

## SHORT COMMUNICATION

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**Low chitinase activity in *Acacia* myrmecophytes: a potential trade-off between biotic and chemical defences?**

Received: 16 August 2000 / Accepted in revised form: 28 October 2000

**Abstract** We determined chitinase activity in leaves of four myrmecophytic and four non-myrmecophytic leguminous species at the plants' natural growing sites in Mexico. Myrmecophytic plants (or 'ant plants') have obligate mutualisms with ants protecting them against herbivores and pathogenic fungi. Plant chitinases can be considered a reliable measure of plant resistance to pathogenic fungi. The myrmecophytic *Acacia* species, which were colonised by mutualistic ants, exhibited at least six-fold lower levels of chitinase activity compared with the non-myrmecophytic *Acacia farnesiana* and three other non-myrmecophytes. Though belonging to different phylogenetic groups, the myrmecophytic *Acacia* species formed one distinct group in the data set, which was clearly separated from the non-myrmecophytic species. These findings allowed for comparison between two recent hypotheses that attempt to explain low chitinase activity in ant plants. Most probably, chitinases are reduced in myrmecophytic plant species because these are effectively defended indirectly due to their symbiosis with mutualistic ants.

**Introduction**

Chitinases (EC 3.2.1.14) are abundant proteins widely distributed in higher plants (for reviews see, for example, Punja and Zhang 1993; Neuhaus 1999). There is increasing evidence that they play an important role in the defence of plants against fungal pathogens (Sahai and Manocha 1993; Iseli et al. 1996; Jackson and Taylor 1996). Many chitinases are produced in large amounts when plants are wounded or attacked by pathogens. These chitinases have therefore been included in the group of 'pathogenesis-related' (PR) proteins (van Loon 1997; Neuhaus 1999). Purified chitinases exhibit pronounced antifungal activity, particularly in combination with  $\beta$ -1,3-glucanases (e.g. Schlumbaum et al. 1986; Mauch et al. 1988). Moreover, plants over-expressing chitinase can show decreased susceptibility to infection by some fungi that have a chitin-containing cell wall (Broglie et al. 1991; Datta and Datta 1999).

While hydrolytic enzymes and other forms of direct biochemical defence are widespread throughout the plant kingdom, plants of several genera have evolved indirect defensive strategies which include the action of animal mutualists representing 'enemies of the plants' enemies' (Price et al. 1980; Baldwin and Preston 1999; Dicke 1999). In most cases, these defence responses act against herbivorous insects or mites. Myrmecophytic plants form one functional group within this context. They are defended by the action of ants, which are housed and often nourished by their host plants (Buckley 1982; Beattie 1985; Hölldobler and Wilson 1990; Davidson and McKey 1993). Recent studies revealed that ants can also reduce fungal infections of their host plants (Letourneau 1998; Heil et al. 1999), both by direct protection and by reducing herbivore-caused wound sites, which can facilitate fungal attack.

*Macaranga* plants, which are efficiently defended by mutualistic ants (Heil et al. 2000a), showed only weak chitinase activity in comparison with a number of non-myrmecophytic plants (Heil et al. 1999). Two hypotheses have been raised to explain this finding:

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1. Theory predicts that defence against pathogens or herbivores does incur costs (Simms and Rausher 1987; Herms and Mattson 1992). Plants therefore should avoid superfluous costs resulting from functionally redundant defensive strategies. Myrmecophytes, which are defended indirectly by their mutualists, should therefore reduce their corresponding direct defences.
2. In the case of *Macaranga*, however, plants may also have been forced to reduce their chitinase activity to avoid negative effects on their mutualistic ants (Heil et al. 1999), since these have to gnaw entrance holes through living plant tissue and thus might be negatively affected by chitinolytic activity of plant sap. Harmful effects of chitinase on insects have already been reported by Broadway et al. (1998) and Inbar et al. (1999).

The present study was designed to distinguish between these two hypotheses. We investigated the chitinase activity in leaves of four Mexican myrmecophytes of the genus *Acacia* (Mimosoideae, Fabaceae), all of which are defended by mutualistic ants that are housed in 'swollen' thorns and are nourished by specialised food bodies produced by their plant hosts (Janzen 1966, 1967, 1974). For comparison, chitinase activities were determined in leaves of a non-myrmecophytic *Acacia* species and three other non-myrmecophytic species belonging to the family Fabaceae. In the interactions with *Acacia*, the inhabited thorns are already hardened and lignified when ants gnaw their entrance holes (Janzen 1974; and observations by M. Heil). The ants therefore do not come directly into contact with the plant sap of fresh tissue. A direct negative effect of chitinase on these ants is therefore less likely. Rejection of this alternative hypothesis allows for a direct test of the consequences of redundant defences.

## Materials and methods

The study was conducted during a field stay in Mexico (March and April 2000). We collected fresh plant material from untreated plants to ensure that only chitinase activities occurring in plants growing under fully natural conditions were measured. In order to exclude site effects as far as possible, we chose a site where several 'ant' *Acacia* species co-occurred. At the Isthmus of Tehuantepec we found four different ant *Acacia* species. Ant *Acacia* and non-ant *Acacia* species differ in their site requirements, e.g., ant acacias grow on much wetter soils than the non-ant species of this genus (Janzen 1974). It was therefore not possible to collect non-ant *Acacia* species from the same site, and non-*Acacia* species of the family of Fabaceae had to be included in the study. For comparison, we used two closely related non-*Acacia* shrub species and one weedy species of the Fabaceae growing as close as possible to the sampled ant *Acacia* trees and being in the same developmental stage (flowering). Moreover, material of one closely related non-ant *Acacia* species (Seigler and Ebinger 1995) was collected. Additional material of one of the ant *Acacia* species was obtained from a third site to check whether site effects may have biased the measured data.

Plant material from the myrmecophytic shrubs belonging to the genus *Acacia* (Mimosoideae, Fabaceae), i.e. *A. hindsii* Benth., *A. cornigera* (L.) Willdenow, *A. globulifera* Safford and *A. chia-*

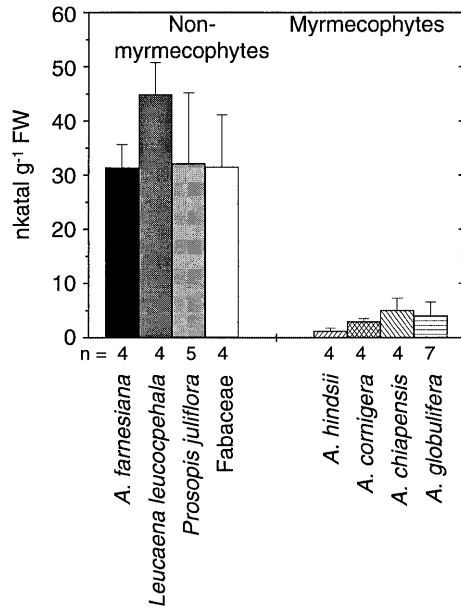
*ensis* Safford, was obtained at the Isthmus of Tehuantepec (State of Oaxaca). Samples of the non-myrmecophytic shrubs *Prosopis juliflora* (Swartz) DC. (Mimosoideae, Fabaceae) and *Leucaena leucocephala* (Lamarck) de Wit (Mimosoideae, Fabaceae), and of a weedy species belonging to the subfamily Faboideae (Fabaceae) originated from the same site. Samples of the non-myrmecophytic shrub *Acacia farnesiana* (L.) Willd. originated from the valley of Oaxaca (State of Oaxaca). Additional samples of *A. globulifera* were collected from another site near Coba, on the Yucatan peninsula (State of Quintana Roo). Ant *Acacia* species were identified following Janzen (1974) and Seigler and Ebinger (1995); the other genera and species after Standley (1920) and Hughes (1998). Voucher specimens are deposited at the Herbario MEXU at UNAM (Mexico City), and additional specimens are held by M. Heil. Detailed descriptions of the ecology and growing sites of the myrmecophytic *Acacia* species are given by Janzen (1974).

Total chitinase activity of freshly harvested leaves was measured according to Boller (1992). To include wound-inducible chitinase isoforms, only those leaves were harvested that had already been wounded naturally by herbivores. Only leaves with damage levels of 5–10% missing leaf area were used. About 1 g of fresh leaf material was ground with sand and extracted in 15 ml of an ice-cold extraction buffer containing 50 mM Tris-HCl (pH 8), 250 mM NaCl, 0.2% (w/v) Triton X 100, 0.5% (w/v) polyvinylpyrrolidone and 0.5% (w/v) bovine serum albumin. The extract was filtered through paper filters and then further cleared by micro-membrane filtration (pore volume 45  $\mu$ m, FP 030/0.45 Schleicher & Schuell, Dassel, Germany). Samples of between 5  $\mu$ l and 100  $\mu$ l of the extract (in steps of 5, 10, 20, 50 and 100  $\mu$ l) were incubated for 30 min at 37 °C in a reaction solution consisting of 145, 140, 130, 100 or 50  $\mu$ l sodium citrate buffer (100 mM sodium citrate adjusted with HCl to pH 5), 50  $\mu$ l sodium acetate buffer (0.1 M sodium acetate adjusted with HCl to pH 5) and 50  $\mu$ l colloidal,  $^3$ H-labelled chitin. The samples were incubated for 30 min and the reaction was stopped by adding 250  $\mu$ l of 10% trichloric acid. All samples were frozen and thereafter transported to Würzburg, where they were centrifuged at 14,000 rpm for 30 min. After this, the supernatant containing soluble chitin oligomers was filtrated through a micromembrane (pore volume 45  $\mu$ m, FP 030/0.45 Schleicher & Schuell) and centrifuged for another 15 min. An aliquot of 250  $\mu$ l of the remaining supernatant was taken to measure the radioactivity of the chitin oligomers using a Wallac 1409 Liquid Scintillation Counter. Enzyme activity was calculated based on calibration curves established separately for each extract and expressed in nkat (nanomol of *N*-acetylglucosamine equivalents released per second) per gram fresh weight.

For each plant species, four to seven extractions originating from different plants were prepared. Five parallel enzymatic reactions using different amounts of extract were conducted for each extraction. One mean was calculated for each sample, and species were compared using one-way ANOVA using the GLM procedure of SPSS. Post hoc comparisons of all species were done following Games-Howell, since there was no homogeneity of variances (Sachs 1992).

## Results

'Species' was a significant source of variation in the data set (univariate ANOVA:  $n=36$ ,  $df=7$ ,  $F=32.3$ ,  $P<0.001$ ). Chitinase activities from all four myrmecophytic *Acacia* species were more than six-fold lower than those of the non-myrmecophytic *A. farnesiana* (Fig. 1). The other three non-myrmecophytic plants exhibited chitinase activity at a similar high level (Fig. 1). While the mean activities of the myrmecophytes ranged from 1.2 nkat  $g^{-1}$  FW in *A. hindsii* to 5.0 nkat  $g^{-1}$  FW in *A. chiapensis*, mean activities of the non-myrmecophytic species were 30 nkat  $g^{-1}$  FW or higher. Statistical



**Fig. 1** Chitinase activity of four myrmecophytic *Acacia* species, the non-myrmecophytic *Acacia farnesiana* and three other non-myrmecophytic plants of the family Fabaceae. Chitinase activity is expressed in nkat per gram fresh weight (1 nkat=1 nanomol of *N*-acetylglucosamine equivalents released per second). Bars represent means, error bars standard deviations. The number of samples (*n*) is indicated under the bars

evaluation revealed that the myrmecophytic species and the non-myrmecophytic species formed two different groups in the data set (Table 1). Most, but not all, single comparisons between myrmecophytic *Acacia* species on one hand and non-myrmecophytic species on the other hand were significant ( $P < 0.05$ ), with all remaining comparisons showing *P* values between  $P = 0.05$  and  $P = 0.07$  (see Table 1). Within the group of myrmecophytes, only one pair of species (*A. hindsii* and *A. cornigera*) showed a significant difference in chitinase activity, while none of the non-myrmecophytic species differed significantly from each other (Table 1).

## Discussion

Chitinase activity can be considered as a reliable measure of plant biochemical defence against fungal pathogens. In this study, we found only very low chitinase activities in myrmecophytic *Acacia* species in comparison with the non-myrmecophytic *A. farnesiana* and three other leguminous plants. Our data strongly indicate that low chitinase activity is related to myrmecophytism. Three of the 'non-myrmecophytes' originated from the same site as the ant acacias, and one of the ant acacias had been sampled at two different sites. Site effects are thus not likely to have strongly influenced the observed patterns in chitinase activity. Moreover, species of at least two independent phylogenetic groups in the genus *Acacia* have evolved myrmecophytism (Janzen 1974; Seigler and Ebinger 1995). Both groups are represented in our study, with *A. cornigera* and *A. hindsii* belonging to one, and *A. chiapensis* and *A. globulifera* belonging to the second group (Seigler and Ebinger 1995). This provides further evidence for the hypothesis that low chitinase activity is a trait directly related to myrmecophytism rather than a general trait of a distinct taxonomic group within the genus *Acacia*.

This report is in line with the restricted number of studies showing that myrmecophytes, which are defended by the action of their mutualistic ants, have reduced levels of plant chemical defences (Janzen 1966; Rehr et al. 1973; Heil et al. 1999). The data reported by Seigler and Ebinger (1987) on cyanogenic glycosides did not support the general trend for some ant *Acacia* species, which in part exhibited cyanogenesis. However, even in the study of Seigler and Ebinger (1987), nine of the 12 *Acacia* species investigated lacked the ability of cyanogenesis and thus confirmed the general pattern.

For the earlier findings on reduced chitinases in the obligate myrmecophytic *Macaranga* plants (Heil et al. 1999), it could not be ruled out that chitinase activity in the plant may have harmful effects on the mutualistic *Crematogaster* ants, which gnaw entrance holes through

**Table 1** Results (*P* values) of multiple comparisons after Games-Howell between all four ant acacias and four non-myrmecophytic species of the Fabaceae. *n.s.* indicates  $P > 0.10$ . See Fig. 1 for sample numbers

		Myrmecophytes				Non-myrmecophytes			
		<i>A. hindsii</i>	<i>A. cornigera</i>	<i>A. chiapensis</i>	<i>A. globulifera</i>	<i>A. farnesiana</i>	<i>Leucaena leucocephala</i>	<i>Prosopis juliflora</i>	Fabaceae (undet.)
Myrmecophytes	<i>A. hindsii</i>	–							
	<i>A. cornigera</i>	0.041	–						
	<i>A. chiapensis</i>	<i>n.s.</i>	<i>n.s.</i>	–					
	<i>A. globulifera</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	–				
Non-myrmecophytes	<i>A. farnesiana</i>	0.004	0.005	0.002	0.002	–			
	<i>Leucaena leucocephala</i>	0.004	0.005	0.002	0.003	<i>n.s.</i>	–		
	<i>Prosopis juliflora</i>	0.046	0.056	0.068	0.061	<i>n.s.</i>	<i>n.s.</i>	–	
	Fabaceae	0.048	0.057	0.063	0.058	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	–

living and not yet hardened plant tissue. In *Acacia*, however, ants are less likely to come in contact with plant sap, which may contain considerable amounts of chitinase. Our data on the myrmecophytic *Acacia* species thus strengthen evidence for the hypothesis that low chitinase activity might be favourable because the defensive function is reliably fulfilled by the ants. Superfluous costs resulting from redundant defences are thus avoided. We suggest that synthesis of chitinase protein can impose relevant fitness costs to the plants, as do other forms of pathogen defence (Smedegaard-Petersen and Tolstrup 1985; Heil 2000; Heil et al. 2000b). However, further studies are needed in order to determine the evolutionary causes for the reported pattern of reduced plant chemical defences in ant plants. These studies should (1) try to quantify directly the allocation costs of defence and (2) include further forms of plant biochemical defence in different 'ant plants'.

**Acknowledgements** We thank K.E. Linsenmair, T. Boller and two anonymous referees for critically reading the manuscript; T. Boller (Botanisches Institut, Basel, Switzerland) for the  $^3\text{H}$ -chitin; R. Krueger and L. Schreiber for practical support; and Alfredo Saynes-Vasques (Jardín Etnobotánico Oaxaca) for help with species determination. Financial support from the DFG (research grant He 3169/1-1 and 1-2) is gratefully acknowledged.

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