

Seedling resistance, tolerance and escape from herbivores: insights from co-dominant canopy tree species in a resource-poor African rain forest

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Summary

1. Although plants can reduce the impacts of herbivory in multiple ways, these defensive traits are often studied in isolation and an understanding of the resulting strategies is incomplete.
2. In the study reported here, empirical evidence was simultaneously evaluated for the three main sets of traits available to plants: (i) resistance through constitutive leaf traits, (ii) tolerance to defoliation and (iii) escape in space, for three caesalpiniaceous tree species *Microberlinia bisulcata*, *Tetraberlinia bifoliolata* and *T. korupensis*, which co-dominate groves within the lowland primary rain forest of Korup National Park (Cameroon).
3. Mesh cages were placed around individual wild seedlings to exclude insect herbivores at 41 paired canopy gap and understorey locations. After following seedling growth and survival for *c.* 2 years, caged and control treatments were removed, leaves harvested to determine nutrient and phenolic concentrations, leaf mass per area estimated, and seedling performance in gaps followed for a further *c.* 2 years to quantify tolerance to the leaf harvesting.
4. The more nutrient-rich leaves of the weakly shade-tolerant *M. bisulcata* were damaged much more in gaps than the two strongly shade-tolerant *Tetraberlinia* species, which had higher leaf mass per area and concentrations of total phenols. Conversely, the faster-growing *M. bisulcata* was better able to tolerate defoliation in terms of height growth (reflushing capacity), but not at maintaining overall leaf numbers, than the other two species.
5. Across gaps, insect-mediated Janzen–Connell effects were most pronounced for *M. bisulcata*, less so for *T. korupensis*, and not detectable for *T. bifoliolata*. The three species differed distinctly in their secondary metabolic profiles.
6. Taken together, the results suggested a conceptual framework linking the three sets of traits, one in which the three co-dominant species adopt different strategies towards herbivore pressure depending on their different responses to light availability. This study is one of the first in a natural forest ecosystem to examine resistance to, tolerance of, and escape from herbivory among a group of co-occurring tropical tree species.

Key-words: canopy gap disturbances, growth–resistance trade-off, herbivory, Janzen–Connell effects, leaf phenolics, plant–insect interactions, tolerance to defoliation, tropical Africa

Introduction

Besides growth and reproduction, a key function in nearly all plants is to adequately defend themselves against animals that eat them (Bazzaz *et al.* 1987). Plants can reduce the negative impacts of attacks from herbivores in three main ways (Boege & Marquis 2005; Marquis 2005; Walters 2011): (i) investing in constitutive traits that resist feeding

by reduced palatability, and hence how fast they are eaten; (ii) tolerating being eaten by compensation of growth losses (van der Meijden, Wijn & Verkaar 1988; Wise & Abrahamson 2007); and (iii) escaping in space from density- or distant-responsive herbivores (Janzen 1970; Connell 1971). These three trait sets, constituting resistance, tolerance and escape, are often evaluated separately, however, making it difficult to determine their possible trade-offs and relevance for different plant species in vegetation

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communities (Marquis 2005). To our knowledge, all three have not been investigated before in a single ecosystem under natural conditions.

Of the three trait sets, by far the most studied to date is resistance in the form of constitutive chemical or structural traits of plant tissues (Coley & Barone 1996; Boege & Marquis 2005; Walters 2011). These two types of traits are thought to be shaped by aspects of species life history but especially intrinsic growth rate, in that faster-growing species tend to invest less in anti-herbivore defences than slower-growing ones, resulting in a growth–resistance trade-off ('resource availability hypothesis', of Coley, Bryant & Chapin 1985; reviewed by Stamp 2003a; Endara & Coley 2011; Massad *et al.* 2011). Chemical and structural traits are also thought to covary in ways that jointly influence both the feeding type and intensity of herbivory (Agrawal & Fishbein 2006). A critical factor for leaf-feeding insects is host plant nutrition, especially the concentrations of nitrogen (N) and phosphorus (P) available to them (Mattson 1980; Scriber & Slansky 1981).

Chemical traits such as the concentrations of polyphenolic compounds in leaves, and 'condensed tannins' in particular, have received much attention because of their role as deterrents of herbivory (Feeny 1970; Dudt & Shure 1994; Dixon, Xie & Sharma 2005). However, the efficacy of these secondary metabolites has been questioned when they were simultaneously evaluated alongside other major plant defence traits (Carmona, Lajeunesse & Johnson 2011; Massad *et al.* 2011; also see Mole, Ross & Waterman 1988; Close & McArthur 2002). In particular, two structural leaf traits, leaf mass per area (LMA) and leaf toughness, which are tightly correlated with each other, have been repeatedly associated with reduced herbivory, the longevity of tree seedlings under shaded conditions, and species demographic rates (Coley & Barone 1996; Hanley *et al.* 2007; Lusk & Warton 2007; Poorter *et al.* 2009; Kitajima & Poorter 2010; Kitajima *et al.* 2012).

Tolerance to herbivory has been studied less than resistance, especially under field conditions and for juvenile tropical trees (Coley & Barone 1996; Marquis 2005). This umbrella trait set can be shown either by continued survivorship under herbivore pressure (Fine *et al.* 2006) or, more traditionally, by the ability to regrow tissues after being damaged. This last is often tested by estimating how plant fitness changes across a range of defoliation levels (Strauss & Agrawal 1999; Stowe *et al.* 2000). Because tolerance is a set of traits that can be inherited, it is probably of adaptive value against herbivory, although it is less clear as yet whether it does, or must, trade off with resistance (van der Meijden, Wijn & Verkaar 1988; Fornoni 2011; Stowe & Marquis 2011). In addition to genotype local gradients in resources, especially those in least supply, should also influence how tolerance is expressed in a plant (Wise & Abrahamson 2007). For example, in tropical rain forests, where low light near the ground is strongly limiting growth in the understorey, high light provided by treefall gaps may mitigate seedling mortality despite the severe defoliation occurring there

(Blundell & Peart 2001; Norghauer, Malcolm & Zimmerman 2008). High light may even ameliorate the full impact of herbivores on tree growth of usually shade-tolerant species, whereas seedlings of shade-intolerant species may soon die if damaged in the understorey (Myers & Kitajima 2007).

In contrast to both plant resistance and tolerance, which are occasionally studied together for one or more plant species, evidence for the third trait set is often sought quite separately. Escape in space underpins the mechanism of the Janzen–Connell hypothesis for explaining tree species diversity in many tropical forests (Janzen 1970; Connell 1971). Dispersal enables juveniles to avoid the severe impact of herbivory that is disproportionately greater closer to parent trees than further away from them. Conspecific seedlings are generally most abundant close to their parents, or where parents are clustered and generate overlapping seedling shadows at more intermediate distances. Tests of Janzen–Connell (JC) effects have so far been largely focused on seed and seedling mortality, but unlike studies involving vertebrates, little empirical evidence has been gathered for invertebrate impact on seedling growth rates, much less so through experimental manipulation that physically excludes these smaller herbivores (Clark & Clark 1984; Hyatt *et al.* 2003; Carson *et al.* 2008).

The primary lowland rain forest in the southern part of Korup National Park (Cameroon) is a highly suitable ecosystem in which to simultaneously investigate these different trait sets for two reasons. First, the predominant wet season at Korup leads to many months of persistently low-light conditions in the understorey which, coupled to the very low nutrient status of the soils at the site (Gartlan *et al.* 1986), suggests potentially high costs in replacing leaf tissues lost due to herbivory (Janzen 1974; McKey *et al.* 1978; Coley, Bryant & Chapin 1985; Givnish 1999). Second, large patches (or 'groves') of forest occur that are characteristically co-dominated by three species in the subfamily Caesalpiniaceae (Leguminosae = Fabaceae), extending over 10's to 100's of hectares and forming up to *c.* 60% of the basal area of trees locally (Newbery *et al.* 2013). This ancient primary forest (Gartlan 1992) offers a simply structured and tractable ecosystem in which to test hypotheses about plant–herbivore interactions.

In this study, the objective was to evaluate seedlings of the above tree species for the three anti-herbivore defence-related trait sets. Specifically, we tested the following hypotheses: (i) leaf traits within and across species are correlated with resistance to herbivory but trade-off with species' intrinsic growth rates; (ii) the species-specific ability of seedlings to survive and grow after defoliation offers an alternative to constitutive defensive traits; and (iii) conspecific adult neighbourhoods can further affect the impact of herbivory in gaps, lending a performance advantage to seedling escape in space (JC mechanism). Finally, we provide a conceptual framework that links the three sets of traits in terms of growth–defence trade-offs in response to insect herbivory at Korup and defines the strategies that each species appears to have adopted.

Materials and methods

STUDY SITE AND SPECIES

The research was conducted in the permanent 82.5-ha 'P-plot' established in 1991 in southern Korup National Park in S.W. Cameroon (5°1'N, 8°5'E; 125 m asl). The locality receives *c.* 5100 mm of rain annually, yet it has one distinct 2- to 3-month dry season from December to February (Newbery, Chuyong & Zimmermann 2006a). Further site details are found in Newbery *et al.* (2010, 2013).

The three study species were *Microberlinia bisulcata* A. Chev, *Tetraberlinia korupensis* Wieringa and *Tetraberlinia bifoliolata* (Harms) Haumann. All of them are mast fruiting, dispersing their discoid seeds ballistically over distances of 20–60 m during the latter half of the wet season (July to October). They are ectomycorrhizal species. Mean \pm SE seed masses of the species are 0.64 \pm 0.02, 1.36 \pm 0.03 and 1.57 \pm 0.02 g, respectively (Green & Newbery 2001). In the 2005 re-enumeration of the P-plot (see Newbery *et al.* 2013), where all trees \geq 50 cm diam were measured, the species' relative abundances were 18.3%, 13.7% and 10.3% of stems with corresponding basal area representations of 38.8%, 12.5% and 10.6% (for *M. bisulcata*, *T. korupensis* and *T. bifoliolata*, respectively).

EXCLUSION EXPERIMENT

Individual cages of mesh (1 \times 4-mm hole size, 40 \times 40 \times 50-cm tall) and control treatments directly quantified the impact of insect herbivores on the seedling performance of the three species in contrasting forest light environments (see Norghauer & Newbery 2013, 2014, for full details). Naturally formed gaps were searched for in early December 2007 and defined as obvious canopy openings overhead with tree/limb fallen debris below. The seedlings used were all newly established (same aged cohort, 2–5 months old, with non-lignified stems), firmly rooted, not overtopped by other seedling vegetation, had both original leaves present for the *Tetraberlinia* spp. (at least four leaves for *M. bisulcata*), and had <5% of their leaf area damaged when first flagged. Between mid-December 2007 and mid-January 2008, across 80 ha of the P-plot, a total of 332 control roofs and 332 cages were installed ($n = 664$). These were split between paired canopy gap and understorey locations ($n = 41$ 'blocks' in total) but split unevenly among species because of seed source and dispersal limitations into gaps. The average distance between paired gap/understorey locations was 37.1 m; the nearest-neighbour distance among gaps, using the centres of seedling distributions in each, was 90.9 m (SE = 5.4 m; min–max = 43.9–202.6 m). Because *T. korupensis* did not have a masting year in 2007, its sample size was the smallest with 104 seedlings in 10 blocks (cf. *T. bifoliolata* with 172 in 18 blocks; *M. bisulcata* with 388 in 34 blocks). After installation, seedlings were again measured for height and leaf number three times: at census 2 (9–14 November 2008), census 3 (11–15 March 2009) and census 4 (4–10 October 2009). The censuses defined three successive time intervals: a late dry-wet season 2008, a second dry season 2009 and a second wet season 2009.

The experiment lasted *c.* 22 months in all, during which light availability above seedlings was measured midway and at the end (%PPFD_{mid} and %PPFD_{end}, where PPFD = photosynthetic photon flux density transmitted through the canopy under overcast conditions; Methods S1, Supporting Information). Light availability was not significantly different between caged and control seedlings for either canopy type (Norghauer & Newbery 2013). Following the approach taken by Fine *et al.* (2006), relative growth rates in height and leaf number (*RGR-HT* and *RGR-LV*) were used to calculate an impact of herbivory (or 'herbivore effect') on seedling growth rates in gaps. After the harvesting of leaves at

census 4 to quantify their traits, a *c.* 27-month 'post-treatment' phase followed until census 5 (3–4 March 2012; refer to Norghauer & Newbery 2013 for a timeline graphic of the experiment).

RESISTANCE

Leaf collection and storage

Leaves were harvested from seedlings at understorey locations on 18–20 October 2009 and in gaps on 27 October through 4 November 2009. In the understorey, all leaves per seedling were taken; most of them were the original same-age leaves already present at census 1, and therefore the oldest ones possible in our experiment, as they were formed during establishment. Because leaf age imposes a biophysical constraint on defensive traits and leaf longevity can vary among species (Coley & Barone 1996), the many more leaves produced in gaps were grouped according to three age classes: 'old', being those produced after census 1 but prior to census 2 (>7 months, max. 21 months in age); 'mature', being those produced since census 2 (1–7 months); and 'young', those recently expanded and not yet fully greened leaves (<1 month). In gaps, very heavily damaged leaves (>50% missing leaf area) were avoided where possible, and leaves (both leaflets and rachises) were collected from plants in an even manner commensurate with their total leaf number by taking every leaf, or every second one, or every third one, and so on. Leaves were kept dry with silica gel in paper or plastic bags and oven-dried once at 45–55 °C for 2–3 days within 2 days of collection and again in Switzerland before chemical analyses were performed.

Leaf mass per area

Subsets of harvested leaves that were completely undamaged, up to eight per seedling, mostly caged, were opportunistically sampled to estimate species-specific LMA. The product of individual leaf length and width at its widest point (in mm) was a strong predictor of a leaf's area for each species ($r^2 = 99.0$ – 99.6%). Total leaf dry mass was divided by total leaf area per seedling to give LMA.

Nutrients

Stored leaves from different seedlings of each species were separately bulked for gap and understorey locations, according to leaf age and treatment (caged vs. control). They were fine-milled, re-dried for 48 h at 50 °C and analysed for five macronutrients. From each sample, 300 mg was digested in a 2.5 ml mixture of selenium, sulphuric acid and salicylic acid and analysed for nitrogen (N) using the modified Bertholet reaction, and for phosphorus (P) with the molybdenum blue one. Concentrations were determined colorimetrically on a Skalar San⁺⁺ continuous flow autoanalyzer (Skalar Analytical B.V., Breda, Netherlands). The same digests were used to determine potassium (K), magnesium (Mg) and calcium (Ca) concentrations on Optima 7000 inductively coupled plasma, optical emission spectrometer (ICP-OES; Perkin Elmer, Waltham, MA, USA).

Secondary metabolites

Sixty dried leaf samples, 20 per species that spanned the three leaf age classes, had been taken from interspersed understorey and gap locations and evenly split across caged and control treatments. This resulted in 75% of the forest blocks being used. The samples were analysed in an untargeted manner for low molecular weight primary and secondary metabolites, namely organic acids, phenols, flavonoids and non-volatile terpenes. To separate and detect

these compounds, ultra-high-pressure liquid chromatography coupled to high-resolution mass spectrometry was used (UHPLC-HRMS; see Methods S2 for details). Ready identification of metabolites was hindered by the fact that the three species' leaf chemistry was largely unknown beforehand.

Total phenols

Concentrations of total phenolic compounds were determined for locations with enough milled leaf material remaining. This was achieved by an automated procedure for a reaction where an alkaline ferric solution is added to leaf material in a mixture of carbonylmethyl cellulose and ethylenediamine tetra-acetic acid (EDTA). This method has been shown to yield near-identical results to the classic, more manually intensive, Folin–Ciocalteu method (de Mattos & Zagal 2010). Phenols were extracted from 16 mg of material in 60% methanol and stored at -20°C . Catechin (+)– was chosen as the standard ($\text{C}_{15}\text{H}_{14}\text{O}_6$, xH_2O ; Sigma-Aldrich, St. Louis, MO, USA) because, according to UHPLC-HRMS profiling, it or its derivatives were prevalent in the leaves of all three species. The more commonly used gallic acid was detected only in *M. bisulcata*. Moreover, species comparisons of total phenolics are more reliable when the standard used is actually present in their tissues (Waterman & Mole 1994); catechin has been used in previous studies quantifying polyphenols in trees (e.g. Booker, Anttonen & Heagle 1996). Samples from some understorey locations were bulked on the basis of their spatial proximity to one another because of low amounts of sample material. Concentrations were expressed in (+)– catechin equivalents (CE, mg g^{-1}).

TOLERANCE

To measure each species' tolerance to simulated herbivory (i.e. leaf harvesting for traits at census 4), and to test whether it was influenced by prior exposure to herbivores, all gap locations were revisited on 3–4 March 2012 (census 5; Norghauer & Newbery 2013). Many of them had closed up since census 4, but a few had stayed open because of renewed disturbance by foraging elephants. Stem height and leaf number were quantified for each surviving seedling from the starting sample (plus some replaced *M. bisulcata* controls, see Norghauer & Newbery 2014).

ESCAPE

For each of the 41 gaps, the centre of the group of treated seedlings was approximated visually. Distances were measured from these centres to the nearest *M. bisulcata* and/or *Tetraberlinia* spp. adult within a 75-m radius ($Dis_{75\text{m}}$). Adults of the two species were trees with stem diameters of ≥ 50 and ≥ 30 cm respectively (unpublished fecundity data). Basal areas of conspecific trees ≥ 10 cm stem diameter were also summed per species, but within a 50-m radius of positions ($BA_{50\text{m}}$). In the last plot censuses of 2005–2007, averaged nearest-neighbour distances between stems ≥ 10 cm diameter were 20, 18 and 15 m for *M. bisulcata*, *T. bifo-liolata* and *T. korupensis*, respectively.

Data analyses

RESISTANCE

Leaf mass per area

Because new leaf production only occurred in gaps (Norghauer & Newbery 2013, 2014), and fully intact control

leaves from gaps were largely lacking (many of them had been partly eaten), linear mixed models (LMMs) in GENSTAT v.14.1 were applied to test for species' differences in LMA with gap location as the random (block) term.

Nutrients

Concentrations for each leaf age class ('young', 'mature' and 'old') were averaged for each species at gap locations for comparison with the understorey. Gap leaves were then separately compared across age classes, species and treatments (caged vs. control). In both analyses, LMMs were used for the five macronutrient concentrations ($\alpha = 0.05/5$ or 0.01 as the family-wide experiment error rate). In the first analysis, model fitting was improved, and assumptions better met, when the variables were log-transformed: only P and K required log-transformation in the second analysis, however. Because of many post-hoc multiple pairwise comparisons involved (15 and 36, respectively), for these $\alpha = 0.05$ was adjusted in a sequential Bonferroni procedure that used *P*-values of *t*-statistics derived from average standard errors of differences between pairs of means (Quinn & Keough 2002).

Metabolite profiling

Metabolic profiles were treated using Markerlynx XSTM (Waters, Milford, MA, USA), which automatically performs feature detection and alignment, noise elimination and de-isotoping. The generated list of features (i.e. variables described by both their retention time and mass-to-charge ratio) were normalized by unit norm (i.e. to the total integrated area per sample). Data were mean-centred and Pareto-scaled prior to multivariate analysis. In Pareto scaling, variables are divided by the square root of their standard deviation. This is commonly used in metabolomic studies as an intermediate between no scaling and dividing variables by their standard deviation (a.k.a. UV-scaling; Boccard, Veuthey & Rudaz 2010); variables of medium to high intensity have more influence on the outcome than when UV-scaling is applied (van den Berg *et al.* 2006). Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA) tested for evidence of sample clustering (Ezinfo Software, Umetrics, Umea, Sweden).

Total phenols

To investigate how the concentrations of total phenols may have been influenced by light levels and correlated with leaf damage, focus was placed on the late dry-wet season 2008 (DW_1). This was when gaps were most open and plant growth the fastest, and the interval for which amounts of leaf material for analysis were also the largest. To better compare aged leaves between the two canopy types, those collected in the understorey were compared with 'old' caged and control ones from gaps, in a single LMM. Caged and control replicates were pooled here

because they had similar concentrations of phenols in gaps and understorey ($P = 0.408$ and 0.865 , respectively). For the gap samples, for each leaf age class, a separate LMM tested for main and interaction effects of species and herbivory treatment. Following Sagers & Coley (1995), possible intraspecific costs of total phenols in leaves on their growth ($RGR-LV$) in the DW_1 interval were investigated using just caged seedlings. Light availability ($\%PPFD_{mid}$) was the first term in each species' LMM (to remove its influence on growth) followed by total phenol concentrations for mature or old leaves (both already averaged at the gap level). An analysis for mature leaves of *T. korupensis* was not possible because there were data available for only three gaps.

Growth–resistance trade-off

Following Fine *et al.* (2006), three terms were derived from the gap-habitat data: (i) 'herbivore effect' on each species (the caged-minus-control difference in means of $RGR-HT$ and $RGR-LV$ in the DW_1 interval, equivalent to an effect size); (ii) each species' inherent growth rate (slope of $RGR-HT$ and $RGR-LV$ regressed on $\%PPFD_{mid}$ for caged and control seedlings pooled for DW_1); and (iii) the concentrations of total phenols in leaves (midpoint of averaged concentrations of 'old' and 'mature' leaves, caged and control together). In the calculation of the herbivore effect on $RGR-LV$ for *T. korupensis*, one extreme outlier was omitted.

TOLERANCE

Individual percentage change in height or leaf number of surviving seedlings in gaps between censuses 4 and 5 was calculated. Following Stowe *et al.* (2000), tolerance was quantified as the regression slope of this percentage change in growth as a function of the amount of simulated herbivory inflicted on seedlings (log-transformed percentage of leaves harvested). LMMs were fitted separately for each species, with gap (block) as the random term; seedling light availability (log-transformed $\%PPFD_{end}$) was first entered into the model to remove its influence on tolerance ability, followed by main and interaction effects of defoliation level and herbivory treatment, caged and control (i.e. each model had five terms in total). Values of percentage change in height or leaf number were log-transformed in all LMMs to meet model assumptions. Models for height could not be fitted for either *Tetraberlinia* spp. without the removal of one confirmed case of stem dieback in each of their samples; for *M. bisulcata*, four very poorly growing seedlings (two caged and two control) were also removed as outliers from the analysis of height.

A greater percentage of leaves were harvested from control than from caged *M. bisulcata* seedlings (LMM, species \times treatment interaction, $F_{2,239.6} = 3.50$, $P = 0.032$, with three extreme outliers of 300%, 325% and 500% omitted). Several cases of >100% harvesting arose because of

new leaves that became fully expanded in the period between the census 4 leaf counts and the subsequent harvest. Means \pm SE (range) of percentage defoliation for control and caged samples, respectively, were $92.2 \pm 7.1\%$ (37.1–500) and $73.4 \pm 2.0\%$ (25.6–120) for *M. bisulcata*; $88.6 \pm 6.0\%$ (37.5–300) and $79.6 \pm 1.9\%$ (33.3–100) for *T. bifoliolata*; and $81.5 \pm 3.6\%$ (58.3–116.7) and $77.4 \pm 2.8\%$ (47.1–100) for *T. korupensis*. The mean stem heights of survivors at census 5 and their total number of leaves were compared among species and treatments using LMMs. For leaf numbers on *T. bifoliolata*, one outlier was omitted.

Trade-offs involving tolerance and resistance were investigated by plotting seedling performance slopes for the control samples only (one outlier removed for *M. bisulcata* in refitted LMMs). Tolerance to mortality risk was expressed as the percentage of controls in the gaps that survived the post-treatment phase.

ESCAPE

On a cohort of seedlings, Janzen–Connell effects are expected to accumulate over time (see Augspurger 1983; Clark & Clark 1984; Leigh *et al.* 2004). These were accordingly investigated using the *gap-level means* of final height and total leaf number of survivors at census 4. If a JC effect was operating, it should have been detectable among the control seedlings in the form of plant performance increasing with distance (Dis_{75m}) and/or decreasing with basal area (BA_{50m} ; one-tailed tests). For the caged seedlings, the prediction was one of no change, although this did not exclude any effects of other unmeasured distance- or density-related processes (two-tailed tests for a slope different from zero).

As BA_{50m} -values were derived from the last plot enumeration in 2005–2007, some neighbourhoods had to be corrected for recent deaths of adult *T. bifoliolata* ($n = 1$ gap) and *T. korupensis* ($n = 3$ gaps) trees. Of those 11 gaps located 50 m inside of the plot boundary ($n = 7$) or just outside of it ($n = 4$), one had no stems within 50 m (from Dis_{75m} search). The neighbourhoods at another eight of these 11 gaps had already been determined (Norghauer & Newbery 2011), leaving just one and two gaps unusable in the analysis using BA_{50m} as a predictor for *Tetraberlinia* spp. and *M. bisulcata*, respectively.

Separate LMMs of Dis_{75m} and BA_{50m} were fitted for each species \times treatment combination, with again mean gap $\%PPFD_{end}$ as the first term in each model to remove any light effect on seedling growth. A test of difference in the slopes between control and caged populations in one model (i.e. treatment \times distance interaction term) could not be made because distance was fully confounded with gap (block). No attempt had been made at the set-up to control for seedling densities across the 41 gaps because our large stratified random design was aimed to account for spatial variation in numbers of newly established seedlings across the forest. Because the three species mast fruit and have ballistic seed dispersal, our assumption that

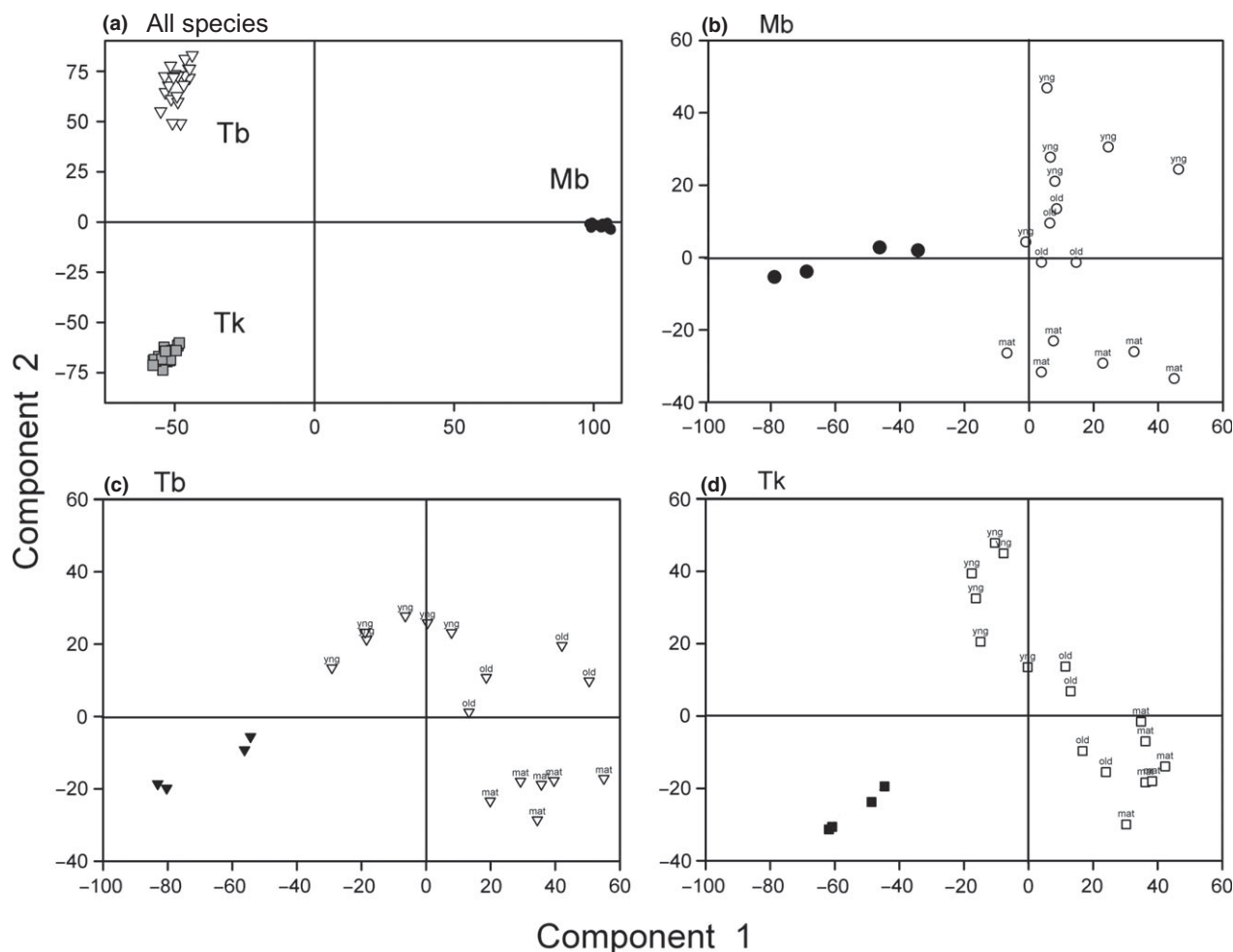


Fig. 1. Multivariate analysis of leaf metabolite profiles of *Microberlinia bisulcata* (Mb), *Tetraberlinia bifoliolata* (Tb) and *T. korupensis* (Tk) seedlings at Korup. (a) Principal component analysis (PCA) of metabolite features. The first and second components explained 41% and 24% of the variance, respectively. Samples for each species, $n = 20$, were split evenly among the caged and control treatments and comprised 16 gap and four understorey locations (see Methods). (b–d) Partial least-squares discriminant analysis (PLS-DA) scores for the leaf metabolite data of *M. bisulcata*, *T. bifoliolata* and *T. korupensis* tree seedlings at Korup. For first and second components, proportions of variance explained were (b) 24%, 12%; (c) 35%, 8%; and (d) 24%, 15%, respectively. Leaf samples from the gaps were of known age classes: young (<1 month; ‘yng’), mature (1–7 months; ‘mat’) and old (>7 months ‘old’); those from the understorey consisted almost entirely of leaf material formed during seedling establishment (i.e. oldest possible; filled symbols in b–d).

associations between total phenols in old leaves and mean damage to control leaves produced in the late dry-wet season 2008 (one-sided test of correlation, three P -values of 0.93, 0.53 and 0.48; Fig. 3b). At the between-species level, however, *M. bisulcata*, with the lowest concentration of total phenols, had the greatest leaf damage on average, followed by *T. korupensis*, and then *T. bifoliolata*, which had the highest concentration of total phenols and least damage (LMM, species term, $F_{2,8.8} = 63.3$, $P < 0.001$, $n = 39$; Fig. 3b inset). As the second term in the LMM, total phenol concentrations were not significant ($F_{1,10.9} = 2.58$, $P = 0.14$). Finally, considering the caged seedlings, there was no significant evidence to suggest a measurable growth cost to total phenols in any of the three species when either old or mature leaves were used in the analyses (P -values: 0.13–0.79).

Growth–resistance trade-off

The three species followed the expected positive correlation between the impact of herbivores on seedling growth rate and species’ inherent capacity to grow (Fig. 4a), and to a lesser extent the negative correlation between leaf defence and impact (Fig. 4b). Only for leaf numbers did the species appear to follow the negative trade-off predicted between putative defence (total phenols) and plant growth (Fig. 4c).

TOLERANCE

The three co-dominant tree species responded differently to leaf removal. For *M. bisulcata*, the effect of prior defoliation on its percentage height change depended on whether its seedlings had been previously caged or not

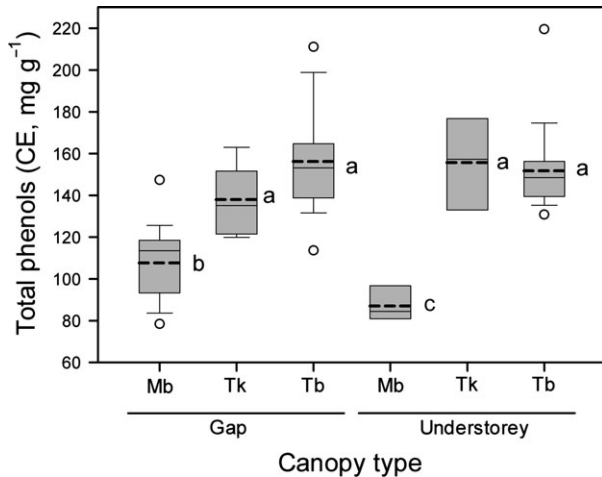


Fig. 2. Phenolic concentrations in leaves of *Microberlinia bisulcata* (Mb), *Tetraberlinia bifoliolata* (Tb) and *T. korupensis* (Tk) tree seedlings in canopy gaps and at understory locations at Korup presented as boxplots. Samples for gaps are 'old' leaves (>7 months in age) pooled per location separately for caged and control seedling. Concentrations are expressed in catechin equivalents (CE). Box boundaries are 25th and 75th percentiles; whiskers indicate 10th and 90th percentiles. Means are indicated by bold dashed lines through boxes; those not sharing the same letters are significantly different at $P \leq 0.05$. Samples sizes, from left to right, were 29, 21, 13, 7, 21 and 3 locations: each location consisted of pooled leaf materials from surviving seedlings (at census 4).

(defoliation \times treatment interaction, $F_{1,83.4} = 5.52$, $P = 0.021$); the slope of the former was significantly different from the near-horizontal slope of the controls (treatment effect, using control as reference = -0.28). Neither this interaction nor the main effects were significant for leaf number, however (P -values: 0.13–0.68). Light availability was, not surprisingly, a highly significant predictor of percentage changes in height and leaf number for *M. bisulcata* ($F_{1,74.7} = 20.8$, $P < 0.001$; $F_{1,85.5} = 9.81$, $P = 0.002$, respectively).

Among *T. bifoliolata* seedlings, there was a significant decline in height change with increased percentage defoliation (slope \pm SE = -0.381 ± 0.165 ; $F_{1,52.1} = 4.37$, $P = 0.041$); a similar pattern, albeit not quite significant, occurred for leaf number ($F_{1,52.1} = 3.46$, $P = 0.07$), while the positive effect of light availability on height and leaf number was weak (slopes, 0.057 ± 0.164 , 1.896 ± 0.085 ; $P = 0.10$ and 0.049 , respectively). For changes in the height of *T. korupensis* – the species with the smallest sample sizes, none of the model terms were significant (P -values: 0.11–0.93). Nevertheless, previously caged seedlings produced more leaves than control ones (treatment term, $F_{1,25.4} = 6.48$, $P = 0.017$), while the remaining three model terms were not significant (P -values: 0.48–0.80).

A trade-off between tolerance (using control values) and resistance was more evident for species' growth in height (linear trend) than in leaf number (modal; Fig. 5a). Substituting our estimates of species-specific LMA for total phenols gave a similar pattern (Fig. 5b). When tolerance was investigated in terms of the long-term mortality risk of

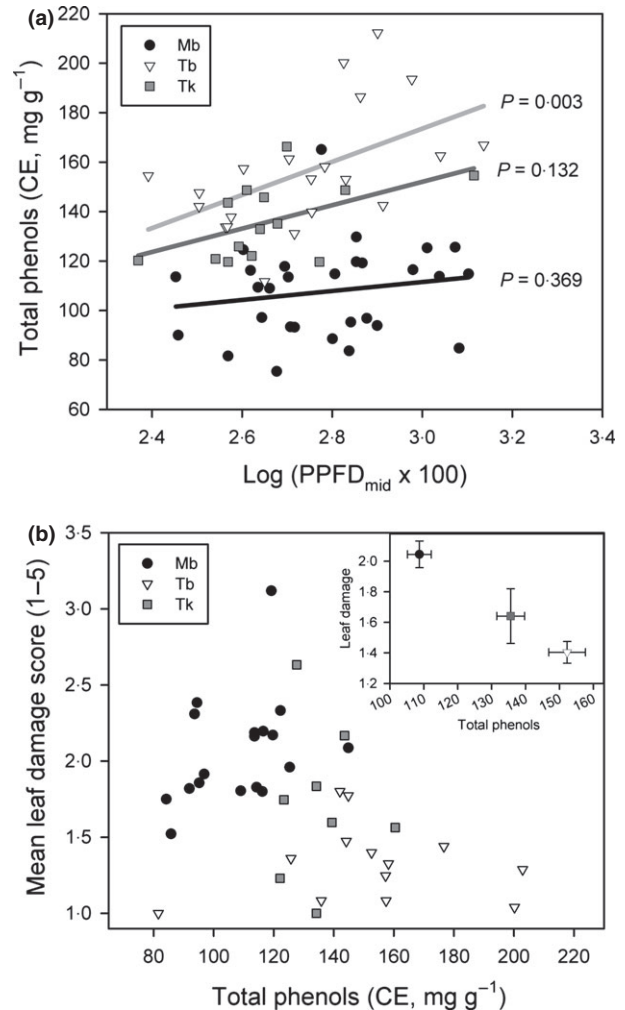


Fig. 3. The relationships between the concentrations of total phenolic compounds in 'old' leaves (>7 months in age) of *Microberlinia bisulcata* (Mb), *Tetraberlinia bifoliolata* (Tb) and *T. korupensis* (Tk) seedlings growing in canopy gaps at Korup and (a) light availability midway through the experiment (see Methods) and (b) the mean static leaf damage levels on controls in the late dry-wet season 2008 (i.e. the first growing interval). In (a) caged and control values are plotted, while in (b) their midpoint concentration was used instead for a given gap, where applicable. In (b) the inset shows the means (\pm SE) of the symbols in the main graphic for each species. Phenol concentrations are expressed in catechin equivalents (CE).

control seedlings, *T. bifoliolata* – the species with the highest concentration of phenols and highest LMA – had also the highest survivorship in the post-treatment period. *Microberlinia bisulcata*, with the lowest levels of this putative defence (and lowest LMA), conversely had the lowest survivorship, while *T. korupensis* was intermediate with respect to both leaf variables and survivorship (Fig. 5a,b). Compared to controls, 68.1%, 88.0% and 73.2% of caged seedlings survived the post-treatment period for *M. bisulcata*, *T. korupensis* and *T. bifoliolata*, respectively. The corresponding treatment differences in percentage survivorship between caged and controls were 16.7%, 7.3% and 26.1%.

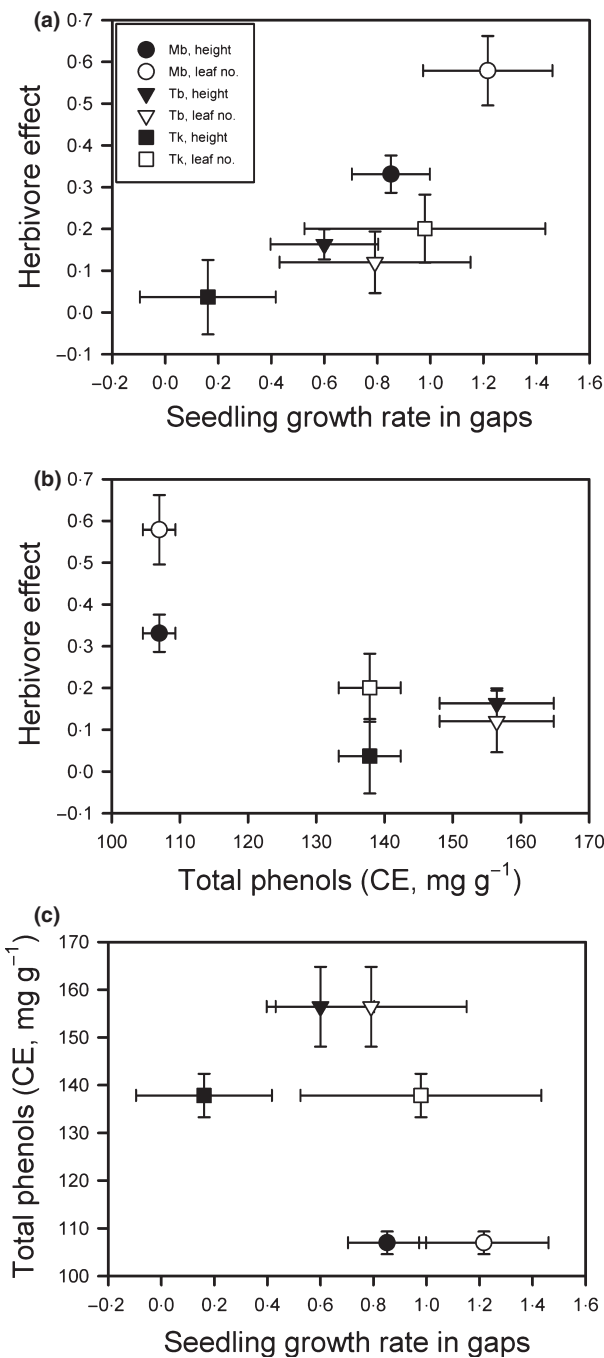


Fig. 4. Pairwise relationships among the species-specific impact of insect herbivores (herbivore effect), each species' growth rate and a putative anti-herbivore defence (concentration of total phenolics, in catechin equivalents [CE]) for tree seedlings of *Microberlinia bisulcata* (Mb), *Tetraberlinia bifoliolata* (Tb) and *T. korupensis* (Tk), in canopy gaps at Korup. The symbols indicate relative changes in heights (RGR-HT) and leaf numbers (RGR-LV). For (a) and (b), the herbivore effect was calculated for the late dry-wet season 2008, that is, the first growing interval (when gaps were most open). Bars are SEs of means. For calculations of the 'herbivore effect' in panels (a) and (b), $n = 30-30$, 18-18 and 9-8 gap locations for height-leaf number responses in Mb, Tb and Tk, respectively; likewise, for 'total phenols' in (b) and (c), $n = 27-27$, 8-8 and 15-15; for 'seedling growth rate in gaps' in (a) and (c), $n = 63-64$, 35-35 and 18-18 gap-level means of caged and/or control seedlings.

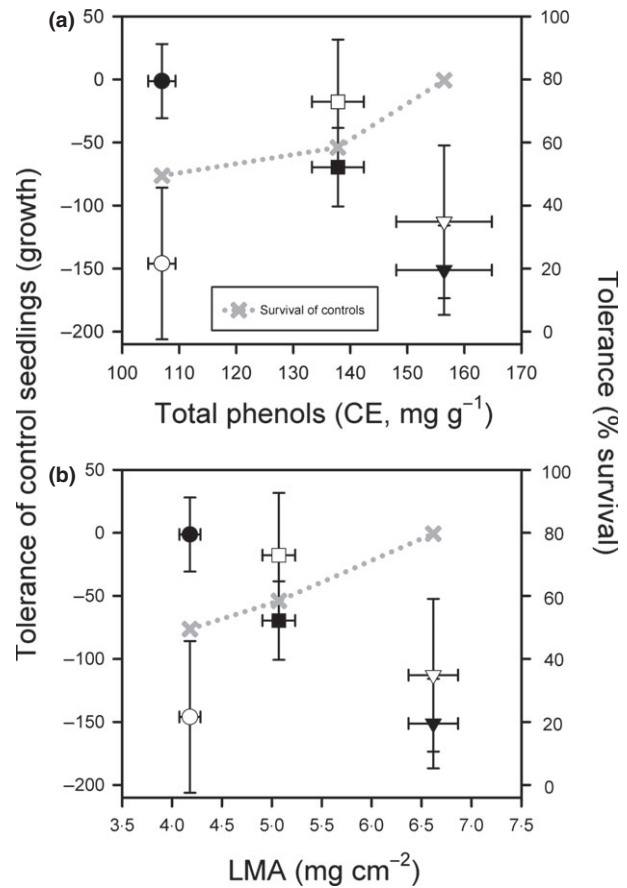


Fig. 5. Tolerance of tree seedlings in the control sample to manual defoliation expressed in terms of growth and survival in the post-treatment phase of the experiment in relation to (a) the putative anti-herbivore defence [concentration of total phenolics, in catechin equivalents (CE)] and to (b) leaf mass per area (LMA), of *Microberlinia bisulcata* (circles), *T. bifoliolata* (triangles) *Tetraberlinia korupensis* (squares) in canopy gaps at Korup. The closed-open symbols are slope values of corresponding percentage changes in heights-leaf numbers regressed on percentage defoliation. Bars are SEs of means. The gray crosses with dotted lines display the survival values. For the three species, respectively: $n = 36-35$, 13-12 and 30-30 control seedlings in gaps that survived to census 5 to calculate height-leaf number responses, and likewise $n = 27-27$, 8-8 and 15-15 gap locations to calculate total phenol concentrations (averaged first across seedlings in each gap).

ESCAPE

By the end of the *c.* 22-month experimental period of insect exclusion, unlike their caged counterparts, surviving *M. bisulcata* control seedlings in gaps had significantly more leaves farther away from nearest conspecific adult ($P = 0.004$), and fewer leaves with increasing conspecific basal area ($P = 0.009$, Table S4, Fig. S1). By contrast, numbers of leaves on *T. bifoliolata* control seedlings were not significantly affected in a negative way by their conspecific neighbourhood (Table S4, Fig. S2). Both caged and control *T. korupensis* seedlings had more leaves farther from nearest conspecific adult ($P = 0.064$, 0.014, respectively), but not the fewer leaves expected when BA_{50m} was the predictor (P -values = 0.306, 0.066; see Table S4,

Fig. S3). For both *M. bisulcata* and *T. bifoliolata*, neither conspecific neighbourhood term had a significant effect on final stem height at census 4 (P -values = 0.099–0.771), whereas *T. korupensis* controls were significantly taller farthest from the nearest conspecific adult (P = 0.007), but not so when caged (P = 0.482; Table S4, Fig. S3).

Discussion

RESISTANCE

The stronger impact – in terms of both lost photosynthetic area and reduced growth rates – of insect herbivores on the faster-growing *M. bisulcata* than on the two *Tetraberlinia* spp. suggests that its foliage had qualities that made it more palatable. Leaves of *M. bisulcata* seedlings showed the highest concentrations of the five nutrients investigated (especially N and P), they were lowest in leaf mass per area (LMA), and they were lowest in their concentrations of total phenols. Although the *Tetraberlinia* species differed little in their nutrient concentrations, they did significantly in terms of LMA. Taken together, the results from the present study lend support to the hypothesis of a growth–resistance trade-off across tree species, which is a central tenet of the resource availability hypothesis (Coley, Bryant & Chapin 1985; Coley 1987). They also concur with a global meta-analysis that found LMA to be positively correlated with the degree of shade tolerance across tree species (Lusk & Warton 2007; see also Kitajima & Poorter 2010). In our experiment, species' shade tolerance was defined as the persistence (survival) of its seedlings in the understorey and found to be ranked *M. bisulcata* < *T. korupensis* < *T. bifoliolata* (see Norghauer & Newbery 2013).

The higher N and P concentrations in the leaves of *M. bisulcata* support previous work at Korup (Green & Newbery 2001). But the extent to which nutrient concentrations could directly affect herbivory within species remains uncertain, given the weak correlations that were found, and that nutrient levels were not experimentally manipulated. It is therefore more likely that LMA and polyphenols as constitutive defences played larger roles than nutrients in determining the patterns of herbivory and its impact on tree seedling growth in gaps at Korup. Among the three species, LMA varied in proportion to the concentration of total phenols, and any effects of these two variables cannot be distinguished from one another. Poorter *et al.* (2009) showed that LMA is generally correlated with soluble phenolics and total structural carbohydrates in plants, and for several Australian rain forest trees, leaf toughness and total phenolics were also positively correlated (Read *et al.* 2009). Both earlier and more recent research suggests that LMA, and hence leaf toughness (Kitajima & Poorter 2010), has a greater influence in deterring herbivory on tropical tree seedlings and saplings than measures of total phenolics or tannins *per se* (Coley 1987; Poorter *et al.* 2004; Hanley *et al.* 2007; Carmona, Lajeunesse & Johnson 2011; Kitajima *et al.* 2012).

Several prominent secondary metabolites in tree leaves were identified from this study, namely flavonoids and their building blocks. Although this general class of compounds has been shown to be highly bioactive in leaves of *Inga* spp. and to factor in their defensive strategies against insect herbivores [(Coley *et al.* 2005; Brenes-Arguedas *et al.* 2006; Kursar *et al.* 2009)], they may serve other ecological functions [(McKey *et al.* 1978; Mole, Ross & Waterman 1988; Close & McArthur 2002; Dixon, Xie & Sharma 2005] in this forest, including a possible role in leaf decomposition rates on nutrient poor soils (L. Njampa, I.M. Alexander & D.M. Newbery, unpublished).

The major difference between *M. bisulcata* and the two *Tetraberlinia* species along the first principal component reflected the former's lack of condensed tannins (larger polyphenols). Along the second component, however, neither chemical polarity nor molecular weight explained the *T. bifoliolata* and *T. korupensis* differentiation. Instead, subtle differences in typical polyphenol structures – flavonoids C₃₀H₂₆O₁₁ vs. C₃₀H₂₆O₁₂, likewise for glucosides C₂₁H₂₂O₁₁ vs. C₂₀H₂₀O₁₁ and for catechin vs. epicatechin – suggest that different metabolic pathways operate in the two *Tetraberlinia* spp.

TOLERANCE

The resource availability hypothesis implicitly suggests that faster-growing species should be better able to maintain their growth when damaged by herbivores than slower-growing species (Coley, Bryant & Chapin 1985). *Microberlinia bisulcata* showed higher tolerance to defoliation in terms of maintaining its growth in height than other species, which can be interpreted as a proxy for its reflushing capacity (normally any height increment is accompanied by leaves being flushed). This in turn might trade off with leaf mass per area (LMA) as a defensive trait. Strong evidence from other studies indicates that the evolution of tolerance might be favoured to mitigate impacts from specialist herbivores (Agrawal & Fishbein 2008), an explanation we cannot discount because *M. bisulcata* was attacked by an unidentified moth caterpillar. Although *M. bisulcata* was the most tolerant of the three species in terms of height growth, it was not so tolerant in replacing its lost leaves, and this may have been due to gaps becoming more light-limited after census 4. Alternatively, it may be more important for this species to maintain height growth (to position new leaves higher above the ground) over its total leaf area *per se*, in order to compete effectively amid the usually dense gap vegetation.

In our field study, ranking of the three species in terms of tolerance in controls was reversed when measured in terms of the risk of seedling mortality: this type of tolerance was lowest for *M. bisulcata*, intermediate for *T. korupensis* and highest for *T. bifoliolata*, matching LMA profiles and degree of shade tolerance shown in a previous study on seedling dynamics (Newbery *et al.* 2006b). The most plausible explanation is that increasing

LMA leads to longer leaf life span, and hence survival, in the more shaded conditions following gap closure.

ESCAPE

Plants can also reduce the impact of herbivory by not being present where attacks are more likely to happen (Root 1973; Feeny 1976). If these attacks come from distance or density-responsive herbivores, as articulated by Janzen (1970) and Connell (1971) more than 40 years ago (reviewed by Clark & Clark 1984; Carson *et al.* 2008), then recruitment into sapling and larger size classes should be limited to some distance away from living conspecific adults trees, or away from where they are more clustered as adults, thus preventing self-replacement of trees over back-to-back generations.

Microberlinia bisulcata had reduced leaf numbers when growing closer to the nearest conspecific adult and in neighbourhoods of higher conspecific basal area. Because no such trend was detected for protected (i.e. caged) seedlings, we interpret this as direct support for a Janzen–Connell mechanism driven by insect herbivores. However, our results also indicate that a JC-type effect caused by insects is much less likely to be operating across gaps for seedlings of *T. bifoliolata*, for which a pathogen- or parent-mediated process, perhaps happening below ground, might have reduced their leaf numbers close to conspecific adults. Interestingly, *T. korupensis* showed a more intermediate pattern of effects, mediated most likely by insect herbivores when the variable was seedling height, but by a non-herbivore biotic factor when it was leaf number – given the near-similar responses between caged and control treatments in the latter case. Taken together, the results suggest that the relative strength of escape from JC herbivores at Korup is as follows: *M. bisulcata* > *T. korupensis* > *T. bifoliolata*. A prevailing JC effect for *M. bisulcata* is also supported by the much reduced leaf damage on, and higher leaf numbers attained by, experimental seedlings grown in gaps further than 75 m from those adult trees forming the grove periphery (Norghauer & Newbery 2011).

We are aware of only one other insect exclusion test of the classic JC mechanism in a primary forest. In 4 ha of Peruvian rain forest, seedling mortality in the shaded understorey was greater near to, compared with far away from, conspecific adults for half the tree species tested, because of insects and/or pathogens (Swamy & Terborgh 2010). Unlike that field study, however, local seedling density could not be manipulated at Korup to determine whether the insects responsible for the JC effect on *M. bisulcata* leaves were either distance- or density-responsive agents. And even when considering non-exclusion types of manipulations, few studies have related tropical tree seedling growth to insect attack of leaves in the context of the JC mechanism (Clark & Clark 1984, 1985; Howe, Schupp & Westley 1985; Blundell & Peart 1998) or broadly tested their impacts at the population

level of multiple conspecific adults in a forest, as done here using BA_{50m} (cf. Norghauer, Malcolm & Zimmerman 2008; Visser *et al.* 2011). One rain forest study in West Borneo did report – not unlike for *M. bisulcata* at Korup – lower seedling leaf numbers of the dominant *Shorea quadrinervis* tree in areas of high conspecific adult density that was attributed to herbivory (Blundell & Peart 2004).

INTEGRATING THE SEEDLING STRATEGIES

A conceptual framework for understanding the three ways that can mitigate the impacts of herbivory is proposed in Fig. 6 for the caesalp species studied at Korup. The framework builds upon a key tenet of the resource availability hypothesis (growth–resistance trade-off), the JC escape model, and the general concept of a trade-off between the ability of seedlings to respond to light and their survival under shaded conditions (Kitajima 1994; Wright *et al.* 2003; Sterck, Poorter & Schieving 2006). Some authors have hinted that such a functional trade-off might interact with either canopy disturbances or JC effects, but as yet no specific predictions or hypotheses have been put forward (see Wright 2002; Leigh *et al.* 2004; Kobe & Vriesendorp 2011). The constitutive traits that contribute to resistance [leaf mass per area (LMA), polyphenol concentrations] and the traits that contribute to tolerance both trade off against seedling growth rate/survival and escape in space from insect herbivores. All four axis variables were inter-correlated with one another across the species to form principal components that could also accommodate other functional traits not quantified here (e.g. leaf toughness). Resistance, tolerance and escape were measured in the present study: the three species' growth responses to light

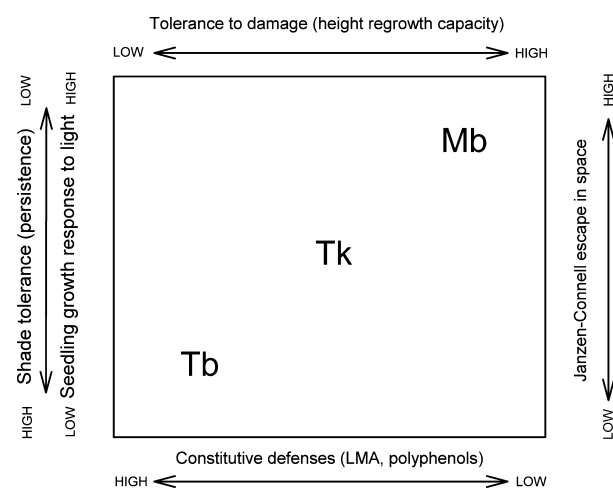


Fig. 6. A conceptual framework that links the three main plant strategies to mitigate impacts from insect herbivores on tree seedlings and saplings of the three study species *Microberlinia bisulcata* (Mb), *Tetraberlinia bifoliolata* (Tb) and *T. korupensis* (Tk), based on the results from the present field exclusion experiment and earlier studies at Korup.

and survival abilities in the shade come from previous work at Korup (Green & Newbery 2001).

Highly resistant leaves are associated with (relatively) low-light response ability (high shade tolerance) and with low tolerance to herbivory. For such species, there is little benefit to new seedlings having long-distance dispersal because their mature leaves are mostly unpalatable to herbivores. This set of traits is best shown among the three species by *T. bifoliolata* (Fig. 6), which also had the heaviest seeds. Conversely, species that are less shade tolerant can respond faster to the high light available in gaps but are necessarily not as resistant, although they are more tolerant, to herbivory. For such plants, their greater palatability makes them sought-after food in this resource-poor forest, and so their escape to mitigate the impacts of herbivores takes on greater prominence. Moreover, far dispersal not only reduces leaf damage to seedlings in gaps but also increases the probability of seeds colonizing new gap sites, as these are necessarily more frequent farther away from source trees than close to them (Augsburger 1983; Newbery *et al.* 2010). *Microberlinia bisulcata*, with the highest dispersal ability of the three species, facilitated by it having the lowest seed mass, exemplifies this strategy precisely (Fig. 6). Between *M. bisulcata* and *T. bifoliolata* lies *T. korupensis*, with an intermediate mix of strategies (as well as intermediate seed mass and relative abundance and basal area in the canopy). Both *Tetraberlinia* spp. had similarly one-third smaller crown sizes than *M. bisulcata* (Newbery *et al.* 2013), which contributed to their relatively lower dispersal abilities. In the posited framework, the defensive sets of traits thought to mainly determine response to herbivores highlight a different strategy for each species. These strategies are determined by a fundamental trade-off in costs of defence versus growth, nested within a more general trade-off in light response versus shade tolerance.

The framework is currently restricted though to tree–insect interactions on growing plants studied in an ecosystem where light availability limits recruitment. We do not yet know whether the JC effects (right axis in Fig. 6) might extend to involving mammals or pathogens, or be different at the seed stage. Being closely related species that dominate the community, a divergence in ecological strategies to mitigate impact from insect herbivory could play a crucial role in facilitating their coexistence and alleviating competition among their offspring.

Conclusions

Plant defence theory has expanded considerably since the 1960s, bringing with it some confusion and many empirical contradictions, partly because of the also many non-mutually exclusive hypotheses involved (Stamp 2003a,b; Walters 2011). Attempts to better integrate and distinguish the various models with respect to their mechanisms and predictions for plant–herbivore interactions are clearly required. The work reported in this paper is one

of the first studies in a primary forest to evaluate together the main ways that impacts of leaf herbivory are ameliorated among a group of co-occurring tree species. The three abundant, taxonomically close and presumably competing caesalp species at Korup displayed clear trait patterns and strategies with regard to their herbivore interactions. It is unlikely that this would have succeeded without the integrated experimental approach adopted under natural field conditions.

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Note

The fate of the caged experimental seedlings in gaps after census 5 could not be reported in this paper. However, in January 2014, all the gap locations were revisited (by J. Norghauer) to restore them to a ‘natural state’. All surviving previously caged and control seedlings were remeasured for their stem heights and leaf numbers counted. In the case of *M. bisulcata* (the only species of the three to show long-term significant effects of protection from herbivores; see Fig. 2d,h in Norghauer & Newbery 2013), caged seedlings were reduced in height to partly undo the growth advantage gained vis-à-vis natural herbivory (and forest dynamics).

This was achieved by reducing the stem height to match that of the control(s) in the same gap (using means of the latter where applicable). If a control plant was no longer alive in that gap, then the mean size(s) of control(s) in similar-sized gaps was used. In gaps with one or more caged seedlings still alive, each was pruned to match the height of the control in one of two ways: (i) If the caged stem had leaves below the designated point of pruning (the new reduced height), the stem was sheared with a knife, as were any lateral branches, to the reduced height. (ii) If no leaves were present below the point, the stem was instead snapped (but not severed) to mimic damage from falling branch debris: the concern here was that if stripped completely of its leaves, the seedling might not be able to resprout.

What was sought in this follow-up work was a height size correction of caged seedlings, to maintain natural regeneration dynamics in the Park, and not their outright death, as these seedlings had naturally established in gaps in this forest.

Data accessibility

Data for this paper have been deposited in the Dryad repository: <http://doi.org/10.5061/dryad.129vg>.

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Supporting Information

Additional Supporting information may be found in the online version of this article:

Methods S1. Light environment data.

Methods S2. Metabolomics techniques.

Table S1. Leaf nutrients.

Table S2. Summary of correlations between leaf nutrients, herbivory and light.

Table S3. Leaf secondary metabolites.

Table S4. Statistical results for tests of the Janzen–Connell mechanism.

Fig. S1. *Microberlinia bisulcata* regressions of the number of leaves on seedlings in gaps against distance and basal area neighbourhood terms investigated.

Fig. S2. *Tetraberlinia bifoliolata* regressions of the number of leaves on seedlings in gaps against the distance neighbourhood term.

Fig. S3. *Tetraberlinia korupensis* regressions of the number of leaves and heights of seedlings in gaps against the distance neighbourhood term.