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Legume nodules from nutrient-poor soils exhibit high plasticity of cellular phosphorus recycling and conservation during variable phosphorus supply

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ABSTRACT

Nitrogen fixing legumes rely on phosphorus for nodule formation, nodule function and the energy costs of fixation. Phosphorus is however very limited in soils, especially in ancient sandstone-derived soils such as those in the Cape Floristic Region of South Africa. Plants growing in such areas have evolved the ability to tolerate phosphorus stress by eliciting an array of physiological and biochemical responses. In this study we investigated the effects of phosphorus limitation on N_2 fixation and phosphorus recycling in the nodules of *Virgilia divaricata* (Adamson), a legume native to the Cape Floristic Region. In particular, we focused on nutrient acquisition efficiencies, phosphorus fractions and the exudation and accumulation of phosphatases. Our finding indicate that during low phosphorus supply, *V. divaricata* internally recycles phosphorus and has a lower uptake rate of phosphorus, as well as lower levels adenylates but greater levels of phosphohydrolase exudation suggesting it engages in recycling internal nodule phosphorus pools and making use of alternate bypass routes in order to conserve phosphorus.

1. Introduction

Phosphorus (P) is an essential nutrient for plant growth and a key structural constituent for nucleic acids, phospholipids, sugar phosphates and other catalytic cofactors, apart from the role it plays in metabolic regulation and energy transfer (Bosse and Köck, 1998). Plants thus depend heavily on P for plant growth and development, especially legume plants since P is required for biological nitrogen fixation (BNF) (Schulze et al., 1999) and has been reported to affect the energy costs of BNF (Valentine et al., 2010), as well as nodule formation and function (Israel, 1987). Soil P is however, limited and its availability is contingent on various factors such as diffusion rates in the soil and solubilisation of P containing compounds (Vance et al., 2003).

Plants have evolved an array of morphological and biochemical mechanisms to obtain adequate P or Pi (the metabolic form of P) under P deficient conditions (Vance et al., 2003; Tran et al., 2010). Morphological responses include transformed root architecture (Williamson et al., 2001), increasing root hair density and length which is common in legumes, and producing specialized roots known as proteoid roots for nutrient acquisition (Johnson et al., 1996; Neumann et al., 1999). Biochemical changes encompass increasing the abundance of Pi transport proteins and alternate enzymes to bypass Pi- or adenylate dependant reactions of glycolysis and mitochondrial respiration (Theodorou and Plaxton, 1993; Plaxton, 2004; Sieger et al., 2005; Tran et al., 2010). These alternate enzymes promote Pi recycling and the synthesis of organic acids, and Pi is a by-product of their reactions. P deficiency causes a decline in cytosolic Pi and adenylates (Rychter et al., 1992) and under these conditions the increased engagement of these alternative routes, eliminate the necessity for adenylates and Pi (Duff et al., 1989; Nagano et al., 1994).

Plants also increase their efficiency of Pi use during P deficiency by inducing phosphohydrolases such as ribonucleases (RNases) and acid phosphatases (APases) which scavenge Pi from P-esters (Raghothama, 1999; Tran et al., 2010; Hurley et al., 2010). APase activity has been used as a marker for P deficiency. APases release P (Miller et al., 2001) and have been implicated in the synthesis of glycolate and glycerate especially those associated with carbon metabolism (Duff et al., 1991; Vance et al., 2003). Extracellular APases cause the cessation of organic phosphate monoesters in the soil, while intracellular APases remobilize and scavenge Pi from internal sources (Duff et al., 1994; Marschner, 1995). Many organic P compounds occur in soil, with soil phytate (inositol hexaphos-

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Fig. 1. *Virgilia divaricata* seedlings (a), plants at 22 days of growth (b) and determinate nodules attached to the root system (c).

phates) forming a major component (around 25%), which could be hydrolyzed by APases or phytases. The latter represent a special group of phosphatases that are able to hydrolyze phytate to myo-inositol and phosphate (Richardson et al., 2000).

The correlation between P deficiency and BNF is not consistent among legumes, and nodular P metabolism is fairly understudied. In addition, many of the legume studies examining the effect of P deficiency on BNF focuses on model legumes (Tang et al., 2001; Le Roux et al., 2006; Schulze et al., 2006; Sulieman et al., 2013; Thuynsma et al., 2014). The P poor soils of the Cape Floristic Region (CFR) in South Africa has a high legume diversity (Goldblatt and Manning, 2002), yet not much is known about the functional adaptations they elicit with nutrient fluctuations.

The aim of this study was therefore to investigate the effects of P stress on BNF through response mechanisms of recycling and conservation inside the nodules of *Virgilia divaricata* (Adamson) (Fig. 1). This legume is native to the CFR and is distributed over a wide range of variably P poor soils, from relatively richer forest margins to poorer Fynbos soils (Coetsee and Wigley, 2013).

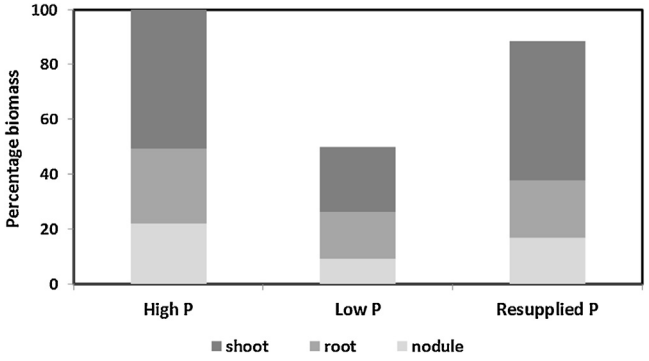

Fig. 2. Percentage biomass of *Virgilia divaricata* (Adamson, Fabaceae) grown under adequate (High P), deficient (Low P) and resupplied (Resupplied P) phosphorus conditions. High P was used as a standard (for optimal growth conditions) and percentages for Low P and Resupplied P indicate differences in mass on a percentage basis from the standard. The different letters indicate significant differences among the treatments ($P \leq 0.05$).

2. Materials and methods

2.1. Seed germination, bacterial inoculation and growth

V. divaricata seeds (Silverhill Seeds, Kenilworth, South Africa) were germinated as described in Vardien et al. (2014). Following the initial leaf emergence (Fig. 1a), seedlings were transferred to and inoculated with a locally sourced strain of *Burkholderia*. Inoculum was prepared as previously documented (Vardien et al., 2014). Three treatment categories, based on P concentration, were used: low P, high P (control), and resupplied P (four weeks of low P followed by three weeks of high P). All plants were supplied with 100 ml of a quarter strength Long Ashton nutrient solution twice a week. The nutrient solution was adjusted to contain either 5 μM P (LP) or 500 μM P (HP) (pH 5.8), and 500 μM NH_4NO_3. Plants were grown for 55 days until they were harvested.

2.2. Nutrient analysis

A subset of the harvested material was dried at 50 °C for 72 h and the dry weights (dw) recorded. The material was milled and analysed for their C, N and P concentrations according to previously established protocols in Vardien et al., 2014.

2.3. Nutrient cost calculations

The specific P absorption rate (SPAR) (mgP g^{-1} dw d^{-1}) and P utilization rate (SPUR) (g dw mg^{-1} P d^{-1}) of plant organs were calculated according to Nielson et al., 2001. These equations were however modified to include nodules instead of roots for SPAR and whole plants for SPUR, in accordance with previous work by Vardien et al. (2014).

Construction costs, C_w (mmol C/ g dw) were determined according to Mortimer et al. (2005), adjusted from the equation by Peng et al. (1993):

$$C_w = [(C + kN14 \times 180)/24] \times (1/0.89) \times (6000/180),$$

where C is the carbon concentration (mmol C/g), k is the reduction state of the N substrate ($k = -3$ for NH_3) and N is the organic nitrogen content of the tissue (g/g dw) (Williams et al., 1987). The constant (1/0.89) represents the fraction of the construction cost which provides reductant that is not assimilated into biomass (Williams et al., 1987; Peng et al., 1993) and 6000/180 converts units of g glucose/g dw to mmol C/g dw.

2.4. Calculations of $\delta^{15}N$

The $\delta^{15}N$ analyses were carried out as described in Vardien et al. (2014). The values obtained were subsequently used to determine the percentage N derived from the atmosphere (NDFA) (Vardien et al., 2014).

2.5. In vitro NMR measurements

Perchloric acid (PCA) extracts were prepared from 8 to 10 g of nitrogen frozen nodules according to the method described by Gout et al. (2000) and divalent paramagnetic cations were chelated by