Correlated evolution of defensive and nutritional traits in native and non-native plants

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Abstract
We performed a comparative analysis of defensive and nutritional plant traits responsible for differential herbivory in a series of experimental feeding trials with generalist herbivores. We measured three defensive traits (leaf strength, leaf mass per unit area and endophytic fungal infection) and two nutritional traits (foliar nitrogen and water) for 26 native and eight non-native plant species from coastal California shrublands. Our feeding trials involved three species of generalist herbivore (beet armyworm, cabbage looper and the garden snail) in two types of laboratory feeding trial (single plant species and preference tests). All traits were significantly related to the amount of leaf area consumed, with foliar nitrogen followed by leaf strength explaining most of the variation in herbivore damage. Defensive and nutritional traits were tightly correlated with one another. These correlations were still apparent after incorporation of the phylogenetic relationships of species using independent contrasts, suggesting that there has been repeated selection for certain trait combinations. Non-native species had lower defensive traits and greater nutrient content and therefore experienced greater herbivory damage than natives. Poorly defended, nutrient-rich species (like most of the non-natives in our study) may be better suited for rapid growth and nutrient acquisition, thus reducing the cost of replenishing leaf material lost to herbivores.

Introduction

Although herbivores may be one of the proximal causes of variation in community structure, variation in plant defence and nutritional quality may predict herbivore food choice. Defensive traits include chemical deterrents (secondary metabolites) synthesized by the plant and/or by endophytic fungi (Clay, 1990; Saikkonen et al., 1998) and leaf structural traits that make the leaf less palatable (Perez-Harguindeguy et al., 2003). Nutritional traits include leaf protein or nitrogen content (Feeny, 1970; Crawley, 1983; Herms & Mattson, 1992) and leaf water content (Scriber, 1977; Tabashnik, 1982; Perez-Harguindeguy et al., 2003). The theory of optimal defence and resource allocation predicts that plants that invest less in defensive traits will have more energy for growth and reproduction (Coley, Bryant & Chapin, 1985; Bazzaz & Grace, 1997; Hamilton et al., 2001). Plants that grow more quickly typically have leaves that are more nutritive (e.g. high in water and nitrogen; Cornelissen et al., 1997). The astounding diversity of plants is partly the result of differential evolutionary solutions to this classic leaf investment dilemma: to invest in defence at the cost of growth or to invest in growth at the cost of decreased defence and increased herbivory. Here, we study these evolutionary outcomes in a vegetation largely considered to be highly mechanically defended: the coastal shrublands of California.

Shrubland communities are widespread in California and are often dominated by a sclerophyllous vegetation adapted to the cool wet winters and hot dry summers of this Mediterranean-type climate (Holland & Keil, 1995). Non-native species have become a common, and often dominant, feature in most plant communities, and the shrublands of California are no exception. There have been multiple mechanisms put forward to explain the rapid spread, competitive dominance and persistence of non-native species in their introduced range. The enemy release hypothesis (ERH) suggests that a major factor contributing to the success of non-native species is the loss of their specialist herbivores (Keane & Crawley, 2002). In addition, ERH suggests that generalist herbivores have a greater impact on native than on non-native species (Keane & Crawley, 2002). However, Agrawal & Kotanen (2003) documented a preference for non-native herbaceous species over native relatives by naturally occurring herbivores in Ontario, Canada. Similarly, Parker & Hay (2005) found that non-native aquatic and terrestrial plants were preferred by native generalist herbivores in laboratory trials. Successfully established non-native species may invest differently in defence and nutrition compared with native, long-term residents in a community, but it is currently unclear as to what defensive and nutritional traits account for the increased palatability of non-native species and/or which defences they typically lack.

Here, we examine differences among species with regard to their susceptibility to generalist herbivores in laboratory feeding trials. We measured three defensive traits [leaf mass per unit area (LMA), leaf strength and the presence of endophytic fungi] and two nutritional traits (foliar nitrogen and water content) in 26 native and eight non-native species (34 species in total) from coastal Californian shrubland vegetation (chaparral and coastal sage scrub). We chose to include LMA and leaf strength in our study because these traits are representative of structural integrity and are important in reducing tissue digestibility (Choong, 1996), and are a dominant feature of

many native shrubland species. Although structural deterrents to herbivores have received considerable attention (Coley, 1983; Choong, 1996; Lucas et al., 2000; Brunt, Read & Sanson, 2006), only recently has the role of endophytic fungi been widely considered. We chose to include endophytic fungi in our analysis because they have been shown to produce alkaloids and other mycotoxins that can deter herbivores (Prestidge & Gallagher, 1988; Siegel et al., 1990; Porter, 1994; Siegel & Bush, 1996; Bush, Wilkinson & Schardl, 1997; Dahlman, Siegel & Bush, 1997), and there is a growing awareness of their ubiquity and diversity in nature (Arnold et al., 2000; Arnold & Herre, 2003; Arnold, 2007; Jumpponen & Jones, 2009). However, our measurement of endophyte occurrence was crude (simply the presence or absence in leaf samples, see Material and methods for further details), and therefore we consider our endophyte analysis to be a preliminary evaluation of whether their presence correlates with herbivore food choice. Lastly, we investigated foliar nitrogen and water content because these nutrients have repeatedly been linked to larval success (Scriber, 1977, 1979; Slansky, 1993; Taylor, Hyde & Jones, 1999; Wheeler & Halpern, 1999) and herbivore feeding preference (Athey & Connor, 1989; Rossi & Strong, 1991). We did not include secondary metabolites in our analysis because there are numerous unique secondary compounds that do not vary continuously across plant species (Barbour et al., 1999).

To measure herbivore preference and feeding, three generalist herbivores were used [beet armyworm (Spodoptera exigua), cabbage looper (Trichoplusia ni) and the garden snail (Helix aspersa)], and herbivory was quantified as the amount of leaf area consumed in replicated timed trials. We used two types of laboratory feeding trial: timed trials with a single plant species present and preference trials in which all of the plant species were present, sometimes called 'cafeteria' feeding trials (Krebs, 1989; Perez-Harguindeguy et al., 2003). Using these experiments, we addressed the following questions. (1) Which defensive and nutritional traits best predict susceptibility to generalist herbivores? (2) Have evolutionary divergences in leaf nutrition been correlated with divergences in leaf defence (this was addressed using phylogenetic independent contrasts)? (3) Does origin (native or non-native) predict susceptibility to herbivore damage and, if so, which traits are typically different for introduced species?

Material and Methods

Mature leaves from 34 species (26 native and eight exotic species; Appendix 1) were collected in San Luis Obispo County, California, USA. Non-native species were sampled exhaustively and native species were picked randomly from a pool of 40 abundant perennial species that were known to occur at six natural areas in the county. The distribution of non-native species within these six natural areas was variable, but, in general, these species were more commonly associated with disturbed areas (roadsides or disturbed ravines). All eight non-native species are documented by the California invasive plant council as species that threaten wildlands. One individual from each non-native and native species was selected randomly at six different field locations [Poly Canyon (35°18'30, 120°39'19), Cuesta Grade (35°20'40, 120°38'33), Montana de Oro (35°15'48, 120°53'14), Reservoir Canyon (35°17'19, 120°37'35), Perfumo Canyon (35°15'53, 120°43'20) and Sycamore Canyon (35°11'16, 120°43'00)]. A branch from one individual at each site with several healthy, fully expanded, sun leaves was collected and transported to the laboratory. Once branches were detached, the cut ends remained in water and the sample was kept cool until the laboratory

analyses began (see below). There were six replicates (one from each field location) per species per analysis and feeding trial; each replicate involved a single leaf detached from one branch. All field collections occurred in spring (between April and June 2005) during the height of insect activity.

Herbivory

Three species of non-native generalist herbivore were used for the laboratory feeding trials. Although it would have been ideal to collect native herbivores, this was not feasible given the large number of individuals needed for the laboratory feeding trials. Third instar larvae of *Trichoplusia ni* and *Spodoptera exigua* were ordered from Benzon Research Facilities (Carlisle, PA, USA). Non-native *Helix aspersa* was collected from abundant local populations and reared on cabbage prior to the feeding trials. All animals were starved for 48 h prior to the feeding trials. Plant tissue was exposed to herbivores via two methods: (1) single species' time trials (SSTTs) were used to quantify the magnitude of leaf damage for each plant species independently of other plant species; this assay reflects palatability; SSTTs were performed in a plastic cup with moist filter paper on the bottom with a single plant and herbivore species; (2) multiple species' preference trials (MSPTs) were performed to simulate a more 'natural' feeding scenario in which herbivores have a choice between several plant species; this assay represents a measure of herbivore preference; in the MSPTs, all 34 plant species were simultaneously placed in cardboard boxes together with one species of herbivore (12 cabbage looper, 12 beet armyworms or eight snails). The SSTTs and MSPTs were replicated six times for each of the three herbivores.

Containers were checked daily; water and fresh herbivores were replenished as needed; however, there were too few cabbage loopers to replace individuals that died during the experiment. Both trials took place at 22 °C with 12 h light and dark cycles. Both the SSTTs and MSPTs lasted for 3 days. Snail chambers from MSPTs were disassembled after 2 days because the snails began feeding on the cage materials (cardboard, masking tape, etc.). For both the SSTTs and MSPTs, we presented approximately the same amount of leaf area (8 cm^2) of each plant species to the herbivores. In the case of small leaves, this meant using multiple leaves from a single individual. To avoid the natural release of phytotoxins in response to wounding, no leaves were cut (Scheidel & Bruelheide, 1999). Herbivore feeding was quantified by comparing the initial leaf area with the final leaf area in each trial. We measured leaf area using an Epson flat-bed scanner and SCION image analysis software (Scion, Fredrick, MD, USA).

Measures of defence

Leaves were tested for mechanical strength using an Imada Force Gauge (Series AXT, Northbrook, IL, USA). The force gauge was lowered at a constant speed of 1.36 mm s^{-1}, and the force required for a flattened probe (2 mm in diameter) to break the leaf surface was recorded (Aranwela, Sanson & Read, 1999). Pilot studies showed strong correlations between maximum force, specific force, work and specific work. As a result, we did not generate force displacement curves or measure leaf thickness; rather, we used the maximum force (measured in newtons) required to break through the leaf tissue as our measure of strength.

LMA was calculated by dividing the dry mass (g) by the leaf area (m^2). The area was calculated by scanning the leaves and analysing the images with SCION image computer software. The dry mass was obtained by drying the leaves for 1 week at 60 °C.

Endophytic fungi

Leaf tissue was sectioned using a 5 mm hole-punch, and then rinsed with a 1% dish soap solution and deionized water. Surface sterilization consisted of washing the leaf segments in an ethanol–hypochlorite–ethanol series. Leaf discs were allowed to dry for 10 min before being plated onto Petri dishes containing 2% malt extract agar (MEA) (Arnold et al., 2003) with 20 μg mL^{-1} chloramphenicol (Broadbent & Terry, 1958; Cao, You & Zhou, 2002). Petri dishes were incubated at room temperature for 2 months. Because endophytic fungi are extremely difficult to identify morphologically, we employed abundance surveys in which the presence or absence of fungi was recorded 2 months following incubation. Endophytes were recorded as present if fungi were found growing radially from the leaf disc.

To ensure that the radial growth pattern was not from epiphytic fungi, we performed two controls for surface contamination. First, the sterilized leaf segment was swabbed to disrupt leaf hairs that could potentially act as refuges for contaminants. A sterilized swab was moistened with water and used to rub the adaxial and abaxial leaf surfaces. The content of the swab was then smeared onto a clean Petri plate with 2% MEA. The second control consisted of pressing both the upper and lower leaf surfaces against a clean Petri plate with 2% MEA for 30 s. Control plates were allowed to incubate under the same conditions as noted above. If surface contamination was found on controls, all plates from that species and sampling date were omitted from the analysis and new leaves were immediately plated.

Measure of nutrition

Leaves collected for LMA calculations were also used to measure water and nitrogen content. Fresh leaf weight was obtained after allowing the leaves to hydrate in a plastic bag with a moist paper towel for 30 min. Following the measurements, the leaves were placed in coin envelopes and dried for 1 week at 60 °C. We measured foliar nitrogen as a proxy for protein content. Dried leaves were ground until leaf material was milled into a fine powder using a wig-l-bug (Crescent, Maple Grove, MN, USA) and metal beads. Tissue was weighed using an analytical balance (Sartorius, Goettingen, Germany) and analysed for carbon and nitrogen using a Carlo-Erba NA1500 (Fisons Instruments, Milan, Italy) with an acetanilide standard.

Statistical analysis

All statistical analyses were performed using R statistical software (R Development Core Team, 2007). Results from the three herbivore species were pooled to obtain a single estimate of herbivory for each of the 34 plant species in the SSTTs and MSPTs. The relationship between the two types of herbivore feeding assay (SSTT and MSPT) was assessed using Pearson's correlation. A univariate regression was performed on each physiological trait measured and herbivory (average of leaf area removed by all herbivores), with each defensive and nutritional trait being regressed independently on herbivory. A classification and regression tree (CART) was used to ascertain which of the measured traits was best able to explain the variation in herbivore damage. Defensive

and nutritional traits were used as the explanatory variables and the percentage of leaf area removed (MSPT) as the response. This multivariate analysis partitions the explanatory variables into two homogeneous groups based on the trait that explains the most variation in leaf area removed by herbivores. Each group can then be split again to further explain the variation in leaf area removed (De'ath & Fabricius, 2000).

Pearson's correlations were used to assess the pair-wise relationships between foliar nitrogen, water content, LMA, endophytic fungi and leaf strength. To determine whether correlated evolutionary trends are represented in our data, we also calculated the phylogenetic independent contrasts using a genus-level phylogeny produced using the Phylomatic web software (Felsenstein, 1985; Webb & Donoghue, 2005, tree version: R20040402). The analysis of traits (AOT) module of Phylocom (Ackerly, 2004) was used to calculate phylogenetically independent contrast correlations (PICr) for all pair-wise comparisons. Branch lengths were set equal for our analysis (Ackerly, 2000).

Differences between introduced and native species for herbivore damage, LMA, nitrogen, water content and endophytic fungi were tested using a two-tailed t-test. The following transformations were used in all statistical analyses, with the exception of the CART analysis (for which untransformed data were used): log transformations were performed on LMA, strength, herbivory and SSTT (1 was added to all values to account for zeros in the dataset), and a fourth root transformation was used on MSPT (1 was added to all values to account for zeros in the dataset). All figures include transformed data.

Results

Herbivory

Average leaf area lost to herbivores varied from 0% to 48% in SSTTs [Eucalyptus globulus Labill. and Genista monspessulana (L.) L.A.S. Johnson, respectively] and from 0% (multiple species) to 44% (Nicotiana glauca Graham) in MSPTs (Appendix 1). In SSTTs, snails, beet armyworm and cabbage looper removed on average 8.2%, 3.3% and 6.1% of leaf area, respectively, whereas, in MSPTs, 9.5%, 4.1% and 2.6% were removed by each of the herbivores, respectively (Fig. 2). A strong positive correlation was found between herbivore damage in SSTTs and MSPTs (Fig. 1; Pearson's correlation, $r_{(32)} = 0.885$, $P < 0.001$). Therefore, all pair-wise comparisons (see Defence, nutrition and herbivory below) were made using data from MSPTs, because the presentation of multiple plant species to herbivores more accurately reflects food choice in nature. Variables that were correlated with herbivory from SSTTs were largely similar to those that were correlated with leaf area lost in MSPTs.

All plants, except E. globulus, were sampled by at least one herbivore species in SSTTs (Fig. 2), and five species were not sampled by any herbivore in MSPTs (Fig. 2). Herbivore damage was well distributed phylogenetically (using a genus-level phylogeny of our plant species, see Fig. 2), with the exception that both Quercus spp. (Fagaceae) experienced little herbivore damage, and both species of Solanaceae (Solanum douglasii Dunal and N. glauca) exhibited relatively high damage (Fig. 2).

Defence, nutrition and herbivory

Generally, as defensive traits increased in magnitude, herbivore damage decreased (Fig. 3). There was a significant negative relationship between LMA and herbivore feeding (MSPT) (Fig. 3A; regression, $r^2 = 46.1\%$, $F_{(1,32)} = 27.32$, $P < 0.001$), strength and herbivore damage (MSPT) (regression, $r^2 = 33.3\%$, $F_{(1,32)} = 15.99$, $P < 0.001$) and the presence of endophytic fungi and herbivore damage (MSPT) (Fig. 3B; regression, $r^2 = 29.3\%$, $F_{(1,32)} = 13.27$, $P < 0.001$). Conversely, positive relationships were found between leaf nutritional traits and herbivore damage. There was a significant positive relationship between leaf nitrogen content and herbivore damage (Fig. 3C; regression, $r^2 = 42\%$, $F_{(1,32)} = 23.2$, $P < 0.001$), and foliar water content held the highest predictive power of all the traits measured, again in a positive direction (Fig. 3D; $r^2 = 47.1\%$, $F_{(1,32)} = 28.49$, $P < 0.001$).

CART analysis revealed that the nitrogen content explained the most variation in herbivore damage (Fig. 4). Species with a leaf nitrogen content of more than 2.25% had an average of 15.27% of leaf area removed by herbivores. Variation for herbivore damage in plants with low nitrogen levels (< 2.254%) was further explained by the strength of the leaves. Structurally poor (< 2.238% nitrogen), low-nitrogen leaves had an average of 1.42% of leaf area removed, whereas stronger leaved species (> 2.238% nitrogen) had the lowest percentage of leaf damage (0.033%) (Fig. 4).

Relationships between traits

Using Pearson's correlation, we found uniformly significant negative relationships between defensive and nutritional traits (Table 1). These negative relationships were also significant when tested using independent contrasts (Table 1), indicating that there has been a correlated evolution of defensive and nutritional traits. Therefore, selection pressures favouring greater defence were associated with the evolution of lower nutrient levels. For example, leaf nitrogen content was negatively correlated with both LMA and endophytic fungal infection (Pearson's correlation, $r = -0.730$, $P < 0.05$ and $r = -0.487$, $P < 0.05$, respectively), and these comparisons were also significant when using independent contrasts (PICr, $r = -0.751$, $P < 0.05$ and $r = -0.426$, $P < 0.05$) (Table 1). Water content was negatively related to both LMA and endophyte infection, and these comparisons were also significant when using independent contrasts (Table 1).

We found positive correlations within the class of defensive or nutritive traits (Table 1). For example, there were positive correlations between nitrogen and water content, and between LMA and leaf strength (Table 1). Variables that correlated with LMA had similar relationships with leaf strength (Table 1). There was no significant relationship between endophytic fungal infection and LMA or strength (Table 1). The positive correlations found within trait classes (defence or nutrition) were robust when tested using independent contrasts (Table 1).

Non-native vs. native species

There was a significant difference in herbivore damage between non-native and native species (Fig. 5, t-test: MSPT; $t = 2.94$, $P < 0.05$; SSTT; $t = 1.96$, $P = 0.085$). The top three species consumed by herbivores in both feeding trials were non-native species (N. glauca, Delairea odorata Lem. and G. monspessulana). Solanum douglasii was the only native species to have more than 5% of leaf area

lost in either of the herbivore feeding trials (Appendix 1, Fig. 2). In contrast, five of the eight non-native species had more than 5% of leaf tissue removed.

Differences in the amount of leaf area removed may result from the higher nutritive quality and lower defence found in non-native species. There was a trend for LMA to be lower in non-native species than in native species (Fig. 6A, t-test, $t = -1.77$, $P = 0.107$). The leaves of non-native species were less likely to be colonized by endophytic fungi (Fig. 6B, t-test, $t = -2.17$, $P = 0.053$). Non-native species had a higher nitrogen content (Fig. 6C, t-test, $t = 2.16$, $P < 0.05$) than natives. The nitrogen content in introduced species averaged 2.33%, whereas that in native species averaged 1.82%. Water content was significantly higher in non-native species than in natives (Fig. 6D, t-test, $t = 2.61$, $P < 0.05$). Unlike the other non-native species, E. globulus had low herbivore damage, strong leaves with high LMA, high levels of endophytic infection and low leaf water and nitrogen content.

Discussion

Using a community-based sampling approach, we determined that structural deterrents, endophytic fungi and nutritional attractants are all good predictors of plant susceptibility to herbivory. The tight relationships between these traits make it difficult to separate and identify whether a sole plant trait may be driving resistance to herbivory or whether the suite of traits is essential. Any one of the traits measured in this study may be a good proxy for future work investigating plant species' susceptibility to herbivores, yet our CART analysis identified nitrogen followed by leaf strength to be the best indicators of which plants would be impacted the most by generalist herbivores. Other studies have yielded similar results. Wardle et al. (1998) found that the nitrogen content of the stem was the only trait that was significantly correlated with levels of herbivory in an ecophysiological analysis of grassland species. Perez-Harguindeguy et al. (2003) performed an analysis of 52 angiosperms in central Argentina, and found that grasshoppers and snails preferentially consumed species with high leaf nitrogen, low C : N ratio and low tensile strength. Their work supports our finding that highly nutritious leaves with lower levels of defence are preferentially consumed by generalist herbivores.

Phylogenetic independent contrast analyses suggested that defensive and nutritional traits (with the exception of endophytic fungi and leaf structure) evolve together, but in opposing directions. We found that, across the 27 evolutionary divergences that we were able to reconstruct, as leaf nutritive quality increases, investment in defence decreases, and vice versa. Co-evolution of these traits is likely to be a result of selection pressure by herbivores and selection for optimal physiological function and carbon gain within a given environment. These findings support the work of others, which suggests a trade-off between resource acquisition and conservation (Grime, 1977; Poorter & Bergkotte, 1992; Cornelissen et al., 1997; Westoby et al., 2002; Díaz et al., 2004) and its relation to herbivore defence (Coley, 1980, 1988; Coley et al., 1985; Bryant et al., 1989). The resource availability hypothesis of plant–herbivore defence highlights this trade-off, whereby slow-growing species in nutrient-poor habitats invest more in defence than fast-growing species in nutrient-rich habitats (Coley et al., 1985). The species analysed in our study were randomly sampled from shrubland environments and, although we did not quantify the abiotic variables associated with each species at each site, it is likely that certain species were more frequently found

in microsites that selected for one plant strategy over the other. This may be especially true for the poorly defended non-native species in our study. A few of the species (Nicotiana glauca and Ricinus communis L.) were more common in frequently disturbed sites, such as road cuts, whereas others (Delairea odorata and Vinca major L.) were more commonly associated with more mesic sites. These microsite differences (which include more frequent disturbance and increased water availability) may contribute to the dissimilar traits found between native and non-native species.

In general, non-native species had lower defensive traits (LMA, leaf strength and endophytic fungi) and higher nitrogen and water contents (Fig. 6). Studies conducted using non-native and native Hawaiian plants found that non-native species were better suited for efficient resource use when compared with natives (Pattison, Goldstein & Ares, 1998; Baruch & Goldstein, 1999). Non-native species had higher specific leaf area (SLA, lower LMA), lower leaf construction costs and higher nitrogen content than native species (Baruch & Goldstein, 1999). Although the work performed in Hawaii did not assess the susceptibility of non-native species to herbivores, it suggests that, when compared to natives, non-native species may trend toward the rapid resource acquisition–fast growth spectrum of species. Following the predictions put forward by the resource availability hypothesis, non-native species (with rapid resource acquisition and growth) would be expected to invest minimally in defences against herbivores (Coley et al., 1985; Coley 1988).

Our findings support this hypothesis. In California shrublands, non-native species were poorly defended when compared with the native vegetation and, as a result, were preferred by generalist herbivores in our feeding trials. Although herbivores selectively feed on non-natives, it does not appear that this form of biotic resistance is sufficient to resist the establishment or slow the growth of non-natives in these shrubland communities. Coupling our results with the widespread distribution of non-native species in the field suggests that the fast growth and low leaf replacement costs of non-native species buffers them from the effects of herbivores. An alternative to this hypothesis may be that the herbivore pressure at our field sites (where the non-native species were abundant) was low, and thus herbivore pressure in the field is minimal when compared with our laboratory trials. Surveys of herbivory in the field will be essential in the future to gain a more realistic assessment of the patterns and predictors of herbivory.

Although our data suggest that multiple traits are driving herbivore food choice in the laboratory, herbivore preference may be confounded by biotic interactions in the field. Reassuringly, some studies have compared herbivore consumption in the field and laboratory and have found similar results. For instance, Perez-Harguindeguy et al. (2003) found a nonsignificant relationship between generalist herbivory in cafeteria experiments and field observations, yet the plant traits (nitrogen content, C : N ratio and tensile strength) driving herbivore preference in the field and laboratory were the same. Similarly, Scheidel & Bruelheide (1999) found that, for their species of interest, Arnica montana L., laboratory and field herbivory were well matched, yet some herbivore species altered their feeding behaviour when in the laboratory. These results are encouraging for controlled experiments like ours.

The protective role of endophytes against plant predators has been well established in grasses (Cheplick & Clay, 1988; Breen, 1994; Brem & Leuchtmann, 2001; Vicari, Hatcher & Ayres, 2002), and this mutualistic relationship has been shown to affect competitive interactions and to alter

species' richness within grass communities (Rudgers & Clay, 2008). There is also evidence linking endophytes with protection in algae (Cubit, 1975), gymnosperms (Miller et al., 2002) and dicots (Webber, 1981; Arnold et al., 2003; Braun et al., 2003). However, Faeth (2002) questioned the generality of the 'defensive mutualism concept' (DMC), suggesting that other mechanisms may be just as important as DMC for explaining the presence of endophytic fungi, such as sexual parasitism, endophyte acquisition of limiting leaf nutrients or exploitation of leaf resources during senescence, with neutral or even negative interactions during the physiologically active portion of the leaf life span. Therefore, more research needs to be performed to understand the ecological roles played by these fungi.

In our study, endophytic fungi were rarely found growing in the leaves of non-native species (with the exception of E. globulus). In addition, native plant species had unique endophytic fungal morphotypes; it did not appear that there were 'generalist' fungi colonizing the tissues of multiple species. Therefore, it is possible that fungal symbionts were not simultaneously introduced together with their hosts. This question will be answered as we gain a greater understanding of the degree of species' specificity of endophytic fungi, and with more rigorous sampling efforts. The implications of losing endophytic fungi could be substantial for non-native species (Mitchell & Power, 2003). The loss of endophytic fungi may make non-natives more susceptible to herbivores in their introduced range and thus slow down the spread of the species. However, the non-natives assessed in this study tended to be relatively widespread, thus leading to a contrasting hypothesis that the loss of endophytic fungi could be beneficial to the species. If the success of an introduced species relies on the rapid acquisition of nutrients and fast growth (as discussed above), perhaps not having to support endophytes allows for more energy to be invested in growth and reproduction, thereby allowing the species to proliferate quickly across the landscape. The presence/absence data collected in our study did not allow us to determine the identity of the isolated endophytes. Future projects like ours should include environmental sequencing of the phyllospere, if possible, and should use DNA barcoding and operational taxonomic unit (OTU) classification to estimate the identity and diversity of endophytic fungi.

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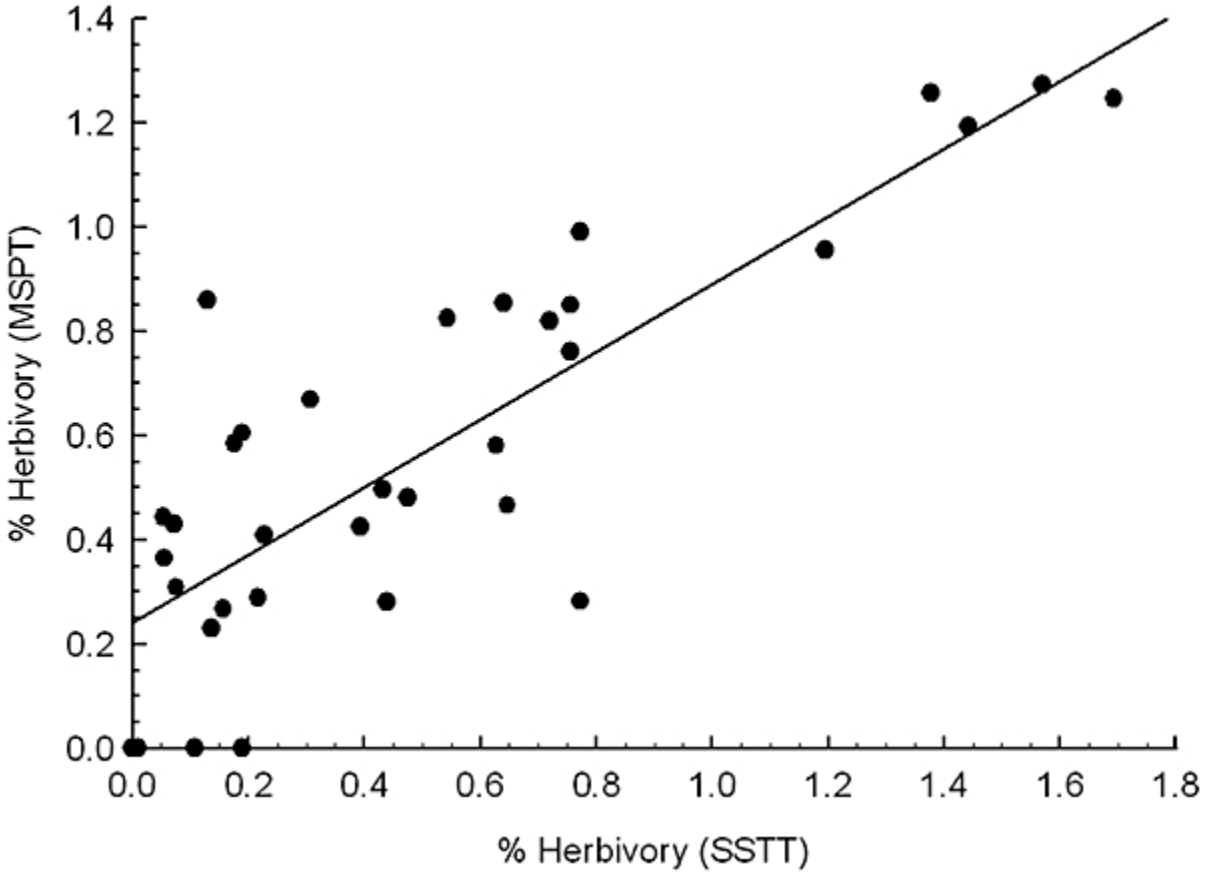
Appendix 1

Trait values for the species sampled. Non-native species are indicated with an asterisk. Single species' time trial is abbreviated SSTT and multiple species' preference trial is abbreviated MSPT. Values listed under SSTT and MSPT are the percentages of leaf area consumed during the trial. Endophytic fungal infection is the percentage of leaf discs in which fungi were observed. Values listed under water content and nitrogen content are a percentage of wet and dry biomass, respectively.

Species	SSTT (%)	MSPT (%)	Strength (N)	LMA (g m^{-2})	Endophytic infection (%)	Water content (%)	Nitrogen content (%)
1. *Ailanthus altissima (Mill.) Swingle	14.69	6.59	1.11	56.43	33.30	71.25	2.46
2. Arctostaphylos obispoensis Eastw.	0.69	0.06	4.97	335.59	50.00	44.04	0.68
3. Baccharis pilularis DC.	3.43	0.12	1.73	55.67	16.70	70.33	2.40
4. Ceanothus cuneatus Nutt,	0.65	0.01	2.56	251.23	60.00	53.73	1.33
5. Ceanothus griseus (Trel. ex B.L.Rob.) McMinn	4.91	0.01	1.14	124.92	75.00	56.37	1.18
6. Ceanothus oliganthus Nutt.	4.26	2.60	1.77	133.47	50.00	52.83	1.51
7. Cercocarpus betuloides Nutt. ex Torr. & A.Gray	3.37	3.33	2.15	119.27	16.70	52.16	1.92
8. Cornus sericea L.	3.24	0.38	1.00	56.25	0.00	63.87	1.67
9. *Delairea odorata Lem.	22.88	39.93	0.76	24.53	0.00	91.61	2.34
10. Dendromecon rigida Benth.	0.13	0.10	5.59	159.94	0.00	59.66	1.64
11. Eucalyptus globulus Labill.	0.00	0.00	6.89	294.55	100.00	45.29	1.19
12. Garrya veatchii Kellogg	0.13	0.04	6.42	249.66	66.70	42.84	0.97
13. *Genista monspessulana (L.) L.A.S.Johnson	48.35	37.71	0.82	63.68	16.70	62.85	2.44
14. Heteromeles arbutifolia (Lindl.) M.Roem.	0.37	0.00	4.29	228.25	16.70	46.28	1.37
15. Holodiscus discolor (Pursh) Maxim.	4.70	3.22	0.81	54.68	66.70	53.72	2.17
16. *Marrubium vulgare L.	1.03	0.82	0.70	47.96	25.00	73.05	2.80
17. *Nicotiana glauca Graham	36.06	43.86	1.98	75.63	0.00	81.91	2.62
18. Prunus ilicifolia (Nutt. ex Hook. & Arn.) D.Dietr.	1.75	0.01	3.97	221.12	0.00	43.71	1.65
19. Quercus agrifolia Née	0.02	0.01	4.49	159.91	100.00	43.06	1.33
20. Quercus durata Jeps.	0.19	0.02	5.22	194.61	100.00	36.60	1.15
21. Rhamnus californica Eschsch.	1.98	0.15	2.51	152.61	83.30	57.33	1.71
22. Rhamnus crocea Nutt.	0.28	0.00	2.56	130.35	80.00	52.04	1.57
23. Ribes speciosum Pursh	0.50	0.40	2.11	95.01	80.00	61.17	1.57
24. *Ricinus communis L.	4.92	8.16	1.56	74.89	0.00	69.64	2.84
25. Rubus parviflorus Nutt.	0.54	0.48	0.90	51.92	100.00	66.69	1.69
26. Rubus ursinus Cham. & Schltdl.	4.70	1.70	1.20	57.05	83.30	63.03	2.00
27. Salix laevigata Bebb	1.70	0.17	1.21	80.42	16.70	58.69	2.66
28. Salix lasiolepis Benth.	1.47	0.08	1.37	78.21	33.30	61.73	2.70
29. Salvia mellifera Greene	0.44	0.01	1.63	84.17	83.30	73.43	1.69
30. Salvia spathacea	0.18	0.08	0.83	44.38	50.00	80.71	1.89
31. Sambucus mexicana C.Presl ex DC.	2.50	2.67	1.74	64.52	16.70	75.67	3.56
32. Solanum douglasii Dunal	26.71	27.82	0.83	34.16	33.30	84.26	3.64
33. Umbellularia californica (Hook. & Arn.) Nutt.	0.55	0.00	3.58	154.98	66.70	42.36	1.72
34. *Vinca major L.	0.35	3.46	1.76	54.54	0.00	78.52	1.95

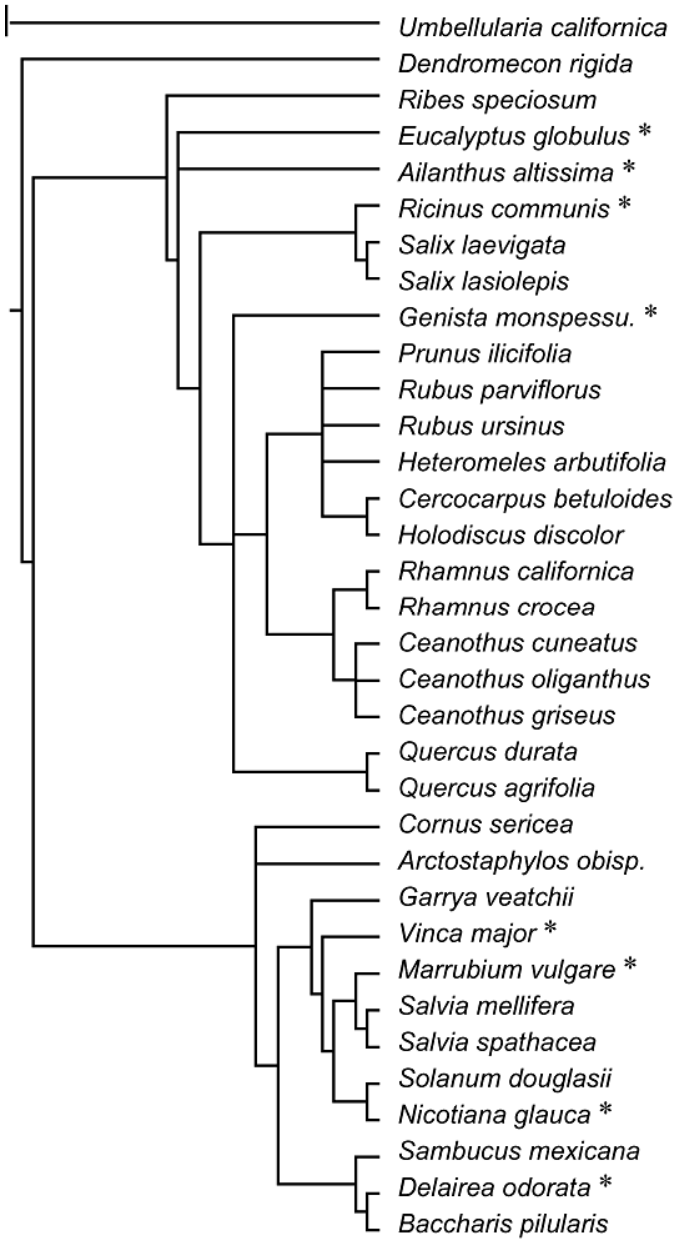
Figures

Figure 1



Percentage of leaf area removed by herbivores in the multiple species' preference trial (MSPT) and the single species' time trial (SSTT). Pearson's correlation coefficient, $r = 0.876$, $N = 34$. ***$P < 0.001$.

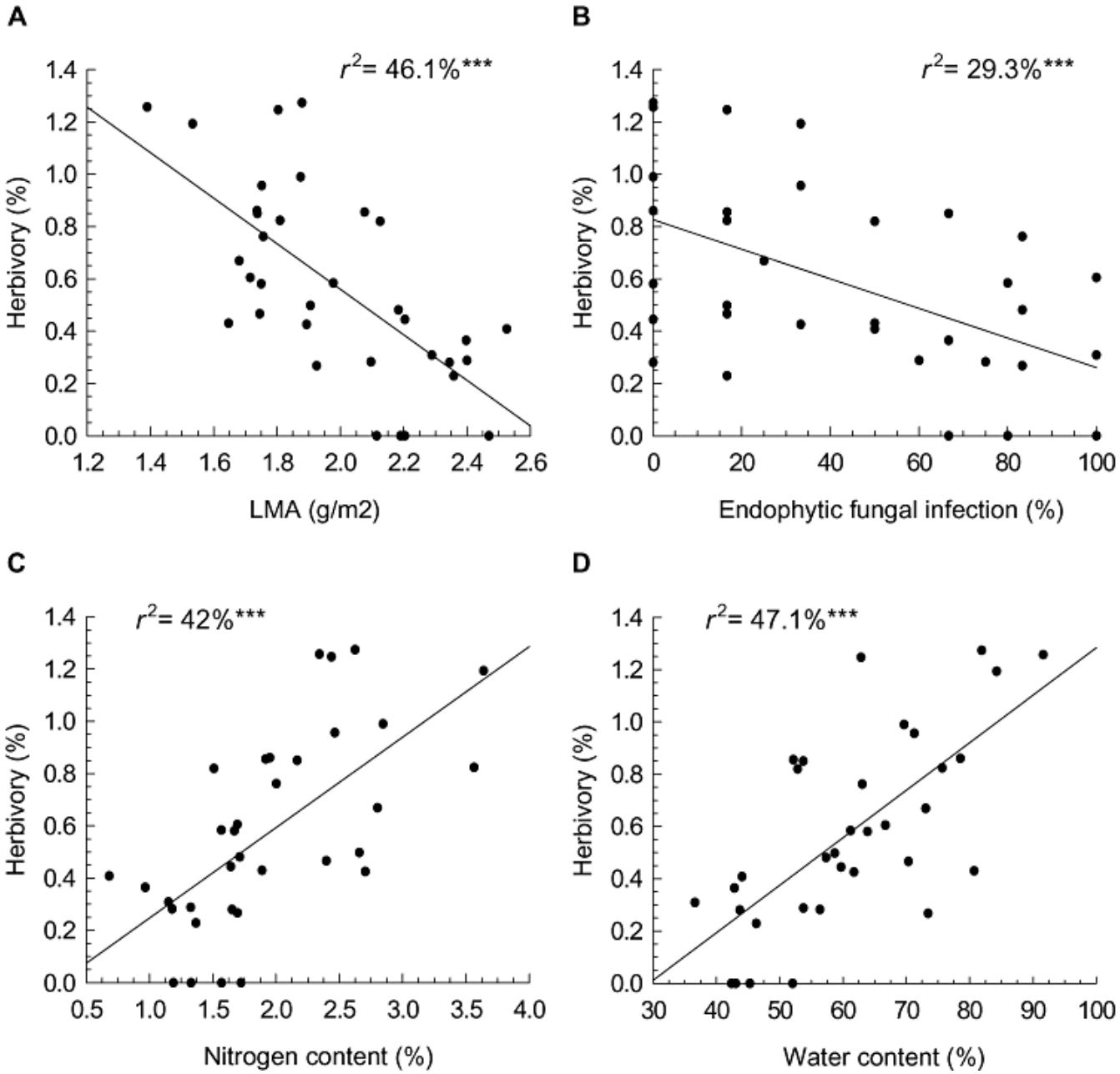
Figure 2



	Garden snail		Beetarmy worm		Cabbage looper	
	SSTT	MSPT	SSTT	MSPT	SSTT	MSPT
Umbellularia californica	<0.5	0	<1.0	0	0	0
Dendromecon rigida	<0.5	<0.5	<0.5	<0.5	0	0
Ribes speciosum	<0.5	1.0	<1.0	<0.5	<0.5	0
Eucalyptus globulus *	0	0	0	0	0	0
Ailanthus altissima *	26.7	17.7	3.4	2.1	<0.5	0
Ricinus communis *	3.7	<0.5	6.2	24.3	0	<0.5
Salix laevigata	1.5	<0.5	2.3	<0.5	<1	<0.5
Salix lasiolepis	<1.0	<0.5	2.9	<0.5	<0.5	<0.5
Genista monspessu. *	95.8	79.2	6.7	33.9	19.2	0
Prunus ilicifolia	0	0	3.1	0	2.7	0
Rubus parviflorus	<0.5	<0.5	1.2	1.3	0	0
Rubus ursinus	3.3	0	7.1	5.1	<0.5	0
Heteromeles arbutifolia	<0.5	0	<1.0	0	0	0
Cercocarpus betuloides	3.6	8.8	4.3	<0.5	0	0
Holodiscus discolor	7.5	<0.5	3.0	9.2	2.9	<0.5
Rhamnus californica	1.1	<0.5	4.1	<0.5	0	0
Rhamnus crocea	<0.5	0	<0.5	0	0	0
Ceanothus cuneatus	<0.5	<0.5	1.3	<0.5	<0.5	0
Ceanothus oliganthus	2.0	7.6	4.8	<0.5	6.4	<0.5
Ceanothus griseus	<0.5	<0.5	2.6	<0.5	33.7	0
Quercus durata	0	<0.5	<0.5	0	<0.5	0
Quercus agrifolia	0	0	<0.5	0	0	0
Cornus sericea	<0.5	<0.5	6.9	<1.0	1.3	<0.5
Arctostaphylos obisp.	0	<0.5	1.6	<0.5	0	<0.5
Garrya veatchii	<0.5	<0.5	0	0	<1.0	0
Vinca major *	<0.5	10.4	<1.0	0	0	0
Marrubium vulgare *	1.1	<1.0	1.1	1.4	0	0
Salvia mellifera	<1.0	<0.5	<0.5	0	<0.5	0
Salvia spathacea	<0.5	<0.5	<0.5	<0.5	0	0
Solanum douglasii	40.6	71.6	9.0	<0.5	31.4	11.8
Nicotiana glauca *	58.2	84.5	19.3	39.9	4.0	7.2
Sambucus mexicana	1.1	7.1	4.7	<1.00	<0.5	<0.5
Delairea odorata *	27.5	32.7	2.8	18.6	100	68.5
Baccharis pilularis	<0.5	0	7.9	<0.5	<0.5	0
Average	8.15%	9.52%	3.26%	4.08%	6.05%	2.60%

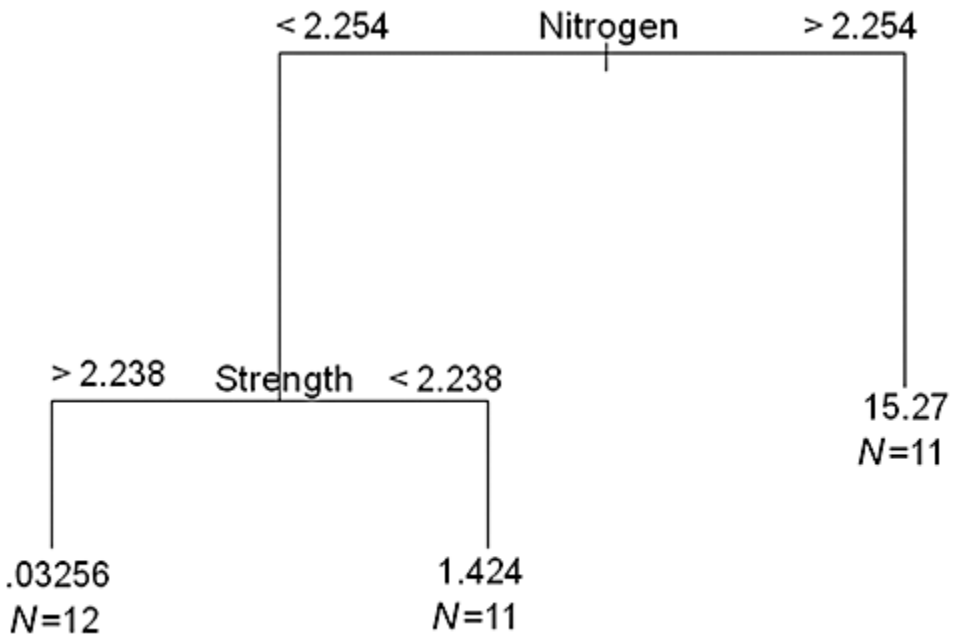
The distribution of herbivory is displayed for all three herbivores and overlaid onto a phylogeny of the 34 plant species sampled. The black bars represent plant species sampled during the single species' time trial (SSTT) and the grey bars represent sampling during the multiple species' preference trial (MSPT). The average percentage of leaf area removed by each herbivore is listed inside the black or grey bar for each species. The average leaf area eaten per herbivore is reported at the bottom of the figure. *Non-native plant species.

Figure 3



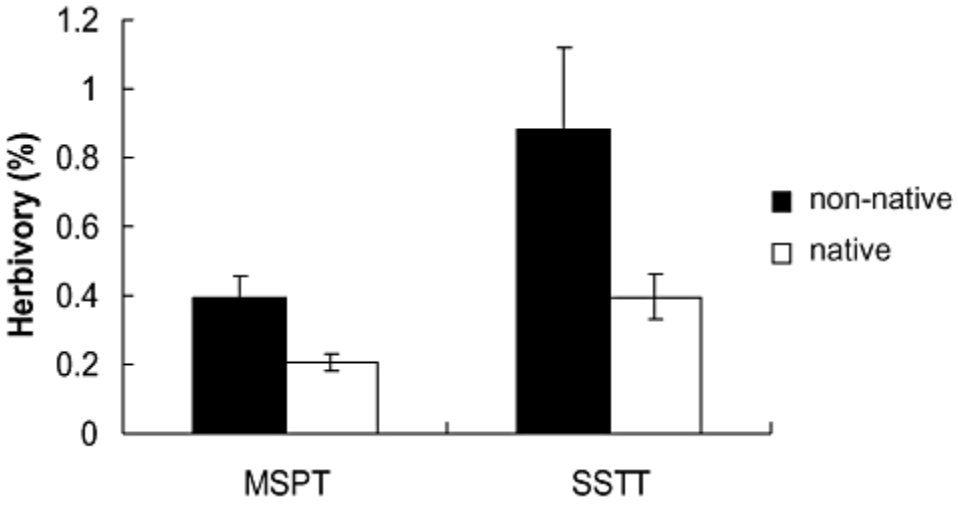
The relationship between leaf traits and the percentage of leaf area lost to herbivores during the multiple species' preference trial (MSPT). Regression statistics (r^2) are reported for each figure. Each datum point represents the average for a species (N = 34). The percentages of leaf area lost to all three herbivores in MSPTs were averaged together to obtain herbivory (%). ***P < 0.001.

Figure 4



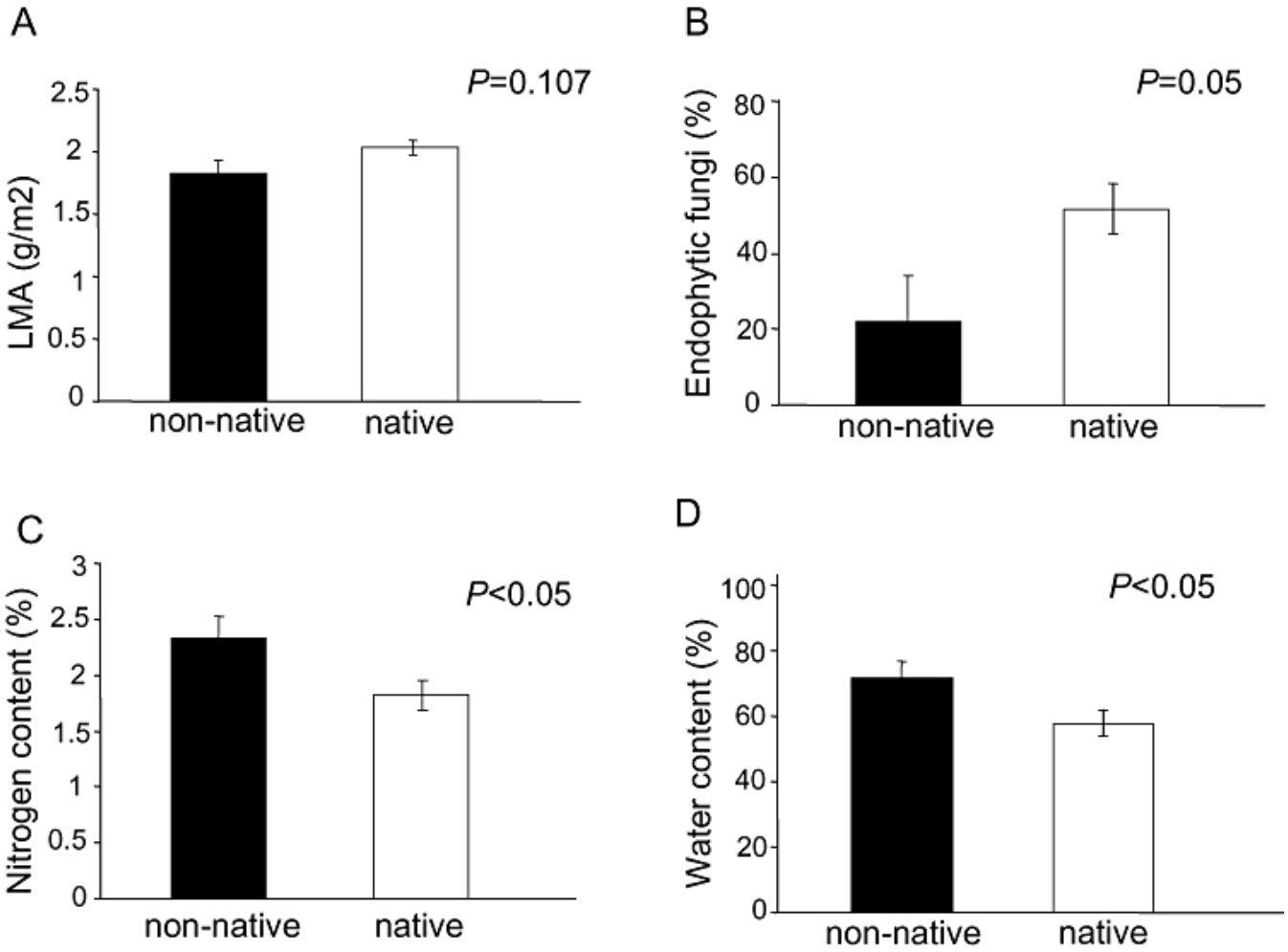
Category and regression tree identifying the plant traits most closely associated with the leaf area removed by herbivores. Plant species are initially partitioned according to their foliar nitrogen content. Eleven species (right branch) had high nitrogen content (> 2.254%) and high levels of herbivore damage (averaging 15.27%). The 23 plant species with low foliar nitrogen (left branch) were further split according to their leaf strength. Eleven species had low foliar nitrogen (< 2.254%) and high structural integrity (> 2.238 N) leading to the lowest level of herbivore damage (averaging 0.033%).

Figure 5



Percentage of leaf area removed by herbivores on non-native and native plant species. A *t*-test was performed on each type of herbivory trial [multiple species' preference trial (MSPT) and single species' time trial (SSTT)] separately. Non-native species had significantly greater herbivory in MSPT ($t = 2.71$, $P < 0.05$, $N_{(exotic)} = 8$, $N_{(native)} = 26$). Similarly, non-native species were eaten more in SSTT, but this difference was not significant ($t = 1.96$, $P = 0.085$, $N_{(exotic)} = 8$, $N_{(native)} = 26$).

Figure 6



Exotic and native species' comparisons for leaf mass per unit area (LMA, g m^{-2}) ($t = -1.77$, $P = 0.107$) (A), percentage of leaves tested with endophytic fungal infection ($t = -2.17$, $P = 0.05$) (B), percentage foliar nitrogen content ($t = 2.16$, $P < 0.05$) (C) and percentage foliar water content ($t = 2.61$, $P < 0.05$) (D). Eight non-native species and 24 native species were included in the analysis.

Table 1

Pearson correlation coefficients (bold) and phylogenetic independent contrast correlation coefficients (PICr) (not bold) for all pairwise relationships

	Nitrogen (%)	Water (%)	Endophyte (%)	LMA (g m^{-2})	Strength (N)
Nitrogen	1	0.744	−0.426	−0.751	−0.657
Water	0.701	1	−0.564	−0.87	−0.742
Endophyte	−0.487	−0.425	1	0.361NS	0.307NS
LMA	−0.730	−0.859	0.279NS	1	0.932
Strength	−0.623	−0.722	0.245NS	0.889	1

The PICr values were calculated using the phylogeny in Figure 2, the traits in Appendix 1 and the AOT software package. NS indicates correlation coefficients that were not significant. Correlation coefficients without NS were significant at the level of $P < 0.05$ or lower. LMA, leaf mass per unit area.