Responses to competition for nitrogen between subtropical native tree seedlings and exotic grasses are species-specific and mediated by soil N availability

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Competitive interactions between native tree seedlings and exotic grasses frequently hinder forest restoration. We investigated the consequences of competition with exotic grasses on the growth and net nitrogen (N) uptake capacity of native rainforest seedlings used for reforestation depending on soil N availability and N source. Tree seedlings and grasses were grown in the greenhouse in different competition regimes (one tree species vs one grass species) and controls (grass monocultures or single tree seedlings) at low and high soil N. After 8 weeks, we quantified net N uptake capacity using ¹⁵N-labelled organic (i.e., glutamine and arginine) and inorganic (i.e., ammonium and nitrate) N sources and biomass indices. Depending on soil N availability, we observed different species-specific responses to growth and N acquisition. Tree seedlings generally increased their net N uptake capacity in response to competition with grasses, although overall seedling growth was unaffected. In contrast, the responses to competition by the grasses were species-specific and varied with soil N availability. The different N acquisition strategies suggest the avoidance of competition for N between trees and grasses. Overall, the results highlight that quantifying underlying mechanisms of N acquisition complements the information on biomass allocation as a measure of responses to competition, particularly with varying environmental conditions.

Keywords: ammonium, interspecific competition, net nitrogen uptake capacity, nitrate, nitrogen acquisition, organic nitrogen, reforestation, subtropics.

Introduction

Plant–plant interactions shape the structure of plant communities (Callaway and Walker 1997, Brooker 2006, Tylianakis et al. 2008). Their magnitude and direction are influenced by factors including life stage, physiological requirements as well as environmental conditions (Callaway and Walker 1997). The interplay between these biotic and abiotic factors determines the outcome of species interactions which in turn affects the coexistence and spatial distribution of species (Brooker et al. 2008, Soliveres and Maestre 2014). A main interaction is the competition for resources such as soil nutrients. The ability of plants to acquire nutrients is determined by morphological and physiological

traits, such as uptake rates of nutrients (Casper and Jackson 1997). By modifying nutrient uptake in response to environmental cues, such as nutrient availability, plants can be more competitive but the extent of such response varies (Hodge 2004).

Competitive interactions between native and exotic plant species are relevant in the context of global environmental change, because there is evidence that the predicted changes in the abiotic environment will likely influence competition in favour of exotic species (Bradley et al. 2010, Verlinden et al. 2014). The superior competitive ability of exotic plant species is frequently a key factor promoting invasions, with exotic species able to better exploit limited resources, to the detriment of native species

(D'Antonio and Vitousek 1992, Gioria and Osborne 2014). Changing nutrient status, such as increased nitrogen (N) availability with atmospheric deposition, can alter plant growth and biomass allocation in a species-specific manner, and consequently results in shifts in the outcome of interspecific competition (Rennenberg et al. 2009). Here, we investigate the competition between exotic and native plants comparing the response strategies of different species, and how resource availability modulates these interactions. A focus on N physiology is warranted because N is quantitatively the most important of soil-acquired nutrients (Marschner 1995), limits plant growth in most ecosystems (Agren et al. 2012), drives competition (Eller and Oliveira 2017), and N availability and uptake can vary considerably in response to environmental change (BassiriRad 2000, Kuster et al. 2016). Since most studies focus on biomass production as indicator of the magnitude and direction of competitive interactions, even though other factors besides competition may influence biomass, (Trinder et al. 2013), we also examined net N uptake as a process that underlies the interaction between native and exotic species, because exotic species might alter soil N availability, show strong preferences to certain N forms and/or interfere with the N acquisition of native species, therefore affecting their performance (Fraterrigo et al. 2011, Huangfu et al. 2016, Eller and Oliveira 2017).

While earlier studies have focused mainly on the acquisition of inorganic N, organic N uptake has received increasing attention due to its potential to drive niche differentiation and species coexistence, and therefore ecosystem stability (Schmidt and Stewart 1999, McKane et al. 2002, Kielland et al. 2006, Näsholm et al. 2009, Ashton et al. 2010). A plant's ability to use a wider array of N sources can result in competition avoidance (e.g., Miller et al. 2007, Simon et al. 2010, Simon et al. 2014, Li et al. 2015). Studies that have quantified the uptake of organic and inorganic N forms found species-specific preferences for different N forms (e.g., Persson et al. 2003, Weigelt et al. 2005, Simon et al. 2010, Li et al. 2015). For example, European beech (*Fagus sylvatica*) shows a preference for organic rather than inorganic N, whereas the opposite is true for sycamore maple (*Acer pseudoplatanus*) (Li et al. 2015). In a study on excised roots of native and exotic grasses of Australian savannas, a general preference for ammonium was found, but preferences for nitrate and the amino acid glycine were species-specific (Rossiter-Rachor et al. 2009). Furthermore, the uptake capacity of different N sources is influenced by interspecific competition (e.g., Miller et al. 2007, Simon et al. 2010, Simon et al. 2014, Li et al. 2015). For example, when grown in competition with sycamore maple, the capacity for organic N uptake of European beech seedlings was reduced (Simon et al. 2014). However, the responses of competition with regard to N acquisition are also influenced by environmental factors (Simon et al. 2014, Li et al. 2015). Overall, most studies that have examined the acquisition of different N forms in context of competition have

focused either on herbaceous or woody species in temperate ecosystems, rather than the interactions between trees and grasses. These, however, are important in the context of conservation and ecosystem restoration, because the successful establishment of tree seedlings (commonly planted in order to restore a plant community) often depends on the outcome of their interaction with grasses (Erskine et al. 2005, Doust et al. 2008, Elgar et al. 2014), where N is likely to play a determinant role (Coll et al. 2004, Barbosa et al. 2014).

Tree–grass interactions have been broadly studied in savanna ecosystems measuring the influence of abiotic factors (e.g., light, water and nutrient availability) that mediate the interaction (e.g., Ludwig et al. 2001, van der Waal et al. 2009, Moustakas et al. 2013) and in studies investigating the long-term coexistence of these contrasting life forms (e.g., Jeltsch et al. 2000, Sankaran et al. 2004). With regard to forest regeneration, tree–grass interactions have been studied in the context of secondary succession, tree regeneration in forest gaps after disturbances, or meadows (e.g., Coll et al. 2004, Bloor et al. 2008a, Elgar et al. 2014), where tree seedlings interact with the already established plant community commonly composed of early successional life forms, including grasses. However, research on the effect of herbaceous vegetation on tree seedlings mainly focusses on seedling survival and growth (e.g., Davis et al. 1998, Bloor et al. 2008b, Meli and Dirzo 2013), physiological aspects such as water relations (e.g., Quinteros et al. 2017) or photosynthesis (e.g., Davis et al. 1999, Loik and Holl 2001), while competition for nutrients, specifically N, has received little attention despite the potential of belowground competition with grasses to influence tree growth and establishment (e.g., Cramer et al. 2010) and the influence of soil N availability on the competition outcome (e.g., Cramer et al. 2012).

Here, we studied the interactions between native tree seedlings and exotic grasses because ecological forest restoration efforts are frequently hindered by competition from exotic grasses (Kanowski et al. 2003, Erskine et al. 2005). In reforestation programs, grasses successfully establish even after the planting of seedlings without regular weeding. To mimic these conditions, we used nursery-grown tree seedlings and grasses grown from seed in our study. We selected tree species that are relevant in the context of restoration of riparian zones in subtropical Australia (Catterall and Harrison 2006, Gageler et al. 2014), including an N_2 fixer. Tree species with the capacity to fix atmospheric N_2 via symbiotic rhizobacteria in root nodules are commonly used in restoration programs with the intent to improve the overall N supply at a site (Erskine et al. 2005). Their N_2 fixing ability would make them largely independent from external soil N supply (Messina and Barton 1985) and potentially alleviate competition for N (Epron et al. 2006). We conducted a greenhouse experiment to advance the understanding of whether competition for N plays a role in the interaction between seedlings of two native tree species and two exotic grass species, and the potential effects of soil N availability.

Our objectives were to (i) determine the effect of interspecific competition on inorganic and organic N acquisition and biomass indices as response strategies of tree seedlings and grasses, and (ii) evaluate the impact of low vs high soil N availability on the competition outcome in terms of N acquisition. We hypothesized that tree seedlings and grasses potentially avoid competition by using different N forms (i.e., fast-growing grasses using inorganic forms and relatively slow-growing trees using organic forms), that growth and net N uptake capacity of tree seedlings is negatively affected in the presence of competing exotic grasses, and that this negative effect is lessened at high soil N availability.

Materials and methods

Study species and plant material

We selected two native tree species used in riparian reforestation in the Maleny region (26.7° S, 152.8° E, Queensland, Australia), angiosperm *Acacia melanoxylon* R.Br (Fabaceae) and gymnosperm *Podocarpus elatus* R.Br. ex Endl. (Podocarpaceae). *Acacia melanoxylon* is an early successional, N₂ fixing species native to south-east Australia (Smith et al. 2008, Birnbaum et al. 2012) that occurs in rainforests and wet sclerophyll forests (Doran and Turnbull 1997). *Podocarpus elatus* is a late-successional endemic species that grows in subtropical rainforests (Smith et al. 2008). Seedlings of *A. melanoxylon* (5 months old, ~40 cm tall) and *P. elatus* (10 months old, ~30 cm tall) were obtained from a local nursery (Brush Turkey Enterprises, Maleny, Australia). We selected tree species with contrasting successional status to explore potential differences in their N acquisition strategies related to growth rate (Li et al. 2015), and species adaptation to N forms available in soils of different successional status (e.g., higher nitrate availability in early successional sites compared to late sites) (Kronzucker et al. 1997, Britto and Kronzucker 2013). The exotic grasses *Pennisetum clandestinum* Hochst. ex Chiov. (Poaceae) and *Cynodon dactylon* (L.) Pers. (Poaceae) are perennial, originate from Africa and have been introduced to tropical areas around the world (Judd 1979). Spreading via rhizomes, stolons and seeds, these grasses naturally occur in grasslands and are common in pastures and lawns in their introduced range (Judd 1979). In subtropical Australia, these two grass species are reported as a threat to restoration efforts, and their negative effects on the growth of native tree seedlings have been observed in the field (M. Amos, personal communication). Grass seeds were purchased from Royston Petrie Seeds (Mudgee, Australia). From here on, species will be referred to by their genus, i.e., *Acacia*, *Podocarpus*, *Pennisetum* and *Cynodon*.

Experimental setup

Tree seedlings were planted and grasses were sown from seed into 1 l pots (125 mm diameter, Anova Solutions, Brisbane, Australia) with a 1:1 mixture of grade A pit sand (grain size:

0.5 mm) and G2 vermiculite (grain size: 1–2 mm) in interspecific competition or under control conditions at low and high soil N availability. Competition treatments consisted of one tree individual surrounded by 10–12 individual grasses that were uniformly distributed ~4 cm from the seedling stem. The four species combinations were *Acacia* vs *Pennisetum*, *Acacia* vs *Cynodon*, *Podocarpus* vs *Pennisetum* and *Podocarpus* vs *Cynodon*. Control treatments consisted either of a single tree seedling or 12 individuals of one grass species (i.e., monoculture). Tree seedlings were not large enough to result in shading for the grasses (A. Bueno, pers. obs.). Because competition for N is highest under conditions of N limitation (Wilson and Tilman 1991, Casper and Jackson 1997), all competition regimes received the low soil N treatment. To ensure sufficient replication, the high soil N treatment was only applied for tree species vs *Pennisetum* because *Cynodon* had poor germination rates. Therefore, it was excluded from the high soil N treatment to ensure sufficient replicates in the low soil N treatment. For each combination of competition regime and soil N availability treatment, ten replicates were established. To control for the variation in initial size of the seedlings, we recorded the initial above-ground size of all tree seedlings (i.e., stem length measured from root crown, number of leaves and length of largest leaf) before planting. Pots were watered with tap water every second day to field capacity until the grasses germinated which occurred within 2 weeks of planting. Soil N availability treatments therefore commenced 2 weeks after planting. Subsequently, once a week 100 ml of nutrient solution was applied, with low or high N concentrations, for low N: 1 µM NH₄Cl, 100 µM KNO₃, 25 µM glutamine and 25 µM arginine; high N: 40 µM NH₄Cl, 400 µM KNO₃, 100 µM glutamine and 100 µM arginine, based on soil N availability in previous low vs high soil N studies (Simon et al. 2013) based on a low and high N forest site (Dannenmann et al. 2009, Stoelken et al. 2010, respectively), and on the occurrence of glutamine and arginine in subtropical Queensland soils (Holst et al. 2012). Other nutrients applied at the same rate in both N treatments were: 10 µM AlCl₃, 90 µM CaCl₂, 7 µM FeSO₄, 6 µM K₂HPO₄, 50 µM KCl, 24 µM MnCl₂, 20 µM NaCl and 70 µM MgCl₂. The experiment was conducted in a naturally lit greenhouse at The University of Queensland, St. Lucia Campus (Brisbane, Australia), between April and June 2015. Average temperature recorded in the greenhouse was 24.7 ± 3.7 °C during the day and 20.3 ± 1.8 °C at night, reaching a minimum of 13.5 °C and a maximum of 35.3 °C.

¹⁵N uptake experiments and harvest

Eight weeks after commencing the N treatments (and 10 weeks after planting), the net inorganic (i.e., ammonium, nitrate) and organic (i.e., glutamine, arginine) N uptake capacity of fine roots of tree seedlings and grasses was quantified following the ¹⁵N enrichment technique described by Gessler et al. (1998) as modified by Simon et al. (2010) using fine roots still attached to

the individuals. For this, we carefully removed the plants from the pots and washed the roots to remove adherent soil particles. Fine roots were then incubated for a 2 h period in 4 ml artificial nutrient solution containing all four N sources (as described above), but with one of the sources labelled as either ^{15}N -ammonium, ^{15}N -nitrate, $^{13}\text{C}/^{15}\text{N}$ -glutamine or $^{13}\text{C}/^{15}\text{N}$ -arginine. Amino acids were $^{13}\text{C}/^{15}\text{N}$ double-labelled to determine whether they were taken up as intact molecules or degraded over the time of incubation (Simon et al. 2011). Additionally, we used artificial soil solution without ^{15}N -label to account for the natural abundance of ^{15}N in the roots of each species for all treatment levels. Plants were incubated in either low or high N artificial soil solution matching the soil N availability treatment to which they had been subjected. Root incubation occurred between 10 a.m. and 2 p.m. to avoid diurnal variation in N uptake (Gessler et al. 2002), i.e., the incubation period of 2 h for any given individual sample started between 10 a.m. and 12 p.m. and ended between 12 and 2 p.m. For the duration of the ^{15}N incubation experiments, the remaining seedling's roots were carefully wrapped in wet tissue to prevent drying out. A total of six replicate root samples were set up for each of the four N sources (plus controls for natural abundance) per species, competition regime and soil N availability level. Depending on the size of the root system for an individual plant, one to four N sources plus control were tested per individual. After the incubation period, fine roots were carefully cut, washed twice in 0.5 M CaCl_2 solution to remove the incubation solution from the root surface, and dried with tissue. Following the ^{15}N uptake experiments, we separated the plants into above- and below-ground organs, i.e., the tree seedlings into leaves, stem and roots, and the grasses into shoots and roots, since no stem had developed. We determined the fresh weight (fw) of all plant tissues and the incubated fine roots, oven dried the samples at 65 °C for 72 h and determined their dry weight (dw) to obtain biomass indices.

Quantification of total N and C, ^{15}N and ^{13}C in fine roots

To quantify ^{15}N and ^{13}C enrichment the dried fine roots were ground using a ball mill (TissueLyser, Retsch, Haan, Germany). Aliquots of 1.2–2.5 mg of the resulting homogeneous fine powder were weighed into 4 × 6 mm tin capsules (IVA Analysentechnik, Meerbusch, Germany), and analysed with an isotope ratio mass spectrometer (Delta V Advantage, Thermo Electron, Dreieich, Germany) coupled to an elemental analyser (Euro EA, Eurovector, Milan, Italy). Δ values were calculated using a laboratory standard (acetanilide) that was part of every sequence in intervals, and also used in different weights to determine isotope linearity of the system. The laboratory standard was calibrated against different international standards from IAEA (Vienna): for ^{15}N USGS 40 ($\delta^{15}\text{N}_{\text{Air}} = -4.5\text{‰}$), IAEA 600 ($\delta^{15}\text{N}_{\text{Air}} = +1.0\text{‰}$), IAEA N2 ($\delta^{15}\text{N}_{\text{Air}} = +20.3\text{‰}$), USGS 41 ($\delta^{15}\text{N}_{\text{Air}} = +47.6\text{‰}$), USGS 26 ($\delta^{15}\text{N}_{\text{Air}} = +53.7\text{‰}$), and USGS 32 ($\delta^{15}\text{N}_{\text{Air}} = +180\text{‰}$), and for

^{13}C IAEA 600 ($\delta^{13}\text{C}_{\text{V-PDB}} = -27.77\text{‰}$), IAEA 3 ($\delta^{13}\text{C}_{\text{V-PDB}} = -24.72\text{‰}$), and IAEA CH6 ($\delta^{13}\text{C}_{\text{V-PDB}} = -10.45\text{‰}$). ^{15}N results of highly enriched samples were finally corrected with different enriched ^{15}N standards (from 0.437 to 0.734 at %) from Fischer Analysen Instrumente (Leipzig, Germany). Δ values are defined as $\delta [\text{‰}] = (R_{\text{SA}}/R_{\text{Std}} - 1) * 1000$, where R is the ratio of heavy isotope against light isotope (i.e., $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$), SA is sample and Std the international reference for the respective element (V-PDB for ^{13}C and air- N_2 for ^{15}N). For each of the four N sources, we then calculated net N uptake capacity ($\text{nmol N g}^{-1} \text{ fw h}^{-1}$) based on the incorporation of ^{15}N into root fresh weight according to Gessler et al. (1998): net N uptake capacity = $((^{15}\text{N}_i - ^{15}\text{N}_c) * N_{\text{tot}} * \text{dw} * 10^5) / (\text{MW} * \text{fw} * t)^{-1}$, where $^{15}\text{N}_i$ and $^{15}\text{N}_c$ are the atom% of ^{15}N in labelled (N_i) and control plants (N_c , natural abundance), respectively, N_{tot} is the total N percentage, MW is the molecular weight ($^{15}\text{N g mol}^{-1}$), and t represents the incubation time (120 min). Based on ^{13}C incorporation, net uptake capacity of glutamine and arginine was ~60% and ~95%, respectively, compared to that based on ^{15}N incorporation, which indicates (1) the degradation of amino acids in the solution or on the root surface, and/or (2) the respiration of amino acid-derived C inside the roots (Simon et al. 2011).

Statistical analysis

For all species, we tested for differences between treatments for total biomass and root:shoot ratio as well as net uptake capacity of the four N sources by performing Permutational ANOVAs (PERMANOVA) based on a Euclidean resemblance matrix between samples (Anderson et al. 2008). PERMANOVA was chosen as a non-parametric univariate test equivalent to ANOVA (Anderson et al. 2008). We performed two-way PERMANOVAs using 'competition regime' and 'soil N availability' as orthogonal factors. For the tree species, the 'competition regime' factor had three levels: control, competition with *Pennisetum*, and competition with *Cynodon*. Moreover, for the tree seedlings biomass and root:shoot ratio analyses, initial size measurements of the tree seedlings were included as a covariate. For the grass species, the three levels were: control, competition with *Acacia*, and competition with *Podocarpus*. The 'soil N availability' factor included two levels: high N and low N for *Acacia*, *Podocarpus* and *Pennisetum*. For the grass *Cynodon*, we performed one-way PERMANOVAs, considering the factor 'competition regime' with three levels: control, competition with *Acacia*, and competition with *Podocarpus*, all at low soil N availability as described above. When a significant interaction between factors was found, post hoc PERMANOVA pairwise comparisons were performed. To test for differences in net N uptake capacity between species, one-way PERMANOVAs were performed on the control data (i.e., no competition), using species as factor, for each N form. To test for preferences in net uptake capacity of the four N sources for a given species, one-way PERMANOVAs were performed, using N source as factor, for each combination of competition regime and soil N availability treatment. All analyses

were performed using PRIMER 6.0 with the PERMANOVA+ add-on (PRIMER-E Ltd, Plymouth, UK), and figures were prepared in SigmaPlot 13.0 (Systat Software Inc., San Jose, USA).

Results

Effects of competition regime and soil N availability on biomass and root:shoot ratio

Native tree seedlings and exotic grasses responded differently in biomass production depending on competition regime and soil N availability (Figure 1). For the tree species, neither *Acacia* nor *Podocarpus* differed significantly in total biomass between single and competing seedlings regardless of soil N availability (Figure 1; see Supplementary Table S1 available as Supplementary Data at *Tree Physiology* Online). Positive correlations were found for *Acacia* between total biomass and the initial leaf number, for *Podocarpus* between total biomass and both initial leaf number and initial stem length (both $p\text{-perm} < 0.001$, data not shown). This indicates that total biomass at the end of the experiment was a function of initial tree seedling size. For the grass *Pennisetum*, total biomass was significantly

reduced in competition with *Acacia* or *Podocarpus* compared to the control at high soil N availability, and it was also significantly reduced at low compared to high soil N availability irrespective of the competition regime (Figure 1; see Table S2 available as Supplementary Data at *Tree Physiology* Online). For the grass *Cynodon*, competition with *Acacia* significantly reduced total biomass compared to competition with *Podocarpus* or control (at low soil N availability) (Figure 1; see Table S2 available as Supplementary Data at *Tree Physiology* Online).

Root:shoot ratio was not affected by soil N availability or competition regime for both tree species as well as competition regimes for the grass *Cynodon* at low soil N availability (Figure 1). For the grass *Pennisetum*, root:shoot ratio was significantly lower in competition with *Podocarpus* compared to competition with *Acacia* or controls irrespective of soil N availability (Figure 1).

Effects of competition regime and soil N availability on inorganic and organic net N uptake capacity

Inorganic and organic net N uptake capacity of all species increased significantly with high compared to low soil N

Figure 1. Total biomass and root:shoot ratio of *Acacia melanoxylon*, *Podocarpus elatus*, *Pennisetum clandestinum* and *Cynodon dactylon*, at low and high soil N availability under different competition regimes. For *C. dactylon*, only low soil N data is available. comp *Pennisetum* = competition with *P. clandestinum*; comp *Cynodon* = competition with *C. dactylon*; comp *Acacia* = competition with *A. melanoxylon*; comp *Podocarpus* = competition with *P. elatus*. Box plots show mean (dotted line) and median (straight line). Different letters indicate significant differences between competition regimes within a specific soil N availability treatment, and asterisks indicate significant differences between soil N availability treatments, detected using permutational analysis of variance ($P < 0.05$).

availability, whereas the responses to competition regime differed depending on the species (Figures 2 and 3; see Table S3 available as Supplementary Data at *Tree Physiology* Online). Inorganic and organic net N uptake capacity of *Acacia* seedlings increased with higher soil N availability regardless of competition regime. At high soil N availability, net ammonium uptake capacity of *Acacia* seedlings competing with the grass *Pennisetum* increased significantly compared to seedlings growing without competition (Figure 2; see Supplementary Table S4 available as Supplementary Data at *Tree Physiology* Online), but no change for nitrate (Figure 2), glutamine-N or arginine-N (Figure 3) was found at either low or high soil N availability. Similarly, inorganic N (Figure 2) and arginine-N (Figure 3) net uptake capacity of *Podocarpus* increased with high compared to low soil N availability irrespective of competition regime. However, net glutamine-N uptake capacity increased with higher soil N availability only for *Podocarpus* seedlings grown in competition with *Pennisetum* (Figure 3; see Table S4 available as Supplementary Data at *Tree Physiology* Online). At high soil N availability, competition with *Pennisetum* increased net glutamine-N uptake capacity of

Podocarpus seedlings compared to seedlings growing alone (Figure 3); however, net ammonium, nitrate (Figure 2), and arginine-N (Figure 3) uptake capacity were not affected. For the grass *Pennisetum*, net ammonium and organic N, but not nitrate uptake capacity were significantly higher at high compared to low soil N availability (Figures 2 and 3). For *Pennisetum*, competition regime had no significant effect on organic (Figure 3) and inorganic (Figure 2) net N uptake capacity at either low or high soil N availability. At low soil N availability, nitrate, but not ammonium or organic net N uptake capacity of the grass *Cynodon* was significantly higher when grown in competition with *Podocarpus* compared to that with *Acacia*, or control (Figures 2 and 3; see Table S4 available as Supplementary Data at *Tree Physiology* Online).

Differences between species with regard to inorganic and organic N acquisition

In the absence of interspecific competition (i.e., single grown tree seedlings or grasses growing in monocultures), species differed in their inorganic and organic N acquisition strategies

Figure 2. Ammonium (NH_4^+) and nitrate (NO_3^-) net uptake capacity ($\text{nmol N g}^{-1} \text{fw h}^{-1}$) by fine roots of *Acacia melanoxylon*, *Podocarpus elatus*, *Pennisetum clandestinum* and *Cynodon dactylon* at low and high soil N availability under different competition regimes. For *C. dactylon*, only low soil N data is available. comp *Pennisetum* = competition with *P. clandestinum*; comp *Cynodon* = competition with *C. dactylon*; comp *Acacia* = competition with *A. melanoxylon*; comp *Podocarpus* = competition with *P. elatus*. Box plots show mean (dotted line) and median (straight line). Different letters indicate significant differences between competition regimes within a specific soil N availability treatment, and asterisks indicate significant differences between soil N availability treatments detected using permutational analysis of variance ($P < 0.05$).

Figure 3. Glutamine-N (Gln-N) and arginine-N (Arg-N) net uptake capacity ($\text{nmol N g}^{-1} \text{fw h}^{-1}$) by fine roots of *Acacia melanoxylon*, *Podocarpus elatus*, *Pennisetum clandestinum* and *Cynodon dactylon* at low and high soil N availability under different competition regimes. For *C. dactylon*, only low soil N data is available. comp Pennisetum = competition with *P. clandestinum*; comp Cynodon = competition with *C. dactylon*; comp Acacia = competition with *A. melanoxylon*; comp Podocarpus = competition with *P. elatus*. Box plots show mean (dotted line) and median (straight line). Different letters indicate significant differences between competition regimes within a specific soil N availability treatment, and asterisks indicate significant differences between soil N availability treatments detected using permutational analysis of variance ($P < 0.05$).

depending on the N source: seedlings of *Podocarpus* took up significantly less nitrate than seedlings of *Acacia* and the grass *Pennisetum*, irrespective of soil N availability (Table 1). However, glutamine-N acquisition of *Pennisetum* was significantly lower than that of *Podocarpus*, which in turn was lower than that of *Acacia* seedlings at both low and high soil N availability (Table 1). No differences were found between any of the species with regard to ammonium and arginine-N acquisition (Table 1).

General preference patterns of the different N forms were found for each species within each competition regime depending on soil N availability: At low soil N availability, both tree species preferred nitrate and glutamine-N over ammonium and arginine-N (see Table S5 available as Supplementary Data at *Tree Physiology* Online). However, at high soil N availability, this pattern was reversed with ammonium and arginine-N being favoured over nitrate and glutamine-N for both tree species (Table 2; see Table S5 available as Supplementary Data at *Tree Physiology* Online). For the grasses, inorganic N sources,

particularly nitrate, were preferred over organic N sources (regardless of soil N availability for *Pennisetum*) (Table 2; see Table S6 available as Supplementary Data at *Tree Physiology* Online).

Discussion

Our study provides novel insight into the species-specific growth and N acquisition patterns in native tree seedlings and exotic grasses competing for N. Depending on soil N availability, native tree seedlings responded to competition with increases in N acquisition only, whereas the responses of the grasses in terms of biomass and N acquisition varied with species and soil N availability. These results highlight that quantifying underlying mechanisms complements biomass indices to advance the understanding of interspecific competition (Trinder et al. 2013). Furthermore, the lack of response with regard to biomass in the tree seedlings compared to the grasses suggests that N storage capacity plays a role in the growth response as well.

Table 1. PERMANOVA results of the differences in ammonium (NH_4^+), nitrate (NO_3^-), glutamine-N (Gln-N) and arginine-N (Arg-N) net uptake capacity of fine roots between *Acacia melanoxylon*, *Podocarpus elatus*, *Pennisetum clandestinum* and *Cynodon dactylon*. Significant values are indicated in bold.

Main test	NH_4^+		NO_3^-		Gln-N		Arg-N	
	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)	Pseudo-F	P(perm)
Species	1.630	0.170	4.248	0.013	7.469	<0.001	0.573	0.648
Soil N availability	16.749	<0.001	0.674	0.419	8.259	0.006	65.941	<0.001
Species $\times$ Soil N availability	2.184	0.098	0.011	0.991	2.545	0.092	1.307	0.295
Pairwise comparisons	t	P(perm)	t	P(perm)	t	P(perm)	t	P(perm)
<i>Acacia</i> vs <i>Podocarpus</i>	Not applicable		3.843	<0.001	2.493	0.022	Not applicable	
<i>Acacia</i> vs <i>Pennisetum</i>			1.180	0.251	4.766	<0.001		
<i>Acacia</i> vs <i>Cynodon</i>			0.803	0.441	1.771	0.096		
<i>Podocarpus</i> vs <i>Pennisetum</i>			3.0987	0.005	2.207	0.041		
<i>Podocarpus</i> vs <i>Cynodon</i>			1.760	0.066	1.401	0.179		
<i>Pennisetum</i> vs <i>Cynodon</i>			1.125	0.271	1.509	0.157		

Table 2. Differences between ammonium (NH_4^+), nitrate (NO_3^-), glutamine-N (Gln-N) and arginine-N (Arg-N) net uptake capacity of fine roots of *Acacia melanoxylon*, *Podocarpus elatus*, *Pennisetum clandestinum* and *Cynodon dactylon*, at low and high soil N availability. Only significant differences are presented.

	<i>Acacia</i>	<i>Podocarpus</i>
1) Low soil N availability		
Control	NO_3^- , Gln-N > NH_4^+ > Arg-N	Gln-N > NH_4^+ , NO_3^- , Arg-N NO_3^- > Arg-N
Competition with <i>Pennisetum</i>	NO_3^- , Gln-N > NH_4^+ , Arg-N	NO_3^- , Gln-N > NH_4^+ , Arg-N
Competition with <i>Cynodon</i>	Gln-N > NH_4^+ , Arg-N	Gln-N > NO_3^- , Arg-N > NH_4^+
2) High soil N availability		
Control	No differences	NH_4^+ > NO_3^- , Gln-N, Arg-N
Competition with <i>Pennisetum</i>	NH_4^+ > NO_3^- , Gln-N, Arg-N Arg-N > Gln-N	NH_4^+ > NO_3^- , Gln-N, Arg-N Gln-N > NO_3^-
	<i>Pennisetum</i>	<i>Cynodon</i> ¹
1) Low soil N availability		
Control	NO_3^- > NH_4^+ , Gln-N, Arg-N	NO_3^- , Gln-N > NH_4^+
Competition with <i>Acacia</i>	NO_3^- > NH_4^+ , Gln-N, Arg-N Gln-N > Arg-N	NO_3^- > NH_4^+
Competition with <i>Podocarpus</i>	NO_3^- > NH_4^+ , Arg-N NH_4^+ , Gln-N > Arg-N	NO_3^- > NH_4^+ , Gln-N, Arg-N Gln-N > Arg-N
2) High soil N availability		
Control	NH_4^+ > NO_3^- , Gln-N, Arg-N Arg-N > Gln-N	
Competition with <i>Acacia</i>	NH_4^+ > Arg-N > Gln-N NO_3^- > Gln-N	
Competition with <i>Podocarpus</i>	NH_4^+ > Arg-N > Gln-N	

¹At low soil N availability.

Native tree seedlings increase N acquisition in competition with exotic grasses at high soil N availability

At high soil N availability, both tree species responded similarly to competition with the exotic grass *Pennisetum* by increasing N acquisition. However, growth and biomass allocation were unaffected, which indicates that they might allocate the newly acquired N to storage rather than investing into growth, thereby reducing N availability for their competitors (Millard and Grelet 2010). These similarities between *Acacia* and *Podocarpus* in

terms of net N uptake capacity and growth responses to competition with grasses indicate that both tree species use a common strategy, which is rather unexpected given the different successional status, growth strategies and physiological characteristics of the two tree species. *Acacia* is an early successional, fast-growing and N_2 fixing species, while *Podocarpus* is a slow-growing, late-successional species (Smith et al. 2008). Thus, since fast-growing species would require higher amounts of N to meet their growth rate (Poorter et al. 2012), the observed

increase in net N uptake capacity when growing in competition would be expected in *Acacia* and not in *Podocarpus*. However, previous studies have shown that *Acacia* seedlings are capable of N-fixation (Sun et al. 1992), and that root nodulation in *Acacia* species is stimulated by competition with grasses (Cramer et al. 2007). Therefore the increased requirements of *Acacia* due to competition would probably be mitigated by its ability to fix atmospheric N₂ (not quantified in our study) and thus reduce to an extent its dependency on external N supply. Moreover, the lack of response of *Acacia* to competition in terms of N uptake at low soil N availability in our study might also be attributed to a greater reliance on N₂ fixation under limiting N conditions (Pfautsch et al. 2009).

Tree species differed in the acquired N forms: in competition fast-growing *Acacia* acquired more ammonium compared to single grown individuals, while slow-growing *Podocarpus* used glutamine-N. This preference of inorganic vs organic N forms when competing might reflect different N acquisition strategies between fast- and slow-growers as reported for temperate tree seedlings (Simon et al. 2010, 2014, Li et al. 2015). The similar responses found here might be an early indication that, across biomes, N acquisition of trees is linked to growth strategy. These increases in net N uptake capacity as a response to competition were found only at high soil N availability suggesting mediation of N acquisition by soil N availability (e.g., Simon et al. 2013, Li et al. 2015). For *Acacia*, this could be related to a reduced nodule activity at high soil N concentrations, as it has been suggested that N acquisition from the soil, rather than N₂ fixation, is energetically more efficient for this species when inorganic N is readily available (Pfautsch et al. 2009). With regard to *Podocarpus*, an increase in organic N acquisition with competition was also reported for a temperate slow-growing tree species at high, but not low soil N (Li et al. 2015). Our results constitute a further example of increased N acquisition under competition with high N availability, but in a subtropical environment. Particularly in the view of increased atmospheric N deposition in the future (Rennenberg and Dannenmann 2015, Jia et al. 2016), the outcomes of competition in currently N-limited habitats are likely to change.

At low soil N availability, competition with grasses did not result in changes in N acquisition or biomass production and allocation for both tree species. Similarly, previous studies have reported a lack of effects of competition with the grass *Cynodon dactylon* on the growth (i.e., biomass, basal diameter and stem height) of a different *Acacia* species (*A. smallii* Isely) on non-supplemented native soil (Cohn et al. 1989). Nevertheless, this grass species hinders the growth of native tree seedlings in the field and is reported as a threat to restoration efforts in subtropical Australia (M. Amos, personal communication). Since we did not observe these negative effects in our study, it is likely that the impacts of the competition with *Cynodon* on tree seedling growth are evident at later developmental stages of both the

grass and the tree species than the ones considered here. The lack of response to competition of the tree seedlings regarding total biomass could also be due to the short duration of our experiment, considering the relatively slow growth of tree seedlings compared to grasses. Previous studies reporting negative effects of grasses on *Acacia* measured responses over longer periods, e.g., 3 years (Messina and Barton 1985), and 8 years (Florentine and Westbrooke 2004). Furthermore, the effect of the grass on tree seedlings may be mediated by soil N availability: in our study, the effects of competition with *Cynodon* were not investigated at high soil N availability, but it has been reported that competition with *Cynodon* combined with an increase in soil nutrient availability results in 70–90% reduction in growth of *A. smallii* (Cohn et al. 1989).

Exotic grasses respond to competition for N depending on the competitor and soil N availability

For the exotic grasses, four strategies were identified which depended on the competing species and soil N availability: (1) *Pennisetum* biomass was reduced in competition with both tree species compared to the control at high, but not low soil N availability suggesting that the outcome of competitive interactions between native tree seedlings and *Pennisetum* depends on soil N availability. Moreover, the biomass of *Pennisetum* at high soil N availability, even if reduced by competition with the tree seedlings, was still higher than its biomass at low soil N in the absence of interspecific competition, suggesting that, at least in the short-term, the grass is more successful than the tree seedlings at high soil N availability, despite the observed negative effects of competition. (2) The grass *Cynodon* showed a similar strategy as *Pennisetum*, i.e., a reduction in biomass but no change in N acquisition, but only when competing with *Acacia*, but not *Podocarpus*, indicating that for both grass species the response to competition depends on the competitor. (3) Resource allocation shifted to aboveground at the expense of belowground tissue production (Aerts et al. 1991) for *Pennisetum* competing with *Podocarpus* at low and high soil N. This suggests that, despite the fact that shading from the tree seedlings was not apparent, the potential competition for light is more relevant for *Pennisetum* than competition for belowground resources (Poorter et al. 2012), thus not leading to an increase in N acquisition. However, these results were not observed when grown in competition with *Acacia* or for the grass *Cynodon* indicating that this biomass allocation strategy of *Pennisetum* also depends on the competing species, but not soil N availability. (4) Competition with *Podocarpus*, but not *Acacia*, resulted in increased net nitrate uptake in *Cynodon*, but not *Pennisetum* suggesting different N acquisition strategies between the two exotic grass species that vary with the competing tree species. Overall, our results suggest that the outcome of competitive interactions between native tree seedlings and exotic grasses are species-specific and mediated by soil N availability.

Differences in N acquisition strategies between species suggest potential for niche differentiation

All four species took up both inorganic and organic N forms, although specifics varied according to N forms. Nitrate acquisition was higher in *Pennisetum* and *Acacia* compared to *Podocarpus*, consistent with their successional status and the low abundance of nitrate in late-successional forest soils as well as a reduced nitrate use capacity in late-successional tree species (Kronzucker et al. 1997, Britto and Kronzucker 2013). At sites with higher soil nitrate availability, e.g., disturbed areas, the grass *Pennisetum* would have a competitive advantage over *Podocarpus*, but not *Acacia*, highlighting the importance of species-specific consideration for reforestation programs. Moreover, glutamine-N acquisition was higher for *Acacia* than *Podocarpus*, contrary to what we expected according to their successional status, since soil amino acid content generally increases as succession progresses (Werder-Pfisterer et al. 2009). Nevertheless, glutamine-N acquisition was higher in both tree species compared to *Pennisetum* indicating a potential for avoidance of competition via the use of the different N forms (Simon et al. 2017). Overall, that plants take up organic N forms is now well established and has been reported for numerous plant species and ecosystems (Näsholm et al. 2009). Moreover, its ecological relevance is increasingly recognized due to the resulting niche differentiation and consequences in terms of species coexistence (Näsholm et al. 2009, Simon et al. 2014, Li et al. 2015, Andersen et al. 2017, Uscola et al. 2017).

Comparing within tree and grass species, higher soil N availability resulted in increased inorganic and organic N acquisition in general, which for *Pennisetum* was combined with increased biomass production (for *Cynodon* data is only available for low soil N). This increase in grass biomass production with increasing soil N availability while there was no short-term response of the tree seedlings suggests a potential advantage for the grass in habitats on N-rich soils or in future scenarios with increased atmospheric N deposition compared to the native trees. This link between high resource availability and success of invasive species has been explored in previous studies (e.g., Badgery et al. 2005, Gao et al. 2014, Yelenik et al. 2017), and has been linked to invader traits related to exploitation and efficient use of resources, which allows for better growth (Huangfu et al. 2016, Yelenik et al. 2017). In our study, although competition with seedlings of both tree species reduced the total biomass of *Pennisetum* compared to *Pennisetum* grown in controls, the increase in *Pennisetum* biomass in response to higher soil N availability was of greater magnitude. Increases in the productivity of *Pennisetum* with N fertilization resulting in its dominance at fertilized sites have been reported before (Mears 1970, Barton et al. 2008), and under such conditions, *Pennisetum* might be more competitive than tree seedlings. Moreover, the potential of *Acacia* to induce increases in soil N availability over time as a consequence of its N₂ fixing ability has been shown before in

silvopastoral systems (Power et al. 2003). Thus, increased N supply as a consequence of the use of N₂ fixing tree species such as *Acacia* might favour the growth and potential dominance of *Pennisetum* at restoration sites.

Methodological considerations

Our experimental approach has several limitations. First, the competitor grasses were grown from seeds, whereas the trees were purchased as seedlings. This set up was selected because our aim was to mimic the actual timing in a restoration setting under field conditions for which tree species are planted as seedlings for reforestation and grasses arrive at a later time. Thus, a comparison between trees and grasses cannot exclude differences due to variation in developmental stages between tree species and grasses. Specifically, the lack of response in seedling biomass reported here might not persist with older or larger grasses. Second, tree seedlings differed in their age at the start of the experiment which might have influenced their responses to competition and/or soil N availability in our experiment. However, given the differences in growth rates between the tree species used in this study, seedlings of similar age would have likely differed in their initial above- and belowground dimensions, thereby introducing another variable. Third, the experiment ran for 8 weeks which might not be sufficient time for a response in tree seedling biomass, however they did respond with regard to N acquisition from the soil. Finally, the artificial N solution used in this study is based on a low and high N forest site (Dannenmann et al. 2009, Stoelken et al. 2010, respectively) as well as the occurrence of glutamine and arginine in soils of subtropical Queensland (Holst et al. 2012), but not actual measured concentrations at subtropical restoration sites.

Conclusions

In our study, native tree seedlings and exotic grasses responded to competition with two distinct response types: (1) a reduction in biomass, but no change in N acquisition, (2) an increase in N acquisition without a change in biomass. This outcome depended on the competing species and soil N availability. Thus, we suggest that these aspects be considered also for ecosystem restoration and management, with special attention to the potential feedbacks between native trees possessing traits such as N₂ fixing ability and exotic grasses that may benefit from the resulting increase in soil N availability. Exotic grasses such as *Pennisetum* and *Cynodon* are considered a high priority for control in Australia and their suppression can promote the reestablishment of woody vegetation, thus determining the success of subtropical rainforest restoration programs (Florentine and Westbrooke 2004, Downey et al. 2010, Shoo and Catterall 2013). We found no common N acquisition strategy across species in response to competition between native trees and invasive grasses indicating the need to consider species-specific approaches in restoration management plans.

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Conflict of interest

None declared.

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