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REGULAR ARTICLE

Nodulation promotes cluster-root formation in *Lupinus albus* under low phosphorus conditions

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Abstract
Background and aims Phosphorus deficiency often limits crop productivity, while phosphate rock, which is used to produce phosphorus fertilisers, is a non-renewable resource. Formation of cluster roots is an adaptation to nutrient-poor soils in *Lupinus* species, including *L. albus*. *Lupinus* species also produce nodules, which require a large investment of phosphorus. Our aim is to test whether nodulation promotes cluster-root formation in *L. albus*.
Methods Seedlings of *L. albus*, either inoculated with rhizobia or non-inoculated, were grown in nutrient solution with a low phosphorus supply. Non-inoculated plants were provided with the same amount of nitrogen in the form of nitrate as the nodulated ones acquired, from both air and nutrient solution, based on preliminary experiments. We measured biomass, phosphorus and nitrogen concentrations as well as photosynthesis just prior to each harvest.
Results Nodulated plants and non-nodulated control plants produced the same amount of biomass. Nodulated plants had, on average, 86% more cluster roots than non-nodulated ones at the four harvests. As hypothesised, nodulation significantly promoted cluster-root formation; it also enhanced rates of photosynthesis.
Conclusions Nodulation promoted cluster-root formation and photosynthesis, presumably because nodules are significant sinks for phosphorus and photosynthates. Our results do not provide evidence for a trade-off between investment of resources in nodules and cluster roots.

Keywords Nitrogen fixation · nodules · proteoid roots · rhizobium · white lupin · trade-off

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Introduction

Phosphorus (P) is an essential macronutrient for plant growth and P deficiency often limits crop yield, especially in Australia, Africa, South America and South Asia (Lynch 2007; Raghothama 1999; Stutter et al. 2012). Therefore, a large amount of P fertiliser is applied to crops to sustain high yields (Lynch 2007). However, the resource to produce P fertiliser, rock phosphate, is non-renewable (Cordell et al. 2009; Gilbert 2009), although it may not be depleted as rapidly as previously estimated (Scholz and Wellmer 2013). Moreover, P applied to crops may lead to eutrophication (Sharpley et al. 2001), algal blooms, low oxygen concentrations in rivers and lakes, and loss of biodiversity (Carpenter et al. 1998; Lambers et al. 2013a; Smith and Schindler 2009). Therefore, it is urgent to manage P fertilisation properly, and develop crops that have greater P-acquisition efficiency so as to reduce the use of P-fertiliser (Belay et al. 2002; Fixen and Johnston 2012; Simpson et al. 2011).

To decrease the use of P fertiliser, different plant strategies can be considered (Richardson et al. 2011). Among those strategies, root structures have received considerable attention. Root structures in many species respond to P starvation (e.g., the formation of cluster roots in *Lupinus* and *Banksia*; the longer and higher density of root hairs, and increased lateral root development in *Arabidopsis*) (Lynch 1995; Niu et al. 2013; Peret et al. 2011; Williamson et al. 2001). Cluster roots, or proteoid roots, as a specialised root structure, are found in several plant families (Betulaceae, Casuarinaceae, Fabaceae, Myricacea, and Proteaceae) as an adaptation to nutrient-poor soils (Lambers et al. 2003; Shane and Lambers 2005). Cluster roots enhance the uptake of P, which is mobilised from sparingly available sources as a result of the release of carboxylates (Florez-Sarasa et al. 2014; Gardner et al. 1983; Gerke et al. 2000; Lambers et al. 2012). Cluster-root formation is regulated by shoot P concentration and sugar signals in white lupin (Shane et al. 2003; Wang et al. 2015).

Lupinus albus (Fabaceae) is a model plant for studies on cluster roots (Florez-Sarasa et al. 2014; Gardner et al. 1983; Keerthisinghe et al. 1998; Neumann et al. 1999; Shane and Lambers 2005). Being a legume, *L. albus* can form nodules and symbiotically fix nitrogen (N_2), as well as cluster roots to acquire P (Lambers et al. 2013b). Nodules are a significant P sink (Drevon and Hartwig 1997; Schulze et al. 2006); therefore, P plays an important role in nodule functioning (Tang et al. 2001). The rate of N_2 fixation declines when P is in short supply (Schulze et al. 2006). This is partly due to the role of rRNA in nodules which comprises a large fraction of organic P (Raven 2013b). Ribosomal RNA is required to sustain rapid synthesis of nitrogenase, which is turned over rapidly (Raven 2013a). In addition, N_2 fixation also costs more respiratory energy (ATP) than that when N is acquired from soil (Lambers et al. 2002).

The formation of cluster roots may be affected by the presence of nodules, because additional P is required for the functioning of nodules. Alternatively, the high carbon (C) cost of nodules may lead to competition with the formation of cluster roots, whose production and functioning are also a C cost (Lambers et al. 2006). Thus, rather than an increase in cluster-root formation in N_2-fixing plants, a decrease might be anticipated. Our hypothesis is that: the formation of nodules increases the plant's demand for P, and thus promotes cluster-root production in *L. albus* under low P supply. Our objective was to test the relationship between formation of nodules and cluster roots, and explore the priority of C and P investment, if any, in either of these plant structures.

Materials and methods

Similar-sized seeds of *L. albus* L. cv. Kiev were soaked in 5% (*v*/v) sodium perchlorate solution for 20 min, then thoroughly washed with deionised water and soaked in deionised water overnight. Then, the seeds were sown in pots filled with washed and sterilised river sand in a greenhouse. The seeds were evenly divided into two groups: a nodulated group, which was inoculated with Group G® (Bradyrhizobium sp. (*Lupinus*) WU425), purchased from New-Edge Microbials Pty. Ltd., Albury, Australia, and a control group without inoculation. Though we did not inoculate the control plants, some had a few small nodules. We removed these from the control plants every day to make sure that they did not fix nitrogen. Cotyledons were removed as soon as the first leaf expanded to eliminate the effect of the nutrients in the cotyledons. At 15 days after sowing, uniformly-sized seedlings from each of the two groups were carefully removed from the pots, and the roots were washed free of sand. The stem of each seedlings was placed in the centre of a grey foam disk, which formed the centre lid of a 4-l black plastic pot with continuously aerated nutrition of the following concentration, similar to the one below in preliminary experiment (except N, P and the K in the KNO_3 and KH_2PO_4): 100 μM Ca^{2+}, 100 μM K^+, 154 μM SO_4^{2-}, 54 μM Mg^{2+}, 0.24 μM Mn^{2+}, 0.1 μM Zn^{2+}, 0.018 μM Cu^{2+}, 2.4 μM H_3BO_3, 0.03 μM Mo^{4+}, 200 μM Cl^-, 10 μM Fe-Na-EDTA. The plants were individually grown in containers half-immersed in a temperature-controlled root-cooling tank maintained at 18–22 °C. The experiment was carried out in a glasshouse at the University of Western Australia in a factorial, completely randomised design.

In a preliminary experiment, 20 days after sowing, 54 seedlings of *L. albus* inoculated with rhizobium were placed in 4-l black plastic pots with continuously aerated nutrient solution of the composition: 100 μM NO_3^-, 10 μM $H_2PO_4^-$, 210 μM K^+, 100 μM Ca^{2+}, 154 μM SO_4^{2-}, 54 μM Mg^{2+}, 0.24 μM Mn^{2+}, 0.1 μM Zn^{2+}, 0.018 μM Cu^{2+}, 2.4 μM H_3BO_3, 0.03 μM Mo^{4+}, 200 μM Cl^-, 10 μM Fe-Na-EDTA. Plants were harvested at 20, 26, 30, 35, 40, 44, 49, 54, 56 and 59 days after sowing, with 4–8 plants harvested at each time, and the fresh weight of each plant were recorded, then oven dried to measure total biomass, total N and total P concentrations. An exponential equation was used to describe the increase in fresh weight, where x represents day after sowing:

$$Y = 0.4265e^{0.0813x}, R2 = 0.974$$

We used a linear equation to describe the increase in total plant N concentration with time, where x means fresh weight:

$$Y = 1.9546x + 7.2907, R2 = 0.9772$$

These two equations were used in the actual experiment to decide the amount of N and P that was to be added to the nodulated and the control group.

The nutrient solution was replaced every three days. We used the equations given above and measured the fresh weight of plants in the nodulated group to calculate the amount of N accumulated in plants of the nodulated group during the next three days. Then we added the same amount of N (in the form of KNO_3) to the control group during the three-day interval and kept the added N to P (in the form of KH_2PO_4) ratio at 18.9 to provide a balanced N:P ratio. We also added a basic N concentration for the nodulated group which was the same as the level we gave to the control group at the beginning, and 100 μM NO_3^- when the nitrogen concentration provided to the control group exceeded 100 μM. The amount of N and P added each time was shown in Table S1.

Plant treatments

Nitrate in solutions, before these were replaced, was measured using a microplate nitrate test kit (The Nitrate Elimination Company, Inc. Lake Linden, MI, USA) to calculate the amount of nitrogen absorbed from solution by nodulated plants and to calculate the nitrogen fixed. Four plants from both nodulated and control groups were harvested before the treatment to measure the initial biomass as well as N and P concentrations. Before each harvest, three single leaves from the first, second and third mature compound leaves of five plants from each group were chosen to measure photosynthesis using a Li-Cor 6400 (Li-Cor, Lincoln, NE, USA). We harvested at least six plants from each group during the 13 (D13), 23 (D23), 30 (D30) and 36 (D36) days after transferring into the hydroponic system. Each harvested plant was gently removed from its pot and then rinsed with deionised water. For each plant, cluster roots and nodules were separated from the rest of the root system. Mature leaf area of each plant was measured before weighing. Fresh weight of stems (including petioles), leaves and roots (nodules, cluster and non-cluster roots) was determined at each harvest, and the dry biomass was measured after drying at 70 °C for a week. Plant parts were separately weighed before and after drying, and the total biomass and biomass allocation (all the biomasses below are dried biomasses) were calculated as follows:

Total biomass = leaf biomass + stem biomass + non-cluster-root biomass + cluster-root biomass + nodule biomass
Shoot biomass = leaf biomass + stem biomass
Root biomass = non-cluster-root biomass + cluster-root biomass + nodule biomass
Cluster-root biomass ratio = cluster-root biomass / total biomass

Dried material was then ground into a fine powder using GenoGrinder (SPEX SamplePrep LLC, New Jersey, USA), and the plant total N and total P concentrations were measured by ChemCentre, Perth, WA, Australia. Dry sample powder (0.5 g) was digested with 6.25 mL salicylic and sulphuric acid mixture (50 g salicyclic acid dissolved in 1 L concentrated sulfuric acid) and 3.5 ml hydrogen peroxide (30% *w*/w). After digestion, sodium nitroprusside was added to react with nitrogen (Anonymous. 1977), ammonium molybdite and ammonium vanadates were added to react with phosphorus (Rayment et al. 2010). Colours represent N and P were produced during reaction and then measured by Technicon A11 Auto Analyser (Technicon Instrument Co. Ltd., Basingstoke, Hants, UK).

N:P ratio (mass basis) was calculated as total N concentration / total P concentration.

Statistics

Data were compared using T tests ($p \leq 0.05$) using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). All figures were drawn with the Origin 8.0 software (Originlab Co., Northampton, MA, USA). Means are presented with standard errors to indicate the variation of each measurement. For statistical comparisons, differences were considered statistically significant at $p \leq 0.05$.

Regressions were carried out and results were plotted in curvilinear graphs using a polynomial fitting method using Microsoft Excel 2016 MSO (16.0.8625.2121) in exploring the pattern between cluster-root biomass and nodule biomass.

Results

Plant growth

As intended, following our preliminary experiment, at all harvest times, nodulation did not significantly affect total biomass (Fig. 1a) and shoot biomass (Fig. 1b) of *L. albus*.

At the D30 and D36, nodulated *L. albus* plants had slightly more root biomass (including nodules and cluster roots) than the control, whereas plants of both groups had a similar root biomass at the D13 and D23 (Fig. 1c). The non-cluster-root biomass of *L. albus*, excluding nodules and cluster roots, was similar in the two groups at the D13, D23 and D36, while that of the control plants was slightly larger than the nodulated group at the D30 (Fig. 1d).

Cluster roots

Inoculation with rhizobia enhanced the production of cluster roots, at all harvest times: 134%, 68%, 101% and 42% more at D13, D23, D30 and D36, respectively (Fig. 2a). Nodulated plants also produced significantly more cluster-root biomass as a proportion of total biomass than control plants at all four harvest times (Fig. 2b).

Relationship between cluster-root biomass and nodule biomass

For the nodulated plants, cluster-root biomass was curvilinearly correlated with nodule biomass. The slope became flatter at higher nodule mass; this means that larger plants had less cluster root biomass per nodule biomass (Fig. 3). The percentage of N fixed by the nodulated group was 64% ± 2%, 44% ± 2%, 37% ± 2% and 41% ± 4% respectively at D13, D23, D30 and D36.

Nutrient concentrations and photosynthesis

Nodulated plants had a significantly higher total N concentration than control plants at the D13, D23 and D30, while there was no significant difference at the D36 (Fig. 4a). Control plants had a slightly higher total P concentration than nodulated plants at the D23 and D30, while no significant differences were seen at the D13 and D36 (Fig. 4b). Nodulated plants had a significantly higher N:P ratio than control plants at the D13, D23 and D30, while there was no significant difference at the D36 (Fig. 4c).

At the D13 and D36, no significant difference was found in net photosynthetic rate of *L. albus*, while at the D23 and D30, control plants had a significantly lower net photosynthetic rate than inoculated plants (Fig. 4d).

Discussion

Previous studies have shown that investment of resources in cluster roots and nodules may exhibit a trade-off (Thuynsma et al. 2014a, 2014b). Cluster roots are formed when *L. albus* plants experience P deficiency (Johnson et al. 1996; Shu et al. 2007). Nodules are a major P sink (Schulze et al. 2006), because a large amount of organic P is associated with ribosomal RNA that is required to replace damaged nitrogenase enzymes (Raven 2012). On one hand, nodules as a sink for P increase the need for P of the plants, and thus may stimulate the production of cluster-root. On the other hand, plants that have nodules require more energy and C than non-nodulated plants, and this may compete with and decrease the production of other plant parts, including cluster roots. To test the effect of N_2-fixing nodules on the production of cluster roots, confounding factors should be eliminated. Plants with similar growth and nutrient levels should be compared, to ensure that the results are only due to the source

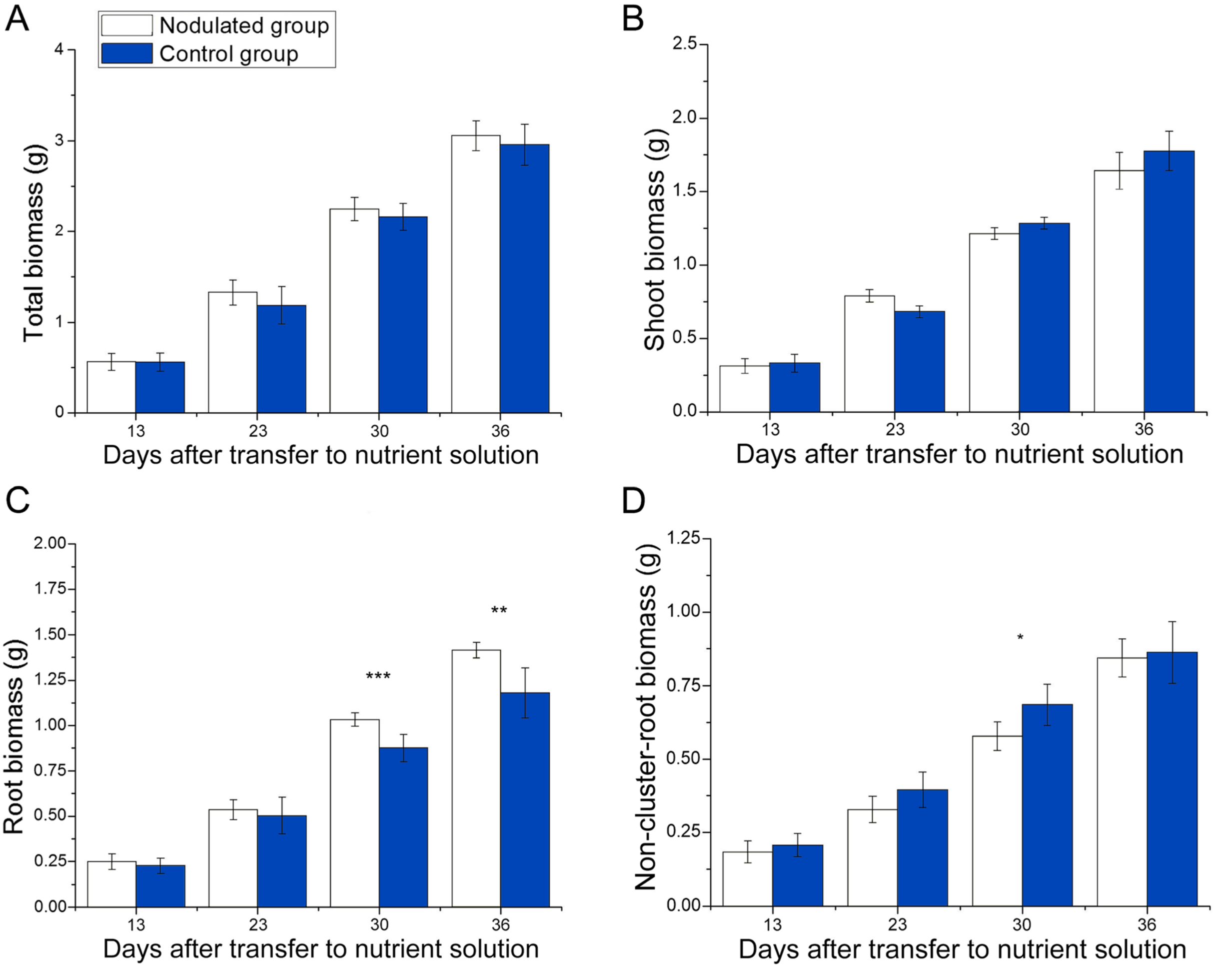

Fig. 1 Total biomass (**a**), shoot biomass (**b**), total root biomass (**d**) and non-cluster-root biomass (without nodules and cluster-roots) (**d**) of *Lupinus albus* in nodulated and control groups at the 13, 23, 30, 36 days after treatment. Error bars refer to standard errors ($n = 6$). Asterisks above the bars indicate significant effects: *** $p \leq 0.001$, ** $p \leq 0.01$ and * $p \leq 0.05$ with T tests

of N. This is what we aimed for and achieved in the present study, and, increased cluster-root formation was observed in nodulated group.

Similar growth in both groups of *L. albus*, except for cluster roots and nodules

In a preliminary experiment, we determined the N concentration of inoculated *L. albus*. That information was then used to grow plants without nodules, but with nitrate as a source of N, so the source of N, rather than the N amount was the main difference between the two groups. Plant total biomass and non-cluster-root biomass of both groups were similar, as intended. However, nodulated *L. albus* produced more root biomass, due to having more cluster-root and nodule biomass. The N source did not affect the shoot growth, confirming the results of White and Robson (1989). The P concentrations of both groups were also similar. So, the major factor that induced differences in the formation of cluster root was the source of N and associated cost of P, as nitrogenase synthesis requires major investment of P in rRNA (Raven 2013a).

Nodulated *Lupinus albus* allocated more P to nodules and produced more cluster roots

Nodulated *L. albus* plants had more cluster roots under similar growth condition, but larger plants had less cluster-root biomass per nodule biomass.

In previous studies, nodulated roots (excluding nodules) achieved a higher or similar P concentration

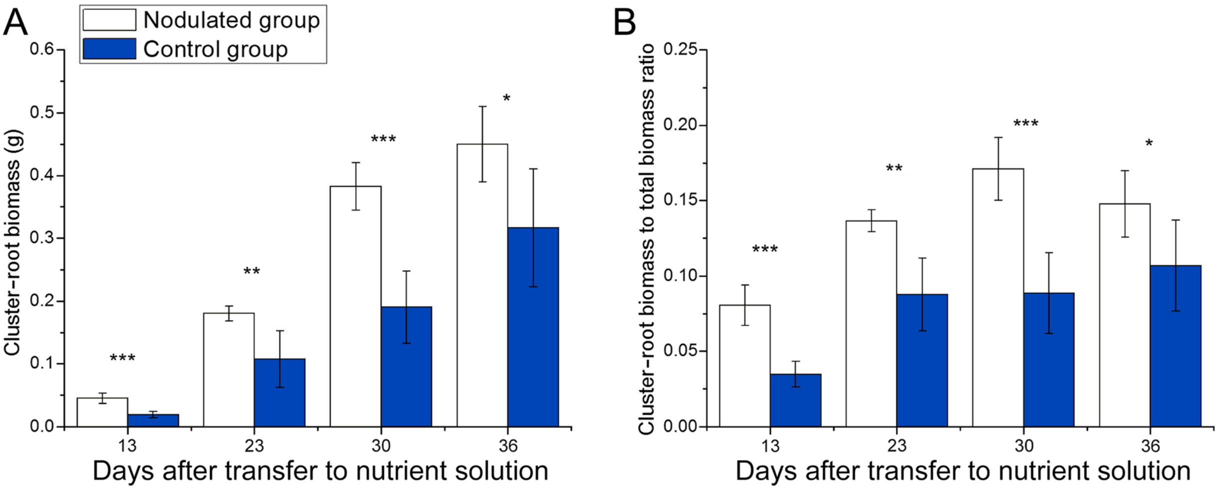

Fig. 2 Cluster-root biomass (**a**) and cluster-root biomass to total biomass ratio (**b**) of *Lupinus albus* in nodulated and control groups at the 13, 23, 30, 36 days after treatment. Error bars are standard errors (n = 6). Asterisks above bars indicate significant effects: *** $p \leq 0.001$, ** $p \leq 0.01$ and * $p \leq 0.05$ with T tests

compared with shoots in *L. albus* under various rates of P supplies (Li et al. 2008; Sas et al. 2002; Schulze et al. 2006; Shen et al. 2005; Shu et al. 2007; Wang et al. 2013). Nodules are strong P sinks with a high P concentration (Drevon and Hartwig 1997; Sa and Israel 1991; Schulze et al. 2006). In our study, though whole plant P concentrations were similar in nodulated and non-nodulated plants, it is likely that nodulated plants allocated more P to nodules at the expense of P concentration in leaves. The whole root (including nodules and cluster roots) also had more biomass in the nodulated group than in the control. Based on this we infer that nodulated *L. albus* allocated more P to its nodules, and this led to a lower leaf P concentration than in the control group. According to previous reports, cluster-root formation is negatively correlated with leaf P concentrations in *L. albus* (Abdolzadeh et al. 2010; Shane et al. 2003; Shen et al. 2003; Wang et al. 2013). A lower leaf P concentration would explain why more cluster roots were produced in nodulated *L. albus* plants.

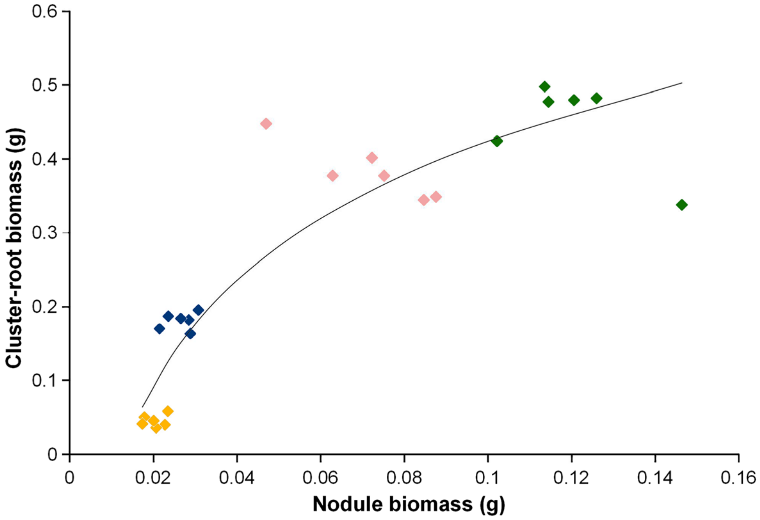

Fig. 3 Relationship between cluster roots and nodules (based on dry mass) at four harvest times of *Lupinus albus*. The curve represents the relationship between cluster-root biomass and nodule biomass in inoculated *L. albus*. The yellow, dark blue, pink and dark green symbols refer to the data from 13, 23, 30, 36 days after treatment, respectively. The solid line represents the fitted curve based on the scatter plot. The fitted equation of cluster-root biomass to nodule biomass, using a polynomial fitting method, is $Y = 0.2061 \ln x + 0.8986$, $R^2 = 0.8474$

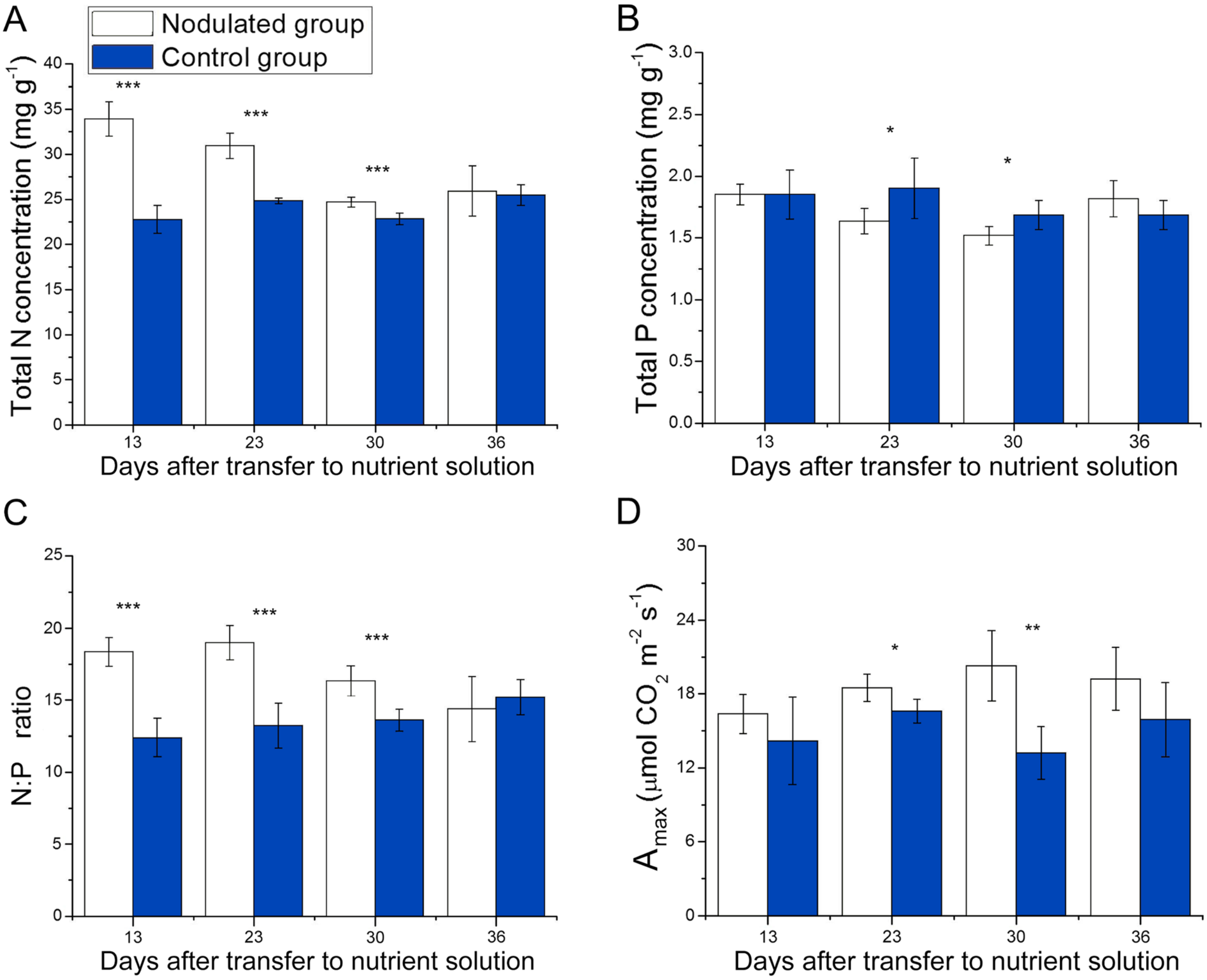

Fig. 4 Total nitrogen (N) (**a**) and phosphorus (P) (**b**) concentration, (N:P) ratio (**c**) and photosynthesis (A_{max}) (**d**) of *Lupinus albus* plants that were either nodulated or grown with nitrate (control) at the 13, 23, 30, 36 days after treatment. Error bars are standard error (n = 6). Asterisks above the bars indicate significant effects: *** $p \leq 0.001$, ** $p \leq 0.01$ and * $p \leq 0.05$ with T tests

Less cluster-root biomass per nodule biomass was found in older plants compared with younger ones. Many previous studies have shown the importance of P during nodule growth and in the N_2-fixation process (Israel 1987). It costs more oxygen, i.e. respiratory energy, to fix N_2 for plants under P deficiency (Schulze and Drevon 2005), and P deficiency would lead to a decrease of specific nitrogenase activity (Sa and Israel 1991). N is remobilised from senescing leaves (Schulze et al. 1999), especially in legumes under P deficiency (Valentine et al. 2017), as is common in plants in general (Vergutz et al. 2012). P is also remobilised from senescing leaves (Li et al. 2017; Vardien et al. 2016; Vergutz et al. 2012). It is likely that P was remobilised during leaf senescence in older *L. albus* plants, reducing the need for P uptake by roots and thus for investment in cluster roots.

Signals involved in regulation of the formation of cluster root and photosynthesis

White lupins in the nodulated group exhibited faster photosynthesis rates (11% and 54% faster at the D23 and D30, respectively) than those in the control group. The rhizobial symbiosis may increase photosynthetic rates (Kaschuk et al. 2009). Photosynthesis has little dependency on leaf N concentration in many N_2-fixing legume species (Adams et al. 2016). N_2 fixation may increase photosynthetic rate through a decrease of feedback-inhibition of photosynthesis by sugars (Jones

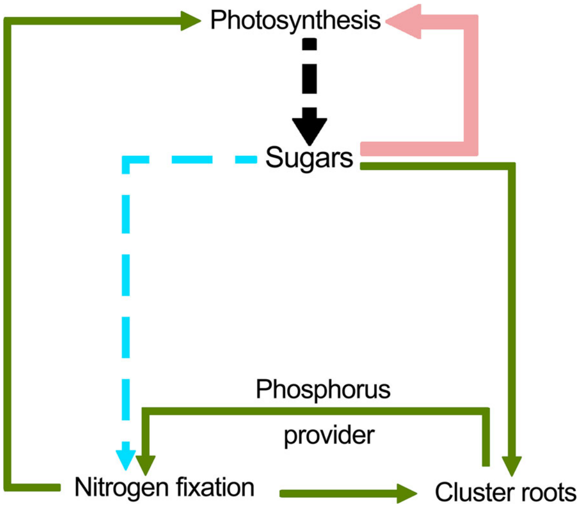

Fig. 5 Schematic diagram on the relationship of source and sink metabolism. These relationships include products/substrate and negative/positive effects. The black dotted bolding line refers to products, the light blue dotted line refers to substrate pink solid line refers to a negative effect, while dark green solid bolding line refers to positive effects

et al. 1996; Patrick et al. 2013; Ruan et al. 2012), and in turn increase the plant's photosynthesis rate (Fig. 5) (Kaschuk et al. 2009; Paul and Foyer 2001; Sharkey et al. 1986). The greater source and sink strength of plants in the nodulated group may have led to a variation in the strength of sugar signals with time, and have affected cluster-root formation (Fig. 5) (Liu et al. 2005; Muller et al. 2007). As plants became larger, reproduction also started to consume sugars (Bustan and Goldschmidt 1998). At the D36 harvest, all plants had reached their reproductive stage. Therefore, the consumption of sucrose may have lowered the leaf sucrose concentration. We speculate that lowered leaf sucrose concentration may have been a reason for older nodulated plants producing less cluster-root biomass per nodule biomass, in addition to a decreased P demand, as a result of P remobilisation from senescing leaves.

Concluding remarks

Previous studies considered that there is a trade-off between nodule growth and cluster-root formation (Thuynsma et al. 2014a, 2014b). We decided that their hypothesis about a trade-off needed to be tested using nodulated plants as well as an appropriate control. In our study, using both nodulated and control groups, *L. albus* plants had similar total biomass, shoot biomass and root biomass (not including nodules or cluster roots). However, nodulated plants at all harvests had both more nodules and cluster-root biomass than control plants. Whatever the signalling may have been, nodules enhanced the formation of cluster roots, rather than there being a trade-off, confirming our hypothesis that nodule activity promotes the growth of cluster roots.

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