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Long- and short-term induction of defences in seedlings of *Shorea leprosula* (Dipterocarpaceae): support for the carbon:nutrient balance hypothesis

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Abstract: The induction of carbon-based secondary metabolites in leaves following damage has been proposed to be a result of a shift in the carbon:nutrient balance, when growth is limited by nutrients in relation to carbon. Here we test this hypothesis using seedlings of a tropical tree, *Shorea leprosula* (Dipterocarpaceae). In the short term, we analysed the phenolic content of leaves 7 d after damage on seedlings grown under differing light and nutrient treatments. In the long term, we examined the effect of nutrients, over 12 mo, on leaf phenolic concentration and seedling growth. In both the long and short term, levels of phenolics increased in damaged leaves under low nutrient treatments. No changes in leaf phenolics were detected under high nutrient regimes, or in the short term under low light. In addition, it was found that defoliation of seedlings in high-nutrient environments led to greater rates of leaf production than in undamaged seedlings, suggesting compensation.

Key Words: carbon:nutrient balance hypothesis, compensation, defence induction, dipterocarp, herbivory, Malaysia, phenolics

INTRODUCTION

It has long been known that herbivore damage to leaves can induce increases in carbon-based secondary metabolites (Haukioja & Hanhimaki 1985, Schultz & Baldwin 1982), although the mechanism by which this occurs remains a topic of debate (Hamilton *et al.* 2001, Koricheva *et al.* 1998, Lerdau & Coley 2002, Ryan 2000). The occurrence and magnitude of short-term induction i.e. responses within hours or days of damage, or long-term induction, i.e. over months or even years of continuous damage, is thought to depend greatly on the environmental conditions in which the plant is growing. Theories explaining the mechanisms by which defence induction occurs in plants fall into two main categories; resource-driven changes and active defence. There is experimental evidence to support both of these theories and they are not mutually exclusive in the explanation of chemical induction following damage.

The principal theory explaining resource-driven defence induction is the carbon:nutrient balance hypothesis (CNBH) (Bryant *et al.* 1983), which states that a plant will invest in carbon-based secondary metabolites when growth is limited by nutrients, primarily nitrogen, rather than light. Under these conditions, tissue lost to defoliation is hard to replace because growth is limited (Ruohomaki *et al.* 1996), and thus carbon is diverted to secondary metabolites, such as phenolic compounds. If however, the plant is in a more fertile environment (where replacement of lost tissue is not limited by nitrogen and plant resources are used for growth), or in a low-light environment (where photosynthetic rates and thus carbon acquisition rates are low), the production of secondary compounds will be constrained (Bryant *et al.* 1993, Hunter & Schultz 1995). The hypothesis relates to resource limitation (Tuomi *et al.* 1984) and does not assume that the secondary compounds produced are necessarily an active response to deter further herbivory.

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There is also substantial evidence to show that induction of secondary chemicals, such as tannins and phenolics, may be an active defence mechanism by which the plant deters further herbivore damage. Wound signalling pathways, which induce chemical defences in leaves, have been identified in some plants following both mechanical (Pearce *et al.* 1991, Ryan 2000) and herbivore damage (Ryan 2000, Walling 2000). Further to this, elicitor compounds have been isolated in the oral secretions of herbivores that induce specific changes in leaf chemistry, through changes in gene expression not brought about by mechanical damage (Alborn *et al.* 1997, Baldwin & Preston 1999). Changes in plant chemical composition in response to these elicitors can reduce nutritional quality of leaves, deter further herbivore attack and reduce the performance of insect herbivores (Agrawal 1999, Lempa *et al.* 2004, Mutikainen *et al.* 2000).

The focus of the current debate surrounds the usefulness of the CNBH as a tool for predicting plant responses to changes in environmental factors (Hamilton *et al.* 2001). Many studies over the past two decades have sought to refine and focus the scope of the CNBH (see reviews by Hamilton *et al.* 2001, Koricheva *et al.* 1998), to the extent that some workers are now suggesting it has lost its generality and should be abandoned. However, the CNBH has been shown, in a recent meta-analysis, to successfully predict changes in phenolic levels due to decreases in nutrients or increases in light (Koricheva *et al.* 1998). Since damage is likely to affect the relative levels of nutrients and carbon within a plant, it is possible that changes in the allocation to secondary compounds fit the predictions of the CNBH. Previous studies have found that some species do show increased levels of secondary compounds when nutrient levels are low (Dudt & Shure 1994, Larsson *et al.* 1986, Stout *et al.* 1998). Here we assess the effectiveness of the CNBH to predict changes in allocation to phenolic compounds in seedlings of a fast-growing tropical tree following defoliation in a range of light and nutrient environments.

Much of the work previously carried out on induced secondary metabolites has taken place in temperate regions, particularly with birch, willow and oak trees (Hunter & Schultz 1995, Mutikainen *et al.* 2000, Ruuhola *et al.* 2001). This project examined the occurrence of both long- and short-term induction of secondary metabolites in a tropical tree, *Shorea leprosula* Miq. *Shorea leprosula* is a relatively light-demanding dipterocarp found extensively throughout the forests of Borneo, and has been the subject of previous ecological and physiological studies (Howlett & Davidson 2001, Leakey *et al.* 2003, Scholes *et al.* 1997, Zipperlen & Press 1996). The investment in phenolic compounds by *S. leprosula* is known to be greater in gaps than in understorey sites (Massey 2002),

and it is a fast-growing species that suffers relatively high levels of herbivore damage.

We tested the hypothesis that, in the short term, damage would increase the levels of phenolic compounds in high-light environments, but that in low-light environments, where carbon is limited, no increase in levels of phenolic compounds in response to damage would occur. In high-nutrient environments, where nitrogen does not limit growth to the extent that carbon does, the CNBH predicts that carbon will be invested in growth and not in increased defences. Over the longer term, damaged plants would be predicted to invest more in defences than undamaged plants, or where possible, compensate for lost tissue with increased leaf production. The active defence theory would predict that levels of secondary defences would increase after damage regardless of the growth conditions.

METHODS

Study site

The study was carried out between September 2000 and September 2001 in a tree nursery, in a cleared area of logged forest adjacent to Danum Valley Conservation Area, Sabah, East Malaysia.

Experiment 1: Short-term induction

Experimental design. *Shorea leprosula* seedlings were grown in 0.5-litre pots, from locally collected seeds, under 50% shade and watered regularly. After 2 y, seedlings were transplanted into 1.5-litre pots and placed under two shade levels: 60 plants received 80% incident light (high-light treatment), and 30 plants received 7% incident light (low-light treatment), through shade netting placed 2 m above seedlings. Half of the plants in the high light had a fertilizer treatment added 5 wk before the beginning of the experiment (30 g of NKP 18:5:10 slow release fertilizer to each pot). The seedlings of all treatments were then divided into experimental and control groups. Plants were left for 5 wk to acclimatise to new conditions. To each seedling in the experimental group, nine holes were punched into the lamina in each of 10 mature leaves, with a 5-mm-diameter stationery hole punch, avoiding the mid-rib to remove approximately 30% leaf area. Leaves on the control plants were handled in the same way though no damage was made. All plants were approximately the same height with a similar number of leaves.

Chemical analysis. Immediately prior to damage and at intervals of 1, 3 and 7 d, after damage, one leaf was removed from each plant, at the base of the petiole,

oven dried at 60 °C for 48 h. After which leaves were milled through a 1-mm mesh, before being analysed for total phenolic compound content using the Folin–Ciocalteau method (Kerslake *et al.* 1998). 10 mg of each sample was extracted in 10 ml 50% methanol at 80 °C for 30 min. Samples were then centrifuged for 5 min and 0.1 ml of the supernatant added to 2.9 ml of distilled water together with 0.25 ml Folin–Ciocalteau reagent and 1 ml of saturated Na_2CO_3. After 1 h the absorbance of each sample was measured at 760 nm using a spectrophotometer (Camspec™, UK) and compared against a standard curve of a range of tannic acid concentration. This method was chosen over other defence measures as a non-specific, easily repeatable measure of resource allocation that is known to change under different environmental conditions.

Experiment 2: Long-term induction

Experimental design. *Shorea leprosula* seedlings were grown in 0.5-litre pots, from locally collected seeds, under 50% shade and watered regularly. After 3 y, 260 seedlings were transplanted into 2-litre pots and placed in a 10×10-m insect exclosure, of 50% shade netting, using a factorial design, with artificial defoliation and fertilizer addition treatments. Plants, selected to be 40–50 cm tall, were randomly allocated to a treatment. For the artificial defoliation treatment, approximately 30% of leaf area was removed, from every new leaf, as it reached full size, using a 5-mm-diameter hole punch, avoiding the mid-rib. The fertilization treatment consisted of the application of 30 g of NPK (18:5:10) slow release fertilizer every 3 mo to the top of the soil.

Growth measurements and chemical analysis. At the beginning and end of the 12-mo study period, the following growth measurements were made on each plant: height to apical meristem, relative growth rate as the rate of change in seedling height relative to its initial height, total length of branches, number of branches and number of leaves. Leaf samples from 10 seedlings in each treatment were analysed for total phenolics, as described above. Total foliar nitrogen and phosphorous concentrations were also determined, after a Kjeldahl digestion procedure, by a colorimetric assay using a flow injection analysis system (Tecator 5042 detector and 5012 analyser Tecator, UK) as described in Bungard *et al.* (1999).

Statistical analysis. Short-term induction of phenolics was analysed by comparing the phenolic concentration of damaged and undamaged seedlings, within each growth treatment, immediately prior to damage and 7 d after using two-sample t-tests. In the long-term induction

experiment, growth, as height and total branch lengths, were analysed using general linear models to compare the effects of fertilizer, damage and the interaction between these factors. Comparisons of total leaf numbers and total branch numbers were made using Kruskal–Wallis tests and the *post-hoc* Fisher's individual error rate analysis to locate where differences between treatments lie. As in the growth data, the total phenolic, phosphorus and nitrogen concentrations within leaves were compared between treatments using general linear models.

RESULTS

Experiment 1: Short-term induction

There were no differences in the levels of leaf phenolics between leaves on damaged and undamaged *S. leprosula* seedlings at the beginning of the study for any treatment (Figure 1a). There was no evidence of phenolic induction in low light after 7 d following damage (Figure 1b). In high light, *S. leprosula* significantly increased levels of phenolic compounds over 7 d following damage (Figure 1a). However, in high light with addition of nutrients, no induction was observed (Figure 1c).

Experiment 2: Long-term induction

Seedling growth. At the beginning of the experiment no differences were found in the height, branching, leaf number, foliar phenolics, nitrogen or phosphorus content of seedlings between treatments. After 12 mo, fertilized seedlings had greater rates of growth than unfertilized across measurements of height ($F_{1,98} = 105$, $P < 0.001$, Figure 2), branch length and relative growth rate ($F_{1,98} = 13.6$, $P < 0.001$, $F_{1,98} = 29.6$, $P < 0.001$ respectively, data not shown). However, no differences were observed in seedling growth between defoliated and un-defoliated seedlings within nutrient treatments (Figure 2a). In the high-nutrient treatments, significantly more leaves had been produced on damaged seedlings (Kruskal–Wallis: $H = 56.6$, $df = 3$, $P < 0.001$) than on undamaged trees, although the same trend occurred in the damage treatments in low nutrients, it was not significant (Figure 2b).

Leaf chemical composition. In the low-fertilizer treatments, higher levels of total leaf phenolics were found in the damage treatment when compared with the control treatment after 12 mo. However, no induction of phenolics was found in either of the treatments at high nutrient levels (Figure 3a, Table 1). As expected, the levels of foliar nitrogen were higher in fertilized seedlings than unfertilized. No differences were found between damaged
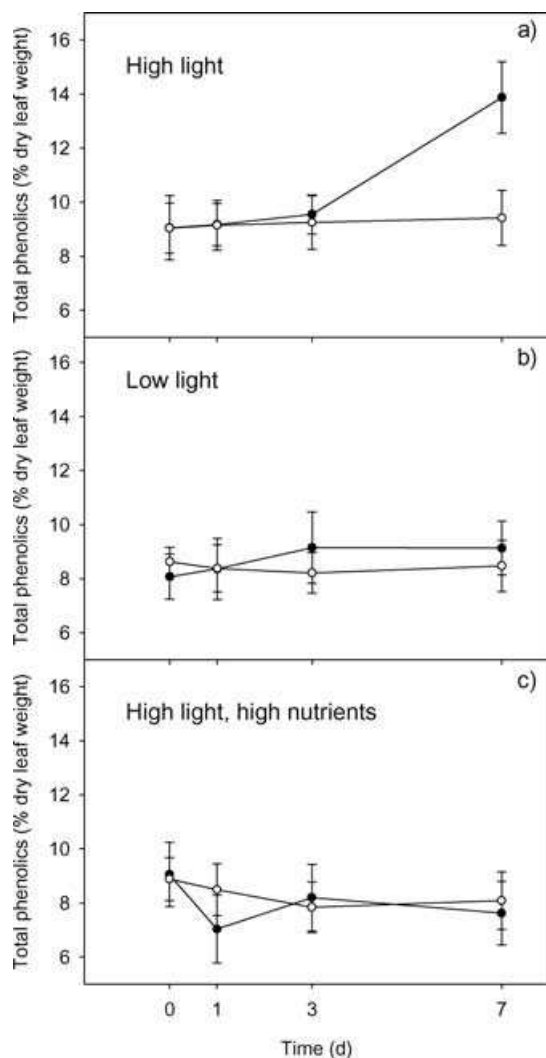
Figure 1. Total phenolic concentration of *Shorea leprosula* over 7 d following damage for mechanically damaged (●) and control leaves (○). Seedlings in (a) high light, (b) low light and (c) high light with nitrogen fertilization. Values are means (± SE). Two-sample t-test 7 d after damage in high light: t = 10.3, df = 16, P < 0.0001; low light: t = 1.52, df = 17, P = 0.15; high light, high nutrients: t = 1.83, df = 18, P = 0.084.

Table 1. Results of General Linear Model for total phenolic, nitrogen and phosphorus concentrations across damage and fertilization treatments after 12 mo.

Leaf quality variable	F	df	P
Total phenolic concentration			
Damage	55.4	1	0.001
Fertilization	6.51	1	0.021
Damage × Fertilization	3.97	1	0.047
Error		16	
Total nitrogen concentration			
Damage	10.9	1	0.005
Fertilization	704	1	< 0.001
Damage × Fertilization	20.0	1	< 0.001
Error		16	
Total phosphorus concentration			
Damage	2.08	1	0.156
Fertilization	18.1	1	< 0.001
Damage × Fertilization	0.21	1	0.646
Error		48	

Figure 2. (a) Seedling height and (b) number of leaves of *Shorea leprosula* seedlings under control (C), fertilizer (F), damage (D), and fertilizer and damage (FD) treatments after 12 mo. Values are means (± SE). Bars not sharing a common letter differ significantly: (a) Tukey Test P < 0.05; (b) Fisher's Test P < 0.05.
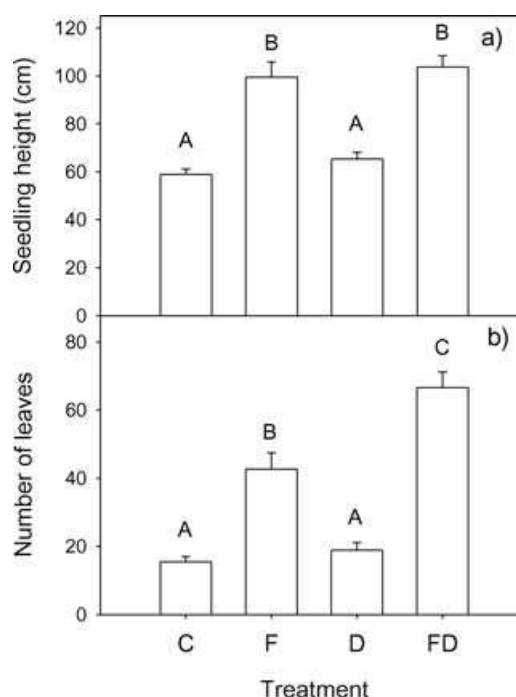
and control plants at low levels of fertilization, but at high fertilization seedlings in the defoliation treatment had significantly lower foliar nitrogen levels than undamaged controls (Figure 3b, Table 1). A similar trend was found in the foliar phosphorus concentrations where fertilizer application increased levels, but damage to fertilized plants reduced levels to the point where they were no different than leaves on control seedlings (Figure 3c, Table 1).

DISCUSSION

Short-term induction of leaf phenolic content, due to mechanical defoliation, was found to occur in *S. leprosula*

seedlings in high-light environments, with increases of 55% over the initial concentrations. However, no induction occurred in low-light, nor in high-light with the addition of nutrients. These results are consistent with predictions made by the CNBH, where damage to leaves resulted in increased levels of secondary compounds in high-light environments, and mitigation of induction in low-light or high-nutrient conditions (Bryant *et al.* 1983). The results are also consistent with findings that phenolic
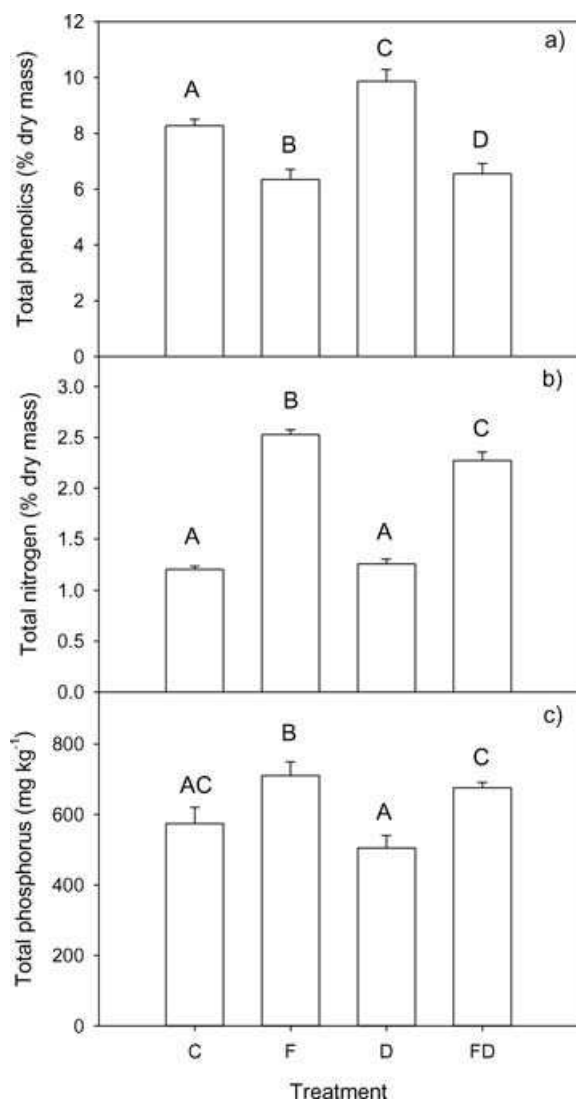
Figure 3. (a) Total phenolic, (b) nitrogen and (c) phosphorus concentration in leaves of *Shorea leprosula* seedlings, under control (C), fertilizer (F), damage (D), and fertilizer and damage (FD) treatments after 12 mo. Values are means ($\pm$ SE). Bars not sharing a common letter differ significantly (Tukey Test P < 0.05).

levels are naturally higher in higher-light environments, but lower in increased-nutrient environments (Larsson *et al.* 1986, Stout *et al.* 1998). Under low light, where the light levels are limiting carbon production, carbon was limiting growth to a greater extent than nitrogen, and no increase in phenolic content was observed. In the high light levels with the addition of nutrients, lost tissue is readily replaced, so no increase in allocation to carbon-based secondary compounds was observed.

In natural forest conditions, the fact that nutrient and light levels influence both the allocation to defence and response to defoliation may result in differential herbivory in gap and understorey light environments, or in areas differing in soil nutrient availability. Previous studies have found damage to be greater in understorey

environments compared with gaps or the canopy (Gilbert 1995, Lowman 1985, 1992). In the case of *S. leprosula* seedlings, defoliation was 39.2% in the understorey compared with 29.1% for canopy gaps (Massey 2002), although such changes have not been linked to levels of induced defences as yet.

This experiment used mechanical damage rather than insect herbivores, so the responses of the plant could be greater under natural herbivory. Generally, the use of insect herbivores elicits higher levels of phenolic induction than artificial damage (Hartley & Lawton 1991), and mechanical damage alters gene expression in a different way to damage by natural herbivores (Korth & Dixon 1997, Reymond *et al.* 2000). Therefore, the levels of induction to seedlings in the forest by insect herbivores may be greater than the responses recorded here. However, as mechanical damage removes resources from the plant without producing the specific chemical signals found with herbivore damage (Baldwin & Preston 1999, Walling 2000), it may favour resource-driven responses rather than defence induction.

In the long-term growth experiment, additions of nutrients increased the growth of *S. leprosula* in all measured variables: height, branch lengths, relative growth rate and branch and leaf numbers. However, 30% defoliation did not affect height, branching or relative growth rate for either low or high nitrogen levels, suggesting that the levels of herbivory imposed upon seedlings were still below a threshold of tolerance and compensation (Strauss & Agrawal 1999). Since *S. leprosula* is a light-demanding and fast-growing species, it may have been able to compensate for damage (Stowe *et al.* 2000). This study found evidence for compensatory growth in response to defoliation, in terms of increased leaf production, when levels of nutrients were high.

These results suggest that changes to leaf phenolic content cannot be assumed to be an active defence mechanism in either the long- or short-term response to defoliation, as they can be mitigated by the addition of nitrogen or decreases in light levels (Hunter & Schultz 1995). Rather, the results are consistent with the hypothesis that changes in whichever resources are in excess of those for growth will be moved to secondary chemicals (Chapin *et al.* 1990).

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