Phytoplasma infection alters morpho-anatomical and physiological traits in *Ugni molinae* Turcz.

La infección con fitoplasmas altera los rasgos morfo-anatómicos y fisiológicos de *Ugni molinae* Turcz.

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ABSTRACT

The "murtilla" (*Ugni molinae* Turcz., Myrtaceae), present in Contulmo Natural Monument (Araucanía Region, Chile), is affected by witches' broom disease, caused by the presence of a phytoplasma in the sieve tubes of the phloem. This phytoplasma affects plants morphoanatomically, producing evident symptoms. Infected branches show reddening of leaves and general dwarfism. In the present study, we compared morphoanatomically and physiologically branches (modules) of murtilla with and without symptoms of witches' broom disease. This was done in order to quantitatively evaluate the actual effect of the infection on adult plants. We cut branches of similar age, with and without symptoms, where we measured fluorescence, chlorophyll content, specific leaf area, stem size, pith size, and vessel size and number. We also measured fruit traits in symptomatic and asymptomatic plants. We did not found differences in the Fv/Fm (maximun efficiency of the PSII) and total chlorophyll content between symptomatic and asymptomatic modules. However, symptomatic modules had lower pith area, vessel size, and leaf area, and dry weight than asymptomatic modules. Infected plants also had less fruits per plant and with less total soluble solutes (TSS) in the fruits compared to healthy plants, although average individual fruit weight was similar between symptomatic and asymptomatic plants. Witches' broom disease affects the anatomy, morphology, and quality and quantity of fruits of murtilla plants. However, the disease does not seem to affect some physiological parameters. The murtilla in Chile has high economical potential as an edible fruit and in the cosmetic and pharmaceutical industry. Future studies should focus on the vector of the disease and the possible effects of the infection on other commercially-relevant traits.

Keywords: *Ugni molinae*, murtilla, phytoplasma, Contulmo Natural Monument.

RESUMEN

La murtilla (*Ugni molinae* Turcz., Myrtaceae), presente en el Monumento Natural Contulmo (Región de la Araucanía, Chile), se encuentra afectada por la enfermedad "escoba de bruja", causada por la presencia de un fitoplasma en los tubos cribosos del floema. Este fitoplasma afecta morfoanatómicamente a las plantas, produciendo síntomas apreciables a simple vista. Las ramas afectadas presentan enrojecimiento de las hojas y enanismo generalizado. En este estudio se compararon morfoanatómica y fisiológicamente ramas de murtilla (módulos) con y sin síntomas de la enfermedad escoba de bruja. Esto con el objetivo de evaluar cuantitativamente el efecto real de la infección en plantas adultas. Se extrajeron ramas de edad similar, con y sin sintomatología, donde se evaluaron parámetros de fluorescencia, contenido de clorofila, área foliar específica, tamaño de tallos, área de médula del tallo y tamaño y número de vasos. Además se evaluaron las características de los frutos de plantas sintomáticas y asintomáticas. No se encontraron diferencias significativas en el Fv/Fm (eficiencia máxima del PSII) y contenido total de clorofilas entre módulos asintomáticos y sintomáticos. Sin embargo, los módulos sintomáticos presentan menor área de medula, tamaño de vasos, área foliar y peso seco que módulos asintomáticos. Las plantas enfermas, además, presentaron menos frutos por planta y con menos sólidos solubles totales (SST) que las plantas sanas, aunque el peso individual promedio de los frutos no varió entre plantas con y sin síntomas. La enfermedad escoba de bruja afecta la anatomía y morfología de las plantas de murtilla y la calidad y cantidad de los frutos, sin embargo, la enfermedad no parece afectar algunos parámetros fisiológicos. La murtilla en Chile tiene un alto potencial económico como alimento y en la industria cosmética y farmacéutica, por lo que futuros estudios debiesen considerar el vector de transmisión y los efectos que podría producir esta enfermedad en otras características de interés comercial.

Palabras clave: *Ugni molinae*, murtilla, fitoplasma, Monumento Natural Contulmo.

INTRODUCTION

Ugni molinae Turcz. (Myrtaceae) is a Chilean shrub that produces an edible berry with great productive potential (Landrum & Donoso 1990). This evergreen plant is distributed from Maule to Aysen district in Chile (Seguel & Torralbo 2004). The berries have high antioxidant and analgesic properties (Seguel & Torralbo 2004, Tacón *et al*. 2006, Delporte *et al*. 2007, Suwalsky *et al*. 2007, Rubilar *et al*. 2011), and the plant extract has anti-inflammatory activity (Goity *et al*. 2013). There is a growing interest in producing the plants commercially (Landrum & Donoso 1990), but commercial initiatives are limited in extension and further research is required.

Many natural populations of *U. molinae* show symptoms of a disease called witches' broom, caused by a phytoplasma (Andrade *et al.* 1984, 2009, Arismendi *et al*. 2010, 2011). Different types of phytoplasma infect many cultivated plant species causing reduced yield and plant quality (Seemüller *et al*. 1994, Lee *et al*. 2000, Endeshaw *et al*. 2012). They are usually transmitted by phloem-feeding insects (Camarena & De la Torre 2008). Plants affected by the disease show increased branching, dwarfism, and color change in affected branches (Lee *et al*. 2000, Camarena & De la Torre 2008, Andrade *et al*. 2009). The phytoplasm infects the phloem of the plant (Andrade *et al.* 2009, Arismendi *et al*. 2010, 2011), altering normal vegetative development. Fruit set is decreased and fruits, when present, have bad flavor (personal observation, Andrade *et al*. 2009). To our knowledge, no studies have evaluated fruit quality of healthy and infected plants of *Ugni molinae*. Phytoplasma infection can also affect plant physiology. It has been shown to reduce pigment content, maximum efficiency of photosystem II (Fv/Fm), photosynthesis, stomatal conductance, and fruit yield in symptomatic plants (Endeshaw *et al*. 2012). Thus the overall fitness of infected plants is reduced compared to non-infected plants. This could have negative ecological consequences at the population-level and economical consequences for productive initiatives.

In the present study we aim to quantitatively compare the morphology, anatomy and physiology of branches of *Ugni molinae* with and without symptoms of the witches' broom disease. We also evaluated the incidence of Phytoplasma infection in the population. This knowledge could help quantify the effect of the infection on adult plants and to estimate the possible ecological and economical costs of phytoplasma presence on *Ugni molinae*. We address the following hypothesis: a. Phytoplasma infection alter morphology, physiology and anatomy of *Ugni molinae*. b. Phytoplasma infection also alters fruit quality of *U. molinae*. The studied individuals were obtained from a natural population growing in Contulmo Natural Monument in the Araucanía Region, Chile.

MATERIALS AND METHODS

Study site

Contulmo Natural Monument is a protected area belonging to the SNASPE (National System of Protected Areas of Chile). Located in the Nahuelbuta mountain range (Araucanía district, Chile), close to the town of Contulmo (38° 01' S; 73° 10' W). The vegetation correspond to a temperate forest were *Ugni molinae* is a common shrub dominating semi-open sites. The climate is temperate with high marine influence.

Population analysis and sampling

To estimate the frequency of symptomatic plants, we registered *U. molinae* individuals along 4 transect of 50 m. We found 250 individuals in total, with and without symptoms of phytoplasma infection. To estimate the number of infected branches per plant we randomly selected 24 symptomatic individuals.

We collected symptomatic and asymptomatic branches of *Ugni molinae* from plants showing witches' broom disease. Sampling was conducted from December 2011 to January 2012, corresponding to the summer season. We randomly sampled individuals (coin toss) along four 50 m transects. Before collecting, we measured fluorescence parameters *in situ* on 40 symptomatic and 40 asymptomatic branches using a Pocket PEA (Hansatech, UK). After that, branches were bagged and transported to the laboratory for morphological and anatomical measurements.

24 symptomatic and 24 asymptomatic branches were used for the morphological analysis. We measured basal diameter of the branch using a digital caliper (Mitutoyo, Japan). We estimated specific leaf area (SLA) as total leaf area divided by total dry weight of leaves. We digitalized all leaves and measured leaf area with image software (AxioVision 4.8, Zeiss, Germany). The dry weight was obtained by weighting the leaves in a scale after drying for 48 h at 70° in an oven. Leaf thickness was measured with a digital caliper (Mitutoyo, Japan) in 20 symptomatic and 20 asymptomatic individuals.

We conducted anatomical measurements in 24 symptomatic and 24 asymptomatic branches were we performed transversal sections of the base of the branch. We photographed the sections using an optical microscope with a digital camera attached (Carl Zeiss Primo Star microscope with a Canon EOS Rebel XS camera). Using image software (AxioVision 4.8, Zeiss, Germany) we measured pith area, conduit radius, and conduit number.

Chlorophyll content was measured spectrophotometrically in 4 branches per symptom using a modified Hansmann method (Hansmann 1973). We measured absorbance at 663 and 645 nm with a UV-visible spectrophotometer (Genesys 10UV, Thermo Scientific, USA).

Since symptomatic branches had no fruits, we analyzed fruit traits in plants with and without evidence of phytoplasma infection. Fruit quality was analyzed in 4 plants per symptom. In March-April 2013, we counted fruit number per plant and we measured average fruit weight with an analytic scale. We also measured total soluble solids (TSS) using a portable refractometer (0-32° Brix refractometer). It was impossible to increase sample size because after a second visit to Contulmo National Monument, fruits were collected and consumed by visitors, as reported by the CONAF staff.

Statistical analysis

We used a t-test, when possible, to compare between symptomatic and asymptomatic branches (or plants for fruits comparisons). In order to normalize the data, branch diameter, leaf thickness, SLA, Fo/Fm, and pith area were Ln-transformed. For leaf weight, leaf area, number of vessels and vessel radius we used a Mann-Whitney test (non-parametric).

RESULTS

Approximately 1/3 (90 out of 250) of the sampled individuals showed symptoms of Witches' Broom disease (Fig. 1). They usually had between 1 to 5 symptomatic branches per plant (2.5 in average). Symptomatic branches were shorter (data not shown), with thinner stems than non-symptomatic branches (Table I, t-test $p<0.05$). Leaves were smaller (Table I, Mann-Whitney test $p<0.05$) and had a higher SLA (Fig, 2, t-test $p<0.05$). Leaf thickness was similar in symptomatic and non-symptomatic branches (Table I, t-test $p>0.05$).

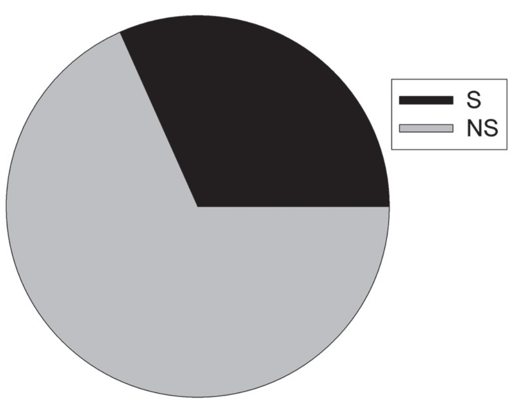

Figure 1. Proportion of plants of *Ugni molinae* with (black) and without (gray) symptoms of witches' broom disease in Contulmo Natural Monument (Araucanía district, Chile). *n*=250 individuals.

Figura 1. Proporción de plantas de *Ugni molinae* con (negro) y sin (gris) síntomas de la enfermedad "escoba de bruja" en el Monumento Natural Contulmo (Región de la Araucanía, Chile). *n*=250 individuos.

Physiologically, there were no differences between branches evidencing and not-evidencing phytoplasma infection (Fig. 2 and Table I, t-test $p>0.05$). However, symptomatic plants had lower chlorophyll a content (Table I, t-test $p<0.05$) and Chl a/Chl b ratio (Fig. 2, t-test $p<0.05$). There were no differences in Chl b content between symtomatic and asymtomatic plants (Table I, t-test $p<0.05$).

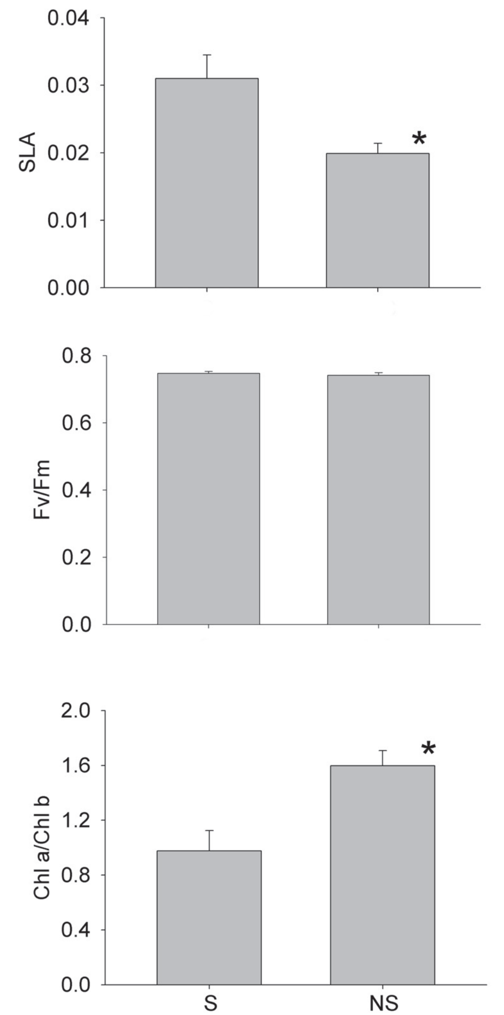

Figure 2. Average (± SE) specific leaf area (SLA), Fv/Fm, and Chl a/Chl b ratio of symptomatic (S) and asymptomatic (NS) branches of *Ugni molinae*. For SLA, data was Ln-transformed for normalization. * = statistically significant differences between symptomatic and asymptomatic branches (t-test, $p<0.05$). For SLA and Fv/Fm *n*=24 branches per symptom (48 in total). For Chl a/Chl b ratio). *n*=4 branches per symptom (8 in total).

Figura 2. Promedio (± ES) del Área foliar específica (SLA), Fv/Fm y Chl a/Chl b de plantas con (S) y sin (NS) síntomas de *Ugni molinae*. Para el SLA, los datos se transformaron por Ln para normalizarlos. * = diferencias significativas entre ramas con y sin síntomas (test de t, p<0,05). Para SLA y Fv/Fm, *n*=24 ramas por síntoma (48 en total). Para Chl a/Chl b, *n*=4 ramas por síntoma (8 en total).

Table I. Morphological, physiological and anatomical traits in symptomatic (S) and non-symptomatic (NS) branches of *Ugni molinae*. Traits average ± SE are shown. Different letters represent statistically significant differences (t-test, $p<0.05$ / * = Mann-Whitney test, $p<0.05$). Branch diameter, leaf thickness, Fo/Fm, and pith area were Ln-transformed for normalization. n=24 per symptom, except for Chl a and Chl b (n=4 per symptom).

Tabla I. Rasgos morfológicos, fisiológicos y anatómicos en ramas sintomáticas (S) y no-sintomáticas (NS) de *Ugni molinae*. Se muestran los promedios de los rasgos ± ES. Letras distintas representan diferencias significativas (test de t, $p<0,05$ / * = test de Mann-Whitney, $p<0.05$). Diámetro de la rama, grosor foliar, Fo/Fm y área de médula fueron transformadas con Ln para normalizar. n=24 por síntoma, excepto para Chl a and Chl b (n=4 por síntoma).

Symptom	Branch diameter (mm)	Leaf thickness (mm)	Total leaf weight (mg)*	Leaf area (cm^2)*	Fo/Fm	Chl a (ug/mL)	Chl b (ug/ml)	Pith area (mm^2)	Conduit number*	Conduit radius (µm)
S	2.85 ± 0.20^a	0.62 ± 0.07^a	7.2 ± 0.6^a	0.18 ± 0.21^a	0.25 ± 0.01^a	50.8 ± 14.3^a	63.0 ± 24.50^a	0.15 ± 0.01^a	721.2 ± 42.7^a	5.66 ± 0.22^a
NS	1.95 ± 0.14^b	0.60 ± 0.06^a	121.9 ± 10.3^b	2.25 ± 0.22^b	0.27 ± 0.01^a	155.5 ± 19.5^b	96.4 ± 27.47^a	0.17 ± 0.02^a	535.8 ± 30.0^b	8.00 ± 0.38^b

Table II. Average of fruit traits ± SE of symptomatic (S) and non-symptomatic (NS) individuals of *Ugni molinae*. Different letters represent statistically significant differences (t-test, $p<0.05$, n=4 per treatment).

Tabla II. Promedio ± ES de rasgos del fruto de plantas sintomáticas (S) y no-sintomáticas (NS) de *Ugni molinae*. Letras distintas representan diferencias significativas (test de t, $p<0,05$, n=4 por tratamiento).

Symptom	Fruit weight (g)	Fruits per plant	TSS (°Brix)
S	0.29 ± 0.04^a	18.5 ± 4.3^a	7.25 ± 0.62^a
NS	0.33 ± 0.03^a	57.3 ± 5.7^b	10.75 ± 0.48^b

When comparing the anatomy of the cross section of the stem, we found that symptomatic branches had more vessels than non-symptomatic branches (Table I, Mann-Whitney test $p<0.05$), but they were significantly smaller in size (Table I, Mann-Whitney test $p<0.05$). There were no differences in pith area between branches types (Table I, t-test $p>0.05$).

Symptomatic individuals had less fruits per plant and lower TSS in their fruits than non-symptomatic plants (Table II, t-test $p<0.05$). Individual fruit weight, however, was similar between both plant types (Table II, t-test $p<0.05$).

DISCUSSION

Phytoplasma infection can affect up to 1/3 of the *Ugni molinae* population in Contulmo National Monument (Fig. 1). Nevertheless, there could be even more individuals in the population infected by phytoplasma, but not showing symptoms (see Endeshaw *et al*. 2012). Infected plants have a relative low proportion of symptomatic branches. The consequences for the whole-plant carbon balance, and ultimately, the fitness needs to be further evaluated.

Phytoplasma are transmitted by phloem-feeding insects (Aguilera *et al*. 2005, Camarena & De la Torre 2008). In *U. molinae* it has been described that Hemiptera (genus *Carelmapu*), Coleoptera, and Lepidoptera insects could be the most important vectors for phytoplasms infections (Aguilera *et al*. 2005, 2009). The future control of the phytoplasma disease in *U. molinae* could involve control of the vectors. Further studies are required in order to understand the biology of this insect-plant interaction.

Phytoplasma infection usually results in dwarfism and leaf alterations (Camarena & De la Torre 2008, Andrade *et al*. 2009). Here we found that symptomatic branches of *U. molinae* were smaller, thinner, and had smaller leaves (Table I). Leaves were of similar thickness but differed in SLA. This could be due to increased leaf density and more sclerenchymatic tissues in symptomatic leaves. Symptomatic leaves were, in fact, harder to the tact, but quantitative analyses are required to probe this.

Despite the striking morphological differences, there were no differences in fluorescence parameters between symptomatic and asymptomatic plants (Table I, Fig. 2). Fv/Fm corresponds to the maximal efficiency of the photosystem II (PSII). This trait is proportional to the quantum yield (Lambers *et al*. 2008) and it gives information about the status of the PSII. The lack of differences in Fv/Fm does not necessarily imply similarity in carbon gain. Due to gas exchange differences and mesophyll limitations, symptomatic branches could have reduced photosynthesis. Previous studies with Bois noir, a phytoplasma-caused disease, reported reduced Fv/Fm and carbon gain (Pn) in symptomatic leaves compared to healthy leaves (Endeshaw *et al*. 2012). In this study, the changes in Fv/Fm and Pn were seasonally variable. Our fluorescence results could change if measured at different times of the year. We found differences in Chl a content and Chl a/Chl b ratio. Previous studies have also found a reduction in total leaf chlorophyll content and general yellowing of symptomatic leaves (Camarena & De la Torre 2008, Endeshaw *et al*. 2012). Lower Chl a, and Chl a/Chl b ratio implies lower proportion of reaction centers relative to the antenna complexes, since Chl a is more abundant in PS than in antennae (Lambers *et al*. 2008). This could result in lower carbon gain in symptomatic plants due to lower photochemical capacity and higher risk of photo-inhibition.

Symptomatic plants had anatomical differences in the xylem of the stem. Infected plants had smaller, yet more abundant, conduits in the stem cross section (Table I). Phytoplasma usually infects phloem cells of plants. Nevertheless, xylem conduits are probably altered due to the differences in water demand. Symptomatic branches are smaller and have smaller leaves, thus possibly resulting in the formation of smaller vessels to comply with the reduced water demand. Despite the increased conduit number, the hydraulic conductivity (K_h) could be smaller in symptomatic plants because it is proportional to conduit's radius to the fourth power (Hagen-Poiseuille's law). If this occurs, photosynthesis in infected plants could be also limited by water supply, since Pn is proportional to K_h (Meinzer & Grantz 1990, Sperry & Pockman 1993, Hubbard *et al*. 2001).

Fruits quality and quantity differed between symptomatic and non-symptomatic plants (Table II). Other phytoplasma infection can cause fruit loss and quality reductions in important crops such as grapevines (Endeshaw *et al*. 2012). If *U. molinae* is to be considered a commercial berry, we would need to understand and control the phytoplasma infection that affects natural populations.

Ugni molinae has high potential as a commercial crop in Chile (Landrum & Donoso 1990) due to the many benefits and products that can be obtained from the fruit and the whole plant (Seguel & Torralbo 2004, Tacón *et al*. 2006, Delporte *et al*. 2007, Suwalsky *et al*. 2007, Rubilar *et al*. 2011, Goity *et al*. 2013). It is also used as an ornamental plant and the fruits are consumed as food, jam, and as alcoholic beverage (Riedemann & Aldunate 2011). To our knowledge, this is the first study that evidenced that phytoplasma infection can alter plant morphology, anatomy, physiology, and fruit quality of *U. molinae*. This knowledge could help understand the fitness costs of phytoplasma infection, and its ecological and economical impact. Future productive initiatives should include some sort of control of the phytoplasma, especially control of the natural vectors of the disease. Further studies should address the infection mechanisms and the genetic alterations that phytoplasma induce in plants.

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