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ORIGINAL PAPER

Leaf herbivory and calcium oxalate crystal production in *Prunus avium*

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Abstract

Plants require mechanisms of defense to limit the amount of damage by herbivores. Calcium oxalate crystals (COC) in the leaves can serve as inducible defenses against chewing insects, such as *Caliroa cerasi* larvae. We studied the relationship between leaf COC from *Prunus avium* and herbivory by *C. cerasi* larvae, to assess the defensive role of inducible responses. We examined from cafeteria choice experiments the *C. cerasi* larvae preference towards *P. avium* cultivars (Bing, Lapins and Van) and larvae preference towards leaves from infested and non-infested trees. The number of damaged leaves per meter of branch and the total non-damaged area and the area removed by the insects was evaluated in the field. We also determined the presence, location, size, and number of crystals in leaves from all studied cultivars. Van and Lapins cultivars were the most preferred by *C. cerasi* and exhibited a greater number of COC in their leaves, possibly due to being more eaten by the insect. This increase in COC possibly contributes to protect the leaf vein from chewing larvae, maintaining this portion of the vascular transport functionality. Potential manipulations of this induced response could be used in the future as a tool for the pest management against herbivore insects.

Keywords Calcium oxalate crystals · *Caliroa cerasi* · Plant defense · *Prunus avium* · Sweet cherry

Introduction

Inducible responses to herbivory are direct and indirect defenses that vary in plants following injury or stress and finally tend to reduce the performance and/or preference of herbivores (Aljbory and Chen 2018; Chen 2008; War et al. 2012). The amount of secondary metabolites and physical defenses of the damaged plant tissue may increase and/or its nutritional quality may decrease because of herbivore attack (Chen 2008; War et al. 2012). Once this type of defense occurs, inducible responses are assumed to have a defensive function due to their heavy effect on herbivore performance (e.g.,Chen 2008; Cornelissen and Fernandes 2001).

Calcium oxalate crystals (COC) can be an effective inducible defense against attacks by herbivores (Molano-Flores 2001; Nakata 2003; Xiang and Chen 2004). The COC occur quite commonly in the plant kingdom; they are found in over 200 plant families and distributed in the different organs such as roots, leaves, stems, seeds, and floral structures (Meric 2009; Nakata 2003). Vacuoles of cells called crystal idioblasts are specialized for crystal formation (Nakata 2003; Pelden and Meesawat 2019). In some plants, crystals accumulate in the vacuoles of other cell types such as storage parenchyma, mesophyll, and epidermal cells (Franceschi and Horner 1980). Physical, biological, and chemical conditions such as light, temperature, pH, ion concentration, and herbivory may affected the size, location, and other properties of the crystals in plants (Franceschi and Horner 1980; Kuo-Huang et al. 2007; Molano-Flores 2001). The COC form macro patterns in leaves and they appear to be important for *Prunus* systematics and also of significant

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interest for comparative plant anatomy (Lersten and Horner 2000). However, to date, knowledge of the function of COC is limited (Nakata 2003; Xiang and Chen 2004) and little is known regarding its relationship with herbivory (Molano-Flores 2001; Ruiz et al. 2002; Ward et al. 1997).

In this study, we investigated the relationship between leaf COC from commercial cultivars, *Prunus avium* and herbivory by *Caliroa cerasi* larvae, to assess the defensive role of induced responses. This species may serve as a good model system for the study of induced defenses, because *C. cerasi* herbivory occurs during a single period of the year. Lastly, we examined from cafeteria choice experiments the *C. cerasi* larvae preference towards *P. avium* cultivars (Bing, Lapins and Van).

Materials and methods

Location and study species

The study was carried out in February 2011 in El Porvenir Ranch located in Los Antiguos, Argentina (46° 19′ S, 71° 62´ W, altitude 220 m). At this site, several sweet cherry varieties are cultivated (San Martino and Manavella 2004).

Cherry slugworm, *C. cerasi* (Hymenoptera: Tenthredinidae), is one of the most significant pests of sweet cherries in the Northern Hemisphere and has migrated to many South American countries. Larvae feed on leaf mesophyll and maintain the larger leaf veins intact (Peschiutta et al. 2016). Window feeding is a way the larvae avoid the larger leaf veins and they rarely penetrate the abaxial leaf surface (Carl 1972; Naumann et al. 2002).

Lapins, Bing, and Van cultivars of sweet cherry trees (*P. avium* L.) were selected for the study. These plants are the most commonly cultivated in Southern Patagonia (Cittadini and San Martino 2007). The trees selected for the study were 7 years old as minimum, planted as free standing trees aligned in rows (280 trees ha^{-1}) and where irrigated by the traditional gravity method (Muñoz 2004).Three to nine infested and non-infested individuals for each cultivar were randomly chosen resulting in a total of 18–54 trees across all three cultivars depending of the variable measured. An initial grouping based on infested and non-infested trees was used to classify them, by the close monitoring of herbivory presence over a year period before this study began. The infested threshold was 50% of damaged leaves for the infested category, while the control group of non-infested trees had less than 1% of damage (Peschiutta et al. 2018a). All trees where close to each other, so in practice all of them were growing under similar environmental conditions over time. The sampling method consisted in collecting leaves from both groups the same day and from plants of similar age.

Field consumption

Images of 20 full expanded fresh leaves from infested and non-infested trees were acquired using a scanner. The images were analyzed for leaf size using the ImageJ 1.47 k software. The total non-damaged area and the area removed by the insects were also determined in each leaf using the same program. The number of damaged leaves per meter of branch and the number of *C. cerasi* larvae per leaf from infested trees were estimated.

Herbivore preference

Stationary olfactometers (olfactory responses) were used to quantify preference of *C. cerasi* larvae by different cultivars. Olfactometers were built with Petri dishes of 9.5 cm diameter, which was offered to each insect two different stimuli (leaf Sects. 2 cm^2). Larvae were placed in the middle of the Petri dish and allowed to selectively migrate into the food. Dual tests of selection for different cultivars were: (1) Van vs Lapins, (2) Van vs Bing, and (3) Bing vs Lapins (15 replicates per test). All leaves used in this experiment were fully intact and from non-infested trees. Also a dual test was performed to evaluate the existence of differences in preference between leaves from infested plants and leaves from non-infested plants (55 replicates). All leaves were kept in sealed bags at 4–5 °C until processed the same day that they were harvested. Herbivores used for the experiment were collected from the same sampling site and were starved for 24 h before the experiment. Pretrial starvation is considered important in cafeteria experiments, because they avoid biased results due to preconditioning (Pérez-Harguindeguy et al. 2003). All experiments were performed under standard conditions of constant temperature (25 °C) and similar irradiance. In addition moistened filter paper was introduced to Petri dish in order to maintain constant leaf fragment quality throughout the time that the tests lasted.

Determination of crystals content in leaves

Leaf from infested and non-infested trees were cleared following the methods of Bailey and Nast (D'Ambrogio de Argüeso 1986) to determine the presence, location, size, and number of crystals. Fresh material was placed in 3% NaOH to prevent evaporation and placed at 55 °C oven until the material remained completely transparent. Crystal density was defined as number of crystals/area counted and crystal size were determined in 2–3 fields of the middle region from leaf abaxial side using a light microscope (Zeiss Axioplan, Germany). Solubility tests following Molano-Flores 2001 were conducted to determine the chemical composition of the crystals. Leaf Sects. (25 cm^2) were immersed in one of the following reagents: 1, 2, and 5% acetic acid, 10% hydrochloric acid, 70% ethanol, 3% nitric acid, 4% sodium hydroxide, and 4% sulfuric acid. The COC are not soluble in 1, 2, or 5% acetic acid, 70% ethanol, or 4% sodium hydroxide. However, they are soluble in 4% sulfuric acid, 3% nitric acid, and 10% hydrochloric acid.

Statistical analysis

All data were analyzed to assess normality using the Shapiro-Wilks test and homogeneity of variances using the Levene test before performing an ANOVA. When data were not normal, a nonparametric test was applied as the Kruskal Wallis or the Chi-square test. Student *t* test was used to compare means of leaves from infested and non-infested trees within a cultivar. All data were analyzed using the R software (version 3.6.3).

Results

Olfactory response tests showed a greater selection of *C. cerasi* larvae towards Van cultivar when intact leaves from non-infested trees were used (Fig. 1). Van cultivar was more preferred than Bing ($\chi^2 = 4.76$, df = 1, $P < 0.05$) and tended to be more preferred than Lapins ($\chi^2 = 1.67$, df = 1, $P > 0.05$), while Lapins cultivar tended to be less preferred than Bing ($\chi^2 = 0.69$, df = 1, $P > 0.05$; Fig. 1). Also, we found that intact leaves from non-infected trees were more preferred than damaged leaves from infested trees (65.45% and 34.55%, respectively; $\chi^2 = 5.25$, df = 1, $P < 0.05$), independent of cultivar.

In the field, Lapins cultivar tended to exhibit larger percentage of damaged leaves per meter of branch than any of the other two cultivars, reaching up to 74% of affected leaves (F = 1.78, $P = 0.22$; Fig. 2a). Lapins and Van cultivar had larger percentage of damaged area per leaf (F = 12.18, $P < 0.01$; Fig. 2b). But, the number of insects per leaf did not differ among cultivars, with a mean of about two larvae per leaf (H = 0.35, n = 5, $P = 0.86$) (data no shown).

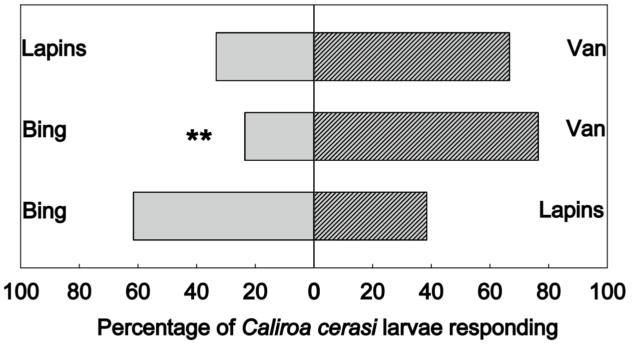

Fig. 1 Olfactory responses of *Caliroa cerasi* larvae towards intact leaves from non-infested Bing, Lapins, and Van cultivars. The bars indicate larvae percentage that made a choice for one of the two odor sources that were offered simultaneously (n = 15). Asterisks indicate significant differences ($P < 0.05$) between the choice tests (χ^2 test)

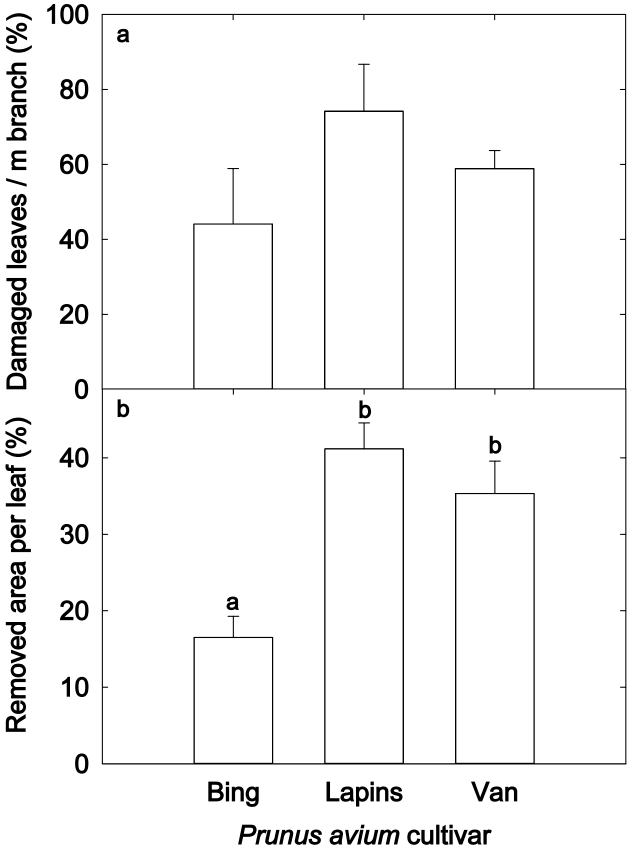

Fig. 2 **a** Percentage of damaged leaves by *Caliroa cerasi* per meter of branch (damaged leaves/ m branch) and **b** percentage of removed area per leaf from each of the three *Prunus avium* cultivars. Bars represent mean + SE (n = 3–8 trees per cultivar) from infested trees. Different letters between bars indicate significant differences ($P < 0.01$; Fisher's LSD test)

Changes in foliar calcium oxalate crystals induced by Caliroa cerasi larvae

The crystals found in *P. avium* were a mixture of prisms and druses. All crystals of *P. avium* leaves were made of calcium oxalate (COC). They were found associated with veins and were usually placed in straight lines (Fig. 3). Number of COC was significantly higher in damaged leaves from infested trees in relation to intact leaves from non-infested trees in all cultivars. In Lapins cultivar, for example, COC of leaves from infested trees was 92.2% higher in relation to leaves from non-infested trees (Fig. 4a). Bing cultivar had fewer COC than Van cultivar, both in intact leaves from non-infested trees and in damaged leaves from infested

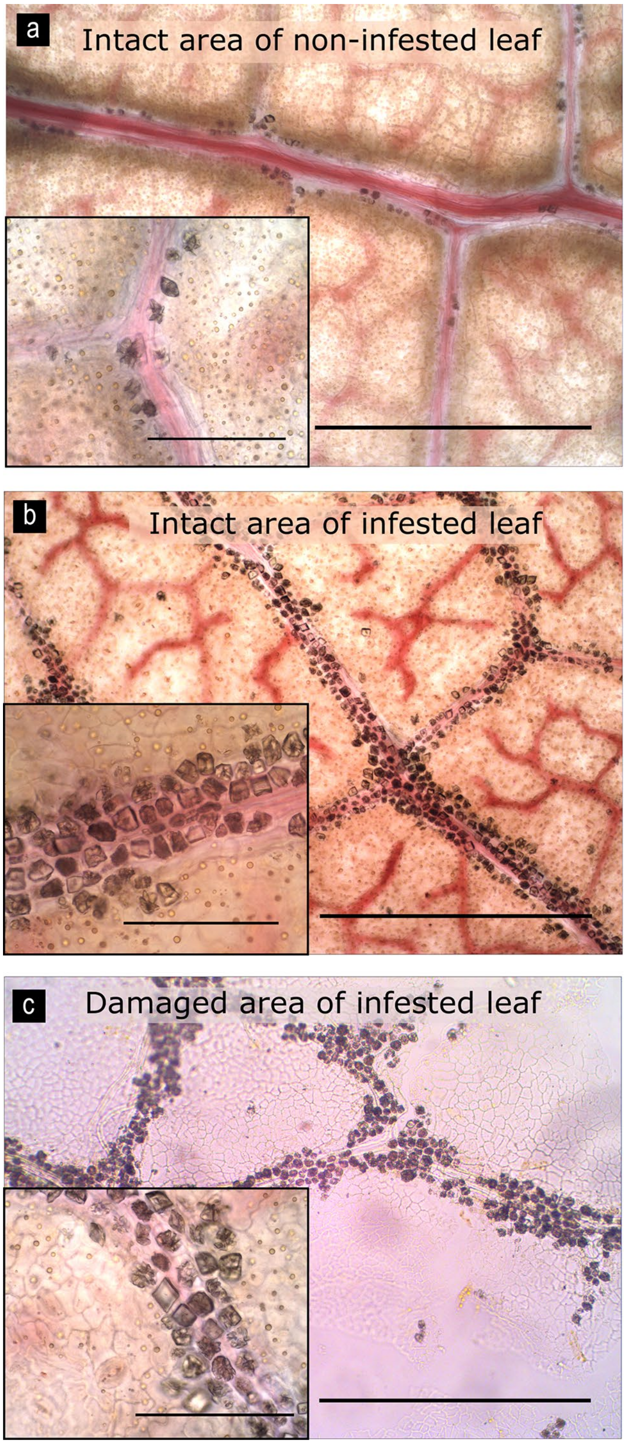

Fig. 3 Presence of calcium oxalate crystals (COC) in cleared leaves of *Prunus avium*. The inserts shown prism and druse images with more detail and larger scale **a** intact area of non-infected leaf where few COC were observed, **b** intact area of infected leaf showing COC bordering veins, and **c** portion of leaves eaten from infested plants in the area of damage where the insects after feeding only leave the veins intact with the COC. The scale bar represents 0.5 mm (**a**, **b** and **c**) and 0.1 mm (all inserts)

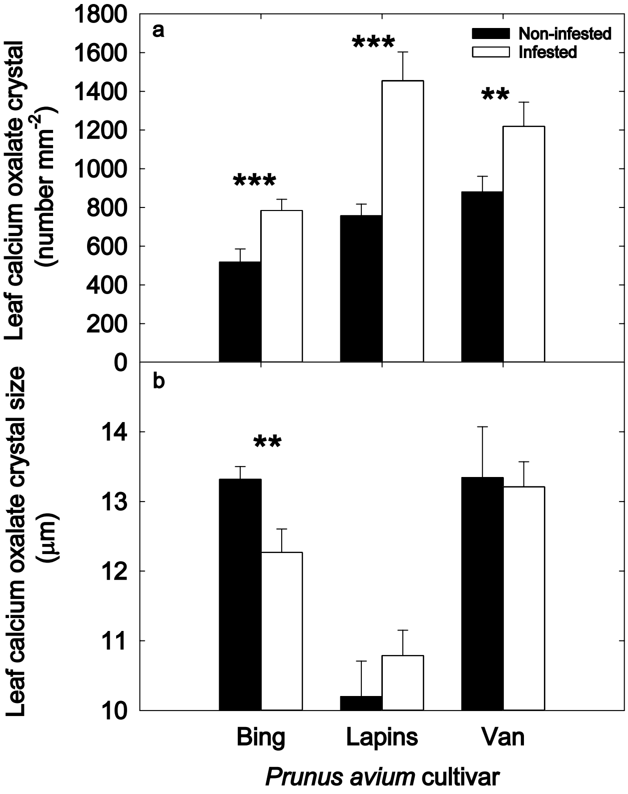

Fig. 4 **a** Number of COC per mm^2 of blade surface, **b** COC size from intact leaves from non-infested trees (black bars) and damaged leaves (white bars) from infested trees of three *Prunus avium* cultivars. Bars are mean + SE (n = 3–4 trees per cultivar) from non-infested trees and infested trees. Significant differences between infested and non-infested trees are indicated as ** $P < 0.05$ and *** $P < 0.01$

trees (F = 5.47, $P < 0.01$ and F = 7.50, $P < 0.01$, respectively). These crystals were similar in size in both infested and non-infested trees in Van and Lapins cultivars, while infested trees from Bing cultivar had smaller COC than non-infested trees (Fig. 4b). Also, Lapins cultivar had smaller crystals than the other cultivars both in intact leaves from non-infested trees and in damaged leaves from infested trees (F = 8.73, $P < 0.01$ and F = 12.40, $P < 0.01$, respectively).

Discussion

Evolutionary interactions between plants and herbivores have resulted in an extraordinary variety of adaptations, and herbivory pressure has led to the evolution of phenological, chemical, and mechanical defenses in plants (Coley 1983; Hahn et al. 2019; Maron et al. 2019). This work suggests that *P. avium* foliar resistance to herbivory may substantially be influenced by the feeding of *C. cerasi* larvae. Many plants are able to perceive touch and respond with induction defense that may affect the insect selection and the behavior of natural enemies (Markovic et al. 2014). We found that intact leaves from non-infested Van and Lapins trees were the most preferred by *C. cerasi* larvae in relation to Bing leaves. These leaves were the least chosen possibly because they are nutritionally poor, with more scleromorphic and hard blades (Peschiutta et al. 2013, 2018b). Leaves from non-infested Bing trees also had less COC than the leaves of the other cultivars studied, possibly related to their lower selection by the herbivore. In the same way, infested Bing cultivar showed fewer removed area per leaf than infested Van cultivar; thus, leaves from infested Bing cultivar had fewer COC than infested Van cultivar ones.

We found that *C. cerasi* preferred intact leaves of non-infested trees over the damaged leaves from infested trees independent of cultivar probably because damaged plants increased their resistance to pests (Chehab et al. 2012). This could repel partially larvae and, therefore, reduce the feeding potential damage. Damaged leaves of *P. avium* had higher amount of COC than leaves from non-infested trees. For example, *Spodoptera exigua*, has preference for the COC-deficient mutants of *Medicago truncatula* in relation to wildtype plants (with COC)(Doege 2003). Nevertheless, in that study Doege (2003) found that younger *S. exigua* larvae tend to feed around the secondary veins from *M. truncatula*, normally avoiding the tissue that contains the COC. Because of this feeding pattern, it is likely that these larvae would have no preference for genotypes with or without COC because they would not be ingesting large amounts of this insoluble mineral. COC have an abrasive effect on insect mandibles that suggest that calcium oxalate acts, mainly, by a physical means to deter insect chewing (Korth et al. 2006) and it could be related to the protection of its veins. *Prunus* species commonly have druses and prisms of COC around all leaf veins (Lersten and Horner 2000) and in this study the three *P. avium* cultivars exhibited a significant increase in the number of COC along the veins from affected leaves. This could provide significant protection to them against the action of herbivores. Consistent with the protective role of COC on veins, in a previous study we observed that damaged leaves maintained their leaf hydraulic conductance at similar levels than intact leaves from non-infested trees (Peschiutta et al. 2016). These COC associated with veins, also could be responsible for the "skeleton appearance" observed in the eaten leaves from infested trees (Raffa and Lintereur 1988). These patterns of larval feeding are consistent with the hypothesis of selective feeding, which predicts herbivore selects only the most nutritious sites or with minor anti-herbivory defenses (Scheirs et al. 2001). The COC represents an underappreciated form of effective induced defense plant that prevents future feeding of the insect and gives immediate protection to leaf veins (Korth et al. 2006).

COC can be considered an inducible defense in *Prunus* because its amount is enhanced with the herbivore attack. Inducible defenses may confer an advantage over constitutive defenses because it makes the plant a more unpredictable environment for insect herbivores. In conclution, Van cultivar was the most selected by *C. cerasi,* showed more removed area per leaf and exhibited a greater number of COC in their leaves than Bing cultivar. This increase in COC contribute to protect the leaf veins of chewing larvae, maintaining their hydraulic functionality. Although few studies have provided evidence of COC effects on herbivorous insect and mammalian (Molano-Flores 2001; Ruiz et al. 2002; Ward et al. 1997), recent studies suggest that the amount and spatial distribution of COC formation can be manipulate (Nakata 2003). Thus, induced resistance could have a great potential in agriculture as a management tool to minimize the damages caused by herbivore insects.

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Author contributions MLP, FGS, and GG conceived and designed the experiments. MLP performed the experiments. MLP, FGS, and SJB analyzed the data. MLP, FGS, SJB, and GG wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors also declare that there is no conflicts of interest.

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