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Intra- and Interspecific Variations in the Balance between Ant and Non-Ant Defenses in *Macaranga*

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Abstract - We measured inter- and intraspecific variations in the balance between ant and non-ant defenses in *Macaranga* (Euphorbiaceae) species. We found that there were wide inter- and intraspecific variations in the intensities of ant and non-ant defenses, and that there was a negative correlation between the intensities of ant and non-ant defenses. These results suggest that the two modes of defense mechanisms face a trade-off between them under resource limitation.

I. Introduction

Plants have evolved various mechanisms for anti-herbivore defense. Many plant species are known to depend on other organisms for anti-herbivore defense [1 - 4]. Some plant species utilize ants for anti-herbivore defense [3, 5 - 7]. In this type of defense system, plants attract or nourish ants, and in turn get protection from attacks by herbivores. The plants that provide nest sites for ants and have symbiotic relationship with them are called myrmecophytes [8]. Here, anti-herbivore defenses with ants are called ant defenses.

In contrast, it is widely known that plants defend themselves against herbivores by using chemical defenses, such as toxins and repellents, and by physical ones, such as trichomes and thick cell walls [9 - 11]. We call these types of defense mechanisms non-ant defenses, as opposed to ant defenses.

Both ant and non-ant defense mechanisms require metabolic costs. For the maintenance of ant defenses, plants have to provide their symbiont ants with food that contains large amounts of lipids, amino acids and carbohydrates [12 - 16]. For the maintenance of non-ant defenses, plants have to synthesize secondary metabolic compounds, including toxins and fibers [17, 18]. High investment in each anti-herbivore defense mechanism may increase the efficiency of the defense mechanism against herbivores, however, it does not always increase a plant's fitness because limited resources are diverted from other life history aspects, such as growth and reproduction [18, 19 - 21].

How do plants balance the two costly but contradictory

defense mechanisms? Janzen [22] hypothesized that non-ant defenses have been lost in the myrmecophyte, because maintenance of both ant and non-ant defenses places an unnecessary metabolic burden on the plants.

The genus *Macaranga* consists mainly of pioneer trees and diversified mainly in tropical areas of Southeast Asian [23, 24]. The genus is known for a wide variation in the intensity of mutualistic relationship with ants from non-myrmecophytes to facultative, transitional and obligate myrmecophytes [25-28]. Obligate myrmecophytes provide nest sites and food bodies for their symbiont ants, and in turn, they benefit from protection offered by the ants against herbivores [25 - 32].

Obligate myrmecophytic species without ant colonies suffer serious herbivory damage [31]. Thus, both obligate myrmecophytes and their symbiont ants depend on each other for their survival. However, even obligate myrmecophytic species do not always maintain symbiosis with ants throughout their life cycle. In *Macaranga*, symbiosis with ants starts in young seedlings when foundress ant queens colonize the seedlings inside the hollow stems. About one month after settlement by ant queens, adult ant workers emerge from the hollow stems and then start defending the host plants. If obligate myrmecophytic *Macaranga* defend themselves perfectly against their herbivores throughout their life cycle, it is predicted that they defend themselves mainly by non-ant defenses before the beginning of colonization by symbiont ants.

The purpose of this study is to examine whether Janzen's hypothesis [22] of a trade-off between ant and non-ant defenses is applicable to inter- and intraspecific variations in anti-herbivore defense strategies in *Macaranga*. Our hypotheses are:

1. The intensity of ant defenses is lower in the *Macaranga* species in which non-ant defense is more intensive.
2. Myrmecophytic *Macaranga* defend themselves by intensive non-ant defenses before symbiosis with ants, and that the intensity of non-ant defenses decreases after the symbiont ant colonies become established.

II. Study Site

This study was conducted in a lowland mixed dipterocarp forest in Lambir Hills National Park, Miri, Sarawak, Malaysia (4°2'N, 113°50'E, altitude 50 - 150m). The region is situated in the humid tropics with no pronounced dry season and seasonal variation in temperature [33, 34].

III. *Macaranga*

At least 15 species of *Macaranga* occur at the study site [35], and at least nine species are myrmecophytes. We focused on ten species, *Macaranga winkleri* Pax. and Hoffm., *M. trachyphylla* Airy Shaw, *M. bancana* (Miq.) Muell. Arg., *M. hypoleuca* (Reichb. F. and Zoll.) Muell. Arg., *M. beccariana* Merr., *M. lamellata* Whitmore, *M. kingii* Hook., *M. hullettii* King ex Hook., *M. gigantea* (Reichb. F. and Zoll.) Muell. Arg. and *M. praestans* Airy Shaw. The former eight species are obligate myrmecophytes, and the latter two species are non-myrmecophytes. Myrmecophytic species provide nest sites and food bodies for their symbiont ants. Symbiont ants patrol the leaves of their host plants and protect them from herbivores [25 - 32]. Most of the ant species that are symbionts of *Macaranga* belong to *Crematogaster* (Hymenoptera: Formicidae: Myrmicinae) [27, 36]. The partnership between *Macaranga* myrmecophytes and symbiont ants is highly species-specific [28, 36]. Symbiont ants use food bodies as their main food and they seldom leave their host plants. Obligate myrmecophytes cannot survive without symbiosis with their specific ants, and almost all seedlings 40 cm or more in height of obligate myrmecophytic species harbor symbiotic ant colonies in the field. However, even obligate myrmecophytes do not always maintain symbiosis with ants throughout their life. In *Macaranga*, symbiosis with ants starts when seedlings reach about 10 - 30 cm in height. At that time, the stems begin to swell and the piths degrade so that foundress ant queens can settle inside the hollow stems. After confining themselves within the stems, the ant queens begin to feed their initial ant workers with regurgitated food. About one month after queen settlement, the initial ant workers emerge from the stems as adults and start defending the host plant. We defined three growth stages of seedlings (saplings) with reference to the time of initiation and establishment of symbiosis with ants. The first stage is the "pre-symbiosis" stage, when seedlings have not yet received ant queens. Seedlings about 10 - 30 cm in height were used to represent plants in this growth stage. The second is the "pre-defense" stage, soon after the initial ant workers emerge but before ant defense has well established. Seedlings about 40 cm in height and with only one exit hole on the stem were used to represent plants in this growth stage. Saplings about 1.5 - 2.2 m in height were used to represent plants in the "ant-defending growth stage, a stage when saplings are defended by many ant workers.

M. gigantea and *M. praestans* are non-myrmecophytic species. They often attract non-specific ant species with

extrafloral nectar (EFN), which are secreted from leaf surface. Ants that are attracted to EFN also defend the plants from herbivores, although the effectiveness of the defense is remarkably weaker than that in myrmecophytic species [29]. In *M. praestans*, only newly developing leaves have EFN, which then deteriorate as the leaves mature. To measure the intensities of non-ant defenses, leaves collected from 1.5 - 2.2 m tall saplings of *M. praestans* and 3 - 8 m tall saplings of *M. gigantea* were used.

Although there is interspecific variation in habitat preference with reference to light intensity among eight myrmecophytic species [37], their habitats overlap closely each other. Five myrmecophytic species, *M. winkleri*, *M. bancana*, *M. trachyphylla*, *M. hypoleuca*, *M. beccariana*, prefer moderate shade-intensity conditions and they are observed at treefall gaps and riversides in the forest. Three myrmecophytic species, *M. kingii*, *M. lamellata* and *M. hullettii*, prefer slightly shadier conditions than do the other myrmecophytic species. Therefore, the former five species are called "shade-intolerant myrmecophytes" and the later three species are called "shade-tolerant myrmecophytes". *M. gigantea* prefers slightly brighter conditions, although its habitat also overlaps closely with that of the shade-intolerant myrmecophytes. *M. praestans* prefers much shadier conditions than do the other species, such as the forest floor.

We randomly selected seedlings (saplings) that showed no obvious damage. Only mature apical leaves of each selected seedling (sapling) were used for the measurements of the intensities of non-ant defenses.

IV. Interspecific Variation in the Intensity of Ant Defense

Interspecific variation in the intensities of ant defenses in *Macaranga* has been partly described [16, 25 - 32, 39]. Itioka et al. [31] and Nomura et al. [38] measured ant aggressiveness to artificial damage on host plants at the ant-defending stage. There were significant differences in all measures of ant aggressive behaviors among eight species of obligate myrmecophytes (Table 1). Ant aggressiveness on the five shade-intolerant myrmecophytes tended to be higher than that on the three shade-tolerant myrmecophytes. There were significant differences in ant aggressive behaviors even within five shade-intolerant myrmecophytes (Kruskal-wallis test, adjusted $H = 46.095, 44.409, 37.510$ and 44.312 for aggregated ants at leaf tip, climbers, workers on the hand, and biters, respectively; $P < 0.0001$ for all; Table 1), and three shade-tolerant myrmecophytes (Kruskal-wallis test, adjusted $H = 6.825$ and 6.300 for climbers and workers on the hand, respectively; $P < 0.05$; Table 1). To estimate the intensity of ant defense, the sum of the

Table 1 The average number of ants that showed each of four type of behaviors when we cut off a leaf tip (1 cm²) and put it close to the hole in second node by holding it with forceps (\pm SD) (adopt from Nomura et al. [38]).

Species	No. of ants				
	Total	Aggregated at leaf tips	Climbers	On the hand	Biters
<i>M. winkleri</i>	86.1 \pm 36.0	38.6 \pm 13.3	26.9 \pm 13.8	18.4 \pm 10.4	2.2 \pm 1.6
<i>M. trachyphylla</i>	33.3 \pm 17.0	21.7 \pm 12.3	9.3 \pm 4.9	2.0 \pm 2.0	0.3 \pm 0.5
<i>M. beccariana</i>	17.7 \pm 18.7	7.8 \pm 4.2	7.7 \pm 11.8	1.9 \pm 3.6	0.2 \pm 0.6
<i>M. bancana</i>	13.4 \pm 13.7	9.2 \pm 6.2	3.3 \pm 5.6	0.9 \pm 2.3	0
<i>M. hullettii</i>	11.1 \pm 9.6	8.6 \pm 7.9	2.1 \pm 1.7	0.4 \pm 0.6	0
<i>M. hypoleuca</i>	9.9 \pm 5.8	7.2 \pm 4.1	1.8 \pm 1.7	0.9 \pm 1.2	0
<i>M. kingii</i>	5.6 \pm 6.0	5.0 \pm 5.7	0.6 \pm 0.7	0	0
<i>M. lamellata</i>	3.0 \pm 1.4	2.3 \pm 1.0	0.8 \pm 1.0	0	0

The types of behaviors were as follows: aggregating around the leaf tips, climbing on the forceps, walking on the hand of the experimenter who held the tips with forceps, and biting the experimenter's hand. There were significant differences among the eight species (Kruskall-Wallis test, $H = 58.68$ for Aggregated at leaf tip, $H = 64.48$ for Climbers, $H = 53.50$ for On the hand, and $H = 66.62$ for Biters, respectively; $P < 0.0001$).

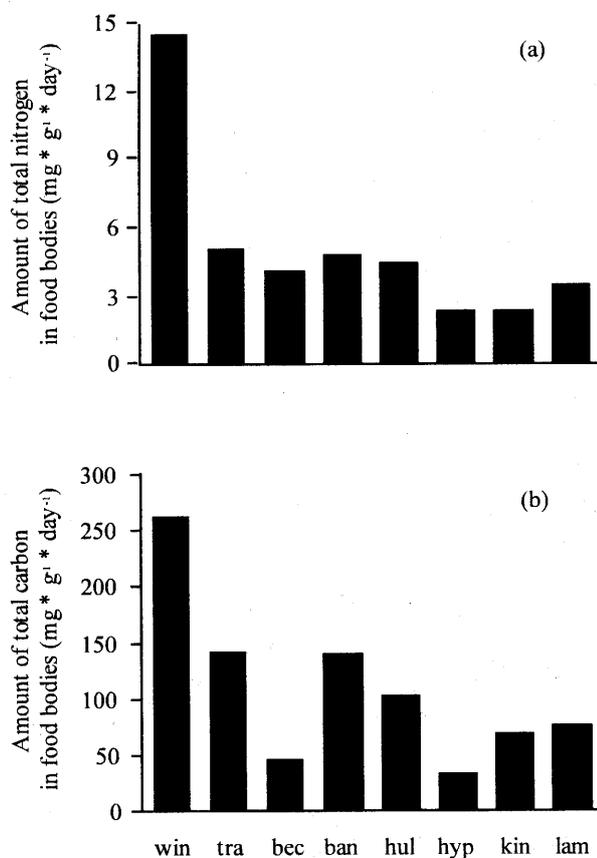


Fig. 1. Total amounts of carbon and nitrogen in food bodies per day per leaf biomass in the eight *Macaranga* species, determined by the production rate of food bodies and nitrogen and carbon contents of food bodies: (a) nitrogen, (b) carbon. win = *M. winkleri*; tra = *M. trachyphylla*; bec = *M. beccariana*; ban = *M. bancana*; hul = *M. hullettii*; hyp = *M. hypoleuca*; kin = *M. kingii*; lam = *M. lamellata*. Redrawn from Hatada et al. [16].

average number of ants that showed any of the four types of behavior was calculated (i.e., total in Table 1). The order of these values from highest to lowest was as follows: *M. winkleri*, *M. trachyphylla*, *M. beccariana*, *M. bancana*, *M. hullettii*, *M. hypoleuca*, *M. kingii* and *M. lamellata*.

Hatada et al. [15, 16] reported that there were interspecific variations in the total amounts of nitrogen and carbon in food bodies per day per leaf biomass among eight myrmecophytic species. The order of the nitrogen investments in food bodies from highest to lowest was as follows: *M. winkleri*, *M. trachyphylla*, *M. bancana*, *M. hullettii*, *M. beccariana*, *M. lamellata*, *M. hypoleuca* and *M. kingii* (Fig.1(a)). The order of the carbon investments in food bodies from highest to lowest was as follows: *M. winkleri*, *M. trachyphylla*, *M. bancana*, *M. hullettii*, *M. lamellata*, *M. kingii*, *M. beccariana* and *M. hypoleuca* (Fig.1(b)). There was a significant positive correlation between carbon and nitrogen investments in food bodies (Kendall's coefficient of rank correlation, adjusted $\tau = 0.786$, $P < 0.01$). Moreover, there were significant positive correlations between intensity of ant defense and carbon investment in food bodies (Kendall's coefficient of rank correlation, adjusted $\tau = 0.571$, $P < 0.05$) and between the bodies (Kendall's coefficient of rank correlation, adjusted $\tau = 0.786$, $P < 0.01$). These results suggest that the more intensive ant defenses are supported by more resources invested in food bodies by the plants.

V. Interspecific Variation in the Intensity of Non-Ant Defenses

The variations in the intensities of non-ant defenses in *Macaranga* have been partly described [38- 48]. Nomura et al. [38] measured the interspecific variation in the intensity of total non-ant defenses among ten *Macaranga* species,

including eight myrmecophytic species at ant-defending stage by comparing the inhibitory effects on the growth performance on a generalist herbivorous insect, the common cutworm (*Spodoptera litura* Fabricius (Lepidoptera: Noctuidae)) when larvae fed on fresh leaves of each *Macaranga* species. Curves of cumulative survival rates of the cutworm larvae were significantly different among ten species (Log rank test, $\chi^2 = 1135.818$, $P < 0.0001$; Fig. 2). The hazard curve of cumulative survival rate was higher for the five shade-intolerant myrmecophytes than for the three shade-tolerant myrmecophytes. Cumulative survival rate of cutworm larvae on *M. praestans* was lower than that on the other species, except for *M. hulleitii*. The cutworm larvae were able to penetrate leaves of all species with their mandibles, except for *M. praestans*, on which all larvae died of starvation due to their inability to break the leaf surface. Therefore, harmful influences on cutworm growth were higher in *M. praestans* than *M. hulleitii*. Cumulative survival rate on *M. gigantea* was intermediate between those on shade-intolerant and shade-tolerant myrmecophytes. Curves of cumulative survival rates were not significantly different among *M. gigantea*, *M. hypoleuca* and *M. beccariana*. However, some larvae survived until 3rd instar on *M. beccariana* and *M. hypoleuca*, whereas all larvae on *M. gigantea* died as 2nd instars. Therefore, harmful influences on cutworm growth were higher in *M. gigantea* than in *M. beccariana* and *M. hypoleuca*. Curves of cumulative survival rates of cutworm larvae were different even among the five shade-intolerant myrmecophytes and the three

shade-tolerant myrmecophytes (Log rank test, $\chi^2 = 522.281$ and 89.995 , for shade-intolerant and shade-tolerant myrmecophytes, respectively; $P < 0.0001$; Fig. 2). The order of inhibitory effects of fresh leaves on the growth of the cutworm larvae, from lowest to highest was as follows; *M. winkleri*, *M. trachyphylla*, *M. bancana*, *M. beccariana*, *M. hypoleuca*, *M. gigantea*, *M. lamellata*, *M. kingii*, *M. hulleitii* and *M. praestans*. There was a significant negative correlation between the intensities of ant and non-ant defenses (Kendall's coefficient of rank correlation, adjusted $\tau = -0.629$, $P < 0.05$). This result supports Janzen's hypothesis of a trade-off between ant and non-ant defenses. Moreover, this result suggests that even sympatric species have different defense strategies, with a trade-off between the two modes of defense mechanisms.

Non-ant defense mechanisms can be separated into physical and chemical components. To assess the intensity of physical defense, Nomura et al. [38, 46] measured leaf toughness. Since the leaves that were used in this study are covered with very few trichome and spines, it was assumed that leaf toughness is a good character that determines and indicates the intensity of physical defense of *Macaranga* species. Leaf toughness differed significantly among the ten species (ANOVA, $F = 84.033$, $P < 0.0001$; Fig. 3). Leaf toughness tended to be lower in the five shade-intolerant myrmecophytes than in the three shade-tolerant myrmecophytes and the two non-myrmecophytes. The leaf

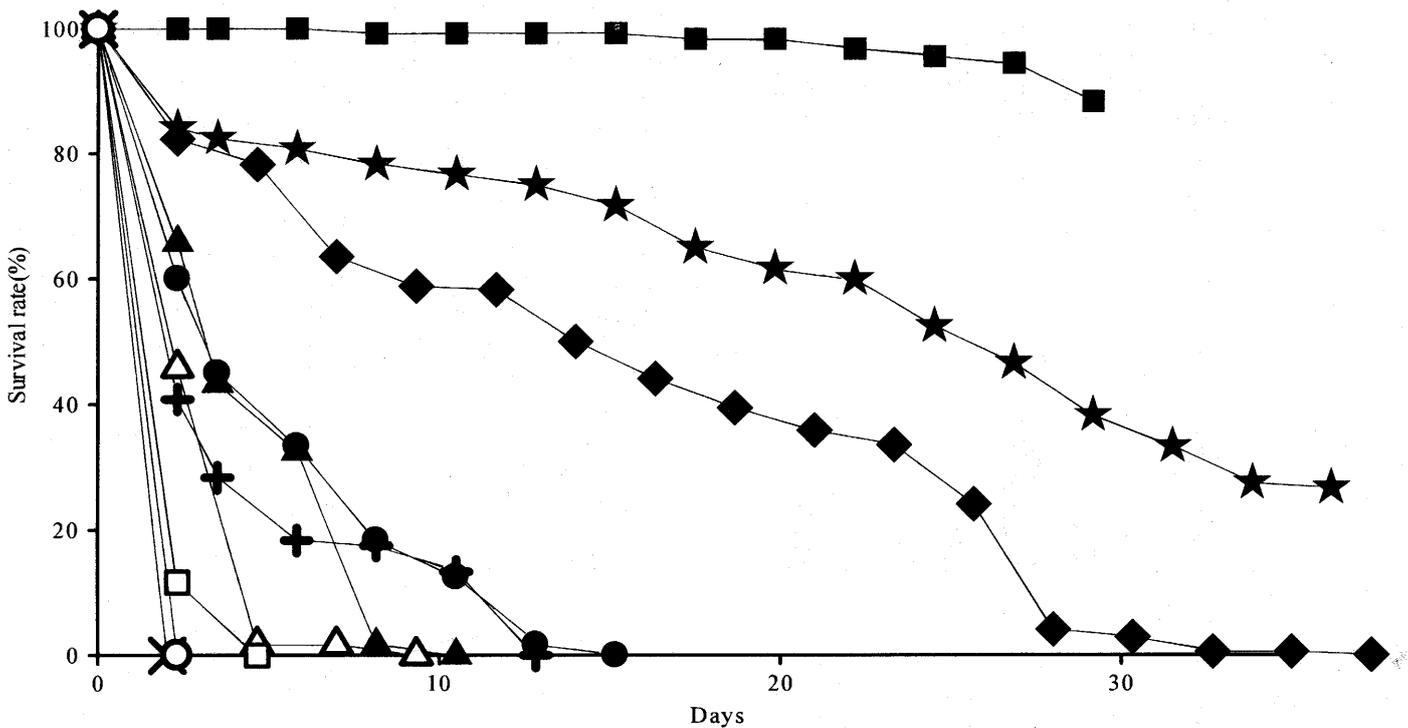


Fig. 2. Curves of cumulative survival rates of larvae of the common cut worm (*Spodoptera litura*) fed fresh leaves of ten *Macaranga* species: (■) *M. winkleri*, (★) *M. trachyphylla*, (◆) *M. bancana*, (●) *M. beccariana*, (▲) *M. hypoleuca*, (+) *M. gigantea*, (△) *M. lamellata*, (□) *M. kingii*, (○) *M. hulleitii* and (×) *M. praestans* (adapted from Nomura et al.[38]). Curves of cumulative survival rates of the cutworm were significantly different among the ten species (Log rank test, $\chi^2=1135.818$, $P<0.0001$)

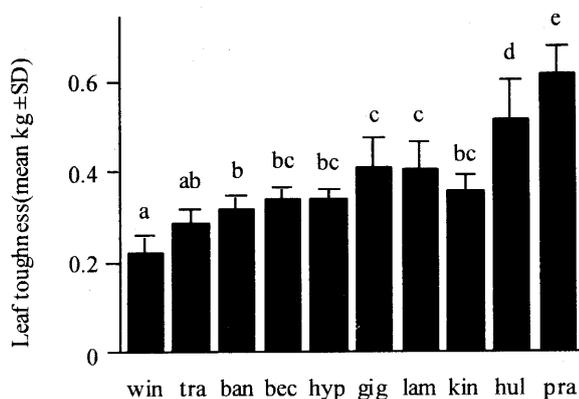


Fig. 3. Leaf toughness in the ten *Macaranga* species. Toughness was determined from the weight needed to penetrate a fresh leaf by 3 mm-diameter penetrometer column (adopt from Nomura et al. [38]). There were significant differences among ten species (ANOVA, $F = 84.033$, $P < 0.0001$). Weight followed by different letters are significantly different from each other (Scheffé F test: $P < 0.05$). Abbreviations as in Fig. 1 legend.

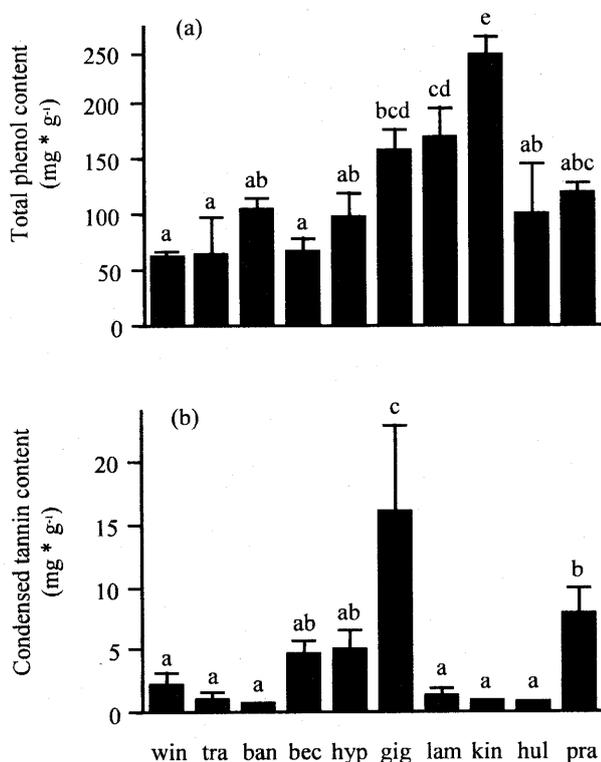


Fig. 4. Total phenol and condensed tannin contents in the ten *Macaranga* species: (a) total phenol, (b) condensed tannin (adopt from Nomura et al. [38]). There were significant differences in total phenol and condensed tannin contents among ten species (ANOVA, $F = 31.164$, 24.575 for total phenol and condensed tannin, respectively; $P < 0.0001$). Weight followed by different letters are significantly different from each other (Scheffé F test, $P < 0.05$). Abbreviations as in Fig. 1 legend.

toughness of *M. praestans* was significantly lower than that in the other species (Scheffé F test, $P < 0.05$; Fig. 3). The leaf toughness in *M. gigantea* was intermediate between those in shade-intolerant and shade-tolerant myrmecophytes. Leaf toughness differed significantly even among sympatric species (ANOVA, $F = 20.088$ and 25.700 for shade-intolerant and shade-tolerant myrmecophytes, respectively; $P < 0.0001$, Fig. 3). The toughness of *M. winkleri* leaves was significantly lower than that of *M. hypoleuca*, *M. beccariana* and *M. bancana* leaves (Scheffé F test, $P < 0.05$). The leaf toughness in *M. kingii* was significantly lower than that in any shade-tolerant myrmecophyte (Scheffé F test, $P < 0.05$). There was a significant positive correlation between leaf toughness and intensity of total non-ant defense (Kendall's coefficient of rank correlation, adjusted $\tau = 0.854$, $P < 0.001$).

To assess the intensity of chemical defense, Nomura et al. [38] measured total phenol and condensed tannin contents in leaves. Total phenol contents differed significantly among ten species (ANOVA, $F = 31.164$, $P < 0.0001$; Fig. 4(a)). However, there was no visible tendency in total phenol contents among shade-intolerant myrmecophytes, shade-tolerant myrmecophytes and non-myrmecophytes.

Condensed tannin contents differed significantly among ten species (ANOVA, $F = 24.575$, $P < 0.0001$; Fig. 4(b)). However, there was no visible tendency in condensed tannin contents among shade-intolerant myrmecophytes, shade-tolerant myrmecophytes and non-myrmecophytes. There were no significant correlations between the intensity of total non-ant defense and phenol content and between the intensity of total non-ant defense and condensed tannin content. These results suggest that leaf toughness is more important for total non-ant defense than for chemical defense.

VI. Intraspecific Variation in the Intensity of Non-Ant Defenses in Myrmecophytic *Macaranga* before and after the Initiation of Symbiosis with Ants

Changes in the intensity of total non-ant defense of seedlings before and after symbiont ants colonized were estimated by measuring the survival rate of the cutworm when larvae were fed on fresh leaves from seedlings (saplings) of the three species at three growth stages [48].

Within each *Macaranga* species, the curve of the cumulative survival rate of larvae fed pre-symbiosis leaves was significantly lower than that of larvae fed ant-defending leaves (Log rank test, $\chi^2 = 276.231$, 427.388 and 38.408 , for *M. winkleri*, *M. trachyphylla* and *M. beccariana*, respectively; $P < 0.0001$ for all; Fig. 5). The curve of the cumulative survival rate of the cutworm larvae on pre-defense leaves was intermediate between those on pre-symbiosis and ant-defending leaves in both *M. trachyphylla* and *M. beccariana*. Sufficient seedlings in the pre-defense stage in *M. winkleri* were not available at the study site.

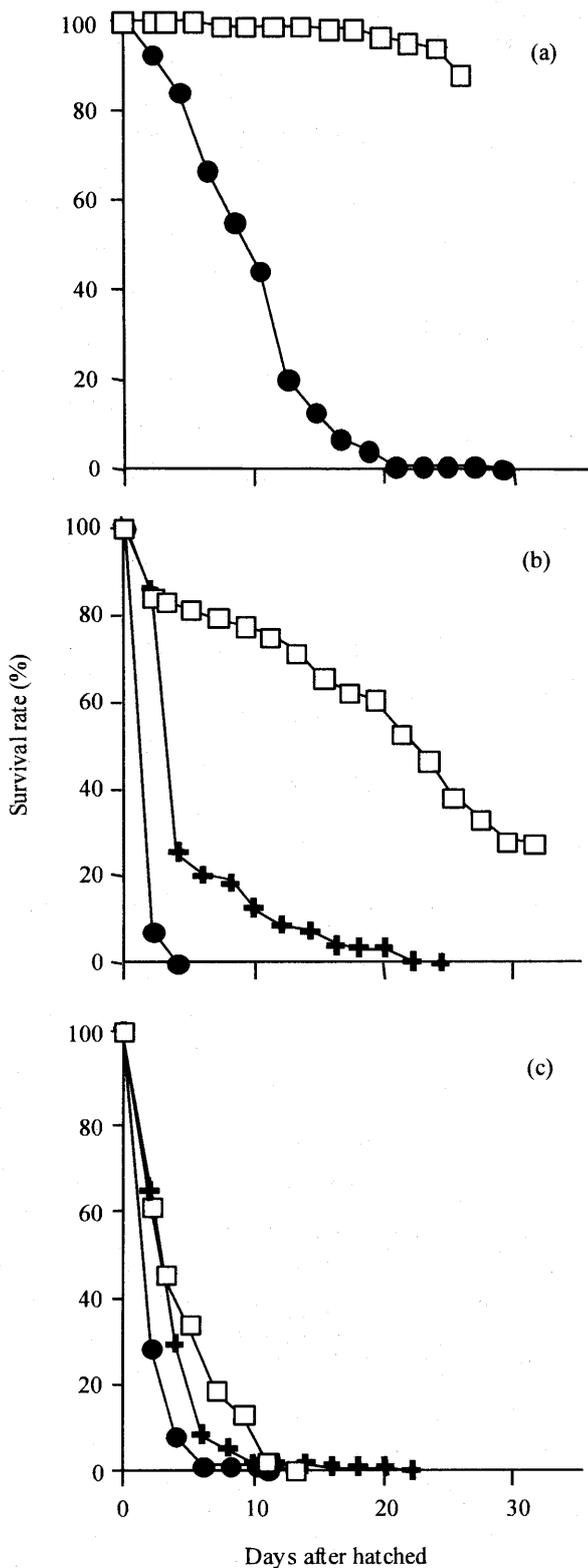


Fig. 5. Hazard curves of the cumulative survival rates of the larvae of the common cutworm (*Spodoptera litura*) fed on fresh leaves at three growth stages of three *Macaranga* species: (a) *M. winkleri*, (b) *M. trachyphylla*, (c) *M. beccariana* □, "ant-defending" stage; +, "pre-defense" stage; ×, "pre-symbiosis" stage. Redrawn from Nomura et al. [48].

There was a wide interspecific variation in the degree of difference between the cumulative survival rates on the pre-symbiosis and ant-defending growth stages. All larvae died on pre-symbiosis leaves of all *Macaranga* species. On *M. beccariana*, all larvae died early on ant-defended leaves. On *M. winkleri* and *M. trachyphylla*, however, some larvae completed their growth on ant-defended leaves.

Curves of the cumulative survival rate of cutworm larvae on pre-defense leaves differed significantly between *M. trachyphylla* and *M. beccariana* (Log rank test, $\chi^2 = 23.881$, $P < 0.0001$, Fig. 5). Although all larvae fed with pre-defense leaves died on both *M. trachyphylla* and *M. beccariana*, they survived longer on *M. trachyphylla* than on *M. beccariana*. Curves of the cumulative survival rate of cutworm larvae fed on pre-symbiosis leaves differed significantly among three species (Log rank test, $\chi^2 = 331.194$, $P < 0.0001$, Fig. 5). On pre-symbiosis leaves, larvae survived longest on *M. winkleri* and shortest on *M. trachyphylla*. All larvae fed on pre-symbiosis leaves of *M. trachyphylla* died at 1st-instar, whereas some larvae fed on pre-symbiosis leaves of *M. beccariana* reached 3rd-instar. These results support our hypothesis that myrmecophytic *Macaranga* may defend themselves against herbivores by intensive non-ant defenses before symbiosis with ants, and that the intensity of non-ant defense decreases significantly as ant colonies develop.

VII. Conclusions

There were wide interspecific variations in the intensities of both ant and non-ant defenses. In addition, there was a significant negative correlation between the intensities of ant and non-ant defenses. These results suggest that each *Macaranga* species has different defense strategies, with a trade-off between the two modes of defense mechanisms.

Total non-ant defenses of a plant consist of physical and chemical components. We measured leaf toughness as a plausible major agent of physical defense. In addition, we measured condensed tannin and total phenol contents as a plausible major agent of chemical defense. We found that there was a significant positive correlation between the intensities of total non-ant and physical defenses, however, there was no significant correlation between the intensities of total non-ant and chemical defenses. These results suggest that physical defense contributes more to non-ant defense in *Macaranga* than chemical defense.

The intensity of the non-ant defense when seedlings had not yet received symbiont foundress queens was significantly higher than that after ant defense was well established. This result suggests that the resources that plants can invest in each anti-herbivore defense mechanism are limited, and that the intraspecific change in the balance of the two modes of defense mechanisms in the ontogenetic process is present in the trade-off. Almost all *Macaranga* myrmecophytes are pioneer trees [38], and they must grow and reproduce as soon as possible. Thus, optimal resource allocation to ant and non-ant defenses would lead to

increased fitness in *Macaranga* seedlings because they can invest the saved resources in other life history aspects, such as growth and reproduction.

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