Feeding preferences of the Christmas beetle *Anoplognathus chloropyrus* (Coleoptera: Scarabaeidae) and four paropsine species (Coleoptera: Chrysomelidae) on selected *Eucalyptus grandis* clonal foliage

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Summary

Christmas beetles (Coleoptera: Scarabaeidae) (*Anoplognathus* spp.) and paropsine leaf beetles (Coleoptera: Chrysomelidae) are common defoliators of eucalypts, including several important plantation species, throughout south-eastern Australia. It has already been demonstrated that populations and individuals of several eucalypt species vary in susceptibility to defoliation by these leaf-chewing beetles. The objective of this study was to determine whether significant variation in resistance to insect herbivory was present in *Eucalyptus grandis* genotypes selected from the hardwood plantation program of State Forests of NSW.

Grafted clones from three young trees with significantly less crown damage than neighbouring trees in a plantation established near Byron Bay, NSW, were tested for attractiveness to *Anoplognathus chloropyrus* adults in a binary choice experiment in the laboratory. Shoots from the resistant grafted ramets were paired with shoots from susceptible seedlings of similar age, and caged with feeding *A. chloropyrus* adults for 24 h. The Christmas beetles consumed significantly ($P \leq 0.05$) less leaf area from the resistant shoots, compared to that consumed from the shoots from susceptible seedlings, when given access to both. The binary choice test method was also used to test feeding preferences of four paropsine chrysomelid species: *Paropsis atomaria* (adults and larvae), *P. variolosa* (larvae only), *Paropsisterna beata* (adults), and a *Chrysophtharta* sp. (adults), using the same *E. grandis* genotypes. No significant differences ($P > 0.05$) in feeding preferences were detected for *P. variolosa* larvae, *P. atomaria* adults, *P. beata* adults or the *Chrysophtharta* sp. adults. In contrast, *P. atomaria* larvae displayed a significant preference for foliage from the susceptible seedlings. In a larval development study using *P. atomaria* larvae, those reared on the susceptible foliage developed approximately five days faster and had significantly higher survival than those reared on resistant foliage.

Several leaf traits were also compared between the two types of foliage. Leaves of similar age from the resistant clones did not differ significantly from the susceptible seedlings in terms of carbon to nitrogen content or concentrations of the monoterpene, 1,8-cineole. They did, however, have significantly higher specific leaf weight (SLW). SLW may have the potential for use in rapid screening of *E. grandis* genotypes for susceptibilty to defoliation by leaf-chewing beetles that prefer young soft foliage.

Keywords: insect resistance; defoliation; Christmas beetles; chrysomelids; *Eucalyptus grandis*

Introduction

Eucalyptus grandis is viewed in many overseas countries as a desirable plantation species (e.g. Brazil, China and South Africa). In Australia, however, the areas planted with *E. grandis* are in decline. A major disincentive to planting this potentially fast-growing species is its susceptibility to defoliating and stem-boring insects. *Eucalyptus grandis* is host to a wide range of herbivorous insects including Christmas beetles (*Anoplognathus* spp.) and paropsine chrysomelids (Coleoptera: Chrysomelidae) (Carne *et al*. 1974; Stone *et al*. 1997). Both these groups of insects regularly cause extensive damage to *E. grandis* plantations in north-eastern NSW (A. Carnegie, State Forests of New South Wales (SFNSW), *pers. comm*.). The only management options for minimising the impact of these defoliating insects available at present is the timely application of insecticides and the use of optimal silvicultural practices to maximise growth. The impetus for this study was the observation that during heavy Christmas beetle infestations in an *E. grandis* plantation in north-eastern NSW, occasional trees sustained little damage compared with surrounding trees (A. Carnegie, SFNSW, *pers. obs*.). If this apparent resistance to Christmas beetle attack is heritable, incorporation of this material into a breeding program could help decrease the susceptibility of *E. grandis* plantations to insect herbivory. This could be even more beneficial if the genotypes proved resistant to feeding by other insect herbivores, such as chrysomelid beetles (Floyd and Farrow 1994).

Several chemical and physical leaf traits are known to affect the extent of eucalypt defoliation by insects (e.g. Edwards *et al*. 1993; Steinbauer 2001), but the underlying causal mechanisms are still being elucidated (e.g. Lawler *et al*. 1998). Many of these leaf traits are intercorrelated and are often associated with leaf phenology (e.g. Ohmart *et al*. 1987; Steinbauer *et al*. 1998), confounding simple interpretation. Host quality, in terms of factors such as leaf toughness and nitrogen content, changes as leaves

mature (e.g. Steinbauer *et al*. 1998). Older eucalypt leaves tend to be tougher, less nutritious and better defended (e.g. Landsberg 1990; Ohmart 1991). We chose to measure three leaf traits previously shown to be related to insect feeding behaviour on eucalypts: foliar concentration of the two terpenes 1,8-cineole and α-pinene (Edwards *et al*. 1993; Li 1993; Stone and Bacon 1994); foliar carbon : nitrogen ratio (C : N) and specific leaf weight (g cm^{-2}) (SLW). In general, fast-growing plants tend to have lower C : N ratios than slower but better defended plants (Coley *et al*. 1985), while SLW is a leaf parameter indicative of leaf toughness (Steinbauer 2001) and leaf sclerophylly (Landsberg and Gillieson 1995) in eucalypts. While these three traits may not directly govern susceptibility of *E. grandis* to defoliation from Christmas beetles and chrysomelid leaf beetles, they are relatively easy and cheap to measure.

Shepherd *et al*. (2000) assessed variation to defoliation by Christmas beetles across a series of eucalypt clones including *E. grandis* and *E. grandis* x *E. urophylla* hybrids. He reported that the interspecific hybrids were more susceptible than the *E. grandis* individuals tested. Our study extends Shepherd's findings by comparing routine *E. grandis* seedlings with clonal material originating from three field trees exhibiting partial resistance to Christmas beetle defoliation.

In particular, we addressed the following questions:

1. Is the original resistance to Christmas beetle defoliation maintained in grafted clones?
2. Is foliage that is unattractive to Christmas beetles similarly unattractive to paropsine leaf beetles?
3. If resistance to paropsine feeding is shown, is it consistent between species and between life stages within species?
4. Does foliage from resistant clones and susceptible seedling stock differ significantly in nutritional, physical and/or chemical traits such as C : N ratio, SLW and monoterpene concentration?

Materials and methods

Collection and maintenance of insect colonies

Beetles, larvae and eggs were collected from three young SFNSW Joint Venture plantations situated close to Gloucester and Maitland in north-eastern NSW. Christmas beetles (*Anoplognathus chloropyrus*) were collected from *E. dunnii*, and paropsines *Chrysophtharta* sp., *Paropsisterna beata, Paropsis variolosa* and *Paropsis atomaria,* from *E. dunnii* and *E. grandis.*

Adult beetles were initially enclosed with field-collected foliage in cages. Additional foliage was later provided for feeding (and oviposition sites for paropsines) in the form of potted *E. grandis* seedlings from a seed source separate from that used in the feeding preference trials. Christmas beetles were maintained from late January 1999 until mid-February 1999. Cages of paropsines were initially kept in an air-conditioned glasshouse, and were later moved into an insectary when night-time temperatures decreased. Indoor temperatures were maintained at about 22°C.

Paropsine egg batches and early instar larvae were kept on moistened filter paper in Petri dishes, with later instars moved into larger jars. Fresh foliage was added as needed, with containers cleaned every 1–2 days. Food material consisted of young shoots from *E. grandis* from a source separate to that used in feeding preference trials. When larvae reached the pre-pupal stage, a layer of vermiculite (1–2 cm thick) was added to the jar below the filter paper, thus providing a substrate into which they could pupate. When adults emerged they were added to stock colonies.

Eucalyptus grandis test material

In April 1997, shoots from three 2-y-old plantation trees exhibiting noticeably less damage from Christmas beetle feeding in the field were grafted onto potted juvenile seedling rootstocks, grown from seed originating in the same seed orchard as the original field material. We refer to these grafted ramets as the Byron Bay resistant clones. The total amount of foliage available from each of the Byron Bay clones was limited, so the clonal material was pooled for testing and we did not make any comparisons between the individual clones. The susceptible seedlings were grown as potted stock plants from seedlots of families from the same seed orchard. All foliage used in the feeding preference trials was from plants of similar age.

To confirm that the original trees exhibiting resistance were *E. grandis*, fresh leaf samples from both the resistant clones and the susceptible test seedlings were sent to Dr Michael Gillings (Key Centre for Biodiversity and Bioresources, Macquarie University, Sydney). DNA fingerprinting analysis using the LP-RAPD method was carried out on the leaf samples (Gillings and Holley 1997). The resistant clonal genotypes were confirmed to be genuine members of *E. grandis* (M. Gillings, unpublished data).

Feeding choice tests using Christmas beetles

Preliminary feeding tests using Christmas beetles indicated that 24 h was sufficient for the beetles to forage over all the plant material present and to consume up to, but not exceeding, two-thirds of the foliage. Shoots of similar age with fully expanded leaves were randomly selected from the resistant Byron Bay clones and paired with shoots of similar age selected randomly from the susceptible seedlings. Shoots within pairs were matched such that each had fully expanded leaves of an equal number and size. Mature leaves were chosen because these are the leaves fed upon preferentially by *A. chloropyrus* in the field.

Shoot pairs were held in a water-filled test tube and placed in full contact, with both stems and leaves touching, to facilitate movement of the beetles onto all available foliage. Two beetles were used per trial, one being placed onto the lowest leaf of each shoot at the beginning of the trial period. All trials were conducted over 24 h, beginning and ending in the late afternoon to control for possible diurnal fluctuations in feeding. In total, 12 trials were carried out, in eight of which enough damage was sustained to permit a meaningful comparison of feeding between shoots.

Photocopies of each shoot were made before and after beetle feeding to record total leaf area per shoot and the area consumed. The stem sections were cut from these photocopies and the remaining leaf images scanned. Leaf area per shoot was determined using the program Delta-T Scan®. Shoots used in feeding preference tests were air dried for later measurement of foliage traits.

Feeding choice tests using chrysomelid beetles

Feeding choice trials pairing shoots randomly selected from the Byron Bay clonal plants and the susceptible plants were conducted on *P. atomaria* and *P. variolosa* fourth-instar larvae, and on *P. atomaria*, *P. beata* and *Chrysophtharta* sp. adults. The larval stages were of interest as they are the most damaging in the field, with third- and fourth-instar larvae known to be responsible for around 90% of larval feeding damage (Carne 1966). However, as larvae are restricted in mobility and are unlikely to actively choose host trees, the feeding preferences of adults were also examined.

Feeding choice tests using paropsines differed from those of Christmas beetles in three respects. First, the foliage type used consisted of immature and expanding leaves only, because these are the preferred food source of these species in the field. Second, the length of experiments was altered to suit the different species and life stages. Tests using *P. atomaria* and *P. variolosa* larvae lasted 24 h and 48 h respectively. Tests using *Chrysophtharta* sp. adults, *P. beata* adults and *P. atomaria* adults lasted 24, 36 and 48 h respectively. Third, it was noted that the immature and expanding leaves used in these trials grew in size over the duration of the feeding tests. To account for this, extra pairs of shoots (controls) were set up in the same manner as the test shoots, but with insects excluded. Growth was calculated from these control shoots as a percentage increase, and factored into post-feeding measurements of leaf area.

***Paropsis atomaria* larval development experiment**

Development times, mortality, pre-pupal weights and newly-emerged-adult weights were measured for 12 groups of *P. atomaria* larvae reared exclusively on resistant clonal foliage compared with 12 groups reared on susceptible seedling foliage.

Egg batches from the stock colony of *P. atomaria* were placed into a petri dish on slightly moistened filter paper, and kept at 22°C until hatching. Batches of hatchlings were divided evenly between two treatments. Larvae were provided with fresh shoot tips *ad libitum*. Dishes were partially sealed with Parafilm® to prevent foliage from dehydrating, with small holes pierced around the edge of each dish for ventilation. As the larvae grew, larger leaves were provided, with food material replenished and frass removed from each dish daily. All larvae were maintained in an incubator at 25°C, with a 12 L:12 D photoperiod. Petri dishes were repositioned daily within the incubator to control for possible position effects.

The number of larvae per dish was recorded daily. The duration of each instar, as indicated by head capsule width, was also recorded. The beginning of an instar was defined as the date when two-thirds (67%) or more of the surviving larvae in a dish had moulted out of the previous instar. Once the larvae reached the fourth instar the number per dish was limited to 10 to reduce mortality due to infectious disease.

When each larva reached the pre-pupal stage, it was allocated a unique number, weighed to the nearest 0.1 mg and sealed with Parafilm® into a glass petri dish filled with vermiculite. The date of pupation was noted, as well as the number of days until adulthood. Adults were weighed and sexed on the day they emerged.

Mortality at the end of each instar was calculated as:

$$m = 1 - (s/i),$$

where m is mortality, s is the number of larvae surviving per petri dish at the end of an instar, and i is the number of larvae counted three days after hatching.

Foliage trait measurements

Specific leaf weight

Shoots used to calculate SLW were dried at 65ºC for 48 h. One immature tip leaf (where available), and one expanding leaf (four to five positions from the shoot tip) per shoot, from equivalent positions between paired shoots, were weighed (n = 19 for tips, n = 28 for expanding leaves). Leaf area of each weighed leaf was estimated using Delta-T-Scan from photocopies as described above.

Monoterpenes

Concentrations of α-pinene and 1,8-cineole were determined for foliage randomly sampled from each of the Byron Bay resistant clones and from the routine test seedlings, using a Varian Star 3400® gas chromatograph (methods as in Edwards *et al.* 1993) at CSIRO Division of Plant Industry. Samples consisted of fully-expanded leaves from the current season's growth.

C:N ratio

Leaves were dried at 65°C for 48 h and ground. C:N ratios were determined by mass spectrometry at CSIRO Plant Industry using an Europa 20-20 isotope ratio mass spectrometer with an ANCA preparation system. Tip and expanding leaf (leaves from the fourth or fifth position down the shoot from the tip) samples were analysed for each of the resistant clones, and for the susceptible seedlings used in the feeding preference trials.

Data analysis

Growth of young leaves occurred during the feeding trials conducted for the paropsine species (range = 0.96% – 1.04%). This meant that in some cases where little or no feeding occurred on a shoot, damage was recorded as a negative number. This was corrected, prior to analysis, using data from control shoots (caged without beetles), with adjusted consumption C calculated as follows:

$$C = b + b(a/100) - c,$$

where a is the growth in area (as a percentage) measured in the control foliage, b is the initial area (mm^2) of the trial shoot, and c is the post-feeding area (mm^2) of the trial shoot.

Feeding preferences were analysed by paired sample t-tests. SLW, C:N, total *P. atomaria* larval development times and monoterpene concentrations were compared between resistant and standard foliage using independent sample t-tests. Expanding leaf SLW and α-pinene values were ln-transformed, to satisfy normality requirements. *Paropsis atomaria* larval mortality (arcsin transformed) and development times in each instar were compared

between foliage types using two-factor repeated measures ANOVAs (factors = foliage type and life stage). Pre-pupal and adult weight for *P. atomaria* reared on the two foliage types were analysed by a three-factor ANOVA (factors = foliage type, dish and sex).

Results

Feeding trials

Anoplognathus chloropyrus displayed a significant feeding preference for foliage from the susceptible seedling stock over the Byron Bay resistant clones in paired feeding choice trials ($t = 2.88$, $P = 0.024$, df = 7). Overall, an average of 83% of total damage, in terms of leaf area, occurred to the routine shoots and 17% to the Byron Bay clonal shoots (Fig. 1). *Paropsis atomaria* larvae also exhibited a highly significant preference for susceptible foliage over the resistant foliage ($t = 4.68$, $P < 0.001$, df = 29, Fig. 1). In contrast, no significant differences between consumption of susceptible and resistant foliage were displayed by *P. variolosa* larvae, or by *P. atomaria*, *P. beata* or *Chrysophtharta* sp. adults.

P. atomaria larval development

Significantly higher mortality occurred in *P. atomaria* larvae developing on the Byron Bay clonal foliage compared with the susceptible foliage, particularly during the first two instars (life stage $F_{(5,110)} = 26.5$, $P < 0.0001$; life stage x foliage type $F_{(5,110)} = 6.49$, $P = 0.018$, Fig. 2). Three of the groups reared on Byron Bay clonal foliage had no survivors into the second instar. Total development times for larvae that survived until adulthood were significantly longer ($t = 3.23$; $P = 0.0065$; df = 13) for *P. atomaria* larvae reared on resistant foliage (mean = 33.0 ± 3.37 days, $n = 7$), compared to susceptible foliage (26.5 ± 4.28 days, $n = 8$). Development times of individual life stages were also longer but there was no significant interaction between foliage type and life history stage (foliage type $F_{(1,52)} = 11.1$, $P = 0.0054$, foliage type x life stage $F_{(4,52)} = 2.11$, $P = 0.092$, Fig. 3).

Foliage type did not significantly affect pre-pupal or adult weights (Fig. 4). There was a significant interaction between sex and foliage type, with a larger difference between male and female weights occurring on resistant foliage for both pre-pupae and adults (pre-pupae: $F_{(2,86)} = 5.17$, $P = 0.007$; adults $F_{(2,86)} = 3.28$, $P = 0.04$).

Foliage traits

C:N ratio did not vary significantly between foliage types ($t = 0.21$, df = 4, $P = 0.85$) (Fig. 5a).

Concentrations of the monoterpene 1,8-cineole did not vary significantly between the two foliage types (Fig. 5b) but α-pinene was in significantly higher concentrations in the routine seedling foliage ($t = 4.06$, $P = 0.002$, df = 11). SLW was significantly higher for shoots from the Byron Bay clones in both tip and expanding leaves (tip: $t = –3.12$, $P = 0.004$, df = 36; expanding: $t = –2.10$, $P = 0.039$, df = 54, Fig. 5c).

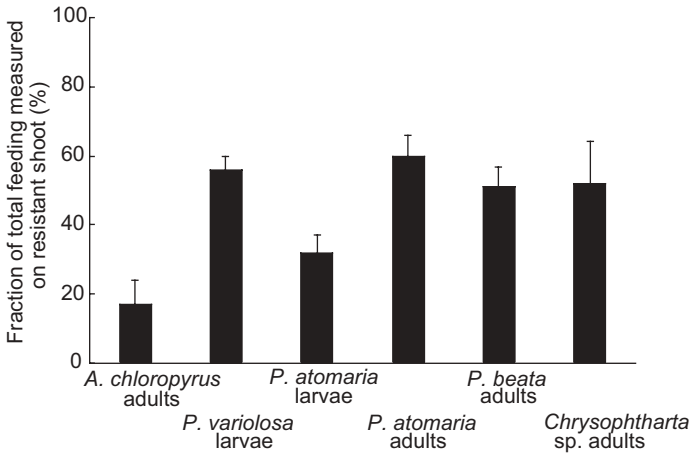

Figure 1. Percentage of feeding damage, in terms of leaf area (both foliar age-class types combined), measured on the Byron Bay clonal foliage in choice trials (*A. chloropyrus*, $n = 8$; *P. variolosa* larvae, $n = 30$; *P. atomaria* larvae, $n = 30$; *P. atomaria* adults, $n = 34$; *P. beata* adults, $n = 25$; *Chrysophtharta* sp. adults, $n = 29$) (mean ± s.d.)

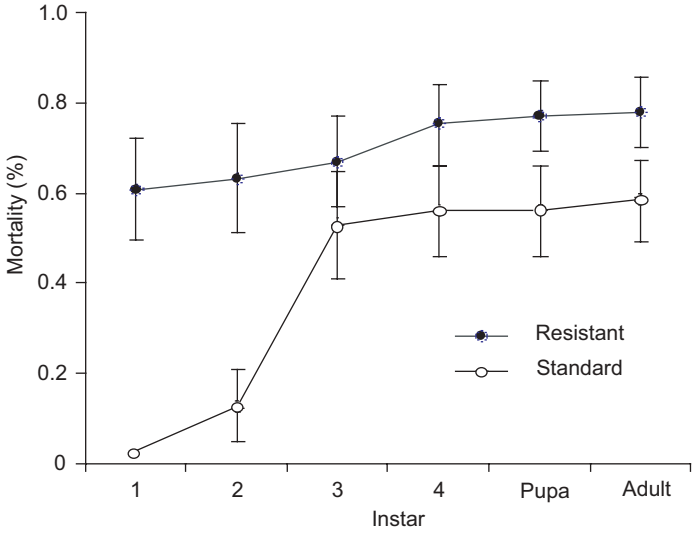

Figure 2. Mortality of *P. atomaria* larvae, as a proportion of initial population size per group, feeding on the Byron Bay clonal and susceptible seedling foliage (mean ± s.d., $n = 12$ groups for each foliage type)

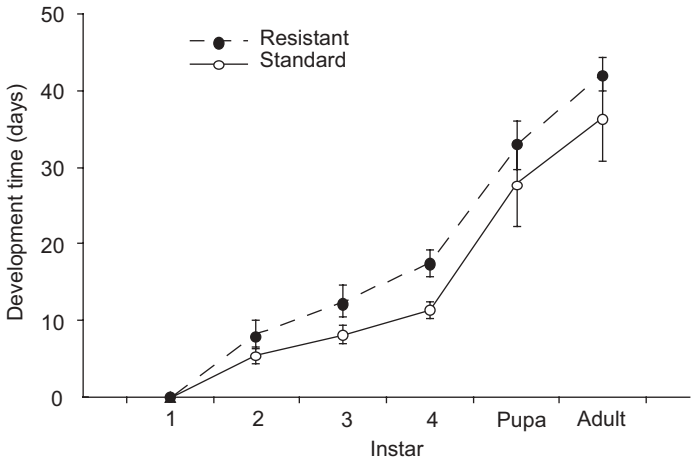

Figure 3. Development times of *P. atomaria* larvae feeding on the Byron Bay clonal and susceptible seedling foliage (mean ± s.d., $n = 12$ groups for each foliage type)

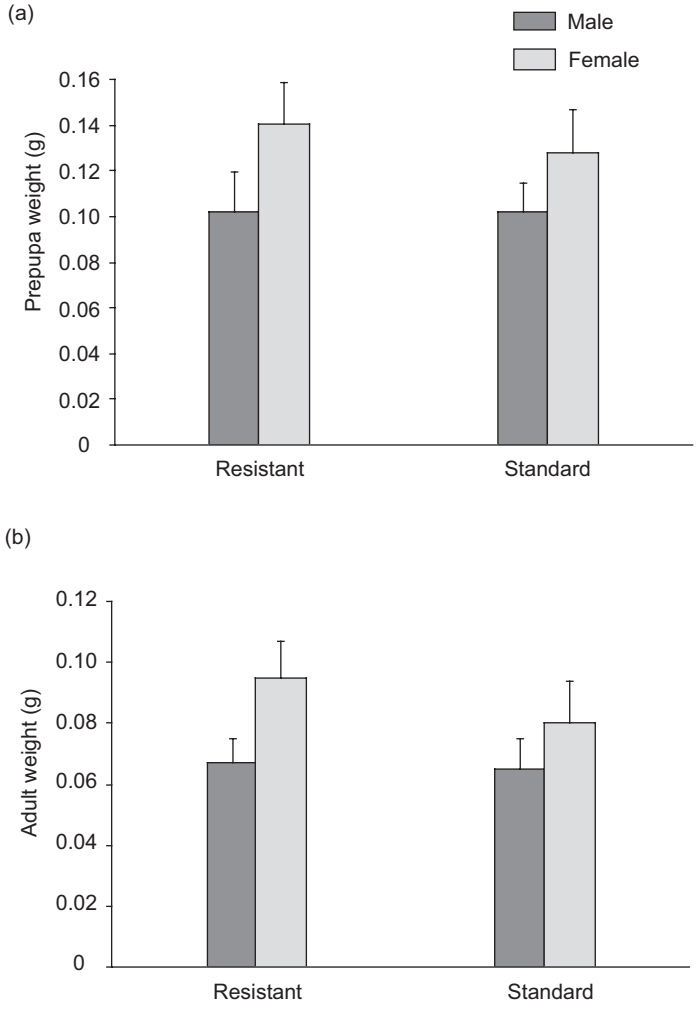

Figure 4. Weight of male and female *P. atomaria* (a) pre-pupal stage and (b) adults developing on Byron Bay clonal (resistant) and susceptible (standard) seedling foliage (Pre-pupae: resistant males $n = 20$; resistant females $n = 17$; standard males $n = 27$; standard females $n = 24$. Adults: resistant males $n = 22$; resistant females $n = 17$; standard males $n = 27$; standard females $n = 24$).

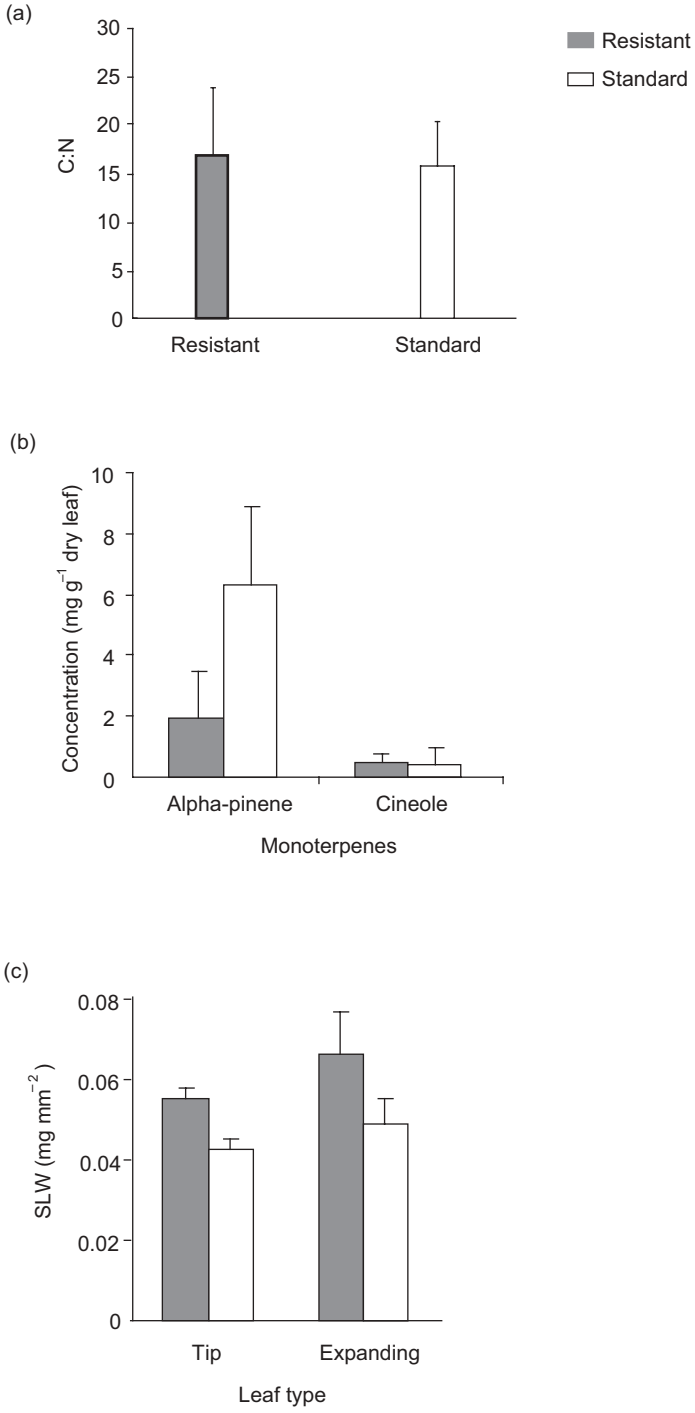

Figure 5. Traits of Byron Bay clonal and susceptible seedling foliage (a) C:N ($n = 3$, data for tip and expanding leaves pooled) (b) Concentration of α-pinene and 1,8-cineole ($n = 3$ resistant, $n = 10$ routine) (c) SLW ($n = 19$ tips, $n = 28$ expanding leaves for each foliage type).

Discussion

This study has verified that the observed field resistance of individual young *E. grandis* trees to defoliation from Christmas beetles was maintained in clonal material propagated from those trees, indicating that this resistance trait(s) is under genetic control rather than due to site characteristics. Incorporating this clonal material into a breeding program might enable the planting of *E. grandis* on sites currently considered at high risk from Christmas beetle defoliation. Before this proceeds, however, any potential trade-offs in other economic factors and implementation costs need to be examined. The expression of plant resistance to herbivores can be costly in terms of ecological fitness and trade-offs between resource allocations to the processes of growth, reproduction and defence by the tree (Strauss *et al*. 2002). With respect to management, there would be economic and environmental benefits arising from a reduced need for insect pest monitoring and insecticide application. These economic savings may, however, be off-set by the costs associated with incorporating an additional trait into the selection process of a tree breeding program. Any additional trait used in the selection process must be reliable, quantifiable and cheap to assess.

Of the four foliar traits— SLW, C:N ratio, 1,8-cineole and α-pinene — measured in this study, only SLW was significantly correlated with feeding preference by the Christmas beetles. Edwards *et al*. (1993) and others have found significant correlations between Christmas beetle herbivory on a range of eucalypt species and the 1,8-cineole content in the foliage. The amount of 1,8-cineole in

E. grandis foliage is low compared to other species of *Symphyomyrtus* (Boland *et al*. 1991). This may have contributed to the lack of significant difference between the resistant clonal foliage and the susceptible seedling foliage.

Lawler *et al*. (1998) showed that foliar terpenes such as 1,8-cineole and α-pinene can act as negative smell or taste cues to the concentration of the real deterrent to eucalypt herbivory from browsing marsupials, a group of chemicals known as the diformylphloroglucinol compounds (DFPCs). Specific DFPCs have been isolated and identified from *E. grandis* (W. Foley, the Australian National University, *pers. comm*.). In addition, Floyd and Foley (2001) reported a high correlation between Christmas beetle feeding and one of the DFPCs, sideroxylonal A, in several species of eucalypts. While the relative content of 1,8-cineole may be correlated to DFPC content, these compounds are not formed by the same biosynthetic pathway and so it is unknown why they should co-vary (Lawler *et al*. 1998). A more direct measure of DFPC content might therefore be more desirable than relying on terpene content (Foley *et al*. 1998; Lawler and Foley 1999).

Christmas beetles are large robust insects with strong mandibles capable of chewing tough foliage such as mature eucalypt leaves. Paropsine species, especially the early instar larvae, are much smaller and feed preferentially on young, soft foliage. Our study found that *P. atomaria* larvae displayed a significant preference for young foliage from the susceptible seedlings compared with similar-aged foliage from the Byron Bay resistant clones. Mortality was higher, particularly in the first two instars, for larvae reared on the resistant foliage, and development times longer. Lengthened larval development time in the field has important consequences for survival because of the increased exposure to threats such as predators and parasitoids (e.g. de Little 1983). These findings were associated with the fact that the SLWs of both tip and expanding leaves were significantly greater in the resistant clonal foliage. This is consistent with Ohmart *et al*. (1985) who found that leaf toughness was a major cause of mortality of *P. atomaria* larvae reared on *E. blakelyi*, particularly for the early instar larvae. Response of the paropsines to the tougher young leaves of resistant clonal foliage, however, was not consistent among the other paropsine species tested or between life-stages within species. It appears, therefore, that the critical threshold of leaf toughness differs for different paropsine species. It has also been reported that a threshold value of 1.0% nitrogen is required for the survival of leaf chewers on eucalypts (Fox and Macauley 1977; Ohmart *et al*. 1987). Nitrogen content did not appear limiting for the paropsine larvae tested in our study.

If SLW was strongly related to the rate of leaf development in *E. grandis*, this characteristic could be a form of general resistance to all insect herbivores requiring young soft foliage. Assuming this to be the case, then overall the amount of young soft foliage suitable for insect herbivores such as chrysomelid larvae on resistant tree crowns would be less than that on tree crowns supporting more immature leaves for longer periods of time. This argument complements that presented by Steinbauer *et al*. (1998) who proposed that eucalypts that initiate and expand leaves rapidly will be less preferred for oviposition by the paropsine, *Chrysophtharta bimaculata*, than those that develop and expand leaves slowly, because of the smaller area of its canopy with less sclerophyllous foliage. If the correlations between SLW, leaf age and herbivory by insects known to be susceptible to leaf toughness are confirmed, then the measurement of SLW of young *E.grandis* leaves at a standard age could be considered as a technique for screening of planting stock in the nursery.

The results from this study suggest that several foliar traits may be required to confer resistance across the range of insect herbivores known to attack *E. grandis* in north-eastern NSW. Only further careful experimentation will disentangle the causal mechanisms of resistance to herbivory in specific eucalypt species, including *E. grandis*. This is a prerequisite for developing a more precise understanding of the genetics involved. The clonal material tested, however, appeared to exhibit partial resistance to the two key pests of *E. grandis*, *A. chloropyrus* and *P. atomaria*. The individual clones are, therefore, worthy of detailed evaluation as a source of planting material on sites at risk from these insect pests.

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