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Effects of light on direct and indirect defences against herbivores of young plants of *Mallotus japonicus* demonstrate a trade-off between two indirect defence traits

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• *Background and Aims* Although most studies on plant defence strategies have focused on a particular defence trait, some plant species develop multiple defence traits. To clarify the effects of light on the development of multiple defence traits, the production of direct and indirect defence traits of young plants of *Mallotus japonicus* were examined experimentally under different light conditions.

• *Methods* The young plants were cultivated under three light conditions in the experimental field for 3 months from May to July. Numbers of ants and pearl bodies on leaves in July were examined. After cultivation, the plants were collected and the developments of trichomes and pellucid dots, and extrafloral nectaries (EFNs) on the leaves were examined. On plants without nectar-collecting insects, the size of EFNs and the volume of extrafloral nectar secreted from the EFNs were examined.

• *Key results* Densities of trichomes and pellucid dots did not differ significantly among the plants under the different light conditions, suggesting that the chemical and physical defences function under both high and low light availability. The number of EFNs on the leaves did not differ significantly among the plants under the different light conditions, but there appeared to be a trade-off between the size of EFNs and the number of pearl bodies; the largest EFNs and the smallest number of pearl bodies were found under high light availability. EFN size was significantly correlated with the volume of extrafloral nectar secreted for 24 h. The number of ants on the plants was smaller under low light availability than under high and moderate light availability.

• *Conclusions* These results suggest that direct defence traits function regardless of light conditions, but light conditions affected the development of indirect defence traits.

Key words: Physical defence, chemical defence, biotic defence, trichome, pellucid dot, extrafloral nectary, pearl body, light condition, *Mallotus japonicus*, indirect defence, direct defence, trade-off.

INTRODUCTION

Herbivores often impact plant growth or reproduction, and plants have therefore evolved various defensive traits against herbivores. Plant defences can be classified into direct and indirect types. Direct defences include chemical defences such as secondary compounds and toxins and physical defences such as trichomes and leaf toughness (Howe and Westley, 1988; Schoonhoven *et al.*, 1998). Indirect defences include the biotic defences in which plants bear extrafloral nectaries (EFNs), food bodies such as pearl bodies and Müllerian bodies, and/or domatia to attract predators and bodyguards against herbivores (Koptur, 1992; Rico-Gray and Oliveira, 2007; Heil, 2008) or emit volatile compounds to attract parasitic wasps or predatory mites against herbivores (Heil, 2008).

The growth and resource availability of plants are influenced by abiotic environmental factors of the habitat, and the development of defence traits varies with growth stage and/or resource availability (Coley and Barone, 1996; Schoonhoven *et al.*, 1998). Light condition, in particular, is an important factor in plant growth because it directly influences the

photosynthetic rate and also strongly impacts the development of defence traits (Coley *et al.*, 1985; Herms and Mattson, 1992; Nicotra *et al.*, 1999; Agrawal, 2007). For example, species adapted to low resource availability often evolve low inherent growth rates along with quantitative chemical defence (Coley and Barone, 1996; Agrawal, 2007). In contrast, species adapted to high resource availability often evolve higher inherent growth rates and qualitative chemical defence (Coley and Barone, 1996). Leaf toughness as a physical defence develops better under low light conditions than high light conditions (Coley and Barone, 1996; Agrawal, 2007).

Light condition also influences the evolution of the biotic defence by ants. It has been suggested that ant-plants that have obligate mutualism with ants have evolved under high light in the tropical rain forests of south-east Asia (Beattie, 1985; Coley and Barone, 1996). In addition, EFNs are found more frequently on plants under high than low light conditions (Schupp and Feener, 1991). Webber *et al.* (2007) noted that food bodies are found on shade-tolerant plant species. Folgarait and Davidson (1994), however, reported that in *Cecropia*, the Müllerian body as a kind of food body developed under high light conditions, but that under low light

conditions another food body, pearl body, developed. There has therefore been no consistent description on the effects of light on the development of biotic defence traits. This may be due to differences in experimental conditions, light intensity level, other environmental factors and plant species among these studies.

Although it has been reported that light condition brings about intraspecific variations in the development of defence traits (Gromer, 1937; Mole *et al.*, 1988; Coley and Barone, 1996; Wilkens *et al.*, 1996), the effects of light on the intraspecific variation in the development of biotic defence traits have been less studied.

Although most studies on plant defence strategies have focused on a particular defence trait, some plant species have developed multiple defence traits. For example, some species of ant-plants with less effective ant defences develop chemical and physical defences (Folgarait and Davidson, 1994; Eck *et al.*, 2001). Some species of EFN-bearing plants that have facultative association with ants also develop chemical and physical defences (Steward and Keeler, 1988; Agrawal and Spiller, 2004; Rudgers *et al.*, 2004; Kobayashi *et al.*, 2008). However, the effects of light on the development of multiple defence traits have not been sufficiently elucidated.

In ant-plants with food bodies as a reward for ants, such as *Cecropia* and *Macaranga*, it has been reported that there appears to be a trade-off between the biotic defence by ants and the chemical defence by secondary metabolites or the physical defence by leaf toughness (Koptur, 1985; Heil *et al.*, 1999, 2002; Eck *et al.*, 2001). In contrast, on EFN-bearing plants that have a facultative ant–plant mutualism, there has been no reported trade-off between the biotic defence by EFNs and the chemical and/or physical defences (Steward and Keeler, 1998; Rudgers *et al.*, 2004).

To clarify the effectiveness of each of multiple defence traits and the relationships among them, it is necessary to investigate the effects of light on the development of defence traits in those plant species that have multiple defence traits.

To clarify the effects of light on the development of direct and indirect defence traits, the production of multiple defence traits of young plants of *Mallotus japonicus* (Euphorbiaceae) under different light conditions were examined experimentally. The present study also investigated how effectively the plants combine these defence traits in association with their ecological requirements under different light conditions.

MATERIALS AND METHODS

Study species

Mallotus japonicus Müll. Arg. is a pioneer plant that grows in gaps and disturbed areas in the temperate regions of eastern Asia. It is known to have EFNs on its leaf edges and food bodies (pearl bodies) on its leaf surface and stems as indirect defence traits against herbivores (Roupert, 1926; Pemberton, 1990). The plants usually carry ants on their leaves, and EFNs and pearl bodies function as biotic defence traits (Yamawo, 2009). Furthermore, this species has trichomes and pellucid dots on the leaf surface as direct defence traits.

The trichomes function as a physical defence trait. The pellucid dots that contain toxic secondary metabolites function as a chemical defence trait. These defence traits may be important on young plants of *M. japonicus* because the impact of herbivory is greater for young plants than for older plants (Yamawo, 2009). Young *M. japonicus* plants are therefore suitable for studying the effects of light condition on the development of multiple defence traits such as EFNs, pearl bodies, trichomes and pellucid dots.

Effects of light condition

Fifty seeds of *M. japonicus* were collected from trees growing on the campus of Okayama University of Science, Okayama (34°41′N, 133°55′E), in September to October, 2007. A plastic container (45 cm wide, 35 cm long, 5 cm deep) was filled with wet germ-free soil 5 cm in depth, and 50 seeds were sown into this soil at a depth of 1 cm on 1 November. The plastic container was placed in a growth chamber at 40 °C under light for 12 h, because *M. japonicus* seeds can germinate after experiencing high temperature (Washitani and Takenaka, 1987). Thereafter, the plastic container was maintained at 25 °C under a 12-h light–dark photoperiod in the growth chamber for 29 d. The seeds or seedlings that emerged were watered every other day. On 30 November, when the seedlings had emerged, the plastic container was set in the field and the plants were over-wintered.

Healthy plants that had reached approx. 3·5 cm in height were selected for the experiment on 1 March, 2008, when their leaves had not yet begun to flush. The plants were transplanted into plastic pots ($20 \times 20 \times 25$ cm) containing 70 % sand and 30 % Perlite soil. The pots were placed in an experimental field at Okayama University of Science; the site was an open area of 50×20 m, surrounding by natural woodlands that consisted mainly of deciduous broadleaved trees and pines. To prevent drying, the pots were embedded to a depth of approx. 5 cm. The experiment was started on 1 May, when the plants had grown to $4 \cdot 2 \pm 1 \cdot 1$ cm (mean $\pm$ SD) in height.

The pots were set under three light conditions because young plants of *M. japonicus* grow in habitats with various light conditions, such as open areas (high light), forest edges (middle light) and tree-fall gaps (low light). Under the high light condition, ten plants were exposed at approx. 90 % full sun using a white shading cloth with a 1-mm mesh. Under the middle light condition, ten plants were exposed at approx. 50 % full sun using a black shading cloth with a 1-mm mesh. Under the low light condition, ten plants were exposed at approx. 30 % full sun using two layers of black shading cloth with a 1-mm mesh. The plants were watered every other day. Three grams of fertilizer (N/P/K = 8 : 8 : 8; APM JAPAN, Tokyo, Japan) were applied directly onto the soil surface surrounding the stems of the experimental plants once a month.

The number of ants on each plant was counted once during the period 0900–1200 h, when ant activity was high, on sunny days (21, 23, 25, 27 and 29 July).

It is difficult to accurately estimate the number of pearl bodies produced on each leaf because some of the pearl bodies have been removed by ants. Thus, the productivity

of the pearl bodies on each leaf was examined on 31 July. The ants on the plants were removed. To prevent the ants from climbing on the plants from the ground, adhesive flycatcher ribbons were bound on the shoots at approx. 5 cm above the ground. All pearl bodies on the leaves were removed using a fine brush. The number of pearl bodies on every leaf was counted after 24 h, when the plants had produced a sufficient number of pearl bodies.

Chlorophyll content was estimated as an indicator of the photosynthetic ability of the fifth and greater fully expanded leaves from the apex of the plants using a chlorophyll meter (SPAD-502, Konica Minolta) on 31 July. Chlorophyll content (C) was estimated from the SPAD-502 meter values (M) using the following equation: $C = 10^{(M0.265)}$ (Markwell *et al.*, 1995). Photosynthetic rate could be assessed from the SPAD-502 values because chlorophyll content correlates directly with photosynthetic rate (Emerson, 1929; Fleischer, 1934). Measurements were replicated three times on one leaf.

The plants were cultivated for 92 d. The plants were then collected on 31 July pressed in newspaper for 14 d. They were then divided into roots and shoots and dried at 80 °C for 3 d. The dried roots and shoots were weighed to a precision of 10^{-4} g using a micro-balancer. The fifth and greater fully expanded leaves from the apex of the plants were used to measure the development of defence traits on the leaves, in order to exclude the effects of leaf age on the development of defence traits. The densities of trichomes and pellucid dots on the under surface of all leaves were estimated under a microscope with a 40× lens. The numbers of trichomes and pellucid dots in two circular areas of 23·7 mm^2 located halfway between the midrib and first lateral vein from the leaf base on both sides of the midrib were counted. The number of EFNs was counted on all leaves. EFN size was estimated by measuring the lengths of the long axis and short axis of every EFN using a hand lens (Peak loupe) with a ruler to a precision of 10^{-1} mm and calculating the product of the long axis multiplied by the short axis.

Correlation between EFN size and volume of extrafloral nectar

On five plants growing in the glasshouse without nectar-collecting insects, the size of EFNs and the volume of extrafloral nectar secreted from the EFNs on the third leaves from the apex of plants were examined, because nectar secretion was active on the third leaf (Yamawo, 2009). EFN size was estimated in the same manner as described above. All leaves were washed with water to remove the existing extrafloral nectar. Twenty-four hours later, the extrafloral nectar which had been secreted for 24 h was collected using 2-μL micro-capillary tubes, and the volume was estimated by multiplying the proportion of the length of the tube filled with EFN to the total length of the tube by 2 μL.

Statistical analysis

Plant biomass, densities of trichomes and pellucid dots, number and size of EFNs, estimated chlorophyll content and number of ants on the plants under different light conditions were analysed using the Kruskal–Wallis test (Zar, 1999), and multiple comparisons were carried out using Dunn's procedure. Correlations between EFN size and the volume of extrafloral nectar secreted or the estimated chlorophyll content were analysed using least squares regression.

RESULTS

Shoot, root and total biomasses differed significantly among the plants under the different light conditions (Table 1; Kruskal–Wallis test, shoot: $P = 0.0004$, root: $P = 0.0004$, total: $P = 0.0003$). Shoot, root and total biomasses of the plants cultivated under the low light condition were one-third to one-seventh smaller than those of the plants cultivated under the high and middle light conditions, but did not differ significantly between the plants cultivated under the high and middle light conditions (Table 1; Dunn's procedure, shoot: $P = 0.80$, root: $P = 0.44$, total: $P = 0.52$).

The densities of trichomes and pellucid dots did not differ significantly among the plants cultivated under the different light conditions (Kruskal–Wallis test, trichomes: $P = 0.14$, pellcid dots: $P = 0.85$, Fig. 1A, B).

The number of EFNs also did not differ significantly among the plants cultivated under the different light conditions (Kruskal–Wallis test, $P = 0.46$, Fig. 1C). However, EFN size did differ significantly among the plants cultivated under the different light conditions (Kruskal–Wallis test, $P = 0.0014$, Fig. 1D). Plants cultivated under the high and low light conditions had the largest and smallest EFNs, respectively (Dunn's procedure, $P = 0.0027$).

The number of pearl bodies differed significantly among the plants cultivated under the different light conditions (Kruskal–Wallis test, $P = 0.0256$), and that of the plants cultivated under the high light condition was smaller than that of the plants cultivated under the middle and low light conditions (Dunn's procedure, middle: $P = 0.0143$, low: $P = 0.0451$, Fig. 1E), but did not differ significantly between the plants cultivated under the middle and low light conditions (Dunn's procedure, $P = 0.54$).

Workers of two ant species, *Tetramorium tsushimae* and *Formica japonica*, visited the plants. There was a high frequency of visits by *T. tsushimae* workers, but the frequency of visits by *F. japonica* workers was low. The numbers of ants on the plants at each investigation date were pooled because they did not differ among investigation dates (Kruskal–Wallis test, low: $P = 0.6674$, middle: $P = 0.7569$, high: $P = 0.7569$). The number of ants on the plants differed significantly among the plants cultivated under the different light conditions (Kruskal–Wallis test,

TABLE 1. *Shoot, root and total biomasses (g, mean ± s.d.) of* M. japonicus *plants cultivated under low, middle and high light conditions*

	Low light	Middle light	High light
Shoot	7·0 ± 3·9[a]	19·0 ± 4·1[b]	20·6 ± 8·0[b]
Root	1·1 ± 0·7[a]	7·8 ± 4·3[b]	9·9 ± 1·9[b]
Total	8·2 ± 4·6[a]	27·0 ± 7·2[b]	30·4 ± 9·0[b]

Different letters indicate significant differences within the column (Dunn's procedure, $P < 0.01$).
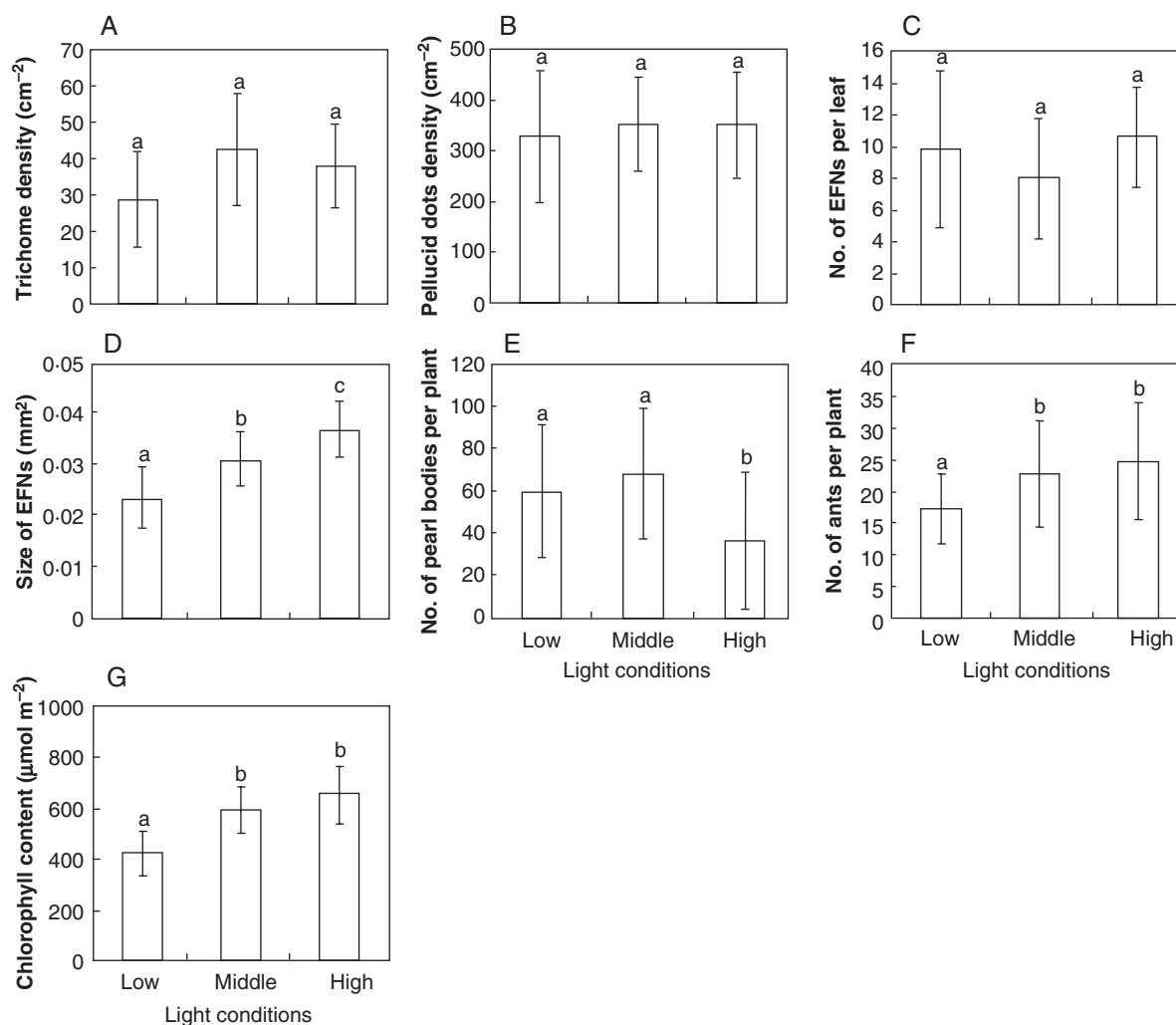
FIG. 1. Leaf defence traits against herbivores in *M. japonicus* plants under low, middle and high light conditions. (A) Trichome density, (B) pellucid dot density, (C) number of EFNs per leaf, (D) size of EFNs, (E) number of pearl bodies per plant, (F) number of ants per plant, and (G) estimated chlorophyll contents. Bars represent standard deviation. Different letters indicate significant differences (Dunn's procedure, *P* < 0·05).

P = 0·0030), with about 25–30 % fewer visiting ants on the plants cultivated under the low light condition than under the high and middle light conditions (Dunn's procedure, high: *P* = 0·0028, middle: *P* = 0·0073, Fig. 1F). However, the numbers did not significantly differ between the plants cultivated under the high and middle light conditions (Dunn's procedure, *P* = 0·28).

Estimated chlorophyll contents differed significantly among the plants cultivated under the different light conditions (Kruskal–Wallis test, *P* < 0·0005). The contents on the plants cultivated under the low light condition were less than on the plants cultivated under the high and middle light conditions (Dunn's procedure, *P* = 0·0002, Fig. 1G), but there was no significant difference in the estimated chlorophyll contents between the plants cultivated under the high and middle light conditions (Dunn's procedure, *P* = 0·13).

The size of the functional EFNs that secreted the extrafloral nectar was significantly correlated with the estimated

chlorophyll contents (*r* = 0·655, *P* < 0·0001) and with the volume of extrafloral nectar secreted for 24 h (*r* = 0·920, *P* < 0·0001).

DISCUSSION

Many studies have discussed the chemical responses to light (Koricheva *et al.*, 1998). Several have indicated that the chemical defence by carbon-based metabolites or that physical defence decline in power on plants under low light availability (Mole *et al.*, 1988; Wilkens *et al.*, 1996; Hemming and Lindroth, 1999). Murase *et al.* (2003) reported that in *Macaranga*, a myrmecophytic species with an obligate ant–plant mutualism, chemical and physical defences developed under high light. In this study, however, light availability did not affect the chemical defence by pellucid dots and physical defence by trichomes. Similar results were reported by Taniguchi *et al.* (2006), who showed that the tannin content of *Aleurites forlii* (Euphorbiaceae) did not differ among different

light availabilities under relatively low light conditions in a growth chamber compared with natural condition in the field. These results suggest that chemical and physical defences under low light availability function similarly as under high light availability.

Here, high light resulted in an increase in the size of the EFNs, implying an increase in volume of the extrafloral nectar secreted. We suggest that EFNs can become enlarged and the amount of extrafloral nectar secreted increased under high light availability, because the extrafloral nectar seems to result from photosynthesis. This is supported by the increase in estimated chlorophyll contents under the high light condition (Fig. 1G). In contrast, under low light, with lower photosynthetic activity, young plants of *M. japonicus* may reduce the size of EFNs and restrict the secretion of extrafloral nectar.

Although several studies have reported that the development of EFNs is genetically determined (Mitchell, 2004; Wooley *et al.*, 2007), *M. japonicus*, a pioneer plant that grows in the environments where light conditions are variable and unpredictable, may have a plasticity that allows individual plants to manipulate the development of EFNs in response to changing light conditions. This suggestion is supported by Kersch and Fonseca (2005), who reported that ant defence was more effective under high than low light conditions. Several studies have reported that plasticity in the development of EFNs results from biotic factors such as ant abundance (Heil *et al.*, 2000, 2009) and herbivory (Pulice and Packer, 2008). Therefore, plasticity in the development of EFNs of *M. japonicus* mediated by biotic factors should be examined in future studies.

The present results appear to show a trade-off between EFN size and the number of pearl bodies in association with light. To our knowledge, the present study is the first to reveal a trade-off between the development of two indirect defence traits in plants with a facultative ant–plant mutualism.

In contrast to the development of EFNs under high light, low production of pearl bodies on plants cultivated under high light could be explained as an economy of cost. We suspect that the pearl body, which is rich in lipids and/or proteins, is more expensive than extrafloral nectar, which contains primarily sugars (O'Dowd, 1982; Heil *et al.*, 2001), and the development of EFNs sufficiently attract ants under high light. Reduced production of pearl bodies under high light was reported in *Cecropia* (Moraceae) by Folgarait and Davidson (1994). A high cost due to the production of food bodies was also reported by Heil *et al.* (1997), who showed that the cost of food body production accounted for 5 % of the total energy in *Macaranga* plants. By contrast, O'Dowd (1979) reported that the cost in EFN production was 1 % of the leaf emergence energy in *Ochroma pyramidale* (Bombacaceae). Folgarait and Davidson (1994) reported that in *Cecropia*, a myrmecophytic species with an obligate ant–plant mutualism, Müllerian bodies as an indirect defence trait rich in glycogen developed under high light, but another indirect defence trait, pearl bodies, which are rich in lipids, developed under low light. These results indicate that the costs of defence differ in relation to the different materials of defence traits such as carbohydrates and lipids.

The number of ants on the plants, an important factor in the effectiveness of ant defence (Oliveira *et al.*, 1999; Apple and Feener, 2001; Giusto *et al.*, 2001; Heil *et al.*, 2001;

Katayama and Suzuki, 2004), was larger on plants cultivated under high light than under low light conditions (Fig. 1). This may be because *T. tsushimae* and *F. japonica*, the ants observed in the present study, mainly inhabit open areas (Terayama, 1997; Yamawo *et al.*, 2008), and might be attracted to the increased extrafloral nectar under high light conditions. Yamawo (2009) reported that young plants of *M. japonicus* growing in environments with various light availability attracted 15 ant species. We thus suspect that the pearl bodies of *M. japonicus* function as a biotic defence trait against herbivores by attracting workers of ant species other than *T. tsushimae* and *F. japonica*, presumably those inhabiting low-light environments.

These results suggest that direct defence traits function regardless of light conditions, but light conditions affected the development of indirect defence traits.

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