Reduced chemical defence in ant-plants? A critical re-evaluation of a widely accepted hypothesis

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Since its original formulation by Janzen in 1966, the hypothesis that obligate ant-plants (myrmecophytes) defended effectively against herbivores by resident mutualistic ants have reduced their direct, chemical defence has been widely adopted. We tested this hypothesis by quantifying three classes of phenolic compounds (hydrolysable tannins, flavonoids, and condensed tannins) spectrophotometrically in the foliage of 20 ant-plant and non-ant-plant species of the three unrelated genera *Leonardoxa*, *Macaranga* and *Acacia* (and three other closely related Mimosoideae from the genera *Leucaena*, *Mimosa* and *Prosopis*). We further determined biological activities of leaf extracts of the mimosoid species against fungal spore germination (as measure of pathogen resistance), seed germination (as measure of allelopathic activity), and caterpillar growth (as measure of anti-herbivore defence).

Condensed tannin content in three of four populations of the non-myrmecophytic *Leonardoxa* was significantly higher than in populations of the myrmecophyte. In contrast, we observed no consistent differences between ant-plants and non-ant-plants in the Mimosoideae and in the genus *Macaranga*, though contents of phenolic compounds varied strongly among different species in each of these two plant groups. Similarly, among the investigated Mimosoideae, biological activity against spore or seed germination and caterpillar growth varied considerably but showed no clear relation with the existence of an obligate mutualism with ants. Our results did not support the hypothesis of 'trade-offs' between indirect, biotic and direct, chemical defence in ant-plants.

A critical re-evaluation of the published data suggests that support for this hypothesis is more tenuous than is usually believed. The general and well-established phenomenon that myrmecophytes are subject to severe attack by herbivores when deprived of their ants still lacks an explanation. It remains to be studied whether the trade-off hypothesis holds true only for specific compounds (such as chitinases and amides whose cost may be the direct negative effects on plants' ant mutualists), or whether the pattern of dramatically reduced direct defence of ant-plants is caused by classes of defensive compounds not yet studied.

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Most ecological theories on plant antiherbivore defence (McKey 1974, 1979, Feeny 1976, Coley et al. 1985) assume resistance to be costly, i.e. production and maintenance of defensive compounds can reduce plant fitness under enemy-free conditions in which the resistance has no beneficial effects (Simms and Fritz 1990). Costs can occur, for example, because limited resources, once allocated to defence, cannot be used for other fitness-relevant processes such as growth and reproduction (Coley et al. 1985, Bazzaz et al. 1987, Herms and Mattson 1992, Bazzaz and Grace 1997). This concept, among others, is used to explain both variation in the level of constitutive defence and the evolution of induced defences (Karban and Baldwin 1997, Baldwin and Preston 1999, Tollrian and Harvell 1999, Heil and Baldwin 2002).

The same concept leads to the hypothesis that selection will favour plants that do not maintain redundant defences. This hypothesis has been repeatedly tested using ant-plants as model systems. Originally, the hypothesis of reduced chemical defence in ant-plants was formulated by Janzen (1966) who observed that foliage of ant-acacias does not have 'the bitter taste that characterises other members of the genus *Acacia*'. He therefore proposed that chemical defence has been lost in the 'biotically' defended ant-acacias, probably to avoid 'superfluous' costs resulting from redundant defences (Janzen 1966, Rehr et al. 1973).

Obligate ant-plants (myrmecophytes) house and often nourish specific ant colonies that live inside 'domatia', i.e., specialised hollow structures provided by the host plant (Janzen 1967b, 1974, Buckley 1982, Beattie 1985, Hölldobler and Wilson 1990, Fiala and Maschwitz 1992, Davidson and McKey 1993). These ants patrol the plant surface and remove all kind of foreign material, thereby defending their host against herbivores and climbers (Janzen 1969, 1972, Fiala et al. 1989, Rocha and Bergallo 1992, Agrawal and Dubin-Thaler 1999, Heil et al. 2001a). In some cases this defence is extended against shoot borers and pathogenic fungi (Letourneau 1998, Heil et al. 1999, 2001a), and even allelopathic effects, i.e. removal of surrounding vegetation by the resident ants, have been described (Janzen 1966, 1967a, 1969, 1974). All these behaviours can provide important fitness benefits to the host plant. However, defence via mutualistic ants is costly (Heil et al. 1997) and can be limited by soil nutrient content (Heil et al. 2001b). The production of ant food thus might compete metabolically with other resource-demanding processes. It therefore has been assumed that ant-plants might be characterised by a lowered direct, chemical defence.

The first experimental study on chemical defence of ant-plants was that by Rehr et al. (1973) who reported a strong negative impact on growth of caterpillars fed on a diet containing leaf powder from a non-ant-acacia (*A. farnesiana*), but much lower effects when caterpillars received leaf powder from the ant-acacia, *A. cornigera*. Later, Seigler and Ebinger (1987) reported that foliage of most ant-acacias contained no or only low amounts of cyanogenic glycosides, which are a widespread defence of non-myrmecophytic species in the genus *Acacia*. Even the enzymatic anti-fungal defence of ant-plants in the genera *Macaranga* and *Acacia* was found to be much lower than in closely related non-myrmecophytes (Heil et al. 1999, 2000). Recent studies conducted in the context of this hypothesis focused on condensed tannin content in ant-plants and non-ant-plants of the genus *Macaranga* (Eck et al. 2001) and on herbivore-deterrent amides in inhabited and ant-free individuals of *Piper cenocladum* ant-plants (Dyer et al. 2001).

However, some of the published studies seem to draw an oversimplified picture. Rehr et al. (1973) were not able to explain the higher quality of ant-acacias as food for herbivores by the differences observed in chemical defences. The phytophagous insect they tested was not deterred by cyanide. In fact, southern armyworm has later been shown to prefer to feed on cyanogenic glucoside-containing plants and even to grow better on cyanide-containing diet (Brattsen et al. 1983). The strong effects observed by Rehr et al. (1973) remained unexplained. Also, some myrmecophytic *Acacia* are cyanogenic. Three out of twelve ant-acacias investigated by Seigler and Ebinger (1987) showed at least some cyanogenic activity. Moreover, these authors used herbarium material, in which 'either the cyanogenic glycosides or the hydrolytic enzymes present in fresh material may be destroyed' (Seigler and Ebinger 1987). In the study of Eck et al. (2001) only three out of six 'early' myrmecophytes (i.e. myrmecophytic species that are inhabited by resident ant colonies already at the seedling stage) differed significantly from non-myrmecophytes in condensed tannin content. While all studies mentioned thus far used interspecific comparisons, one recent paper focused on phenotypic plasticity within a species. In *Piper cenocladum*, ant-inhabited plants had a slightly lower content of four different amides than did ant-free ones, but still some redundancy in defences was observed (Dyer et al. 2001).

We conducted the present study to provide a larger data set suitable for testing the hypothesis of lowered chemical defence in obligate ant-plants. Leaves from myrmecophytes and non-myrmecophytes of the genera *Leonardoxa*, *Macaranga*, *and Acacia* (and three other related Mimosoideae from the genera *Mimosa*, *Leucaena* and *Prosopis*), were collected from natural populations in the field. We quantified their contents of phenolic compounds (flavonoids, hydrolysable tannins [i.e. gallic acid derivatives], and condensed tannins [i.e. proanthocyanidins or flavan-3-ols]) in extracts by spectrophotometry. Several compounds belonging to these groups are considered to be defences against phytophagous insects (Mole and Waterman 1987a, b,

Mueller-Harvey 2001, Schofield et al. 2001), pathogens (Hain et al. 1993, Taiz and Zeiger 1998, Hammerschmidt 1999a, b), and competing vegetation (Rice 1987).

Phenolic compounds thus seem to fulfil the same three defensive functions (i.e. anti-herbivore defence, pathogen resistance, and allelopathy) as described above for 'biotic' defence via resident ants. Moreover, phenolic synthesis directly competes with protein biosynthesis (Jones and Hartley 1999), the latter being required for growth and reproduction, but also for ant food production (Heil et al. 1998). It therefore appeared reasonable to expect trade-offs between phenolic compounds and ants. In order to obtain additional data on defensive activity that are independent from quantification of selected chemical compounds, we determined the biological activity of extracts of the mimosoid species. As test for general pathogen resistance, activity was tested against spore germination of a widespread plant-colonising fungus (*Gliocladium roseum*). Allelopathic activity was checked for by testing activity against seed germination of cress (*Lepidium sativum*), and anti-herbivore defence was measured as activity against growth of caterpillars of a polyphagous lepidopteran (*Spodoptera littoralis*). Our study is the first in this context that combines chemical analyses with biotests against three classes of 'target' organisms, and that covers three phylogenetically unrelated genera of ant-plants from three different continents (*Acacia*: Fabaceae (Mimosoideae), Central America; *Leonardoxa*: Fabaceae (Caesalpinioideae), Central Africa; *Macaranga*: Euphorbiaceae, South East Asia).

Material and methods

Plant material

We collected leaves of five ant-acacias, three non-ant-acacias and three other non-myrmecophytic mimosoid shrub species in Mexico in March and April 2000 and in April 2001. In 2000, material from the *Acacia* myrmecophytes, *A. hindsii* Benth., *A. cornigera* (L.) Willdenow, *A. globulifera* Safford and *A. chiapensis* Safford, was collected from sites on the Isthmus of Tehuantepec (state of Oaxaca). Samples of the non-myrmecophytic mimosoid shrubs *Prosopis juliflora* (Swartz) DC., *Leucaena leucocephala* (Lamarck) de Wit, and *Mimosa tenuiflora* (Willd.) Poir. originated from the same site. Samples of the myrmecophyte *A. collinsii* Safford and of the non-myrmecophyte *A. macracantha* Humb. et Bonpl. were collected near Coba, on the Yucatan peninsula (state of Quintana Roo). *A. chiapensis* is regarded as an ant-acacia in this study, since all plants found at the study site produced extrafloral nectar and food bodies and were inhabited by resident ant colonies. According to the information

given by Janzen (1974) this species shows 'intermediate' characteristics. Samples of the non-myrmecophyte *Acacia farnesiana* (L.) Willd. originated from the valley of Oaxaca (state of Oaxaca). We collected additional samples of *A. farnesiana* (hereinafter '*A. farnesiana* Isth.') in 2001 at the Isthmus of Tehuantepec (the two populations of *A. farnesiana* are hereinafter treated as two 'species' for statistical and graphical purposes), and samples of another non-myrmecophyte, *A. cochliacantha* Humb. et Bonpl. ex Willd. in 2001 near Puerto Escondido on the Pacific coast (state of Oaxaca). All sites were extensively used pastures or similarly structured, open secondary shrublands. All plants used were shrubs of heights between 1.5 and 2.5 m and grew in the full sun. We determined species following Standley (1920), Rudd (1966), Janzen (1974), Seigler and Ebinger (1995), and Hughes (1998) and by comparison with specimens held at the Herbario MEXU at UNAM (Mexico City). Voucher specimens are deposited at the Herbario MEXU and in the personal collection of M. Heil.

Within the species *Leonardoxa africana* (Baill.) Aubréville, four subspecies differing in their form of mutualism with ants are currently distinguished by McKey (2000). We collected leaf material from five different populations of the myrmecophyte *L. a. africana*, at Bissiang, Boundé, Bipaga, For and Mbodé. Populations of the non-myrmecophyte *L. a. gracilicaulis* were sampled in Boundé (three populations) and Mont Kala. All sites were located in humid tropical forest (all lowland sites except for Kala [1000 m]) in Cameroon, Central Africa.

Leaf material from four myrmecophytic and three non-myrmecophytic *Macaranga* species was collected in September and October 2000 in a valley between Kuala Pilah and Rembau, Peninsular Malaysia. All plants grew within 10 km along a roadside in a secondary forest. Myrmecophytes were *Macaranga triloba* (Bl.) Muell. Arg., *Macaranga hullettii* King ex Hook., *Macaranga hosei* King ex Hook., and *Macaranga hypoleuca* (Reichb. f. & Zoll.) Muell. Arg.; non-myrmecophytes were *Macaranga gigantea* Muell. Arg., *Macaranga heynei* I. M. Johnston and *Macaranga tanarius* (L.) Muell. Arg. Young plants of *M. tanarius* produce ant-attractant food (extrafloral nectar and food bodies). Because insects attracted facultatively to extrafloral nectaries can effectively reduce herbivory (Heil et al. 2001c), we sampled young, ant-attracting individuals and adult individuals which had lost this trait during their ontogeny separately and treated them hereinafter as two different 'species' for statistical and graphical purposes. We determined species determined according to Whitmore (1967, 1973), but *M. triloba* as described there probably must be re-named as *M. bancana* (Mig.) Muell. Arg. (T.C. Whitmore and S. J. Davies, pers. comm.; see also Slik 1998).

Sample collection, storage and preparation for chemical analysis

We sampled leaves of all *Acacia* and *Macaranga* specimens so as to obtain a mixture of age classes representing the natural distribution of age classes present on the plants. Each sample comprised leaves from several branches of one individual plant (see Figures for sample sizes). We excluded leaves bearing extensive mechanical or herbivore-caused damage. About 20 g fresh material were stored in 20 ml ethanol p.a. (100%) and transported in this form to Europe. The samples then were filtered; leaves were ground with sand and extracted in further 20 ml of ethanol p.a. After 15 min extraction in a sonar bath (Bransonic 220, Roucaire, France) this material was filtered and the filtrates were combined. Filtrates were diluted 1:10 (v/v) in methanol p.a. and were used for quantification of phenolic compounds as described below.

Leaves of the *Leonardoxa* samples were dried at ambient temperatures in the field and then were stored in silica gel. Leaf material was ground and extracted by aqueous (80%) acetone: aqueous (80%) methanol (1:1) (100 ml solvent per 3 g dry leaf powder). We filtered this material after 15 min extraction in a sonar bath and used these extracts for quantification of phenolic compounds as follows. Solvents were purchased from Merck (Darmstadt, Germany).

Chemical analysis

For quantification of condensed tannins, we further diluted the extracts 1:10 in methanol (1 ml extract + 9 ml methanol). Aliquots (100 µl) of the diluted extracts were thoroughly mixed with 1 ml DMCA solution (0.1% DMCA in methanol-HCl 9:1 v/v) following Mc-Murrough and McDowell (1978) and Treutter (1989). DMCA (4-dimethylaminocinnamaldehyde) was purchased from Fluka (Neu-Ulm, Germany). We measured absorption at 640 nm (reference: 1 ml DMCA solution mixed with 100 µl ethanol: methanol 1:10 (v/v)) after 5 min of colour development at room temperature in a UNICAM spectrophotometer (UV/Vis Spectrophotometer, UNICAM Ltd., Cambridge, UK), and calculated content of condensed tannins based on a calibration curve established with + catechin (Sigma, St. Louis, USA) as a standard (calibration curve: catechin [µg ml^{-1}] = $12.88 \times x^2 + 36.6 \times x$, r^2 = 0.997, x = absorption relative to 1 ml DMCA solution mixed with 100 µl ethanol: methanol 1:10 (v/v)). In contrast to other colorimetric methods used to quantify phenolic compounds (Martin and Martin 1982, Mole and Waterman 1987a, b, Schofield et al. 2001), the method used here has the advantage that the reaction product of DMCA with catechin shows an absorbance maximum between 632 and 640 nm, while other aldehydes lead to absorption at shorter wavelengths. The DMCA reagent therefore can be used for specific chemical reaction detection of catechin and proanthocyanidins (Treutter 1989).

For the quantification of flavonoids and hydrolysable tannins, we diluted 100 µl of the diluted extracts further in 1 ml methanol. After addition of 100 µl Neu's reagent ('Naturstoffreagent A', $C_{14}H_{16}BNO$, Roth, Karlsruhe, Germany) we measured absorption immediately at room temperature in a UV-1605 (UV-visible) spectrophotometer (Shimadzu, Kyoto, Japan) at 325 nm (hydrolysable tannins) and at 404 nm (flavonoids). Contents were calculated based on calibration curves with gallic acid (purchased from Sigma, St. Louis, USA) and luteolin (purchased from Extrasynthèse, Genay, France) as standards (calibration curves: luteolin [µg ml^{-1}] = $2.72 \times x$, r^2 = 0.989 and gallic acid [µg ml^{-1}] = $0.479 \times x$, r^2 = 0.996, x = absorption relative to 100 µl Neu's reagent mixed in 1 ml methanol).

Sample collection and extract preparation for biotests

We collected additional leaf material of all mimosoid species mentioned above at the same days and from the same individuals as the material for chemical analysis. Leaves to be used for biotests were dried in the dark at air temperatures between 25 and 35°C within 48 h to constant weight, first openly and then in air-tight boxes over silica gel. We then transported this material to Germany, ground it with an IKA MF 10 mill (IKA GmbH, Germany) to fine powder and sieved it (mesh size 1 mm).

We extracted three grams of dry powder in 40 ml methanol (48 h at ca 25°C). After filtration, the filtrate was re-extracted twice, each time using 20 ml methanol. We combined these three extracts and evaporated at 40°C and under light vacuum (continuously decreasing from ca 400 mbar to 150 mbar). The residue was re-dissolved in 40 ml methanol.

Biotests with caterpillars

For the tests on biological activity against herbivores, we used neonates (newly hatched first-instar caterpillars) of Egyptian cotton worm, *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae). This species is a generalist herbivore that is not expected to show specific adaptations to the defensive compounds of *Acacia*. The animals had been reared for at least four generations on artificial diet consisting of finely homogenised white beans saturated with an equal amount (v/v) of water. To this diet, we added 18 mg g^{-1} ascorbic acid, 18 mg g^{-1} Nipagin, 8 mg g^{-1} formaldehyde and 1 mg g^{-1} Gentamycin to inhibit bacterial and fungal infection.

We used the same diet for the biotests. Three ml of extract were allowed to dry overnight at room temperature, and the residue was mixed thoroughly with 3 g of diet in order to create a homogenous distribution of tannins in the artificial diet. We placed twenty neonates on this food for a period of two weeks and added each day some water (ca 0.5 ml) to keep the food moist. The number of surviving caterpillars was recorded and their total weight was measured after two weeks. Controls (20 caterpillars on 3 g diet without extract) were kept in the same rooms and for the same time span. We calculated relative weight gain by subtracting the total weight of control caterpillars from the total weight of caterpillars fed with extract-containing diet. Three replicates per extract and three controls were performed and their results were averaged. Results of biotestes using the same methods and the same herbivore have already been reported for the *Macaranga* species investigated here (Eck et al. 2001).

Biotests with fungi

For the tests on biological activity against fungi we used cultures of *Gliocladium roseum* (Link) Bainier (Ascomycota: Pezizomycotinaseae, see Schroers et al. 1999 for a recent taxonomic re-classification and ecological description) reared in glass tubes on agar medium (30 g agar, 15 g malt extract and 10 g potato-dextrose agar in 1 l water). *G. roseum* is a widespread, soilborne, mycoparasitic fungus occurring both on living plants and in decomposing plant material and capable of growing on leaves and flowers of a broad spectrum of plant species. *G. roseum* is widely used as biocontrol agent (Perello et al. 1997, Yu and Sutton 1997). Due to its ecology, no specific adaptations to chemical defences of *Acacia* were to be expected.

Preliminary experiments were conducted to check for the ecological relevance of the method as applied here. We prepared spore suspensions as described below and tested the activity of several fungicides and of extracts of several plant species which differed in the level of their phenotypic resistance against fungal infections. Both extracts and fungicides were used in different concentrations. The results of the biotest described here matched the expected patterns very well, both with respect to dose-response relations and with respect to the known anti-fungal resistance of the used plant species (unpubl. data).

To prepare spore suspensions, 10 ml of sterile water were put daily into the culture tube and shaken gently. We checked the resulting suspension of spores for spore density and if necessary diluted further with sterile water to adjust to a final density of 10^5 to 10^6 spores ml^{-1}. We diluted extracts 1:10 with water. Aliquots of 20 µl were placed in microtiter plates and mixed with 180 µl spore suspension (three replicates per sample).

Controls consisted of 180 µl spore suspension mixed with 20 µl of methanol: water (1: 10 v/v). After incubation in the dark at 30°C for 24 h, we counted numbers of germinated and non germinated spores using Malassez counting chambers (10 cells each containing ca 25–30 spores were counted per sample). From these data we calculated relative germination rates and averaged them for each sample.

Biotests with seeds

Cress (*Lepidium sativum* L., Brassicaceae) seeds were used for the biotests on allelopathic effects due to their rapid and easy germination. These tests were conducted on the same extracts as those used for the tests on caterpillar growth, but were limited to the subset of mimosoid species collected in 2000. We prepared four different dilutions of extracts and one control consisting of pure methanol to test for biological activity. To maintain equivalency of amounts of methanol used in all casses (1 ml), 0, 100, 250, 500 and 1000 µl of extract were mixed with 1000, 900, 750, 500, and 0 µl of methanol, respectively. We conducted three replicates per sample and per extract concentration as follows. Round pieces of filter paper were placed in petri dishes, and 1 ml of the diluted extracts was pipetted on this filter paper, which was allowed to dry for at least 1 h. We then moistened the filter paper with 2 ml water and placed 20 seeds on it. The petri dishes were closed, covered for 1 d with dark foil, and kept at room temperature (approximately 22°C) for a total of 3 d. The number of germinated seeds was then counted and relative germination rates calculated for each sample. Mean values were then calculated for the three replicates conducted per sample and per extract concentration.

Statistical analysis

We analysed the data separately for each taxonomic group within which differences among myrmecophytes and non-myrmecophytes were to be expected. Significant effects of species were tested using a univariate ANOVA, and post-hoc tests (LSD) were conducted to determine which species pairs significantly contributed to the overall effects. Data were tested in advance whether they met the requirements of the tests applied. All calculations were conducted with SPSS 10.1

Results

Acacia and related Mimosoideae

Hydrolysable tannins occurred in all species investigated (Fig. 1A). Their average content ranged from ca

1 mg g^{-1} FW in *P. juliflora* up to 4 mg g^{-1} FW in *L. leucocephala*. Both extreme values were thus found in the group of non-ant-plants. The 'species' was a source of significant variation in the data set (univariate ANOVA: n = 94, df = 11, F = 8.27, p < 0.001).

We detected flavonoids in six species (Fig. 1B), four of which were ant-acacias. In the other six species or populations (one ant-acacia, three non-ant-acacias, *L. leucocephala* and *P. juliflora*) we could detect no flavonoids by the method used. Their absence was confirmed by TLC analysis (data not shown). The 'species' was a source of significant variation in the data set (univariate ANOVA: n = 94, df = 11, F = 56.46, p < 0.001). Average (median) amounts present in the leaves of different species ranged from 5.1 mg g^{-1} FW (fresh weight) in the non-myrmecophyte *M. tenuiflora*

up to about 54 mg g^{-1} FW in the non-ant-acacia *A. macracantha*. Thus, as for hydrolysable tannins, non-myrmecophytes included both extreme values. Amounts found in the four flavonoid-containing ant-acacias were between these two extreme values.

Though being present in all species, contents of condensed tannins varied strongly among the single samples within species, and among different species investigated in this study (Fig. 1C). The 'species' was a source of significant variation in the data set (univariate ANOVA: n = 94, df = 11, F = 10.93, p < 0.001). Lowest contents of condensed tannins were found in leaves of *A. farnesiana* (mean of seven samples: 0.02 mg g^{-1} FW). Similarly low values appeared in the ant-acacia *A. cornigera*. Highest contents were found in *A. macracantha* (on average 29 mg g^{-1} FW).

We could detect no systematic differences between ant-acacias and non-ant-plants in any of the three classes of substances investigated (Fig. 1).
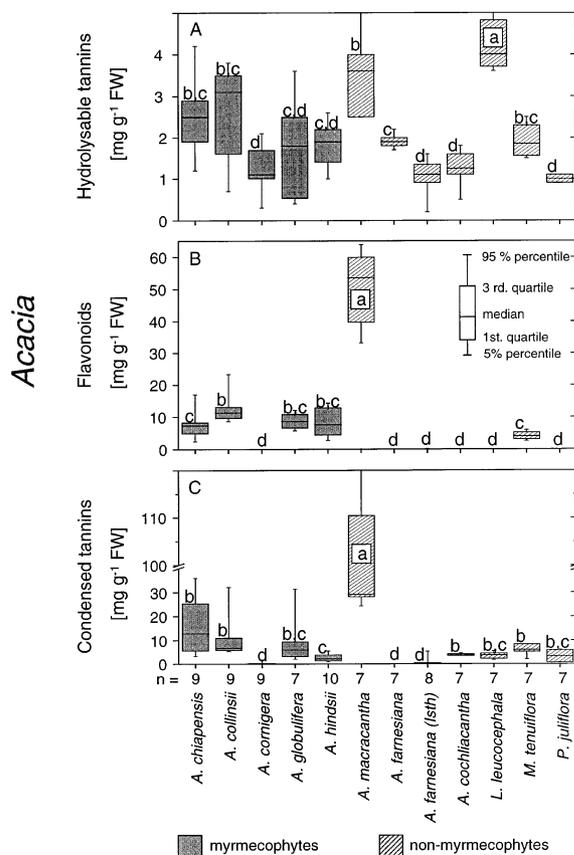

Fig. 1. Contents of phenolic compounds (A. hydrolysable tannins, B. flavonoids, C. condensed tannins) in leaf extracts of different myrmecophytic and non-myrmecophytic *Acacia* species and closely related non-myrmecophytic Mimosoideae. Species (or populations in the case of *A. farnesiana*) differing significantly from each other (p < 0.05 according to LSD post-hoc tests, see 'Results' section for results of univariate ANOVAs) are marked by different letters on the top of boxes. See insert in B for explanation of boxplots. Sample sizes (n) appear at the bottom of Fig. C.

Leonardoxa

We detected no hydrolysable tannins and no flavonoids in the studied *Leonardoxa* samples. The absence of these compounds was confirmed by TLC analysis (results not shown). In contrast, condensed tannins were present in all samples. Average contents in the populations of the myrmecophyte, *L. a. africana*, ranged from 2.76 mg g^{-1} DW to 5.53 mg g^{-1} DW, while leaves of different populations of the non myrmecophyte, *L. a. gracilicaulis*, contained on average 3.45 mg g^{-1} DW up to 28.52 mg g^{-1} DW. The 'population' was a source of significant variation in the data set (univariate ANOVA: n = 53, df = 8, F = 40.85, p < 0.001). Three of the four *L. a. gracilicaulis* populations differed significantly from the fourth population and from all populations of *L. a. africana* (Fig. 2).

Macaranga

In general, the investigated *Macaranga* species had lower contents of all measured phenolic compounds than the *Acacia* species and other Central American Mimosoideae (Fig. 3). Average (median) contents of hydrolysable tannins ranged from 0.2 mg g^{-1} FW in *M. triloba* to 0.9 mg g^{-1} FW in *M. hypoleuca* (Fig. 3A). Both species are myrmecophytes. Average flavonoid contents (Fig. 3B) ranged from less than 1 mg g^{-1} FW in the myrmecophyte *M. hosei* and the non-myrmecophyte *M. gigantea* to ca 18 mg g^{-1} FW in the non-myrmecophyte *M. heynei*. Very low contents of condensed tannins (on average less than 0.2 mg g^{-1} FW) were present in leaves of four 'species', namely *M.*
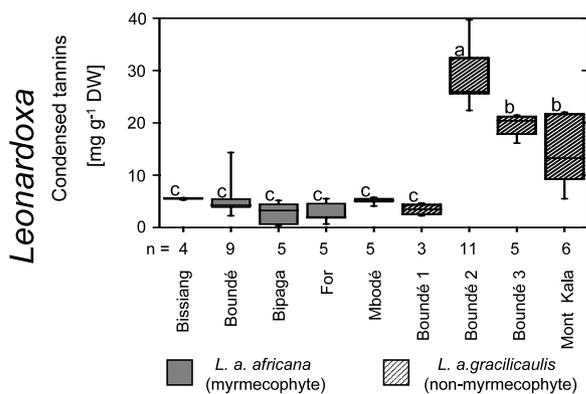
Fig. 2. Contents of condensed tannins in leaf extracts of different populations of the myrmecophyte, *L. a. africana*, and of the non-myrmecophyte, *L. a. gracilicaulis*. Populations differing significantly from each other (p < 0.05 according to LSD post-hoc tests, see 'Results' section for results of univariate ANOVAs) are marked by different letters on the top of boxes. See insert in Fig. 1B for explanation of boxplots. Sample sizes (n) appear at the bottom of the figure.

hulletti, *M. triloba*, *M. tanarius* 'young' and *M. tanarius* 'adult' (Fig. 3C). The first two species are myrmecophytes, while *M. tanarius* is a non-myrmecophyte. Considerable tannin contents were found in leaves of *M. heynei* (ca 4 mg g^{-1} FW), *M. gigantea*, *M. hypoleuca* (both ca 5.5 mg g^{-1} FW) and *M. hosei* (ca 21.5 mg g^{-1} FW). The two first species are non-myrmecophytes, the two latter myrmecophytes.

'Species' was a source of significant variation in the data sets on flavonoids (univariate ANOVA: n = 40, df = 7, F = 20.45, p < 0.001) and condensed tannins (univariate ANOVA: n = 40, df = 7, F = 36.31, p < 0.001), but not for hydrolysable tannins (univariate ANOVA: n = 40, df = 7, F = 1.93, p = 0.099). No systematic difference between myrmecophytes and non-myrmecophytes could be detected (Fig. 3). Contents of flavonoids and condensed tannins were nearly identical in leaves of 'young' and 'adult' *M. tanarius* plants, while the contents of hydrolysable tannins showed a slight, but non-significant tendency to higher concentration in leaves of the 'adult' plants.

Biotests with mimosoid species

We observed strong differences among extracts of the various mimosoid species in the biological activity against spore germination (Fig. 4B); among-species variation was significant (n = 94, df = 11, F = 4.010, p < 0.001). Less extreme (but still highly significant: n = 94, df = 11, F = 5.043, p < 0.001) differences were observed in the effects on caterpillars (Fig. 4A). Extracts of all species tested had a strong inhibitory effect on seed germination, i.e. the negative effect on seed germination increased with the amount of extract added to germinating seeds (Fig. 5A).

Yet, as in the data on phenolic compounds we saw no systematic differences between ant-plants and non-ant-plant species. Strongest inhibitory effects on caterpillar growth were exhibited by extracts of the two non-ant-acacia species *A. farnesiana* (Isth) and *A. macracantha*, while extracts of two other non-ant-species (*A. cochliacantha* and *L. leucocephala*) had the most positive effects on caterpillar growth. Similarly, spore germination was most strongly inhibited by extracts of the non-ant-acacia *A. macracantha*, while extracts of the non-ant-species *A. farnesiana* and *P. juliflora* had nearly no effect on germination rates. The significant differences in effects on seed germination (ANOVA for results after addition of 1000 µl extract: n = 7, df = 9, F = 3.15, p = 0.003) resulted only from the fact that *A. farnesiana* differed significantly from all other species (Fig. 5B).
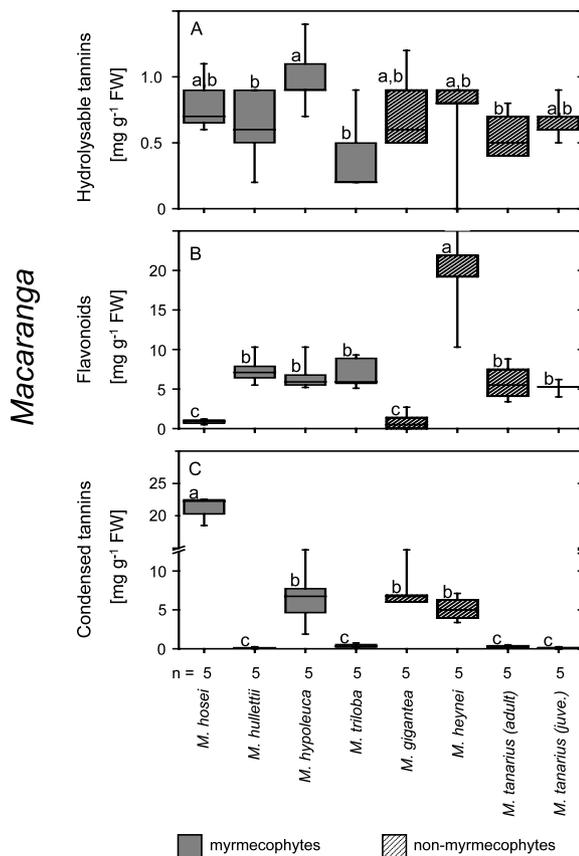

Fig. 3. Contents of phenolic compounds (A. hydrolysable tannins, B. flavonoids, C. condensed tannins) in leaf extracts of different myrmecophytic and non-myrmecophytic *Macaranga* species. Species (or ontogenetic stages in the case of *M. tanarius*) differing significantly from each other (p < 0.05 according to LSD post-hoc tests, see 'Results' section for results of univariate ANOVAs) are marked by different letters on the top of boxes. See insert in Fig. 1B for explanation of boxplots. Sample sizes (n) appear at the bottom of Fig. C.
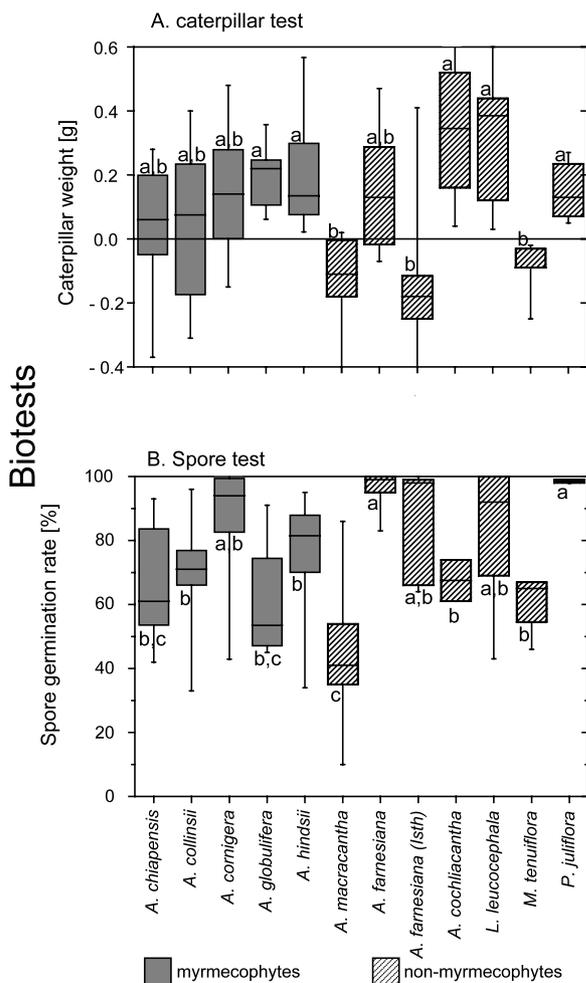
Fig. 4. Effects of extracts of different myrmecophytic and non-myrmecophytic *Acacia* species and closely related non-myrmecophytic Mimosoideae on growth of *Spodoptera* caterpillars (A) and spore germination of the fungus *Gliocladium roseum* (B). Results are expressed as difference in mean weight of caterpillars reared for 14 days on extract-containing food and of control caterpillars reared on extract-free food in A, and as percentage germination rate of spores after 24 h in extract-containing spore suspension in B. Species (or populations in the case of *A. farnesiana*) differing significantly from each other (p < 0.05 according to LSD post-hoc tests, see 'Results' section for results of univariate ANOVAs) are marked by different letters. See insert in Fig. 1B for explanation of boxplots. Sample sizes (n) appear at the bottom of Fig. 1C.

Discussion

Our results are in striking contrast to the hypothesis that ant-plants have reduced their chemical defence in order to avoid 'superfluous' costs resulting from redundant defences. We quantified three classes of phenolic compounds (hydrolysable tannins, flavonoids, and condensed tannins) in the foliage of 20 species belonging to the unrelated genera *Acacia*, *Leonardoxa* and *Macaranga*. In *Acacia* and *Macaranga*, their contents

varied strongly among species (see Fig. 1 and 3), indicating a large within-genus variability. This variability appears to be largely independent of phylogenetic relationships. *M. triloba*, *M. hullettii* and *M. hypoleuca* are all members of a single clade within the genus *Macaranga*, distinct from those including the other species (Blattner et al. 2001, Davies et al. 2001). Similarly, the ant-acacias *A. chiapensis* and *A. globulifera*
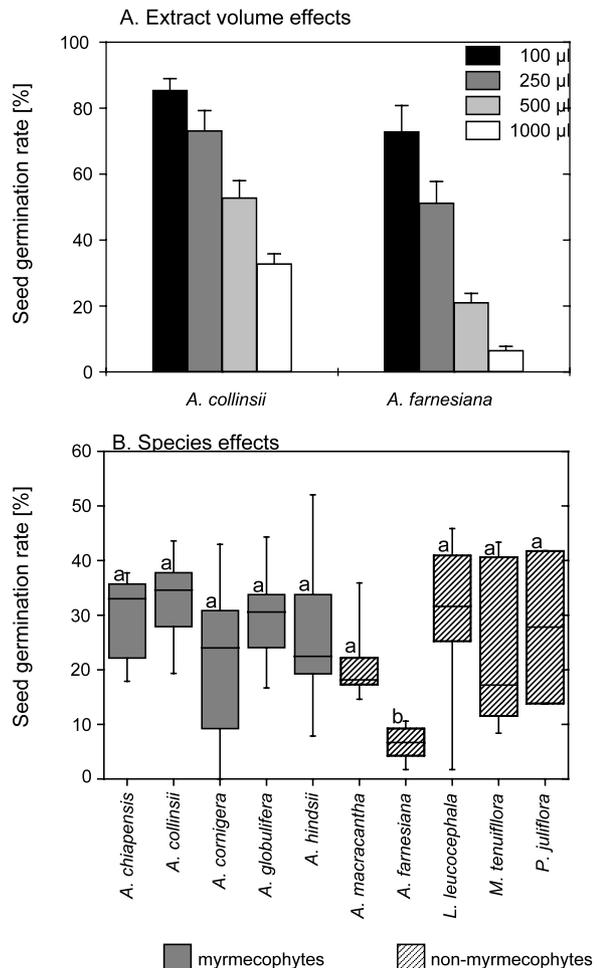

Fig. 5. Effects of extracts of different myrmecophytic and non-myrmecophytic *Acacia* species and closely related non-myrmecophytic Mimosoideae on germination of seeds of *Lepidium sativum*. Results are given in percentage of seeds germinated. (A) Dose-response relation in effects of different amounts of extracts of two representative species (note that the same amount of solvent, methanol, has been added in all cases). Patterns of effects were similar for all other species (data not shown). Sample size (n) = 9 for *A. collinsii* and 7 for *A. farnesiana*. (B) Effects of the highest amount of extract tested (1000 µl, the most right column in A) for all Mimosoideae tested. Species (or populations in the case of *A. farnesiana*) differing significantly from each other (p < 0.05 according to LSD post-hoc tests, see 'Results' section for results of univariate ANOVAs) are marked by different letters. See 'Results' section for results of univariate ANOVAs and insert in Fig. 1B for explanation of boxplots. Sample sizes (n) appear at the bottom of Fig. 1C.

and the non-ant-species *A. macracantha*, *A. farnesiana* and *A. cochliacantha* are characterised by globose inflorescences and thus might form a phyletic group within the genus, distinct from species with elongate inflorescences such as *A. cornigera*, *A. collinsii* and *A. hindsii* (Janzen 1974). In a recent taxonomic revision, *A. macracantha* and *A. cochliacantha* were placed close to the ant-acacias (Seigler and Ebinger 1995). Again, closely related species showed great variation in contents of phenolics. Within the group with elongate inflorescences, for example, flavonoids and condensed tannins were present in high amounts in *A. collinsii* and *A. hindsii*, but were nearly or completely absent in *A. cornigera*.

The considerable variability in phenolic contents among these related species indicates that phylogeny imposes but little constraint on the evolution of the traits studied here. Yet, no clear pattern emerged in any of these genera that would point to a difference in phenolic chemical defence between myrmecophytes and non-myrmecophytes. Similarly, biological activity of plant extracts against a generalist herbivorous caterpillar (*Spodoptera littoralis*), against spore germination of an ecologically widespread fungus (*Gliocladium roseum*) and against germination of cress seeds varied strongly among the tested mimosoid species (Fig. 4 and 5). As in the study by Rehr et al. (1973), the quantified chemical defence could not explain differences in biological effects on caterpillars (compare Fig. 1 to Fig. 4A). The same holds for effects against seed germination (Fig. 5). In contrast, contents of condensed tannins and flavonoids (Fig. 1C and Fig. 1B) appear to give a reverse-image pattern compared to spore germination rates (Fig. 4B). We therefore conclude that phenolic compounds in the investigated mimosoid species might have anti-pathogen rather than anti-herbivore effects. More importantly in the context of the present study, the screenings for biological activity revealed strong differences among the investigated species which, however, could not be related to the presence or absence of ant-plant mutualisms.

It was only for *Leonardoxa africana* that three out of four populations of the non-myrmecophyte, *L. a. gracilicaulis*, had significantly higher contents of condensed tannins than the five populations of the myrmecophyte, *L. a. africana* (Fig. 2). However, even in this case tannin contents in leaves of the 'Boundé 1' population of *L. a. gracilicaulis* were within the range covered by *L. a. africana*. Our data thus do not support the widely accepted hypothesis of trade-offs between chemical and biotic anti-herbivore defence.

A critical re-evaluation of published studies indicates that existing data also give a pattern that is less clear than generally assumed. Only three out of the eight studies on 'trade-offs' between chemical and biotic defence in ant-plants which are reviewed here have revealed unequivocally clear patterns (Heil et al. 1999,

2000, Dyer et al. 2001). No trade-offs between ant-mediated and direct defence were reported by Steward and Keeler (1988) and Letourneau and Barbosa (1999). In the study of Eck et al. (2001) only three out of six 'early' myrmecophytes (i.e. species that are inhabited by resident ants already at the seedling stage) and none of the six 'late' myrmecophytes (i.e. species developing myrmecophytic traits later during their ontogeny) differed significantly from the four non-myrmecophytes in tannin content. No systematic difference among these functional groups appeared in biotests conducted to determine effects of caterpillar growth rates (Eck et al. 2001). Rehr et al. (1973) regarded *A. chiapensis* as a non-ant-acacia, though individuals in a considerable part of naturally occurring populations develop hollow thorns, produce ant food and in many cases are inhabited by resident ant colonies on which they may partly rely for survival (Janzen 1974, and M. Heil, pers. obs). Two of the ant-acacias investigated by Seigler and Ebinger (1987) were polymorphic in their chemical defence, in that 116 of 280 specimens (*A. hindsii*) and 62 of 66 specimens (*A. globulifera*) were cyanogenic. Furthermore, all samples of *A. chiapensis* investigated by these authors showed active cyanogenesis. Though it can be discussed whether this species should be regarded as an ant-acacia or a non-ant-acacia (see above), the picture is not clarified by placing it in either of the two groups, in the data set presented by Seigler and Ebinger (1987) or in our study (Fig. 1).

The hypothesis that ant-plants reduce all their chemical defences obviously represents a severe oversimplification. Yet, it remains true that for a taxonomically and ecologically diverse set of ant-plants many studies have found a strikingly low defensive ability of obligate myrmecophytes when these are deprived of their ants (for the genera investigated here see Janzen 1966, 1967b, 1974, McKey 1984, Fiala et al. 1989, 1994, Gaume et al. 1997, Heil et al. 2001a). One possible explanation for this situation is that the studies focused on trade-offs have not determined those direct defences that are responsible for the considerable differences in defensive ability among ant-plants and non-ant-plants evident in experimental studies in natural populations. Studies published so far have concentrated on one or a few classes of chemical defence compounds. However, trade-offs in the expected context can occur between indirect, 'biotic' defences and all forms of direct defences, as long as they are functionally redundant or make use of the same limited resources. Direct defence might be achieved by defensive chemicals, but it can also result from mechanical traits. Janzen (1966) observed that "the new green shoot tips of swollen-thorn acacias lack the fibrous material which makes the shoot tips of other acacias very tough and resistant.... presumably to insect feeding". By concentrating on chemical defence instead of all direct defences, most of the studies that have attempted to test Janzen's (1966)

hypothesis thus might have used the 'wrong' defensive traits. For example, in a study of 46 rain forest tree species in Panama, Coley (1983) found that herbivory was strongly negatively correlated with toughness, while there was only a non-significant tendency in the same direction for total phenolics. Costs of high fibre contents (in either metabolic terms, or the constraints imposed by lignification of young tissue) that are not incurred in ant-acacias are indicated by Janzen's observation that "The shoot tips of other acacias grow much more slowly" (Janzen 1966).

Moreover, many secondary compounds have multiple effects. Redundancy in one of these effects thus does not necessarily lead to selection favouring a quantitative reduction, as long as these compounds perform other, vitally important functions. Phenolics might be active in defence against pathogens (Hammerschmidt 1999a), and they have important roles in defence against many physical stresses, such as ultraviolet radiation (Shirley 1996, Bieza and Lois 2001). In all studies published thus far, clear patterns have been found only for very specific compounds such as chitinases (Heil et al. 1999, 2000) and amides (Dyer et al. 2001). For these compounds, plausible alternative explanations can be formulated as to why they are reduced in ant-inhabited myrmecophytes. Chitinases might have detrimental effects on resident ants, and their reduction in ant-plants may function to avoid these effects rather than to avoid production of redundant defences (Heil et al. 1999). The same might be true for the amides investigated by Dyer et al. (2001), since it was demonstrated in the same study that these compounds have deterrent effects on ants.

Conclusions

Although the reduced direct defence of obligate ant-plants has been demonstrated in many independent field studies, most studies focusing on distinct chemical defences have found no clear evidence of trade-offs between biotic and chemical defence in ant-plants. A widespread phenomenon is thus still in search of an explanation. Based on the published data we can formulate two alternative hypotheses. (i) By focusing on some selected chemical defences most studies conducted so far have disregarded mechanical traits. Because the latter appear to be costly, they may be subject to strong counterselection if they become redundant. (ii) The maintenance of very low contents of some chemical defences (chitinases and amides) in obligate myrmecophytes might be necessary to avoid negative effects of these compounds on resident ants.

Further studies taking into account larger sets of putative defensive traits (and distinguishing between the different defensive and other functions of the regarded traits) are required to decide whether the present study, and others that have failed to demonstrate clear trade-offs between biotic and direct defence, have simply concentrated on the 'wrong' defensive traits, or whether the hypothesis must be restricted to very specialised defences which however have strong effects on herbivory under natural conditions.

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