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ORIGINAL PAPER

Young leaf protection in the shrub *Leea glabra* in south-west China: the role of extrafloral nectaries and ants

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Abstract Field experiments on *Leea glabra* in its natural forest habitat of southern Yunnan, China were conducted to study the effects of artificial damage of young and old leaves on extrafloral nectaries (EFNs) secretion quantity and sugar concentration, as well as the effects on ant abundance on the plants following the damage treatments. We found there were no rapid changes in extrafloral nectar volume or nectar sugar concentration which would indicate an induced reaction following artificial damage. However, both cutting and punching of young leaves resulted in a significant increase (2–4-fold) of ants within 6 h after damage compared to undamaged controls. In another experiment, disks of fresh young *L. glabra* leaves that were pinned on young leaves of another *L.glabra* plant also resulted in a significant increase in the number of ants compared to treatment with paper disks, indicating that ants were most probably attracted by volatile organic compounds (VOCs) released from damaged young leaves. Furthermore, we found that portion of damaged leaf area of young leaves was significantly lower than that of old leaves and the concentration of tannins was significantly higher in young than in medium and old leaves. In conclusion, our results show that young leaves of *L. glabra* are protected against attacks by herbivores by multiple mechanisms, which include: (1) the activity of EFNs, which attract different ant species from the surrounding ground; (2) a mechanism induced by the damage of young leaves, which leads to rapidly increased ant recruitment and is most probably caused by the release of volatiles from damaged leaf and (3) a higher allocation of tannins in young than in older leaves.

Keywords Ant–plant interactions · *Leea glabra* · Plant cues · Simulated herbivory · Tannin · VOCs

Introduction

Extrafloral nectaries (EFNs) are found in more than 90 plant families and about 300 genera. They have a high diversity of shapes, patterns of distribution on plants and secretion products (Koptur 1992). EFNs are generally considered as structures established to attract defending arthropods by providing nectar as a food resource, especially for ants (Heil 2008). Based on a meta-analysis of ant–plant protection mutualisms, Chamberlain and Holland (2009) concluded that ant effects on plants are routinely positive for plants, and only occasionally neutral. The most pronounced effects of ants as biotic defenses are from tropical systems and for true myrmecophytic plants, even though ants consume extrafloral nectar when no herbivores are present, decreasing plant fitness (Rosumek et al. 2009).

An interesting question is whether the quantity or quality of extrafloral nectar production can be increased or improved following damage in order to increase patrolling by defending ants. This issue was reviewed by Agrawal and Rutter (1998) who showed an increase in extrafloral nectar volume and the number of EFNs in different EFN-plant species after artificial plant damage, indicating a mechanism of induced defense. However, there are also

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studies which showed no significant effects of damage on nectar production or quality (Koptur 1989; Smith et al. 1990). Other studies demonstrated that ants recruit specifically to damaged leaves and respond rapidly to herbivores in obligate ant–plant systems (Agrawal 1998a; Agrawal and Dubin-Thaler 1999; Grangier et al. 2008; Romero and Izzo 2004), but the potential cues for the rapid recruitment of ants were not always clear due to the few studies that measured both ant activity in response to damage and nectar flow in the field.

Furthermore, a question remains as to whether constitutive or induced defense mechanisms differ between plant tissues in relation to the value of the tissue for the plant, i.e., whether plants invest more defenses in higher value parts (e.g., young leaves) than in those of lower value (e.g., old leaves). Such conditions are discussed in the optimal defense hypothesis, with the basic assumption that defense is costly for plants and therefore deployment among tissues is directly related to their value and likelihood of herbivore attack (McKey 1979; Rhoades 1979).

In addition to the attraction of defending arthropods to deter herbivory, many plant species with EFNs employ a diverse array of other chemical and physical defenses (Agrawal and Rutter 1998), especially the production of chemical compounds such as tannins and phenolics (Coley 1986). Young leaves of various plant species are often endowed with higher concentrations of secondary metabolites which form a combined defensive strategy for the adaptation of selective pressure from different herbivore communities (Coley et al. 2005; Raupp and Denno 1983).

The present study deals with the shrub *Leea glabra* and the defensive function of the EFNs found at leaf petioles in relation to their interactions with ants under different conditions. Field experiments with naturally grown *L. glabra* plants were conducted in a tropical forest plot of southern Yunnan (south-west China) to answer the following major questions: (1) is the quantity of EFN secretions and the sugar concentration of the nectar in artificially damaged young leaves higher than in old leaves? (2) Are ant abundances higher after damage on young leaves than damage on old leaves? If so, is ant recruitment related to changes in EFN production or to the release of VOCs after artificial damage? (3) Are there differences in tannin concentrations in leaves of different ages, indicating tannins are an additional mechanism of herbivore defense?

Methods

Study site

The study area is located in the Naban River Watershed National Nature Reserve (NRWNNR) within the Dai autonomous prefecture of Xishuangbanna, southern Yunnan Province, south-west China (22°10′N, 100°38′E). The climate is humid northern marginal tropical monsoonal with three distinct seasons: cool-dry (October–January), hot-dry (February–May) and a rainy season (June–September) when most of the mean annual precipitation of almost 1,600 mm occurs.

The specific study site was a middle-slope forest area located at about 900 m asl. It is a disturbed fragment of a lower hill tropical seasonal rainforest with a maximum tree height of about 30 m. The forest structure is characterized by two to three strata, and *Pometia tomentosa*, *Bauhinia variegata* and *Kydia calycina* are the dominant tree species. The understorey is characterized by *Litsea monopetala* and the herb *Curculigo capitulata* together with the seedlings of canopy tree species. All plant samples observed in this study were growing under roughly uniform soil and light conditions.

Study species

Leea glabra C. L. Li (Leeaceae: Li 1996) is an erect shrub species with a height of 1.5–3.5 m and is one of about 70 *Leea* species. It is native to the southern parts of Yunnan and Guangxi Provinces of south-west China and is mainly distributed in the lower layer of forests up to 1,200 m asl. (Flora of China, eFloras.org). In our study site, about 500 *L. glabra* plants were distributed over an area of 5 ha.

According to our field observations on *L. glabra* in the study site over a period of one year (May 2008–April 2009), the plants produce flowers only once per year between April and May before the rainy season. Fruits are produced in August at the end of the rainy season. Production of new leaves was observed throughout the year on branches already bearing old leaves. Single units of extrafloral nectaries (EFNs) of four to six parallel strips not exceeding 1 cm in length are developed on the stem on the opposite side of the buds of new leaves or flowers. When the young leaves are mature and change their color from red to green, the EFNs become inactive and stop nectar secretion. It generally takes young leaves almost four weeks to mature. A lepidopteran larva is the major herbivore of mature leaves, on which it produces many clear holes in parallel strips.

We observed about 10 species of ants assembling at the EFNs with *Camponotus singularis, Oecophylla smaragdina* and *Crematogaster rothneyi* being the most common, but usually there was only one ant species present at a time on one plant. The ants do no nest on the plant, but visit the plant from the surrounding ground, patrolling the leaves and consuming nectar from the EFNs. Ants were never found on inflorescences during the flowering period of about five days.

Experiment 1. EFN measurements

To test the effects of simulated herbivore damage on EFN production and sugar content, field experiments were conducted in April 2009 before the flowering period. A total of 20 plants that were almost three years old and approximately 2.5 m tall were selected randomly for experiments. All plants were individually enclosed in white nylon nets (mesh size 0.5 mm), supported by woody sticks to avoid contact with the plant: this was done to protect plants from herbivore attacks. Each selected experimental plant stood at least 10 m apart from the next. Because all the experimental plants were covered from the top down but the nylon net did not touch the ground, it should not have influenced ant activity.

To study the effects of different types of herbivore damage on extrafloral nectar quantity and sugar concentration, we surrounded the active EFNs with grease on one young or one old leaf with no signs of previous herbivore damage, and artificially damaged the leaves by either cutting or punching. Cutting was conducted by removing the distal half (50%) of each leaflet using scissors. Punching was conducted by removing 50% of the each leaflet area over the whole leaf surface using a 3-mm-diameter hole-punch. These methods of artificial leaf damage were adapted from Pulice and Packer (2008). The two damage methods mimic the most common natural damage form found on the plants. In total, the following treatments to net-covered plants were conducted: (1) cutting young leaves; (2) cutting old leaves; (3) punching young leaves and (4) punching old leaves. Each treatment was applied to four plants, and four undamaged net-covered plants served as controls.

The young leaves used for damage experiments were two weeks past leaf bud development, and old leaves were six weeks past the leaf color change from red to dark green. The old leaves selected for treatments were adjacent to a young leaf, sharing the same branch with the EFNs selected for nectar measurements. Quantitative nectar measurements after damage by cutting or punching were conducted in the following way: the total volume of nectar produced by the EFN units of the branch was collected using micro-capillaries with a minimum scale of 0.5 μL (Abel Industries Inc., USA). Nectar was drawn into the micro-capillary tubes by means of capillary action. In addition to nectar quantity, the sugar concentration in Brix units was measured by a refractometer (B + S Instruments, UK). The method used to measure the sugar concentration within nectar was adapted from Heil et al. (2000). The grease did not interfere with the nectar measurements. Measurements were conducted prior to damage, then 10 min after damage, and followed by 10 further records at time intervals of 6 h for a total of 12 records within a period of almost 60 h. The same measurements were also made on the four control plants on the same days.

Experiment 2. Ant behavior

Effects of simulated herbivore damage on ant recruitment to young *L. glabra* leaves were analyzed in June 2010 by counting numbers of ants on artificially damaged and undamaged young and old leaves of *L. glabra*. The following treatments were conducted on selected plants with both young and old leaves present and without signs of recent herbivore damage: young leaves of 20 plants were damaged by removing the distal half of a leaf by cutting, and 20 plants were damaged by removing half of the leaf area by hole punching, as described for the previous experiment. The same two types of damage were also applied to old leaves using 20 separate plants each. Each selected plant stood at least 10 m apart from the next. A total of 20 undamaged plants located 50 m from the plot of damaged plants were used as controls for the respective treatments. All plants observed were of a similar age, almost three years old. Numbers of ants on the leaves of the experimental and the control plants were counted prior to treatment, and 10 min, 6 and 24 h after treatment. All treatment and control plants were monitored during the same period, so there would not be temporal differences in ant activity.

To look for an indication that ants might be attracted by VOCs released by wounded plants (Agrawal and Dubin-Thaler 1999), a further experiment was conducted: six disks of freshly cut young *L. glabra* leaves of 6 cm^2 each were pinned randomly on leaflets of one young undamaged compound leaf of another *L. glabra* plant. Leaf disks were from another three-year-old plant. These treatments were conducted on 20 plants, and another 20 control plants were located in another plot which stood 50 m from the plot of treatment plants. The controls were treated with paper disks applied in the same way.

Experiment 3. Tannin and leaf damage measurements

To test for a biochemical difference between leaves of different development stages, leaf samples of three age categories were collected and analyzed for tannin concentration: (1) young leaves, (2 week old); (2) medium-aged leaves (4 week old) and (3) old leaves (8 week old). The three categories of leaves were collected from the same plant at same time from a total of 12 plants randomly selected. Damage to leaves at different development stages was measured using graph paper in the field to calculate the damaged leaf area ratio of leaves at different ages. All leave samples used were fully expanded at the time of measurement.

Total tannin concentrations were then determined colorimetrically by spectrophotometric techniques in the laboratory. The Folin–Ciocalteau (Laborclin) method was used to determine the tannin content of the leaf samples. First, calibration curves were made with different concentrations of the pyrogallol standard. Then, a water coloration reagent (Folin–Ciocalteau) and sodium carbonate solution were proportionally added to a prepared extract solution of crushed leaf powder in ethanol. Finally, the blended solution was shaken and its optical density measured at 760 nm after coloration for 30 min.

Statistical analysis

All data were analyzed using SPSS statistical software 13.0 version (SPSS Inc. 2004). To ensure that there were no differences among the treatment groups of plants prior to damage, initial plant height and number of leaf twig layers of plants used for the EFN production and sugar concentration measurements were compared with one-way ANOVA. Repeated measures ANOVA procedures were used to compare EFN production and sugar concentration between different treatments and controls over time. In these procedures, time was considered as the factor of repetition, and treatments and plants were considered fixed and random effects, respectively. Then *post hoc* tests were used for the paired comparison between different treatments on young and old leaf with a Bonferroni correction. Differences among the number of ants after treatments were analyzed by repeated measures ANOVA followed by Dunnett's contrast with a Bonferroni correction also. To account for violations of the sphericity assumption of variances, the Greenhouse–Geisser (*G–G*) and Huynh–Feldt (*H–F*) correction was applied to the degrees of freedom based on the ε value which is more or less than 0.75 produced by Mauchly's test before (Zar 1996). Tannin concentrations and the damaged leaf area ratio of leaves at different ages were compared using MANOVA with leaf age as the independent variable and the plant was included as a random factor.

Results

EFNs and sugar

Nectar production did not differ significantly between the four different treatments and controls within 60 h after simulated damage (Fig. 1a; repeated measures ANOVA: $F_{4,\ 15} = 0.971$, $P = 0.452$). No significant effects on EFN production over time were found in comparison to control plants after the *G–G* correction (Fig. 1a; repeated measures ANOVA: $F_{3.13,\ 46.90} = 2.73$, $P = 0.052$). Plants differed within the young leaf cutting treatment (Fig. 1a; $P < 0.01$). However, this among plant variation was not observed within the three other types of treatment (Fig. 1a; $P > 0.05$). There was no significant effect of leaf age on nectar production for either damage treatment (paired comparison with a Bonferroni correction; Fig. 1a; $P > 0.05$).

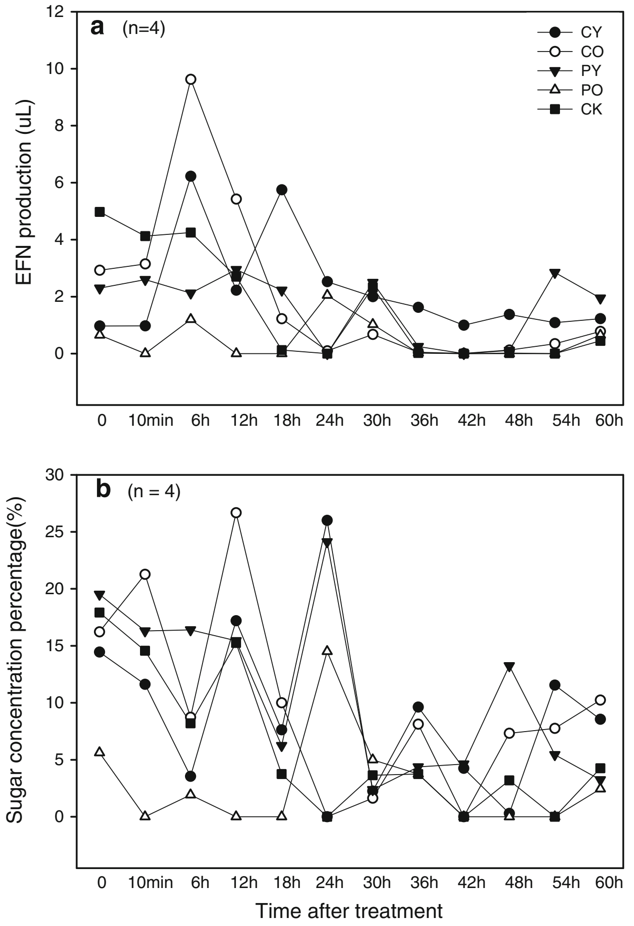

Fig. 1 Mean values of EFN production (**a**) and sugar concentration percentage (**b**) within nectar after damage treatment on young and old *L. glabra* leaves by cutting and punching at different times compared to undamaged control leaves. t_0 corresponds to the values immediately prior to damage (CY, CO, PY, PO and CK represent four treatments of cutting young leaves, cutting old leaves, punching young leaves, punching old leaves and control, respectively, and it is same with Fig. 2)

Sugar concentrations did not differ significantly between the four treatments and controls within 60 h after simulated damage (Fig. 1b; repeated measures ANOVA: $F_{4,\ 15} = 1.26$, $P = 0.273$). No significant effects on sugar concentration over time were found in comparison to control plants after the *G–G* correction (Fig. 1b; repeated measures ANOVA: $F_{4.26,\ 63.86} = 1.05$, $P = 0.187$). Nectar quality also did not differ between the young and old leaves following either damage treatment (Fig. 1b; $P > 0.05$, with Bonferroni correction).

There were no significant differences in plant height ($F_{4,\ 15} = 1.38$, $P = 0.29$) and number of leaf twig layers ($F_{4,\ 15} = 2.78$, $P = 0.07$) on plants prior to simulated herbivory damage.

Ant recruitment on leaves

Both cutting and punching of young leaves resulted in a 2–4-fold increase in the number of ants compared to undamaged controls (Fig. 2; repeated measures ANOVA: $F_{4,\ 95} = 15.10$, $P < 0.001$). In both treatments, ant recruitment varied significantly over time (Fig. 2; repeated measures ANOVA: $F_{2.95,\ 279.79} = 84.76$, $P < 0.001$) and reached a peak 6 h after damage and remained elevated until the end of the 24-h observation period. Interactions between treatment and time were highly significant for both types of treatment. Cutting and punching damage of young leaves had similar effects ($P > 0.05$). The two damage treatments of old leaves did not result in any significant changes in ant numbers compared to controls (Fig. 2; Dunnett's contrast, $P > 0.05$). There were significantly more active ants in the cutting treatment ($F_{1,\ 38} = 15.027$, $P < 0.001$) as well as in the punching treatment ($F_{1,\ 38} = 23.521$, $P < 0.001$) of young leaves than old leaves.

Disks of fresh young *L. glabra* leaves that were pinned on young leaves of another plant led to a significant increase in the number of ants compared to controls with fixed paper disks (Fig. 3; repeated measures ANOVA: $F_{1,\ 38} = 16.306$, $P < 0.001$).

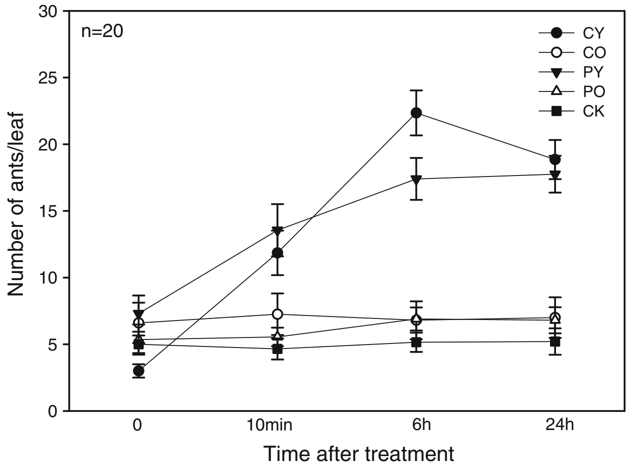

Fig. 2 Mean numbers of ants (±SE) on young and old *L. glabra* leaves damaged by cutting and punching at different times after treatment compared to undamaged control leaves. t_0 corresponds to the numbers immediately before damage

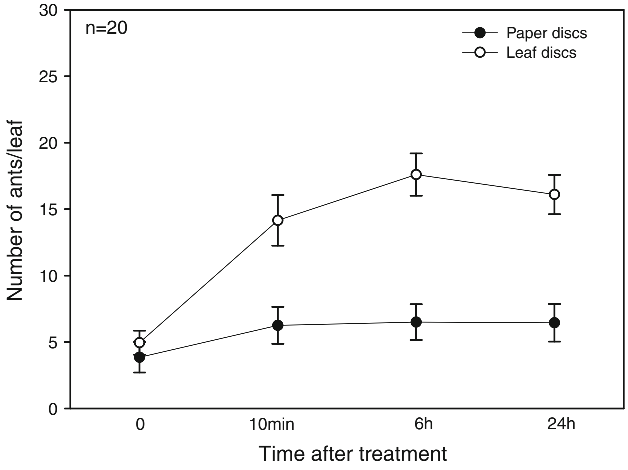

Fig. 3 Mean numbers of ants (±SE) on young *L. glabra* leaves with fixed disks from young leaves of another *L. glabra* plant and with fixed paper disks. t_0 corresponds to the numbers immediately before treatments

Tannin concentrations and herbivory

The tannin concentration of young leaves was significantly higher than that of both medium-aged and old leaves (MANOVA: $F_{2,\ 33} = 67.074$, $P < 0.001$) exceeding 100% in both comparisons (Fig. 4a). The portion of damaged leaf area of young leaves was significantly lower than that of both medium-aged and old leaves (Fig. 4b; MANOVA: $F_{2,\ 33} = 45.890$, $P < 0.001$).

Discussion

Induced changes of extrafloral nectar quantity or quality following natural or artificial tissue damage have been reported from a number of EFNs plants (Heil et al. 2000, 2001; Ness 2003; Wäckers et al. 2001; Wäckers and Bonifay 2004; Wäckers and Wunderlin 1999), but there are also plants that fail to show such induction (Koptur 1989; Smith et al. 1990). Similarly, in our experiments with *L. glabra*, we found no temporal changes in extrafloral nectar volume or nectar sugar concentration within 60 h after artificial damage, indicating there was no induction following the treatments. The nectar production differences between plants with cut young leaves can most probably be attributed to biotic or abiotic factors. Environmental conditions including the air humidity and temperature in the experiment site are the most probable factors which influenced the nectar quality and quantity measured. We can exclude the possibility that ants consumed nectar, though it was not possible to completely prevent the access of ants to the experimental plants in the field. In total, we found no indication that plants react to the damage of leaves by

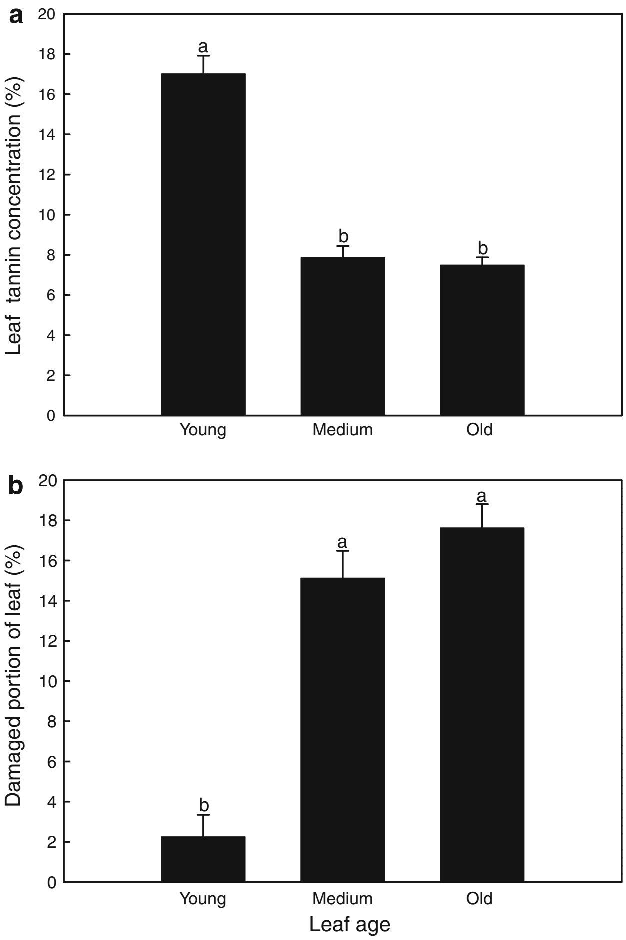

Fig. 4 Total leaf tannin concentration (**a**) and damaged leaf area ratio (**b**) in leaves of different age (percent; mean + SE). Columns marked with different *letters* are significantly different at $P < 0.001$ (MANOVA)

changes in extrafloral nectar quantity or quality. We acknowledge that the power of our tests is relatively low and results should be considered within this context. Though it did not completely remove the relatively low power of the test with a sample size of four plants in each treatment in this measurement, there were no significant differences in the main effect within the tests.

Despite this lack of nectar changes, our field experiments showed a significant increase in ant abundance on plants with damaged young leaves within 24 h after damage, although not on plants with damaged old leaves. This indicates that herbivore-specific elicitors are not required for induction in *Leea*-ants system. These results are consistent with those of Heil et al. (2001) who found induction of plants by artificial damage. They differ slightly from those of the Amazonian ant–plant *Hirtella myrmecophila* which showed that leaf wounds induced ant recruitment regardless of the leaf's age (Romero and Izzo 2004).

In our experiments, we did not observe a direct link between EFN production and ant abundance on damaged leaves. According to field observations, the ants attracted to damaged plants directly moved to the damaged young leaves and ignored the extrafloral nectaries at the leaf petioles. Extrafloral nectar production of *L. glabra* therefore seems to be a constitutive defense mechanism by the plant, suitable to create a general attractiveness to ants by the consistent production of extrafloral nectar provided as food. This interpretation is supported by our observation that extrafloral nectaries are only active at young leaves, which are most resistant to herbivore damage.

As suggested by the results of the leaf disk experiment, the increased recruitment of ants is most probably caused by the release of VOCs by damaged young leaves of *L. glabra,* indicating an inducible mechanism of herbivore defense. A number of other studies also clearly indicate that ants are able to detect VOCs of damaged leaves and can distinguish between damaged plant species (Agrawal 1998b; Bruna et al. 2004; Inui and Itioka 2007).

Furthermore, the tannin concentration of young leaves is more than 100% higher than in medium-aged and old leaves, and the portion of damaged leaf area of young leaves is lower than for both groups of medium-aged and old leaves; this maybe indicates the existence of an additional constitutive protective mechanism of the plant. There is evidence that tissues with high tannin content have lower numbers of herbivorous insects and lower damage levels than tissues with lower tannin content (Bialczyk et al. 1999; Coley 1986).

Our results support the general assumption of the optimal defense hypothesis that the most valuable tissues of a plant receive a higher proportion of the overall defensive investment than tissues of lower value. Young leaves of *L. glabra* which suffer low-level damage by herbivores are more defended than old leaves. This appears to contradict some other tropical trees, which suffer more damage by herbivores on young leaves than on old leaves (Kursar and Coley 2003). Maybe it was attributed to the plant tissues using divergent defensive strategies such as "escape" as well as "defense."

Many plants have multiple defense mechanisms for adaptation to different selection pressures (Coley et al. 2005) and there are not always trade-offs among them (Koricheva et al. 2004). Our results show that young leaves of *L. glabra* are protected against attacks by herbivores by different mechanisms which include: (1) the constitutive activity of EFNs during the period of young leaf production, which attract ants from the surrounding ground; (2) a mechanism induced by the damage of young leaves, which leads to increased ant recruitment, most probably caused by the release of VOCs and (3) a higher allocation to tannins in young leaves than in older leaves. Young leaves of the

L. glabra plants have multiple defense mechanisms, which indicate that these young tissues are of significant value to the plant. Further detailed studies are still needed to fully understand the production of defenses in *L. glabra*, including experiments to reveal the factors influencing EFN production and leaf tannin and anthocyanin concentration such as the effects of different nutrient and light conditions. Many plants have multiple defense mechanisms for adaptation to different nutrient and light conditions.

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