south-central Chile (33°–40° S, Oehrens and González 1974). After three years, Oehrens and González (1977) reported that the effect on *R. constrictus* (that we call here as *R. cf. constrictus*) was significantly greater than on *R. ulmifolius*, concluding that the former is more susceptible to the rust. Seed viability of *R.*

cf. constrictus plants was reduced by 45% in cases of severe infection (Oehrens and González 1977). Moreover, an accession of *R. praecox* from Chile, showed to be susceptible to *P. violaceum* in greenhouse experiments (Bruckart III et al. 2017). It is not known when *P. violaceum* was released on RCI, and whether it was intentional or accidental. It was only first detected on the island around 1984–1985 by a Japanese scientist (Pers com, Ivan Leiva, ex-National Park administrator). In this study, we quantified the pathogenicity of *P. violaceum* on *R. ulmifolius* ca. 32 years after the rust detection on RCI. We included field sampling and exploratory experiments in the laboratory. In addition, considering that *R. cf. constrictus* from the mainland is highly susceptible to *P. violaceum*, we tested *P. violaceum* isolates from the island on this species in order to understand their pathogenicity level. The aims were to: (a) evaluate the rust pathogenicity over *R. ulmifolius* in different forest sites of RCI; (b) identify environmental conditions related to higher infection of *P. violaceum* on RCI; and (c) test the pathogenicity of *P. violaceum* isolates from RCI on both *R. ulmifolius* from RCI and *R. cf. constrictus* from the mainland.

Materials and methods

Study area

Robinson Crusoe Island (RCI) is located in the South Pacific Ocean (33°38'29"S; 78°50'28"O) 670 km west of San Antonio, in central Chile (Fig. 1). The climate is warm-temperate and humid, with short dry summers. Annual average temperature is 15.3 °C with an average annual rainfall of 1150 mm (Marzol et al. 2001). Average temperature during the period comprising one month before the start of the growing season up to the month we sampled (i.e., August to March inclusive), over the last 17 years, registered a minimum of 13.1 °C (2016) and a maximum of 15.9 °C (2009). The year our study was undertaken, the average temperature was 14.6 °C. Accumulated precipitation over these eight months was more variable, ranging from a minimum of 464.5 mm to 1126.5 mm (2013 and 2002, respectively). In the latter year, a strong ENSO climatic fluctuation affected RCI. Throughout the study year precipitation was 607.5 mm, which is close to the average in years without the ENSO phenomena (on average 672.8 mm in La Niña years). It is significant that over 13 years (between 2002 to 2015) no rainfall characteristic of El Niño had occurred on the island (Dirección Meteorológica de Chile 1860–2016).

RCI formed over a volcanic hotspot about 4.2 million years ago (Stuessy et al. 1984). The soils are developed from colluvial sediments and ash, presenting in general, sandy loam textures and a large, highly erodible skeleton proportion. The upper and lower montane forest covers ca. 1015 ha,

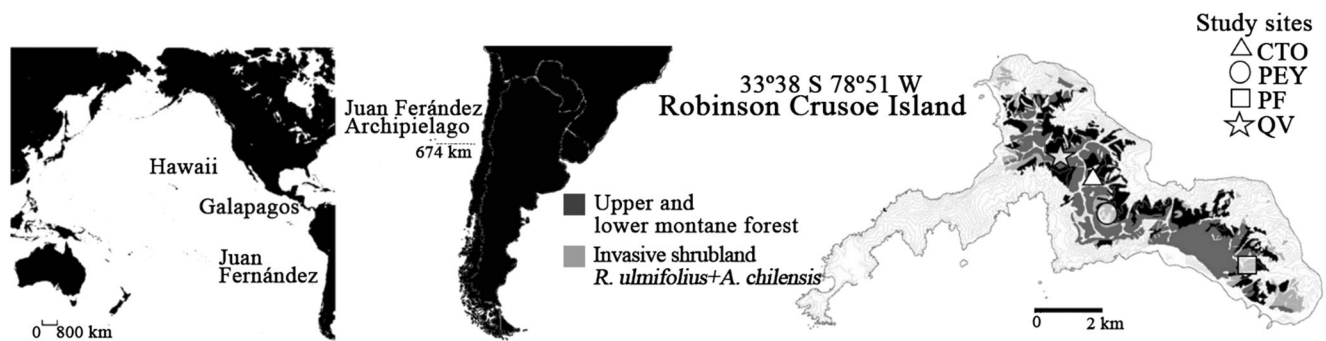


Fig. 1 Location of Juan Fernández Archipelago. Robinson Crusoe Island (RCI) is shown at right with main vegetation associations and approximate location of study sites, CTO = Carbonera de la Torre,

PEY=Plazoleta el Yunque, PF=Puerto Francés, QV = Quebrada Villagra (Adapted from Bernardello et al. 2006)

which represents about 22% of the island surface and ca 32% of the island's vegetation cover (Díaz 2013).

R. ulmifolius was intentionally introduced into RCI as hedge fence between 1922 and 1927 by Desiderio Charpentier, a settler of Cumberland Bay, where the only town of RCI is located (Skottsberg 1953; Looser 1927; Oehrens and Garrido 1986). Around 1950, *R. ulmifolius* already covered ca 300 ha on RCI (Skottsberg 1953). After more than 90 years, this blackberry species has expanded considerably, infesting about 808 ha of the total 4794 ha of the island by 2010 (Díaz 2013). Thus, this invasive presence of *R. ulmifolius* increases on average ca. 0.36 ha/yr. (Díaz 2013), and is highly likely to colonize and invade forest canopy gaps that occur at a rate of 0.04 ha/yr. (Smith-Ramírez et al. 2017).

Field sampling

Due to the high variability of *Rubus* species used in this study, *Rubus* leaves from RCI were first identified by specialists; however the identification was only possible by using flow cytometry on seeds from herbarium specimens that were available; we detected diploidy at somatic level, confirming the species as *R. ulmifolius*. In the case of the species considered as *R. constrictus* by Oehrens and González (1974), which has an ample distribution in the province of Valdivia, we were not able to completely confirm its identity due to differences in flower color and prickles shape with respect to the established description of *R. constrictus*. As a result, we referred to this *Rubus* as *R. cf. constrictus*.

To characterize rust incidence in the field, we sampled four forest sites on RCI during March 2014: Carbonera de La Torre (CTO), Plazoleta El Yunque (PEY), Puerto Francés (PF) and Quebrada Villagra (QV) (Fig. 1). In each forest site, we established four to six transects, each separated by at least 50 m, extending from the shrubland to the forest (ca. 220–350 m above sea level). We defined three zones along these transects: shrubland, ecotone, and forest. Within a 100 m² sample area set in each zone, we established four 4 m² plots. When infection was detected, we randomly selected four

plants per plot and visually inspected the last 20 cm of two branches from each plant. Each inspected section presented around 5 to 13 leaves and 1 to 2 nodules. We chose the last 20 cm in order to include only leaves from the most recent growth season because they are more susceptible to infection (Evans and Bruzese 2003, Evans et al. 2011). New leaves and shoot expansions in *R. ulmifolius* adult plants are mainly produced during the austral spring (author's pers. obs.). Thus, in March 2014, new leaves were 4 to 6 months old. In total, we examined 182 plots, evaluating around 728 plants in the field, inspecting ca. 1456 branches, and consequently more than 7000 leaves. For each plot the observations were averaged in the following categories: low (approx. < 33% of the leaves visibly infected), intermediate (>33% < 66% of the leaves visibly infected) and high (approx. > 66% of the plant leaves infected). Thus, we assigned an incidence category to each plot. Given that *R. ulmifolius* is a clonal plant, we selected branches originating from the same basal trunk, sampling trunks separated at least 30 cm from each other, in order to estimate severity in different individuals.

In addition, we measured four types of variables in each plot: (i) *abiotic variables*: elevation (m.a.s.l), aspect (°), slope (%) and solar radiation transmittance (mol/m²/day). To estimate solar radiation, hemispherical photos were taken under continuous cloud cover at the center of each plot (taken at 1.45 m above ground, using a Nikon® 5100 camera fitted with a SIGMA®10 mm f/2.8 EX DC HSM). Photos were analyzed using the Gap Light Analyzer software© (Frazer et al. 1999) to estimate total light (mol/m²/day). (ii) *micro-site variables*: including moss and liverwort coverage (%), litter (%), coarse woody debris (> 5 cm in diameter, %), exposed mineral soil (%) and rocks (%), were measured. (iii) *soil variables*: the skeleton proportion (%) was visually estimated by digging a small hole of ca. 500 cm³, quantifying rocks >3 cm; soil depth was assessed by extracting a soil sample with an iron core of 120 cm at the center of each plot (Schlatter et al. 2015). (iv) *vegetation cover*: visually estimated, differentiating native and exotic vascular plant species (%) using Danton and Perrier (2006) nomenclature.

Laboratory methods

In September 2015, *R. ulmifolius* leaves infected by *P. violaceum* were collected from individuals at three sites on RCI (La Pólvara, Pasillo and PEY). The leaves were transported to the Phytopathology Laboratory of the Universidad Austral de Chile, Valdivia, and stored in a refrigerator at 5 °C for two weeks. Subsequently, urediniospores were removed from the abaxial surface of the leaves and stored separately at −12 °C for three weeks, prior to pathogenicity tests. To test the pathogenicity of *P. violaceum*, uninfected seedlings of *R. ulmifolius* were collected from PEY on RCI (10 seedlings, 33° 39' 32" S 78°50' 40"W) and from the highly susceptible species *R. cf. constrictus*, on mainland Chile (Valdivia, 10 seedlings, 39°48'00"S 73°14'00"W). The seedlings of both origins were planted and maintained under common greenhouse conditions (18–28 °C). Two months after planting, one three-week-old compound leaf was randomly selected and cut from three vigorous healthy seedlings of *R. ulmifolius*, per origin (RCI and Valdivia) (whole compound leaves were used). A urediniospore suspension (4×10^4 spores ml^{−1} water) obtained from the infected plants was atomized onto the leaves contained in Petri dishes (10 × 15 mm). The urediniospores were removed from the leaves using a scalpel and placed in microcentrifuge tubes. The Petri dishes (one per leaf) were incubated in a growth chamber at 18 °C; they were kept in the dark for the first 12 h to enhance spore germination and subsequently at a 16 h photoperiod. Approximately 18 h after inoculation, the dishes were opened for about 1 h to allow droplet evaporation. Seven days before leaves were inoculated, the leaves were transferred to 1% water-agar dishes at 18 °C and 16 h photoperiod (Evans et al. 2011). The trial comprised three replicates, including the two *Rubus* species and the three accessions of *P. violaceum* (i.e., La Pólvara, Pasillo and PEY) from RCI; a water-inoculated control was included and installed in a complete randomized block arrangement. The viability of urediniospores was assessed by spraying the spore suspension on Potato Dextrose Agar (PDA) and incubating in the dark for 12 h at 24 °C and 80% humidity. Percentage germination was assessed using a light microscope, based on the count of 50 spores. We also measured urediniospore size, both leaf spot and uredinium diameter and counted uredinia. The uredinia density was calculated as the number of uredinia per cm² of leaf. Measurements of leaf spots and uredinium size were taken 21 days after inoculation, according to procedures used by Morin et al. (2011).

Statistical analyses

Firstly, we compared disease incidence of *R. ulmifolius* by *P. violaceum* among forest sites by computing the Kruskal-Wallis test (Croarkin et al. 2006). Secondly, we focused on

identifying the variables that influence disease incidence probability. Concordantly, we modeled the infection probability of *R. ulmifolius* plants by *P. violaceum* by fitting several logistic regression models, where different predictor variables were assessed (Table 1). All models were fitted by maximum likelihood. We assessed each model by comparing the statistical goodness-of-fit, as well as the statistical significance of the corresponding predictor variables.

Model comparisons were carried out by computing the Akaike Information Criterion (AIC) as a goodness-of-fitness index. Furthermore, model behavior was assessed by representing its biological performance as a function of fixed values of the predictor variables, as recommended by Salas et al. (2008). All the statistical analyses were carried out in the software R (v. 3.0.2., R Development Core Team 2013).

Results

Disease incidence in the field The sites evaluated presented a range of different abiotic, micro-site, and soil attributes. In all forest sites, most of the exotic plant species coverage was composed of *R. ulmifolius* (Table 1). We found low infection incidence of *P. violaceum* in the field, considering that 120 of 182 plots (66%), presented no infection. The remaining, 62 (34%) of 182 plots presented at least one individual of *R. ulmifolius* visibly infected by rust (Table 2). We did not find statistically significant differences in disease incidence among the forest sites analyzed ($P > 0.05$). In general, of the plots analyzed, four presented highly infected individuals (i.e., > 66% of leaves visibly infected), and most of the plots with infection symptoms in the field presented low infection (< 33% infection, $n = 37$). *R. ulmifolius* plants on southern slopes were more infected than those located on sites with a northern aspect, although differences were not statistically significant ($P = 0.19$; Table 2).

Notwithstanding, when we analyzed incidence probability through logistic regression, we found that among all the predictor variables tested (Table 1) our proposed model was a function of two environmental attributes: soil depth and aspect, both being statistically significant. The model behavior shows that disease incidence probability decreases with soil depth, and is lower on northern aspects than southern aspects (Fig. 2). Overall, infection incidence probability decreased with soil depth, being higher on shallow soils (< 30 cm). Furthermore, disease incidence probability was 10 % higher on southern aspects than on northern aspects.

Infection under laboratory conditions The rust isolates infected *R. ulmifolius* leaves but not those of the highly susceptible *Rubus* that is *R. cf. constrictus* (Fig. 3). No infection was observed in the water inoculated control. The number of uredinia per leaf varied between 3 and 28. Pathogenicity was

Table 1 Description of forest sites (mean \pm SE) CTO = Carbonera de la Torre, PEY=Plazoleta El Yunque, PF=Puerto Francés, QV = Quebrada Villagra

Environmental variable/ Site	CTO	PEY	PF	QV	N	S
(a) Abiotic variables						
Elevation (m a.s.l)	237 \pm 4.7	345 \pm 13	268.6 \pm 12.5	261.7 \pm 7.8	305.2 \pm 11.6	253.5 \pm 5.0
Slope (%)	64 \pm 2.2	76 \pm 2.1	58 \pm 4	77 \pm 1.9	69.7 \pm 5.3	69 \pm 1.5
Solar radiation transmittances (mol/m ² /day)	29.1 \pm 1.7	32 \pm 0.9	13.7 \pm 1.5	23 \pm 1.5	24.3 \pm 1.9	23.6 \pm 1.1
(b) Micro-sites (%)						
Mosses and liverworts	12.0 \pm 1.5	13.3 \pm 3.6	23.7 \pm 2.0	19.2 \pm 1.3	15.8 \pm 1.94	17.6 \pm 1.0
Litter	44.7 \pm 2.2	63.3 \pm 5.1	47.4 \pm 2.7	53.6 \pm 2.2	54.2 \pm 4.9	49.3 \pm 1.4
Coarse woody debris	53.7 \pm 3.5	56.7 \pm 9.6	56.3 \pm 5.8	39.4 \pm 2.6	49.2 \pm 6.4	48.7 \pm 2.2
Exposed mineral soil	33.3 \pm 2.1	31.7 \pm 2.6	7.4 \pm 0.5	28.9 \pm 2.2	20.8 \pm 2.7	26.8 \pm 1.5
Rocks	26.0 \pm 3.1	16.7 \pm 2.8	46.1 \pm 2.3	20.6 \pm 1.6	29.2 \pm 4.9	27.2 \pm 1.7
(c) Soil characters						
Skeleton (%)	9.4 \pm 1.8	5.0 \pm 0.0	47.6 \pm 2.3	7.8 \pm 1.6	22.9 \pm 4.6	15.5 \pm 1.6
Soil depth (cm)	80.4 \pm 2.4	76.7 \pm 1.5	83.4 \pm 1.8	70.9 \pm 2.2	70.8 \pm 2.4	78.1 \pm 1.4
(d) Vegetation (%)						
Total Cover	71.1 \pm 6.4	94.6 \pm 13.5	57.9 \pm 13.1	79.0 \pm 7.4	84.7 \pm 10.7	70.9 \pm 5.4
Native cover	38.6 \pm 6.4	49.9 \pm 11.5	12.0 \pm 3.5	44.7 \pm 8.3	43.2 \pm 8.5	34.0 \pm 4.9
Exotic Cover	32.4 \pm 8.3	44.7 \pm 20.8	45.8 \pm 13.8	34.3 \pm 8.7	41.4 \pm 13.8	36.8 \pm 6.1
<i>R. ulmifolius</i> cover	21.6 \pm 6.7	38.9 \pm 19.3	15.2 \pm 5.7	29.9 \pm 7.7	30.8 \pm 12.2	23.9 \pm 4.6

N = North = plots at any site of dominant aspect to north, S = plots at any site of dominant aspect to south

low, with an average spot diameter of 1.0 ± 0.3 mm (Fig. 3). The germination percentage of urediniospores was 19, 6 and 17% in La Pólvara, Pasillo and PEY, respectively. The rust fungus produced no more than 0.5 uredinia/cm² (Table 3). The diameter of uredinia varied per site, between 0.38 to 0.67 mm (Fig. 3) and of urediniospores, between 24 to 27 μ m (Table 3).

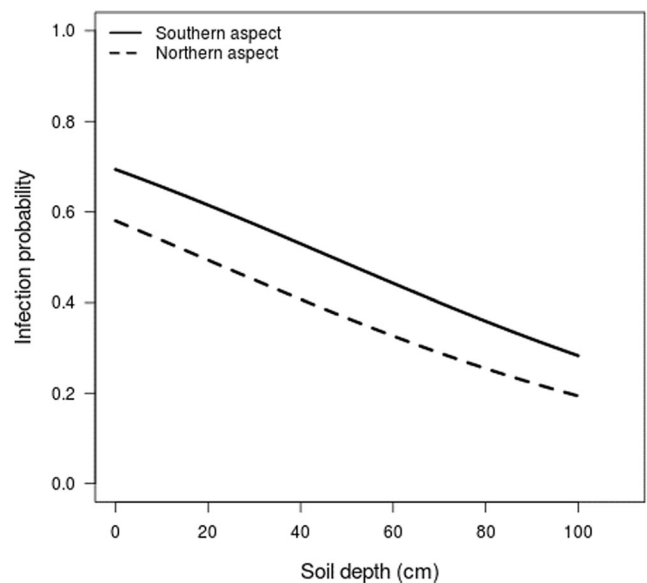
Table 2 Rust incidence on *R. ulmifolius* individuals at different forest sites of RCI (N = 182 plots)

Site	Infected (n = 62)				
	Incidence (%)				
	Not infected (%) (n = 120)	Low	Intermediate	High	Total
CTO	60.0	26.7	11.7	1.7	40.1
PEY	83.3	8.3	8.3	0	16.6
PF	79.0	7.9	13.2	0	21.1
QV	61.1	23.6	11.1	4.2	38.9
North aspect sites	79.2	16.7	4.2	0	20.9
South aspect sites	63.9	10.8	22.8	2.5	36.1
Overall	65.9	20.3	11.5	2.2	34.1

Considering infection on branches of *R. ulmifolius*, disease incidence was averaged and attributed to each plot (N = 182) using the following categories, low (>0% < 33% of leaves visibly infected), intermediate (>33% < 66% of leaves visibly infected) and high incidence (> 66% of plant leaves infected). CTO, Carbonera de la Torre; PEY, Plazoleta el Yunque; PF, Puerto Francés; QV, Quebrada Villagra

Discussion

We found low *P. violaceum* pathogenicity on *R. ulmifolius* plants from RCI, both in the field and under laboratory conditions. In the field, two thirds of the plots analyzed with *R. ulmifolius*, presented no infection.

**Fig. 2** Expected infection probability of *P. violaceum* over *R. ulmifolius* given soil depth and aspect. The predictions are based on our best fitted logistic regression model

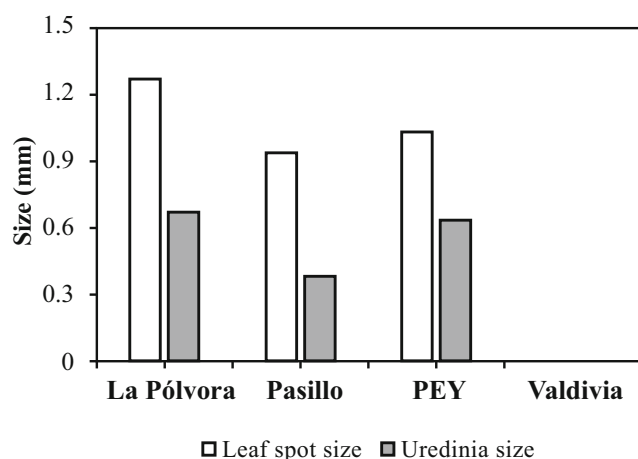


Fig. 3 Leaf spot and uredinia diameter (mm) on leaves of *R. ulmifolius* quantified after 21 days of infection by *P. violaceum* under laboratory conditions. Three isolates from RCI (i.e., La Pólvara, Pasillo, PEY) were tested over uninfected plants from RCI and mainland (Valdivia). * no uredinia were produced

When infection was detected, most individuals presented few affected leaves. The incidence categories used were designed to evaluate rust in the mainland, where infection is considerably higher than that found on RCI (Oehrens and Garrido 1986). Thus it is probable that the categories used in this study could be masking the extremely low infection found on RCI. This, because in many cases plants classified with low incidence (< 33% of leaf numbers visibly infected) presented less than 5 to 10% leaf area infected (authors pers. obs.). On RCI 60 to 83% of the plots analyzed did not present any infection. In comparison to RCI, two years after the release of the rust for biological control purposes in mainland Chile, high incidence of its presence occurred in almost the entire *Rubus* distribution (Oehrens and González 1977). We found a significant trend of higher rust infection on *R. ulmifolius* when plots were located on southern aspect slopes and associated with shallow soils. We do not

know whether the wetter conditions that predominate in southern slopes are positive or negative for *R. ulmifolius* growth and *P. violaceum* infection. It is also not known if the stressed plants that grow in shallow soils may affect the *P. violaceum* development. In southeastern Australia a low infection of *P. violaceum* over *R. leucostachys* under field conditions has been associated with climate (Evans et al. 2005). However, the relationship between stressed plants and fungus in the *Rubus-Phragmidium* association remains unclarified. Here we found more infection on southern aspects, and shallow soils, but further studies are required to determine if this increased infection is due to site conditions affecting *R. ulmifolius* growth. It has been described that the leaf-emergence rate in *Rubus* can influence disease severity in shoots (Evans et al. 2005); in our case, we have no information about this rate and how it is influenced by topographic, soil and climatic variables.

Moreover, the low pathogenicity observed in the field could be associated with the low genetic variability of the *P. violaceum* isolates that were released in Chile in the seventies, all of which probably originated from one provenance in West Germany (Oehrens and González 1974). If the rust appeared on RCI spontaneously, not from the Chilean mainland, it is also possible that its genetic variability could be low. Also, *R. ulmifolius* could have generated some tolerance to the rust established on RCI, as has been shown in the native distribution of other *Rubus* species in Europe (Borcean et al. 2016). It has been determined that increasing the genetic diversity of rust can improve its capability to adapt to different environments where *Rubus* species occur (Morin et al. 2011). For example, in Australia, new strains of *P. violaceum*, brought from France, increased infection over *R. polyanthemus*, determining a significant reduction in its biomass and reproductive capacity (Gomez et al. 2008; Mahr and Bruzzese 1998). *P. violaceum* is an autoecious rust fungus that can rapidly form physiological races as shown in previous experiments (Oehrens and Garrido 1986), which may explain why the isolates from RCI did not infect *R. cf. constrictus*.

We conclude that the low uredinospore germination observed in our exploratory laboratory evaluations concurred with the low infection incidence found in the RCI forest sites evaluated. We hypothesize that releasing new strains of *P. violaceum* with well-documented and increased pathogenicity from European sources, could improve infection capability on RCI. Undoubtedly, a Biological Control Program on RCI is necessary and, in this context, the use of *P. violaceum* must still be considered, in spite of the negative results found in this study, especially because the aim is not to eradicate *R. ulmifolius*, but to decrease its invasiveness.

Table 3 *P. violaceum* uredinospore germination, density of uredinia and diameter of uredinospores on leaves of *R. ulmifolius* from RCI (mean \pm SE)

Isolate	Germination (%)	Uredinia/cm ²	Uredinospore diameter	
			Length (μ m)	Width (μ m)
La Pólvara	19.0 \pm 9.9	0.2	29.3 \pm 2.9	25.8 \pm 2.6
Pasillo	6.0 \pm 0.0	0.5	25.2 \pm 2.2	22.8 \pm 2.1
PEY	17.0 \pm 1.4	0.1	25.8 \pm 3.3	23.0 \pm 2.8
Mean	14.0 \pm 3.8	0.3	26.8 \pm 2.8	23.9 \pm 2.5

Results contrast three rust isolates from RCI, based on a sample of three leaves of *R. ulmifolius* measured 21 days after inoculation under laboratory conditions

Acknowledgments We are grateful to I. Leiva, (Juan Fernandez National Park administrator 2004–2017) for help in collecting and sending samples to the mainland; to Ana Abarzúa (Universidad Austral de Chile) for collaborating with seedling collection, and Diego Pennekamp who contributed to the field methods. Additionally, we thank Marcia Barrientos and Carla Manríquez (Universidad de La Frontera) who collaborated with formatting matters. Our thanks to Dr. Michael Sohor for performing the ploidy analysis of seeds and to ElSharif Ramires for helping us with the pictures to confirm the identification of *R. constrictus*. We also acknowledge the collaboration of Susan Angus in the English edition of the manuscript. We especially thank the two anonymous reviewers who significantly improved this document. RVG thanks FONDECYT 11170987 and the Vicerrectoría de Investigación y Postgrado, Universidad de La Frontera (UFRO) for funding this study. CSR thanks the IEB (Instituto de Ecología y Biodiversidad-Chile) projects PF-23, 05-002 and CONICYT AFB 170008 for their financial support.

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