



Original article

Compensatory responses in plant-herbivore interactions: Impacts of insects on leaf water relations

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ABSTRACT

Herbivore damage to leaves has been typically evaluated in terms of fractions of area removed; however morpho-physiological changes in the remaining tissues can occur in response to removal. We assessed the effects of partial removal of the leaf mesophyll by *Caliroa cerasi* (Hymenoptera) on leaf hydraulic conductance (K_{leaf}), vascular architecture, water relations and leaf size of three *Prunus avium* cultivars. The insect feeds on the leaf mesophyll leaving the vein network intact (skeletonization). Within each cultivar there were trees without infestations and trees chronically infested, at least over the last three years. Leaf size of intact leaves tended to be similar during leaf expansion before herbivore attack occurs across infested and non-infested trees. However, after herbivore attack and when the leaves were fully expanded, damaged leaves were smaller than leaves from non-infested trees. Damaged area varied between 21 and 31% depending on cultivar. The non-disruption of the vascular system together with either vein density or capacitance increased in damaged leaves resulted in similar K_{leaf} and stomatal conductance in infested and non-infested trees. Non-stomatal water loss from repeated leaf damage led to lower leaf water potentials in two of the infested cultivars. Lower leaf osmotic potentials and vulnerability to loss of K_{leaf} were observed in infested plants. Our results show that skeletonization resulted in compensatory changes in terms of water relations and hydraulics traits and in cultivar-specific physiological changes in phylogenetic related *P. avium*. Our findings indicate that detrimental effects of herbivory on the photosynthetic surface are counterbalanced by changes providing higher drought resistance, which has adaptive significance in ecosystems where water availability is low and furthermore where global climate changes would decrease soil water availability in the future even further.

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1. Introduction

Leaf injury by insects (either miners or mass consumers) can induce a wide range of metabolic and physical changes in host-plants (Oleksyn et al., 1998). Plant responses to insect injury are highly dependent on leaf phenological state, intensity of damage, type of plant tissue attacked, type of injury (that is, the feeding guild of the herbivore), species and environmental conditions (Welter, 1989; Peterson and Higley, 2001). Research on biotic stress

traditionally has focused on the biological agents causing the infestation, rather than on the physiological responses of plants to tissue removal (but see McNaughton, 1983; Raimondo et al., 2003; Aldea et al., 2005; Kerchev et al., 2012). In particular the influence of herbivory on plant water relations and hydraulic traits is less understood (Raimondo et al., 2003; Aldea et al., 2005; Nardini et al., 2010; Pittermann et al., 2014).

The removal of leaf tissue by herbivores can result in a reduction of the total photosynthetic surface; while the remaining leaf tissue can experience changes in liquid phase water movement and stomatal conductance, which could affect whole leaf functioning (Delaney and Higley, 2006; Turnbull et al., 2007). When the leaf vasculature is damaged, in particular the main veins, leaf hydraulic conductance drastically decreases, and this effect persists even

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after the damage has healed. Nevertheless, redundancy in the venation can buffer leaf water transport against the damage by providing alternative pathways for water transport to the mesophyll (Sack et al., 2008). Mechanical damage to inter-veinal tissue (skeletonization) produces substantial water loss from cut edges and surfaces (Ostlie and Pedigo, 1984; Aldea et al., 2005) affecting the leaf extra-vascular water transport. This higher water loss may result in leaf water potentials decline and eventually in total leaf dehydration. Some of the few studies that have examined the effects of defoliation on whole-plant water relations, however, have shown improved plant water status in response to increased water supply per unit leaf area (Hart et al., 2000; <http://jxb.oxfordjournals.org/content/64/6/1625.full> Quentin et al., 2011).

Leaf size can be genetically determined or can be regulated by consumption (e.g. herbivores), mechanical damage (e.g. wind) or physiological processes (e.g. water deficits). High levels of herbivore pressure apparently favor the evolution of small leaf size (Brown et al., 1991; Moles and Westoby, 2000). The loss of part of the leaves during leaf expansion by herbivores, not only implies a loss of structural components but also of proteins and lipids associated with photosynthesis and leaf growth (Kursar and Coley, 1992). Simultaneously, the excessive loss of water from edges after defoliation may have a negative effect on both leaf expansion rates and final leaf size (Mazzoleni and Dickmann, 1988; Metcalfe et al., 1990).

The objective of this study was to investigate the effects of leaf infestation by *Caliroa cerasi* L. (cherry slugworm) on three sweet cherry (*Prunus avium* L.) cultivars. This insect is one the most important pests in sweet cherries crops in the Argentinean Patagonia and their larvae produce skeletonization of the leaves leaving relatively intact the vein network of the eaten leaves. We asked whether: i) the herbivory by *C. cerasi* during the leaf expansion phase of *P. avium* impacts on the leaf size, ii) leaf damage affects leaf water potential components, and iii) responses to herbivory can counter/offset the effects of herbivore attack, in particular anatomical and physiological changes related to leaf hydraulics. To respond those questions we used intact leaves of non-infested trees as control and damaged leaves from chronically infested trees occurring at least during the last three years before this study was done.

2. Materials and methods

2.1. Location and study species

The study was conducted in El Porvenir Ranch located in Los Antiguos town in northwest Santa Cruz, Argentina at the foot hills of the Andes (46° 19' S, 71° 62' W, altitude 220 m), one of the main areas for commercial cherry growth in southern Patagonia. The climate can be characterized as temperate or cold-temperate. Mean monthly temperatures range from 2.5 to 14.9 °C and the chilling requirements of cherries are easily satisfied. This area is dominated by Pacific air masses and strong westerly winds. The Andes mountain range presents an orographic barrier for these humid masses of air coming from the Pacific Ocean which precipitate on the western side of the Andes, resulting in a very low annual precipitation in the eastward of Andes. The mean annual precipitation in the study site is 192 mm falling mostly in the fall and winter (April–September). The cherry slugworm, *C. cerasi* (Hymenoptera: Tenthredinidae), is conspicuous in many South America countries and it is one of the most important pests in sweet cherries. The larvae feed in such a way that the leaf veins remain intact. They rarely remove the abaxial leaf surface determining the characteristic skeletonized appearance. *Prunus* is an important host genus (Carl, 1972; Naumann et al., 2002). *P. avium* is a deciduous woody

species that flowers before new leaves are produced. Three cultivars of sweet cherry trees (*P. avium*) were selected for the study: Lapins, Bing and Van. These cultivars are the most commonly used in Southern Patagonia (Cittadini and San Martino, 2007). In the study area and in the three cultivars new leaves expand from November to January and they are fully developed in February. The study was carried out before tree infestation by *C. cerasi*, (November 2009) and during the month of most intense defoliation by the insect (February 2010). Three to six infested (at least during the three previous years before this analysis) and non-infested individuals in each cultivar were randomly chosen resulting in a total of 18–36 trees across all three cultivars depending on the variable measured. These grouping (infested and non-infested trees) used to classify trees were determined by the presence or absence of herbivory during three years before this study began. The infested trees had more than 50% of damaged leaves (eaten to some degree), while non-infested trees (control group) had less than 1% of damaged leaves. All the trees were seven years old with similar total leaf surface area and height, planted as free standing trees (280 trees ha⁻¹) and irrigated by gravity (Muñoz, 2004). All trees from the three cultivars were grown under similar environmental conditions. Leaves within each group were sampled on the same calendar date and were of similar age. In this study we will use hereafter “damaged leaves” and “intact leaves” to refer to leaves with substantial tissue removal by the insect from infested trees and intact leaves from non-infested trees, respectively.

2.2. Leaf area, leaf dry mass per unit area and venation architecture

To determine the effects of herbivory on leaf size, twenty full expanded damaged leaves and twenty full expanded intact leaves were collected both in February (end of the leaf expansion phase) from six trees per cultivar and condition. A similar number of leaves (n = 20) from five infested and non-infested trees were collected in November (prior to infestation and when leaves were expanding). Fresh leaves images were acquired using a scanner and the images analyze for leaf size was performed with the ImageJ 1.47k software. The total non-damaged area and the area removed by the insects were also determined in each leaf. The leaves collected in February were oven-dried at 70 °C until constant weight, and dry mass was used for leaf dry mass per area (LMA) determination.

To evaluate the effects of infestation on leaf vasculature several intact leaves recently expanded from infested trees were marked. The next day those leaves infested by *C. cerasi* were identified. At least five damaged leaves after five days of infestation and five intact and recently expanded leaves from non-infested trees in each cultivar were cut off under distilled and filtered water. Then their petioles were placed in contact with filtered aqueous Phloxine B for 30 min to check whether the veins in damaged leaves were still intact as revealed by their light red staining. Leaves were then cross-sectioned using fresh razor blades and major and minor veins were observed under a microscope for their structural and functional integrity (Raimondo et al., 2003). Leaves of infested and non-infested trees per cultivar were cleared for studying venation traits. Leaf major vein density and vein diameter were determined from not damaged sections cut centrally on the right-hand side of each leaf. The major vein density was determined as the sum of 1°, 2°, and 3° vein densities (Scoffoni et al., 2011). Images of approximately 0.5 mm² per section were obtained with a color microscope camera (TCA-3C Tucsen, China) mounted on a light microscope (Axioplan ZEISS, Germany). The images were then obtained with TSVIEW v 6.1.3.9 Software and the veins were measured and counted using the ImageJ 1.47k software.

Twenty four to fifty three sections of the middle part of the leaf midrib from infested and non-infested trees were obtained using a

razor blade and the sections were stained with safranin to increase visual contrast. Images of the cross-sections were obtained with a color microscope camera (TCA-3C) mounted on a light microscope (Axioplan). Images were then acquired with the TSView v 6.1.3.9 Software. The photographs were obtained at 400× to calculate vessel density and the midrib diameter. Vessels were measured and counted using the ImageJ 1.47k software.

2.3. Leaf hydraulic conductance and vulnerability curves

Predawn leaf hydraulic conductance (K_{leaf}) was measured using a vacuum chamber (Nardini et al., 2001) in damaged leaves from infested plants and in intact leaves from non-infested plants. A total of 3 leaves from 5 trees per cultivar and tree condition (infested and non-infested) were severed in the field under water before dawn, and connected to plastic HPLC tubing while the large petioles were still under water. The tubing passed through the rubber seal of a 1 L vacuum chamber to a graduated micro-pipette containing 30 mM KCl solution. A vacuum pump maintained pressures (P) in the vacuum flask at 80, 60, 40 and 20 kPa below atmospheric pressure. At each pressure the water volume collected was determined with graduated micro-pipette at 60 s intervals, thus allowing measurements at the corresponding flow rates (F). The average F was calculated from at least ten readings after F became stable. The flow was first recorded at 80 kPa below atmospheric pressure, and then measurements proceeded at 60, 40 and 20 kPa. Final F values were done at atmospheric pressure. Previous work had shown that such a procedure stabilizes F in a matter of a few minutes (Nardini et al., 2001). The absolute leaf hydraulic conductance (K) was calculated as the slope of the F to P relationships ($K = F/\Delta P$ where ΔP is the pressure difference applied). Then, K was normalized by leaf dry mass (DW) to obtain leaf hydraulic conductance (K_{leaf}) on a mass basis ($K_{\text{leaf}} = K/DW$) and by leaf area (LA) to obtain K_{leaf} on an area basis ($K_{\text{leaf}} = K/LA$). All K_{leaf} measurements were performed on leaves at zero photosynthetic photon flux density (PPFD) to induce stomatal closure.

Stomatal conductance to water vapor (g_s) was measured using a portable photosynthesis system (model LI-6400, LI-COR, Lincoln, NE). Fifteen damaged and fifteen intact leaves from five trees per cultivar and condition (three leaves per tree). All measurements were done on fully developed sun-exposed leaves during mid-morning on sunny days at 400 ppm CO_2 , generated from a CO_2 cylinder connected to the LI-6400. The photon flux density (PPFD) was held constant at 1200 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ to ensure light saturation.

Leaf hydraulic vulnerability curves were developed by measuring K_{leaf} in full expanded leaves rehydrated from a range of initial Ψ_L . This K_{leaf} was measured by assessing the kinetics of Ψ_L relaxation upon leaf rehydration, as described by Brodribb and Holbrook (2003). Four branches per infested and non-infested tree from each cultivar ($n = 4$) were collected at predawn, on the same day, and immediately bagged to avoid water loss. The standard rehydration period varied between 15 and 45 s depending on dehydration leaf level. Gradual bench desiccation of excised shoots under laboratory light conditions (low irradiance) was used to provide a range of Ψ_L before being bagged to induce the stomatal closure and to ensure uniform Ψ_L across the leaf. For each sample, K_{leaf} was calculated as:

$$K_{\text{leaf}} = C \ln \left[\frac{\Psi_{L_i}}{\Psi_{L_f}} \right] / t$$

where C is the leaf capacitance, Ψ_{L_i} is Ψ_L before rehydration and Ψ_{L_f} is Ψ_L after rehydration. The mean of the two leaves samples was used as the K_{leaf} at a specified Ψ_L value.

2.4. Leaf water relations

To determine relative water content (RWC), eighteen new fully expanded were collected by cultivar and plant condition (6 trees and 3 leaves per tree) at midday (1300 h). Damaged leaves were collected from infested trees. The leaf RWC was determined as

$$\text{RWC} = 100 \times [(FW - DW) / (SW - DW)]$$

where FW is the leaf fresh mass, SW is the leaf saturated mass after rehydrating the leaves for 24 h and DW is the dry mass after drying the leaves in an oven at 70 °C until constant weight.

Leaf water potential ($\Psi_{L \text{ min}}$) was measured with a pressure chamber (PMS; Corvallis, OR, USA) on three damaged leaves per infested tree ($n = 3-6$) and three intact leaves per non-infested tree ($n = 3-6$) in each cultivar. Leaves were collected at midday (1300 h) and were immediately sealed in plastic bags upon excision and kept in a cooler until balancing pressures were obtained within 1 h of the excision.

Pressure–volume (P–V) curves were determined in fully developed and exposed leaves (3–6 trees per cultivar and condition, and three leaves per tree) in order to determine the osmotic potentials at zero turgor (π^0), full turgor (π^{100}) and leaf capacitance. Leaves were cut at the base of the long petioles in the field, re-cut immediately under water and the entire leaves covered with black plastic bags with the cut end kept under water for less 2 h until measurements began. After each determination of balancing pressure with the pressure chamber, the leaves were immediately weighed to the nearest 0.001 g, and between readings they were left to transpire freely on the laboratory bench. After all balancing pressure–weight measurements were obtained (down to about -4 MPa), the branches were oven-dried at 70 °C to a constant mass and weighed. Pressure values, fresh mass at each reading, saturated mass and dry mass for each leaf were used to determine π^0 , π^{100} and leaf capacitance. Leaf capacitance (C_{leaf}) was expressed in absolute terms and normalized by leaf area:

$$C_{\text{leaf}} = \Delta \text{RWC} / \Delta \Psi_L \times (DW/LA) \times (WW/DW) / M$$

where DW is leaf dry weight (g), LA is leaf area (m^2), WW is mass of leaf water at 100% RWC (g), and M is molar mass of water (g mol^{-1}).

2.5. Statistical analysis

All physiological and morphological trait data were normally distributed (Kolmogorov–Smirnov test). A one-way analysis of variance (ANOVA) was used to test statistical differences in the percentage of removed area per leaf among cultivars. Student's *t*-test was used for mean comparisons between non-infested and infested trees within a cultivar. Linear regression analysis was used for leaf traits data. All data was analyzed using the R software (Version 2.15.1).

3. Results

During the leaf expansion phase and before insect herbivory began (November), leaves of similar age from chronically infected and non-infected trees of Lapins and Van cultivars did not show differences in size, while leaves of infested Bing trees were larger than similar age leaves from non-infested Bing trees (Fig. 1a). At the end of the expansion phase (February), damaged leaves but active were substantially smaller than intact leaves from non-infested trees across all cultivars (Fig. 1b, Table 1), although the differences were not statistically significant in Lapins cultivar. To determine leaf size (cm^2) at the end of the expansion phase damaged

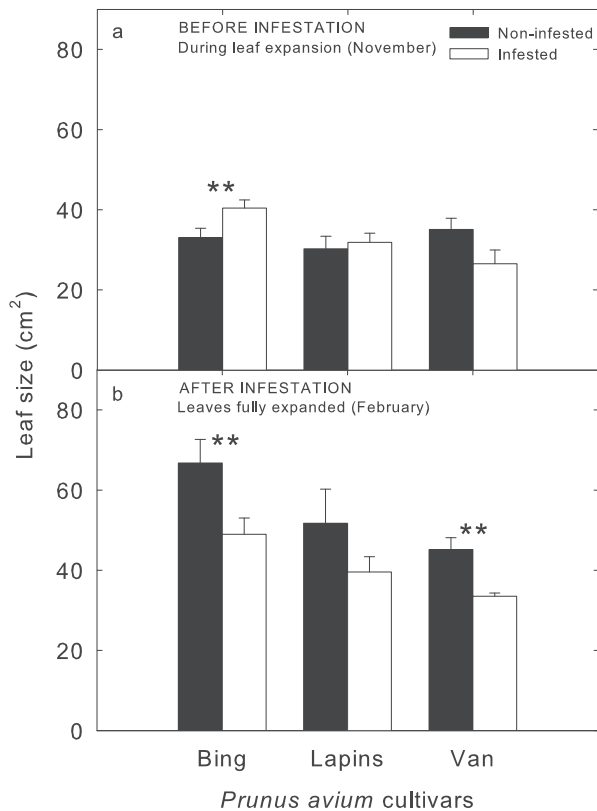


Fig. 1. Leaf size during (a) the early leaf expansion phase (November) in non-infested and infested trees (these last trees will be infested latter before full expansion), and (b) damaged fully expanded leaves from infested trees and intact leaves from non-infested trees (February). Significant differences between non-infested and infested plants for each cultivar are indicated as $^{**}P < 0.05$. Bars are means + SE of five to six trees infested and five to six non-infested trees per cultivar (20 leaves per tree).

Table 1
Leaf size, leaf dry mass per unit area (LMA), leaf vein density and diameter, midrib vessel density and diameter, the water potential at 50% of loss of hydraulic leaf conductance (P^{50}), in each non-infested and infested *Prunus avium* L. cultivars. In infested trees only results from damage leaves were included in the table. Values are means \pm SE ($n = 3-6$ trees per cultivar and plant condition). Significant differences between non-infested and infested plants are indicated with different letters ($P < 0.05$).

	Leaf size (cm ²)	LMA (g m ⁻²)	Leaf vein density (mm mm ⁻²)	Leaf vein diameter (μ m)	Midrib vessel density (vessel mm ⁻²)	Midrib vessel diameter (μ m)	P^{50} (MPa)
Bing							
Non-infested	66.7 \pm 5.9 ^a	89.5 \pm 7.7	7.2 \pm 0.4 ^a	32.9 \pm 1.5 ^a	2476 \pm 8 ^a	13.8 \pm 0.2 ^a	-1.25
Infested	49.0 \pm 4.1 ^b	111.1 \pm 6.5	5.1 \pm 0.3 ^b	43.4 \pm 2.3 ^b	1360 \pm 4 ^b	18.7 \pm 0.2 ^b	-1.43
Lapins							
Non-infested	51.7 \pm 8.5	84.9 \pm 4.9	5.1 \pm 0.1	43.3 \pm 2.5 ^a	1278 \pm 7 ^a	16.3 \pm 0.5 ^a	-0.54
Infested	39.6 \pm 3.8	103.2 \pm 6.6	5.4 \pm 0.2	36.9 \pm 1.6 ^b	1551 \pm 8 ^b	14.3 \pm 0.4 ^b	-1.29
Van							
Non-infested	45.1 \pm 3.0 ^a	73.0 \pm 6.3	4.9 \pm 0.1 ^a	33.50 \pm 1.55 ^a	1521 \pm 5 ^a	15.7 \pm 0.3	-1.02
Infested	33.5 \pm 0.8 ^b	84.7 \pm 5.4	5.7 \pm 0.2 ^b	43.65 \pm 3.14 ^b	1300 \pm 7 ^b	16.4 \pm 0.5	-0.86

leaves were considered as intact leaves (i.e. taken into account whole area within leaf perimeter). The insect infestation removed $21.16 \pm 4.51\%$, $22.90 \pm 4.00\%$ and $31.01 \pm 4.74\%$ of the leaf surface in Bing, Van and Lapins cultivars respectively.

Anatomical studies of damaged leaves showed that *C. cerasi* only removed soft tissues of the adaxial leaf surfaces maintaining intact the abaxial epidermis and the vascular system. Damaged leaves fed with Phloxine B dye showed red-stained veins on both intact and damaged leaf areas, similar to dye pattern observed in intact leaves (Fig. 2). Smaller veins also remained apparently functional but it is more difficult to observe in this image.

Leaf vein density was higher in infested Lapins and Van cultivars

compared to non-infested cultivars (Table 1). Otherwise indicated comparison will be done between intact leaves of non-infested trees and partially damaged leaves in chronically infested trees within each cultivar. Average leaf vein diameter was smaller in infested compared to non-infested Lapins cultivars while it was larger in the other two cultivars of infested trees (Table 1). Infested Lapins also exhibited higher midrib vessel density and lower size midrib diameters compared to non-infested trees (Table 1). Leaf dry mass per area (LMA) of damaged leaves was 16–24% higher than LMA of leaves from non-infested trees but the differences were no significant across all cultivars ($P > 0.05$) (Table 1).

Leaf hydraulic conductance (K_{leaf}) measured at predawn, on both mass and area basis, and leaf stomatal conductance (g_s) did not differ between infested and non-infested trees across cultivars (Fig. 3a–c). Results from leaf vulnerability curves of infested and non-infested trees (see leaf vulnerability curves in online supplement) indicated that leaf water potential (Ψ_L) at 50% loss of K_{leaf} (P^{50}) in Lapins cultivar decreased from -0.54 MPa in non-infested trees to -1.29 MPa in infested trees (Table 1).

Chronically infested trees tended to have lower midday leaf water potentials ($\Psi_{L\ min}$) compared to non-infested trees, particularly in Bing and Van cultivars (Fig. 4a). The $\Psi_{L\ min}$ was positively correlated to RWC across cultivars and degree of infestation (infested and non-infested trees) ($r^2 = 0.66$; $P < 0.05$; $y = 0.017x - 2.568$). Consistent with the difference in $\Psi_{L\ min}$, midday relative water content (RWC) was significantly lower in leaves of infested trees compared to leaves of non-infested trees (Fig. 4b). The osmotic potential at zero turgor (π^0) and full turgor (π^{100}) of leaves from infested Lapins trees were similar to leaves from non-infested trees; however infested trees of the Van cultivar had lower leaf π^0 and π^{100} than leaves from non-infested trees, and infested trees of the Bing cultivar had lower leaf π^0 compared to non-infested trees (Fig. 5a and b). Leaves of the Lapins cultivar had higher leaf capacitance (C_{leaf}) than leaves from non-infested trees,

while there were no significant differences in the other two cultivars (Fig. 5c).

Minimum leaf water potentials, π^0 and RWC were positively correlated to full expanded leaf size across infested and non-infested trees and across cultivars (Fig. 6a–c). Damaged leaves were smaller and exhibited lower $\Psi_{L\ min}$, π^0 and RWC than leaves from non-infested trees.

4. Discussion

Vein density is the primary determinant of water transport efficiency in leaves (e.g. Sack and Holbrook, 2006) since that xylem

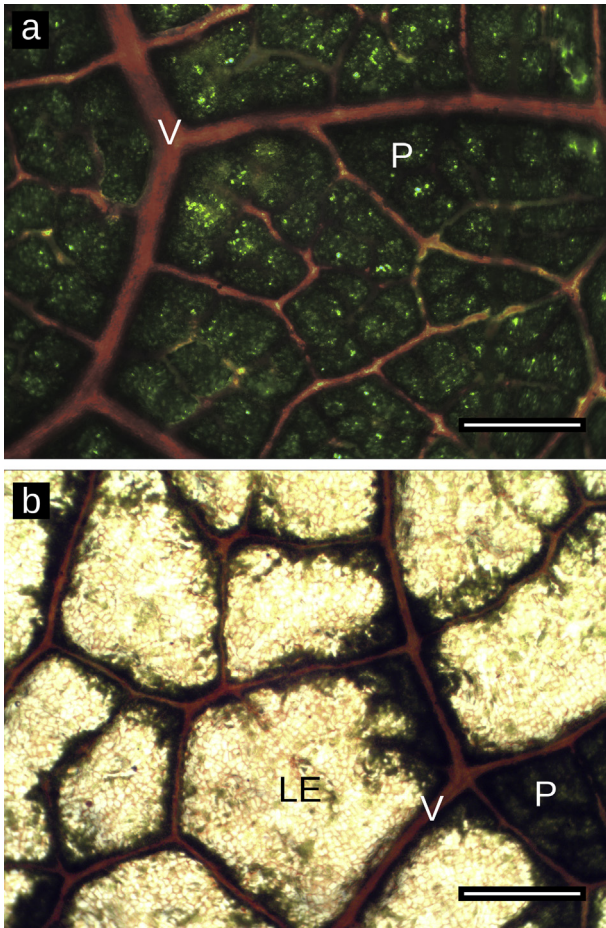


Fig. 2. Micrographs of *Prunus avium* leaf sections from (a) non-infested leaf and (b) infested leaf by *Caliroa cerasi*. Parenchyma (P), lower epidermis (LE) and red-stained network veins (V) in infiltrated leaf with Phloxine B. In panel b, white areas are the sections eaten by *C. cerasi* of adaxial face with lower epidermis intact (LE). Scale represents 0.5 mm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

conduits provide an efficient shortcut through the very resistive leaf mesophyll tissue (Brodribb, 2015). The slow hydraulic pathway through living tissue between the xylem and the stomata can be a source of variation in water transport properties among leaves of different species. Leaf sclereids and bundle sheath extension cells can help in water delivery to leaf tissues outside the xylem conduits (Brodribb, 2015). Removal of part of the mesophyll tissue by herbivore insects leaving the veins intact can be used as an experimental treatment to assess the relative effect of modifying the internal non-vascular pathways of water movement within the leaves on their physiological and morphological characteristics. Plants may respond to herbivory through various morphological, biochemical, and molecular mechanisms to counter/offset the effects of herbivore attack (War et al., 2012). In this study we thoroughly investigated anatomical and physiological changes related to leaf hydraulics elicited after insect attack. We strongly believe that the observed changes between infested and non-infested trees were in response to herbivory. We discharged the possibility of genotypic differences in the resistance or susceptibility to infestation due these changes occurred within each cultivar. Individuals within a cultivar have common genetic traits and identity and reproducibility are inherent features of cultivars (Raheel et al., 2015). In addition, we corroborated the lack of genetics differences in the resistance to infestation by *C. cerasi* because depending

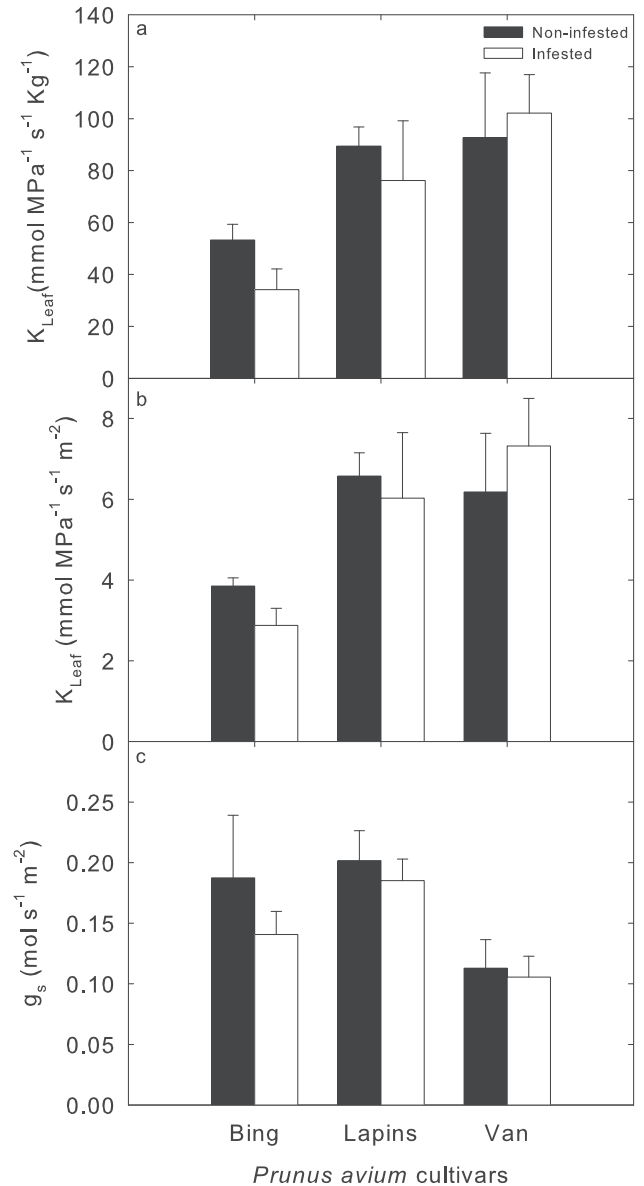


Fig. 3. (a) Predawn leaf hydraulic conductance (K_{leaf}) on a mass basis, (b) Predawn leaf hydraulic conductance (K_{leaf}) on an area basis, and (c) stomatal conductance of non-infested (black column) and infested (white column) trees from the three *Prunus avium* cultivars. Each column represents the mean value + SE of five trees three leaves per tree.

on the year some of the control plants were attacked by insects, which were removed from studies in the following year.

4.1. Herbivore damage and leaf size

The size of fully expanded leaves varied between chronically infested and non-infested *P. avium* trees, being the average area of fully expanded leaves a 25% smaller in infested trees compared to non-infested trees. This leaf size reduction observed in *P. avium* infested cultivars appears to be a common response to herbivory (Rautio et al., 2002; Huttunen, 2008). These trees have been infested by *C. cerasi* at least during the three previous years before the current analysis and in each year these trees can experience two or three events of infestation by *C. cerasi* (Bado, 2010). We assume that this chronic infestation during the period of expansion and the

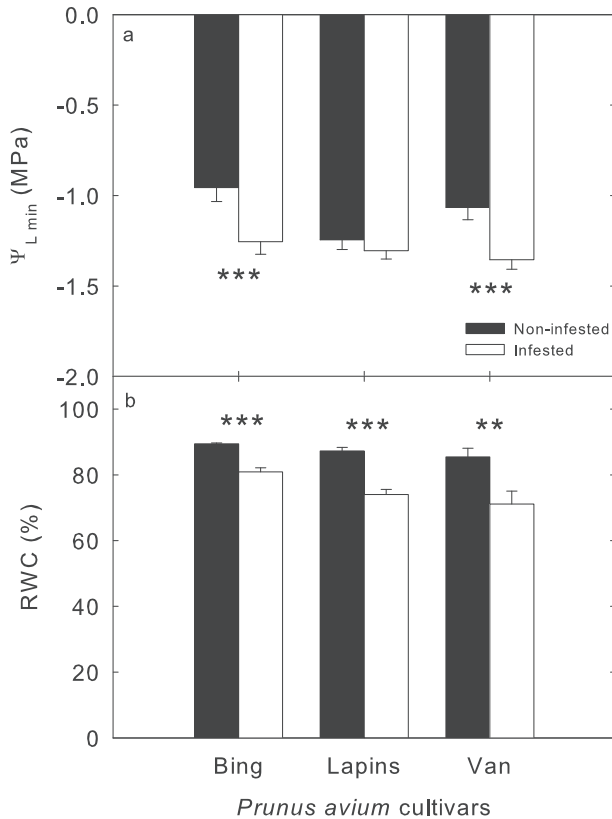


Fig. 4. (a) Minimum leaf water potential ($\Psi_{L\min}$) and (b) relative water content (RWC) for non-infested (black bar) and infested (white bar) trees of the three study *Prunus avium* cultivars. Each bar represents the mean value \pm SE from 3 to 6 trees and three leaves per tree. Significant differences between non-infested and infested plants are indicated as ** $p < 0.05$ and *** $p < 0.01$.

consequent drop in minimum leaf water potential observed in infested trees due in part to the water loss through extra vascular damaged tissue, resulted in smaller leaves than those of non-infested trees. Zhang et al. (2011) found that water deficit in leaves undergoing expansion reduces the leaf relative growth rate and leaf size because cell wall extensibility and turgor pressure threshold increase. This negative effect of water deficit on leaf size observed by Zhang and coauthors occurred even in the presence of osmotic adjustment, similar to findings in the present study in which Bing and Van cultivars exhibited lower π^0 in infested trees. An alternative explanation of this phenomenon may be related to permanent changes in leaf resources partitioning (higher production of compounds for structural and chemical defenses) in trees experiencing chronic defoliations. For example, damaged leaves of *P. avium* have higher amount of phenolic compounds than intact leaves (Peschiutta, 2015).

4.2. Effects of defoliation on leaf water relations

Studies assessing water loss after photosynthetic leaf surface removal by herbivores, have shown that stomatal conductance and transpiration are reduced (Pincebourde et al., 2006; Nability et al., 2009), increased (Quentin et al., 2011; Eyles et al., 2013), or maintained constant (Gálvez, 2012). Although both variables are generally correlated (Motzer et al., 2005) transpiration may be decoupled from stomatal conductance variations due to water loss from mesophyll tissue removal by the insects (Aldea et al., 2005). The non-stomatal water loss in infested *Prunus* cultivars could contribute to the decrease in RWC and in $\Psi_{L\min}$, while stomatal

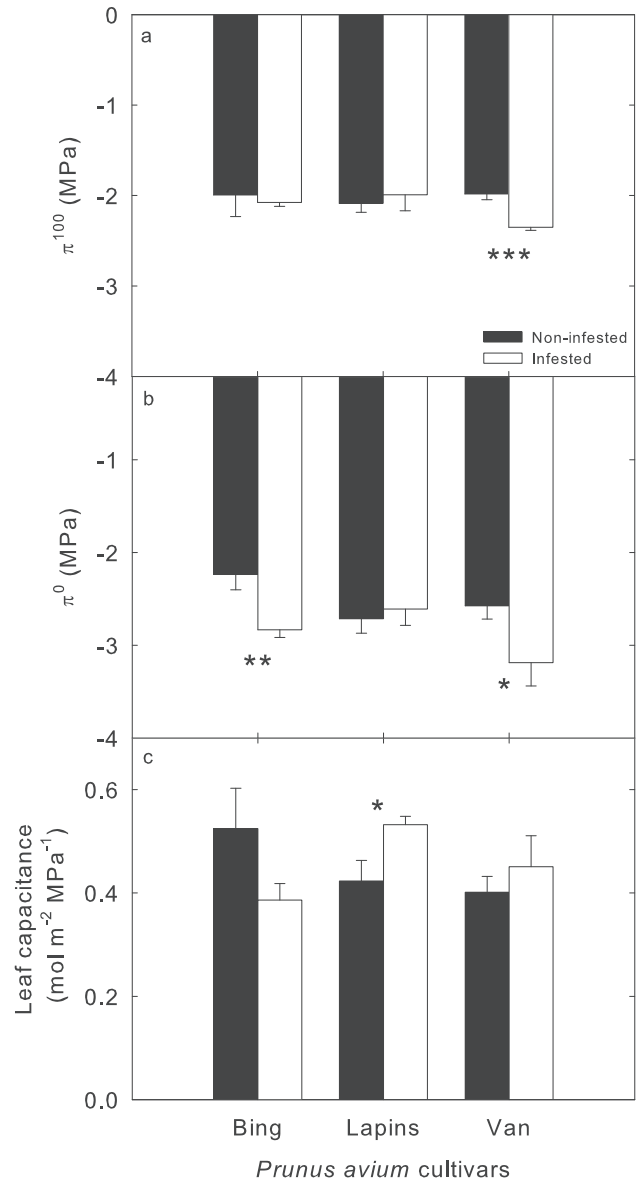


Fig. 5. (a) Osmotic potential at full turgor (π^{100}), (b) osmotic potential at the turgor loss point (π^0) and (c) leaf capacitance in the three *Prunus avium* cultivars. Black bars represent non-infested trees and white bar represent infested trees by *Caliroa cerasi*. Bars are means \pm SE from 3 to 6 trees depending on the cultivar (three leaves per tree). Significant differences between non-infested and infested plants are indicated as * $p < 0.1$, ** $p < 0.05$ and *** $p < 0.01$.

conductance remained constant. Increased vein density in damaged leaves may help to compensate the extravascular water loss and delivery of water to the stomatal cavities. Although it is expected that water loss from cut edges can be substantially reduced by lignifications of the wounded edges after feeding (Tang et al., 2006) avoiding leaf dehydration, in some species non-stomatal water loss is significant even after several days of the infestation (Aldea et al., 2005). This non-stomatal water loss may be substantially increased in *P. avium* due to the continuous attacks by new young *C. cerasi* larvae (up to three events per season), which delay the complete cicatrization of the damaged tissues by several days leading to higher dehydration. While leaves from non-infested trees start to senesce, damaged leaves seem dehydrated but remain attached to the trees during a longer period of time (Cittadini and San Martino, 2007).

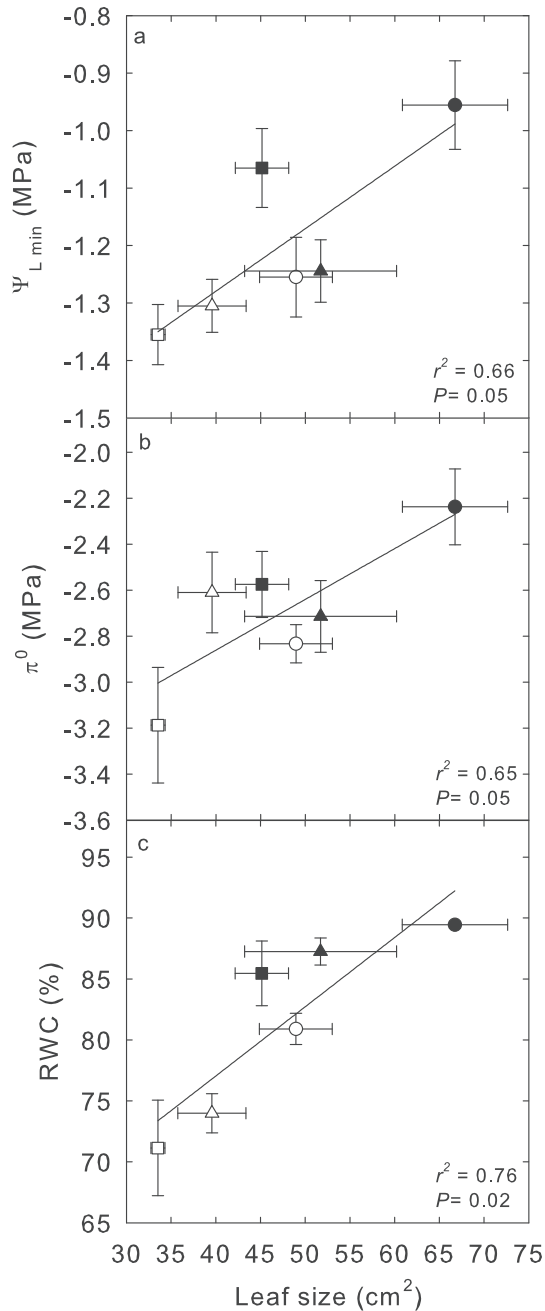


Fig. 6. (a) Minimum leaf water potential ($\Psi_{L\min}$), (b) osmotic potential at the turgor loss point (π^0) and (c) relative water content (RWC) in relation to leaf size. The lines are the linear regressions fitted to the data, (a) $y = 0.004x - 1.71$, (b) $y = 0.022x - 3.745$ and (c) $y = 1.35x - 61.88$. Values are means \pm SE of three to six infested (open symbols) and non-infested (closed symbols) trees within each cultivar (three leaves per tree). Cultivars: Bing (circles), Lapins (triangle), Van (square).

Although other tree species show homeostasis in $\Psi_{L\min}$ (Ohno et al., 2008; Gálvez, 2012; Quentin et al., 2012) or even increase in $\Psi_{L\min}$ after defoliation (Oren et al., 1999), our results, which are consistent with the findings of Eyles et al. (2013), show a negative effect of leaf surface removal on the tree water status. Plants that experience higher water deficits, such as infested Bing and Van trees, may avoid the turgor loss by incurring in a large energy costs associated with the increase in the amount of osmotically active solutes. However, osmotic adjustments observed in infested Bing and Van trees apparently were not involved in maintaining cell

turgor because $\Psi_{L\min}$ was about 1.5 MPa higher (less negative) than the osmotic potential at the turgor loss point (π^0) and thus cell turgor loss is unlikely to occur in all cultivars. We hypothesized that osmotic adjustment in defoliated trees of Van and Bing cultivars is an anti-herbivory response related to the production of chemical compounds to deter herbivores (Sharpe et al., 1986).

The cultivar with larger removal of leaf area (Lapins) exhibited an isohydric behavior, (i.e. the $\Psi_{L\min}$ of damaged leaves did not vary with respect to leaves from non infested trees). Mechanisms contributing to homeostasis of minimum water potential include stored water use, leaf shedding, high capacitance, partial stomatal closure, high hydraulic conductance or deep root systems (e.g. Jones, 1990; Saliendra et al., 1995; Bucci et al., 2004, 2013; Scholz et al., 2011; Bucci et al., 2013). In the case of Lapins cultivar a higher leaf capacitance could contribute to maintain its water status for a longer period of time.

4.3. Hydraulic changes in response to defoliation

Partial removal of the mesophyll in the three studied cultivars by *C. cerasi* did not affect its leaf hydraulic conductance since skeletonization leaves the veins relatively intact. Bundle sheath extensions observed in *P. avium* (Nikolopoulos et al., 2002) may help in water delivery outside the xylem conduits. Nardini et al. (2010) observed that leaf hydraulic conductance remained unchanged after extensive disruption of the palisade tissue by leaf miners in *Aesculus hippocastanum* suggesting that water flow bypasses this tissue in some way.

Changes in cell turgor of the extra-vascular tissue in *P. avium* trees might act together with the changes in density and size of vasculature to maintain constant K_{leaf} . Some studies have shown that lower π^0 results in decreased leaf hydraulic vulnerability (Blackman et al., 2010; Scoffoni et al., 2011; Nardini et al., 2012; Villagra et al., 2013) suggesting that leaf hydraulic dysfunction is more related with extra-xylary leaf tissues than to xylem anatomy. Changes in p^{50} between damaged and intact leaves were not observed in *P. avium* cultivars, despite changes in properties of tissues outside the xylem (e.g., π^0).

The increased vasculature (higher vein density) observed in Lapins and Van damaged leaves from *P. avium* infested cultivars suggests that herbivory elicit the development of leaves with a hydraulic system based on an expanded vein network. Larger vein density could increase xylem pathways and thus enhance water delivery to the mesophyll and epidermal tissue (Sack and Scoffoni, 2013). The increase in vein density only compensated the reduction in the water pathway outside the xylem. This compensatory response as a consequence of skeletonization may help to maintain delivery of water to the remaining leaf tissue. Independence of K_{leaf} from leaf vein density has been reported by Flexas et al. (2013) across diverse species.

Higher LMA observed in damaged leaves compared to leaves from non-infested trees of Lapins and Van cultivars could be the result of higher carbon investment in a denser vasculature such as was suggested by Blonder et al. (2011, 2014); but see Sack et al. (2013, 2014). K_{leaf} expressed on area basis ($K_{leaf\ area}$) is orthogonal (i.e. statistically independent) to traits related with carbon gain (Sack et al., 2003; Simonin et al., 2012); nevertheless it exhibits a strong and negative correlation with those traits when is expressed on mass basis ($K_{leaf\ mass}$) (Simonin et al., 2012; Nardini et al., 2012; Villagra et al., 2013). Consistent with these previous studies we found a relationship between LMA and $K_{leaf\ mass}$ ($K_{leaf\ mass} = -1.41 LMA + 202.93$; $r^2 = 0.56$, $P < 0.06$), which suggests that there is a carbon cost associated with water transport in infested *P. avium* trees as a result of compensatory changes due to herbivore damage. The increased leaf vein density has been suggested to confer

tolerance to mechanical damage (Sack et al., 2008) but also to embolism-induced blockage of the vascular pathway (Scoffoni et al., 2011), by providing alternative water flow pathways in a redundant network (Corson, 2010). Leaf hydraulic vulnerability decreased in defoliated trees of Lapins, which may be correlated also with smaller leaf vein as has been observed in this cultivar. Although this response to herbivory could imply a reduction in K_{leaf} , this reduction was counterbalanced by an increase in vein density as described in other species by Nardini et al. (2012).

In conclusion our results show that skeletonization of leaves in the expansion phase by *C. cerasi* resulted in morphologic and physiologic changes in *P. avium*, similar to responses to drought: smaller and more scleromorphic leaves (higher LMA), osmotic adjustments, and leaf xylem vessels of smaller diameter conferring more resistance to loss of leaf hydraulic conductance. Because the trees of all cultivars were growing under similar environmental conditions and were of similar age, the different responses reflect genetic differences among closely related plants of the same species. Infestation of the *Prunus* cultivars impacted the leaf size, decreased leaf water potentials and partially offset the effects of herbivore mesophyll removal with anatomical and physiological changes related to leaf hydraulics.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.actao.2016.03.005>.

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