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Reduced Chitinase Activities in Ant Plants of the Genus *Macaranga*

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Abstract. Many plant species have evolved mutualistic associations with ants, protecting their host against detrimental influences such as herbivorous insects. Letourneau (1998) reported in the case of *Piper* that ants defend their plants principally against stem-boring insects and also reduce fungal infections on inflorescences. *Macaranga* plants that were experimentally deprived of their symbiotic *Crematogaster* ants suffered heavily from shoot borers and pathogenic fungi (Heil 1998). Here we report that ants seem to reduce fungal infections actively in the obligate myrmecophyte *Macaranga triloba* (Euphorbiaceae), while ant-free plants can be easily infected. We also found extremely low chitinase activity in *Macaranga* plants. The plants' own biochemical defense seems to be reduced, and low chitinase activity perhaps may represent a predisposition for the evolution of myrmecophytism. These plants are therefore highly dependent on their ants, which obviously function not only as an antiherbivore defense but also as an effective agent against fungal pathogens.

Symbiotic associations between ants and plants have been reported for many different genera and families and have evolved throughout the tropics. Most of these plants are "myrmecophiles," that is, they produce ant food in the form of extrafloral nectar or food bodies and thereby attract ants from the vicinity. Myrmecophytic species are more closely adapted to the association. They produce hollow plant structures (domatia) used by specialized ant colonies as nesting space and in many cases also offer ant food (for overviews on ant-plant interactions see Buckley 1982; Beattie 1985; Hölldobler and Wilson 1990; Davidson and McKey 1993). Most studies that have thus far been carried out in these systems have focused on taxonomic questions or have investigated the protective effect of ants in short-term studies by comparing inhabited and uninhabited plants with regard to the leaf damage done by herbivores (see, e.g., Janzen 1972; Letourneau 1983; McKey 1984; Schupp 1986; Fiala et al. 1989; Fowler 1993). Only few studies have been conducted over longer time spans (Vasconcelos 1991; Fonseca 1994; Heil 1998; Letourneau 1998).

A comparison of results derived from short-term experiments with those of long-term studies indicates that the detrimental effects of pathogenic fungi or shoot borers on plant fitness become fully obvious only in studies covering several months of ant exclusion. Ant exclusion experiments in two different *Macaranga* species have demonstrated that ant-free trees suffer strongly from shoot borers and infections by pathogenic fungi; these two factors dramatically reduced the fitness of ant-free plants (Heil 1998). Similarly, the decrease in seed set and pollen production in ant-free specimens of three different myrmecophytic *Piper* species from Costa Rica resulted mainly from ant disruption of stem-boring insects and reduced fungal infections on the inflorescences (Letourneau 1998).

The observation that ant-free myrmecophytes suffer strongly from fungal infections leads to the view that the "intrinsic" physiological defense against pathogenic fungi may be less well developed in these plants than in others. As early as in 1966 Janzen reported that myrmecophytic acacias seemed to have a reduced defense via secondary plant compounds compared to other species of the same

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genus. Some years later Rehr et al. (1973) demonstrated that leaves of some myrmecophytic *Acacia* species had at least lower contents of cyanogenic compounds than related nonmyrmecophytic species. To our knowledge, no studies have been performed on the possible reduction in other kinds of chemical defense, and in particular of antifungal defense, as a result of the presence of symbiotic ants.

In this study we measured chitinase activity of three different *Macaranga* species and compared it to that of other plants. Chitinases occur in several different plant taxa (Schneider et al. 1996). Due to their chitinolytic activity they play an important role in the physiological defense against fungi (Boller et al. 1983; Schlumbaum et al. 1986; Sahai and Manocha 1993; Iseli et al. 1996; Jackson and Taylor 1996). The induction of chitinases and β-1,3-glucanases by wounding and by the stress hormone ethylene has been studied in particular and has been verified for a variety of plant species (Boller et al. 1983; Vierheilig et al. 1994, and literature cited therein). Chitinases are one class of so-called pathogenesis-related proteins which are involved in the establishment of systemic acquired resistance (SAR; Hunt and Ryals 1996; Hunt et al. 1996; Ryals et al. 1996; Schneider et al. 1996). Although the field of SAR has attracted substantial scientific scrutiny in recent years, no information is available on costs in terms of reduced fitness resulting from the production of chitinases or other pathogenesis-related proteins (Heil 1999). However, to compare the relative efficacy and ecological significance of various types of induced defense, their respective costs and benefits must be evaluated. We propose that SAR – as with other types of chemical defense (Janzen 1966; Rehr et al. 1973) – is reduced in ant plants to the extent that its function is fulfilled by mutualistic ants.

The Southeast Asian genus *Macaranga* (Euphorbiaceae) comprises both obligate myrmecophytes and myrmecophilic species (for detailed information on the *Macaranga/Crematogaster* system see Fiala et al. 1989; Fiala and Maschwitz 1990, 1992a,b; Fiala 1996).

Our study was conducted on plants growing at their natural site in a secondary forest near the Ulu Gombak Field Studies Center on the Malay Peninsula. In our first experiment using the obligate myrmecophyte *M. triloba* ten trees were chosen, and in five trees the inhabiting ant colonies were poisoned by injection of a 1% watery solution of Spruzit (Neudorff, Emmerthal, Germany) into the inside of the hollow twigs and stems (domatia). Spruzit is a pyrethroid functioning as neurotoxic contact insecticide. In preliminary experiments which had been conducted over 2 months, Spruzit had no detectable detrimental effect on *M. triloba* over the entire course of the experiment when injected as a 5% solution into the domatia (Heil et al., unpublished data). Negative effects on the plants occurred only in cases in which the solution remained inside the domatia for a longer period. We avoided this by blowing air into the domatia soon after the injection of Spruzit.

We injured each tree at ten spots on the main stem and several twigs by removing about 2–3 cm^2 of the bark with a sharp knife. These wound sites were infected artificially using fungal hyphae and ripe spores derived from dead *Macaranga* wood. A few hours after wounding, high ant activity was observed at the wound sites of the inhabited plants, and ants seemed to remove actively at least a part of the hyphae. One day later all wound sites were checked visually for remaining fungal material. No hyphae or spores were visible on plants with ants. In contrast, hyphae still were present on 42 of the 50 wound sites of ant-free trees.

A further check 3 weeks later revealed that fungi were growing well on the ant free trees (Table 1). Between five and nine wound sites per plant were covered with hyphae, and on 12 injuries fungi had already developed new spores. On ant-occupied plants only three wound sites on two plants showed hyphae at all, which most probably had developed from spores overlooked in the above optical control. Fungi had developed new spores on only one of these wound sites, and all three infected wound sites had been afflicted to the lowest

Table 1. Fungal persistance and sporulation in the ant exclusion experiment. The first two columns indicate plant number and treatment (A: ant free, B: untreated control). In the third column, the number of wound sites carrying fungal hyphae three weeks after infection is given for all individual plants. In the fourth column, numbers of infected wound sites on which fungi had already produced ripe spores are listed. Total number of infected wound sites was ten per plant

Plant	Treatment	Number of injuries	
		With hyphae	With ripe spores
1	A	6	2
2	A	7	2
3	A	5	1
4	A	6	3
5	A	9	4
6	B	0	0
7	B	1	0
8	B	0	0
9	B	2	1
10	B	0	0

part of the stem where ant activity in general is very low. The differences between ant-free and inhabited plants were statistically significant for both the number of wound sites with fungi and the number of wound sites on which fungi had produced new spores (Mann-Whitney U test, $P < 0.01$ for both variables). The ants thus seemed to confer a very effective protection against fungal infections to the plants, while *M. triloba* on its own seems to be highly vulnerable, possibly because of a reduced chemical antifungal defense.

To test this hypothesis we measured chitinase activity in tissues of three *Macaranga* species (the myrmecophytes *M. triloba* and *M. hosei* and the nonmyrmecophytic *M. tanarius*). For comparison we included five additional, nonmyrmecophytic plant species from other genera [*Mallotus floribundus* (Euphorbiaceae), *Musa* sp. (Musaceae), one Fabacea, *Piper aduncum* (Piperaceae), and one Solanacea]. All plants were collected from natural sites in the vicinity of the Ulu Gombak Field Studies Center. Analyses were carried out using fresh plant material. The chitinase assay followed Boller (1992), with some modifications due to the necessity of

performing the tests under field conditions: Plant extracts (about 0.5 g fresh plant material extracted in 5 ml ice-cold watery 0.1 M Na-citrate buffer) were filtrated instead of centrifuged, and for incubating the colloidal radioactive chitin with plant extract, samples were shaken by hand at air temperatures (28–32° C) instead of using a shaking waterbath at 37° C. The reaction was stopped 30 min later by addition of 250 µl 10% TCA. Two days later all samples were transported to Germany, centrifuged, and the radioactivity in the supernatant (resulting from chitin monomers and therefore a measure of the chitinolytic activity in the extract) measured using a liquid scintillation counter (1900 CA Tri-Carb Liquid Scintillation Analyzer, Packard, Meriden, USA). Enzyme activity was calculated using a calibration curve and expressed in nanomoles of N-acetylglucosamine equivalents released per second per gram of fresh weight. Preliminary experiments demonstrated that samples may be stored up to 1 week after addition of TCA without detectable effects on the measured activity. For each sample three parallels were carried out using 2×20 µl and 1×60 µl plant extract, and the median of the three parallels was taken.

All three *Macaranga* species had lower chitinase activities than any other plant species studied (Table 2). This difference was statistically significant for both leaf tissue and shoot tissue and was highly significant when combining all samples (Table 2). Within the genus the myrmecophilic but non-myrmecophytic *M. tanarius* had the highest chitinase activity. However, activity was still lower than that of the other species. In contrast, *Mallotus floribundus,* as a member of a closely related but totally nonmyrmecophytic genus, showed high activities.

Due to the low sample sizes these results should be regarded with caution. However, the differences between the two plants groups were rather high, and two different hypotheses may be adduced to explain these results: (a) chitinase may have been reduced secondarily in plants with a successful ant defense, or (b) low chitinase activity may be a primary property of *Macaranga* and as such constitutes a preadaptation to the evolution of myrmecophytism.

(a) The results of our first experiment demonstrate that ants can provide an effective protection against fungal infections. A reduction in the plants' own chemical defense against fungi must therefore be expected if this reduces the overall costs of defense. In obligate myrmecophytes ants depend much more on the survival and fitness of their host, and consequently ant defense is more effective in these than in myrmecophilic species (Heil 1998). Consistent with the higher intensity of defense, ant nutrition by myrmecophytes seems to be metabolically more expensive than ant attraction by myrmecophilic species (Heil et al. 1997, 1998). Plants in general should avoid costs resulting from the maintenance of redundant defenses, and myrmecophytes should reduce their investment in chemical defense more than the myrmecophilic species. This hypothesis derives some support from the observation that *M. tanarius* on average tend to have higher chitinase activities than the two myrmecophytic species.

(b) High chitinase activity may also have direct negative effects on symbiotic ants, at least when it occurs in the wooden parts of myrmecophytes. In the myrmecophytic associations within the genus *Macaranga* the ants must chew entrance holes through the living plant tissue and thus inevitably wound it. The cuticle of all insects consists of chitin, and a variety of surface structures, such as parts of the ants' antennae, mouth parts, and digestive surfaces, are covered with very thin or even monomolecular layers of chitin (Gullan and Cranston 1994). High chitinase activities in the plant tissue could potentially harm the ants, especially when chitinolytic plant sap comes into contact with these delicate structures. Therefore it may be hypothesized that low chitinase activity may be a prerequisite of whole plant taxa for the evolution of myrmecophytism.

Our data seem to support the second of these two hypotheses: *M. tanarius* is less well defended than the two myrmecophytes (Heil 1998). Nevertheless it had much lower chitinase activity than the plants belonging to other genera (Table 2). If chitinase loses its importance only due to the antifungal effects of the ants and is thus reduced only after the establishment of myrmecophytism, it would be expected that the nonmyrmecophytic

Table 2. Chitinase activities [nkat/g] (nMol N-acetylglucosamin equivalents released per second, per gram fresh plant tissue) in tissues from three *Macaranga* and five other species from different taxonomic groups. For each species, the mean $+/-$ stdv. is given separately for leaf and shoot tissue as well as for all samples. The number of samples (n) comprised by each mean is given in brackets, each single sample consisted of leaves or parts of shoots of three to five individual plants. Differences between the two groups ('*Macaranga*' and 'other species') were tested with Mann-Whitney-U-test

	Species	leaves	stem	all samples
Macaranga	*M. tanarius*	2.45 ± 2.04 (6)	1.85 ± 1.44 (4)	2.21 ± 1.74 (10)
	M. triloba	1.69 ± 3.01 (9)	0.16 ± 2.70 (4)	1.26 ± 2.70 (13)
	M. hosei	0.06 ± 0.54 (4)	1.69 ± 1.35 (5)	1.01 ± 1.35 (9)
	Mallotus spec.	15.11 ± 22.85 (3)	4.55 ± 1.51 (2)	10.85 ± 17.19 (5)
	Musa spec.	19.10 ± 0.64 (2)	– (0)	19.01 ± 0.65 (2)
other species	*Piper aduncum*	16.35 (1)	– (0)	16.35 (1)
	Fabacea	69.74 (1)	– (0)	69.74 (1)
	Solanacea	22.19 ± 11.80 (3)	19.85 ± 3.13 (2)	21.02 ± 7.18 (5)
	P	<0.01	<0.05	<0.001

M. tanarius would still have high activities.

Our results strongly suggest a further reason for the effective protection ants can provide for their host plants. Although ants are a comparably expensive defense mechanism (Heil et al. 1997), they seem to be extremely valuable since they can fulfill many different defensive functions. However, the exact mechanisms by which ants reduce fungal infections still are not known (Letourneau 1998). Additional studies are needed to determine whether low chitinase activity is a general property of ant plants, and whether plant ants in general are successful defenders against pathogenic fungi. Further studies should also examine other types of defense against pathogens which may be affected by the presence of "biotic defense" via mutualistic ants. It is not yet possible definitively to confirm either of the two hypotheses discussed as the explanation for the low chitinase activity in the investigated *Macaranga* species.

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