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Evidence for allelopathy as a mechanism of community composition change by an invasive exotic shrub, Chrysanthemoides monilifera spp. rotundata

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REGULAR ARTICLE

Evidence for allelopathy as a mechanism of community composition change by an invasive exotic shrub, *Chrysanthemoides monilifera* spp. *rotundata*

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Abstract Chemical interference is increasingly suggested as a mechanism facilitating exotic plant invasion and plant community composition. In order to explore this further, we employed a comprehensive extract-bioassay technique that facilitated detection and demarcation of phytotoxicity, direct allelopathy and indirect allelopathy of bitou bush (*Chrysanthemoides monilifera spp. rotundata*) compared to an indigenous dominant of the invaded system, acacia (*Acacia longifolia* var. *sophorae*). Extracts of the leaves and roots of both species exhibited phytotoxic effects against five indigenous plant species. Evidence for allelopathy between co-evolved indigenous plants was detected between acacia and *Isolepis nodosa*. Allelopathy between bitou bush and four indigenous plant species was also detected. Therefore we propose that both the acacia and bitou bush have the potential to chemically inhibit the establishment of indigenous plants. Eventual dominance of bitou bush is predicted, however, based on more ubiquitous effects on seedling growth.

Keywords Chemical interference competition · Exotic plant invasion · Non-polar compounds · Community structure

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Introduction

Despite the naturalization of many exotic plants, comparatively few become invasive and form monocultures (Williamson 1996). The mechanisms facilitating the invasion of exotic plants, resulting in the displacement of indigenous flora, are often cited as direct or indirect resource and interference competition (Williamson 1996; Amarasekare 2002). Resource competition is often cited without adequate experimentation or exploration for possible underlying interference mechanisms (Levine et al. 2003; Schenk 2006). Direct interference via allelopathy (Molisch 1937) or indirect interspecific interference via abiotic or biotic modification of plant derived soil compounds, are less accepted as mechanisms of invasion, although mounting evidence supports the occurrence of these phenomena (Reigosa et al. 1996; Wardle et al. 1998; Hierro and Callaway 2003; Inderjit et al. 2006). Allelopathy, i.e., referred to

here as the process whereby one plant releases compounds which affect the growth and development of another plant (Molisch 1937).

Historically, arguments against allelopathy as a mechanism of direct interspecific interference are based on methodological inadequacies including the use of bioassays, insufficient controls and the lack of convincing field studies (Harper 1977; Stowe 1979; Keeley 1988). More recently, significant improvements in methodology and technology have facilitated the demonstration of allelochemical exudation (Tang 1986; Inderjit and Nilsen 2003; Bais et al. 2004) and the biochemical mechanisms of action (Einhellig 1986; Dayan et al. 2000; Duke and Oliva 2004). However exploratory allelopathy studies incorporating bioassays can be useful precursors to these more detailed analyses of distinct allelochemicals (Blum 1999). Preliminary bioassay studies can direct research to the type of compounds likely to be allelopathic, where in the plant the allelopathic compounds originated, the possible biochemical pathways leading to allelopathy, and to suitable soil remediation methods.

This study adopted a bioassay-guided, fractionation procedure that incorporated parallel extractions from the leaves, roots and soil of an exotic plant and from a dominant indigenous plant. Extracts were obtained using a series of solvents of increasing polarity in order to solubilise potential allelochemicals with a range of polarities. This comprehensive protocol aimed to distinguish between phytotoxicity, allelopathy and indirect soil effects of an exotic shrub using bioassays. Many past criticisms of allelopathy studies were based on confusion between phytotoxicity and allelopathy and the failure to incorporate the soil media, which were therefore regarded as lacking field relevance. Most plant-derived compounds are likely to flow into the soil except volatile compounds from shoots and leaves. Hence analysis of the soil chemistry is integral to studies of allelopathy (Inderjit and Weiner 2001). We differentiated between allelopathic and phytotoxic effects as phytotoxic compounds may exist in plant parts but are not exuded or released into the surrounding environment. Laboratory-based bioassays of root and shoot extracts are useful indicators of plant phytotoxins: however, the inclusion and comparison of plant *and* soil extracts is imperative if possible allelopathic effects on indigenous species are to be assessed.

The shortcomings of allelopathy bioassay studies, which often include the single application of extract and exclusion of abiotic and biotic manipulators of potential allelochemicals that would be encountered in the field, are acknowledged. We addressed the ecosystem abiotic and biotic manipulation of potential allelochemicals by testing the effect of extracts derived from soil beneath bitou bush and acacia canopies, which therefore should contain the root exudates and/or leaf derived allelochemicals and any abiotically or biotically transformed derivatives. We have also addressed the possibility that interference competition may occur in the non-invaded system as well as in the invaded system by comparing the effects of solvent extracts from the exotic plant and soil with those from the dominant indigenous plant and soil. By comparing the effect of plant and soil extracts of the exotic system to those of the indigenous system at a range of concentrations predicted to occur in the field, some of the criticism that allelopathy studies have attracted in the literature (Williamson and Richardson 1988; Inderjit and Weston 2000) are overcome and we present a critical test of potential allelopathy as a mechanism facilitating bitou bush invasion in Australia.

The objectives of this study were to determine whether bitou bush is allelopathic in the Australian environment and whether chemical interference is unique to this system by comparing the effects of exotic bitou bush (*Chrysanthemoides monilifera* spp. *rotundata* (L.) T. Norl.) extracts on indigenous test species to those of extracts from the dominant indigenous species in the system, coastal acacia (*Acacia longifolia* var. *sophorae* (L.) F. Muell).

Method

Exotic species

Bitou bush (*Chrysanthemoides monilifera* spp. *rotundata* L.; Asteraceae) is a South African woody shrub which was planted on the sand dunes of the New South Wales (Australia) coast to stabilize the sand dunes following mining from 1946 to 1964 (Barr 1965; Department of Environment and Conservation 2006). However by 2000, bitou bush had invaded approximately 80% of the New South Wales coast and spread into relatively undisturbed tracts of native vegetation

(Department of Environment and Conservation 2006). Many plant species, populations and communities are currently threatened by the bitou bush invasion which was declared a key threatening process under the New South Wales *Threatened Species Conservation Act* (1995) in 1999. Bitou bush invasions have also been shown to limit the recruitment of several indigenous plant species (Ens and French 2008) and there is some evidence to suggest this is a function of bitou bush allelopathy. Bitou bush litter and soil have been shown to inhibit the seedling growth of acacia (Vranjic et al. 2000). Copeland (1984) also found that the germination and seedling growth of three woody heath species appeared to be differentially inhibited by bitou bush root and shoot water leachates. However the outcomes of this bioassay study were affected by fungal attack. A third study has also shown that bitou bush leaf litter inhibited the germination of *Hardenbergia comptoniana* and *Lepidium sativum* (cress) and that the water soluble bitou bush leaf extract decreased the germination of *Schoenia filifolia* and *L. sativium* (Hughes 1998). Congruent to these rudimentary studies of bitou bush allelopathy we aimed to conduct a comprehensive assessment of different fractions of bitou bush leaves, roots and soil in comparison with similar extracts from the native dominant of the invaded system, *A. longifolia* var. *sophorae*, against six test species.

Bioassay test species

Five endemic species of the bitou bush invaded region of the New South Wales coast were selected: *Acacia longifolia* var. *sophorae* (woody shrub; Fabaceae); *Banksia integrifolia* (tree; Proteaceae); *Actites megalocarpa* (herb; Asteraceae); *Lomandra longifolia* (rush; Lomandraceae); and *Isolepis nodosa* (sedge; Cyperaceae). Utilisation of taxonomically and morphologically distinct species facilitated generalization of results. Additionally, we employed *Lactuca sativa* as a universal indicator of phytotoxicity (see Escudero et al. 2000; Iqbal et al. 2002). The lettuce seed was purchased from a commercial supplier (Mrs. Fothergills's, "All season" lettuce) and the native seeds were collected and pooled from at least five different sites along the New South Wales south coast from Moruya (35°91′ S 150°15′ N) to Kurnell (34°0′ S 151°21′ N). Bitou bush could not be germinated and grown successfully in the laboratory.

Extraction procedure

Fresh bitou bush and acacia roots (500 g), leaves (500 g) and soil (2 kg) from 10 to 20 cm beneath five plants of each species (within 10 cm from live, visible roots) were collected from a coastal site at North Wollongong, New South Wales, Australia in July 2004. Voucher specimens are deposited at the Janet Cosh Herbarium, University of Wollongong: (*Chrysanthemoides monilifera* spp. *rotundata*) (9872-WOLL) and *Acacia sophorae* var. *longifolia* (9871-WOLL). As the coastal dune soil of this area is highly mobile, we collected samples from 10 to 20 cm below the surface to ensure that the soil had been exposed to the plants of interest for some time. The soils of this region are loose, loamy, quartz sands of low fertility and high permeability and the pH ranges from neutral to slightly acidic (Hazelton and Tille 1993).

The samples of roots, leaves and soil for each extract species were pooled to give a total of six different raw materials that were treated separately. The fresh leaf and root (lightly brushed to remove soil) material was chopped with scissors (to approximately 1–5 mm pieces in order to aid extraction of the compounds) and the soil sample was sifted (1 mm mesh) to remove all biological material. The raw materials were placed into separate conical flasks and dichloromethane (DCM; HPLC grade; 1 L for roots and leaves; 2 L for soil) was added. After 30 h the DCM was decanted from each flask (supernatant) and replaced sequentially with acetone (AR grade), methanol (AR grade) and distilled water (all in equal volumes as used for the DCM extraction) in 30-h cycles. After removal of the supernatant and before adding the next solvent, each solvent was evaporated under reduced pressure from a water bath (temperature <40°C; Büchi Rotavapor). The resultant residues are hereafter referred to as the solvent (DCM, acetone, methanol and water) extracts. DCM extracts alkaloids, aglycones and volatile oils; acetone extracts alkaloids, aglycones and glycosides; methanol extracts glycosides and sugars; and water extracts glycosides, sugars and amino acids (Houghton and Raman 1998).

Extract concentrations

To incorporate the probable temporal and spatial variation in concentrations of soil allelochemicals we

tested a range of concentrations. Specifically, the effect of each solvent extract on seedling growth was assessed by utilizing the dose response of six concentrations: 0, 10, 100, 500, 1,000 and 2,000 ppm (parts of solvent extract/million parts of solvent (distilled water)). These concentrations were based on the concentrations (*w*/*w*; weight of extract/weight of original soil used) of various bitou bush and acacia DCM soil extracts which were approximately 100–900 ppm. Hence, were selected a priori and overcome past criticism of high, unrealistic extract concentrations (Keeley 1988). The soil samples were taken in June during the peak flowering period of bitou bush and the peak vegetative growth period of acacia.

Bioassay procedure

For application in the Petri dish bioassays, the methanol and water extracts were readily re-dissolved in distilled water (2 mL). The DCM and acetone extracts were first dissolved in DCM (1 mL) and added to each Petri dish fitted with filter paper (Whatman number 1). The DCM was then allowed to evaporate from the filter paper (15 min) before distilled water (2 mL) was added to each Petri dish. Four replicate bioassays of each extract at each concentration were conducted with 20 equidistant seeds set in each of four glass Petri dishes (8 cm diameter). The pH of all Petri dish solutions (extract plus water) was recorded using an electronic pH meter (Activon model 209). Controls comprised 20 seeds grown in Petri dishes fitted with filter paper and distilled water (2 mL). The response of all species to DCM controls compared to the water controls was also tested. The DCM controls consisted of 20 seeds in each of four replicate Petri dishes fitted with filter paper to which DCM (2 mL) had been applied then evaporated from (15 min), followed by the addition of distilled water (2 mL).

Replicates were placed in an incubator set to a diurnal (12/12 h) temperature (15/25°C) and light (8 W fluorescent tubes) regime. After 7, 23, 40, 48, 53 and 59 days for lettuce, *I. nodosa*, *B. integrifolia*, *A. longifolia* var. *sophorae*, *A. megalocarpa* and *L. longifolia* respectively, germination and seedling shoot and root length were recorded.

To test whether applying the DCM and acetone extracts to the Petri dish/filter paper with DCM had a confounding effect on seedling growth, we determined whether lettuce seedlings grown on filter paper, to which DCM had been applied then evaporated, differed in length to those grown on regular filter paper.

Protocol for determination of phytotoxicity, allelopathy or indirect allelopathy

We measured germination success and the root and shoot length of all seedlings and assessed whether there was a 50% reduction in these parameters compared to the controls (LC_{50}) using graphical information. Statistical significance of dose response and inhibition to at least 50% of the control were the two criteria used to determine either phytotoxicity or allelopathy. Phytotoxicity was suggested if (a) there was a statistically significant effect of the leaves or root extract and (b) the LC_{50} was reached for the leaves or root extract and (c) there was no significant effect of the comparable soil extract (Table 1). Allelopathy was indicated by (a) significant root or leaf extract inhibition, (b) comparable soil extract having a significant effect and (c) the LC_{50} being reached for the roots or leaves and soil extracts (Table 1). If a soil extract elicited a significant effect on a growth parameter (seedling germination or root or shoot length) and reached the LC_{50}, and comparable shoot and root extracts did not elicit a significant effect, then it was suggested that the plant (bitou bush or acacia) induced an indirect effect on the soil chemistry (through biotic or abiotic pathways), which in turn affected the seedling growth parameter of the test species (Table 1). This latter condition is referred to as indirect allelopathy (sensu Muller 1966).

Statistical analysis

Probit analysis (SPSS 2003) was used to determine whether increasing concentrations (covariate) of comparable extracts of the exotic and native species (factor) differed in their effects on germination of each test species. Pearson's goodness of fit test was used to ascertain whether the regression models adequately fit the data. A *Z* score was used to investigate whether the slopes differed from zero and a parallelism test was conducted to determine whether the slopes of the relationship between germination and concentration of each extract were similar. If the two slopes were not parallel, then the relationship between germination and concentration was analysed to determine significance for each extract separately.

Table 1 Protocol for assessing the presence of phytotoxicity, direct allelopathy and indirect allelopathy of native compared to exotic species extracts (E) using the dose response curve (C), a two-factor ANOVA testing the effects of E, C and C×E and attainment of LC_{50} for ecologically relevant concentrations of extracts

	Indicators			
	Statistically significant factor ($P<0.05$)			
Mechanism	C	E	C×E	LC_{50}
Phytotoxicity	Roots or leaves	Roots or leaves	Roots or leaves	Exotic and/or native
Allelopathy	Roots or leaves and soil	Roots or leaves and soil	Roots or leaves and soil	Exotic and/or native
Indirect soil effects	Soil only	Soil only	Soil only	Exotic and/or native

A two factor ANCOVA (SPSS 2003) was conducted to assess whether the root and shoot length of any of the test species elicited different responses to the bitou bush and acacia extracts (Extract), and whether there was a significant dose response when both extracts were combined (Concentration) or whether there was a different response to different extract species at different concentrations (Extract×Concentration). Extract species was a fixed factor and concentration was a covariate in the model. Data was ln (x+1) transformed to satisfy data normality and variance homogeneity if these assumptions of the ANOVA were violated.

Results

Bitou bush and acacia extracts

The pH of the methanol and acetone extracts of the acacia shoots and roots and the pH of the acacia soil methanol extract significantly decreased with concentration (Table 2). For bitou bush extracts, only the methanol extract of the shoots showed a significant decrease in pH with increasing concentrations (Table 2). At 2,000 ppm, the highest mean pH (7.27) was demonstrated by the DCM extract of the bitou bush soil, and lowest mean pH (5.00) was demonstrated by the acacia leaf acetone extract (Table 2).

We found no significant effect of filter paper type (DCM evaporated or standard) on lettuce germination ($F_{(1,6)}$=0.43; P=0.537), shoot ($F_{(1,6)}$=0.83; P=0.431) or root ($F_{(1,6)}$=0.07; P=0.804) length.

Effects on germination

High unexplained variability in germination resulted in significant deviations in most of the Goodness of fit tests, indicating that the models did not tightly fit the data (analyses not presented). Despite this high variability, regression coefficients and tests of differences between slopes of extract species yielded significant differences indicating that while only a small proportion of the variability is explained by the treatments, it is nevertheless a predictable component.

A significant effect on the germination of at least one of the test species was found for most of the bitou bush leaf extracts, none of the acacia leaf extracts, and all of the root extracts from both the acacia and bitou bush (Table 3). Although, no extract had an effect across a broad range of species, the DCM extract of the bitou bush root was most inhibitory to the species studied (Table 3). Furthermore, the bitou bush root extracts (acetone and water) exhibited allelopathic affects against the germination of three of the test species (Table 3), as suggested by the inhibition of comparable soil extracts (acetone and water). The DCM extracts of the bitou bush and acacia soils also significantly affected the germination of *B. integrifolia* and *L. longifolia*, respectively.

Effects on shoot and root length

All of the leaf extracts from both species inhibited the growth (root or shoot length) of at least one of the test species (Table 4). Approximately half of the acacia and bitou bush leaf extracts were inhibitory to the same species; however, this effect was not seen in the comparable soil extracts, suggesting the effects are from chemicals within leaves that are not released into the soil. The DCM and acetone root and soil extracts were more inhibitory than the methanol and water extracts and more species were affected by the bitou bush extracts than comparable acacia extracts (Table 4).

Table 2 Mean pH range of extract concentrations (10 to 2,000 ppm) and the significance values of an ANOVA testing whether the pH differed with extract concentrations

Extract species	Plant part	Solvent extract	$F_{(1,4)}$	P	Mean pH range (10–2000 ppm)
Acacia	Leaves	DCM	0.75	0.574	7.23–7.05
		Acetone	3.41	0.036*	5.78–5.00
		Methanol	4.25	0.017*	6.34–5.21
		Water	0.56	0.698	6.44–6.48
	Roots	DCM	0.91	0.486	7.20–6.97
		Acetone	4.95	0.010*	6.97–5.46
		Methanol	4.61	0.013*	6.92–5.49
		Water	0.74	0.580	6.53–6.18
	Soil	DCM	0.45	0.772	7.24–7.24
		Acetone	0.45	0.077	7.11–6.35
		Methanol	5.91	0.005**	6.60–5.22
		Water	0.54	0.706	6.33–6.33
Bitou bush	Leaves	DCM	0.86	0.508	7.28–6.83
		Acetone	2.89	0.059	6.34–6.01
		Methanol	3.55	0.031*	6.61–5.33
		Water	0.60	0.670	6.98–6.38
	Roots	DCM	1.71	0.804	6.74–7.17
		Acetone	0.40	0.201	6.65–6.08
		Methanol	1.56	0.235	6.56–5.18
		Water	0.96	0.457	6.09–6.69
	Soil	DCM	0.35	0.838	7.07–7.27
		Acetone	1.65	0.215	7.68–6.76
		Methanol	0.82	0.533	6.86–6.20
		Water	1.91	0.161	6.16–7.07

*$P<0.05$, **$P<0.01$

Phytotoxic, allelopathic and indirect soil effects

From the germination and seedling growth bioassay results, each extract from the bitou bush and acacia had a phytotoxic effect on at least one of the test species (Table 5). Overall, the bitou bush extracts were more phytotoxic, allelopathic and had more indirect negative soil effects than the acacia extracts (Table 5). Furthermore, the DCM and acetone extracts of the bitou bush root and soil appeared to demonstrate allelopathy and indirect soil effects on the seedling growth of all indigenous test species (Table 5).

Discussion

By comparing the effects of extracts of an exotic invasive plant leaves, roots and soil with comparable extracts from the dominant indigenous shrub against five indigenous species, we have found evidence to suggest that although both indigenous and exotic species have the potential to inhibit the establishment of resident indigenous plant species via direct and indirect allelopathy, exotic bitou bush affected a broader range of species, including the dominant indigenous acacia (*A. longifolia* var. *sophorae*). The broader allelopathic effect of bitou bush is a suggested as a mechanism invasion, which is likely to partly explain why bitou bush tends to form monocultures on the New South Wales coast. The comprehensive bioassay scheme adopted, which tested the biological effects of different plant parts and soil extracts of an exotic invasive plant to those of the dominant indigenous species, allows inferences as to whether chemical interference competition is likely to occur within the non-invaded and invaded habitats. Inclusion of soil extracts (Inderjit 2001; Inderjit and Weiner 2001) and exotic versus indigenous comparisons, is imperative to this end. This is the first documented research, to our knowledge, that incor-

Table 3 Coefficients, parallelism tests and goodness of fit of the probit regression comparing the relationship between increasing concentrations of extracts from each extract source species (acacia and bitou bush) and the germination success of six species

Extract		Bioassay species	Regression coefficient and *Z* score		Regression parallelism test (*df*=1)		*Z* score for each regression		LC_{50}
Plant part	Solvent		Coefficient±SE ($\times 10^{-5}$)	*Z*	χ^2	*P*	Acacia	Bitou	
Leaves	DCM	*L. sativa*	*−53±11*	*−4.67*	0.00	1.000			
		A. longifolia	*−12±6*	*−2.14*	0.27	0.604			
		B. integrifolia	−2±6	−0.40	*9.78*	*0.002*	*−2.63*	*2.10*	Bitou
		A. megalocarpa	−31±6	−5.08	*11.29*	*0.001*	−1.24	−5.91	
		L. longifolia	−11±7	−1.96	0.14	0.713			
		I. nodosa	*14±7*	*2.08*	1.47	0.226			
	acetone	*L. sativa*	−28±16	−1.79	1.00	0.317			
		A. longifolia	*28±6*	*4.78*	2.00	0.157			
		B. integrifolia	*−14±6*	*−2.47*	0.10	0.758			
		A. megalocarpa	13±7	1.89	0.50	0.480			
		L. longifolia	*25±6*	*3.79*	*7.68*	*0.006*	1.74	*3.76*	
		I. nodosa	*−14±6*	*−2.18*	0.38	0.537			
	Methanol	*L. sativa*	4±18	0.24	0.09	0.769			
		A. longifolia	*−15±6*	*−2.65*	3.43	0.064			
		B. integrifolia	*−19±6*	*−3.40*	0.04	0.84			
		A. megalocarpa	−6±6	−1.00	*5.83*	*0.016*	1.17	−2.61	
		L. longifolia	−9±6	−1.58	*4.38*	*0.036*	0.56	*−2.79*	Bitou
		I. nodosa	−3±6	−0.45	2.81	0.094			
	Water	*L. sativa*	−8±14	−0.59	1.45	0.228			
		A. longifolia	5±6	0.88	*22.70*	*<0.001*	*4.22*	*−2.89*	Bitou
		B. integrifolia	8±6	1.26	*8.94*	*0.003*	*2.74*	−0.85	
		A. megalocarpa	−11±6	−1.82	*5.21*	*0.022*	−3.17	0.65	
		L. longifolia	*24±6*	*4.13*	0.00	1.00			
		I. nodosa	5±6	0.81	2.98	0.084			
Roots	DCM	*L. sativa*	18±19	0.93	0.00	1.000			
		A. longifolia	*−16±6*	*−2.89*	2.24	0.134			Bitou, acacia
		B. integrifolia	*−13±6*	*−2.35*	0.01	0.704			
		A. megalocarpa	*−21±6*	*−3.50*	1.78	0.182			Bitou, acacia
		L. longifolia	−6±6	−1.11	*5.66*	*0.017*	1.06	*−2.59*	Bitou
		I. nodosa	11±6	1.83	3.08	0.079			
	Acetone	*L. sativa*	−1±14	−0.58	1.43	0.232			
		A. longifolia	*−14±6*	*−2.52*	*6.65*	*0.01*	0.23	*−3.78*	Bitou
		B. integrifolia	*−41±6*	*−7.11*	*10.46*	*0.001*	*−7.24*	*−2.75*	Acacia
		A. megalocarpa	−1±6	−0.23	1.43	0.232			
		L. longifolia	−11±6	−1.95	0.135	0.713			
		I. nodosa	−7±6	−1.15	*5.46*	*0.020*	*−2.05*	0.56	
	Methanol	*L. sativa*	−25±13	−1.92	*7.64*	*0.006*	*−2.52*	0.59	
		A. longifolia	*−24±6*	*−4.20*	*5.07*	*0.024*	−1.21	*−4.70*	Bitou
		B. integrifolia	−6±6	−1.11	0.01	0.917			
		A. megalocarpa	*−41±6*	*−6.52*	1.29	0.257			
		L. longifolia	−6±6	−1.13	1.17	0.280			
		I. nodosa	*−17±6*	*−2.77*	0.00	1.000			
	Water	*L. sativa*	−7±14	−0.50	*4.03*	*0.048*	0.75	−0.97	
		A. longifolia	*−23±6*	*−3.97*	*7.76*	*0.005*	*−4.95*	−0.62	Acacia
		B. integrifolia	−7±6	−1.20	0.73	0.392			
		A. megalocarpa	−3±6	−0.54	*16.57*	*<0.001*	*2.54*	*−3.16*	Bitou
		L. longifolia	2±6	0.42	*3.57*	*0.021*	*2.39*	−1.77	Bitou
		I. nodosa	−12±6	−1.94	*25.75*	*<0.001*	*−3.55*	0.79	

Table 3 (continued)

Extract		Bioassay species	Regression coefficient and *Z* score		Regression parallelism test (*df*=1)		*Z* score for each regression		LC_{50}
Plant part	Solvent		Coefficient±SE ($\times 10^{-5}$)	*Z*	χ^2	*P*	Acacia	Bitou	
Soil	DCM	*L. sativa*	12±27	0.44	1.20	0.273			
		A. longifolia	−7±6	−1.17	0.10	0.751			Bitou, acacia
		B. integrifolia	*−16±6*	*−2.74*	0.09	0.760			Bitou
		A. megalocarpa	*−25±6*	*−4.16*	0.32	0.570			Bitou, acacia
		L. longifolia	−6±6	−1.09	*37.14*	*<0.001*	*−6.55*	0.2	Acacia
		I. nodosa	−4±6	−0.64	0.17	0.677			
	Acetone	*L. sativa*	−6±18	−0.34	*6.22*	*0.013*	0.59	−0.97	
		A. longifolia	*−13±6*	*−2.28*	0.30	0.584			Bitou
		B. integrifolia	−7±6	−1.20	0.73	0.392			
		A. megalocarpa	−5±6	−0.82	1.31	0.252			
		L. longifolia	*−26±6*	*−4.57*	*21.88*	*<0.001*	*−5.24*	*3.79*	Acacia
		I. nodosa	−4±6	−0.61	0.81	0.368			
	Methanol	*L. sativa*	−12±16	−0.71	*13.16*	*<0.001*	−1.49	0.38	
		A. longifolia	1±6	0.16	0.00	1.000			
		B. integrifolia	*−30±6*	*−5.26*	*5.42*	*0.020*	0.54	0.26	
		A. megalocarpa	*−22±6*	*−3.56*	0.71	0.398			
		L. longifolia	7±6	1.26	*16.42*	*<0.001*	*3.36*	−1.44	
		I. nodosa	*23±7*	*3.27*	*6.90*	*0.009*	*3.61*	0.89	
	Water	*L. sativa*	*−50±18*	*−2.76*	2.96	0.085			
		A. longifolia	*−17±6*	*−3.00*	0.165	0.684			
		B. integrifolia	*3±6*	*0.57*	0.04	0.839			
		A. megalocarpa	*−14±6*	*−2.28*	0.07	0.787			Bitou
		L. longifolia	−10±6	−1.68	0.017	0.895			Bitou
		I. nodosa	−12±7	−1.72	0.01	0.940			

Values in italics are significant at α=0.05

porated all of these factors into a bioassay based investigation into potential exotic plant allelopathy.

Our study of the chemical interference between plants that are endemic to low resource environments, found that non-polar rather than polar compounds are likely to influence community composition and species dominance. The DCM extracts of both the indigenous acacia and exotic bitou bush were the most inhibitory to all indigenous test species. Compounds soluble in DCM such as plant waxes, fatty acids, oils, sterols, terpenes and high molecular weight alkanes, are likely to occur in leaves (Yokouchi 1991), roots (Pomilio et al. 2000) and vegetated soil (Franco et al. 2000; Chefetz et al. 2002; Lin et al. 2007). Non-polar compounds have the ability to regulate plant establishment (Langenheim 1994; Barney et al. 2005; Nishida et al. 2005), and are also known to have antimicrobial properties (Deans 1991; Karamanoli 2002; Scher et al. 2004), which has ramifications for plant growth, particularly in low resource environments where plant–microbe mutualisms are common (Ernst 1985; Logan et al. 1989; Abe and Ishikawa 1999). Hence, similar studies on the microbial effects of bitou bush may lend further insight into the mechanisms of bitou bush invasion as this study suggests that non-polar compounds may be an important driver of community change.

The DCM extracts of the acacia roots and soil were found to inhibit the seedling growth of *I. nodosa* while the water soluble acacia soil extract inhibited the growth of *B. integrifolia* and *L. longifolia*. The pH of the DCM and water soluble extracts of the acacia roots and soil did not significantly change with increasing concentration of extract. This suggests that other characteristics of the constituent compounds were responsible for the observed inhibition of growth. We did not find any inhibitory effects of comparable acacia leaves and soil extracts; however, decomposing *Acacia* spp. leaves have been shown to inhibit plant growth (Gonzalez et al. 1995; Bernhard-Reversat

Table 4 Probability values from an ANOVA testing the effect of extract species (E), concentration (C) and the interaction between extract species and concentration (E×C) on seedling shoot and root length of six species for each solvent extract of each plant part

Extract		Bioassay species	Effects on shoot length			Effects on root length			Influential extract species (LC_{50})[a]	
			E	C	E×C	E	C	E×C	Shoot	Root
Leaves	DCM	*L. sativa*	*0.042*	0.110	0.502	*<0.001*	*0.008*	*0.018*	b	a
		A. longifolia	0.189	*0.016*	0.946	0.613	0.374	0.444	b, a[a]	
		B. integrifolia	0.618	0.066	0.264	0.376	*0.032*	0.229		b, a
		A. megalocarpa	0.880	0.462	0.742	0.421	0.564	0.541		
		L. longifolia	0.303	*0.019*	0.386	0.713	*0.001*	0.632	b[a], a[a]	b[a], a[a]
		I. nodosa	0.388	*0.037*	0.644	*0.046*	*<0.001*	0.174	b[a], a[a]	b[a], a[a]
	Acetone	*L. sativa*	0.593	0.947	0.989	*0.007*	*0.001*	0.478		a
		A. longifolia	0.515	0.163	*0.033*	0.741	0.571	0.278	b, a	
		B. integrifolia	0.224	0.661	0.252	0.062	0.867	0.816		
		A. megalocarpa	0.729	0.549	0.532	0.059	*0.010*	0.554		
		L. longifolia	*<0.001*	0.182	*0.004*	*<0.001*	0.063	*0.003*	a[a]	a[a]
		I. nodosa	0.600	0.080	0.408	*0.009*	*<0.001*	0.174		b[a], a[a]
	Methanol	*L. sativa*	0.356	0.480	0.701	0.136	0.087	0.909		
		A. longifolia	0.448	0.142	0.331	0.686	0.552	0.802		
		B. integrifolia	0.596	0.831	0.825	0.405	0.282	0.285		
		A. megalocarpa	0.226	0.466	0.305	0.057	*0.019*	*0.019*		
		L. longifolia	0.187	0.357	0.350	*0.001*	0.435	0.199		a[a]
		I. nodosa	0.990	0.161	0.125	*0.001*	*<0.001*	0.180		b[a], a
	Water	*L. sativa*	0.665	0.137	0.610	0.532	0.458	0.489		
		A. longifolia	0.273	0.997	0.141	0.526	0.439	0.266		
		B. integrifolia	0.822	*0.047*	0.978	0.824	0.678	0.904		
		A. megalocarpa	0.548	0.741	0.760	0.375	0.536	0.681		
		L. longifolia	0.211	*0.002*	*0.005*	*0.029*	*0.002*	*0.035*	b[a], a	b[a], a[a]
		I. nodosa	0.947	0.097	0.542	0.302	0.235	0.689		
Roots	DCM	*L. sativa*	0.077	*0.016*	0.643	*0.002*	0.167	0.366	b[a]	a[a]
		A. longifolia	*0.050*	0.933	0.122	0.143	0.990	0.224	b[a]	
		B integrifolia	0.783	0.087	0.444	0.441	0.102	0.092		
		A. megalocarpa	0.465	*0.015*	0.310	0.234	0.922	0.125	b[a]	
		L. longifolia	*0.014*	*<0.001*	0.104	0.526	*<0.001*	0.628	b[a], a	b[a], a
		I. nodosa	0.115	*<0.001*	0.200	*0.047*	*<0.001*	0.374	b[a], a[a]	b[a], a[a]
	Acetone	*L. sativa*	0.573	0.609	0.494	*<0.001*	0.460	*0.015*		a
		A. longifolia	0.486	0.462	0.294	0.894	0.895	0.968		
		B. integrifolia	*0.050*	*<0.001*	0.067	0.141	*<0.001*	0.782	b[a], a[a]	b[a], a[a]
		A. megalocarpa	0.533	0.210	0.899	0.920	0.762	0.509		
		L. longifolia	*0.041*	0.096	0.165	0.318	0.105	0.475		
		I. nodosa	0.278	0.139	0.792	*0.042*	*<0.001*	0.357		b[a], a[a]
	Methanol	*L. sativa*	0.261	0.410	0.868	*0.021*	0.775	0.133		a[a]
		A. longifolia	0.493	0.133	0.200	0.248	0.192	0.315		
		B. integrifolia	0.552	0.881	0.976	0.936	0.511	0.998		
		A. megalocarpa	*0.038*	0.422	0.488	0.694	0.706	0.451	b	
		L. longifolia	0.515	0.057	0.222	0.619	0.266	0.096		
		I. nodosa	0.961	0.529	0.963	*0.006*	*0.001*	*0.038*		b[a]
	Water	*L. sativa*	0.465	0.872	0.820	0.140	0.997	0.696		
		A. longifolia	0.151	*0.012*	0.320	0.456	0.259	0.890	b[a], a[a]	
		B. integrifolia	*0.029*	0.741	0.320	0.856	*0.032*	0.620	b[a]	b, a
		A. megalocarpa	0.639	0.687	0.770	0.858	0.712	0.168		
		L. longifolia	0.257	0.448	0.706	0.334	0.475	0.449		
		I. nodosa	0.529	0.122	0.958	*0.003*	0.439	0.532		
Soil	DCM	*L. sativa*	0.986	0.829	0.238	0.493	0.554	0.136		

Table 4 (continued)

Extract	Bioassay species	Effects on shoot length			Effects on root length			Influential extract species (LC_{50})[a]	
		E	C	E×C	E	C	E×C	Shoot	Root
	A. longifolia	0.916	0.659	0.994	0.775	0.597	0.816		
	B. integrifolia	*0.029*	*0.047*	*0.044*	0.059	*0.028*	0.450	b[a]	b[a], a[a]
	A. megalocarpa	0.208	*0.035*	0.505	0.489	0.712	0.204	b[a]	
	L. longifolia	0.173	*0.040*	0.101	0.461	0.810	0.038	b, a	b[a]
	I. nodosa	0.368	0.088	0.979	0.647	*<0.001*	0.789		b[a], a[a]
Acetone	*L. sativa*	0.113	0.786	0.747	0.222	0.937	0.925		
	A. longifolia	*0.009*	0.760	0.851	0.198	0.600	0.560	b[a]	
	B. integrifolia	0.617	*<0.001*	0.066	*0.013*	*0.023*	0.084	b, a	b[a]
	A. megalocarpa	0.808	0.130	0.300	0.344	0.678	0.905		
	L. longifolia	0.311	0.191	0.159	0.226	0.090	0.073		
	I. nodosa	0.336	0.702	0.795	0.065	0.734	0.599		
Methanol	*L. sativa*	0.708	0.407	0.871	0.448	0.387	0.987		
	A. longifolia	0.528	0.530	0.329	0.661	0.783	0.951		
	B. integrifolia	0.909	0.209	0.503	0.954	*0.036*	0.217		
	A. megalocarpa	0.596	0.487	0.506	0.074	0.623	0.430		
	L. longifolia	0.054	*0.016*	0.691	0.896	*0.004*	0.824	a	
	I. nodosa	0.774	0.729	0.521	0.060	0.939	0.816		
Water	*L. sativa*	0.655	0.221	0.614	0.406	0.852	0.934		
	A. longifolia	0.123	0.757	0.396	0.475	0.769	0.434		
	B. integrifolia	0.592	0.407	0.481	0.443	*0.008*	0.301		
	A. megalocarpa	0.875	*0.043*	0.410	0.060	0.210	0.308	b	
	L. longifolia	0.333	0.486	0.251	0.180	0.640	0.534		
	I. nodosa	0.130	*0.001*	0.750	0.186	0.942	0.752		

Values in italics are significantly different at α=0.05

a acacia, *b* bitou bush

[a] Occurrence of LC_{50} in dose response curves

1999). Therefore our findings suggest that the interference between co-evolved acacia and indigenous plants is likely to arise from root exudates rather than leaf derived compounds. Gas chromatography–mass spectrometry (GC-MS) studies have shown that the DCM extracts of acacia roots and soil have similar chemical profiles containing largely a high molecular weight alkane series (C19–33), phenolic compounds, plant sterols and a low concentration of terpenes (Ens et al. 2008). High concentrations of alkanes in the soil from both acacia roots and those derived from leaf waxes, are likely to induce water repellency, especially in the sandy soils (Franco et al. 2000; Roper 2005) where this acacia grows. Water repellency is likely to affect seedling growth via reduced soil water availability (Franco et al. 2000; Roper 2005). Phenolic compounds are recognized plant (Gross 1975; Williams and Hoagland 1982) and microbial (Hattenschwiler and Vitousek 2000; Souto et al. 2000) growth regulators and are likely to be primarily responsible for the inhibition of *I. nodosa*, *B. integrifolia* and *L. longifolia* by acacia roots and soil in this study, and potentially in the field. The presence of phenolic compounds in situ may have further ecological ramifications in relation to their potential effects on nutrient cycling and decomposition via direct effects on the microbial community (Hattenschwiler and Vitousek 2000). Therefore, further growth trials and microbial studies in the field are required to confirm the ecological relevance of the present laboratory based findings.

The DCM and acetone extracts of bitou bush roots and soil had significant inhibitory effects on *A. megalocarpa*, *B. integrifolia*, *L. longifolia* and *I. nodosa* establishment. Again, we detected no substantial changes in the pH of increasing concentrations of these bioactive root and soil extracts, suggesting that pH was not responsible for the observed seedling growth inhibition. GC-MS analyses revealed that bitou bush roots and soil both contained high concentrations of terpenes, particularly sesquiterpenes (Ens et al. 2008).

Table 5 Summary of inhibition by extract phytotoxicity, allelopathy or indirect soil effects (+ denotes stimulatory effect) on the test species

			Type of effect		
Extract species	Plant part	Solvent extract	Phytotoxic	Allelopathic	Indirect soil effect
Acacia	Shoots	DCM	L, A	I	
		Acetone	L, I		
		Methanol	L		
		Water	L		
	Roots	DCM	A, Ac, Le	I	
		Acetone	B, I		
		Methanol	Ac, Le		
		Water	A		
	Soil	DCM			B, L
		Acetone			L
		Methanol			
		Water			+I
Bitou bush	Shoots	DCM	A, L	I	
		Acetone	I		
		Methanol	Ac, L, I		
		Water	A, L,		
	Roots	DCM	Le, A, L	Ac, I	
		Acetone	I	B	
		Methanol	A, Ac, I		
		Water	A, B	Ac, L	
	Soil	DCM			B
		Acetone			A
		Methanol			
		Water			+I

A A. longifolia var. *sophorae*, *Ac A. megalocarpa*, *B B. integrifolia*, *I I. nodosa*, *L L. longifolia*, *Le L. sativa*

Sesquiterpenes are also exuded by *Pinus* spp. roots (Lin et al. 2007) and have documented allelopathic (Fischer 1986; Cumanda and Marinoni 1991), antimicrobial (Melin and Krupa 1971; Scher et al. 2004) and herbivore deterrent (Theis and Lerdau 2003) effects. Further fractionation of the DCM and acetone extracts is required to isolate putative allelochemicals as the sesquiterpenes identified in our studies cannot be purchased from commercial sources. Following isolation of these compounds, modes of action could be ascertained.

The inhibitory and stimulatory effect of some of the soil derived extracts of this study were not paralleled by similar effects from the comparable (derived from the same solvent) plant part extracts. The activity of the soil extracts alone, may be due to either the accumulation of plant derived compounds in the soil, the indirect modification (biotic or abiotic) of plant derived compounds or by plant alteration of the microbial community which subsequently lead to changes in the soil chemistry. The identification of indirect soil chemical effects is one of the advantages of comparing both soil and plant based extracts on a range of test species. The indirect soil effects detected in the present bioassays are also likely to prevent the re-establishment of indigenous plants after bitou bush removal. A regeneration lag time (of approximately 6 months) following bitou bush control has been observed (Andresen, personal communication) and is suggested prior to replanting with native stock. Alternatively, fire could be used to speed up the volatilisation of the putative non-polar allelochemicals found in this study.

Based on this comprehensive bioassay approach, we suggest that chemical interference between co-evolved species may occur and is also likely to be a mechanism facilitating the bitou bush invasion of the eastern Australian coast. Bitou bush root and soil extracts were more inhibitory to a broader range of species, including the indigenous dominant acacia. This suggests that the plant growth inhibition caused

by bitou bush root components is likely to be one of the mechanisms facilitating the bitou bush invasion of the eastern Australian coast. Further research into the details of bitou bush allelopathy is planned.

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