Abrahão Anna (Orcid ID: 0000-0001-9295-2292)

Phosphorus- and nitrogen-acquisition strategies in two *Bossiaea* species (Fabaceae) along retrogressive soil chronosequences in south-western Australia

Anna Abrahão[a,b,*], Megan H. Ryan[c], Etienne Laliberté[b, d], Rafael S. Oliveira[a,b], Hans Lambers[b]

[a]Departamento de Biologia Vegetal, Institute of Biology, University of Campinas – UNICAMP, Campinas, 13083-862, Brazil

[b]School of Biological Sciences, University of Western Australia, Crawley (Perth), WA 6009, Australia

[c]School of Agriculture and Environment, University of Western Australia, Crawley (Perth), WA 6009, Australia

[d]Centre sur la biodiversité, Institut de recherche en biologie végétale, Département de sciences biologiques, Université de Montréal, 4101 Sherbrooke Est, Montréal, Québec H1X 2B1, Canada

Correspondence

* Corresponding author,

e-mail anna.abrahao@gmail.com

During long-term ecosystem development and its associated decline in soil phosphorus (P) availability, the abundance of mycorrhizal plant species declines at the expense of non-mycorrhizal species with root specialisations for P-acquisition, such as massive exudation of carboxylates. Leaf manganese (Mn) concentration has been suggested as a proxy for such a strategy, Mn concentration being higher in non-mycorrhizal plants that release carboxylates than in mycorrhizal plants. Shifts in nitrogen (N)-acquisition strategies also occur; nodulation in legumes is expected at low N availability, when sufficient P is available. We investigated whether two congeneric legume species (*Bossiaea linophylla* and *B. eriocarpa*) occurring along two long-term chronosequences on the south-western Australian coast and grown in a glasshouse at varying N and P supply exhibited plasticity in nutrient-acquisition strategies. We hypothesised that the shifts in nutrient limitation and nutrient-acquisition strategies at the community level would also be found at the species level. Leaf N: P ratios and the responses to nutrient availability suggested that growth of both species exhibited P-limitation in all treatments, due to the very high leaf [N] of legumes afforded by symbiotic N fixation. Mycorrhizal

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colonisation was not greater at higher P supply, and root exudation of carboxylates was not stimulated at low P supply; both were unrelated to leaf [Mn]. However, nodule production declined with increasing N supply. We conclude that intraspecific variation in nutrient acquisition and use is low in these species, and that the variation at the community level, observed in previous studies, is likely driven by high species turnover.

Abbreviations – CV, coefficient of variation

Introduction

Nutrient limitation is an agronomic concept adopted in ecology, and difficult to quantify in native ecosystems without disrupting the systems (Chapin et al. 1986). Nutrient-limited crop plants respond to the addition of growth-limiting nutrients with an increased production of biomass (Vitousek et al. 2010, Meharg and Marschner 2012). In contrast, growth of native plants in nutrient-impoverished ecosystems often hardly responds to nutrient addition, and these plants may even experience nutrient toxicity (Chapin et al. 1986, Specht 1963). This suggests that their adaptation to nutrient impoverishment, e.g. by inherently slow growth, may restrict their ability to respond to addition of nutrients. Understanding the response of native plants to changes in nutrient availability is important to understand the factors controlling their distribution and abundance in landscapes where soil nutrient levels vary greatly, and when subjected to eutrophication, for example increased atmospheric nitrogen (N) deposition (Phoenix et al. 2006, Bustamante et al. 2012) or phosphorus (P) arriving in run-off or dust (Lambers et al. 2013). Nutrient availability is one of the main drivers of plant species distributions (John et al. 2007, Perry et al. 2008), in part because plants can only germinate, grow and reproduce where they are able to tolerate local environmental conditions. Therefore, the ability to acquire nutrients is crucial for plant establishment.

Nutrient-acquisition strategies at the community level vary with soil age and associated changes in growth-limiting soil nutrients (Walker and Syers 1976, Turner and Condron 2013, Hayes et al. 2014, Wardle et al. 2008, Zemunik et al. 2015). A chronosequence is a sequence of soils of different ages developed on similar parent materials and relief under the effect of constant climatic and

biotic factors (Stevens and Walker 1970). Pedogenic processes cause P loss from the soil, while N is absent from most parent materials and is accumulated by biological fixation, so that plant growth on young soils tends to be N-limited, and that on old soils P-limited (Walker and Syers 1976). The type and strength of nutrient limitation for plant growth can be assessed through nutrient addition experiments (Lannes et al. 2016), or by analysing foliar nutrient ratios, such as N: P (Güsewell 2004, Richardson et al. 2004, Vitousek et al. 2010, Wardle et al. 2004, Redfield 1958, Hayes et al. 2014). Such nutrient-addition experiments and analyses of foliar N: P ratios have highlighted shifts in nutrient limitation from N to P limitation of plant growth with increasing soil age and degree of weathering along long-term soil chronosequences (Laliberté et al. 2012, Vitousek and Farrington 1997, Richardson et al. 2004). Assessing nutrient-limitation and nutrient-acquisition strategies along gradients of nutrient availability can help explain species distributions across these gradients (Laliberté et al. 2013, Laliberté et al. 2014).

Nutrient limitation of plant productivity influences the relative abundance of species with different nutrient-acquisition strategies. Plants can acquire P directly through their roots or through symbiosis with mycorrhizal fungi (Smith and Read 2008). Mycorrhizal plants are more prominent on younger, or more fertile soils, while morphological and physiological specialisations in non-mycorrhizal cluster-rooted, or functionally similar, plants are more abundant on older, severely P-impoverished soils (Zemunik et al. 2015, Oliveira et al. 2015, Abrahão et al. 2014, Zemunik et al. 2018). Each strategy entails carbon (C) costs, which must be balanced against benefits in terms of acquisition of growth-limiting nutrients (Lynch and Ho 2005, Kaiser et al. 2015). Non-mycorrhizal morphological specialisations such as cluster roots exude a large amount of carboxylates (physiological specialisation) that may require half of daily photosynthates, if growth and respiration are included (Lambers et al. 2006); however, the highly-localised exudation of organic anions can desorb strongly-bound forms of P (Lambers et al. 2002, Jones 1998, Oburger et al. 2009, Ryan et al. 2001). As a result of rapid rates of carboxylate release, soil manganese (Mn) is mobilised (Grierson and Attiwill 1989, Dinkelaker et al. 1995, Gardner et al. 1982) and can accumulate in leaves (Gardner and Boundy 1983, Shane and Lambers 2005, Muler et al. 2014). Arbuscular mycorrhizal fungi, one of several mycorrhizal types, also require a large carbon supply from the plant to build and maintain their hyphae, but these hyphae extend the P-acquisition volume well beyond P depletion zones around fine roots (Kikuchi et al. 2016, Bitterlich and Franken 2016, Raven et al. 2018). Arbuscular mycorrhizal colonisation significantly decreases the amount of Mn transferred to the host plant (Kothari et al.

1991, Nogueira et al. 2007, Bethlenfalvay and Franson 1989, Nazeri et al. 2014). Therefore, leaf [Mn] may be used as a proxy to understand if plants rely on carboxylate release or mycorrhizal associations for P-acquisition in P-poor soils (Lambers et al. 2015). Additionally, plants can exhibit root morphological plasticity, such as greater root length per unit dry mass of root (specific root length), or greater investment in root biomass relative to total biomass (root: mass ratio) in response to changes in nutrient availability (Kramer-Walter and Laughlin 2017). The shifts in relative abundance of mycorrhizal and non-mycorrhizal plants with changes in P availability are well studied (Zemunik et al. 2015, Oliveira et al. 2015), while the within-species shifts in P-acquisition strategies with changes in P availability are relatively poorly known (Albornoz et al. 2016, Png et al. 2017, Pang et al. 2010, de Campos et al. 2013). Intraspecific variation in nutrient-acquisition can play a fundamental role in plant community responses to environmental change and community assembly (Kichenin et al. 2013, Kramer-Walter and Laughlin 2017).

Investment in N-acquisition strategies also varies with soil N and P availability. Nitrogen can be acquired directly or through symbioses with N-fixing bacteria in root nodules, allowing plants to acquire atmospheric N (Vitousek et al. 2002). Nodule formation is inhibited by sufficient N supply (Streeter and Wong 1988), and increased by high P supply (Albornoz et al. 2016), due to plant growth stimulation increasing N demand (Robson et al. 1981). Nodule functioning represents large C and P costs (Ryle et al. 1979), but the additional N supply compensates for this C investment by allowing faster rates of photosynthesis (Tjepkema and Winship 1980). Some species use more than one nutrient-acquisition strategy, e.g. some legumes like *Kennedia* (Fabaceae), native to the south-western Australian *kwongan*, release carboxylates and form mycorrhizal associations and nodules (Adams et al. 2002, Ryan et al. 2012). It is likely that other co-occurring legumes also present all three strategies. Very few studies have tested the effects of nutrient addition on how species are positioned along the trade-off axis between C investment in mycorrhizas versus carboxylates (Ryan et al. 2012), or between mycorrhizas and nodules (Larimer et al. 2014).

In this study, we determine the effect of increasing N and P supply on four nutrient-acquisition strategies (i.e. mycorrhizas, carboxylate release, root morphological plasticity and nodule formation) within two congeneric woody legumes, native to shrublands of south-western Australia. The species belong to the genus *Bossiaea*, are mycorrhizal, and associate with *Bradyrhizobium* spp. to fix N (Lange 1959, Parker 2015, Thrall et al. 2011, Brundrett and Abbott 1991). The species occur along two retrogressive soil chronosequences in Australia, on sand-dunes covering 2-million years of

pedogenesis (Turner et al. 2018). These chronosequences offer excellent model systems to test for changes in nutrient-acquisition-strategies with soil nutrient availability, while controlling for host identity. Total soil P concentration declines 40-fold along the Jurien Bay and 10-fold along the Warren chronosequence from the youngest to the oldest soils (Turner et al. 2018, Turner and Laliberté 2015). We also grew these species in a glasshouse in order to remove possible confounding factors driving nutrient-acquisition strategies in the field, such as biotic interactions and differences in soil pH. *Bossiaea linophylla* R.Br. occurs along all the stages of the Warren chronosequence, while *B. eriocarpa* Benth. occurs only at the three oldest stages of the Jurien Bay chronosequence. We examined four hypotheses. (a) Low P supply, regardless of N supply, would stimulate carboxylate release and increase leaf [Mn], inhibit arbuscular mycorrhizal colonisation, and increase investment in roots (e.g., increase specific root length and root: mass ratio). (b) High N supply at low P supply would inhibit nodulation in the glasshouse, whereas high P supply would enhance nodulation, because of the high P requirements of nodules. (c) Leaf nutrient concentrations would increase with increasing N and P availability, and suggest a switch from N to P limitation along the chronosequences in the field, but plants would have nutrient-conserving traits such as slow growth and little growth response to nutrient addition in the glasshouse. (d) *B. eriocarpa* would present less variation in nutrient-acquisition and -use strategies between chronosequence stages and also between glasshouse treatments than *B. linophylla* as it only occurs in the oldest stages of the Jurien Bay chronosequence, which present less variation in soil pH and total P.

Methods

Field sampling

Study area. We conducted the study along two retrogressive, long-term soil chronosequences on the south-western Australian coast (Fig. 1), each consisting of a series of parallel sand dunes deposited over more than 2 million years (Turner et al. 2018). Stages 1 to 3 correspond to dunes deposited during the Holocene (up to 6 500 years old), stages 4 and 5 correspond to dunes deposited during the Middle Pleistocene (120 000 to 500 000 years old) and stage 6 corresponds to Early Pleistocene dunes (up to 2 million years old) (Playford et al. 1976, Turner et al. 2018, Turner and Laliberté 2015). There are ten permanent 10 m x 10 m plots installed within each chronosequence stage at the Jurien Bay chronosequence and five 20 m x 20 m plots per stage at the Warren chronosequence (Laliberté et al.

2014, Turner et al. 2018). Minimum distance between plots was 2 km at the Jurien Bay chronosequence and 50 m at the Warren chronosequence (Hayes et al. 2014). The Jurien Bay chronosequence (30.29° S, 115.04° E) has a warmer (mean annual temperature, MAT, 19°C) and drier (mean annual precipitation, MAP, 533.2 mm) climate than the Warren chronosequence (34.62° S, 115.89° E; MAT 15°C, MAP 1184 mm) (Turner et al. 2018). Changes in soil N and P availability across both sequences are consistent with predictions of long-term soil and ecosystem development (Walker and Syers 1976, Turner and Condron 2013). Total soil [P] is highest at the youngest stages and is lowest at the oldest stages (Fig. 2); total soil [N] is greatest at intermediate stages (Turner et al. 2018). The total soil [P] of the soil is considerably higher at the Jurien Bay chronosequence (266 mg kg^{-1}, 10 cm depth) than at the Warren chronosequence (25 mg kg^{-1}). However, both chronosequences reach the same low levels of total soil [P] at the oldest stages (6 mg kg^{-1}) (Turner et al. 2018). The proportion of P as organic P is higher along the Warren than at the Jurien Bay chronosequence (Turner et al. 2018). The pedogenic processes include loss of carbonates and a pH decline from approximately 8 to 4 at both chronosequences. Complete soil and vegetation descriptions can be found in Turner et al. (2018) and detailed information about the Jurien Bay chronosequence in Turner and Laliberté (2015). An interesting feature of these landscapes is that they present high vascular plant species diversity and high endemism at very low nutrient availability (Hopper 2009, Hopper and Gioia 2004, Hopper et al. 2016, Zemunik et al. 2016).

Species selection. We selected two congeneric native legume species that occur along the chronosequences and tested if the species switched nutrient-acquisition strategies according to N or P limitation along the chronosequences, and also in a glasshouse experiment. *Bossiaea linophylla* is an erect shrub, 0.4 to 2.2 m tall, growing in sandy soils, coastal limestone, dunes and granite rocks of the South-West Province of Australia, from Bunbury to Albany (FloraBase 2017). It occurs across all stages of the Warren chronosequence, with relative canopy cover of 5, 3, 9, 8 and 1% for stages 1, 2, 3, 4 and 6, respectively (Zemunik et al. unpublished data). *Bossiaea eriocarpa* is an erect or spreading shrub to 1 m tall that grows on sandy soils of the South-West Province from Geraldton to Albany (FloraBase 2017); it grows within stages 4 to 6 of the Jurien Bay chronosequence, with relative cover of 4, 2 and 1%, in stages 4, 5 and 6, respectively (Zemunik et al. 2016). Both *B. linophylla* and *B. eriocarpa* are legumes and associate with N_2-fixing *Bradyrhizobium* spp. to form nodules (Lange 1959, Parker 2015, Thrall et al. 2011). *Bossiaea* is also colonised by arbuscular mycorrhizal fungi (Zemunik et al. 2015, Brundrett and Abbott 1991). Some south-western Australian herbaceous

legumes release large amounts of carboxylates (Ryan et al. 2012), and we expected *Bossiaea* to do the same, because of the relatively high leaf [Mn] of *B. eriocarpa* along the Jurien Bay chronosequence (Zemunik et al., unpublished data).

Plant material. We collected mature, fully-expanded leaves of *B. linophylla* from three individuals per plot within 30 m, for five plots per chronosequence stage in November 2015 (Table S1). *Bossiaea linophylla* occurred at five stages (1-4 and 6). No plots were set up in the stage 5 described in Turner et al. (2018). Since *B. eriocarpa* does not occur along the entire chronosequence, we collected leaves from four individuals per plot in a total of 10 plots from three stages in September 2016 (Table S1). *Bossiaea eriocarpa* only occurred in one plot of stage 5, which is pedologically very similar to stage 4, both belonging to Spearwood dune system (Turner et al. 2018), so samples from stages 4 and 5 were pooled for statistical analyses. Leaves from both species were bulked per plot, oven-dried for 96 h at 70°C and ground with a Geno/Grinder 2010 (SPEX SamplePrep, Metuchen, NJ) using ceramic bearings for 3 min at 1500 rpm. Leaf nutrient analyses were conducted at the ChemCentre, Perth, Australia. A subsample was digested in concentrated HNO_3: $HClO_4$ (3: 1 v/v) and Mn and P concentrations were analysed in an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Varian Australia Pty Ltd, Australia). All digests were first analysed using a simultaneous Varian Vista Pro (Varian Australia Pty Ltd, Australia), radially configured ICP-OES equipment fitted with an Auto Sampler (CETAC ASX-510, Omaha, NE). A second subsample was analysed for total leaf [N]. Samples were digested using the Kjeldahl method with a mixture of salicylic and sulfuric acid with hydrogen peroxide (Bradstreet 1965), diluted and analysed by the Berthelot colourimetric determination (Searle 1984, Varley 1966) using a two-channel autoanalyser (Technicon AAII, Technicon Instrument Corporation, Tarrytown, NY).

Field mycorrhizal colonisation. To measure arbuscular mycorrhizal colonisation, we collected at least 30 cm of young roots from the same plants from which we collected leaves and immediately cleaned them with water and stored them in ethanol (50% v/v). In the laboratory, we cleared the roots in KOH (10 % w/v) at room temperature for 7 days and further heated them in a water bath at 90°C for 1-3 h. We acidified the roots in HCl (1% v/v) overnight and transferred them to Parker blue ink in white vinegar (5% v/v) for 3 hrs at room temperature (Vierheilig et al. 1998). We thoroughly rinsed the samples to remove excess ink and stored them in acidified glycerol (50% v/v with 5% HCl). We assessed mycorrhizal colonisation by counting the number of arbuscular mycorrhizal hyphae, arbuscules and vesicles in 30 cm of root using the slide method at 200 x magnification (Giovannetti

and Mosse 1980). We divided each 1 cm root fragment in 10 visual fields and then recorded the presence/absense of mycorrhizal structures in each field. The percentage was the average proportion of fields where mycorrhizal structures were present.

Glasshouse experiment

Set up. We set up a factorial design with four P treatments (0, 0.5, 5 and 50 mg P kg^{-1} soil) and three N treatments (10, 30 and 50 mg N kg^{-1} soil) with eight replicates for each treatment. The treatments we chose covered the range of total soil P and N along both chronosequences (Turner et al. 2018). We planted two plants per pot to allow measurement of carboxylates on one plant and mycorrhizal colonisation on the other (total number of pots per species: 4 x 3 x 8 = 96). We also had two non-planted pots per treatment combination to measure microbial carboxylates. Since carboxylate concentration in non-planted pots was negligible, we did not discuss this data any further.

Germination. We purchased seeds of *B. linophylla* and *B. eriocarpa* from Nindethana Seed Service, Western Australia. To break dormancy, we boiled the seeds for 60 s (Bell et al. 1993) and then soaked them overnight in aerated sterile deionised (DI) water. We surface-sterilised the seeds in 70% (v/v) ethanol for 10 s, rinsed three times in sterile DI water, submerged seeds for 10 s in 2% (v/v) sodium hypochlorite and then rinsed in DI water a further three times. We spread the seeds on two 18.5 cm diameter filter papers in 20 cm diameter Petri dishes and placed them in a controlled-temperature room in the dark at 15°C and sprayed daily with DI water (Bell et al. 1995). After the radicle emerged (21 to 48 days), seeds were transferred into steam-pasteurised washed coarse river sand in seed trays (cell dimensions: 36 × 38 × 60 mm) and kept in the dark until cotyledons emerged. As soon as the cotyledons emerged, the seed trays were transferred to a glasshouse with a shade cloth reducing the light intensity to 30% in January 2016. To keep the seedlings moist, seed trays were covered with a plastic cover. Between February and April 2016, the glasshouse temperatures varied between 13 and 34°C during the day and between 13 and 24°C during the night. The relative humidity ranged from 31 to 75% and peak irradiance varied from 700 to 2000 µmol m^{-2} s^{-1}. The seedlings were watered every second day with DI water.

Plant growth. We lined pots of 8.5 cm × 8.5 cm × 18 cm depth with plastic bags and filled with 1.08 kg of air-dried, double steam-pasteurised, washed coarse river sand. We fertilised with a P- and N-free basal nutrient solution containing: K 62 mg kg^{-1}, S 43 mg kg^{-1}, Ca 27 mg kg^{-1}, Fe 14 mg kg^{-1}, Cl 35 mg

kg^{-1}, Mg 7.9 mg kg^{-1}, Cu 1.3 mg kg^{-1}, Zn 2.3 mg kg^{-1}, Mn 9.4 mg kg^{-1}, B 0.12 mg kg^{-1}, Mo 0.09 mg kg^{-1}. We added N as potassium nitrate (KNO_3) at 10, 30 or 50 mg N kg^{-1} soil (N10, N30, N50) and P as monopotassium phosphate (KH_2PO_4) at 0, 0.5, 5 or 50 mg P kg^{-1} soil (P0, P0.5, P5, P50). We did not include a zero N treatment, as native legumes showed poor establishment in such a treatment in previous experiments (Png et al. unpublished data).

On top of the sand was added 120 g of field soil, which was intended to act as inoculum for arbuscular mycorrhizal fungi and rhizobia. We did so to maximise the chances that the inoculum interacts with roots of very small seedlings following emergence. The use of inoculum as top-soil only was effective in previous studies in our group (Png 2016). The field soil was a 1:1 mix of soil collected under *B. eriocarpa* and *B. aquifolium*. We collected the soil under *B. eriocarpa* from 0-10 cm in a sandy soil in bushland at Harrisdale in the Perth metropolitan area (32°10' S, 115°92' E), Australia. We collected the soil under *B. aquifolium* from 0-10 cm in a lateritic soil in bushland at Waroona, ~100 km south of Perth, Australia. We used the field soil mix to represent the diversity of habitats of occurrence of *Bossiaea* in the field. We did not have access to soil under *B. linophylla* at the time of the experimental set-up, but arbuscular mycorrhizal fungi are known to associate with a wide range of plant species hosts (Smith and Read 2008).

Chemical analyses of the soils were conducted at ChemCentre, Perth, Australia (Table 1). Total N was measured after digestion with sulfuric acid, in the presence of a catalyst. Using a Technicon AA11 segmented flow auto-analyser (Technicon Instrument Corporation, City, NY), the digest was diluted, and N was determined by reaction with chlorine and salicylic acid to form a blue compound (Berthelot reaction). Total P was determined using the same digest colourimetrically as the phosphomolybdenum blue complex (Murphy and Riley 1962) with a Shimadzu UV-Vis spectrophotometer (Columbia, MD). Other soil nutrients were measured using the Mehlich-3 extraction followed by ICP-OES analysis in a Arian Vista axial ICP-OES (Rayment and Lyons 2011).

Three months after the first seeds germinated (April 2016 - autumn), seedlings were transplanted to the pots prepared with nutrients and soil inoculum. After transplanting, the soil surface in each pot was covered with 10 g of polyethylene beads to minimise soil evaporation. Over the following three months, we watered the plants with deionised water to 90% field capacity three times a week, and then to 70% once a week from June 2016. We recorded pot weight before each watering.

The position of the pots was re-randomised fortnightly. Between April and August, the glasshouse temperatures varied between 10 and 27°C during the day, and between 10 and 22°C during the night. The relative humidity ranged from 28 to 82% and peak irradiance varied from 900 to 1500 µmol m^{-2} s^{-1}.

Harvest. Plants were harvested in August 2016. We removed and separated the two plants per pot for either carboxylate collection (plant A) or mycorrhizal colonisation analysis (plant B). For plant A, the soil that remained attached to the roots after gently tapping the roots was considered rhizosphere soil (Veneklaas et al. 2003). To collect the rhizosphere carboxylates, we gently immersed the roots covered with rhizosphere soil of plant A in a known volume (~20 ml) of 0.2 m*M* $CaCl_2$ for 1 min. We filtered 1 ml of the rhizosphere extract with a 22-µm syringe filter into 25 µl of concentrated orthophosphoric acid and froze the samples at -20°C. Carboxylate concentrations were determined using HPLC, following Cawthray (2003). Working standards of tartaric, formic, malic, malonic, lactic, acetic, maleic, citric, succinic, oxalic, fumaric, cis-aconitic and trans-aconitic acids were used. After carboxylate collection, we rinsed the plant in deionised water, removed the excess water with a paper towel, and separated the roots, the stem and the leaves. Leaves and stems were dried at 70°C for 4 d. Roots were wrapped in damp paper towels in a zip lock bag for a maximum of 48 h at 4°C. Nodules were then counted and removed and the whole root system floated in a transparent tray in water and scanned, and analysed for root length, root surface area and average diameter with WinRhizo (WinRhizo system, Regent Instruments, Montreal, Canada). Roots were then dried at 70°C for 4 d. Root: mass ratios were calculated as the ratio between root and total plant dry mass. Plants were considered dead when leaves and stem had turned brown. Plants exhibiting these symptoms were rewatered for one month and those that did not recover were confirmed as dead.

Mycorrhizal analysis. To measure mycorrhizal colonisation, roots of plant B were rinsed and treated as for the field samples, except for staining. We treated the stem and leaves as described above for plant A. Root samples were stained in Trypan Blue (0.05% w/v) for an hour in a water bath at 90°C, rinsed and stored as described for field samples. We assessed mycorrhizal colonisation using the intersect method by counting the number of arbuscular mycorrhizal hyphae, arbuscules and vesicles at each intersect (Giovannetti and Mosse 1980) for at least 180 intersects. Note that the assessment method used for the field samples can result in a slightly higher estimate of the level of colonisation than the method used for the glasshouse samples (Giovannetti and Mosse 1980).

Leaf nutrient concentrations. Leaf samples were bulked per pot for nutrient analyses. Dry leaves were ground as described above, a subsample was digested using concentrated HNO_3: $HClO_4$ (3:1) and analysed for P and Mn concentrations in an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES, Perkin Elmer 5300DV, Waltham, MA). A second subsample was used for determination of total N concentration using the combustion method (Elementar Vario Macro, Hanau, Germany). All the glasshouse nutrient analyses were performed at Earth and Environment Analysis Laboratory (EEAL), The University of Western Australia. To estimate the control of nutrient uptake, we calculated coefficients of variation (CV) of leaf nutrient concentrations.

Statistical analyses

All statistical analyses for the field data and glasshouse experiment involved model selection using corrected Akaike Information Criterion (AICc). For the field data, linear models were used to compare plant attributes among stages, modelled as categorical variables to test specific differences among chronosequence stages. A model with intercept only (null model) (Mac Nally et al. 2018) was compared with a model with chronosequence stage as an independent variable. We checked model assumptions graphically (Zuur et al. 2009), and if they were not met, variance was modelled using generalised least square models (gls) with the nlme package (Pinheiro et al. 2017). If the models presented residual heteroscedasticity or non-normality, nutrient concentrations were modelled using generalised linear models (glm) with gamma distribution. Because mycorrhizal colonisation is a percentage, we modelled it using beta regressions with the betareg package (Cribari-Neto and Zeileis 2010), adding 10^{-11} to all the values, because zeros cannot be included in betareg. We selected random terms in gls and fixed terms in all the models using the corrected Akaike Information Criterion (AICc) (Zuur et al. 2009). If the differences in AICc between models were less than two units, we chose the most parsimonious model (Arnold 2010). If the term chronosequence stage was in the selected model, Tukey's post-hoc comparisons were performed with the lsmeans package (Lenth 2016).

For the glasshouse experiment, two-way ANOVAs were used to model differences between the plant attributes measured in N and P supply treatments, modelled as categorical variables. We compared a null model with models with P and N supply, with and without interactions, as independent variables, and with only P or only N supply as independent variables. To meet model assumptions gls, glm and betareg were used as described above. Post-hoc comparisons were

undertaken as described above. All statistics were undertaken in R 3.3.2 (R Development Core Team 2017).

Results

Field data

Leaf [P] of *B. linophylla* followed total soil [P] along the high-rainfall Warren chronosequence, being lowest in younger and older soils (Fig. 2A, Fig. 3A, CV of leaf [P] = 32%, Table S2). It increased from 0.8 ± 0.1 mg P g^{-1} DW (least square mean ± CI) at the younger chronosequence stages (1-2) to 1.3 ± 0.2 mg P g^{-1} DW at the intermediate stages (3-4), and decreased again to 0.6 ± 0.2 mg P g^{-1} at the oldest stage (Fig. 3A). By contrast, *B. eriocarpa* along the low-rainfall Jurien Bay chronosequence maintained a similar, relatively low, leaf [P] (0.4 ± 0.1 mg P g^{-1} DW) at stages 4-6 (Fig. 3B, Table S2, CV = 19%). Leaf [N] of *B. linophylla* was greatest at stage 3 (21 ± 2 mg N g^{-1} DW), being lower on younger and older soils (Fig. 3C, Table S2, CV= 16%). Again, *B. eriocarpa* maintained a similar leaf [N] at stages 4-6 (19 ± 1 mg N g^{-1} DW, Fig. 3D, Table S2, CV=11%). Leaf N: P ratios of *B. linophylla* were similar among stages 1-4 (19 ± 2 mg N g^{-1} DW), and higher at stage 6 (28 ± 3 mg N g^{-1} DW, Fig. 3E, Table S2, CV=109%). Leaf N: P ratios of *B. eriocarpa* were similar among stages 4-6 and greater than those of *B. linophylla* (43-48 ± 2 mg N g^{-1} DW, Fig. 3F, CV= 11%).

Leaf [Mn] varied greatly among chronosequence stages (Fig. 4A-B). In *B. linophylla*, the leaf [Mn] started at 11 ± 2 mg Mn kg^{-1} DW at stage 1, and reached a very high 568 ± 163 mg Mn kg^{-1} DW at stage 6 (Fig. 4A, Table S2, CV=150%). In *B. eriocarpa*, leaf [Mn] varied around 24 ± 8 mg Mn kg^{-1} DW at stages 4 and 5, but increased to 98 ± 8 mg Mn kg^{-1} DW at stage 6 (Fig. 4B, Table S2).

The proportion of root length colonised by arbuscular mycorrhizal fungi was similar at all stages for both species (Fig. 4C-D). In *B. linophylla*, mycorrhizal colonisation varied from 35 ± 9% at the first stages to 58 ± 13% at stage 4 (Fig. 4C, Table S2). In *B. eriocarpa*, mycorrhizal colonisation varied between 24 ± 6% at stage 6 and 32 ± 7% at stages 4-5 (Fig. 4D, Table S2).

Glasshouse experiment

Leaf [P] increased with increasing N and P supply for both species (Fig. 5A, b no P-N interaction). In both species leaf [P] approximately doubled from P0-P50 (Fig. 5B, CV of leaf [P] of *B. linophylla* = 52%, *B. eriocarpa* =39%). Leaf [N] also varied with increasing N and P supplies in both species (Fig. 5C, D, Table S3, no interaction between N and P supply). In *B. linophylla*, leaf [N] increased approximately 30% between P0-P50 and 15% between N10-N50 (CV= 17%). In *B. eriocarpa*, maximum leaf [N] variation was 26% between N10-N50 (CV= 18%). Leaf N: P ratios decreased with increasing P supply, but increased with increasing N supply for both species (Fig. 5E, F, Table S3, no N-P interaction). In *B. linophylla* N: P ranged from 25 ± 5 in P50/N10 to 52 ± 6 in P0/N50 (CV= 27%). In *B. eriocarpa* N: P ranged from 32 ± 5 to 63 ± 7 in the same treatments (CV= 23%).

Total dry biomass was very similar for the two species, and increased more than three-fold between P0 and P50 (Fig. 6, Table S3), but did not vary with increasing N supply (Table S3). Total biomass in *B. linophylla* varied from 0.12 ± 0.03 g in P0 to 0.40 ± 0.11 g in P50; and from 0.12 ± 0.06 g to 0.43 ± 0.13 g in *B. eriocarpa* in the same treatments. Changes in the total biomass were due to increases in leaf, stem and root biomass in both species (Fig. 6). However, root length, root volume and root surface area increased with increasing P supply in *B. linophylla* only (data not shown, Table S3). Root: mass ratio decreased with P supply in *B. linophylla* and decreased with increasing P supply, but increased with increasing N supply, in *B. eriocarpa* (data not shown, Table S3).

While increasing N supply did not affect biomass in *B. linophylla*, specific root length (root length/root dry weight) decreased with increasing P supply and increased with increasing N supply in the P0 and P0.5 treatments, but decreased with increasing N supply in the P5 and P50 supplies (data not shown, Table S3). Specific root length did not vary with P and N supply in *B. eriocarpa* (data not shown, Table S3).

We detected citrate (59-98% of total amount of rhizosphere carboxylates for *B. linophylla* and 0-77% for *B. eriocarpa*), malate (0-38% for *B. linophylla* and 7-74% for *B. eriocarpa*), malonate (0-1% for *B. linophylla* and 0-23% in *B. eriocarpa*) and lactate (undetected for *B. linophylla* and 0-20% in *B. eriocarpa*) in decreasing order of amounts (Fig. 7). We also detected acetate, maleate and fumarate, but in much lower amounts. Total carboxylate amount in *B. linophylla* was positively affected by both increasing P and N supply (P-N interaction), but we did not find differences in pairwise comparisons between treatment combinations (Fig. 7C, Table S3). Total carboxylates in the

rhizosphere varied from 2.4 ± 1.3 μmol g^{-1} root DW at P0/N10 to 13.5 ± 31.7 μmol g^{-1} root DW at P0/N50 in *B. linophylla*. In the P50 treatment, *B. linophylla* showed 5.8 ± 4.6 μmol g^{-1} root DW at N10 and 22.9 ± 23.2 μmol g^{-1} root DW at N50. The total amount of carboxylates in the rhizosphere of *B. eriocarpa* was one order of magnitude less than that in *B. linophylla* (Fig. 7D, Table S3). Total carboxylates decreased with increasing N supply, with no pairwise differences, from 0.39 ± 0.26 μmol g^{-1} root DW at N10 to 0.07 ± 0.08 μmol g^{-1} root DW at N50.

Mycorrhizal colonisation decreased with increasing N supply in *B. linophylla*, but was independent of P supply (Fig. 8A, Table S3). In *B. eriocarpa*, mycorrhizal colonisation decreased with increasing P supply, being highest at intermediate N supply (N30) (23 ± 1% colonisation) and lowest in P0.5 (4 ± 2% colonisation, Fig. 8B, Table S3). Numbers of nodules per plant decreased with increasing N supply for both species, but increased with increasing P supply in *B. linophylla* (Fig. 8C,D, Table S3; note no P-N interaction). In the P0 treatment, *B. linophylla* presented 11 nodules at N10, but only 1 nodule at N50, and in the P50 treatment, 24 nodules at N10 and 14 at N50. In *B. eriocarpa*, only N supply affected nodule formation. Numbers of nodules of *B. eriocarpa* decreased from 10 in N10 to 5 in N50 (Fig. 8D, Table S3).

Leaf [Mn] of *B. linophylla* decreased with increasing P supply, but increased with increasing N supply (Fig. 9A, no N-P interaction). In *B. linophylla*, leaf [Mn] halved from P0 to P50, but increased 30% between N10-N30 (CV= 26%). However, total leaf Mn content (leaf [Mn] multiplied by leaf mass), increased with increasing P supply (data not shown) so the decrease in leaf [Mn] with increasing P supply was due to a dilution effect. In *B. eriocarpa* leaf [Mn] decreased 30% with increasing P supply from P0 to P50, being constant among N treatments (Fig. 9B, CV= 24%) and total leaf Mn content of *B. eriocarpa* did not change with increasing N or P supply (data not shown).

Mortality rate increased with increasing N supply in both species and was unaffected by P supply (Fig. S1, Table S3). In *B. linophylla*, mortality rates increased from 29% at N10 to 75% at N50 (Fig. S1A). In *B. eriocarpa*, mortality rates increased from 23% at N10 to 53% at N50 (Fig. S1B).

Discussion

We investigated whether P and N availability affected P- and N-acquisition strategies of two legume species native to the south-western Australian kwongan by investigating foliar [P], [N] and

[Mn] and root mycorrhizal colonisation along two chronosequences with distinct P and N availabilities (Turner et al. 2018). Additionally, we investigated how varying P and N supply affected foliar [P] and [N], biomass production and allocation, root morphology, rhizosphere carboxylate amount, mycorrhizal colonisation and nodulation in a glasshouse experiment. We observed few differences in physiological (e.g. carboxylate release) and symbiotic P-acquisition strategies, but did observe differences in the specific root length and and root: mass ratio. Our results show that growth of two *Bossiaea* species is usually P-limited due to the supply of N from N-fixation, and that the two species presented different shifts in nutrient-acquisition strategies with change in nutrient-availability.Together, these findings have implications for the occurrence of the two species along the two chronosequences.

Phosphorus-acquisition strategies at different nutrient availabilities

We tested if nutrient-acquisition strategies of *Bossiaea* shifted as observed at the community-level by Zemunik et al. (2015). We rejected our hypothesis that low P supply would stimulate carboxylate exudation, but instead observed a greater reliance on morphological modifications (e.g., greater specific root length) for P uptake. We found that for *B. linophylla,* increasing P supply increased the amount of rhizosphere carboxylates (Fig. 7c). A similar result was observed by D'Angioli et al. (2017) for *Zea mays* and by Huang et al. (2017) for *Agonis flexuosa* (Myrtaceae), which also grows along the entire Warren chronosequence. When grown in a glasshouse, the amount of rhizosphere carboxylates of *A. flexuosa* increased two-fold between 0 and 30 mg P kg^{-1} soil treatments. The increased amount of carboxylates in the rhizosphere of *A. flexuosa* occurred in the treatments with lowest root: shoot ratios and highest photosynthetic rates, possibly indicating release of excess C by the roots (Huang et al. 2017). The amount of rhizosphere carboxylates of *B. linophylla* (maximum ~20 μmol g^{-1} root DW) was of the same order of magnitude as those found in several other glasshouse studies for a range of species, native and exotic, for example, several *Kennedia* species inoculated with arbuscular mycorrhizal fungi (Ryan et al. 2012) and *Cullen australasicum*, *Bituminaria bituminosa*, *Medicago sativa* and *Trifolium subterraneum* (Nazeri et al. 2014). However, they were much less than those presented for *Cicer arietinum* and *Lupinus albus* by Veneklaas et al. (2003) and *K. nigricans* by Suriyagoda et al. (2012) and Ryan et al. (2012) (100-200 μmol g^{-1} root DW). At the lowest P supplies, *B. linophylla* appeared to rely on morphological adaptations such as greater specific root length, rather than on carboxylates. The rhizosphere carboxylate amounts of *B. eriocarpa* were one order of magnitude less than those in *B. linophylla*, and they decreased with increasing N supply, rather than

increasing P supply. Despite the lower rhizosphere carboxylate amounts in *B. eriocarpa*, the leaf [P] and total biomass were similar to those of *B. linophylla*, possibly because *B. eriocarpa* invested in alternative P-acquisition strategies, such as the release of phosphatases into the soil as observed in other legumes which occur along the Jurien Bay chronosequence (Png et al. 2017). *Bossiaea eriocarpa* also invested more in root biomass, as shown by the greater root: mass ratio at low P. The large confidence intervals of total carboxylates in the N50 treatments in *B. linophylla* are likely due to the small sample size, because of the high mortality rate in this treatment. In *B. eriocarpa*, the greatest mortality was observed in the P0/N50, and did not affect sample size of N50 at other P treatments as strongly as in *B. linophylla*.

The proportion of arbuscular mycorrhizal plant species decreases along the Jurien Bay chronosequence (Zemunik et al. 2015), so we expected that the proportion of the root length colonised by the fungi would decrease also along the chronosequence within a single species. We expected that the proportion of the root colonised by mycorrhizal fungi would be low at a very low P supply, high at intermediate P supply and also be low at high P supply. However, we rejected our hypothesis that mycorrhizal colonisation would vary with P availability in the field. In fact, colonisation was constant along the chronosequences for both species. At the oldest stages, the costs of maintaining a mycorrhizal symbiosis are expected to be greater than the nutritional benefits (Lambers et al. 2018, Raven et al. 2018). However, at extremely low soil [P], where *B. eriocarpa* occurs, mycorrhizal fungi might have a different role. Rather than increase the uptake of P, the fungi may provide protection against soil pathogens (Albornoz et al. 2017, Laliberté et al. 2015, Lambers et al. 2018). Because the total soil [P] at the youngest stages of the Warren chronosequence is much lower than that at the youngest stages at Jurien Bay (Turner et al. 2018), it is likely that the role of mycorrhizal fungi in pathogen defence dominates much earlier during pedogenesis. This might explain why colonisation was constant along the two chronosequences.

In the glasshouse experiment, increasing P supply did not affect mycorrhizal colonisation in the roots of *B. linophylla*, similar to that reported for *Viminaria juncea* (de Campos et al. 2013). Instead, root colonisation declined with increasing N supply. The decrease in mycorrhizal colonisation at high N supply can be explained by an increased allocation to photosynthesis, and a lower resource allocation to roots and, particularly, lower C-transfer to root symbionts (Marschner et al. 1996, Johnson 2010). This pattern is expected when enough P is supplied to the plant. The mycorrhizal colonisation response to nutrient addition in *B. eriocarpa* differed from that in *B. linophylla.*

Mycorrhizal colonisation decreased with increasing P supplies and was greatest at intermediate N supplies. Thus, we partially rejected our hypothesis of changes in mycorrhizal colonisation with P supply in the glasshouse. Therefore, the shift in the prevalence of species that acquire nutrients through arbuscular mycorrhizas along the chronosequence observed at the community level in Jurien Bay (Zemunik et al. 2015) did not happen within *Bossiaea* species, either in the glasshouse or in the field. The changes in the proportion of species that use nutrient-acquisition through mycorrhizas in the field must therefore be due to species turnover (Laliberté et al. 2013, Zemunik et al. 2016), rather than within-species changes in nutrient-acquisition strategies.

Leaf [Mn] and nutrient-acquisition strategies

Leaf [Mn] cannot be compared among chronosequence stages due to large differences in soil pH which affect Mn availability. Therefore, we only present within-stage comparisons with co-occurring species. We expected that leaf [Mn] would increase with increased carboxylate release and decrease with increased mycorrhizal colonisation. If *Bossiaea* released more carboxylates than co-occurring species at each stage, we would expect them to have greater leaf [Mn] (Lambers et al. 2015); thus, we only partly accepted our hypothesis based on field data. Co-occurring carboxylate-releasing species, including Proteaceae, along the Warren chronosequence (Huang et al. 2017) and along the Jurien Bay chronosequence (Hayes et al. 2014) present similar leaf [Mn] to those of *Bossiaea*; this may indicate facilitation by neighbouring plants, as demonstrated in glasshouse experiments (Muler et al. 2014). The similarity between leaf [Mn] of these species may be due to the root intermingling observed in the field, where we rarely found *Bossiaea* roots separated from cluster roots of other species. However, any role of facilitation needs to be confirmed using targeted glasshouse experiments (Lambers et al. 2018). We surmise that the increase in leaf [Mn], relative to that of other species at the same location, along the chronosequences is a combination of increased availability through mobilisation by rhizosphere microbiota and facilitation.

Nitrogen-acquisition strategies at different P and N availabilities

We expected N limitation to stimulate N-fixation, provided enough P was available (Sprent 1999). In fact, we confirmed our hypothesis that increased N supply would reduce nodulation;

however, increased P supply only stimulated nodulation in *B. linophylla*. In the glasshouse experiment, we found that high N supply effectively decreased nodule formation in both species as expected (Streeter and Wong 1988). Nitrate application reduces nitrogenase activity and induces nodule senescence, for example, due to down-regulation of genes involved in mitochondrial ATP generation (Cabeza et al. 2014). In *B. linophylla*, nodule formation was also stimulated at high P supply, as expected due to the high P-cost of nodulation (Raven 2012, Pang et al. 2011). The range of leaf [P] was greater among treatments in *B. linophylla* than in *B. eriocarpa*. This might explain why nodule formation in *B. eriocarpa* responded only to increasing N, but not to increasing P supply. Therefore, we observed that N and P availability can drive changes in N-acquisition strategies in *Bossiaea*.

Leaf P and N concentrations

Leaf [N] and [P] of the glasshouse-grown plants were similar to those of plants in the field. Leaf [P] in the field (0.2-1.3 mg P g^{-1} DW) and in the glasshouse (0.3-0.9 mg P g^{-1} DW) were below the global average leaf [P] (1.43 mg P g^{-1} DW) estimated by Vergutz et al. (2012), while leaf [N] of both field (15-21 mg N g^{-1} DW) and glasshouse-grown plants (15-28 mg N g^{-1} DW) were equal to, or even higher than the global average (18.4 mg N g^{-1} DW) (Vergutz et al. 2012). The high [N] may reflect the inherently high N demand of legumes (Vitousek et al. 2002, Sprent 1999, Hayes et al. 2014).

We hypothesised that leaf [N] and [P] would increase with increasing supply and, indeed, leaf [N] and [P] of both field- and glasshouse-grown plants generally increased with increasing N and P supplies for both species. While leaf [N] and [P] of *B. eriocarpa* in the field were unchanged along the last three chronosequence stages, where it occurs, this was likely due to the similar soil availabilities of these nutrients.

Leaf [P] and [N] of *B. eriocarpa* were similar to those of the N-fixing species, but higher than the concentrations of species with other nutrient-acquisition strategies, at the same chronosequence stages of Jurien Bay (Hayes et al. 2014). Leaf [P] of *B. eriocarpa* in the field was very similar to that of co-occurring legumes from stages 4 to 6, and lower than leaf [P] and [N] of legumes from the younger stages where *B. eriocarpa* does not occur (Png et al. 2017).

In the glasshouse experiment, *Bossiaea* plants increased in leaf [N] to a small extent and seemed to down-regulate N uptake with increasing N availability. However, the high mortality rate of *B. linophylla* (75%) and *B. eriocarpa* (50%) at the highest N supply (50 mg kg^{-1}), especially at low P supply (0 and 0.5 mg P kg^{-1} soil), might indicate a threshold beyond which N uptake cannot be down-regulated. The mortality may therefore be due to a stoichiometric imbalance (Schulze 1989), rather than N-toxicity, because mortality decreased at higher P supplies. Lower mortality at higher P supplies might also be due to the dilution of the N when biomass increased. Also, the total N supplied to the plants was readily available, in the form of potassium nitrate. In the field, total soil [N] can be higher, but is in less available organic forms (Turner and Laliberté 2015), and, thus, less likely to cause toxicity.

Nutrient limitation, leaf N:P ratios and growth

Nutrient limitation is diagnosed by how primary productivity and other biological processes respond to added nutrients (Vitousek et al. 2010). Nitrogen to P ratios above 16 indicate P-limitation of plant growth (Redfield 1958, Wardle et al. 2004). We expected that the growth of *Bossiaea* would switch from N to P limitation along the chronosequences, in accordance with the ecosystem development model of Walker and Syers (1976). Along the Warren chronosequence, N:P ratios of *B. linophylla* ranged between 17 and 19 along the first four stages, and reached 28 at the last stage, indicating P-limitation at all stages. Since *B. eriocarpa* only occurred at the last three stages of the Jurien Bay chronosequence, all of which have low P availability, it was not surprising that N:P ratios between 43 and 48 indicated growth of all plants was P-limited. The apparent P limitation of plant productivity in *Bossiaea* can be due to the high leaf [N], supported by N-fixation in nodules. Therefore, our results did not support our hypothesis that *Bossiaea* would switch from N to P limitation along the chronosequences, due to their high leaf [N].

We assessed the nutrient limitation of young *Bossiaea* plants, measuring variation in growth with varying N and P supply in a glasshouse experiment. We expected that species that occur in P-impoverished soils would present slow growth rates in order to reduce nutrient demand (Lambers and Poorter 1992). Indeed, *Bossiaea* did show conservative nutrient-use traits such as slow growth, but they also did respond to P addition in a modest way. The plants accumulated less than one gram of dry weight in five months, even at the highest nutrient supply. Co-occurring legumes from the same

stages, *Acacia pulchella* and *Jacksonia floribunda*, accumulated up to 5 g at similar nutrient supplies (50 mg P kg^{-1} soil, 25 mg N kg^{-1} soil) and up to 10 g at higher N supplies (300 mg N kg^{-1} soil) in six months (Png 2016). The slow growth of *Bossiaea* reveals a very conservative nutrient-use strategy. Despite the slow biomass accumulation, total biomass of both species more than tripled from P0 to P50, but it did not respond to increasing N supply, probably due to tight regulation of N-uptake. The range of P supplies represented the maximum variation of total soil [P] in the field along the chronosequences (Turner et al. 2018). Thus, our glasshouse results suggest that the growth of *B. linophylla* and *B. eriocarpa* seedlings in the field might be P-limited, not N-limited. The very high N:P ratios of glasshouse-grown plants support this contention (>25 in all treatments). Liebig´s law of the minimum proposes that growth is determined by the scarcest nutrient (Güsewell 2004). Since N-fixation provides the plants with enough N, it is possible that increased N supply also increased P demand, leading to this apparent P-limitation.

Differences in nutrient-acquisition strategies between the two Bossiaea species

We observed very distinct responses to nutrient availability in both species. We emphasise that the responses to multiple nutrients are more complex than those of single nutrient-addition experiments due to possible interactions and stoichiometric effects. *Bossiaea linophylla* occurred at all the stages of the Warren chronosequence, with the greatest relative canopy cover at intermediate stages 3 and 4. Presence at all stages requires the ability to extract nutrients from a wide array of soils, from alkaline to acidic. The availability of many nutrients varies with soil pH (Lambers et al. 2008); therefore, their acquisition requires different strategies, and implies phenotypic plasticity in nutrient-acquisition strategies or the presence of ecotypes adapted to each soil pH and stage of soil development (De Jong 2005, Kramer-Walter and Laughlin 2017). *Bossiaea eriocarpa* was restricted to the older P-impoverished soils, more abundant at intermediate stages. The three stages where it occurred presented similar N and P availabilities and pH, and *B. eriocarpa* also presented the same nutrient-acquisition strategies at all stages where it occurred, probably reflecting the similar leaf [N] and [P] at different stages. When grown in the glasshouse, *B. eriocarpa* grew well with the wide range of N and P supplies with acidic pH, and presented less variable leaf [P]. Therefore, we surmise that its occurrence in the field is due to its low capacity to acquire nutrients at high soil pH, or lower phenotypic plasticity in P acquisition and use. A restricted capacity of *B. eriocarpa* to acquire

nutrients in alkaline soils may indicate a specialisation, as opposed to the generalist capacity of *B. linophylla* to occupy a wider range of soil pH.

Concluding remarks

For the two species studied here, we found that the shifts in nutrient-limitation and in nutrient-acquisition strategies at the species level are not the same as previously observed at the community level. While we acknowledge that the responses to multiple nutrients are more complex than that of single-nutrient addition experiments, we emphasise that the former remain useful, and practical, to aid our understanding of shifts in nutrient-acquisition strategies, and the ability of plants to occur, along natural gradients of soil fertility such as chronosequences. The amount of rhizosphere carboxylate was not up-regulated at low P supply, but the species invested in morphological adaptations for P uptake. Mycorrhizal colonisation of *Bossiaea* did not change along the chronosequences, possibly due to a relatively unimportant role for the fungi in nutrient uptake at very low P availability, and to their provision of defence against soil pathogens. Nodule formation was down-regulated by increased N supply, as expected. We conclude that, although the two species belong to the same genus, they have distinct nutrient demands and phenotypic responses to nutrient additions, probably reflecting their capacity to occupy different soils in the south-western Australian vegetation mosaic.

Author contributions

A.A. designed and performed the experiment and field collections, analysed and interpreted the data, and wrote the manuscript. R.S.O., M.H.R., E.L. and H.L. supervised the experimental design, interpretation and discussion of the results, and wrote the manuscript. All authors read and approved the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Study plot locations within the dune systems of the high-rainfall Warren and low-rainfall Jurien Bay chronosequences.

Table S2. Model selection of the fixed factors in the field-collected nutrient concentrations and mycorrhizal colonisation of *Bossiaea linophylla* and *B. eriocarpa* along the Warren and Jurien Bay chronosequences, respectively.

Table S3. Model selection of the fixed factors in a glasshouse experiment where *Bossiaea linophylla* and *B. eriocarpa* were grown at variable N and P supplies.

Fig. S1. Mortality of *Bossiaea linophylla* (A) and *B. eriocarpa* (B) grown in a glasshouse at a range of nitrogen (N) and phosphorus (P) supplies.

Figure legends

Table 1. Soil chemical analyses of the substrates used in the glasshouse experiment. Nutrient and aluminium (Al) concentrations are reported in mg kg^{-1}. M3 refers to Mehlich-3 extractions. Total nitrogen (N) and phosphorus (P) were determined after extraction with sulfuric acid. Percentages under soil names refer to the proportion of each soil used in the pots.

Fig. 1. Stages of the dune chronosequences in south-western Australia. Warren chronosequence (A, B, C) and Jurien Bay chronosequences (D, E) are a series of dunes deposited along the south-western Australian coast over 2 million years. A) The youngest stage of the Warren chronosequence; dunes are relatively mobile. B) Stages 2 to 5 of the Warren chronosequence; the canopy is more closed and understorey community composed of woody shrubs. C) Last stage of the Warren chronosequence;

open canopy, herbaceous understorey. D) Woody shrubs of stages 4 and 5 of the Jurien Bay chronosequence. E) *Banksia* woodland in stage 6 of the Jurien Bay chronosequence.

Fig. 2. Total soil phosphorus (P) concentrations along the Warren (A) and Jurien Bay (B) chronosequences. The analyses were conducted in five plots per stage. Data from Turner et al. (2017).

Fig. 3. Leaf phosphorus (P) and nitrogen (N) concentrations (in mg g^{-1} dry weight), and N: P ratios of *Bossiaea linophylla* along the Warren chronosequence (A, C, E) and *B. eriocarpa* along the Jurien Bay chronosequence (B, D, F). Points represent least-square means and bars, 95% confidence intervals. Chronosequence age increases with stage.

Fig. 4. Leaf manganese concentrations (mg Mn kg^{-1} dry weight) of *Bossiaea linophylla* along the Warren chronosequence (A) and *B. eriocarpa* along the Jurien Bay chronosequence (B). Percentage of root length colonised by arbuscular mycorrhizal fungi in roots of *B. linophylla* along the Warren chronosequence (C) and *B. eriocarpa* along the Jurien Bay chronosequence (D). Points represent least-square means and bars, 95% confidence intervals. Chronosequence age increases with stage.

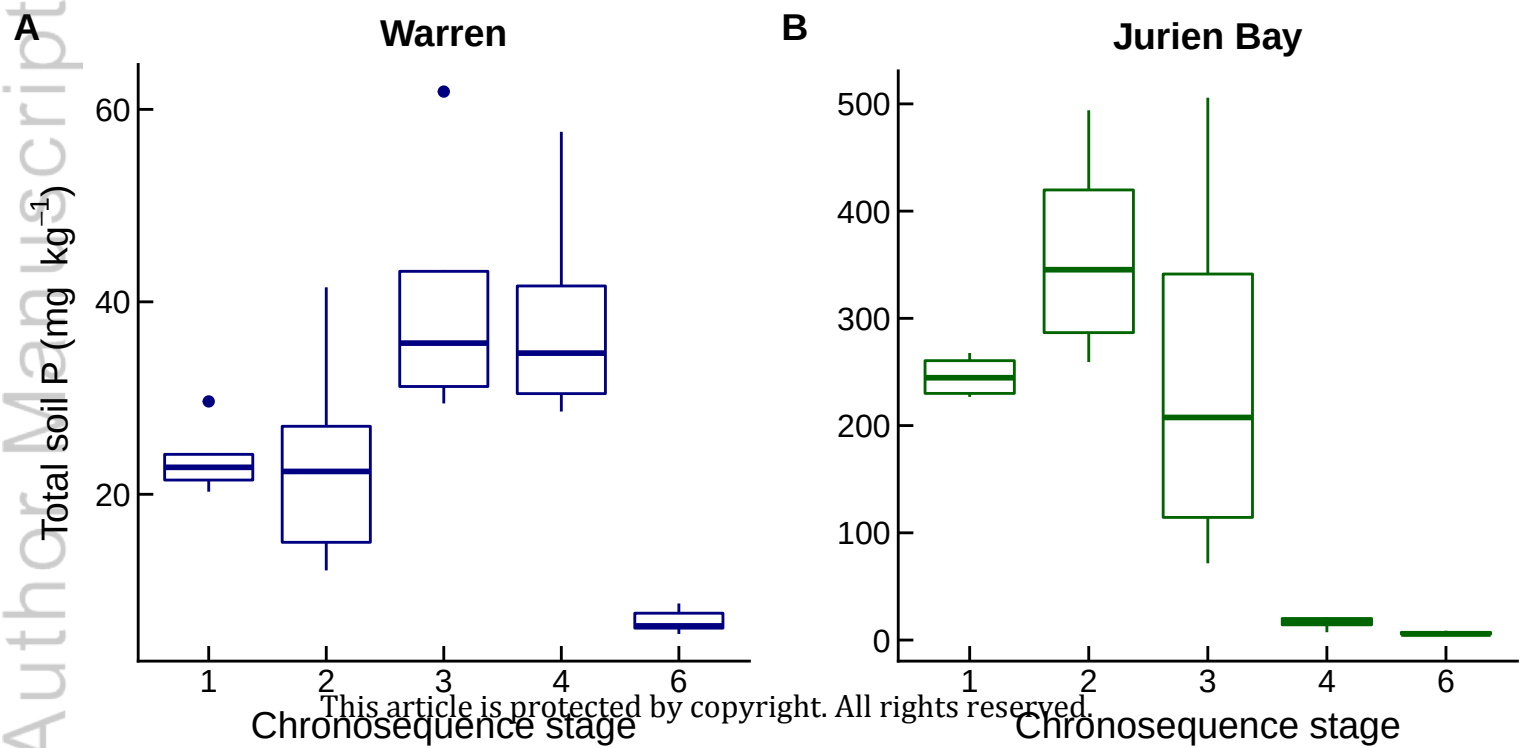
Fig. 5. Leaf nutrient concentrations (mg g^{-1} dry weight) of *Bossiaea linophylla* and *B. eriocarpa* grown in a glasshouse at a range of nitrogen (N) and phosphorus (P) supplies. P concentrations (A) and (B), N concentrations C) and D), leaf N:P ratios (E) and (F). Points represent least-square means and bars, 95% confidence intervals.

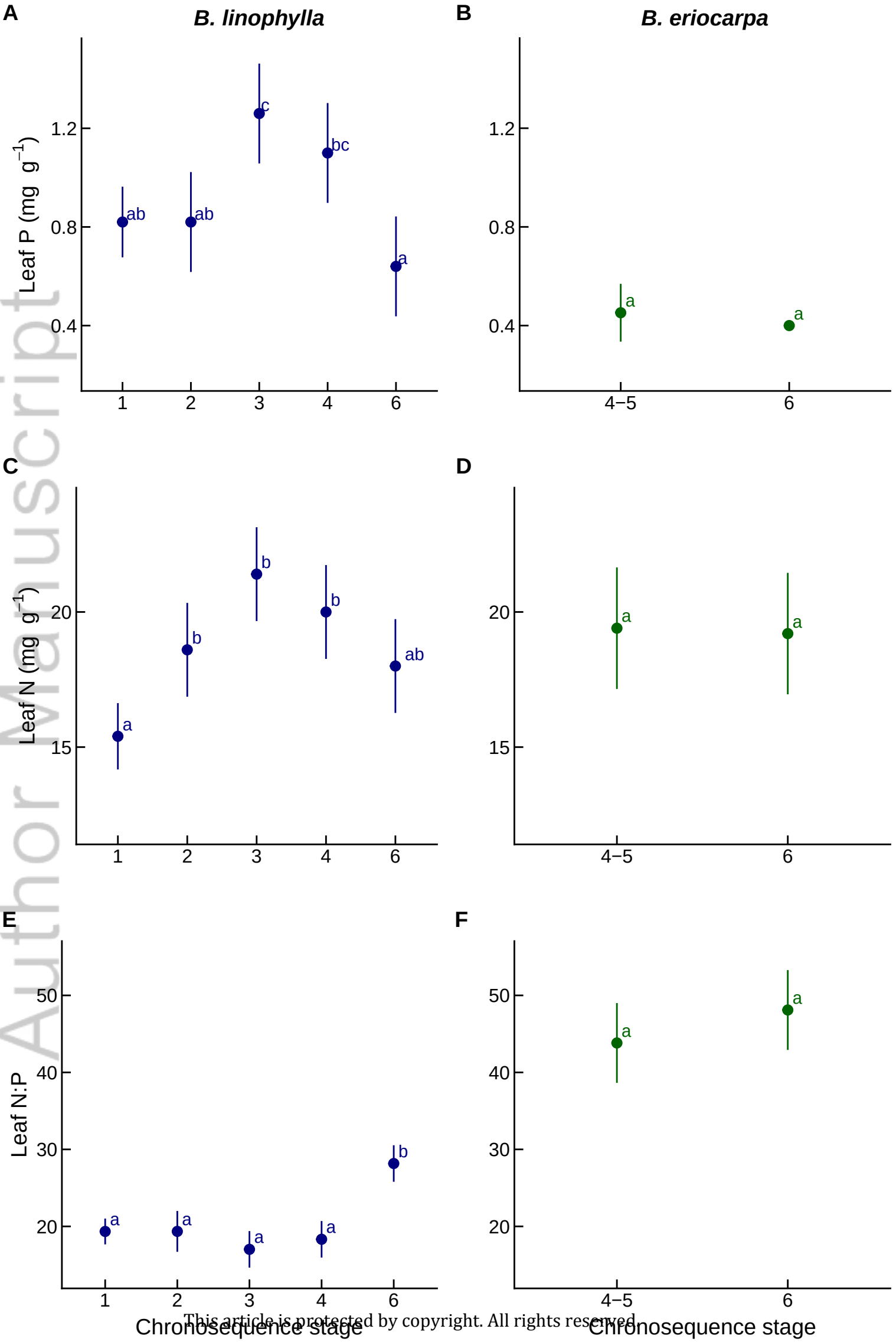
Fig. 6. Total plant biomass divided into leaves, roots and stems of *Bossiaea linophylla* (A) and *B. eriocarpa* (B) grown in a glasshouse at a range of nitrogen (N) and phosphorus (P) supplies. Least-square means and 95% confidence intervals are shown. There was no effect of N supply, so the tissue dry weights were averaged across N treatments, and no interaction of P supply with N supply, except for stem dry weight of *B. eriocarpa* where P and N supply affected stem biomass.

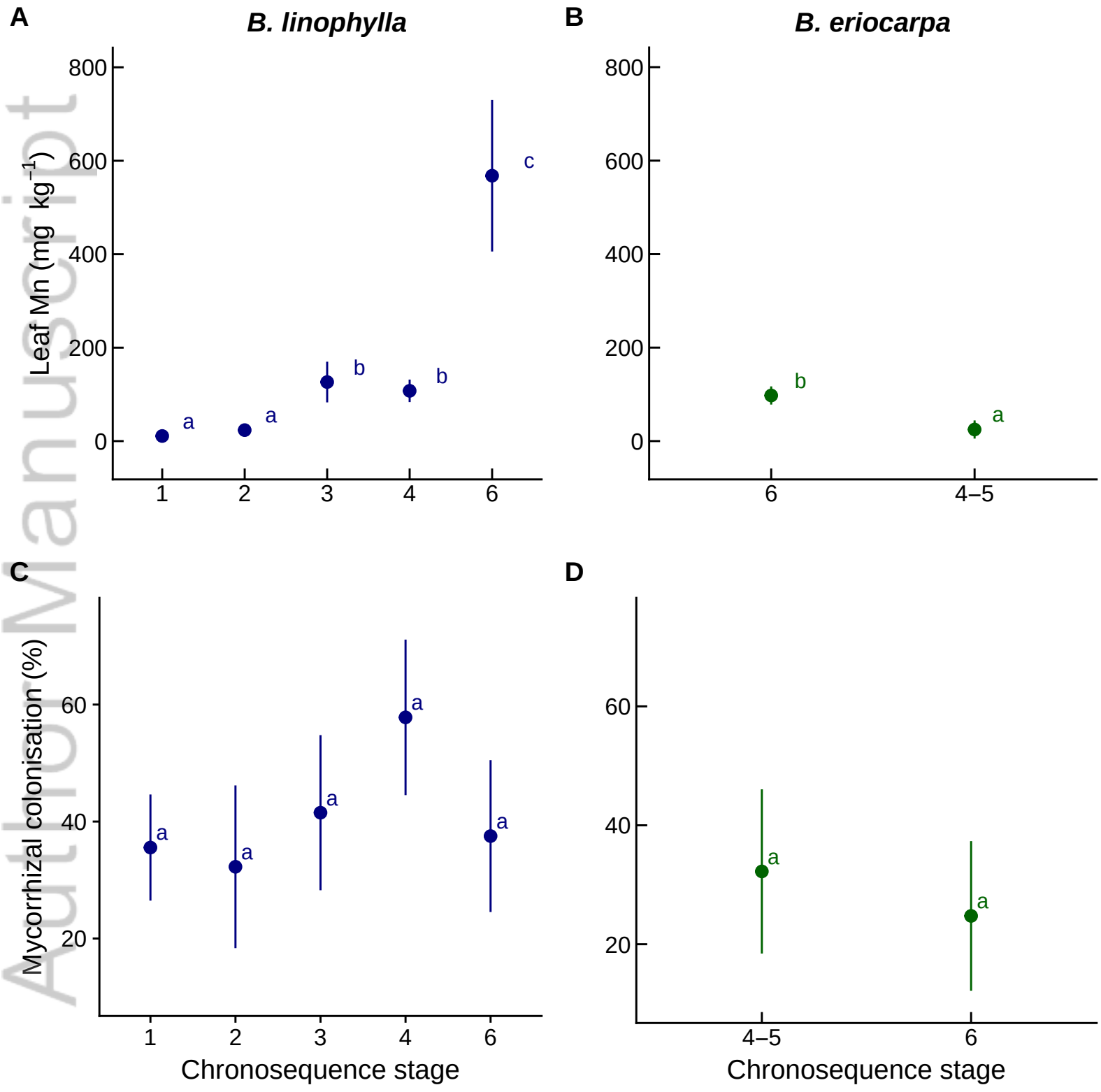
Fig. 7. Proportion of each carboxylate in the rhizosphere of *Bossiaea linophylla* (A) and *B. eriocarpa* (B) grown in a glasshouse at a range of nitrogen (N) and phosphorus (P) supplies. The values are mean per treatment. (C) Total carboxylate amounts in the rhizosphere of *Bossiaea linophylla* (C) and *B. eriocarpa* (C). The insert in (B) shows the same data with a different range of y axis values to represent the variation in rhizosphere carboxylates of *B. eriocarpa* at a smaller scale. Points represent least-square means and bars, 95% confidence intervals.

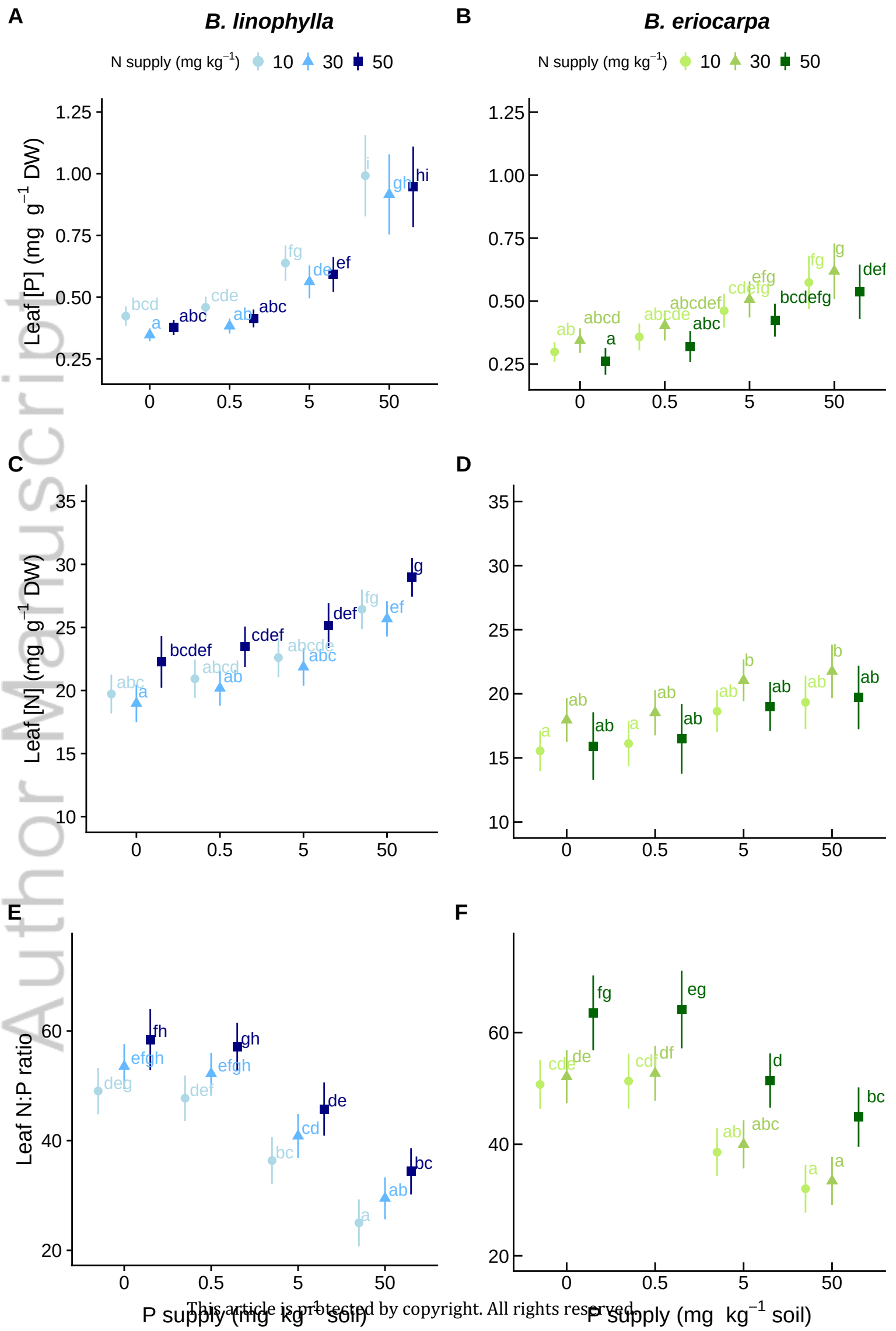
Fig. 8. The percentage of root length colonised by mycorrhizal fungi (A, B) and number of nodules for *Bossiaea linophylla* (A, C) and *B. eriocarpa* (B, D) grown in a glasshouse at a range of nitrogen (N) and phosphorus (P) supplies. Points represent least-square means and bars, 95% confidence intervals. Phosphorus treatments did not affect root length colonised by arbuscular mycorrhizal fungi in *B. linophylla* (A) and number of nodules in *B. eriocarpa* (D); therefore, we grouped P treatments within N treatments.

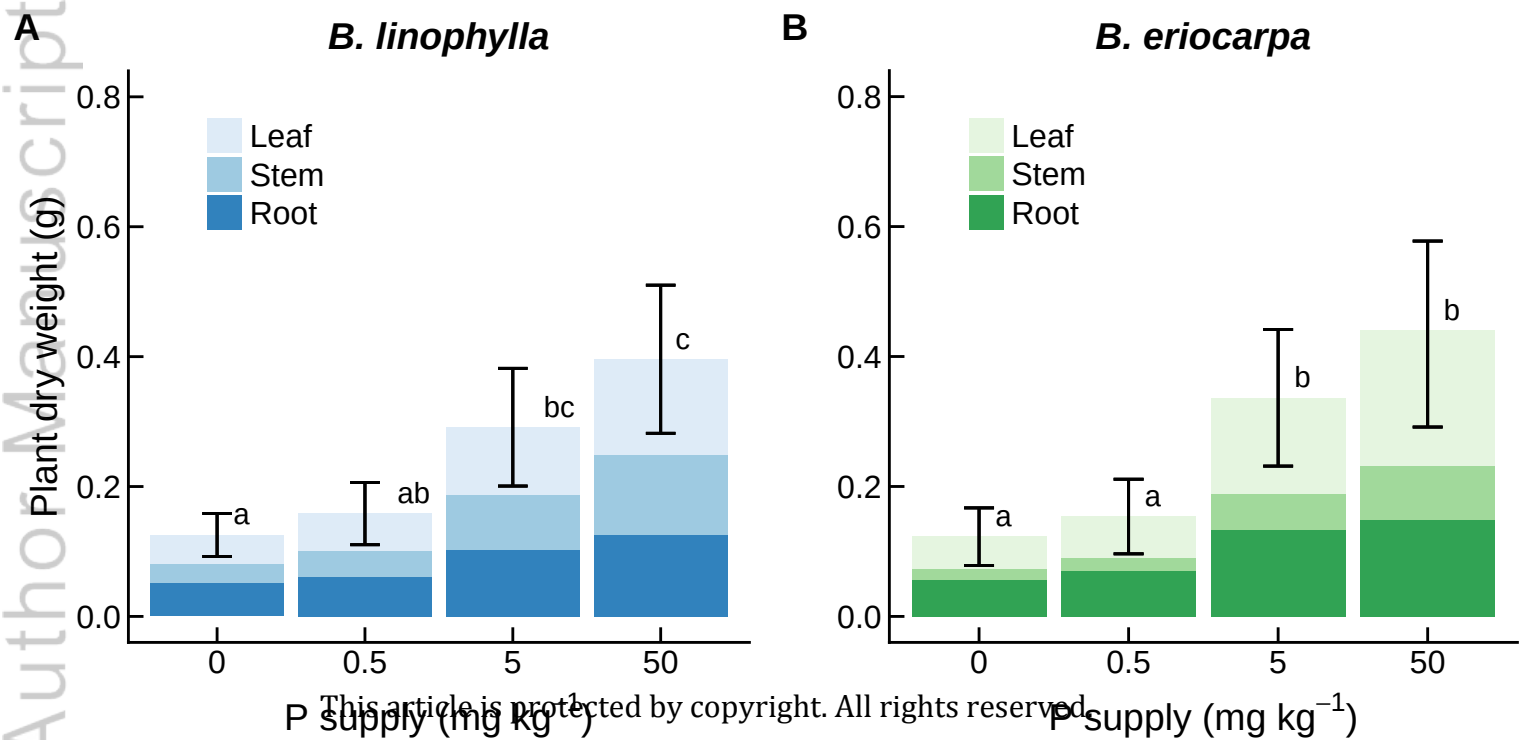
Fig. 9. Leaf manganese (Mn) concentrations of *Bossiaea linophylla* (A) and *B. eriocarpa* (B) grown in a glasshouse at a range of nitrogen (N) and phosphorus (P) supplies. Points represent least-square means and bars, 95% confidence intervals. Nitrogen is only shown in the figures when included in the best model. Nitrogen treatments did not affect leaf [Mn] in *B. eriocarpa*; therefore we grouped N treatments within P treatments.

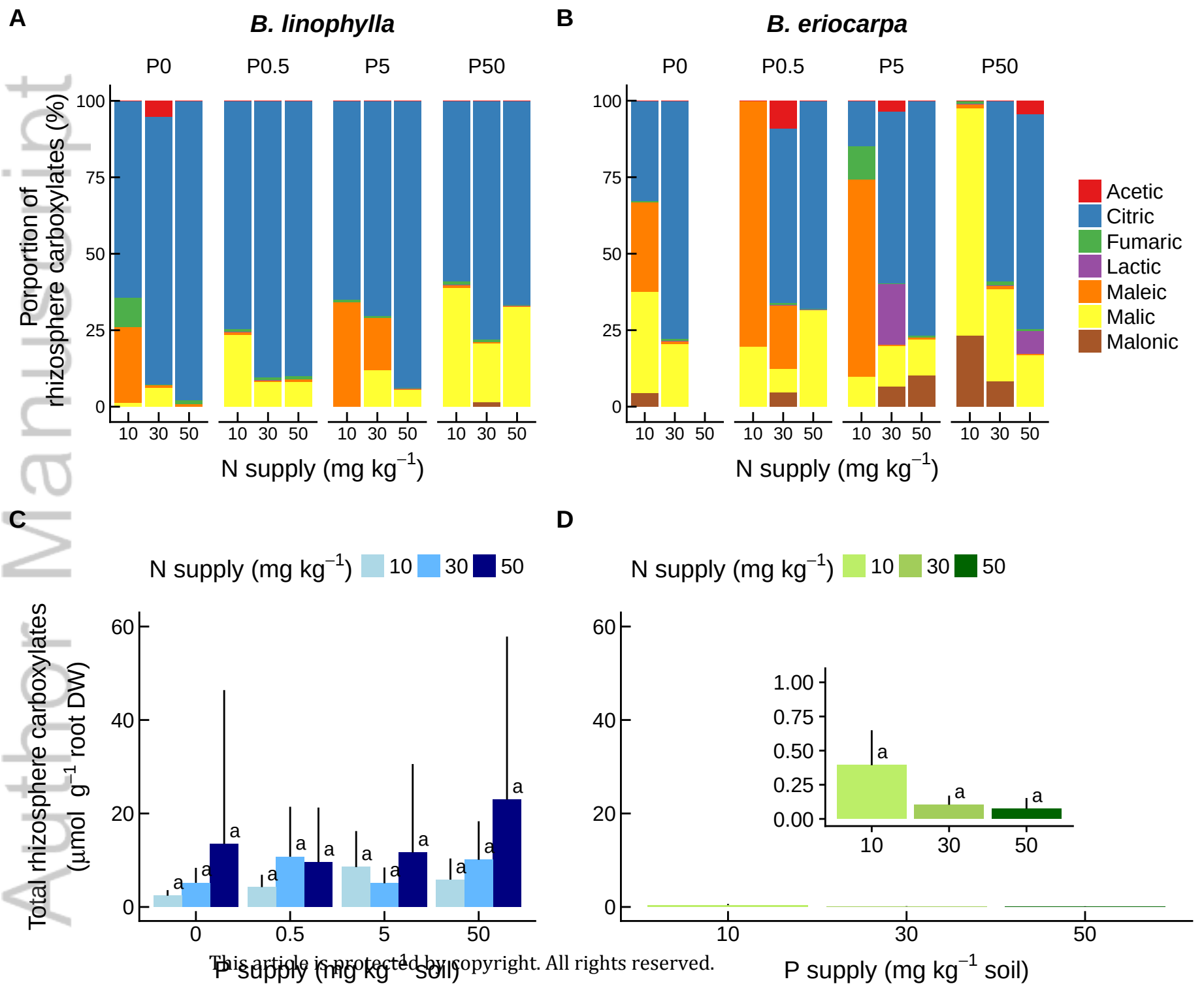


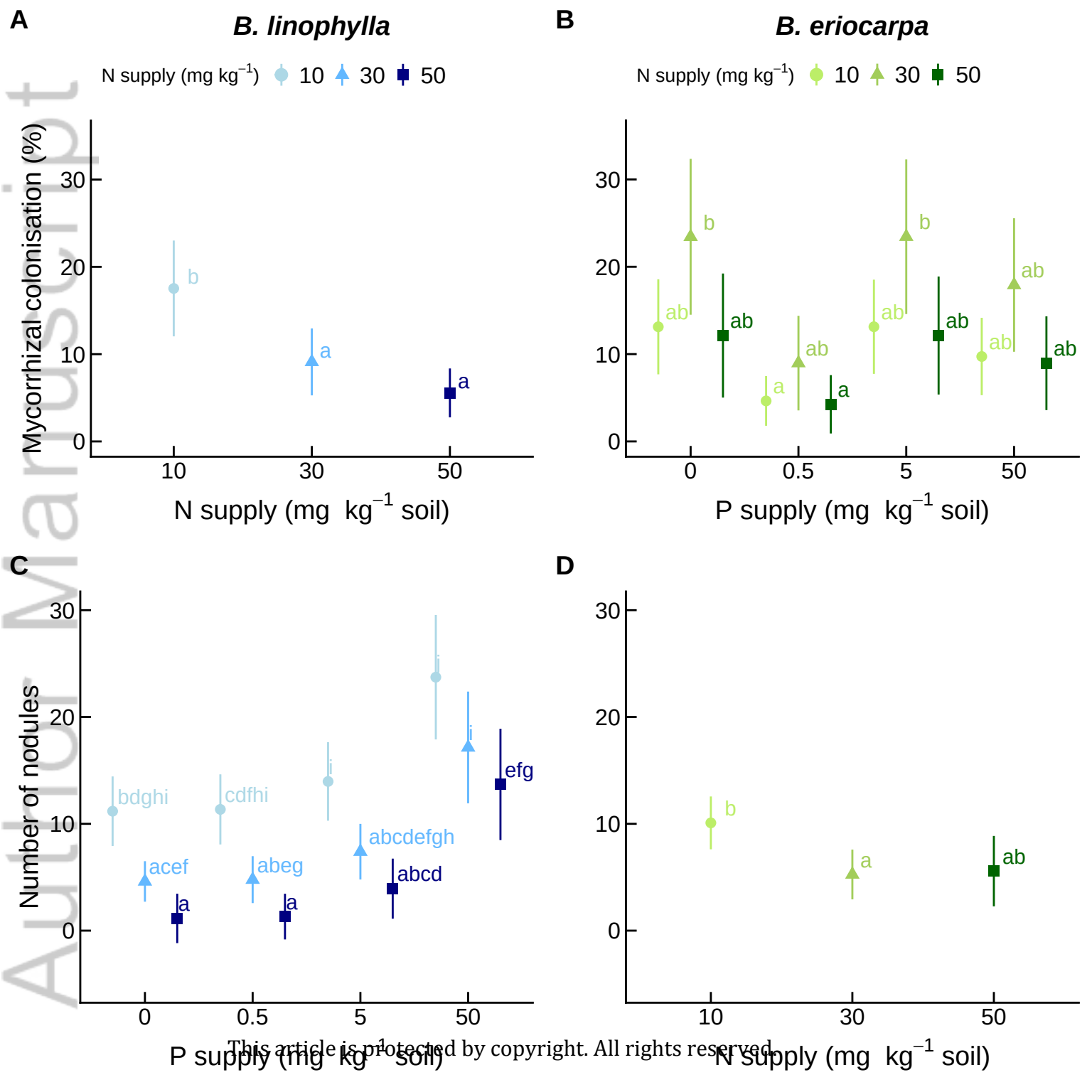












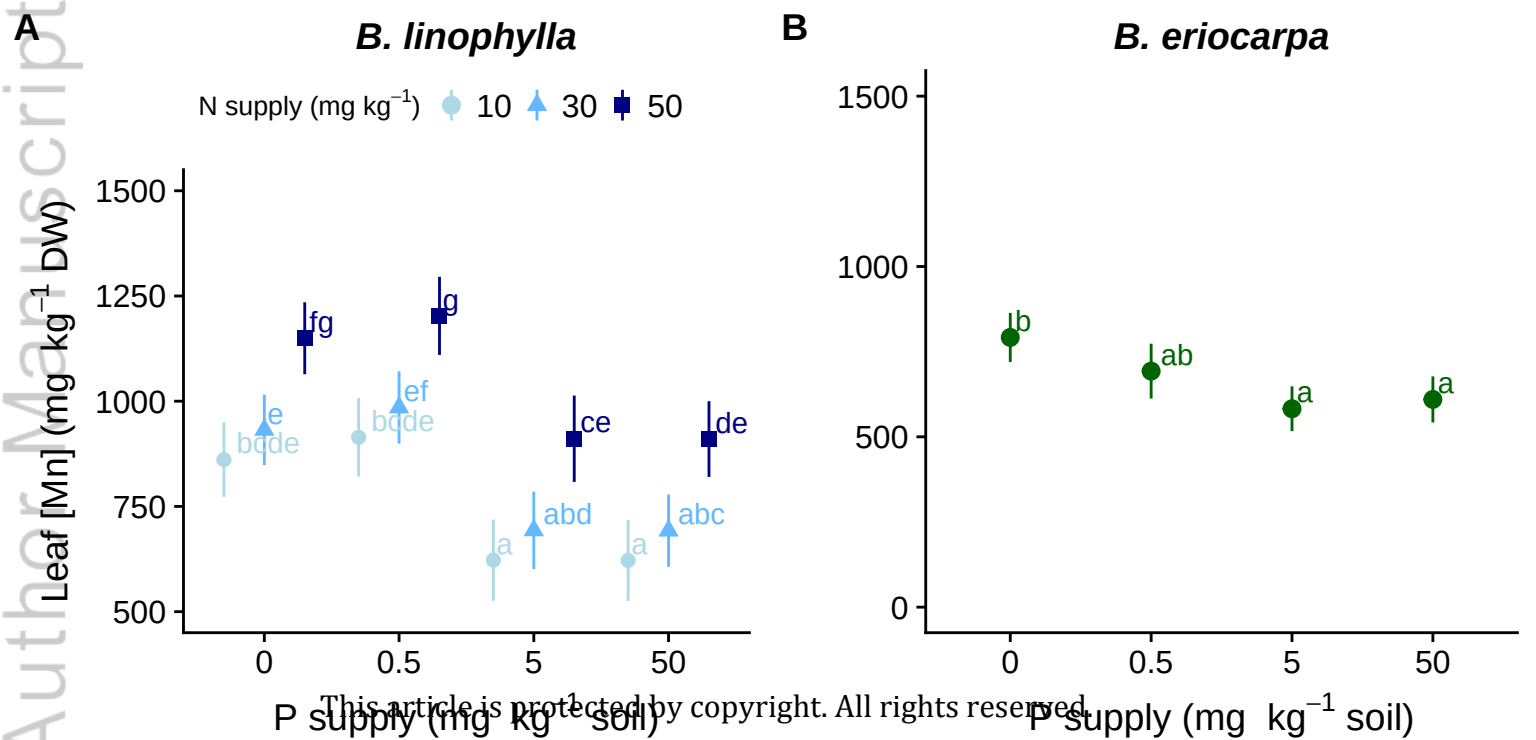


Table 1. Soil chemical analyses of the substrates used in the glasshouse experiment. Nutrient and aluminium (Al) concentrations are reported in mg kg^{-1}. M3 refers to Mehlich-3 extractions. Total nitrogen (N) and phosphorus (P) were determined after extraction with sulfuric acid. Percentages under soil names refer to the proportion of each soil used in the pots.

	pH	pH	N	P	Al	Ca	Cu	Fe	K	Mg	Mn	Zn
	(H_2O)	($CaCl_2$)	(total)	(total)	(M3)	(M3)	(M3)	(M3)	(M3)	(M3)	(M3)	(M3)
Washed river sand (90%)	6.6	6.1	<0.05	31	72	78	0.2	50	9	38	7.4	2.4
Harrisdale sand *Bossiaea eriocarpa* (5%)	5.1	4	0.43	15	46	250	0.1	15	6	31	1.3	0.3
Waroona lateritic soil *B. aquifolium* (5%)	6.1	5.1	1.78	250	550	200	0.5	37	92	53	12	<0.1