Orthophosphate uptake in proteoid roots of naturally occurring *Hakea sericea* Schrad.

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INTRODUCTION

Phosphorus (P) is one of the most important plant macronutrients, playing a key role in many metabolic processes such as in energy transfer, signal transduction, biosynthesis of macromolecules, photosynthesis or respiration (Raghothama, 1999). Despite of this, P is one of the most unavailable and inaccessible mineral nutrients, frequently being the limiting nutrient for plant growth. The form of P most readily accessed by plants is Pi, the concentration of which rarely exceeds 10 µM in soil (Schachtman *et al.*, 1998). Many of the morphological and biochemical changes that are induced in roots growing in Pi-deficient conditions are geared towards enhancing Pi uptake, including not only the ability of increasing soil Pi availability but also the induction of high-affinity Pi uptake systems. Although some progress has been done on the elucidation of phosphate transport in plants, there are still few studies concerning biochemical and molecular characterization of phosphate uptake in proteoid roots. Here we present data on the mechanisms involved in Pi acquisition from soil by *Hakea sericea* Schrad. (Proteacea), an Australian invader of natural habitats, which is able to develop proteoid roots as a response to P deficiency (Fig. 1).

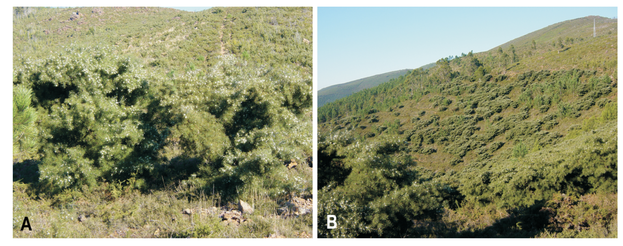

Figure 1. *Hakea sericea* Schrad. schrub (A) and Serra d'Arga (Northern Portugal) landscape where the spreading of *Hakea sericea* has become a major problem (B).

RESULTS

Pi transport

Proteoid roots were harvested from adult *H. sericea* shrubs growing in Serra d'Arga, Northern Portugal (Fig. 2), washed with mineral medium without Pi, and cross-sectioned. To study Pi transport roots were incubated with 2.5-200 µM NaH_2PO_4 and the depletion of Pi from the external medium was determined by the colorimetric method of Adams (1991).

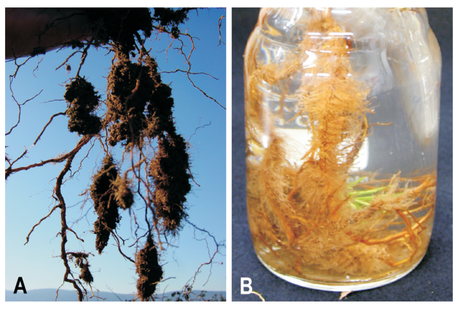

Figure 2. *Hakea sericea* proteoid roots harvested in the field (A) and after being washed in the lab exhibiting densely spaced rootlets (B).

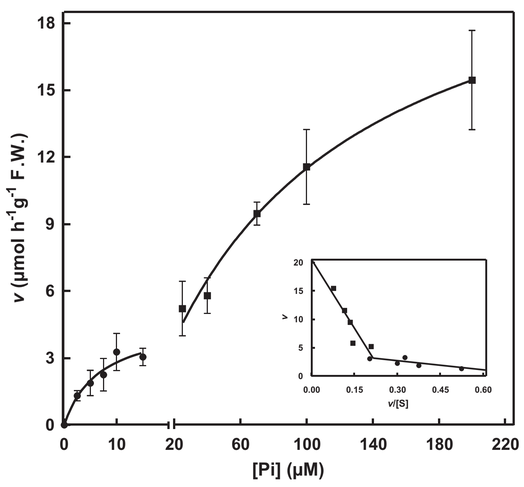

Figure 3. Initial uptake rates of Pi, at pH 6.0, by proteoid roots of *Hakea sericea*. Values are mean ± S.E., N=3. *Insert:* Eadie-Hofstee plot of the initial Pi uptake rates.

Kinetic studies supported the involvement of two Pi mediated transport systems (Fig. 3). The kinetic parameters, were as follows: for the high-affinity system K_m, 6 µM Pi and V_{max}, 5 µmol h^{-1} g^{-1} FW; for the low-affinity system K_m, 100 µM Pi and V_{max}, 24 µmol h^{-1} g^{-1} FW.

The measurement of initial uptake rates of 5-20 µM Pi (high-affinity range) and 20-100 (low-affinity range) µM Pi in the presence of 600 µM phosphite (Phi) showed that this substrate behaved as a competitive inhibitor (Fig. 4 and 5), indicating that it is also a substrate for both Pi transport systems. Mersalyl (100 µM) reduced by 50% the initial uptake rates of 10 µM Pi (Fig. 5).

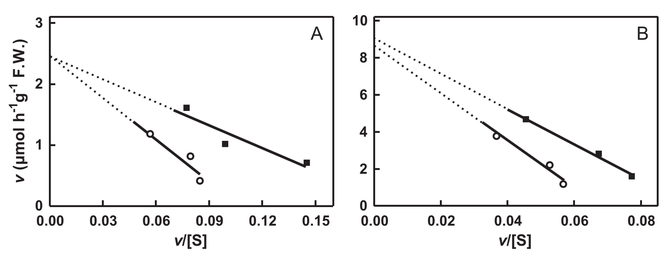

Figure 4. Eadie-Hofstee plots of the initial uptake rates of 5-20 µM Pi (A) and 20-100 µM Pi (B) in the absence (■) or in the presence of 600 µM Phi (O).

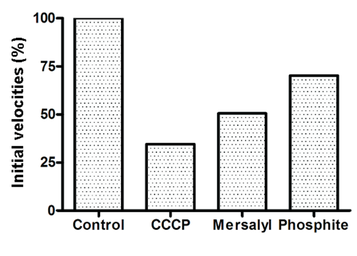

Figure 5. Initial uptake rates of 10 µM Pi in the absence or in the presence of 50 µM CCCP, 150 µM mersalyl and 600 µM phosphite.

The protonophore CCCP (50 µM) inhibited the initial uptake rates of 5-25 µM Pi (high-affinity range) and 25-70 µM (low-affinity range) up to 60%, suggesting the involvement of a H^+-dependent transport (Fig. 5 and 6).

To determine which Pi form is preferentially transported, kinetic studies were conducted at pH 4.5, 5.0, 6.0 and 6.5. For both transport systems, through this pH range, K_m variation is lower when Pi concentration is expressed as $[H_2PO_4^-]$ (Tables I and II), suggesting that $H_2PO_4^-$ is the transported form.

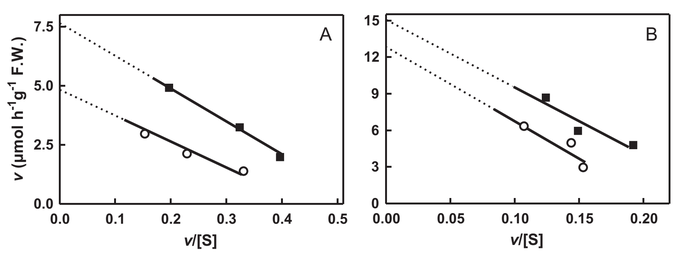

Figure 6. Eadie-Hofstee plots of the initial uptake rates of 5-25 µM Pi (A) and 25-70 µM Pi (B) in the absence (■) or in the presence of 50 µM CCCP (O).

Table I. Michaelis Menten constants (K_m) of *H. sericea* high-affinity Pi transport system at different extracellular pH values (pH_{ext}).

pH_{ext}	K_m (µM)				
	Total phosphate	H_3PO_4	$H_2PO_4^-$	HPO_4^{2-}	PO_4^{3-}
4.5	4.95	6.4×10^{-2}	4.88	3.2×10^{-3}	7.7×10^{-12}
5	6.57	2.7×10^{-2}	6.53	1.2×10^{-2}	1.9×10^{-10}
6.0	6.11	5.8×10^{-4}	5.75	0.35	1.7×10^{-7}
6.5	11.25	4.0×10^{-4}	9.42	1.84	2.8×10^{-6}

Table II. Michaelis Menten constants (K_m) of *H. sericea* low-affinity Pi transport system at different extracellular pH values (pH_{ext}).

pH_{ext}	K_m (µM)				
	Total phosphate	H_3PO_4	$H_2PO_4^-$	HPO_4^{2-}	PO_4^{3-}
4.5	66.79	0.87	65.88	$4{,}1 \times 10^{-2}$	$2.0\ 10^{-10}$
5	78.69	0.33	78.21	0,15	$2.3\ 10^{-9}$
6.0	106.4	1.0×10^{-2}	100.2	6.18	$3.0\ 10^{-6}$
6.5	97.76	$3{,}4 \times 10^{-3}$	81.81	15.95	$2.4\ 10^{-5}$

Search for phosphate transporter genes *(PiT)* in *H. sericea* Schrad.

For the identification of *PiT* genes encoding *H. sericea* Pi/H^+ symporters, a gDNA library was constructed using Lambda DASH II/Bam HI vector kit (Stratagene). In order to obtain homologous *PiT* probes, PCR amplifications of *H. sericea* gDNA were performed in the presence of degenerated primers designed for the conserved regions of *PiT* genes from higher plants (Fig.7).

As a result of the use of degenerated primers, several fragments with the same molecular weight, but corresponding to different *PiT* genes of *H. sericea*, could have been amplified in the same PCR reaction. Electrophoretic and Southern analyses of these fragments were performed after cloning them into pPCR-Script Amp SK (+) (Stratagene) and digesting the recombinant plasmids with *EcoR*I/*Sac*I (Fig. 8). Two distinct restriction patterns were obtained. The first included *PiT1*, *PiT3* and *PiT6* while the second one was observed for *PiT2*, *PiT4* and *PiT5*.

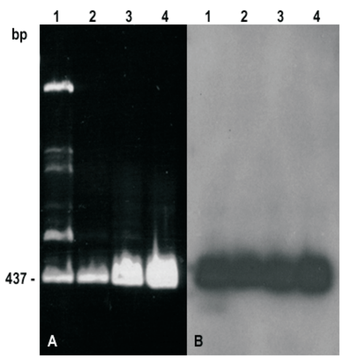

Figure 7. Southern analysis of PCR amplification products of *H. sericea* genomic DNA using degenerated primers for the conserved regions of *PiT* genes from higher plants. Annealing temperatures of 40°C (1); 45°C (2); 50°C (3) and 55°C (4) were used. A - Electrophoretic analysis (1.2% agarose gel); B - Southern blot analysis performed using [-^{32}P]dCTP labeled *Lupinus albus LaPT1* gene.

After sequencing *PiT2* and *PiT6*, two 437-bp fragments sharing 77.4% identity with each other and homologues to phosphate transporters of higher plants were obtained (Figure 9). The deduced amino acid sequences were aligned with amino acid sequences of phosphate transporters from other higher plants and a phylogenetic tree was created (Fig. 10). The hydrophobicity analysis revealed the presence of five transmembrane domains in both PiT2 and PiT6 peptides (Fig. 11).

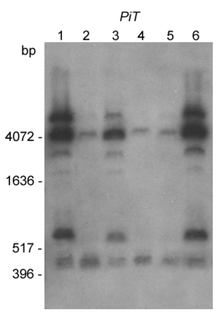

Figure 8. Restriction pattern of the recombinant plasmids containing *PiT* fragments after digestion with *EcoR*I/*Sac*I. Southern blot analysis performed using [-^{32}P]dCTP labeled *Lupinus albus LaPT1* gene.

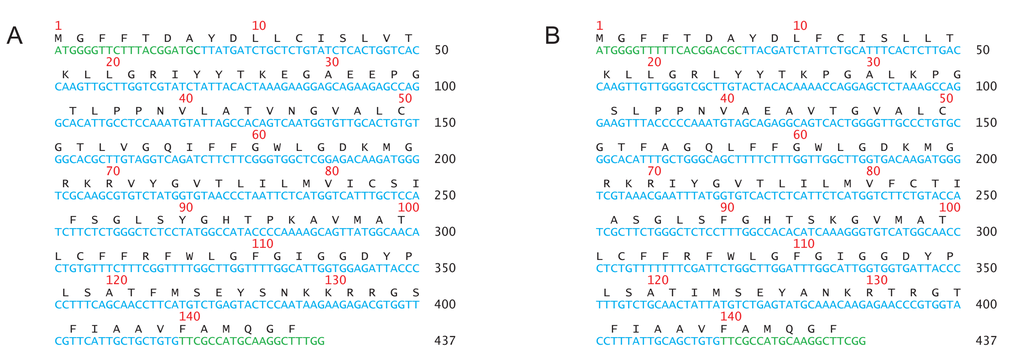

Figure 9. Partial nucleotide and deduced amino acid sequences (*PiT2* - A and *PiT6* - B) identified in *Hakea sericea*. The deduced amino acid sequence is represented above the nucleotide sequence, in the one letter code. The numbers on the right are related with the nucleotides and the numbers above are related with the amino acids. The sequences corresponding to the primers used in the amplification are represented in green.

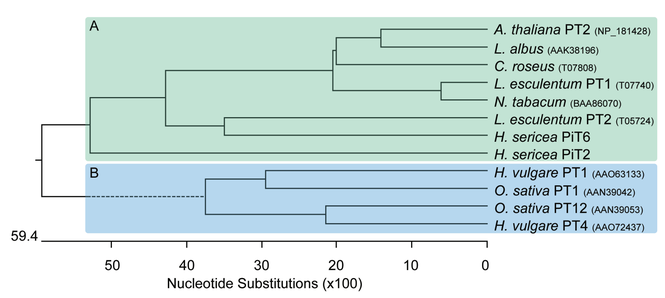

Figure 10. Phylogenetic tree representing the relation between *PiT2* and *PiT6* of *H. sericea* and other phosphate transporters of higher plants. The amino acid sequences were aligned with the program MegAlign (DNAStar). The length of each pair of branches represents the length between pairs of sequences. The scale bellow the tree measures the distances between the sequences. The accession of each sequence follows the species name. A - Eudicotyledons; B -Liliopsida (monocotyledons).

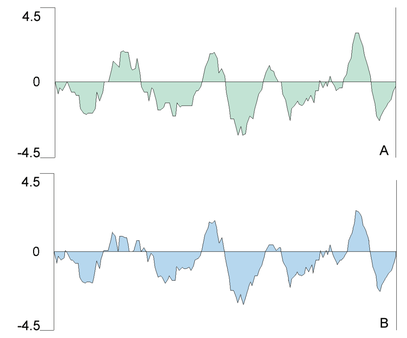

Figure 11. Hydrophobicity plot (Kyte-Doolitle) of *PiT2* (A) and *PiT6* (B) deduced amino acid sequences identified in *H. sericea* genome. Five transmembrane domains are observed in both peptides.

In order to obtain the complete sequences of phosphate transporters, *PiT2* and *PiT6* fragments are currently being used as homologous probes in the screening of the gDNA library of *H. sericea* (Fig. 12).

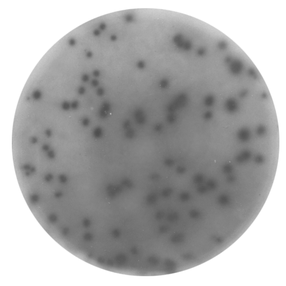

Figure 12. Autoradiogram corresponding to the screening of the gDNA library of *H. sericea* with the homologous probes *PiT2* and *PiT6*.

CONCLUDING REMARKS

- *H. sericea* proteoid roots have highly efficient transporters for acquisition of Pi from soil.
- Pi uptake was inhibited by CCCP, suggesting the involvement of H^+- dependent transport.
- The Pi transported form is likely $H_2PO_4^-$.
- The high affinity Pi transport system has a K_m of about 6 µM, a typical soil Pi concentration.
- Screening of genes encoding *H. sericea* Pi transporters is now under way.

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Acknowledgements: Manuel F. Sousa is supported by FCT, grant ref. SFRH/BD/10899/2002
Paulo Silva is supported by FCT, grant ref. SFRH/BD/13460/2003