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Research paper

# Plant–pathogen interactions: leaf physiology alterations in poplars infected with rust (*Melampsora medusae*)

Fermín Gortari[1,5], Juan José Guiamet[1,2,3] and Corina Graciano[1,3,4,6]

[1]INFIVE (CONICET-Universidad Nacional de La Plata), Diag 113 n° 495, CC 327, 1900 La Plata, Argentina; [2]Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, calle 60 y 122, 1900 La Plata, Argentina; [3]CCT CONICET La Plata, calle 8 n° 1467, 1900 La Plata, Argentina; [4]Facultad de Ciencias Agrarias y Forestales, Universidad Nacional de La Plata, calle 60 y 118, 1900 La Plata, Argentina; [5]Present address: Facultad de Ciencias Forestales, Universidad Nacional de Misiones, Bertoni 124, 3380 Eldorado, Misiones, Argentina; [6]Corresponding author (corinagraciano@agro.unlp.edu.ar)

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Rust produced by *Melampsora* sp. is considered one of the most relevant diseases in poplar plantations. Growth reduction in poplar plantations takes place because rust, like other pathogens, alters leaf physiology. There is not a complete evaluation of several of the physiological traits that can be affected by rust at leaf level. Therefore, the aim of this work was to evaluate, in an integrative way and in the same pathosystem, which physiological processes are affected when *Populus deltoides* Bartr. ex Marsh. leaves are infected by rust (*Melampsora medusae Thümen*). Leaves of two clones with different susceptibility to rust were analyzed. Field and pot experiments were performed, and several physiological traits were measured in healthy and infected leaves. We conclude that rust affects leaf mesophyll integrity, and so water movement in the leaf in liquid phase is affected. As a consequence, gas exchange is reduced, affecting both carbon fixation and transpiration. However, there is an increase in respiration rate, probably due to plant and fungal respiration. The increase in respiration rate is important in the reduction of net photosynthetic rate, but also some damage in the photosynthetic apparatus limits leaf capacity to fix carbon. The decrease in chlorophyll content would start later and seems not to explain the reduction in net photosynthetic rate. Both clones, although they have different susceptibility to rust, are affected in the same physiological mechanisms.

*Keywords*: leaf hydraulic conductance, photosynthesis, plant–pathogen interaction, *Populus deltoides*, stomatal conductance.

## Introduction

Poplars cover around 80 millions hectares of the Earth's surface, 7 million of which are plantations (Ball et al. 2005). The main uses for poplar wood are pulp for paper, sawn timber and plywood, and they are also utilized as bioenergy crops and have environmental uses such as wind and soil protection. Although poplar plantations have high growth rates, their productivity is limited by several stresses that occur along the production cycle. Among biotic stresses, rust produced by *Melampsora* sp. is considered one of the most relevant diseases in poplar plantations (Tabor et al. 2000).

Rust reduces productivity as a consequence of individual growth reduction in many poplar genotypes (May-De Mio et al. 2006, May-De Mio and Ruaro 2008, Cortizo 2014). Growth reduction takes place because rust, like other pathogens, alters leaf physiology. Some plant physiological responses to infections are well documented in some pathosystems. For example, photosynthesis was reduced by 50% in *Salix* sp. leaves infected with rust (Toome et al. 2009) and by 80% in *Phaseolus vulgaris* L. infected with *Uromyces appendiculatus* (Pers.) Unger. (Bassanezi et al. 2001). In the latter case, photosynthesis reduction was associated with a decrease in chlorophyll concentration and stomatal conductance. Net photosynthesis decline can also be due to an increase in respiration rate. For example, the respiration rate increased by 50% in *Triticum aestivum* L. leaves infected with *Puccinia triticina* Erikss. & Henn. (Carretero et al. 2011). The increase in $CO_2$ release can be due to host as well as fungi respiration.

Transcriptomics reveals that, in compatible interactions between *Melampsora* sp. and *Populus* sp., genes associated with photosynthesis

were down-regulated while genes associated with respiration and defense metabolism were up-regulated (Miranda et al. 2007, Rinaldi et al. 2007, Azaiez et al. 2009). Net photosynthesis reduction in infected tissues was associated with a decrease in other assimilation processes and with an increase in respiration and other metabolic pathways involved in defense mechanisms (Major et al. 2010). Moreover, hyphal filaments of the mycelia can alter leaf metabolism to enhance the movement of metabolites to the fungal cells (Berger et al. 2007, Rinaldi et al. 2007). Even though the *Populus* sp.–*Melampsora* sp. interaction has become a model for genetic and molecular studies, there are few physiological evaluations that describe the host response to the pathogen. In this way, one example is that *Populus cathayana* Rehder leaves infected with rust had lower photosynthetic rate and stomatal conductance than healthy leaves, although chlorophyll concentration was not altered (Zhang et al. 2010). However, *Melampsora larici-populina* Kleb. reduced to one-half the net photosynthesis in *P. cathayana* but with only slight photosystem II (PSII) damage (Fv/Fm = 0.84 in healthy leaves and 0.78 in infected leaves) (Zhang et al. 2010). This result suggests that gas exchange can be reduced by the hyphal growth in the substomatal cavity and intercellular spaces (Spiers and Hopcroft 1988). This damage would reduce gas exchange through stomata and might increase resistance to water movement in liquid phase because of the loss of mesophyll integrity. Therefore, photosynthesis and leaf hydration could be affected by rust, independently of any damage to the photosynthetic apparatus.

As there is not a complete evaluation of several physiological traits that can be affected by rust at leaf level, the aim of this work was to evaluate, in an integrative way and in the same pathosystem, which physiological processes are affected when *Populus deltoides* leaves are infected by rust (*Melampsora medusae*). We worked to answer the following questions: how is photosynthesis affected by rust; was the gas exchange limited by rust; and was the photosynthetic apparatus damaged? We tested the hypothesis that photosynthesis could be negatively affected by rust, associated with changes in gas exchange prior to the damage to the photosynthetic apparatus. To test this hypothesis, leaves of two poplar clones with different susceptibility to rust were analyzed.

## Materials and methods

### Plant material

Two rust-susceptible *P. deltoides* clones were used. Clone Onda (hereinafter clone O) a cross between *P. deltoides* var. *missouriensis* × *Populus* sp. (unknown father) was selected in Casale Monferrato (Italy) among seeds from Delta Mississippi River, Stoneville, MS, USA. This clone has few branches, big leaves and is very susceptible to rust. The other clone used is 'Australiano 106/60' (hereinafter clone A), selected in Canberra, Australia, in the Department of Botany, Australian National University, unknown parents. It was selected among *P. deltoides* seeds obtained from College Station, TX, USA. This clone has many branches, small leaves and is less susceptible to rust.

### Field experiment

In the first week of August 2010 (winter) a field experiment was installed near La Plata, Buenos Aires, Argentina (34°59′5.73″ S y 57°59′49.56″ W) with 1-m-length unrooted cuttings of both clones. The soil is a Typic Argiudolls (USDA). The weather is warm, mean annual temperature is 16.3 °C, mean annual air humidity is around 80% and mean annual rainfall is 993.9 mm, distributed evenly throughout the year.

Six plots of each clone (A and O) with 36 plants per plot (6 × 6) were installed. Plants were separated 3 m × 3 m. Three plots of each clone were sprayed with a systemic fungicide (0.258 g tebuconazole/liter) every 15 days, from early sprouting to autumnal abscission (R–). This dose and frequency did not caused phytotoxicity, determined by a visual evaluation of leaf health, and it was well described by Cortizo (2014). The other three plots per clone were naturally infected by rust (R+). Therefore, the experiment had two factors (clone and rust) with two levels each factor, so four treatments were applied: clone O (R–), clone O (R+), clone A (R–) and clone A (R+), with three replications (where each replicate was a single plot of 36 plants, for a total of 432 plants). The 12 plots were randomly distributed because there was no environmental gradient to identify blocks. The presence/absence of rust was recorded every 15 days in all plants.

Physiological measurements were done at midday on three sunny days during summer 2010–11 and repeated in summer 2011–12. Throughout the experiment weeds were controlled with glyphosate around the plant and mechanically between rows. Ants were controlled with baits with sulphuramide when necessary.

### Pot experiment

In the first week of August 2012 (winter) a pot experiment was installed in the same location as the field experiment. Forty-liter pots were filled with a mix of loam soil:sand (1:10 v/v). Unrooted cuttings of 40 cm in length of clones A and O were planted, one cutting per pot. Half of the plants were sprayed with the same fungicide, dose and frequency as in the field experiment. Therefore, four treatments were applied: clone OR–, clone OR+, clone AR–, and clone AR+. Six plants per treatment were used, randomly distributed, so the total number of plants was 24. Pots were watered every other day to ensure adequate water availability. Physiological measurements were done on three sunny days in summer, when rust symptoms (pustules) had fully developed, in five leaves with the higher level of infection in each treatment.

The main difference between field and pot experiments related to leaf physiology is that in the pot experiment plants were watered daily, while in the field experiment plants relied on natural rainfall.

### Detection and quantification of disease

Disease evolution was registered every 15 days in the field experiment and every 7 days in the pot experiment, with the visual inspection of all the plants. In the pot experiment, the first day when rust symptoms were observed was registered, as well as the incidence (number of leaves with rust relative to the total number of leaves of each plant) in each date and treatment. Severity (percentage of the leaf surface affected) was estimated for both experiments. In the field experiment severity was estimated as number of pustules per $cm^2$ in the most affected leaves in 3.5 cm diameter discs. In the pot experiment, severity of rust was estimated as the percentage of area with pustules with a visual scale by analyzing all the leaves of each plant.

After gas exchange measurements (see next section), a 3.5 cm diameter leaf disc was cut, a digital photograph was taken to count pustules and the disc was stored at −80 °C to determine glucosamine concentration (Ekblad and Nasholm 1996). Glucosamine concentration can be used to estimate fungal biomass instead of the number of pustules, which is the traditional approach to quantify rust infection (Pei et al. 2003). As glucosamine is a component of fungi cell wall and it is not present in plant tissues, it can be used to correlate rust severity with the drop in physiological traits. Glucosamine determination has the advantage that it is not affected by environmental conditions like temperature, light, humidity, leaf age and hyperparasite presence (Dowkiw et al. 2003), and is more objective than methods that rely on the experience of the observer. Moreover, the accuracy of glucosamine determination is independent of the presence of reproductive tissues (pustules) and it is directly related to the fungi biomass (Ayliffe et al. 2013).

Determination of glucosamine concentration was done in leaf discs (3.5 cm diameter) that were homogenized with 5 ml acetone, and centrifuged for 10 min (1500*g* at 2 °C) to eliminate the supernatant. The pellet was washed with 10 ml $H_2O$ and centrifuged again; 3 ml KOH was added to the pellet and heated to 130 °C for 60 min. When cold, 8 ml of ethanol 75% was added and kept on ice for 15 min. Then 0.9 ml of a celite suspension was added and the sample was centrifuged for 10 min, 1500*g*, 2 °C. The pellet was washed once with 8 ml ethanol 40% (v/v), centrifuged again in the same conditions, and washed twice with 8 ml $H_2O$. The pellet was diluted in 1.5 ml $H_2O$. Then 0.5 ml $NH_4SO_3NH_2$ was added and shaken for 5 min; 0.5 ml 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) was added and heated for 3 min using a waterbath. When cold, 0.5 ml $FeCl_3$ was added and after 30 min absorbance was read in a spectrophotometer at 650 nm.

To analyze the relationship between the two methods to quantify rust, correlations between the number of pustules and the concentration of glucosamine of each leaf disc were performed with Infostat software (Di Rienzo et al. 2015). Data from the two clones were considered together in the pot experiment and data from both clones in both years were taken together in the field experiment. Pearson's correlation coefficient (*r*) and probability value (*P*) are reported for each correlation.

### Physiological measurements

All the physiological traits were measured at the end of summer in fully expanded apical leaves, when plants were in active growth and rust symptoms were fully developed. Five plants per treatment were measured on each date. In the field experiment, measured trees were chosen from the three plots; therefore, one or two plants were measured in each plot, on each sampling date. The same leaf was never measured twice because a leaf disc was extracted after non-destructive measurements.

Light-saturated net photosynthetic rate (Asat) was measured with an Infra Red Gas Analyzer (IRGA, CIRAS 2, PP Systems, Amesbury, MA, USA), between 10:00 and 15:00 h. To prevent the effect of the hour of the day in Asat, we measured one plant per treatment consecutively, to form a batch. Then we started again with another plant per plot to form the second batch. We repeated this procedure to have five batches. No differences in Asat were observed between 10:00 and 15:00 h with a factorial ANOVA considering clone, rust and the batch as factors, so time of measurement was not taken into account in the analysis. The IRGA leaf chamber was set at 1500 µm photons $m^{-2}$ $s^{-1}$, 25 °C and 360 ppm $CO_2$. The intercellular $CO_2$ concentration (Ci) for each measure was taken. After measuring Asat, the leaf was acclimated in the dark for 5 min and when sample $CO_2$ levels were stable for at least 5 min, *A* was recorded as an approximation of dark respiration. We found that this estimate of dark respiration was comparable to that measured with a Clark type oxygen electrode disc (Hansatech, Norfolk, UK) after overnight dark acclimation for healthy and infected leaves of both clones (OR–: 2.28; OR+ :3.32; AR–: 3.03; AR+: 3.34 µmol $m^{-2}$ $s^{-1}$). However, IRGA measurements approximate leaf day respiration, which is presumably higher than night respiration.

As Asat measurement is affected by the $CO_2$ released by the fungi respiration, to corroborate if the photosynthesis rate drops in infected leaves, the PSII electron transport rate (ETR) was measured with the chlorophyll modulated fluorescence method (FMS2, Hansatech, Norfolk, UK), under natural light conditions, in the same portion of leaf in which Asat was measured. In the field experiment, in 2011–12 ETR measurements were done only on one day due to technical problems with the equipment. In the pot experiment, PSII maximum quantic yield (Fv/Fm) was measured after 30 min of dark acclimatization. After that, in the same leaf sector, total chlorophyll content was estimated with a SPAD-502 Minolta (Spectrum Technologies Inc., Plainfield, IL, USA). Stomatal conductance (gs) was measured with a porometer (Decagon SC1, Pullman, Washington, USA) on sunny days between 10:00 and 11:00 h, on the adaxial side of the leaves,

because both leaf sides had nearly the same gs. After these measurements, the leaf disc to measured glucosamine was extracted (see Detection and quantification of disease).

In another set of leaves with similar position and rust infection as those used to measured Asat, ETR and gs, hydraulic conductance was measured with the pressure drop method (Melcher et al. 2012). This method consists of an hydraulic head connected to the petiole through a silicon microtube. The hydraulic head was 40 cm high and filled with twice distilled, de-gassed water. Between the hydraulic head and the petiole there is a known resistance. A pressure sensor registers the drop in pressure when water starts to exit the leaf through stomata. Water flux was recorded when it was stable for at least for 2 min. During the measurement, leaves were lit to stimulate stomata opening. After flux measurement, the leaf was enclosed in a plastic bag in dark, and water potential was measured with a pressure chamber after 5 min of stabilization. After measuring the equilibrium leaf water potential, the hydraulic conductance of the leaf ($K_{leaf}$) was calculated. $K_{leaf}$ was measured in the field experiment only in the summer 2011–12 and in the pot experiment.

### Statistical analysis

ANOVA was performed considering clone (O or A) and rust (R+ or R–) as factors. If the interaction was significant, means were compared by Duncan test ($P < 0.05$). A multivariate analysis was performed, to analyze the effect of rust in both clones considering all the physiological variables together. Principal components analysis (PCA) was performed with Infostat software (Di Rienzo et al. 2015). Rust and clone were used as categorical factors. Variables were standardized. Two axes were selected for the biplot representation.

## Results

### Detection and quantification of disease

The first symptoms of rust appeared at the beginning of February in 2010–11 and in the middle of March in 2011–12. Plants in sprayed plots (R–) had no rust in any year (0% incidence). All non-sprayed plants (R+) had rust both years (100% incidence). The number of pustules was 51.2 $cm^{-2}$ for clone OR+ and 6.7 $cm^{-2}$ for clone AR+ in the summer of 2010–11, and 44.8 $cm^{-2}$ for clone OR+ and 15.3 $cm^{-2}$ for clone AR+ in the summer of 2011–12. Fungal cell walls are rich in chitin, which is primarily a polymer of glucosamine (Ayliffe et al. 2013). Glucosamine concentration in R– leaves was around 1.2 $\mu g\ cm^{-2}$ in both clones, while it was 1.97–2.07 $\mu g\ cm^{-2}$ in clone AR+ and 2.59–2.89 $\mu g\ cm^{-2}$ in clone OR+ considering both years (Table 1).

In the pot experiment, results are similar to those observed in the field experiment, even though water availability was more controlled. Rust appeared at the beginning of January 2013. Sprayed plants of both clones had no rust (0% incidence). All non-sprayed plants were infected by rust (100% incidence). Severity was higher in clone O (70%) than in clone A (30%). The number of pustules was 106.7 $cm^{-2}$ for clone OR+ and 13.3 $cm^{-2}$ for clone AR+. Glucosamine concentration in R- leaves was around 0.4 $\mu g\ cm^{-2}$ in both clones, 1.47 $\mu g\ cm^{-2}$ in AR+ and 4.14 $\mu g\ cm^{-2}$ in OR+ (Table 1).

### Leaf physiology traits affected by rust

In the field experiment, rust reduced net photosynthetic rate (Figure 1A). The photosynthetic rate reduction was 45% in clone O and 19% in clone A for 2010–11. In 2011–12 the reduction was up to 100%. Respiration rate was not affected by rust (Figure 1E). In the pot experiment, Asat was lower in leaves infected with rust in both clones, it decreased by 52% in the clone O and 29% in the clone A (Figure 1B), but there were no changes in Ci (Figure 1D). Contrary to the results in the field experiment, in which differences were not significant, respiration rate was increased by rust: 240% in clone O and 87% in clone A (Figure 1F).

In the field experiment, rust diminished the electron transport rate at PSII level (ETR), maximum PSII quantum yield (Fv/Fm) and chlorophyll content (only in 2010–11). The ETR reduction was higher in clone O (42%) than in clone A (32%) (Figure 2A). Fv/Fm decreased more in clone O than in A in both seasons (Figure 2C). Chlorophyll content reduction was 22% in clone O and 10% in clone A in 2010–11, but no reductions were observed in 2011–12 (Figure 2E). In the pot experiment, ETR was lower in leaves with rust compared with healthy leaves in both clones. Reduction was about 20% in clone O and 18% in clone A (Figure 2B). Fv/Fm decreased to a lesser extent than in the field experiment, 4% in clone O and 2% in clone A (Figure 2D). In the pot experiment, rust did not reduce chlorophyll content in any clone, although there were differences in chlorophyll content between clones (Figure 2F).

Although there were no differences in gs between healthy and infected leaves in the field experiment (Figure 3A), the movement of water in liquid phase was affected by rust. Leaf hydraulic conductance ($K_{leaf}$) was lower in leaves with rust relative to non-infected leaves (Figure 3C). Reduction was around 45% in the clone O and 50% in the clone A. In the pot experiment, gs and $K_{leaf}$ diminished in leaves with rust compared with healthy leaves. Reduction of gs was 30% in clone O and 25% in clone A (Figure 3B). Reduction of $K_{leaf}$ was 28% in clone O and 38% in clone A (Figure 3D).

### Relationship between rust and physiological variables (PCA)

Principal components analysis (PCA) was used to examine whether our physiological measurements describe the variation between the clone and rust. The total variability of the data is represented in only two axes. Two axes represent very well the total variability: 98.8% in the field experiment and 92.7% in the pot experiment (Figure 4). The variables that quantify rust level,

Table 1. Rust incidence, rust severity, number of pustules, glucosamine concentration and correlation between number of pustules and glucosamine concentration for the field and pot experiments. clone Onda (O), clone 'Australiano 106/60' (A), with rust (R+), without rust (R–), no data (–). Pearson's correlations coefficients (*r*) were calculated considering both clones together, and both years in the field experiment. The *P*-values indicate the significance of the correlation.

| | Field experiment (both years) | | | | Pot experiment | | | |
|---|---|---|---|---|---|---|---|---|
| | OR– | OR+ | AR– | AR+ | OR– | OR+ | AR– | AR+ |
| Rust incidence (%) | 0 | 100 | 0 | 100 | 0 | 100 | 0 | 100 |
| Rust severity (%) | – | – | – | – | 0 | 70 | 0 | 30 |
| Pustules (n°. $cm^{-2}$) | [1]0 | 51.2 | 0 | 6.7 | 0 | 106.7 | 0 | 13.3 |
| | [2]0 | 44.8 | 0 | 15.3 | | | | |
| Glucosamine (μg $cm^{-2}$) | [1]1.20 | 2.89 | 1.20 | 2.07 | 0.35 | 4.14 | 0.41 | 1.47 |
| | [2]1.11 | 2.58 | 1.19 | 1.97 | | | | |
| Correlation between pustules and glucosamine | $r = 0.85$ ($P < 0.001$) | | | | $r = 0.62$ ($P < 0.001$) | | | |

[1]2010–11.
[2]2011–12 season.

i.e., glucosamine concentration and number of pustules, are highly positively correlated between them, because both vectors have the same direction. In both experiments, they are highly negatively correlated with the variables related with photosynthesis (Asat, Fv/Fm, ETR), as can be seen by the opposite directions of the vectors. Respiration (R) is positively related to rust infection, but there is some variability in the PC2, therefore some variability in respiration rate is not explained by the level of rust infection. Chlorophyll concentration (Chl) is not related to rust variables, in any experiment. Stomatal conductance (gs) is not related to rust infection in the field experiment, while it is negatively related in the pot experiment. Rust infection is associated with lower Asat, Fv/Fm and ETR, and higher numbers of pustules and glucosamine content. Plants of both clones without rust are more similar between them than plants with rusts, i.e., infected leaves from each clone are different. Clone A has lower variability between healthy and infected leaves than clone O, because distance between the point AR+ and AR– is shorter than distance between OR+ and OR–, i.e., rust affects to a higher extent leaves from clone O than from clone A.

## Discussion

### *Photosynthesis is affected by rust*

Rust infection causes important changes in poplar's leaf physiology. There is a reduction of net photosynthesis in leaves from both clones when infected with rust (Figure 1A and B), but the differential susceptibility of the clones is evident: in clone A the reduction is about 20% while in clone O it is around 50%, excluding 2011–12 when reduction was up to 100% for both clones. The negative Asat registered in 2011–12 in rust-infected leaves can be explained by the reduction in gross assimilation rate consistent with the low ETR (Figure 2A) and a slight increase in respiration rate and decrease in stomatal conductance, which produced an increment in Ci (Figure 1B). A 30% reduction was observed in *Populus balsamifera* L. leaves infected with *M. larici-populina* (Jiang et al. 2016) and nearly 80–90% in *Populus euphratica* Olivier and *Populus pruinosa* Schrenk., respectively (Zhang et al. 2016). These percentages are in the range of those reported for other plant–pathogen interactions. For example, a reduction of photosynthesis of 50% was observed in *Salix* sp. infected with *Melampsora epitea* (Toome et al. 2009), a reduction between 25% and 47% in *Picea abies* (L.) Karsten infected with *Chrysomyxa rhododendri* (DC.) De Bary (Mayr et al. 2001), the same percentages of reduction in leaves of *Saccharum* sp. hybrids infected with *Puccinia kuehnii* (W. Kruger) E. J. Butler (Zhao et al. 2011), and up to 80% in leaves of *P. vulgaris* infected with *U. appendiculatus* (Bassanezi et al. 2001). The reduction of photosynthesis determined by Asat in our experiments cannot simply be explained by intercellular $CO_2$ concentration, since Ci levels in infected leaves were unchanged or higher relative to healthy leaves (Figure 1C and D), similar to the results observed for *P. balsamifera* (Jiang et al. 2016). At the same time, an increase in respiration was seen in the pot experiment (Figure 1F), more evident in clone O. In general, in both experiments respiration rate was higher in leaves with higher rust infection (number of pustules and glucosamine concentration) as can be observed in the PCA (Figure 4). This result contradicts the observations done in a desert poplar infected with *Melampsora pruinosae* Franz., in which respiration decreased 10 and 20 days after rust infection (Zhang et al. 2016). However, we could not discriminate whether the increase in respiration was a result of an increase in the respiration of the plant, the pathogen or both. In summary, the reduction in net photosynthesis was between 5 and 15 μmol $CO_2$ $m^{-2}$ $s^{-1}$ in infected leaves compared with healthy leaves. However, the respiration increased 0.5–2 μmol $CO_2$ $m^{-2}$ $s^{-1}$ in leaves infected by rust compared with leaves without rust. Thus, the increase in respiration only explains partially the reduction in net photosynthesis and it is evident that gross photosynthesis is also reduced.

### *The photosynthetic apparatus is damaged by rust*

The reduction in net photosynthesis could be partially explained by the dismantling of the photosynthetic apparatus and the

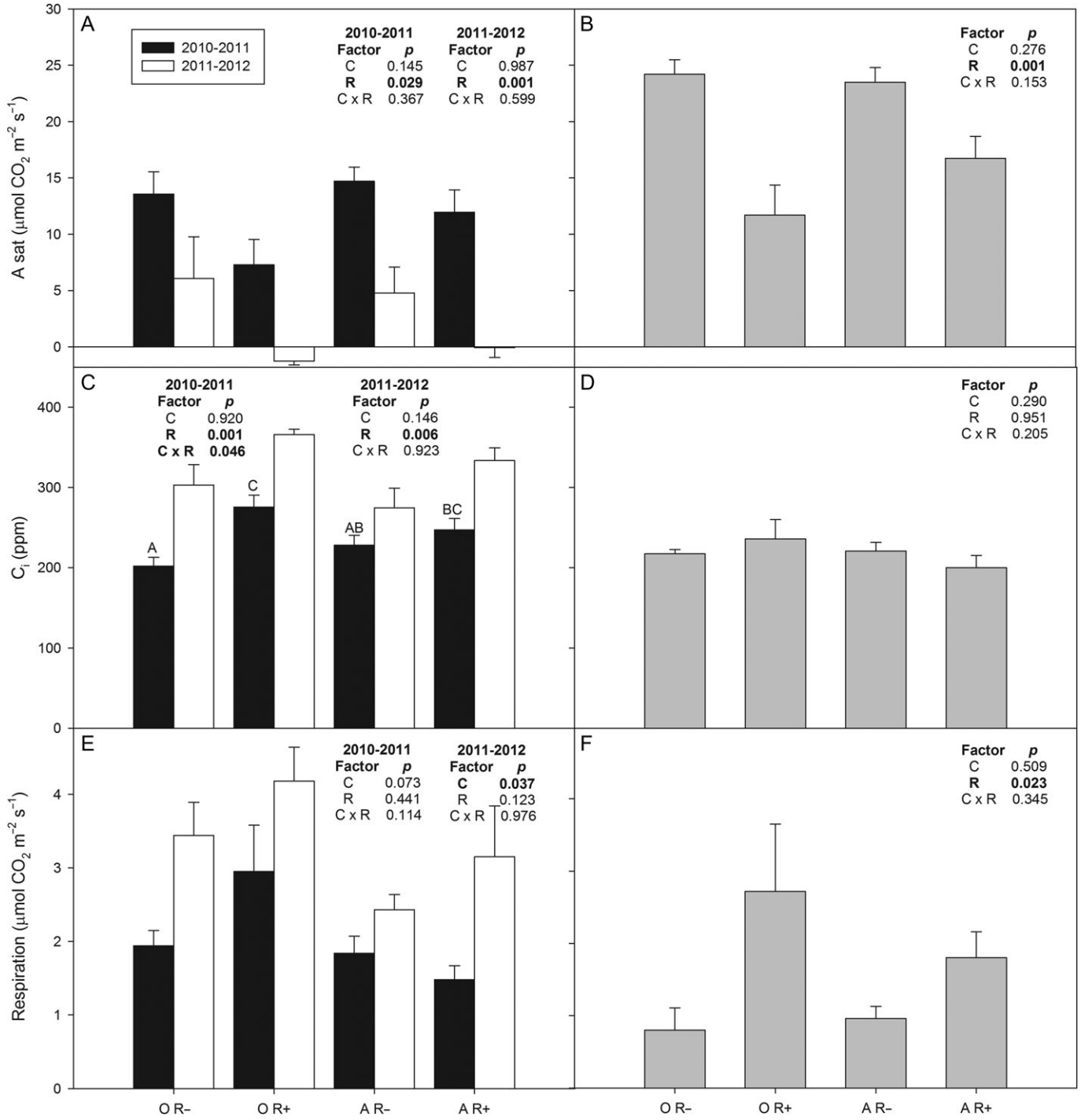


Figure 1. Net photosynthetic rate (Asat, A and B), intercellular $CO_2$ concentration (Ci, C and D) and respiration rate (E and F) in the field experiment (A, C and E) and in the pot experiment (B, D and F) in the clone Onda (O), clone 'Australiano 106/60' (A), with rust (R+) and without rust (R−). Bars indicate standard errors. Inserts in each panel indicate the *P*-value of the ANOVA for each factor and the interaction. Significant factors are in bold letter ($P < 0.05$). Different letter indicated significant differences.

reduction of leaf capacity to fix carbon. Chlorophyll content is reduced between 10% and 20% when leaves are infected with rust (Figure 2E) and a reduction of ETR is observed (Figure 2A and B). This suggests that the functioning of the photochemical apparatus that provides energy and reducing power for carbon reduction is impaired by infection. Moreover, the reduced Fv/Fm in infected leaves is consistent with rust-induced damage to the photosynthetic apparatus (Figure 2C and D). Similarly, 3–8% reduction in Fv/Fm was observed in *P. cathayana* infected with rust, and the reduction was associated with the infection level

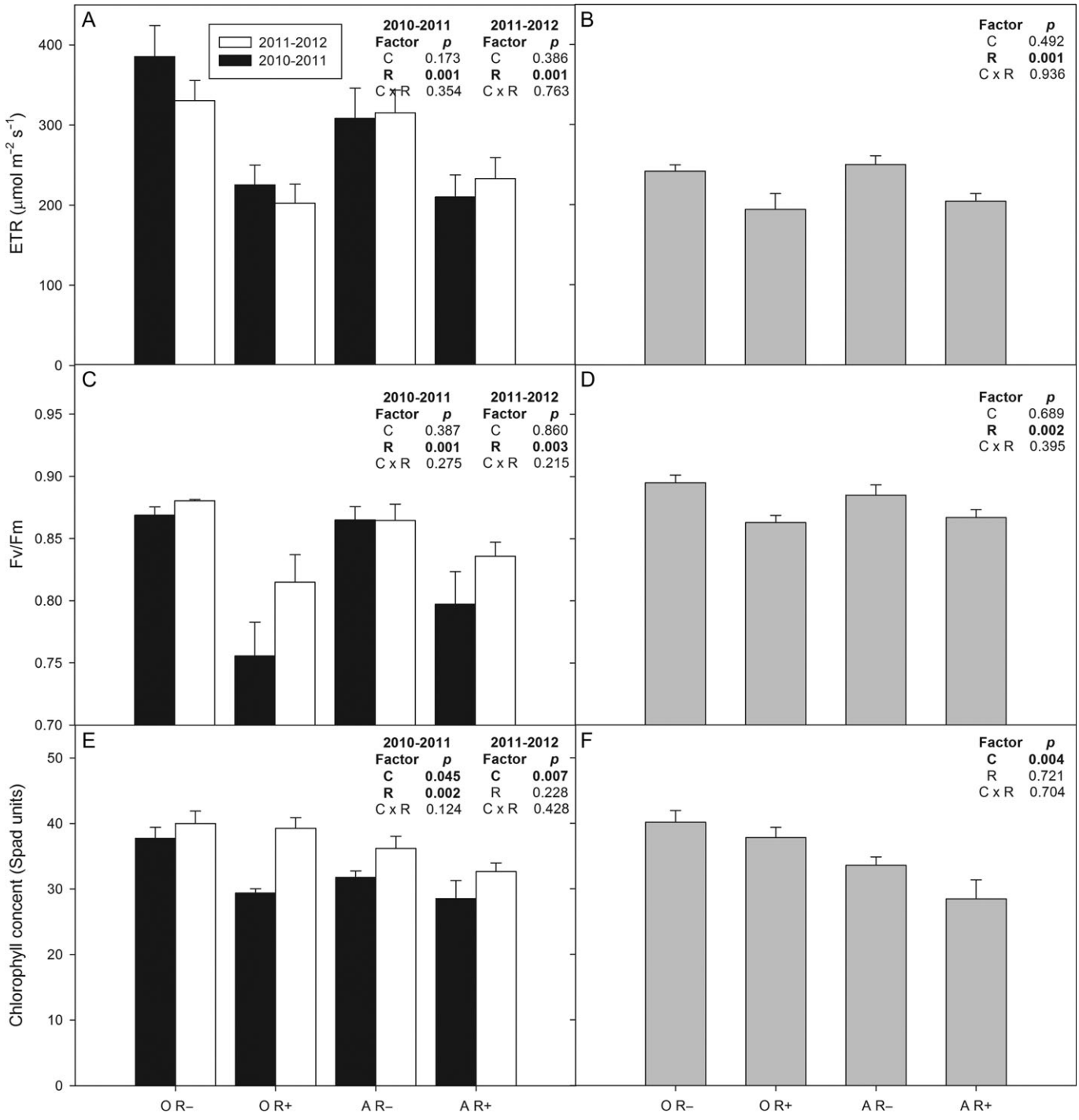


Figure 2. Photosystem II (PSII) electron transport rate (ETR, A and B), maximum PSII quantum yield (Fv/Fm) measured at dark (C and D) and chlorophyll content (Spad units, E and F) in the field experiment (A, C and E) and in the pot experiment (B, D and F) in the clone Onda (O), clone 'Australiano 106/60' (A), with rust (R+) and without rust (R–). Bars indicate standard errors. Inserts in each panel indicate the *P*-value of the ANOVA for each factor and the interaction. Significant factors are in bold letter ($P < 0.05$).

(Zhang et al. 2010). Inasmuch as Fv/Fm represents the balance between the rate of photodamage of PSII and rate of PSII repair (Aro et al. 1993) and it is not related to changes in light absorption (i.e., changes in chlorophyll content or water content) (Fracheboud and Leipner 2003), this suggests that rust infection also interferes with chloroplast protein synthesis. Overall, these observations suggest that rust causes substantial detrimental effects at the chloroplast level. The results obtained by fluorescence techniques complement those obtained by gas exchange methodology (IRGA) to measure net photosynthesis. The gas exchange technique cannot discriminate the origin of the $CO_2$ released, and thus the contribution of fungal respiration

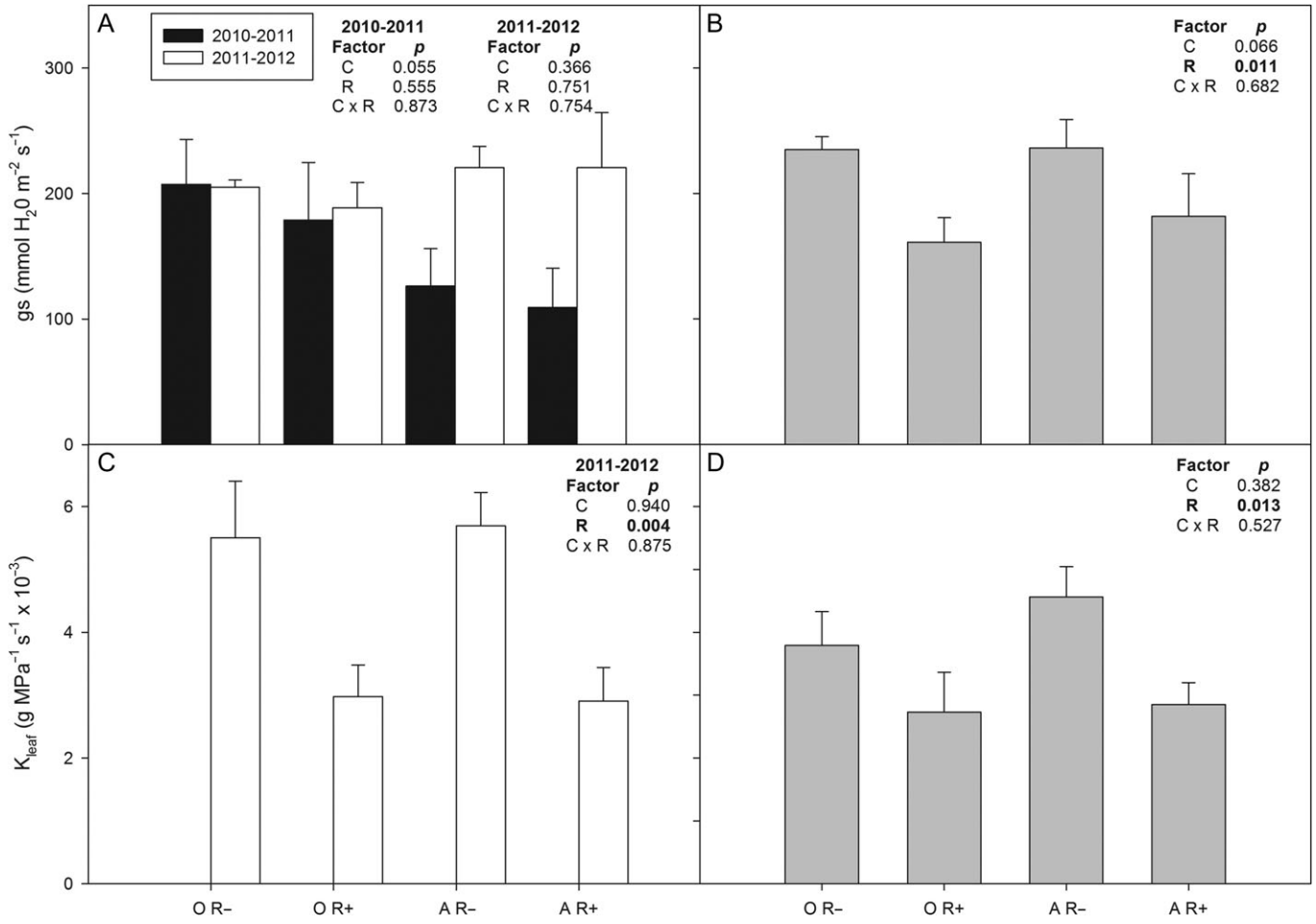


Figure 3. Stomatal conductance (gs, A and B) and leaf hydraulic conductance ($K_{leaf}$, C and D) in the field experiment (A and C) and in the pot experiment (B and D) in the clone Onda (O), clone 'Australiano 106/60' (A), with rust (R+) and without rust (R–). Bars indicate standard errors. Inserts in each panel indicate the *P*-value of the ANOVA for each factor and the interaction. Significant factors are in bold letter ($P < 0.05$). Leaf hydraulic conductance ($K_{leaf}$) was measured only in 2011–12 in the field experiment.

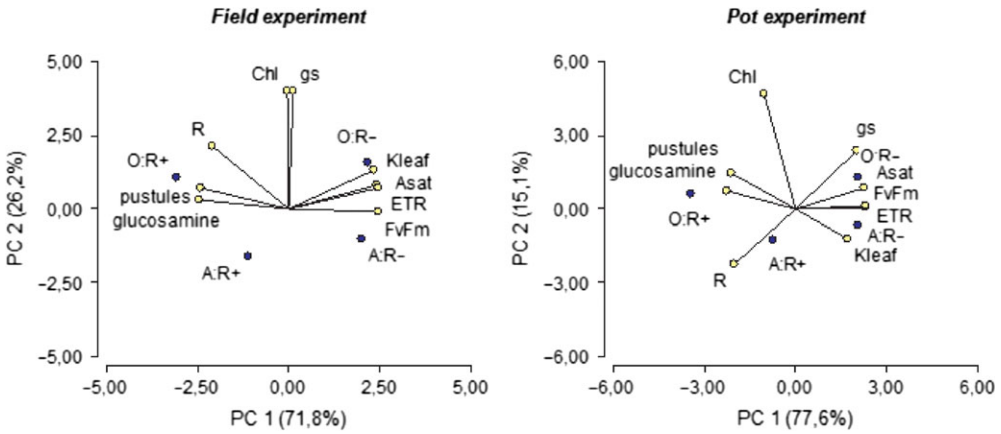


Figure 4. Principal component analysis (PCA) to explore the relationship between physiological variables in the field experiment, considering both years, and in the pot experiment in clone Onda (O), clone 'Australiano 106/60' (A), with rust (R+) and without rust (R–). Vectors with the same direction indicate high positive correlation between variables. The opposite direction indicates negative correlation. Points indicate the relationship between the variables and the treatments.

to the apparent decrease in net photosynthesis of leaves is unknown, although there is a clear positive relation between respiration rate and rust infection (Figure 4). These measurements together demonstrate that the decrease in net photosynthesis by rust infection seems to be of higher magnitude than the reduction in chlorophyll content or ETR. Consequently, it is likely that the disease affects gas exchange also by other mechanisms.

### *Gas exchange and liquid water conductance is limited by rust*

There is a relationship between the reduction in net photosynthesis and a higher difficulty for gas exchange across stomata. In leaves infected with rust, there is a reduction in stomatal conductance compared with healthy leaves (Figures 3B) as well as a clear reduction in the hydraulic conductance of infected leaves compared with healthy ones (Figure 3C and D). These results suggest that the fungi reduce the movement of water in the leaves both in the gas and liquid phases. Consistent with our results, there was a reduction in gs in two *Populus* sp. clones (*Populus nigra* × *Populus maximowiczii* and *P. deltoides* × *P. nigra*) infected with *Marssonina brunnea* (E & E). Magn, from 0.8 to 0.2 mmol $H_2O\,m^{-2}s^{-1}$ (Erickson et al. 2004). It is possible that fungal respiration increases the intercellular concentration of $CO_2$ and this could contribute to reduce the stomatal conductance (Lopes and Berger 2001, Mandal et al. 2009, Zhao et al. 2011). However, it cannot be ruled out that the reduction in hydraulic conductance and gs is due to the colonization of the mesophyll intercellular spaces and the substomatal chamber by fungal hyphae, disrupting the normal circulation of water (Herre et al. 2005). This idea could be supported by the lack of correlation between gs and number of pustules or glucosamine in the field experiment (Figure 4), therefore the partial reduction of gs is not straightforwardly related to the mass of fungi, as it should be if it was related to $CO_2$ release. However, in field and pot experiments there is a negative correlation between $K_{leaf}$ and fungi mass (Figure 4), i.e., as fungi biomass is higher, the damage to the mesophyll structure is higher.

Principal component analysis (PCA) demonstrates that the variables considered described properly the effects of rust on leaf physiology, because they explain a high percentage of the total variation (Figure 4). The number of pustules is highly correlated with the concentration of glucosamine, in both the field and pot experiments, so any of these measurements can be used to estimate rust infection. Although pustules can be affected by environmental conditions, such as heavy rains, temperature and leaf age (Dowkiw et al. 2003), in our experiments both methodologies are highly correlated, so any of them can be used (Table 1). As glucosamine determination is more costly than counting pustules, it makes sense to measure glucosamine if fungal quantification needs to be done from the beginning of the infection to describe fungal mass development until pustules becomes visible. After that, number of pustules seems more convenient.

If the physiological variables are analyzed, rust level of infection is negatively correlated with photosynthetic rates (Asat, ETR) and with the damage to PSII (Fv/Fm) (Figure 4) and positively correlated with respiration rate (R). However, chlorophyll content is not related either with the level of rust infection or with the photosynthetic capacity. This is consistent with the lack of effect of rust on chlorophyll content in the pot experiment and the high variability between clones (Figure 2F). As differences in chlorophyll content between clones are higher that the differences between healthy and infected leaves within each clone, chlorophyll content is not a good indicator of rust infection. It is important to remark that photosynthetic capacity (Asat and ETR) (Figures 1A and B, 2A and B) is consistently reduced by rust in both experiments, as is PSII damage (Fv/Fm) (Figure 2C and D). Therefore, rust reduces photosynthetic capacity before premature leaf senescence is induced, probably because gas exchange and liquid water movement is reduced in infected leaves, as can be seen from the drop in stomatal conductance in the pot experiment (Figure 3B) and leaf hydraulic conductance in both experiments (Figure 3C and D). The fact that stomatal conductance was not significantly reduced by rust in the field experiment (Figures 3A and 4) can be explained because plants in the field are exposed to natural environmental conditions, that involved water shortages and higher evaporative demand than plants in the pot experiment. In the pot experiment, in which plants had high availability of water in the soil, the effect of rust in stomatal conductance was significant (Figures 3B and 4).

The different clones had different masses of fungi in their leaves. In both experiments, glucosamine concentration in R– plants was not zero, therefore fungicide completely inhibits infection and pustule formation but not spore germination and fungi initial growth (Table 1). As the fungicide affects ergosterol synthesis (Sui et al. 2017) some fungi growth is needed in order for it to be effective. Fungi growth was higher in clone O leaves than in clone A leaves, possibly related to clonal differences. It is well documented that *P. deltoides* clones have different susceptibility to rust, even at the level of leaf infection (number of pustules), in terms of the reduction in growth in diseased plants. However, the mechanisms underlying clonal differences are not clear. In other pathosystems, it was observed that modifications in cell wall, fluctuations in the redox state and the effect of different phytohormones contribute to confer moderate resistance to *Chrysoporthe austroafricana* Gryzenh. & M. J. Wingf in some genotypes of *Eucalyptus grandis* Hill ex Maiden (Mangwanda et al. 2015).

Changes in gas exchange occur earlier than the damage to the photosynthetic apparatus. Taking all the results together, in the field experiment, rust reduced leaf hydraulic conductance. Although there was no reduction in stomatal conductance, net photosynthesis and ETR decreased as well as chlorophyll content. Respiration rate did not change significantly. By contrast, in the pot experiment, leaf hydraulic conductance decreased as well as

stomatal conductance. There was a strong reduction in net photosynthesis together with a slight reduction in ETR and Fv/Fm and no reduction in chlorophyll content. There was a high increase in respiration rate. We suggest that rust affects leaf mesophyll integrity so water movement in the leaf in liquid phase is first affected. As a consequence, gas exchange is reduced, affecting both carbon fixation and transpiration. This allowed us to not reject the working hypothesis. However, there is an increase in respiration rate, probably due to both plant and fungal respiration. The increase in respiration rate is important in the reduction of net photosynthetic rate, but more important is the reduction in gas exchange. The damage to the photosynthetic apparatus can limit the leaf's capacity to fix carbon, but the level of damage in functional chloroplasts (reduction in Fv/Fm) is low. The decrease in chlorophyll content, i.e., the dismantling of the chloroplasts, would start later and seems to explain less of the reduction in net photosynthetic rate. Although the clones have different rust tolerance, as is reflected in their different infection level, leaf physiological alterations are similar in both clones and the extent of the damage in physiological traits is directly related to the mass of fungi present in the leaf.

## Conclusion

The results highlight the importance of the fungal growth inside the leaf for the detrimental effect of the rust on leaf physiology, and explain the mechanism involved in the damage. Future evaluations are needed to understand the impact of the damage at leaf level in total plant growth.

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## Conflict of interest

None declared.

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