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N/P imbalance as a key driver for the invasion of oligotrophic dune systems by a woody legume

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Oligotrophic ecosystems, previously considered to be more resilient to invasive plants, are now recognised to be highly vulnerable to invasions. In these systems, woody legumes show belowground ecosystem engineering characteristics that enable invasion, however, the underlying processes are not well understood. Using a Portuguese primary dune ecosystem as an oligotrophic model system, belowground biomass pools, turnover rates and stoichiometry of a native (*Stauracanthus spectabilis*) and an invasive legume (*Acacia longifolia*) were compared and related to changes in the foliage of the surrounding native (*Corema album*) vegetation.

We hypothesized that the invasive legume requires less phosphorus per unit of biomass produced and exhibits an enhanced nutrient turnover compared to the native vegetation, which could drive invasion by inducing a systemic N/P imbalance.

Compared with the native legumes, *A. longifolia* plants had larger canopies, higher SOM levels and lower tissue P concentrations. These attributes were strongly related to legume influence as measured by increased foliar N content and less depleted $\delta^{15}N$ signatures in the surrounding *C. album* vegetation. Furthermore, higher root N concentration and increased nutrient turnover in the rhizosphere of the invader were associated with depleted foliar P in *C. album*.

Our results emphasize that while *A. longifolia* itself maintains an efficient phosphorus use in biomass production, at the same time it exerts a strong impact on the N/P balance of the native system. Moreover, this study highlights the engineering of a belowground structure of roots and rhizosphere as a crucial driver for invasion, due to its central role in nutrient turnover. These findings provide new evidence that, under nutrient-limited conditions, considering co-limitation and nutrient cycling in oligotrophic systems is essential to understand the engineering character of invasive woody legumes.

Invasive plant species are contributing significantly to human-induced global change due to their strong impacts on biodiversity and ecosystem functioning (Simberloff et al. 2013). However, effects of invasion on soil nutrient cycling have long been overlooked and especially comparison studies of native versus invasive species rarely consider belowground plant traits (Smith et al. 2014). Thus, soil properties and nutrient cycling have become major issues in the research of plant invasion (Hulme et al. 2013) with particularly the role of nitrogen (N) and phosphorus (P) relationships between plant and soil requiring further research (Sardans and Peñuelas 2012).

Belowground processes play a crucial role for the invasion of woody plants (Nuñez and Dickie 2014), which is a group of invasive plants receiving increasing attention (Richardson and Rejmánek 2011). Woody legumes in particular can fundamentally alter ecosystem function, e.g. by facilitating succession of tree species in their litter and rhizosphere zone (Bellingham et al. 2001) or by exerting competitive pressure under non-favourable conditions (Watt et al. 2003). While invasive woody legume species are known to be able to provide N to the ecosystem by symbiotic nitrogen fixation (Augusto et al. 2005), this also leads to P limitation in nutrient poor ecosystems (Augusto et al. 2013). However, even though P turnover is a crucial component of ecosystem functions, its relevance for plant invasions has rarely been addressed (Ehrenfeld 2010).

Acacia longifolia is a globally "very widespread" (Richardson and Rejmánek 2011) invasive legume and is also termed an "ecosystem engineer" sensu Badano et al. (2010). It is also one of the most influential invaders in dune systems in several countries worldwide (Marchante et al. 2008), which are both ecosystems with high conservational value and excellent model systems for invasion biology, as they allow for the observation of engineering effects of invasive species on ecological timescales due to their transitional nature and intermediate energy balance (Fei et al. 2014). For example, *A. longifolia* fundamentally transforms the oligotrophic dune systems by promoting monospecific plant communities (Hellmann et al. 2011), profoundly altering

edaphic conditions, increasing soil organic matter (OM) levels (Marchante et al. 2008, Hellmann et al. 2011) and creating a positive feedback loop between plant and soil (Marchante et al. 2008). The success of the *Acacia* genus has been mainly ascribed to high nutrient use efficiency, its ability fix nitrogen, elevated growth rates, larger size and a bigger investment in root mass, both in terms of deep roots and shallow root networks (Morris et al. 2011, Funk 2013). Roots in turn have direct effects on the adjacent rhizosphere, which is increasingly recognized for its role in ecological engineering and exotic plant invasions (Philippot et al. 2013). Also, litter mass plays an important role in *Acacia* spp. invasion, due to their high leaf turnover rates as well as extensive leaf shedding in stress conditions (Rascher et al. 2012), which in the case of *A. longifolia* leads to the accumulation of a thick litter layer underneath the canopy (Marchante et al. 2008).

Not only OM pool sizes, e.g. root, rhizosphere and litter mass, but also flux rates, such as litter and fine root decomposition, increase in invaded ecosystems (Liao et al. 2008). Biomass decomposition rates of plant tissues strongly depend on the constrained stoichiometry between carbon (C), nitrogen (N) and phosphorus (P) requirements of the microorganisms degrading them (Manzoni et al. 2010). While stoichiometric ratios of plant tissues can shed light on nutrient limitation (Güsewell 2004), extracellular enzyme activities (EEA) are useful measures for potential nutrient turnover (Sinsabaugh et al. 2009). The enzymatic turnover of N and P is tightly coupled to each other as well as to carbon release (Manzoni et al. 2010) and can be used as a functional measure of nutrient and energy flow (Sinsabaugh et al. 2009). Among the most widely assessed activities are those of β-1,4-glucosidase, β-1,4-N-acetylglucosaminidase (Nag) and acid phosphatase (Ap) (Sinsabaugh et al. 2009), which are of crucial importance to energy, N and P turnover, respectively.

Invasive and native species have effects on soil nutrient cycling that can be estimated by measuring variables of the surrounding plant community, for example leaf stoichiometry (Sardans and Peñuelas 2012) or ecological tracers such as foliar $\delta^{15}N$ (Hellmann et al. 2011, Rascher et al. 2012). Natural abundance $\delta^{15}N$ measurements can be used to quantify the proportion of N derived from atmospheric N_2 fixation by legumes in plant–soil systems (Unkovich 2013) and foliar $\delta^{15}N$ is a good proxy for soil $\delta^{15}N$, especially under low N availability (Craine et al. 2009). This approach was successfully used in the oligotrophic Portuguese primary dunes, utilizing foliar $\delta^{15}N$ of the non-leguminous native shrub *Corema album* (Hellmann et al. 2011, Rascher et al. 2012). Similar to other ericoid mycorrhizal plants (Craine et al. 2009), *C. album* exhibits very depleted foliar $\delta^{15}N$ values without legume influence, which, together with its high abundance in this system, suggest it to be a good monitoring plant for legume influence (Rascher et al. 2012). As the invasive *A. longifolia* and *Stauracanthus spectabilis*, a native, sclerophyllous, leguminous shrub are the only legumes in this system and the two species co-occur with *C. album* in these very oligotrophic primary dunes with no further sources of OM input, this situation creates a model system that is ideally suited to quantify the impact of *A. longifolia* invasion (Rascher et al. 2012).

It has been shown that *C. album* plants in close proximity of *A. longifolia* exhibit enhanced growth rates, higher foliar N contents and less depleted $\delta^{15}N$ values (Hellmann et al. 2011), these being effects that were not found in the proximity of the native *S. spectabilis*. Because *A. longifolia* will ultimately dominate the invaded ecosystem, these findings are somewhat paradox as they indicate that *C. album* plants seem to benefit from the N supply of the invasive legume. However, woody legumes, like invasive *Acacia* species, have a high N and P demand (Augusto et al. 2013, Mortimer et al. 2013) and as P is mainly released by rock weathering, plant growth can further sink-deplete the residing P pool (Vitousek et al. 2010).

Following this reasoning, legume influence on surrounding *C. album* plants should cause a systemic increase in N availability accompanied by P depletion. It was therefore hypothesized that *A. longifolia*, contrary to the native legume, creates an N/P imbalance by inducing belowground processes that enhance nutrient cycling and increase OM underneath its own canopy.

To test this hypothesis, we quantified the mass and stoichiometry of major belowground OM pools as well as their potential turnover rates for both legumes (native and invasive) and related these variables to changes in the native *C. album* foliar N, $\delta^{15}N$ and total P of the surrounding vegetation.

Material and methods

Study site and species description

Sampling took place from 21 to 23 May 2012 in the coastal sand dune ecosystem of Pinheiro da Cruz, Portugal (38°15.2′N, 8°45.8′W). The site is located in the primary dunes, which are characterized by poor arenosols (FAO classification) and by an open vegetation structure (Rascher et al. 2012). The vegetation is dominated by *Corema album* (Ericaceae), a shrub endemic to the Iberian Peninsula and two nitrogen fixing leguminous species, the native *Stauracanthus genistoides*, subspecies *spectabilis* which is a sclerophyllous leguminous shrub (Barradas et al. 1999) and the invasive non-native leguminous tree *Acacia longifolia* (Fabaceae).

Plant sampling design and in situ measurements

Samples and measurements were taken underneath five *S. spectabilis* and five *A. longifolia* plants. Plant canopy sizes were estimated as ellipses from 2 radii. Underneath each plant, three sampling points were randomly selected and litter height was measured on 12 random spots around each sampling point. Starting from the sampling points, mature, sunlit leaves from 15 *C. album* plants were sampled along three transects (five *C. album* plants per transect) with a mean distance ± standard error of 1.35 ± 0.16 m between each plant (Supplementary material Appendix 1 Fig. A1) and transect lengths of 6.33 ± 1.2 m for *S. spectabilis* and 9.95 ± 0.81 m for *A. longifolia.*

Soil collection and preparation

At each sampling point, soil was collected with a metal tube of 8.5 cm in diameter and 19.5 cm in height. Subsequently, samples were kept at 4°C in airtight plastic bags until analysis. Bulk samples were separated into four fractions (sand, rhizosphere, roots and litter). The top organic layer of the sample up until the onset of the sand layer was removed and termed litter. The sand fraction was removed from the bulk-agglomerated rhizosphere/root in the core and then sieved through a 1-mm sieve. Rhizosphere was separated from roots by gently shaking the adhered particles off the roots and subsequently treating them similar to the soil. Parts of all fractions were oven-dried at 60°C until constant weight and 500 mg were ashed in a muffle furnace (600°C, 24 h) to determine organic matter by the loss on ignition method.

Soil nutrients and pH were analysed in 1:10 (m/v) soil extracts done from both rhizosphere and sand samples in ultrapure water with 30 min extraction time on a shaker at room temperature. To obtain a clear extract, the resulting soil-water suspension was centrifuged (5000 g, 4°C, 15 min) and finally filtered through sterile medical gauze with a pore size of 500 µm. pH was subsequently analysed in the extract with a glass electrode (pH/mV meter).

Colorimetric assays

Concentrations of NH_4^+, NO_3^- and soluble inorganic P of soil extracts was quantified by colorimetric assays. To determine total phosphorus of soil and organic matter, complete samples were ignited, followed by acid-extraction (HCl, 1 M) and inorganic phosphorus determination. All colorimetric assays were done using microscale methods, executed in 250 µl 96-well flat bottom microtiter plates and analysed in a microplate reader. Reaction vessels were acid-washed, bleach-washed and thoroughly rinsed with distilled water before usage. For each single assay a separate triplicate calibration curve was produced with the respective salts (KH_2PO_4, $(NH_4)_2SO_4$, KNO_3) as serial dilutions in ultrapure water. Soluble inorganic phosphorus was analysed using a malachite-green based method described by D'Angelo et al. (2001). Organic phosphorous in soil and rhizosphere was subsequently calculated from inorganic and total phosphorous. NH_4^+ was analysed using a modified Berthelot reaction (Cruz and Martins-Loução 2000) and NO_3^- using the VCl_3/Griess method as described by Hood-Nowotny et al. (2010).

Fluorometric enzyme activity profiling

The procedures, solutions and incubation times were followed as described by Pritsch et al. (2011). Root tips were carefully separated from the attached sand, rinsed in tap water and ca 2 mm sized root parts were transferred into 96-well filter plates (96-filter plate with 30–40 µm mesh size) where they stayed for the whole assay procedure. The enzyme tests were performed using fluorescent substrate analogues labelled with methylumbelliferone (MU): MU-xyloside, MU-glucuronide, MU-cellobiohydrofuran, MU-N-acetylglucosamine, MU-β-glucoside and MU-phosphate and fluorescence measured at 364 nm excitation and 450 nm emission in a fluorescence microplate reader. The values obtained were calibrated against a standard curve and related to the total area of root tips by scanning the root parts and analysing the pictures with Photoshop for surface estimation.

Litter was crushed and homogenized into small pieces using a pair of clean scissors; 20 mg was weighed on a microscale and then transferred to the wells. Rhizosphere samples were mixed thoroughly prior to weighing and also 20 mg was transferred to the wells. The values obtained were calibrated against a standard curve and related to the organic matter weight.

Elemental analysis and isotopic ratios

Dry organic matter samples of foliage, roots and litter were ground to a fine powder in a ball mill. 5 ± 0.2 mg of the powder was weighted into tin capsules and. $^{15}N/^{14}N$ ratios in the samples were determined on a continuous flow stable isotope ratio mass spectrometer coupled to an elemental analyser for online sample preparation by Dumas-combustion. The standards used were IAEA-N1 and USGS-35 for nitrogen isotope ratio, $\delta^{15}N$ results were referred to air. Precision of the isotope ratio analysis, calculated using values from six to nine replicates of laboratory standard material interspersed among samples in every batch analysis, was $\leq 0.2‰$.

Statistical analysis

If not stated otherwise, all tests were performed with the package *stats* using ver. R 3.2.4 (<www.r-project.org>). Comparisons between biomass pools and specific extracellular enzyme activities of rhizosphere and litter of both legumes were performed using Wilcoxon rank sum tests. This test was also used to compare differences between legume influence on *C. album* foliar % N, P and $\delta^{15}N$. To test for significant differences between soil and rhizosphere of both legume species, pairwise Wilcoxon rank sum tests with Holm adjustment were used.

In order to find the spatial threshold of legume influence, the relationship between *C. album* $\delta^{15}N$ and N content, respectively, with the distance to the legume canopy was investigated. Since $\delta^{15}N$ and N content both increased in the vicinity to the legume canopy, a breakpoint was expected reflecting the threshold between the influenced range and the background value. To estimate this breakpoint, segmented regressions were fitted using the function *segmented.lme()* (Muggeo et al. 2014, modified by V. Muggeo to handle nested random effects). Distance to the legume canopy was included as fixed effect and legume and transect were included as random effects, with transect nested in legume (see Supplementary material Appendix 1 Fig. A1 for a schematic illustration of the nested design). The models were fitted by log-likelihood maximisation. Model terms, including breakpoints of all models, were tested for significance with the Wald-test using *anova.lme()*. After breakpoint estimation, plants were grouped into plants growing in the canopy (IN) and plants growing outside (OUT) of breakpoint range. Legume influence was then defined as the difference between IN and OUT (IN - OUT) for $\delta^{15}N$ and the % of change ((IN - OUT)/IN) for total foliar N.

Assumptions for the linear regression between % of change in *C. album* foliar total N and foliar total P (‰) were checked by the Breusch–Pagan test against homoscedasticity and the residuals checked for normality using the Shapiro–Wilk normality test. These assumptions were also verified for all mentioned correlations in the text.

Variables significant to explain legume influence were identified based on partial least squares (PLS) regression and backward selection of significant variables using the *autopls()* function from the *autopls* package (Schmidtlein et al. 2012), which selects variables on the basis of variable importance in the projection (VIP) and significance for prediction The variables used for the selection procedure were: plant size, ΣC litter, Ap litter, Nag litter, ΣC roots, Ap roots, Nag roots, ΣC rhizosphere, Ap rhizosphere, Nag rhizosphere, litter mass, root mass, rhizospere mass, soil OM, rhizosphere OM, soil organic P, rhizosphere organic P, total N litter, total P litter, total N roots, total P roots. The PLSR approach employs linear regression to project observed and predicted variables to a new space, thus creating components that are both explaining variance within the set of predictor variables as well as the variance of the observed variable. The procedure is creating a prediction model using an optimization procedure based on variable significance and is internally validated by leave-one-out cross-validation.

Data deposition

Data available from the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.gc735> (Ulm et al. 2016).

Results

Pool sizes and stoichiometry

Comparison of the native and the invasive legumes, *Stauracanthus spectabilis* and *Acacia longifolia*, revealed differences in size and belowground biomass pools (Table 1), with the canopy of the invasive *A. longifolia* being 12 fold larger than the canopy of the native *S. spectabilis*. The invasive also exhibited a significantly larger root mass and a slightly larger rhizosphere. In terms of soil parameters, both rhizospheric and soil fractions underneath the studied plants showed clear differences between the fractions, but no significant differences between species (Table 2). A deviation from this pattern was the OM content, which was higher in the rhizospheric soil fraction of both legumes than in soil of *S. spectabilis*, while soil and rhizospheric soil under *A. longifolia* were not distinguishable. The rhizospheric compartments of both species were slightly less acidic than the surrounding soil, and exhibited 2-fold higher nitrate, 1.7-fold higher soluble P and 6.2-fold higher organic P concentrations, but also 2.4-fold lower ammonia concentrations. Generally, the quantity of rhizospheric soil was highly correlated with the root mass ($r^2 = 0.89$, $p < 0.001$) and was five times higher underneath the invasive species (Table 1). The rhizospheric soil ratio, describing the amount of soil being influenced by roots, was 0.32 in the uppermost 20 cm of soil underneath *A. longifolia*, contrary to 0.07 underneath *S. spectabilis*. Litter mass was not significantly larger underneath the invasive compared to the native species, but

Table 1. Characterization of the biomass pools associated with the invasive *Acacia longifolia* and the native *Stauracanthus spectabilis* legumes. Three biomass pools were considered: leaf, litter and root. Rhizosphere/soil ratio was calculated as rhizospheric soil relative to the bulk soil sample (w/w). Values are means (n = 5) with standard errors given in brackets, rows in bold show significant differences between groups ($p < 0.05$, Wilcoxon test).

		A. longifolia	*S. spectabilis*	p-value
Plant size	**canopy (m^2)**	**75.0 (18.7)**	**6.5 (0.4)**	**0.008**
Biomass pools	litter (kg m^{-2})	3.3 (0.4)	3.6 (0.7)	1
	root (kg m^{-2})	**2.9 (0.9)**	**0.9 (0.2)**	**0.032**
	rhizosphere (kg m^{-2})	79.2 (27.1)	15.5 (6.7)	0.056
	rhizosphere/soil ratio (w/w)	0.32 (0.11)	0.07 (0.03)	0.056
Leaf biomass	N (%)	1.9 (0.1)	1.8 (0.1)	0.421
	$\delta^{15}N$ (‰)	**–2.6 (0.2)**	**–0.6 (0.2)**	**0.008**
	P (‰)	**0.3 (<0.1)**	**0.9 (<0.1)**	**0.008**
	C (%)	47.5 (1.2)	47.8 (1.3)	0.841
	N/P ratio	**59.6 (7.8)**	**19.9 (0.8)**	**0.008**
	C/N ratio	25.4 (1.7)	27.3 (1.1)	0.548
	C/P ratio	**1512.8 (237.7)**	**540.8 (26.8)**	**0.008**
Litter biomass	N (%)	1.4 (0.2)	1.5 (0.1)	0.548
	$\delta^{15}N$ (‰)	–1.7 (0.4)	–1.6 (0.2)	1
	P (‰)	0.3 (0.1)	0.4 (<0.1)	0.222
	C (%)	42.5 (1.9)	43.5 (0.9)	0.421
	N/P ratio	85.6 (24.8)	43.9 (3.7)	0.222
	C/N ratio	31.9 (2.4)	29.4 (1.7)	0.31
	C/P ratio	2429.5 (566.4)	1295.6 (157.4)	0.095
	thickness (cm)	**14.6 (2.8)**	**28.8 (3.3)**	**0.016**
Root biomass	N (%)	1.2 (0.2)	0.7 (0.1)	0.056
	$\delta^{15}N$ (‰)	**–0.5 (0.1)**	**–1.9 (0.3)**	**0.008**
	P (‰)	0.2 (0)	0.4 (0.1)	0.69
	C (%)	36.6 (1.9)	39.1 (2.3)	0.548
	N/P ratio	83.7 (29.3)	37.3 (12.5)	0.151
	C/N ratio	**33.7 (3.5)**	**74.8 (18.4)**	**0.032**
	C/P ratio	2104.5 (667.6)	2136.2 (569.9)	1

Table 2. Characteristics of the soil and the rhizosphere compartments of the two studied legumes, the invasive *Acacia longifolia* and the native *Stauracanthus spectabilis*. Values are means (n = 5) with standard errors given in brackets. Different letters indicate significant differences ($p < 0.05$, pairwise Wilcoxon test with Holm correction).

	Species	Organic matter (%)	pH	Soluble phosphorus (µg g^{-1})	Organic phosphorus (µg g^{-1})	Nitrate (µg g^{-1})	Ammonium (µg g^{-1})
Soil	*A. longifolia*	0.4 (0)^{ab}	5.6 (0.1)^{a}	0.3 (0.1)^{a}	5 (1.0)^{a}	2.6 (0.4)^{a}	3.3 (0.1)^{a}
	S. spectabilis	0.3 (0)^{a}	5.4 (0.1)^{a}	0.2 (0)^{a}	6.3 (1.7)^{a}	2 (0.3)^{a}	2.8 (0.1)^{a}
Rhizosphere	*A. longifolia*	0.6 (0.1)^{bc}	6.2 (0.1)^{b}	0.8 (0.1)^{b}	32.8 (8.9)^{b}	4.7 (1.1)^{a}	1.1 (0.1)^{b}
	S. spectabilis	1.4 (0.3)^{c}	6.2 (0.1)^{b}	0.7 (0.1)^{b}	37.2 (5.3)^{b}	3.3 (0.7)^{a}	1.4 (0.4)^{b}

litter layers were found to differ significantly in thickness. Total N values did not differ in major tissues, however, *A. longifolia* roots showed a trend for higher total N concentrations compared to *S. spectabilis* roots. Also, while there were significant differences in the $\delta^{15}N$ signatures, both species exhibited values close to the atmospheric standard, which is expected in nitrogen fixing legumes. The foliage of the *A. longifolia* was significantly depleted in total P compared to the native *S. spectabilis*, while roots and litter showed only a slightly lower total P in the case of the invasive.

Specific enzyme activities to assess potential flux rates

For rhizosphere, litter and soil, significant differences of specific enzyme activities were found between species (Table 3). The litter produced by the invasive showed 1.5 times higher C related EEA (ΣC: β-glucosidase + β-xylosidase + β-glucuronidase + cellobiohydrolase) than the litter of the native species, however, β-1,4-N-acetylglucosaminidase (Nag) and acid phosphatase (Ap) activities were not significantly different. Differences of specific enzyme activities were more pronounced and significantly different between the rhizospheres of both species, with 2.7-fold higher ΣC, 2.4-fold higher Ap activity and 1.4-fold higher Nag activity underneath the invasive. Root Ap enzyme activities were significantly higher underneath the invasive species, while root β-glucuronidase activities were higher in the native. Ratios of ln(ΣC)/ln(Nag), ln(ΣC)/ln(Ap) and ln(Nag)/ln(Ap) can be used for stoichiometry considerations (Sinsabaugh et al. 2009), similar to CNP ratios in plant tissues. EEA ratios were not significantly different between the species and ranged around 1 in the litter compartment (SE = 0.1), while they differed to a larger extent in rhizosphere: ln(ΣC)/ln(Nag) = 1.3, ln(ΣC)/ln(Ap) = 1, ln(Nag)/ln(Ap) = 0.8 and the roots: ln(ΣC)/ln(Nag) = 2.7, ln(ΣC)/ln(Ap) = 1.1, ln(Nag)/ln(Ap) = 0.4.

Spatial impact

The impact of the legumes on both foliar total N and $\delta^{15}N$ values of surrounding *C. album* plants was distance dependent and could be modelled using a segmented regression (Fig. 1). Both legumes impacted foliar N or $\delta^{15}N$ of surrounding *Corema album* plants up to certain breakpoint (broken lines) which described the maximum range of influence outside of the canopy. Using this approach, *C. album* plants were grouped into plants growing under

Table 3. Specific extracellular enzyme activities of rhizosphere and litter pools, related to organic matter content. ΣC abbreviates the sum of carbon related enzyme activities (β-glucosidase + β-xylosidase + β-glucuronidase + cellobiohydrolase). Also shown are root potential enzyme activities per projected root area. Values are means (n = 5) with standard errors in brackets. Rows in bold show significant differences between groups ($p < 0.05$, Wilcoxon test).

		Acacia longifolia	*Stauracanthus spectabilis*	p-value
Litter specific enzyme activity (µmol h^{-1} g^{-1} OM)	**β-glucosidase**	**1.9 (0)**	**1.3 (0)**	**0.016**
	β-xylosidase	**0.1 (0)**	**0 (0)**	**0.016**
	β-glucuronidase	0.1 (0)	0.1 (0)	0.222
	cellobiohydrolase	**0.4 (0)**	**0.3 (0)**	**0.032**
	ΣC	**2.5 (0.2)**	**1.6 (0.1)**	**0.016**
	acid phosphatase (Ap)	1.6 (0)	1.2 (0)	0.151
	N-acetylglucosaminidase (Nag)	1.3 (0)	1 (0)	0.151
Rhizosphere specific enzyme activity (µmol h^{-1} g^{-1} OM)	**β-glucosidase**	**346.9 (0.3)**	**103.6 (0.1)**	**0.016**
	β-xylosidase	83.5 (0.1)	40 (0)	0.056
	β-glucuronidase	8 (0)	4.5 (0)	0.095
	cellobiohydrolase	**27.8 (0)**	**14.1 (0)**	**0.032**
	ΣC	**466.1 (52.8)**	**162.2 (21.2)**	**0.016**
	acid phosphatase (Ap)	**202.1 (0.2)**	**93.4 (0.1)**	**0.008**
	N-acetylglucosaminidase (Nag)	**16.3 (0)**	**11.2 (0)**	**0.008**
Root potential enzyme activity (pmol min^{-1} mm^{-2})	β-glucosidase	787 (45.3)	752.8 (17.8)	0.222
	β-xylosidase	197.7 (5.3)	184 (3.5)	0.095
	β-glucuronidase	**10.3 (0.3)**	**14.3 (1.4)**	**0.008**
	cellobiohydrolase	59.4 (3.3)	55 (1.2)	0.222
	ΣC	1054.4 (52.8)	1006.2 (56.5)	0.151
	acid phosphatase (Ap)	**401.2 (3.9)**	**414.3 (3.7)**	**0.032**
	N-acetylglucosaminidase (Nag)	12.2 (0.6)	14.6 (1.5)	0.31

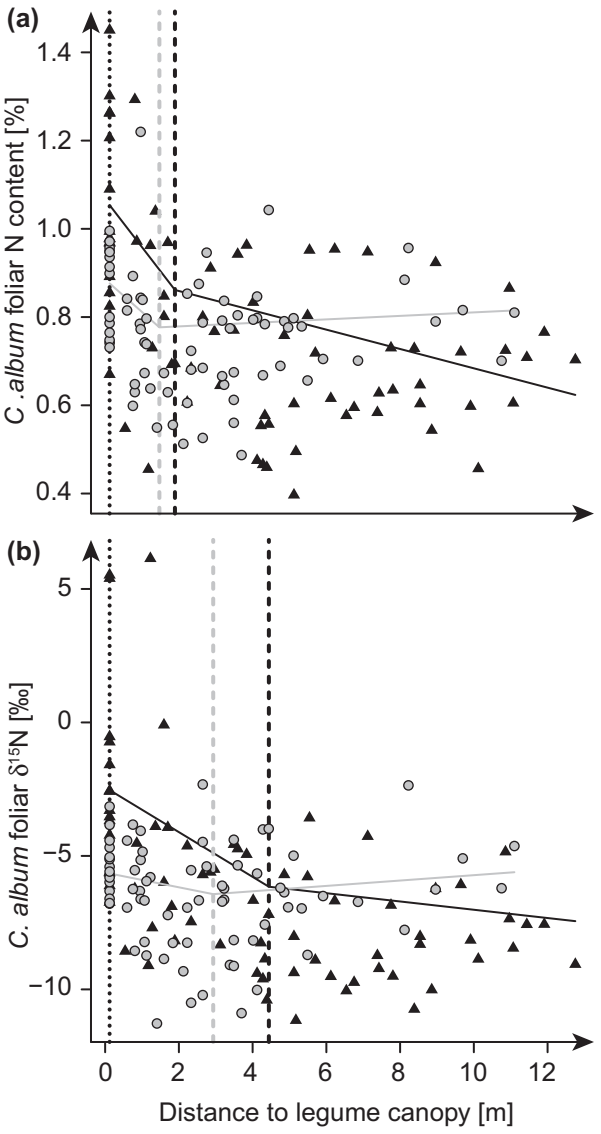

Figure 1. Effect of the distance to the native (*S. spectabilis*) and the invasive (*A. longifolia*) on *C. album* total foliar N (a) and on foliar $\delta^{15}N$ (b). Black triangles indicate *C. album* foliage samples from the surrounding of *A. longifolia*, grey dots samples from the surrounding of *S. spectabilis*. Black dotted lines indicate the canopy of the respective legumes, black and grey broken lines indicate the breakpoint estimated by segmented regression, beyond which the influence of the legumes is not distinguishable from background variation.

the canopy (on the black dotted line, IN), or in distances exceeding the breakpoint, where no significant impact of the legume was evident (OUT). The influence range of *S. spectabilis* on *C. album* was 1.3 m for foliar total N content and 2.7 m for $\delta^{15}N$ values, and thus lower than that of *A. longifolia*, with 1.7 m for total N and 4.1 m for $\delta^{15}N$. Using the radius of the canopy size (Table 1) and adding the breakpoint distance, the impact range of *A. longifolia* on *C. album* was estimated to be 1.9-fold greater for foliar N and 3.8-fold greater for foliar $\delta^{15}N$ than its canopy cover. *S. spectabilis* showed a 3.8-fold larger impact range for impact on *C. album* foliar total N and a 8.2-fold larger impact range on foliar $\delta^{15}N$. As the impact areas are calculated relative to the canopy, they were larger for the native legume, however, the absolute area of influence was far greater for the invasive plant, 134 m^2 for *C. album* foliar total N and 250 m^2 for foliar $\delta^{15}N$, against 23 m^2 and 53 m^2, respectively, for the native. *C. album* foliar total N and $\delta^{15}N$ values were not significantly different between plants growing inside (IN) or outside (OUT) either *A. longifolia* or *S. spectabilis* (Fig. 2) but were generally higher in IN plants compared to OUT plants (Wilcoxon test, $n = 10$, $p < 0.001$). These results were used to express legume influence as % of change in *C. album* total foliar N and ‰ change for *C. album* foliar $\delta^{15}N$ values (Fig. 2, right boxes). In both cases, *C. album* plants growing in the transects measured around *A. longifolia* show a significantly larger change in total foliar N and foliar $\delta^{15}N$ values than plants in the transects measured around *S. spectabilis* (Wilcoxon test, $n = 5$, $p < 0.05$).

Contrary to total N and $\delta^{15}N$ values, no correlation was found between distance to legume canopy and foliar total P concentrations of *C. album*. However, the legume influence on *C. album* foliar total N shows a negative linear relationship ($n = 10$, $r = -0.63$, $p = 0.052$) with *C. album* foliar total P (Fig. 3, left) and legume influence on *C. album* foliar $\delta^{15}N$ exhibits a significant negative monotonic relationship with foliar total P ($n = 10$, $r_s = -0.673$, $p = 0.033$). Also, *C. album* plants in the transects measured around *A. longifolia* were significantly lower in foliar P (Wilcoxon test, $n = 5$, $p < 0.05$, Fig. 3, right).

Partial least-squares regression

Using a partial least-squares regression approach (PLSR) with an automatic backward model selection procedure allowed to determine the most important variables predicting the legume influence on the surrounding *C. album* vegetation. Biplots of the first two components showing sample scores and loadings of the variables can be found in Fig. 4. The first component of the respective models showed a high correlation with % of change in *C. album* total foliar N ($r^2 = 0.95$), *C. album* total foliar P ($r^2 = 0.67$) and ‰ of change for *C. album* foliar $\delta^{15}N$ values ($r^2 = 0.91$), while no significant correlation with the second components was evident. Utilising the loadings of the first component, the impact of each variable can be expressed as a percentage by calculating the loading weights, where loadings are normalised so that the sum of squares of all loadings within each component is summed to one (Carrascal et al. 2009).

In the model describing % of change in *C. album* total foliar N seven variables were retained (Fig. 4a). Variables related to P contents within the system (total P litter, total P roots and soil organic P) and belowground organic matter pools (SOM and root mass) were the most important predictors, accounting for 41% and 27%, respectively, while C turnover rates in the rhizosphere compartment and plant size each accounted for 16%. The model predicting *C. album* total foliar P (Fig. 4b) contained two variables, each accounting for 50% of the variance (root N content and C turnover rates). The model on ‰ of change for *C. album* foliar $\delta^{15}N$ values (Fig. 4c) contained three variables. Here, predictors were soil OM content (33%), root N content (each for 35%), and root mass (31%).

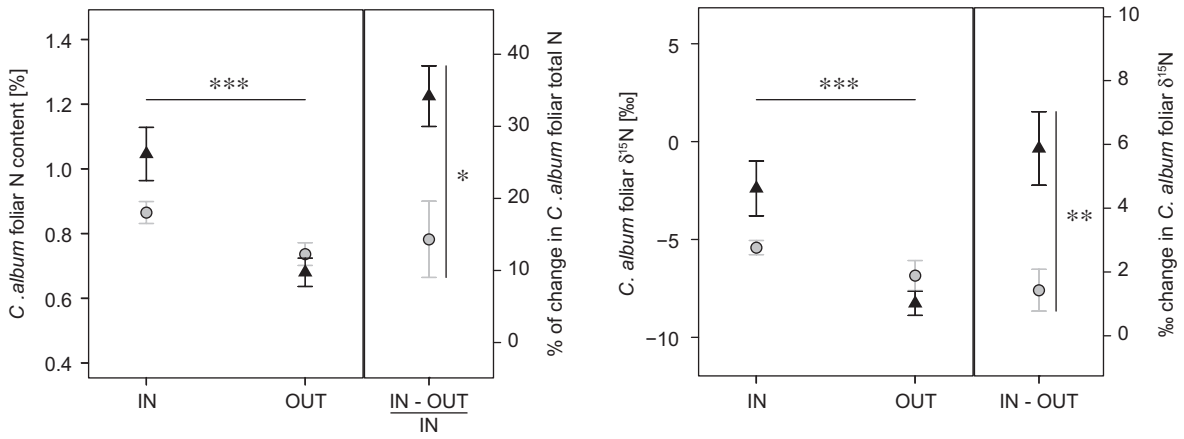

Figure 2. Effect of legume presence on total foliar N (%, a) and $\delta^{15}N$ (‰, b) of *C. album* foliage (mean ± SE, n = 5). Grey dots indicate values for *C. album* plants in the surrounding of *S. spectabilis* and black triangles indicate values from the surrounding of *A. longifolia*. Values for IN and OUT plants were derived from the breakpoint estimation described in Fig. 1, with IN values representing plants growing inside the canopy of the respective legume and OUT values being derived from plants growing outside of the legumes influence, i.e. farther away than the breakpoint (left boxes). Legume influence was then described in % of change in *C. album* total foliar N and ‰ change for *C. album* foliar $\delta^{15}N$ (right boxes). Asterisks indicate significant differences (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.01$; Wilcoxon test with n = 10 in left boxes, n = 5 in right boxes).

Discussion

Understanding plant invasions requires a thorough analysis of belowground processes, not only in terms of quantitative changes of soil OM pools, but also of nutrient stoichiometry and fluxes (Hulme et al. 2013). The work presented here highlights these considerations and adds P as an important nutrient to understand the impact of an ecosystem engineering plant in an oligotrophic model system. Confirming earlier observations (Hellmann et al. 2011, Rascher et al. 2012) of a spatial *Acacia longifolia* impact on foliar N and $\delta^{15}N$ of surrounding *Corema album* vegetation (Fig. 1), the results furthermore show legume influence on *C. album* N and $\delta^{15}N$ are negatively correlated with *C. album* foliar P concentrations (Fig. 3, left). This fits to the hypothesis of induced N/P imbalance, as it indicates the onset of systemic P depletion while N availability increases. The effect is significantly stronger for the invasive (Fig. 3, right), aligning *A. longifolia* with other *Acacia* tree species that have been shown to shift N and P co-limited ecosystems to a stronger P limitation (Sitters et al. 2013).

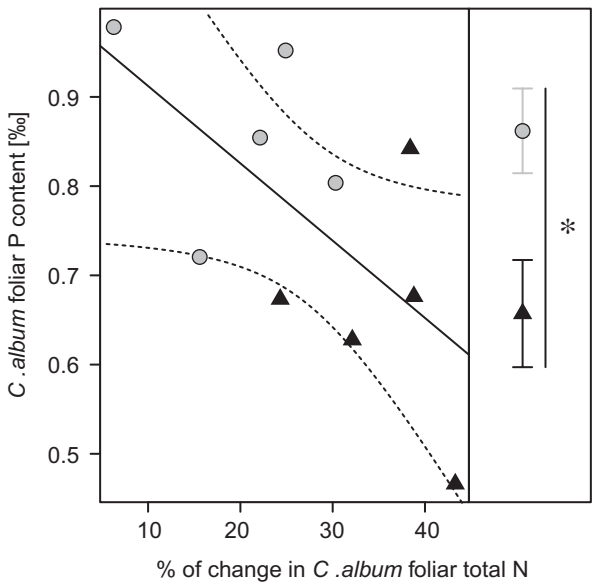

Figure 3. On the left: Relationship between % of change in *C. album* foliar total N and foliar total P (‰), dotted lines indicate the 95% confidence interval. Grey dots indicate values for *C. album* plants in the surrounding of *S. spectabilis* and black triangles indicate values from the surrounding of *A. longifolia*. % of change in *C. album* foliar total N was derived from IN and OUT plants as described in Fig. 2. *C. album* foliar P content was calculated per legume plant as the mean of all surrounding C. album plants (n = 15). On the right: *C. album* foliar total P (‰, mean ± SE, n = 5) of all *C. album* plants from five *A. longifolia* (black triangles) and five *S. spectabilis* (grey dots). Asterisks indicate significant differences (* = $p < 0.05$, Wilcoxon test with n = 5).

The decrease in *C. album* foliar P was related with higher C turnover rates in the rhizosphere and higher root N (Fig. 4b), while the increase in % of change in *C. album* total foliar N was related with depleted legume tissue P, increased SOM and root mass (Fig. 4a). Both root and rhizosphere mass were increased underneath the invasive *A. longifolia*, compared to the native *Stauracanthus spectabilis* (Table 1). Increased root mass is a known trait of invasive *Acacia* species (Morris et al. 2011) and especially important for plant invasions in low resource environments (Funk 2013). Root N content in turn is involved in mediating microbial C turnover efficiency (Carrillo et al. 2014) and microbial nutrient cycling in the rhizosphere (Jones et al. 2009). The rhizosphere is crucial for the understanding of plant invasions, due to its role in establishing soil structure (Philippot et al. 2013) and as a proxy of plant–plant (Sanon et al. 2009) and plant–soil interactions (Callaway et al. 2004). The rhizosphere of *A. longifolia* consistently exhibited higher specific EEA (Table 3), which indicates higher resource availability and microbial growth (Sinsabaugh et al. 2009). In this study, microbial activity, indicated by ΣC EEA values,

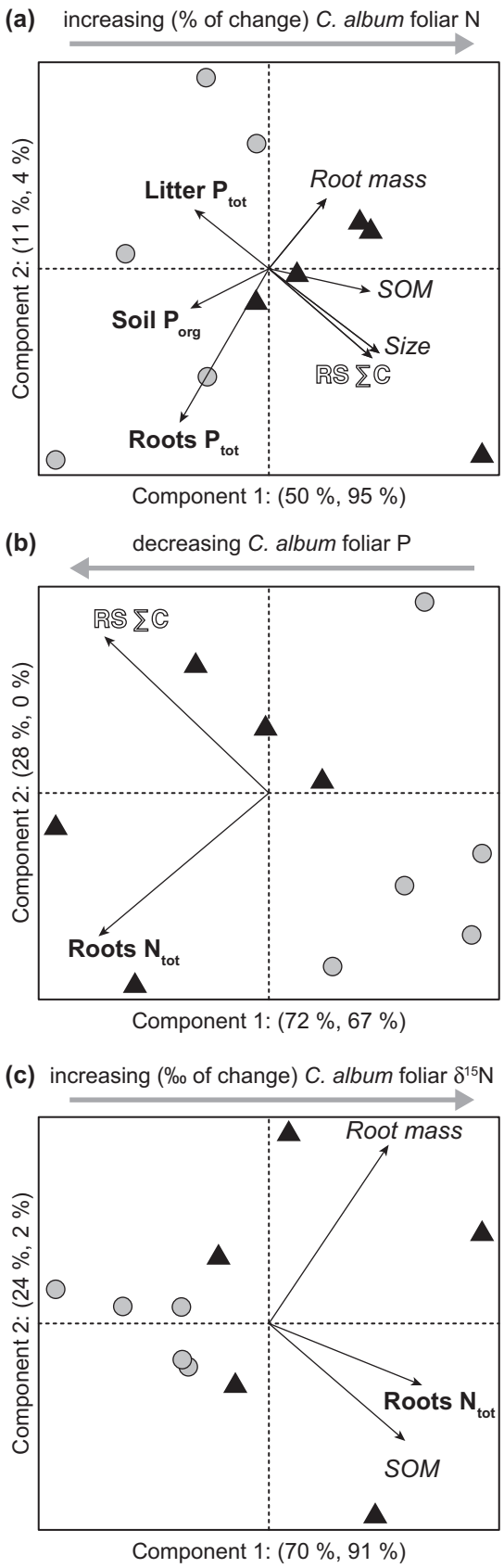

Figure 4. Biplots showing sample scores (dots) and loadings of selected variables (labelled arrows) on the first two components of partial least squares regression (PLSR) models predicting % of change of *C. album* foliar N (a), ‰ total *C. album* foliar P (b) and ‰ of change of *C. album* foliar $\delta^{15}N$ (c). Grey dots indicate samples from the surrounding of *S. spectabilis* and black triangles samples from the surrounding of *A. longifolia*. The models were optimized, selecting significant variables by employing an automatic model selection procedure based on variable importance in the projection (VIP) values and significance for prediction. Percentages given in the axis titles are variances in X (predictor space/independent variables) and Y (dependent variable) explained by the respective component. Variables in bold are related to tissue nutrient concentrations, variables in italic are related to biomass and variables with black contours are related to turnover rates. RS = rhizosphere, ΣC = β-glucosidase + β-xylosidase + β-glucuronidase + cellobiohydrolase, P_{tot} = total phosphorus, N_{tot} = total nitrogen, SOM = soil organic matter, P_{org} = organic phosphorus.

in the rhizosphere of the legumes was found to be a crucial variable in explaining legume influence on *C. album* N and P (Fig. 4a–b). Rhizosphere β-1,4-N-acetylglucosaminidase (Nag) and Ap activity, were also negatively correlated with legume leaf P (Spearman's rank correlation: $r_s = -0.78$, $p < 0.01$ and $r_s = -0.79$, $p < 0.05$, respectively), which might furthermore indicate a strong P demand of the legumes themselves, especially *A. longifolia*, which additionally exhibited significantly higher values of root Ap.

In contrast to root and rhizosphere mass, litter mass was not different between native and invasive legumes (Table 1), even though *Acacia* plants are known to accumulate thick litter layers underneath their canopy (Marchante et al. 2008, Hellmann et al. 2011), which is a frequent plant trait associated with ecosystem engineering (Fei et al. 2014). Also, litter P was not found to be a significantly related with *C. album* foliar P levels, but had a negative relation with legume influence on *C. album* foliar N. This could be due to high P-resorbing efficiencies seen in other *Acacia* spp. (He et al. 2011) and the potentially poor litter quality of both legumes as indicated by C/N and C/P ratios in the litter (Table 1). Poor substrate quality induces lower C use efficiency of the microbial degrader community (Manzoni et al. 2010) and the observed higher ΣC EEA values in the invasive's litter (Table 3) could point to an enhanced litter degradation, which is a frequent occurrence in invaded ecosystems (Liao et al. 2008). During litter degradation, N and P are likely released and while in sandy soils around legume canopies soil N has been shown to be influenced in a spatial manner, soluble P did not follow this pattern (Rodríguez et al. 2009). The high relevance of increased litter degradation and soil nutrient fluxes during the invasion process is further substantiated by the observation that in its native range *A. longifolia* can itself be outcompeted by an invasive weed, *Chrysanthemoides monilifera*, which exhibits even higher litter decomposition rates (Lindsay and French 2005).

Interestingly, while increased ΣC EEA and higher root N values, indicators of increased microbial activity and nutrient turnover, were related with the decrease in *C. album* foliar P, ΣC EEA values increased with legume influence on *C. album* foliar N (Fig. 4a). There is evidence that increased turnover and liberal movement of N between plants could be a mean to decrease competition and increase plant aggregation in a situation where N and P are co-limiting (Teste et al. 2015). Indeed, Mediterranean dunes are known

to be both very N and P limited (Martínez et al. 1998) and extractable P levels of the soil measured here (Table 2) were half of those reported earlier for similar systems (Marchante et al. 2008), thus being indicative of severely nutrient-deficient soil (Funk 2013). However, while liberal movement of N seems to occur in the system observed here (Fig. 1a) which should help sustaining biodiversity (Teste et al. 2015), it is known that *A. longifolia* decreases biodiversity during invasion by increasing monospecific plant cover and outcompeting the native vegetation (Marchante et al. 2008, Hellmann et al. 2011).

This discrepancy might be explained by a high phenotypic plasticity in resource acquisition, coupled with low resource usage in biomass production, which is a strategy of invasive species frequently observed in low-resource ecosystems (Gioria and Osborne 2014). *Acacia longifolia* shows high rhizosphere and litter EEA values and strong investment in belowground biomass, which all contribute to more rapid nutrient cycling and thus resource availability. At the same time, the relation of stronger legume influence on *C. album* N with lower P concentrations in roots and litter (Fig. 4a) could be an indicator for low P usage in biomass production, as *A. longifolia* tissue P concentrations were lower compared to the native legume (Table 1). Species from Australian Mediterranean-type regions often exhibit lower foliar P values as an adaption to their natural low-nutrient habitats (Stock and Verboom 2012). Indeed, *A. longifolia* foliar N concentrations were within range of earlier reports from comparable systems (Marchante et al. 2008, Hellmann et al. 2011), while foliar P concentrations of the invasive were comparable to values reported for several Acacia species under P-impoverished conditions (He et al. 2011). Thus, the resulting foliar N/P ratios between 10 and 20 of the native legume indicate co-limitation for both nutrients (Güsewell 2004) while N/P ratios of *A. longifolia* demonstrate a strong P depletion. However, even though these findings support a P limitation of *A. longifolia*, it is growing to a larger size than the native *S. spectablis* (Table 1), which is related to increasing legume influence on *C. album* N (Fig. 4a). Differences in canopy size also reflect the different growth forms of the legumes, with *S. spectabilis* growing as a shrub, while *A. longifolia* is creating singular, small trees in this early stage of invasion (Hellmann et al. 2011). Furthermore, impact on *C. album* foliar N and $\delta^{15}N$ is related to high SOM beneath *A. longifolia* (Fig. 4a, c), while in contrast, the native *S. spectabilis* accumulates slowly decaying litter underneath its canopy. Furthermore, being a spiny legume with highly reduced leaves (Barradas et al. 1999), *S. spectabilis* likely recycles its shoot N, which would retain N within the plant and make it less available to the surrounding vegetation. This is in line with its foliar $\delta^{15}N$ values, as N retention can lead to an enrichment in foliar $\delta^{15}N$ (Unkovich 2013) and depleted *A. longifolia* foliar $\delta^{15}N$ values compared to *S. spectabilis* were previously reported (Hellmann et al. 2011). Depletion of foliar $\delta^{15}N$ values could furthermore indicate P deficiency (Lazali et al. 2014), however, symbiotic nitrogen fixation is barely affected by insufficient P (Augusto et al. 2013), and the work presented here indicates that *A. longifolia,* like other *Acacia* species (Inagaki and Tange 2014), might show a high P use efficiency compared to other N_2 fixing legumes.

In conclusion, the comparison of a native and an invasive legume yielded the most important predictors for their effect on the surrounding vegetation and thus putative reasons for the invasiveness of *A. longifolia* in this system. In contrast to *S. spectabilis*, *A. longifolia* exhibited an efficient P use in biomass production (low P concentrations) and created a belowground structure consisting of fine roots coupled to a rhizosphere capable of rapid organic matter turnover within its own canopy. In this highly oligotrophic system, the ability of the invasive species to rapidly cycle nutrients may be considered an essential mechanism for its success. As it supplies N to the surrounding vegetation, while requiring substantial amounts of P itself, *A. longifolia* creates an N/P imbalance at community level. Thus, our work gives evidence that the influence on belowground processes and plant–soil-interactions, specifically on community P balance, might substantially contribute to explain the ecosystem engineering character of invasive *Acacia* spp. and their huge ecological impact in dune systems worldwide.

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Supplementary material (available online as Appendix oik-03810 at <www.oikosjournal.org/appendix/oik-03810>). Appendix 1.