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The effects of limiting phosphate on photosynthesis and growth of *Lotus japonicus*

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ABSTRACT

Photosynthesis is an integral part of plant metabolism for the production of carbohydrates, which are in turn used for plant biomass production. Phosphate (P) plays an important role in photosynthesis and the production of carbohydrates; however, it is also one of the most difficult macronutrients for plants to obtain. The effects of P deprivation on photosynthesis and respiration in the model legume *Lotus japonicus* were studied using gas exchange measurements and the A:Ci model of photosynthesis. Phosphate deprivation decreased plant biomass, overall photosynthetic reactions, photosynthesis:respiration ratio and triose-P utilization. The root:shoot ratio, the hallmark of P deprivation, did not show any significant increase under P deprivation, which is in contrast to the bulk of previous findings. Consistent allocation to both roots and shoots could explain this phenomenon. Direct photosynthetic and respiration measurements did not show any significant decrease under P deprivation, although the effect of P on photosynthesis has been shown to be largely species-specific. The ratio of photosynthesis to respiration, however, showed a marked decrease and therefore was affected by P deprivation. Changes in photosynthesis, respiration and triose-P utilization explain the decrease in plant biomass.

1. Introduction

The availability of soil soluble nutrients is a major constraint for plant growth and function (Ragothama, 1999; Vance et al., 2002). Macronutrients are required in large volumes by the plant, but are often in short supply (Ragothama, 1999). One of the least available macronutrients to plants is phosphate (Vance, 2008). Phosphate plays an integral role in many plant processes and its supply is critical to the maintenance of photosynthesis, plant growth and physiology (Plaxton and Carswell, 1999; Ragothama, 1999, 2000). Not only does it fulfil a structural role, but it also plays an important regulatory function in plant carbon metabolism, energy transfer, transformation of sugars and starches, and nutrient movement (Plaxton and Carswell, 1999; Ragothama, 1999, 2000). Plants have thus developed many adaptive strategies, including physiological and metabolic changes, to survive in low P environments (Vance et al., 2002).

Plant growth and function is intimately linked to the acquisition of mineral resources and the functioning of the photosynthetic machinery. The classic end product of photosynthesis is carbohydrates, most prominently sucrose and starch (Rao and Terry, 1995; Flügge et al., 2003). Sucrose is synthesized in the cytosol (Rao and Terry, 1995) and requires the export of triose-P and import of Pi by the well-known chloroplastic phosphate antiport translocator, the triose-phosphate translocator (TPT) (Flügge et al., 2003) located in the inner envelope of the chloroplast membrane. This transporter uses a counter-exchange mechanism to exchange Pi, 3-PGA and triose-P (Rao and Terry, 1995; Flügge et al., 2003).

Orthophosphate (Pi) released into the cytosol is used to drive ATP synthesis via photophosphorylation and consumption of ADP. These reactions occur in the thylakoid membrane itself. The ATPase present here then deposits the ATP into the chloroplast stroma (Flügge et al., 2003). Here, the ATP is consumed in the Calvin/Benson cycle by two separate enzymatic reactions, the activation of ribulose 5-phosphate and the production of 1,3-glycerate-bisphosphate (Flügge et al., 2003). In this way, for every three molecules of CO_2 that are fixed, nine molecules of Pi are consumed (Walker and Robinson, 1978). The generation of large amounts of Pi is thus essential for the maintenance of photosynthetic carbon fixation (Flügge et al., 2003).

The most common limitations on photosynthesis are light (photosynthetically-active radiation [PAR]) and the atmospheric CO_2 concentration. As light (PAR) increases and photosynthesis reaches a maximum, CO_2 becomes the limiting factor. By increasing the CO_2 concentration, CO_2 fixation can be increased to almost double the

Abbreviations: A:Ci, net CO_2 assimilation rate (A): calculated substomatal CO_2 concentration (C_i); PAR, photosynthetically-active radiation; TPU, triose-phosphate utilization.

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normal rate. It can therefore be deduced that at saturating light and CO_2, phosphate in the form of Pi could well be a limiting factor (Sivak and Walker, 1986).

It was demonstrated in isolated chloroplasts of spinach (Cockburn et al., 1976) and in vivo (Chen-Se et al., 1975; Herold et al., 1976) that the limitation of photosynthesis by Pi does indeed occur. When plants were fed Pi-sequestering agents, such as mannose and glucosamine, cytosolic Pi was sequestered in phosphorylated compounds (Sivak and Walker, 1986). These compounds are not easily metabolized, thus limiting the functioning of the photosynthetic machinery. This information was thought to be inapplicable to healthy plants grown in complete nutrient media (Sharkey, 1985), due to the large Pi stores known to be present in the plant cell vacuole and also the lack of knowledge about how the chloroplast may perceive or sense lack of Pi. This has been shown to be untrue and Pi supply can in fact limit photosynthesis (Sivak and Walker, 1986).

Phosphate supply not only limits photosynthesis, but also has various physiological effects on plant growth (Plaxton, 2004; Vance, 2008). The reactive nature of the phosphate molecule with both organic and inorganic substances leads to low soil concentrations of available P, with most soils containing 10 μM or less available P (Plaxton, 2004; Vance, 2008). Plants have developed many physiological strategies to acquire P, with the focus on increased soil exploration and P uptake (Plaxton, 2004; Vance, 2008). This is mainly achieved through an increase in root growth, leading to an increase in the root:shoot ratio. Increased root growth and function is, however, energetically expensive and consumes a large proportion of the daily photosynthate production, which may be further constrained by the limited P supply (Plaxton, 2004; Vance, 2008).

In this paper, the effect of P limitation on plant growth and photosynthesis was investigated in the model legume *Lotus japonicus*, in an unnodulated form. Gas exchange measurements for both light and CO_2 responses were used to determine photosynthetic efficiency and the effect of P deprivation on plant growth and biomass.

2. Materials and methods

2.1. Seed decontamination

L. japonicus MG21 seeds were scarified for 10 min in 3 volumes concentrated sulfuric acid, followed by five washes with sterile dH_2O. The seeds were surface decontaminated in a 30% (*w*/*v*) hypochlorite, 0.1% (*v*/*v*) Tween20 solution, again followed by five washes with sterile dH_2O. The seeds were imbibed overnight in sterile dH_2O and then stratified for three days at 4 °C prior to sowing.

2.2. Plant growth conditions

After stratification, seeds were planted into sterilized quartz sand and allowed to germinate and grow for three weeks. Thereafter, plants were transplanted into 20 cm pots and cultivated in sterile quartz sand for 6 weeks. The plants were divided into two treatment groups, low phosphate (LP) and high phosphate (HP), each receiving a modified Long Ashton (Smith et al., 1983) solution containing either 5 μM (LP) or 500 μM (HP) $NaH_2PO_4 \cdot 2H_2O$ as phosphate source (Keerthisinghe et al., 1998; Le Roux et al., 2006, 2009). The pH of the solution was adjusted to 6.5, and 400 ml was applied to the plants once a week, furthermore, the plants received distilled H_2O every other day. Nitrogen in the form of NH_4NO_3 (0.5 mM) was also added to both the low and high P solutions to prevent nodulation. Plants were grown under glasshouse conditions in a north-facing glasshouse at the University of Stellenbosch between the months of July and September. The range of midday irradiance was between 400 and 600 μmol photons/m^2/s and the average night/day temperatures were 12–18 °C.

2.3. Gas exchange measurements

The youngest fully expanded leaf was used for photosynthetic measurements. Light-response curves were used to determine the appropriate photon flux density (800 μmol photons/m^2/s) at which to conduct photosynthetic measurements. Readings were taken between 11 am and 4 pm, using the LI-6400XT portable photosynthesis and fluorescence system (Li-Cor, Lincoln, Nebraska, USA).

Photosynthetic CO_2 response curves were carried out in order to determine the maximum photosynthesis rate (Pmax), Rubisco activity and electron transport. Measurements were performed on the youngest fully expanded leaves (3 replicates in each treatment per species), using a Li-6400 gas exchange system (LI-COR Inc., IRGA, Lincoln, NE, USA). Measurements were taken between 9 am and 4 pm. A full response curve took 45 min to 1 h to complete. The leaves were enclosed in a leaf chamber (6 cm^2), which received a steady light of 800 μmol photons/m^2/s at a leaf temperature of 24 °C. CO_2 concentrations increased according to the following increments: 50 and 100 ppm. CO_2-response curves were used to calculate electron transport capacity and RUBISCO activity, using the equations of Watanabe et al. (1994). The CO_2-response curves were also used to determine the triose-P utilization as described by Kaschuk et al. (2012) according to the equation:

$$A_P = 3 \times TPU - R_d$$

where A_p is the limitation of photosynthesis by triose-P export and TPU is the rate of triose-P export. It is multiplied by 3 since 3 mol of CO_2 can be fixed for every mol of triose-P made available. $R_{d.}$ is the mitochondrial respiration in the light (mol CO_2/m^2/s), assumed to be directly related to maximum rate of Rubisco carboxylation at 25 °C (Watanabe et al., 1994).

2.4. Dry matter determination

Plants were harvested 6 weeks after transplantation into the sand culture and separated into roots and shoots. The harvested plant material was then placed in a drying oven at 50 °C for 3 days and their dry weights (DW) were recorded.

2.5. Relative growth rate determination

Plant relative growth rate was determined according to Mortimer et al. (2005). Relative growth rate, in the form of growth respiration, was determined using the equation:

$$Rg(w) = Rg(t)/gr(w/t)$$

where Rg (w) is growth respiration, based on dry weight (DW in g), Rg (t) is the daily growth respiration (μmol CO_2/day) and gr is the growth rate of the specific plant organ (root/shoot) (gDW/day).

2.6. Statistical analysis

Plants were grown in a randomized block design, in which P nutrition as a factor, was tested at two levels of supply (low P = LP, 5 μM and high P = HP, 500 μM). This resulted in two treatments (Low P and High P), where the effect of P nutrition was investigated on biomass and photosynthetic gas exchange. For the biomass parameters, the data are presented as the average of six plants from each treatment, Low P (n = 6) and High P (n = 6). For the photosynthetic gas exchange parameters, the data is given as the average of three replicates for both Low P (n = 3) and High P (n = 3) treatments. The effects of the factors were tested with an ANOVA (Kaleidagraph, Synergy Software, Reading, PA, USA). Where the ANOVA revealed significant differences between treatments, the means were separated using a post-hoc Fisher's LSD