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Greater root phosphatase activity in nitrogen-fixing rhizobial but not actinorhizal plants with declining phosphorus availability

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Summary

1. The abundance of nitrogen (N)-fixing plants in ecosystems where phosphorus (P) limits plant productivity poses a paradox because N fixation entails a high P cost. One explanation for this paradox is that the N-fixing strategy allows greater root phosphatase activity to enhance P acquisition from organic sources, but evidence to support this contention is limited.

2. We measured root phosphomonoesterase (PME) activity of 10 N-fixing species, including rhizobial legumes and actinorhizal *Allocasuarina* species, and eight non-N-fixing species across a retrogressive soil chronosequence showing a clear shift from N to P limitation of plant growth and representing a strong natural gradient in P availability.

3. Legumes showed greater root PME activity than non-legumes, with the difference between these two groups increasing markedly as soil P availability declined. By contrast, root PME activity of actinorhizal species was always lower than that of co-occurring legumes and not different from non-N-fixing plants.

4. The difference in root PME activity between legumes and actinorhizal plants was not reflected in a greater or similar reliance on N fixation for N acquisition by actinorhizal species compared to co-occurring legumes.

5. *Synthesis.* Our results support the idea that N-fixing legumes show high root phosphatase activity, especially at low soil P availability, but suggest that this is a phylogenetically conserved trait rather than one directly linked to their N-fixation capacity.

Key-words: Fabaceae, nitrogen paradox, nutrient-acquisition strategies, organic phosphorus, phosphomonoesterase, plant–soil (below-ground) interactions, soil chronosequence

Introduction

Biological nitrogen (N) fixation from N-fixing symbiotic associations involving micro-organisms and vascular plants is the primary source of N input in many terrestrial ecosystems (Cleveland *et al.* 1999). Symbiotic N fixation enhances plant performance on N-poor soils (Vitousek *et al.* 2002; Menge, Lichstein & Ángeles-Pérez 2014), but should not be favoured on phosphorus (P)-impoverished soils (Houlton *et al.* 2008; Hedin *et al.* 2009) because symbiotic N fixation entails a high P cost (Sprent & Raven 1985; Vitousek & Howarth 1991; Hartwig 1998; Sprent 1999; Raven 2012). However, plants possessing the capacity to form a symbiosis with N-fixing bacteria (hereafter referred to as 'N-fixing' plants or species) are abundant in many ecosystems with strongly weathered, P-impoverished soils such as lowland tropical rain forests, where P rather than N is likely to limit plant productivity (Crews 1999; Hedin *et al.* 2009). This has been referred to as the 'nitrogen paradox' (Hedin *et al.* 2009).

One potential explanation for the nitrogen paradox is that the ability to symbiotically fix N could allow a greater investment in extracellular phosphatase enzymes, since enzymes are N-rich organic molecules (Houlton *et al.* 2008). Phosphatase enzymes catalyse the hydrolysis of organic P esters, releasing

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orthophosphate (inorganic P) for uptake by plant roots (Tarafdar & Claassen 1988). The greater root and/or soil phosphatase activity of N-fixing plant species compared with non-N-fixing species could provide N-fixing species with a competitive advantage in P-impoverished ecosystems (Houlton *et al.* 2008), since organic P can represent a major fraction of total P in these soils (Syers & Walker 1969; Harrison 1987; Turner *et al.* 2007; Turner & Laliberté 2015), including lowland tropical forests (Turner & Engelbrecht 2011). However, evidence to support the hypothesis that N-fixing plants show greater root phosphatase activity remains limited, particularly for non-legume, actinorhizal N-fixing species (Giardina *et al.* 1995; Zou, Binkley & Caldwell 1995; Chodak & Niklińska 2010). Unravelling the nitrogen paradox would help to explain the global distribution of N-fixing plants and the controls over biological N fixation in terrestrial ecosystems, which will in turn enhance earth system models aiming to predict the response of terrestrial ecosystems to global change (Cleveland *et al.* 1999; Wang, Houlton & Field 2007; Houlton *et al.* 2008; Vitousek *et al.* 2013; Menge, Lichstein & Ángeles-Pérez 2014).

Although several primary experimental studies (e.g. Giardina *et al.* 1995; Zou, Binkley & Caldwell 1995; Allison, Nielsen & Hughes 2006; Caldwell 2006; Nuruzzaman *et al.* 2006; Nasto *et al.* 2014) support the hypothesis that N-fixing plants have high root phosphatase activity (Houlton *et al.* 2008), these results are difficult to generalise for three reasons. First, plants can up-regulate root phosphatase activity in response to increasing supply of N or decreasing supply of P (e.g. Li *et al.* 1997; Hayes, Richardson & Simpson 1999; Olander & Vitousek 2000; Treseder & Vitousek 2001; Wang, Houlton & Field 2007; Olde Venterink & Güsewell 2010; Olde Venterink 2011; Marklein & Houlton 2012; Keller *et al.* 2013), which confounds the synthesis of results from studies conducted on a broad range of soils and associated nutrient status. Second, the majority of primary experimental studies comparing the phosphatase activity of N-fixing species with non-N-fixing species examined only rhizobial legumes as the N-fixing species (e.g. Hayes, Richardson & Simpson 1999; Allison, Nielsen & Hughes 2006; Caldwell 2006; Nuruzzaman *et al.* 2006; Olde Venterink 2011; Keller *et al.* 2013; Nasto *et al.* 2014), whereas actinorhizal species, which represent the main N-fixing strategy in temperate and boreal forests (Menge, Lichstein & Ángeles-Pérez 2014), have received far less attention – previous studies examined only two species from the same genus (*Alnus rubra* and *Alnus glutinosa*; Giardina *et al.* 1995; Zou, Binkley & Caldwell 1995; Chodak & Niklińska 2010). Third, the direct link between N fixation and the organic P-acquisition strategy based on phosphatase activity has rarely been tested, with the exception of two previous studies (Batterman, Wurzburger & Hedin 2013; Nasto *et al.* 2014). Moreover, the findings in the glasshouse study by Batterman, Wurzburger & Hedin (2013) observed that phosphatase activity of the N-fixing legume examined (*Inga punctata*) decreased slightly in response to increased plant N fixation, which did not support the hypothesis that the capacity to fix N enables a greater investment in phosphatase enzymes (Houlton *et al.* 2008). Thus, it is difficult to establish if the greater phosphatase activity of N-fixing plants is directly related to N fixation itself, or whether it reflects phylogeny.

Nitrogen-fixing plants tend to down-regulate N fixation when soil N is not limiting (e.g. Baker, Hill & Parsons 1997; Hartwig 1998; Pearson & Vitousek 2001; Barron, Purves & Hedin 2011). As such, the highly effective down-regulation of N fixation could offer an alternative explanation for the persistence of N-fixing species in ecosystems with strongly weathered, P-impoverished soils (Crews 1999; Hedin *et al.* 2009; Menge, Levin & Hedin 2009; Menge, Lichstein & Ángeles-Pérez 2014). On the other hand, no apparent down-regulation of N fixation by certain actinorhizal N-fixing species (e.g. Binkley, Cromack & Baker 1994; Menge & Hedin 2009; Chaia & Myrold 2010) on strongly weathered, P-impoverished soils could be a disadvantage (Hedin *et al.* 2009; Menge, Levin & Hedin 2009; Menge, Lichstein & Ángeles-Pérez 2014). However, the down-regulation of N fixation by N-fixing plants when soil N is not limiting does not align with the hypothesis that N fixation directly allows greater investment in phosphatases (Houlton *et al.* 2008). Therefore, how phosphatase activity of N-fixing plants relates to symbiotic N-fixation type, and how phosphatase activity of these plants varies with soil N and P availability require further study.

Natural gradients of soil nutrient availability such as long-term, retrogressive soil chronosequences provide important model systems (Peltzer *et al.* 2010) to understand how N-fixing species differentially invest in organic P acquisition. During long-term soil and ecosystem development, the total amount and availability of soil P decline due to prolonged weathering, and old soils can eventually become P-impoverished (Walker & Syers 1976; Vitousek & Farrington 1997). As such, shifts in soil N and P availability during long-term soil and ecosystem development are expected to influence root phosphatase activity of plants (Li *et al.* 1997; Hayes, Richardson & Simpson 1999; Treseder & Vitousek 2001; Olde Venterink 2011) and rates of N fixation (Baker, Hill & Parsons 1997; Hartwig 1998; Pearson & Vitousek 2001; Vitousek *et al.* 2010; Barron, Purves & Hedin 2011). However, empirical studies comparing root phosphatase activity of N-fixing and non-N-fixing species have not been conducted across a long-term soil chronosequence. This might be partly due to the difficulty of finding a retrogressive soil chronosequence with sufficiently high plant species and functional diversity to enable comparisons of N fixation involving different strategies (i.e. legume-rhizobia and actinorhizal-*Frankia*) and co-occurring non-N-fixing plants in soils of contrasting ages and P availability.

Nitrogen-fixing plants representing the two main N-fixation strategies (i.e. rhizobial and actinorhizal) are common across a long-term retrogressive soil chronosequence in south-western Australia (Zemunik *et al.* 2015, 2016), providing an opportunity to compare phosphatase activity of these N-fixing species with the activity of co-occurring non-N-fixing species. Moreover, this chronosequence represents one of the strongest

local P gradients globally, displaying a 98·5% decrease in total soil [P] from the youngest to the oldest soils (Laliberté *et al.* 2012; Turner & Laliberté 2015). In this study, we examined the hypothesis linking the N-fixing strategy to greater investment in phosphatases to enhance P acquisition from organic sources (Houlton *et al.* 2008) by comparing 'potential' root phosphomonoesterase (PME) activity (including PME produced by root-associates such as mycorrhizas; Turner 2008) across a range of N-fixing (10 species; including eight legumes and two non-legume actinorhizal *Allocasuarina* species) and non-N-fixing (eight species) plant species from eight families. We also explored a possible relationship between the degree of reliance on N fixation for overall N acquisition (estimated using the ^{15}N natural abundance method; Shearer & Kohl 1986; Boddey *et al.* 2000; Unkovich *et al.* 2008) and root PME activity of N-fixing species.

Materials and methods

STUDY SITES

Root and leaf samples of mature N-fixing and non-N-fixing plants were collected from permanent 10 m × 10 m plots in three selected dune systems of different soil ages of contrasting soil P availability (Table 1; see Data S1, Supporting Information) along the >2-million-year Jurien Bay coastal dune chronosequence (Laliberté *et al.* 2012; Turner & Laliberté 2015). This chronosequence is located in south-western Australia, approximately 200 km north of Perth (Laliberté *et al.* 2012; Turner & Laliberté 2015). The study area has a species-rich Mediterranean low shrubland vegetation known as kwongkan (Hopper 2014; Mucina *et al.* 2014), and encounters fire as the main disturbance with approximately <30 years fire-return intervals (Department of Conservation and Land Management 1995). Details about the chronosequence are published elsewhere (see Laliberté *et al.* 2012; Turner & Laliberté 2015).

The study area experiences a Mediterranean climate with hot, dry summers and cool, wet winters (Laliberté *et al.* 2012; Turner & Laliberté 2015). Annual rainfall (1968–2013) is 538 mm, and ~80% of the rainfall occurs between May and September; mean annual maximum temperature (1970–2013) is 25 °C, with the warmest mean monthly maximum temperature in February (31 °C), and the coolest in July (20 °C); mean annual minimum temperature (1970–2013) is 13 °C, with the warmest mean monthly minimum temperature in February (18 °C), and the coolest in July (9 °C) (Australian Bureau of Meteorology, http://www.bom.gov.au/climate/data/). A year prior to root collection (August 2012–July 2013), the annual rainfall of the area was 540 mm and the mean monthly maximum temperature was 26 °C (Australian Bureau of Meteorology, http://www.bom.gov.au/climate/data/).

SAMPLE COLLECTION

Fine roots (≤2 mm diameter) from a total of 10 common species of putative N-fixing and eight common species of non-N-fixing plants of a variety of nutrient-acquisition strategies (Table S1) were collected from the respective soil ages (Table 1; see Data S1). Collections were made over a 2-week period in July 2013 (corresponding to the rainy, cool winter season), when kwongkan ecosystems experience higher rainfall and roots of native plants are actively growing in the surface soil layers in the seasonally dry Mediterranean climate. Mature, individual plants of the respective species were located, and fine roots were collected by tracing the roots from the base of the stem. All samples of fine roots were stored at 4 °C shortly after collection and were analysed within a week.

Healthy, youngest fully expanded leaves of each species were also collected from the same individual plants that were also sampled for the assay of extracellular root PME activity. Leaves were undamaged and exposed to full sunlight. Prior to nutrient analyses, leaves were dried at 60 °C for 72 h before finely ground using a Teflon-coated stainless steel ball mill.

ASSAY OF ROOT PHOSPHOMONOESTERASE ACTIVITY

Root PME activity was measured using *para*-nitrophenyl phosphate (*p*NPP) as substrate. Phosphomonoesterase is a common plant enzyme involved in the hydrolysis of both organic phosphate monoesters (simple) and diesters [which first require hydrolysis by

Table 1. Key features of the three dune systems along the Jurien Bay coastal dune chronosequence that are of different soil ages and contrasting soil phosphorus (P) availability used in this study. Chronosequence stage numbers, names of dune systems, estimated soil ages, likely nutrient (co-) limitation(s) are based on Turner & Laliberté (2015). Soil nutrient data for each dune system are based on the respective plot used in this study (see Data S1), and not for all plots within each dune system as presented in Turner & Laliberté (2015). Values for soil nutrient properties are means ± SEs, $n = 7$

Chronosequence stage	Dune system (~soil age)	Likely nutrient (co-) limitation(s)	Phosphorus		Nitrogen		
			Total (mg kg^{-1})	Organic (% total P)	Total (mg kg^{-1})	pH ($CaCl_2$)	ECEC ($cmol_c$ kg^{-1})
2 (young)	Quindalup medium (Holocene; ~1 ka)	N, P and/or other nutrients	428·1 ± 14·0	3·5	114·7 ± 8·2	7·93 ± 0·02	12·8 ± 3·4
4 (middle-aged)	Spearwood West (middle Pleistocene; ~125 ka)	P	21·6 ± 2·2	35·8	27·3 ± 3·9	5·99 ± 0·16	3·3 ± 0·5
6 (old)	Bassendean (early Pleistocene to late Pliocene; >2000 ka)	P	6·6 ± 1·5	38·6	33·9 ± 6·1	4·45 ± 0·08	3·0 ± 0·3

ECEC, effective cation-exchange capacity.

phosphodiesterase (PDE)] – accounting for most of the soil organic P input (Bowman & Cole 1978; Condron, Turner & Cade-Menun 2005; Turner 2008).

For each sample, roots were rinsed four times in deionised water filtered by Milli-Q® water system (Millipore, Bedford, MA, USA), then blotted on moist paper towels. Approximately 200 mg of fresh roots were added to 9 mL of sodium acetate–acetic acid buffer (pH 5·0). The temperature was equilibrated at 20 °C for 5 min using a shaking (100 rpm) water-bath before 1 mL of *p*NPP substrate (5 mM) was added and incubated for a further 30 min. A second identical sample for each replicate was prepared as a control, in which 1 mL of the buffer was added in place of the *p*NPP substrate. The reaction was terminated by adding 0·5 mL of each test solution to 4·5 mL of sodium hydroxide (0·11 M). The concentration of *para*-nitrophenol (*p*NP) in the final solution was measured by measuring absorbance at 405 nm with UV-Vis spectrophotometers (Shimadzu UV-1601, Shimadzu, Duisburg, Germany and Thermo Scientific Multiskan® Spectrum, Vantaa, Finland). Phosphomonoesterase activity was calculated and expressed on dry and fresh root biomass basis (results based on fresh root biomass are presented in Figs S1 and S2).

DEGREE OF RELIANCE ON SYMBIOTIC N FIXATION

Dry, finely ground leaf subsamples of each species (Zemunik *et al.* 2015; Table S1) were analysed for isotopic composition of $\delta^{15}N$ using a continuous-flow system consisting of a Delta V Plus mass spectrometer connected to a Thermo Flash 1112 via Conflo IV (Thermo-Finnigan, Bremen, Germany).

Degree of reliance on N fixation for overall N acquisition for each N-fixing species was estimated using the ^{15}N natural abundance method (Shearer & Kohl 1986; Boddey *et al.* 2000; Unkovich *et al.* 2008), and the percentage of N content derived from the atmosphere (%Ndfa) was calculated with the following equation: %Ndfa = 100 (average $\delta^{15}N$ of reference species − $\delta^{15}N$ of N-fixing species)/(average $\delta^{15}N$ of reference species − 'B'). Values of %Ndfa that were >100% were re-designated as 100% and these individuals are assumed to possess a high reliance on symbiotic N fixation, while values <0% were re-designated as 0% and these individuals are assumed to possess an extremely low or no reliance on symbiotic N fixation (Unkovich *et al.* 2008).

For each N-fixing species, the reference plant species selected was a co-occurring, non-N-fixing species with the most similar arbuscular mycorrhizal (AM) and/or ectomycorrhizal (ECM) strategy (Zemunik *et al.* 2015; Table S2 and Fig. S4) to account for potential isotopic differences between the forms of soil N accessible by the different mycorrhizal types (Mayor, Schuur & Henkel 2009; Hobbie & Högberg 2012). The reference species selected (Table S2) were also large, woody shrubs so as to be as similar as possible to the examined N-fixing species (i.e. large woody shrub or small tree) in terms of growth form. An average leaf $\delta^{15}N$ value of each reference species was used (Table S2).

The 'B' value to account for the isotopic fractionation within a plant fully reliant on N fixation is an average of several published shoot 'B' values of shrub and tree species (B = −1·4‰; Yoneyama *et al.* 1993; Stock, Wienand & Baker 1995; Boddey *et al.* 2000; Dalal *et al.* 2005; Unkovich *et al.* 2008). This 'B' value is within the range of most species (between 0‰ and −2‰; Andrews *et al.* 2011).

Only youngest fully expanded leaves from the same growing season were collected for analyses to reduce seasonal variation of leaf $\delta^{15}N$ (Shearer & Kohl 1986; Evans 2001). Leaves of N-fixing and non-N-fixing reference plants from each soil age were also collected from individuals that co-occur within the same 10 m × 10 m plot to reduce variation of isotopic consistency of the soil N source (Shearer & Kohl 1986; Boddey *et al.* 2000; Soper, Boutton & Sparks 2015).

STATISTICAL ANALYSES

To test for differences in root PME activity or degree of reliance on N fixation (%Ndfa) among individual species within each soil age, generalised least squares models were used (Zuur *et al.* 2009). Linear mixed-effects (LME) models (Pinheiro & Bates 2006) were used to test for differences in root PME activity among the non-random/'fixed' factors of plant functional groups (N-fixing legume, N-fixing actinorhizal and non-N-fixing), soil ages of contrasting P availability (young, middle-aged and old dunes), and the interaction between these two factors. Species was treated as a random factor in LME models where differences between functional groups were of main interest.

The assumptions of homogeneity of variance, normally distributed residuals with mean around zero, and constant variance for each model were ascertained by visual inspection of standardised residuals. Appropriate variance structures were specified in the subsequent model when required and the appropriate models were identified using Akaike Information Criterion and likelihood-ratio tests (Zuur *et al.* 2009). In a few cases where we could not obtain adequate residual plots after incorporating different variance structures, data were $\log_{10}$-transformed prior to analyses. *Post hoc* Tukey tests were also performed when a main term was significant (Hothorn, Bretz & Westfall 2008). Statistical analyses were performed in 'R' (R Core Team 2015) using 'NLME' (Pinheiro *et al.* 2014) and 'MULTCOMP' (Hothorn, Bretz & Westfall 2008) packages.

Results

Comparisons among individual plant species revealed that the root PME activity of the two non-legume, actinorhizal N-fixing *Allocasuarina* species were consistently low across the soil chronosequence (Fig. 1). By contrast, root PME activity of legumes was generally greater than that of co-occurring non-legume species in the middle-aged and old soils (Figs 1 and 2). The difference in root PME activity between legumes and non-legumes increased with soil age, and the difference was greatest on the most P-impoverished soils (Fig. 2). Indeed, legume species (i.e. *Acacia pulchella*, *Jacksonia floribunda* and *Jacksonia hakeoides*) on the oldest dune had the greatest root PME activity among all species examined (Fig. 1).

Root PME activity showed no clear relationship with the degree of reliance on N fixation for N acquisition (Figs 1, 3 and S3). Indeed, the degree of reliance on N fixation of most legume species was either similar or lower than those of the respective co-occurring, actinorhizal N-fixing *Allocasuarina* species of each soil age (Figs 3 and S3). Most of the putative N-fixing species showed some degree of reliance on N fixation for N acquisition, with the exception of the leguminous *Labichea cassioides*, *A. pulchella* and *J. hakeoides* that showed very little or no reliance on N fixation for N acquisition (Fig. 3).

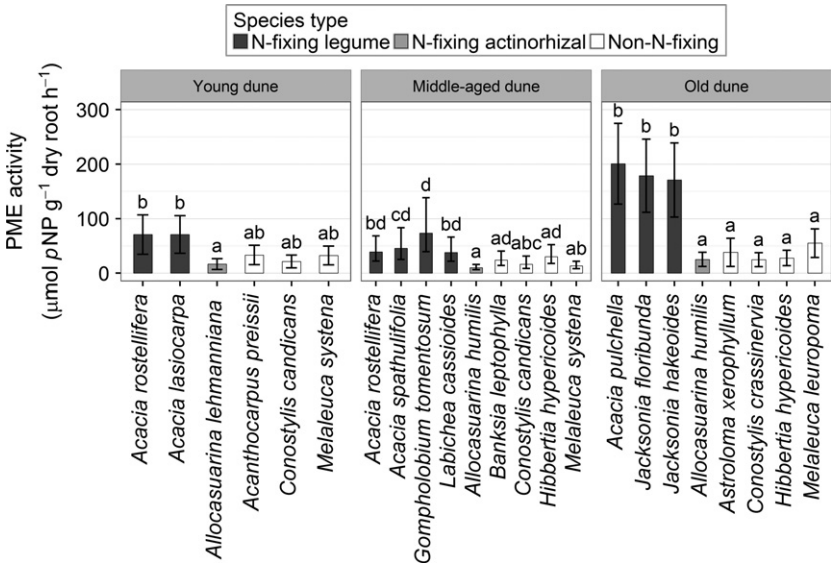

Fig. 1. Root phosphomonoesterase (PME) activity of putative nitrogen (N)-fixing and non-N-fixing individual plants across three soil ages of contrasting phosphorus availability (youngest = highest, oldest = lowest; Table 1). Bars are means and error bars represent 95% confidence intervals from generalised least squares models (young, $P \leq 0{\cdot}01$; middle-aged, $P \leq 0{\cdot}001$; old, $P \leq 0{\cdot}001$; each P-value refers to the species term within the respective panel). The model for the middle-aged soil was based on $\log_{10}$-transformed data. Different letters among species indicate significant differences within each panel (*post hoc* Tukey test, $P \leq 0{\cdot}05$).

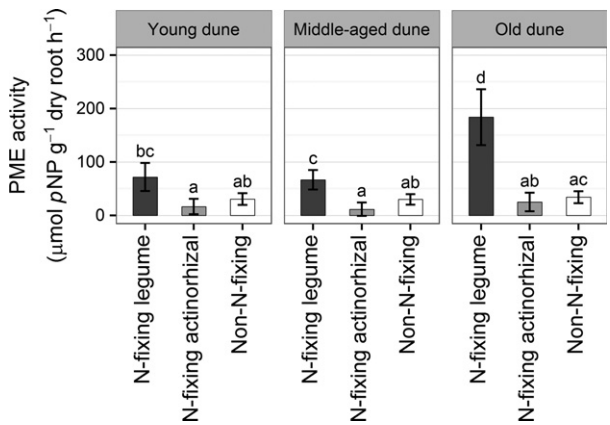

Fig. 2. Root phosphomonoesterase (PME) activity of putative nitrogen (N)-fixing legume, N-fixing actinorhizal and non-N-fixing plant functional groups across three soil ages of contrasting phosphorus availability (youngest = highest, oldest = lowest; Table 1). Bars are means and error bars represent 95% confidence intervals from a linear mixed-effects model (soil age × N-fixing type interaction, $P \leq 0{\cdot}01$). Different letters among functional groups indicate significant differences (*post hoc* Tukey test, $P \leq 0{\cdot}05$).

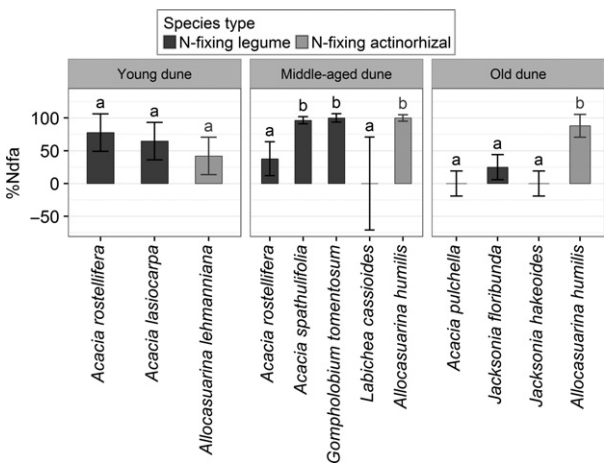

Fig. 3. Degree of reliance on nitrogen (N) fixation for N acquisition of putative N-fixing legumes and actinorhizal plant species across three soil ages of contrasting phosphorus availability (youngest = highest, oldest = lowest; Table 1). Percentage of N derived from the atmosphere (%Ndfa) for each species was estimated using the ^{15}N natural abundance method (Shearer & Kohl 1986; Boddey *et al.* 2000; Unkovich *et al.* 2008). Bars are means and error bars represent 95% confidence intervals from generalised least squares models (young, $P = 0{\cdot}193$; middle-aged, $P \leq 0{\cdot}001$; old, $P \leq 0{\cdot}001$; each P-value refers to the species term within the respective panel; $n = 3$–6). Different letters among species (black letters = legume, grey letters = actinorhizal) indicate significant differences within each panel (*post hoc* Tukey tests, $P \leq 0{\cdot}05$).

Discussion

In support of the hypothesis linking the N-fixing strategy to greater investment in root phosphatases to enhance P acquisition from organic sources (Houlton *et al.* 2008), we found that N-fixing legumes generally showed greater root phosphatase activity than non-legumes and that this difference increased with declining soil P availability. By contrast, root phosphatase activity of non-legume, actinorhizal N-fixing *Allocasuarina* species was consistently low regardless of soil age and P availability. Furthermore, contrary to the hypothesised relationship between N fixation and phosphatase activity (Houlton *et al.* 2008), the similar or lower degree of reliance on N fixation by most legumes than their respective co-occurring actinorhizal species did not correspond to differences in root phosphatase activity. Our results thus suggest that the greater root phosphatase activity of legumes (compared with non-legumes) is likely a phylogenetically conserved trait within the legume family, which may not be directly enabled by their N-fixing capacity. The strength of our study is that our comparisons between N-fixing and non-N-fixing strategies were with co-occurring species growing in the same environment across a wide range of soil N and P availability, whereas previous generalised comparisons were based on

combining responses of species from separate primary experiments of different environmental and soil nutrient conditions (Giardina *et al.* 1995; Zou, Binkley & Caldwell 1995; Allison, Nielsen & Hughes 2006; Caldwell 2006; Nuruzzaman *et al.* 2006).

Based on a previous framework (Houlton *et al.* 2008), actinorhizal N-fixing species, like N-fixing legumes, should also invest more strongly in root phosphatase enzymes than non-N-fixing species as a P-acquisition strategy because of their N-fixing strategy. On the contrary, we found that phosphatase activity in roots of both the actinorhizal N-fixing *Allocasuarina* species examined was consistently low and not different from co-occurring non-N-fixing species, even on extremely P-impoverished soils. This contrasts with three existing studies that separately examined an actinorhizal N-fixing species, which found them to have greater soil phosphatase activity than the respective non-N-fixing species examined (Giardina *et al.* 1995; Zou, Binkley & Caldwell 1995; Chodak & Niklińska 2010). Our study minimised potential confounding phylogenetic responses by examining a greater range of species possessing the legume-rhizobia strategy and two actinorhizal N-fixing plant species from a different clade to those examined in existing studies (Giardina *et al.* 1995; Zou, Binkley & Caldwell 1995; Chodak & Niklińska 2010). Furthermore, given the similar or greater degree of reliance on N fixation by actinorhizal species than that of co-occurring legumes on all soils, the generally lower root phosphatase activity of the actinorhizal species (compared with legumes) cannot be explained by a lower degree of reliance on symbiotic N fixation. As such, our results do not support the hypothesised association between N fixation and root phosphatase activity of N-fixing plants (Houlton *et al.* 2008).

The ^{15}N natural abundance method used in this study was developed for relatively simple systems (e.g. cropping or agroforestry) and has its limitations when applied to natural environments such as the system examined here (Soper, Boutton & Sparks 2015). In this field study, overestimation and/or underestimation of the degree of reliance on N fixation for N acquisition for certain N-fixing species may occur for four reasons. First, we did not account for variation in isotopic fractionation associated with N assimilation that may occur among different N-fixing and non-N-fixing (i.e. reference) species (e.g. Högberg 1997; Boddey *et al.* 2000; Unkovich *et al.* 2008); this would involve a separate glasshouse experiment. Second, leaves of the reference species used were collected from individuals across the plot and may not be immediately next to the N-fixing plant examined. As such, the isotopic consistency of the soil N source spatially may decrease due to differences in N cycling at a localised scale (e.g. Shearer & Kohl 1986; Yoneyama *et al.* 1993; Boddey *et al.* 2000; Soper, Boutton & Sparks 2015). Third, differences in plant rooting depths among N-fixing plants and the non-N-fixing reference species in the field were not accounted for, but they might result in differential access of soil N sources with dissimilar isotopic ratios at different soil depths (Shearer & Kohl 1986). Fourth, the ^{15}N natural abundance method used in this study to estimate the degree of reliance on N fixation is an indirect measure of N fixation and is not as sensitive as methods that directly measure N fixation (e.g. use of labelled $^{15}N_2$; Unkovich *et al.* 2008). Finally, while the degree of reliance on N fixation for N acquisition estimated via the ^{15}N natural abundance method does not indicate the total N content of a plant acquired through symbiotic N fixation, the use of this method still provides a useful approximation for field-based studies (Unkovich *et al.* 2008).

The greater root phosphatase activity of legumes (than non-legumes) could be favoured by their inherently N-demanding lifestyles (McKey 1994) and greater demand for P (Sprent & Raven 1985; Vitousek & Howarth 1991; Hartwig 1998; Sprent 1999; Raven 2012), as opposed to being directly linked to N fixation. Our suggestion that the greater phosphatase activity of legumes than that of non-legumes is a phylogenetically conserved trait is also supported by two previous glasshouse studies (Olde Venterink 2011; Batterman, Wurzburger & Hedin 2013). First, the leguminous *I. punctata* showed decreasing rhizosphere phosphatase activity with increasing levels of N fixation (Batterman, Wurzburger & Hedin 2013). Second, seedlings of leguminous forbs species showed greater root phosphatase activity than non-leguminous species at low soil P availability in the absence of N-fixing nodules and at low soil [N] (Olde Venterink 2011).

The overall greater phosphatase activity of legumes than non-legumes and the observation of the largest difference of phosphatase activity between legumes and non-legumes occurring on the oldest, most P-impoverished soil in this study suggest that phosphatase enzymes and organic P acquisition are of greater importance to legumes than non-legumes. Although only one form of phosphatase (i.e. PME) was measured in this study and different species may also specialise in the use of other forms of phosphatase enzymes, particularly PDE enzymes, it is unlikely that the phosphatase activity for most species has been underestimated. This is because, while phosphodiesters account for most of the organic P input to soils (Bowman & Cole 1978; Condron, Turner & Cade-Menun 2005; Turner 2008), phosphomonoesters are released from phosphodiesters after being hydrolysed by PDE, and PME – a common plant enzyme – is necessary to liberate inorganic phosphate from these phosphomonoesters for plant uptake (Turner 2008). Rather than investing strongly in phosphatase enzymes like the legumes to acquire P, non-legumes may use other strategies to persist and co-exist with legumes in P-impoverished environments (Lambers *et al.* 2008, 2014; Zemunik *et al.* 2015).

Phosphorus-acquisition strategies other than the production of root phosphatases used by non-legumes in this study include cluster-root formation (e.g. Brundrett & Abbott 1991; Diem *et al.* 2000; Lambers *et al.* 2006, 2008), specialising in the acquisition of soluble or insoluble inorganic forms of soil P (e.g. Lambers *et al.* 2008; Turner 2008), symbiotic mycorrhizal associations (e.g. Plassard & Dell 2010; Smith, Anderson & Smith 2015) and/or a higher fine-root biomass to compensate for a lower phosphatase activity per unit root mass. Symbiotic ECM fungi may contribute substantially to organic P acquisition by releasing phosphatase enzymes

(Plassard & Dell 2010; Smith, Anderson & Smith 2015). By contrast, the capacity of AM fungi to hydrolyse organic P is largely insubstantial (Smith & Read 2008). Although ectomycorrhizas are present in many legume and non-legume species examined (Zemunik *et al.* 2015; Table S1), we consider it unlikely that their capacity to hydrolyse organic P was underestimated as a result of hyphal destruction in our sampling design. This is because the average levels of ECM colonisation of species possessing the ECM strategy in this study system were generally low (~1–13%; Zemunik *et al.* 2015; Fig. S4). Furthermore, the similar or lower levels of ECM colonisation of non-legumes than co-occurring legumes possessing the ECM strategy further downplay the possibility of underestimating the capacity of non-legumes to hydrolyse organic P (Fig. S4). Hence, the relative importance of the function of symbiotic ECM associations as an organic P-acquisition strategy on extremely P-impoverished soils requires further study.

Although our study suggests that the relatively greater root phosphatase activity of legumes than non-legumes is likely a phylogenetically conserved trait and not directly linked to N fixation, this trait can still help explain the abundance of N-fixing legumes in P-impoverished ecosystems along this studied chronosequence, as well as elsewhere at lower latitudes (Houlton *et al.* 2008; Menge, Lichstein & Ángeles-Pérez 2014). This is because increasing soil-weathering and P limitation are correlated with decreasing latitude (Huston 2012), and the primary productivity in many low-latitude ecosystems is more likely to be limited by P rather than N (Laliberté *et al.* 2013; Menge, Lichstein & Ángeles-Pérez 2014). Thus, legumes possess a competitive advantage on P-limited soils – where organic P is an important fraction (Syers & Walker 1969; Harrison 1987; Turner *et al.* 2007; Turner & Laliberté 2015) – via greater investment in root phosphatase enzymes, which might explain their abundance at these lower latitudes (Houlton *et al.* 2008; Menge, Lichstein & Ángeles-Pérez 2014).

The consistently low root phosphatase activity of the non-legume, actinorhizal N-fixing *Allocasuarina humilis* was unexpected given its occurrence on strongly weathered, P-impoverished soils. Similarly, its geographical distribution along with other Casuarinaceae at lower latitudes and in ecosystems with strongly weathered, P-impoverished soils (Diem & Dommergues 1990) does not conform to the observed pattern of latitudinal shifts in functional groups with respect to N-fixing strategy (Menge, Lichstein & Ángeles-Pérez 2014). This could be related to the polyphyletic nature of the N-fixing actinorhizal functional group (24 genera from eight dicotyledonous families; Andrews *et al.* 2011), where distinct clades may possess certain traits restricting their distribution to a specific range of environmental conditions along a latitudinal gradient, particularly shifts in soil N and P availability (Laliberté *et al.* 2013). For example, the persistence of members from the Casuarinaceae on strongly weathered, P-impoverished soils and at lower latitudes may be attributable to a number of alternative strategies such as operating efficiently at low leaf [N] and [P] (Fig. S5) and/or cluster roots (Diem *et al.* 2000; Table S1 and Fig. S6), rather than possessing greater root phosphatase activity than co-occurring plant species. While actinorhizal N-fixing plants are not as well represented as legumes in many P-limited, lower latitude ecosystems (Menge, Lichstein & Ángeles-Pérez 2014), including along the studied chronosequence (Zemunik *et al.* 2016), they probably still represent a significant N input in these ecosystems given their relatively high degree of reliance on N fixation for N acquisition (Andrews *et al.* 2011). Therefore, it is vital to identify other mechanisms or adaptations, such as the array of nutrient-acquisition and effective nutrient-use strategies, possessed by the different clades of actinorhizal N-fixing plants to better understand their distribution patterns.

The hypothesis linking N fixation to a greater investment in phosphatases by plants has been useful to help explain N-fixing plant distribution patterns (Wang, Houlton & Field 2007; Houlton *et al.* 2008), which has important implications for efforts to incorporate the N cycle and N-fixation strategies in earth system models (Cleveland *et al.* 1999; Wang, Houlton & Field 2007; Houlton *et al.* 2008; Vitousek *et al.* 2013; Menge, Lichstein & Ángeles-Pérez 2014). However, our results suggest that the greater root phosphatase activity of N-fixing legumes, but not actinorhizal plants, with declining P availability is likely a phylogenetically conserved trait within the family, rather than one directly linked to their N-fixing capacity. Overall, this trait of greater root phosphatase activity could still be applied to modelling the distribution patterns of N-fixing legumes regardless of their N-fixing capacity, but the absence of this trait in the actinorhizal *Allocasuarina* species examined here suggests that the trait of greater root phosphatase activity cannot be applied to N-fixing plants more broadly. Thus, future studies should place more emphasis on identifying other mechanisms or adaptations, not necessarily linked to the N-fixing strategy, to better explain the distribution of non-legume, actinorhizal N-fixing plant clades.

Authors' contributions

G.K.P., B.L.T. and E.L. designed research; G.K.P., F.E.A., P.E.H., H.L. and E.L. performed research; G.K.P., F.E.A. and P.E.H. analysed data; G.K.P., B.L.T., H.L. and E.L. wrote the paper. All authors contributed critically to the drafts and gave final approval for publication.

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Data accessibility

Data available from the Dryad Digital Repository https://doi.org/10.5061/dryad.65vs3 (Png *et al.* 2017).

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Supporting Information

Details of electronic Supporting Information are provided below.

Data S1. Materials and methods for Supporting Information.

Table S1. Common nitrogen (N)-fixing and non-N-fixing plant species found across three soil ages of contrasting phosphorus (P) availability (youngest = highest, oldest = lowest; Table 1) analysed for root phosphomonoesterase (PME) activity, leaf [P] and [N], and mycorrhizal colonisation.

Table S2. Leaf δ15N (‰) of common woody nitrogen (N)-fixing and non-N-fixing tree or shrub species with prior record of arbuscular mycorrhizal (AM) and/or ectomycorrhizal (ECM) root colonisation found across three soil ages of contrasting phosphorus (P) availability (youngest = highest, oldest = lowest; Table 1).

Fig. S1. Root phosphomonoesterase (PME) activity of putative nitrogen (N)-fixing and non-N-fixing individual plants across three soil ages of contrasting phosphorus availability (youngest = highest, oldest = lowest; Table 1) expressed on a fresh biomass basis.

Fig. S2. Root phosphomonoesterase (PME) activity of putative nitrogen (N)-fixing legume, N-fixing actinorhizal and non-N-fixing plant functional groups across three soil ages of contrasting phosphorus availability (youngest = highest, oldest = lowest; Table 1) expressed on a fresh biomass basis.

Fig. S3. Relationship between root phosphomonoesterase (PME) activity and degree of reliance on nitrogen (N) fixation for N acquisition (i.e. %Ndfa) of putative N-fixing plants (including legume and actinorhizal species) across three soil ages of contrasting phosphorus availability (youngest = highest, oldest = lowest; Table 1).

Fig. S4. (a) Arbuscular mycorrhizal (AM) and (b) ectomycorrhizal (ECM) root colonisation (%) of putative nitrogen (N)-fixing and non-N-fixing individual plants across three soil ages of contrasting phosphorus availability (youngest = highest, oldest = lowest; Table 1).

Fig. S5. (a) Leaf nitrogen (N): phosphorus (P) ratio, (b) leaf N and (c) leaf P concentrations of putative N-fixing and non-N-fixing individual plants across three soil ages of contrasting P availability (youngest = highest, oldest = lowest; Table 1).

Fig. S6. *Allocasuarina humilis* seedlings with attached root structures collected along the Jurien Bay Dune Chronosequence, south-western Australia.