

# HOW TO OUTGROW YOUR NATIVE NEIGHBOUR? BELOWGROUND CHANGES UNDER NATIVE SHRUBS AT AN EARLY STAGE OF INVASION

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## ABSTRACT

While it is acknowledged that invasive species are a global driver of land degradation, their effects are often only noticed when the invasion has been going on for a while. However, early stage processes must play a fundamental role in plant establishment until invasive plants are able to outgrow the native vegetation. In ten plots of 100 m<sup>2</sup> each, we tested the hypothesis that belowground properties are associated with early invasion processes aboveground. We examined the early stage of invasion by a woody legume (*Acacia longifolia*), growing in the canopy of native dune shrubs (*Corema album*) as a model system in oligotrophic primary dunes in southern Portugal. Biomass under canopies of invaded and non-invaded *C. album* shrubs as well as organic matter (OM) distribution in various soil fractions was measured. In accordance with our hypothesis, *A. longifolia* presence was related to increased *C. album* foliar  $\delta^{15}\text{N}$ , a proxy for nitrogen derived from the invasive legume. Under invaded canopies, root and rhizosphere biomass were higher, as was OM in the silt-clay fraction. Also,  $\delta^{15}\text{N}$  of the OM in the silt-clay fraction under invaded canopies was enriched, while  $\delta^{13}\text{C}$  was depleted. Finally, we found that the ratio between OM in the biotic versus soil compartment could be a good early indicator for invasion. These findings suggest that even when aboveground invasion pressure on the system is low, it is imperative for ecosystem conservation to remove young plants, as they might alter soil functioning already at an early stage of invasion. Copyright © 2017 John Wiley & Sons, Ltd.

KEY WORDS: dunes; plant invasion; co-facilitation; *Acacia longifolia*; *Corema album*

## INTRODUCTION

The introduction of invasive plant species into novel habitats leads to the degradation of many ecosystems worldwide and poses a fundamental challenge to the conservation of biodiversity (Simberloff *et al.*, 2013). One of the major global gateways for plant invasion is the anthropogenic introduction of non-native plant species for dune stabilization, which might then turn invasive over time (Doody, 2013). However, while some invasive plant species can be more efficient in dune stabilization than native plants, reduction in native plant biodiversity and abundance will ultimately decrease the resilience of these ecosystems (Charbonneau *et al.*, 2017).

Oligotrophic systems, such as dunes, are very vulnerable to plant invasions, for example due to resource competition between native and invading plant species (Martínez & García-Franco, 2008). Especially woody legumes have strong effects on oligotrophic ecosystems due to their longevity and biomass production (Richardson *et al.*, 2014), as well as their ability to fix atmospheric nitrogen (N), which makes them prolific ecosystem engineers (Rundel *et al.*, 2014). As dunes are naturally characterized by intermediate regular disturbance, they are particularly vulnerable to the biogeomorphic effects of ecosystem engineers, for example

soil fixation by roots and soil organic matter (OM) accumulation (Fei *et al.*, 2014). Thus, belowground processes play a crucial role in the invasion process (Nuñez & Dickie, 2014) and plant–soil interactions are particularly important in early facilitation processes as found in oligotrophic systems (Van der Putten *et al.*, 2013).

Very little is known about the early stage of plant invasion with low plant densities (Elgersma & Ehrenfeld, 2011). An important factor to consider in dune systems is facilitation of seedlings by nurse plants (Armas & Pugnaire, 2005), and positive plant–plant interactions seem to be more dominant than competition at an early stage of these ecosystems (Martínez & García-Franco, 2008). However, the shift from facilitation to competition is gradual and dependent on the site of interaction. For example, it was found that the interaction between nursery shrubs and tree seedlings might range from facilitation under environmental stress to competition in less stressful conditions (Muhamed *et al.*, 2013).

In the Portuguese dune systems, one of the most prolific invasive species is *Acacia longifolia*, with highly detrimental impacts on native vegetation and soil (Marchante *et al.*, 2008). The most severe impacts on invaded ecosystems are a strong decline in native species richness (Marchante *et al.*, 2015), as well as the establishment of an abundant seed bank of *A. longifolia*, accompanied by soil eutrophication (Marchante *et al.*, 2008). The invasion process is presumed to start in the nutrient patches developed underneath existing native vegetation, which act as nursery plants (Peperkorn, 2005) and where *A. longifolia* grows

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more predominant than in the open soil. As *A. longifolia* is highly competitive under sufficient nutrient supply, as well as in nutrient poor conditions due to its ability to fix N (Werner *et al.*, 2010), it starts to outgrow its native nursery plant. Subsequently, the ecosystem engineering process continues and changes on soil level strengthen invasion (Marchante *et al.*, 2008) escalating the invasion further along time (Marchante *et al.*, 2015). Medium-term to long-term effects on soil functioning include changes in carbon (C), N (Marchante *et al.*, 2008) and phosphorus (P) pools (Ulm *et al.*, 2017), which are mainly related to altered soil catabolism, as well as a thick litter layer (Marchante *et al.*, 2008) and increased root mass (Morris *et al.*, 2011). These processes ultimately shift the primary dune system from an open vegetation structure of native shrubs to almost exclusive monospecific stands of medium-sized *A. longifolia* trees (Rascher *et al.*, 2012).

Interestingly, while the long-term effects of *A. longifolia* on the native vegetation are clearly negative, it has been shown that native shrubs like the Ericaceae *Corema album* can initially benefit from the increased N availability (Hellmann *et al.*, 2011; Rascher *et al.*, 2012). Using tracers such as foliar  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , it has been shown that *C. album* exhibits higher growth rates and foliar N content with *A. longifolia* presence (Hellmann *et al.*, 2011). These effects are exceeding *A. longifolia* canopy limits (Rascher *et al.*, 2012) and might have secondary effects on *C. album*, such as increased photosynthetic capacity through better plant N nutrition (Hellmann *et al.*, 2016). On the longer term, however, *A. longifolia* dominates *C. album* due to high growth rates and phenotypic plasticity, consequently increasing competition for resources like water (Hellmann *et al.*, 2016) or P (Ulm *et al.*, 2017). Thus, in the invasion process, the coexistence of both plants shifts from facilitation of *C. album* to co-facilitation by both *A. longifolia* and *C. album* and culminates in a competition, which ultimately ends favourably for the invader.

While the later stages of invasion, co-facilitation and competition, are well studied (Hellmann *et al.*, 2011; Marchante *et al.*, 2008, 2015), the initial phase of invasion, changing facilitation to co-facilitation and beginning competition, is less clear. Most of the competitive advantage of the invasive species can be related to its biomass production, for example its litter layer. However, at the beginning of the dune invasion, biomass of *A. longifolia* is still very low and no litter layer has yet developed. Thus, self-facilitation by its own aboveground biomass is therefore not yet viable. The invader nevertheless outgrows its native nursery shrub, which, as no invasive aboveground material is available yet, pinpoints to belowground processes being changed by the presence of the invasive species. Belowground biomass accumulation is a typical process for ecosystem engineering plants (Jones *et al.*, 1996) and apart from roots, also the rhizosphere increases underneath large *A. longifolia* specimen (Ulm *et al.*, 2017), as does soil OM content (Hellmann *et al.*, 2011). Assessing processes occurring in the rhizosphere can be crucial to understand plant invasion

(Philippot *et al.*, 2013), as the rhizosphere is the main site for plant-microbe interactions that mediate OM turnover rates (Ulm *et al.*, 2017). Also, soil OM increase has been shown to be critical in invasion, as it creates a positive feedback loop between plant and soil (Marchante *et al.*, 2008), thus exacerbating the invasion processes. Not only soil OM pools have been shown to be important but also their P concentrations, as *A. longifolia* generates an NP imbalance in plant biomass over time as it increases N input while demanding P for constant growth (Ulm *et al.*, 2017). Also, OM might accumulate through stabilization in certain soil fractions, for example the silt-clay fraction (Six *et al.*, 2002), which is known to increase after plant invasions in other dune-like systems (e.g. salt marshes) (Schwarz *et al.*, 2016). However, not only the quantity of this fraction but also its quality has to be considered, for example C/N ratios or  $\delta^{13}\text{C}$ , which can give information about turnover and residence times (e.g. McClaran *et al.*, 2008).

In the work presented here, we first wanted to confirm that *A. longifolia* plants without developed litter layer have an effect on *C. album* shrubs, using *C. album* foliar  $\delta^{15}\text{N}$  as an ecological tracer. Subsequently, we aimed to connect aboveground responses with belowground changes, focussing especially on plant biomass and soil OM. Due to earlier observations (Ulm *et al.*, 2017), we expected differences in biomass N and P concentrations. Furthermore, we postulated differences in the silt-clay fraction of the soil under invaded plants as a proxy for stabilized soil OM. While we assumed the overall quantity of this fraction to be miniscule due to the extremely oligotrophic nature of this sand dune system, we wanted to explore its potential to indicate biomass flows and soil mechanism changes during early invasion. Lastly, we explored the ratio of belowground plant biomass versus soil OM as a proxy for invasion impact.

## MATERIALS AND METHODS

### *Study Site and Sampling Design*

Sampling took place from 12 to 28 July 2013 in the coastal sand dune ecosystem of Pinheiro da Cruz, Portugal (N38° 15'2", W 8°45'8"), which is invaded by the non-native leguminous tree *Acacia longifolia* (Andrews) Willd. (Fabaceae). The site is located in the primary dunes, which are characterized by poor arenosols (FAO classification) and an open vegetation structure (Rascher *et al.*, 2012), which is dominated by *Corema album* (L.) Don (Ericaceae), a shrub endemic to the Iberian Peninsula.

Sampling took place in a 10,000 m<sup>2</sup> area, using ten plots of 100 m<sup>2</sup> each, laid out randomly, but more than 50 m apart and containing at least one *A. longifolia* plant growing inside the canopy of a *C. album* shrub. Already developed stands or shrubs were not considered, as the aim was to analyse plants in a very early stage of invasion, for example <2 m height and without any aboveground litter. After counting all *A. longifolia* and *C. album* plants in each plot, their canopy sizes were estimated by an ellipsoid and their volumes estimated assuming an upper half spheroid shape

$(4/3 * c_1 * c_2 * \pi * h * 0.5)$ , following recommendations for shrubs in arid landscapes by Ludwig *et al.* (1975).

Per plot, soil was sampled with a metal tube (8.5 cm diameter, 19.5 cm height) once within the canopy of a *C. album* plant growing far away (>5 m) from any *A. longifolia* plant (–Inf) and once within the canopy of a *C. album* plant with an *A. longifolia* plant growing inside its canopy (+Inf). Bulk samples were separated into four fractions: sand, roots, rhizosphere and large OM particles (>2 mm), which were termed coarse OM. The sand fraction was removed from the bulk-agglomerated rhizosphere/root in the core and sieved through a 2 mm sieve. Rhizosphere was separated from roots by gently shaking the adhered particles off the roots and subsequently treating them similar to the soil as described in Ulm *et al.* (2017). Parts of all fractions were oven-dried at 60 °C until constant weight and 500 mg subsequently ashed in a muffle furnace (600 °C, 24 h) to determine OM by the loss on ignition method.

#### Soil Fractionation and Phosphorus Determination

One hundred grams of dry, sieved (2 mm) soil sample was fractionated by using an automatic vibratory sieve shaker (Fritsch Analysette 3, Fritsch, Idar-Oberstein, Germany) and two sieve sizes: 425 and 63 µm. Resulting fractions were termed >425, >63 and <63 µm. The sieving programme used had an interval time of 10 s, with 3 min sieving time and an amplitude of 3 mm; mean sample recovery rate of the procedure was more than 99.84%. Subsequently, each fraction was analysed for OM and total phosphorus (P). Only the fraction <63 µm was analysed for % C, % N,  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ , as they, similar to total soil OM, were below detection limit (Rascher *et al.*, 2012).

For total P of soil and OM, samples were ignited, subsequently acid-extracted (HCl, 1 M) and a malachite-green-based microscale method employed as described by D'Angelo *et al.* (2001). The colorimetric method was executed in 250-µl 96-well flat bottom microtiter plates and analysed in a microplate reader (Rainbow, Tecan, Männedorf, Switzerland). For each single assay, a separate triplicate calibration curve was produced with  $\text{KH}_2\text{PO}_4$  as a serial dilution in ultrapure water.

#### Elemental Analysis and Isotopic Ratios

Dry samples of foliage and the soil fraction <63 µm were ground to a fine powder in a ball mill (Retsch, Haan, Germany). Powder ( $5 \pm 0.2$  mg) was weighted into tin capsules and analysed at the Stable Isotopes and Instrumental Analysis Facility of the Centre for Ecology, Evolution and Environmental Change, University of Lisbon – Portugal. The  $^{13}\text{C}/^{12}\text{C}$  and  $^{15}\text{N}/^{14}\text{N}$  ratios in the samples were determined by continuous flow isotope-ratio mass spectrometry on an Isoprime (GV, UK) stable isotope ratio mass spectrometer coupled to an EuroEA (EuroVector, Italy) elemental analyser for online sample preparation by Dumas-combustion. All values are given in  $\delta$  notation. The standards used were International Atomic Energy Agency N1 (IAEA-N1) and U.S. Geological Survey-35 for N

isotope ratio and IAEA-CH6 and IAEA-CH7 for C isotope ratio;  $\delta^{15}\text{N}$  results were referred to Air and  $\delta^{13}\text{C}$  to PeeDee Belemnite. Precision of the isotope ratio analysis, calculated by using values from 6 to 9 replicates of laboratory standard material interspersed among samples in every batch analysis, was  $\leq 0.2\%$ .

#### Statistical Analysis

All tests were performed with the package 'stats' by using R version 3.3.2 (R Core Team, 2016). Density distribution of plant volume was projected using the *density()* function to compute kernel density estimates. Group wise comparisons were tested for using the pairwise Wilcoxon rank sum test with Holm correction; if only two groups were compared, a simple Wilcoxon rank sum test was used. Linear relationships were tested against heteroskedasticity by using the Breusch-Pagan test (package 'lmtest'), and the residuals were tested for normality using the Shapiro–Wilk normality test.

Multiple factor analysis (MFA) as described by Borcard *et al.* (2011) was used as an exploratory, symmetrical method to test for relationships between the different variable subsets using the function *MFA()* (package 'FactoMineR'). MFA is fundamentally a principal component analysis on the correlation matrix of each subset, which are then weighed and subsequently combined in a global principal component analysis. Variables used were grouped in OM pools (roots, rhizosphere, coarse OM and OM of soil fractions: >425, >63 and <63 µm), characteristics of the soil fraction <63 µm (N/P ratio, C/N ratio,  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ ) and foliar characteristics and plant size (N/P ratio, C/N ratio,  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$  and plant volume). RV coefficients were calculated for the variable sets used in the MFA, as well as on the two variables resulting from the matrix of plant OM contribution to total OM and total P. RV coefficients are a multivariate version of the squared Pearson correlation coefficient ( $r^2$ ) and thus always positive. The RV coefficients were then tested for significance using a permutational analysis from the function *coeffRV()* (package 'FactoMineR').

## RESULTS

#### Invasion Pattern and Aboveground Plant Characteristics

Within the 10,000 m<sup>2</sup> covered here, ten young *A. longifolia* plants were found; thus, from the 170 *C. album* specimen examined, 5.88% were invaded. The density distribution of plant sizes by volume (Fig. 1) showed no specific *C. album* volume that was more prone to invasion in this area. This was further tested by using a Kolmogorov–Smirnov test, which rejected a significant difference between distributions ( $n_{C. album (total)} = 170$ ,  $n_{C. album (invaded)} = 10$ ,  $D = 0.282$ ,  $p$ -value = 0.438).

The invasive *A. longifolia* plants studied here were marginally higher than the native *C. album* shrubs but had smaller canopy sizes, and there were no significant differences found between plant volumes (Table I). As *A. longifolia* was growing within *C. album*, the addition of both plant volumes was used as a conservative estimate of joined



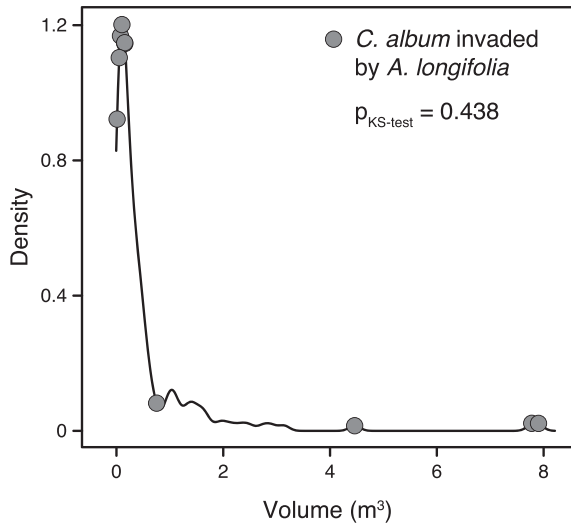


Figure 1. Kernel density estimation of *Corema album* size, expressed in volume (m<sup>3</sup>). The grey spheres show *C. album* shrubs invaded by *Acacia longifolia*. Distribution was estimated from *n* = 170 plants, with ten plants being invaded. The *p*-value depicts results of a Kolmogorov–Smirnov test for a significant difference between distributions of invaded and non-invaded *C. album* shrubs.

plant size, which does not significantly differ from the plant volume of non-invaded *C. album* (Wilcox test, *n* = 10 for combined *A. longifolia* and *C. album* and *n* = 9 for non-invaded *C. album*, *p* = 0.90).

Foliar N concentration was ~3-fold higher in the invasive plant, while total C was significantly lower and C/N ratios were about half of the values observed in native *C. album* shrubs (Table I). The foliage of *A. longifolia* exhibited the least depleted δ<sup>15</sup>N values, while foliage of non-invaded *C. album* was significantly more depleted and values of invaded *C. album* were in between. While P content is similar in both species, *A. longifolia* exhibited about 3 times higher N/P ratios (Table I).

*Belowground Mass, Organic Matter and P Pools in Invaded and Non-invaded C. album*

Various belowground changes were found between invaded and non-invaded *C. album* plants (Fig. 2). As the soil cores

taken had a length of 19.5 cm, only topsoil changes were observed here. Also, the changes in root mass, OM and total P (P<sub>tot</sub>) pools were not attributed to a singular plant but are rather a mix of both *A. longifolia* and *C. album* mass. The presence of *A. longifolia* plants in the canopy of *C. album* was found to significantly increase root mass and OM by a factor of 10, as well as P<sub>tot</sub> by a factor of 40. There was also a 14-fold increase of rhizosphere mass and OM under invaded *C. album* canopies. This significantly increased belowground plant biomass in invaded canopies in general (5.5-fold), as well as plant biomass contribution to total OM (2.4-fold) and P<sub>tot</sub> (4.9-fold). Also, taking all compartments (plant mass and soil fractions) together, total OM and P<sub>tot</sub> were higher underneath invaded plant canopies. Invaded plant canopies also showed marginally higher P<sub>tot</sub> values in the rhizosphere compartment. P<sub>tot</sub> accumulation under both invaded and non-invaded canopies was mostly related to root (*r*<sub>s</sub> = 0.7, *p* < 0.001, Spearman’s rank correlation) and rhizosphere mass (*r*<sub>s</sub> = 0.6, *p* < 0.01, Spearman’s rank correlation), which can also be depicted as a ratio between soil P and plant P (or OM respectively), where the soil under canopies of invaded *C. album* plants exhibited a significantly higher percentage of plant contribution to both total P and OM (Fig. 3c). In contrast to the soil fractions bigger than 425 and 63 μm, the smallest soil fraction (<63 μm) was also significantly increased underneath invaded plant canopies, both in terms of total mass and in terms of OM (both 2.3-fold). While minuscule in comparison to the other soil fractions with 0.1% contribution to total weight, OM content in this fraction doubled under invaded plants, making up 4% of the total soil OM.

Apart from changes in the overall plant biomass, also, the contribution of plant-derived material (roots, rhizosphere and coarse OM) to overall OM (plant-derived OM + OM of soil fractions) was significantly different (Fig. 3c), which was also the case for P<sub>tot</sub>. These values are percentages as they express the relative contribution to total mass in order to indicate the OM and P balance in the root-soil system. The increase of plant OM contribution to total OM was positively correlated with *C. album* foliar δ<sup>15</sup>N (Fig. 3c), which is the only variable to change significantly on foliar level

Table I. Differences between growth form and foliar nutrient content as well as stable isotope values of *Acacia longifolia*, invaded (+Inv) and non-invaded *Corema album* (– Inv)

		<i>A. longifolia</i>	<i>C. album</i> (+Inv)	<i>C. album</i> (– Inv)
Growth form	Height (m)	1.2 (0.2) <sup>a</sup>	0.7 (0.1) <sup>a</sup>	0.7 (0.1) <sup>a</sup>
	Canopy (m <sup>2</sup> )	<b>0.6 (0.1)<sup>a</sup></b>	<b>3.7 (1.6)<sup>ab</sup></b>	<b>3.5 (1.4)<sup>b</sup></b>
	Volume (m <sup>3</sup> )	0.6 (0.2) <sup>a</sup>	2.5 (1.2) <sup>a</sup>	1.5 (0.6) <sup>a</sup>
Foliar nutrient concentration and stable isotope values	δ <sup>15</sup> N	<b>-2 (0.2)<sup>a</sup></b>	<b>-6.1 (0.6)<sup>b</sup></b>	<b>-7.7 (0.6)<sup>c</sup></b>
	δ <sup>13</sup> C	-26.5 (0.5) <sup>a</sup>	-25.8 (0.3) <sup>a</sup>	-25.8 (0.2) <sup>a</sup>
	% N	<b>1.7 (0.2)<sup>a</sup></b>	<b>0.7 (0.1)<sup>b</sup></b>	<b>0.6 (&lt;0.1)<sup>b</sup></b>
	% C	<b>48 (0.9)<sup>a</sup></b>	<b>52.9 (0.3)<sup>b</sup></b>	<b>53.4 (0.2)<sup>b</sup></b>
	% P	0.5 (0.1) <sup>a</sup>	0.6 (0.1) <sup>a</sup>	0.6 (0.1) <sup>a</sup>
	C:N	<b>46 (19.3)<sup>a</sup></b>	<b>81.5 (5.8)<sup>b</sup></b>	<b>95.3 (3.8)<sup>b</sup></b>
	N:P	<b>37.5 (6.7)<sup>a</sup></b>	<b>13 (1.8)<sup>b</sup></b>	<b>10.2 (0.9)<sup>b</sup></b>

The letters indicate significant differences resulting from a pairwise Wilcoxon rank sum test with Holm correction, with *n* = 10 for *A. longifolia*, *n* = 10 for *C. album* (+Inv) and *n* = 9 for *C. album* (– Inv). Significant differences between groups are shown in bold.

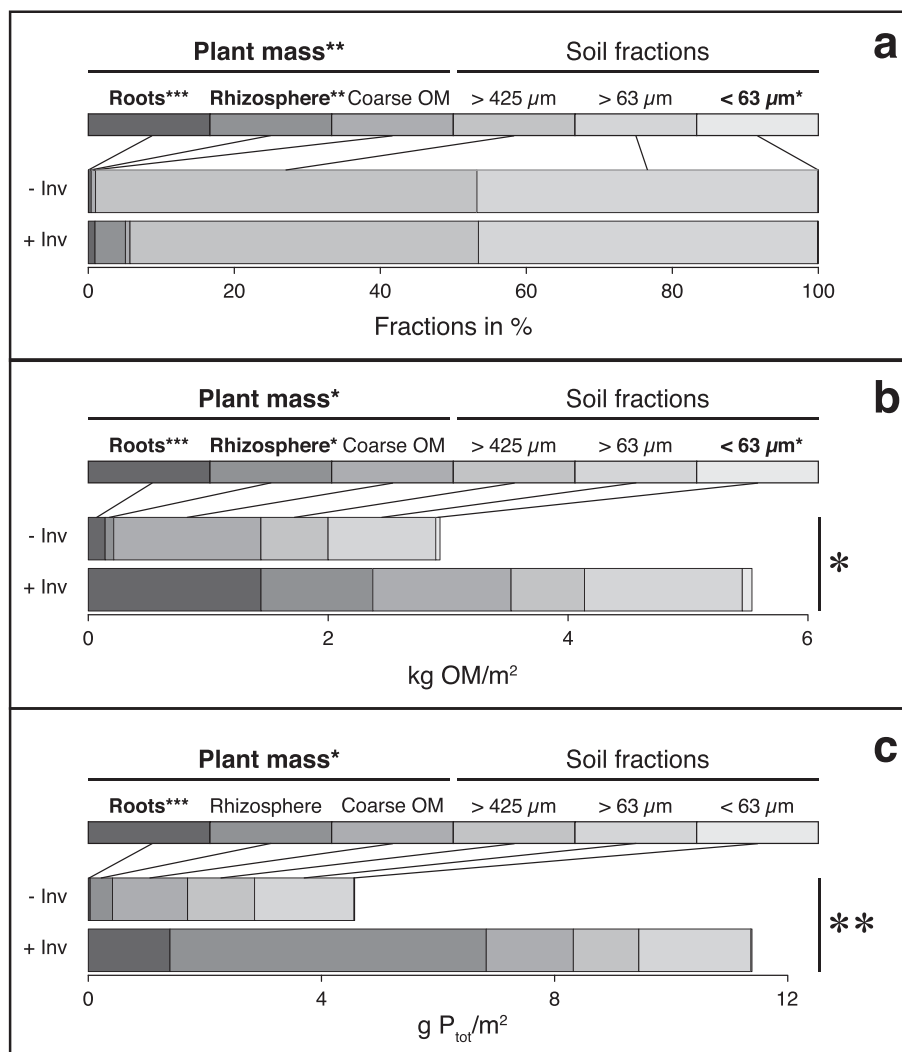


Figure 2. Belowground mass pools underneath non-invaded (–Inv) and invaded (+Inv) *Corema album* plants. (a) The mass balance in dry weight, (b) the mass balance of organic matter (OM) per  $m^2$  and (c) the mass balance of total phosphorus ( $P_{tot}$ ) per  $m^2$ . All these values were measured in the topsoil (20 cm). The asterisks highlight significant differences, \* =  $p < 0.05$ ; \*\* =  $p < 0.01$ ; \*\*\* =  $p < 0.001$  (Wilcoxon rank sum test,  $n = 10$  for +Inv;  $n = 9$  for –Inv).

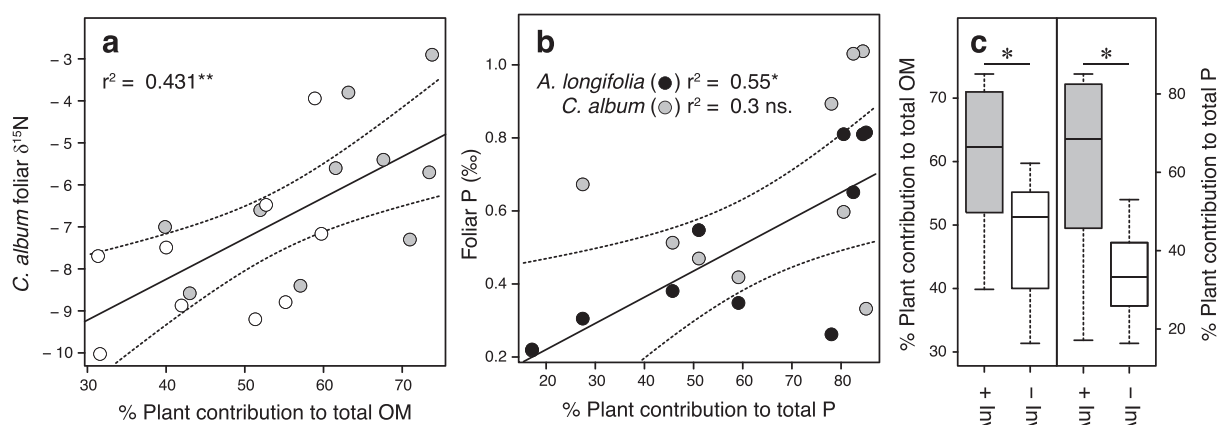


Figure 3. (a) Effect of plant-derived OM (roots, rhizosphere and coarse OM), expressed as % of contribution to total organic matter, on the foliar  $\delta^{15}N$  of *C. album* plants.  $R^2$  is result of a Pearson correlation; the dotted lines depict 95% confidence intervals. The grey spheres show invaded *C. album* plants; the white spheres show non-invaded *C. album* plants. (b) Effect of plant phosphorus (roots, rhizosphere and coarse OM), expressed as % of contribution to total phosphorus, on the foliar P concentration of *Acacia longifolia* (black spheres) and invaded *C. album* plants (grey spheres).  $R^2$  is a result of a Pearson correlation; the dotted lines depict 95% confidence intervals; the correlation was not significant for *C. album* plants. (c) Mass ratios of plant OM contribution to total OM (left box) and to total P (right box) of *C. album* plants invaded (grey) and non-invaded (white) by *A. longifolia*. The asterisks highlight significant differences, \* =  $p < 0.05$  (Wilcoxon rank sum test,  $n = 10$  for +Inv;  $n = 9$  for –Inv).

after *A. longifolia* invasion (Table I). The increase of plant contribution to total P in turn was positively correlated with *A. longifolia* foliar P ratios, but not with *C. album* foliar P (Fig. 3b).

*Nutrient Concentrations and Isotopic Signatures of <63 μm Soil Fraction*

As OM content was in general very high in this soil fraction (36%, no significant differences between species, Wilcoxon Rank Sum test,  $n = 10$  for invaded *C. album*,  $n = 9$  for non-invaded *C. album*,  $p = 0.156$ ), it was possible to analyse nutrient concentrations and isotopic signatures of this material (Table II). While no significant differences between plants were found for C, N, P or stoichiometric relationship of these nutrients, both  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$  of the OM in this fraction were significantly different underneath invaded *C. album* plants in comparison to non-invaded *C. album* plants. Similar to foliar values, invasion led to less depleted  $\delta^{15}\text{N}$  values of  $-0.9\text{‰}$  and therefore close to the atmospheric

standard. In contrast,  $\delta^{13}\text{C}$  values were at  $-27.1\text{‰}$  around  $0.7\text{‰}$  more depleted underneath invaded *C. album* plants.

*Multiple Factor Analysis and RV Coefficients*

The first two dimensions of the MFA can be seen in Fig. 4a, which together explain 48% of the total variance found in the data sets. This variance is split in the first dimension, explaining 30% and second axis, explaining 18%. Variance of dimension 1 is mainly shared between OM pools (contributing with 37%) and characteristics of the <63 μm soil fraction (contributing with 40%), while plant size expressed by volume makes up 15% and foliar-related values 9%. On the contrary, variance of dimension 2 is mainly shared between characteristics of the <63 μm soil fraction (contributing with 39%) and foliar-related values (contributing with 48%), while OM pools contribute with 13% (Fig. 4a). The individual factor map (Fig. 4b) shows that non-invaded *C. album* plants cluster in the lower left part, which is mainly related to less depleted  $\delta^{13}\text{C}$  of the OM in the <63 μm soil fraction, low root OM and more depleted foliar  $\delta^{15}\text{N}$  values, combined with high foliar C/N ratios.

RV coefficients are a multivariate version of Pearson's  $r^2$  and calculate association strength between data matrices, in this case (Table III) the variable subsets used in the MFA and a matrix of the plant contribution to total OM and total P as depicted in Fig. 3c. All matrices were correlated to each other, with the exception of the OM pools, which exhibited no significant association with foliage. On the contrary, plant contribution to OM and P total (termed 'mass ratios') was significantly correlated with all other matrices.

Table II. Nutrient concentrations and isotopic signatures of soil fractions <63 μm of invaded (+Inv) and non-invaded *Corema album* (-Inv)

	<i>C. album</i> (+Inv)	<i>C. album</i> (-Inv)	<i>p</i> -values
$\delta^{15}\text{N}$	<b>-0.9 (0.5)</b>	<b>-3 (0.7)</b>	<b>0.025</b>
$\delta^{13}\text{C}$	<b>-27.1 (0.1)</b>	<b>-26.4 (0.1)</b>	<b>0.002</b>
% N	0.8 (0.1)	0.7 (0.1)	0.411
% C	22.3 (3.8)	16.1 (2.4)	0.278
‰ P	94.4 (17.1)	98.5 (13.3)	0.72
C:N	25.9 (2.5)	23.8 (1.3)	0.842
N:P	103.4 (16.9)	133.2 (72.2)	0.243

Significant differences ( $p < 0.05$ ) between groups are shown in bold, Wilcoxon rank sum test,  $n = 10$  for *C. album* (+Inv) and  $n = 9$  for *C. album* (-Inv).

DISCUSSION

As assumed in the experimental set up, *A. longifolia* invasion density in this area was low and the invasive specimens

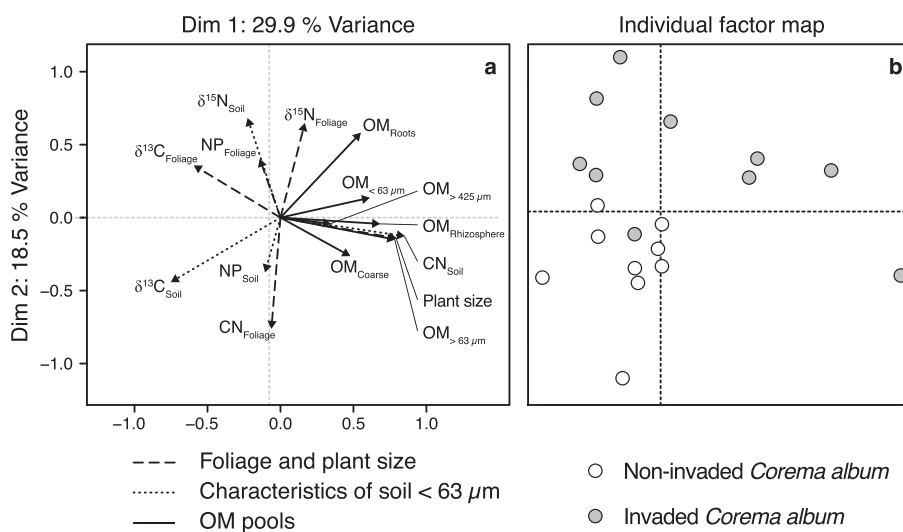


Figure 4. First two dimensions of a multiple factor analysis on parts of the data set from Fig. 2 and Tables I and II. Variable types were grouped in OM pools (OM of soil fractions: >425, >63 and <63 μm and of plant input: roots, rhizosphere and coarse OM), soil characteristics of the fine fraction <63 μm (N/P ratio, C/N ratio,  $\delta^{15}\text{N}$  and  $\delta^{13}\text{C}$ ) and foliar characteristics and plant size (N/P ratio, C/N ratio,  $\delta^{15}\text{N}$ ,  $\delta^{13}\text{C}$  and volume as plant size). (a) Correlation cycle of all variables used in the multiple factor analysis; different line types indicate the different variable types used; axis denote the correlation strength (from -1 to 1). (b) Individual factor map; invaded *Corema album* plants are shown as white spheres and non-invaded *C. album* plants as grey spheres.

Table III. RV coefficients of matrices used in Fig. 4, as well as a matrix of mass ratios (plant contribution to total OM and P total) as shown in Fig. 3c

	Foliage	Soil char. <63 $\mu\text{m}$	OM pools	Mass ratios
Foliage		<b>0.034</b>	0.123	<b>0.006</b>
Soil char. <63 $\mu\text{m}$	<b>0.309</b>		<b>0.03</b>	<b>0.042</b>
OM pools	0.262	<b>0.338</b>		<b>0.004</b>
Mass ratios	<b>0.347</b>	<b>0.238</b>	<b>0.348</b>	

On the bottom left corner, RV coefficients are shown; on the top right corner,  $p$ -values are shown. If  $p$ -values were smaller than 0.05, values were shown in bold.

found were at an early stage of development in terms of plant volume. There was no preference of *A. longifolia* to invade *C. album* plants of a certain volume, which raises the question of how seeds arrive and establish in the canopy of *C. album*. If wind dispersal and initial soil nutrient concentration were the most important factors, canopies of larger *C. album* plants were more likely to be invaded, as there is potentially more OM accumulated (Cushman *et al.*, 2010). However, wind dispersal is unlikely for *Acacia* species, while animal dispersal by ants or birds could be a viable option for seed arrival (Wilson *et al.*, 2011), taking into account that also a wide array of animals disperse *C. album* seeds (Calvino-Cancela, 2005). While the sample size of this experiment was too low to conclude more about nursery plant preference, the lack of pattern found here emphasizes that it is important to conduct further research on this topic in order to better understand introduction pathways of *A. longifolia* seeds.

Despite this early invasion stage and the complete lack of *A. longifolia*-derived litter, there was already a clear influence of *A. longifolia* on *C. album* foliar  $\delta^{15}\text{N}$ , which is in line with our hypothesis about belowground plant–plant interaction. Foliar  $\delta^{15}\text{N}$  is an environmental tracer for N fixation of legumes (Högberg, 1997), and even though there was no effect on foliar N concentration yet, less depleted foliar *C. album*  $\delta^{15}\text{N}$  values indicate that there is an early influence of the invasive legume on its nursery plant. It has been shown that the effect on foliar  $\delta^{15}\text{N}$  exceeds the effect on foliar N concentration, because changes in foliar  $\delta^{15}\text{N}$  are more easily distinguishable due to the extremely low foliar  $\delta^{15}\text{N}$  values of the native vegetation (Rascher *et al.*, 2012). As a legume, foliage of *A. longifolia* is more enriched in  $\delta^{15}\text{N}$  and total N, which creates lower C/N ratios than those observed for the native vegetation. On the contrary, the N/P ratios were much higher in the invasive legume than in the native shrub, indicating a strong P limitation (Güsewell, 2004). These values corroborate earlier findings in the same system and could suggest an onset of systemic P depletion (Ulm *et al.*, 2017). As dunes are often co-limited in N and P, this N/P imbalance is highly detrimental to the native plants on the long term. The alleviation of N limitation by the invasive legume benefits *C. album* initially (Hellmann *et al.*, 2011); however, this comes with an exacerbation of P limitation. This P limitation could mark the shift from facilitation to resource competition, which is a natural process in the oligotrophic dunes (Martínez & García-Franco, 2008),

where resource use efficiency is crucial for successful plant invasion (Funk & Vitousek, 2007). Indeed, *Acacia* species are known to be very P use efficient (Inagaki & Tange, 2014), which, together with their ability to fix atmospheric N, gives them a considerable advantage in oligotrophic ecosystems. The native vegetation, in contrast, is highly adapted to the low resource environment and has a more conservative resource use, which might impede them from benefiting as much from additional N as the invasive legume (Hellmann *et al.*, 2011).

Belowground, invaded *C. album* shrubs did not differ from non-invaded *C. album* shrubs in terms of levels of coarse OM but exhibited increased root and rhizosphere biomass, which were the main sources for OM differences in invaded *C. album* plants. The strong increase in belowground OM was not proportional to the aboveground increase in biomass, indicating that at an early stage, belowground parameters might be more sensitive to invasion in contrast to impacts aboveground, which supports observations from other invasive species that early invasions might develop in a non-linear fashion (Elgersma & Ehrenfeld, 2011). It has been shown that the longer *A. longifolia* invasion persists, the more detrimental its belowground impacts are (Marchante *et al.*, 2008), and the data presented here add a further point in this timeline. While P and OM increase could benefit both plants growing together and the OM shift to the biotic compartment is positively related with the effect on *C. album* foliar  $\delta^{15}\text{N}$ , *A. longifolia* foliar P indicates that it takes more advantage of the P accumulation in the biotic compartment. This might be due to its increased root mass but could also be due to the rhizosphere and the microbial processes therein, as this compartment is crucial in P acquisition, for example through mycorrhizal fungi (Philippot *et al.*, 2013). Changes in microbial community structure and soil conditioning by *A. longifolia* could be highly detrimental to native plant interactions, as they might lead to ‘invasional meltdown’, which describes the co-introduction of non-native symbiotic nitrogen-fixing bacteria (Rodríguez-Echeverría, 2010). It was proposed that the introduction of new soil biota might potentially disrupt native mutualisms and create negative feedback loops; however, it seems that similar to *C. album*, also, native legumes, such as *Ulex europaeus* and *Cytisus grandiflorus*, benefit from the soil alterations by *A. longifolia* (Rodríguez-Echeverría *et al.*, 2009). On the other hand, positive interactions of these native legumes with soil altered by *A. longifolia* could



be a chance for native species restoration, as both species were found to be suitable for revegetation of sites formerly invaded by *A. longifolia* (Rodríguez-Echeverría *et al.*, 2009).

Apart from differences at plant level, soil under invaded *C. album* canopies exhibited an increase in the silt-clay fraction (<63  $\mu\text{m}$ ). This fraction is mainly related to microbially stabilized biomass (Grandy & Neff, 2008), and its increase might be of crucial importance for further soil OM aggregation (Six *et al.*, 2002), which is also evident from the high N, C and P contents in this fraction. Similar to foliar nutrient values, there were no differences in total N, C or P; however,  $\delta^{15}\text{N}$  values and  $\delta^{13}\text{C}$  were already altered in invaded canopies, which is a further example of the usefulness in using stable isotope signatures as early warning signs of ecological change, such as plant invasion (Williams *et al.*, 2007). Less depleted soil  $\delta^{15}\text{N}$  was expected due to nitrogen fixation by *A. longifolia*, and the values observed here were similar to root  $\delta^{15}\text{N}$  values reported in earlier works (Ulm *et al.*, 2017), thus highlighting them as a potential source. Interestingly, also, the  $\delta^{13}\text{C}$  values of this compartment were significantly depleted underneath invaded *C. album* plants. As this compartment must necessarily be relatively recent due to small *A. longifolia* plant volume,  $\delta^{13}\text{C}$  depletion due to age effects, such as leaching, is unlikely. More probable is the accumulation of lignin and aliphatic biopolymers in this fraction (Creamer *et al.*, 2011) and a higher ratio of fungal/bacterial biomass (Kohl *et al.*, 2015). An increase in fungal biomass in turn could have important long-term effects for cross facilitation for other invasive *Acacia* species, as they require mycorrhizal symbionts for P uptake (Nuñez & Dickie, 2014). The difference in  $\delta^{13}\text{C}$  was also highlighted in the MFA as an important factor distinguishing invaded from non-invaded *C. album* canopies, the latter also being associated with low root OM and more depleted foliar  $\delta^{15}\text{N}$  values. Interestingly, the clustering of non-invaded *C. album* plant canopies was visible on both the first and the second axes, which were related to the silt-clay fraction with 40%, stressing this compartment a major factor for their characterization. Also, looking at the relationships between the variable types, it becomes clear that OM pools alone are not related to the foliar changes observed, while both the silt-clay fraction and the soil–plant biomass ratios are correlating well with all other variables measured. As these belowground changes often go unnoticed but precede the aboveground changes observed later in these systems, these data add a further argument for the high ‘invasion debt’, a term describing the delay in invasion impact along time, of *Acacia* species (Richardson *et al.*, 2015).

Summarized, it can be stated that *A. longifolia* exhibits an impact on *C. album* foliar  $\delta^{15}\text{N}$  already at an early invasion phase, accompanied by strong changes belowground, which, at least at this stage, are not related to foliar litter or coarse OM. At soil level, the differences were mainly associated with the silt-clay fraction, which was more abundant with the presence of the invader and constitutes an important pool of OM. We were able to detect differences in both  $\delta^{15}\text{N}$  and

$\delta^{13}\text{C}$  of the OM found in this compartment, underlining its putative invasive origin and highlighting the importance of further research on this soil fraction. Furthermore, we found that roots and rhizosphere play fundamental roles early on in invasion and that the ratio of OM and P in the biotic compartment against the abiotic (soil) compartment is an indicator for differences in soil parameters in invaded and non-invaded *C. album* canopies. As these differences were found in *C. album* plants without aboveground *A. longifolia* biomass accumulation, it is imperative for conservation to tackle *A. longifolia* invasion early on, before soil changes become irreversible. In conclusion, the work presented here adds important evidence to understand the first belowground alterations occurring in the process of *A. longifolia* invasion, which might help to mitigate damages done by this prolific invasive species, which continues to degrade many ecosystems worldwide.

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