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Tese de Doutoramento em Ciências Biológicas

Trabalho efectuado sob orientação da
Professora Doutora Teresa Lino Neto
e do
Professor Doutor Hernâni Gerós

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Do, or do not. There is no 'try'.

***by* Jedi Master Yoda**

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To my parents, my brother and my sister.

To Vanessa, for being the best part of my day...

Abstract

Hakea sericea Schrad. (needlebush or silky hakea) is an Australian Proteaceae that can be found naturalized in South Africa, New Zealand and southern Europe. It presents xeromorphic adaptations in leaves, produces high amounts of long-lived, fire resistant seeds that are easily dispersed by both air and water, and relys on proteoid roots for enhanced nutrient uptake. Such unique abilities allowed *H. sericea* to proliferate and spread throughout its new habitats, being currently considered by regulatory agencies as one of the most agressive invaders established in Portuguese territory. Although mechanical and biological control have already been extensively tested in South Africa to constrain the expansion of *H. sericea*, in Portugal no large scale attempts have been made to control its spreading.

Here we report the identification of a naturally occuring pathogenic fungus able to infect *H. sericea* that could, in a nearby future, be used as part of an integrated control strategy for this invader. The pathogenic agent was isolated from leaf spots of infected wild-growing *H. sericea* found in “Serra de Arga”, in north-western Portugal. Microscopical observation revealed typical *Pestalotiopsis* sp. 5-celled spores, whose members have been described as pathogenic for Proteaceae species. Thermocyclic amplification of the internal transcribed spacer region of the fungal rDNA, using *ITS4* and *ITS5* universal primers, identified the pathogenic agent as *Pestalotiopsis funerea.* Pathogenicity of *P. funerea* on *H. sericea* was confirmed with the infection of leaf-wounded and non-wounded 6-week-old *in vitro*-grown plants. Only the leaf-wounded plants sprayed with *P. funerea* developed lesions identical to those observed in wild-grown specimens.

A major feature of *H. sericea* is the ability to produce proteoid roots in response to mineral scarcity, mainly N and P. Although the enhanced capacity of proteoid roots to absorb inorganic phosphate (Pi) has been mainly attributed to an increased root surface area and higher exudation of organic acids and phosphatases, here we focused on the Pi uptake system of proteoid roots by itself. Uptake experiments with ^{32}Pi suggested the involvement of H^+/Pi co-

transport systems with K_m values of 0.225 and 40.8 μM Pi, both prone to competitive inhibition with the analogs phosphite and arsenate, but not vanadate. This biphasic Pi uptake system with the highest affinity at submicromolar range is likely to confer *H. sericea* the ability to invade and proliferate throughout vast areas of nutrient-poor soils, where survival for most plant species seems harder.

In plants, Pi uptake, translocation through tissues and interorganellar allocation is carried out by members of the phosphate transporter (PhT) family. Here we report the identification of four *PhT* genes (*PiT2*, *PiT6*, *PH5* and *PH7*) from *H. sericea* genome. Through phylogenetic analysis and transmembrane domain (TMD) prediction, all identified genes were placed in the PhT1 family, meaning *PiT2*, *PiT6*, *PH5* and *PH7* are probably high- or low-affinity H^+/Pi symporters involved in Pi transport into the cell. Phylogenetic analysis confirmed the homology between PhT1 members from different plant species, while PhT2 members (chloroplastidial H^+/Pi symporters) were placed in a cluster of their own. Based on phylogeny, we also report the possible existence of new Na^+/Pi symporter family in plants. The correlation between the expression site of each *PhT1* with its deduced amino acid sequence has also been studied. Phylogenetic studies suggest that the amino acid structure of each PhT1 is insufficient to predict the tissue where the protein is likely to be expressed. Expression studies of *PiT2*, *PiT6*, *PH5* and *PH7* in plants grown in high- and low-Pi availabity shall provide insights on the Pi transport regulation mechanism of *H. sericea*.

Resumo

Hakea sericea Schrad. (háquea-espinheiro ou háquea-picante) é uma Proteaceae nativa da Austrália que se encontra actualmente naturalizada na África do Sul, Nova Zelândia e partes do sul da Europa. Apresenta folhas com modificações xerofíticas e produz anualmente uma grande quantidade de folículos contendo sementes aladas. Estas sementes são libertadas apenas após a passagem de fogo ou morte da planta, sendo dispersas quer pelo ar, quer por cursos de água. Associada ao crescimento da *H. sericea* em solos pobres em minerais está a produção de raízes proteóides especializadas na captação de fosfato. Estas características únicas permitiram a fixação e proliferação de *H. sericea* em novos habitats, sendo actualmente considerada pelo Ministério do Ambiente como uma das invasoras mais agressivas em Portugal. Embora na África do Sul tivessem sido testadas formas de controlo mecânico e biológico desta espécie, em Portugal não se efectuou ainda qualquer tentativa em grande escala de controlar a invasão.

Aqui reportamos a identificação de um fungo patogénico capaz de infectar *H. sericea* que poderá, num futuro próximo, ser usado como parte integrante de um programa de controlo desta espécie. O agente patogénico foi isolado a partir de lesões nas folhas de espécimes infectados a crescer nas encostas da Serra de Arga, no noroeste português. Por observação microscópica foram identificados esporos com cinco células típicos do género *Pestalotiopsis* sp. A amplificação termocíclica das regiões ITS do rDNA do fungo, usando os iniciadores oligonucleotídicos universais *ITS4* e *ITS5* permitiu a identificação do agente patogénico como sendo *Pestalotiopsis funerea*. A patogenicidade de *P. funerea* em *H. sericea* foi confirmada através da infecção de plântulas crescidas *in vitro* com ou sem pequenas incisões nas folhas. Apenas plantas lesadas e infectadas com *P. funerea* desenvolveram sintomas idênticos aos observados em plantas contaminadas presentes na Serra de Arga.

Um dos principais atributos de *H. sericea* é a capacidade de produção de raízes proteóides em resposta à escassez de minerais, nomeadamente azoto e fósforo. Apesar da alta eficácia que estas raízes têm em captar fosfato ter sido

associada sobretudo ao aumento da área de superfície da raiz e da grande capacidade de exsudação de ácidos orgânicos e fosfatases, neste trabalho focamo-nos no próprio mecanismo de aquisição de fosfato. Experiências de captação de ^{32}Pi sugeriram o envolvimento de um sistema de co-transporte de fosfato com protões com valores de K_m de 0.225 e 40.8 μM Pi, ambos susceptíveis a inibição competitiva pelos análogos fosfito e arsenato, mas não por vanadato. Este sistema bifásico de captação de Pi com um sistema com uma afinidade invulgarmente alta em plantas deve provavelmente permitir que *H. sericea* seja capaz de invadir e proliferar em áreas pobres em nutrientes, onde a sobrevivência de outras espécies aparenta ser dificultada.

Nas plantas, a captação e translocação de fosfato através de tecidos e organelos está dependente de membros da família dos transportadores de fosfato (PhT). Neste trabalho reportamos a identificação de quatro genes *PhT* (*PiT2*, *PiT6*, *PH5* e *PH7*) no genoma de *H. sericea*. Através de análise filogenética e predição de domínios transmembranares (TMD), todos os genes identificados foram caracterizados como membros da sub-família PhT1. Assim, *PiT2*, *PiT6*, *PH5* e *PH7* são provavelmente simportadores de Pi com protões de alta ou baixa afinidade envolvidos na captação de fosfato ao nível da membrana celular. A análise filogenética também confirmou a homologia entre todos os membros PhT1 analisados. Da mesma forma, todos os membros PhT2, simportadores cloroplastidiais de Pi com protões, formaram o seu próprio agrupamento filogenético. Reportamos também com base na filogenia, a possível existência de uma nova família de simportadores de fosfato com sódio em plantas. O estudo da relação entre o local de expressão de cada *PhT1* com a sua estrutura aminoacídica deduzida sugere que esta é insuficiente para determinar o tecido onde a proteína deverá ser expressa. Assim, para que possamos conhecer mais profundamente os mecanismos de regulação do transporte de Pi em *H. sericea* será necessário realizar estudos de expressão de *PiT2*, *PiT6*, *PH5* e *PH7* em plantas crescidas em altas ou baixas concentrações de fosfato.

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Abbreviations List

Main abbreviations

$A_{###}$	absorbance at ### nm
ACL	ATP-citrate lyase
Aco	aconitase
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pairs
BSA	bovine serum albumin
°C	degrees Celsius
CCCP	carbonyl cyanide *m*-chlorophenylhydrazone
Ci	Curie
CS	citrate synthase
DMSO	dimethyl sulfoxide
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
FW	fresh weight
g	gram
g	gravity acceleration
h	hour
ha	hectare
HK	hexokinase
ICP	inductively coupled plasma atomic emission spectrometry
IPTG	isopropyl-ß-D-thiogalactopyranoside
ITS	internal transcribed spacer
J	Joule
kb	kilobase
L	liter
M	molar
m	meter
min	minute
MDH	malate dehydrogenase
MFS	major facilitator superfamily
MOPS	3-(N-morpholino)propane-sulfonic acid
NCBI	National Center for Biotechnology Information

PCR	polymerase chain reaction
PDA	potato dextrose agar
PEG	polyethylene glycol
PEP	phosphoenol pyruvate
PEPC	phosphoenolpyruvate carboxylase
pfu	plaque-forming units
PGM	phosphoglucomutase
Phi	phosphite
PHT	phosphate transporter
PHYLIP	PHYLogeny Inference Package
Pi	inorganic phosphate
PIPES	piperazine-1,4-bis(2-ethanesulfonic acid)
Po	organic phosphate
PVP	polyvinylpyrrolydone
RNase	ribonuclease
rpm	rotations per minute
s	second
SDS	sodium dodecyl sulphate
SE	standard error
SuSy	sucrose synthase
TCA	tricarboxylic acid cycle
TMD	transmembrane domain
TPP^+	tetraphenylphosphonium
TPT	triose phosphate/phosphate translocator
Tris	tris(hydroxymethyl)aminomethane
Triton X-100	polyoxyethylene-*p*-isooctylphenol
U	unit of enzymatic activity
UV	ultra-violet light
V	volt
v; vol	volume
W	watt
w	weight
X-gal	5-bromo-4-chloro-indolyl-ß-D-galactopyranoside

Nucleic acids

DNA	deoxyribonucleic acid
gDNA	genomic deoxyribonucleic acid
rDNA	ribosomal DNA
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
A	Adenine
T	Thymine
C	Cytosine
G	Guanine
dCTP	2'-deoxyguanosine-5´-triphosphate
dNTP	2'-deoxynucleotide-5´-triphosphate

Amino Acids

A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine

List of publications

- Sousa MF, Façanha AR, Tavares RM, Lino-Neto T and Gerós H, 2007. Phosphate transport by proteoid roots of *Hakea sericea*. Plant Science 173, 550–558.

- Sousa MF, Tavares RM, Gerós H and Lino-Neto T, 2004. First report of *Hakea sericea* leaf infection caused by *Pestalotiopsis funerea* in Portugal. Plant Pathology. 53, 535.

- Sousa MF, Tavares RM, Gerós H and Lino-Neto T. Phosphate transporter sequences from *Hakea sericea*: identification and phylogenetic relation (in preparation)

CHAPTER 1

GENERAL INTRODUCTION

1.1 INTRODUCING *HAKEA SERICEA* AND THE PROTEACEAE FAMILY

1.1.1 The Proteaceae

Proteaceae is a family of woody evergreen plants ranging from almost herbaceous sub-shrubs to trees over 40 m tall, though most exemplars are small trees or shrubs – some of which are prostrate (Myerscough *et al*., 2001) (Fig 1.1). The 80 genera and 1769 species recognized in the family are distributed mainly in the Southern hemisphere and are almost completely restricted to Gondwanic continental blocks and fragments (Weston, 2006). The family is most diverse in Australia, followed by southern Africa, South America, New Caledonia, New Guinea, Malesia, south and east Asia, tropical Africa, Central America, Madagascar, New Zealand, Fiji, southern India, Sri Lanka, Vanuatu and Micronesia (Johnson and Briggs, 1975; Weston and Crisp, 1996; George, 1998) (Fig 1.2). Proteaceae species inhabit a wide variety of habitats, ranging from wet tropical, warm subtropical temperate and cool temperate rainforests, to semi-arid, arid and saline coastal areas of sandstone soils, to Mediterranean climates and alpine areas (Specht *et al*., 1974; Cowling and Lamont, 1998). Furthermore, many Proteaceae are suited with morphological and physiological adaptations that enable them to survive in harsh conditions, such as nutrient deprivation, fire exposure or excess/shortage of light.

Proteaceae family presents diverse leaf morphology and anatomy (Johnson and Briggs, 1975; Carpenter, 1994; Catling and Gates, 1998). With the exception of the mesophytic rainforest species, most Proteaceae are sclerophyllous (from Greek *skleros* - hard and *phullon* - leaf), presenting mature leaves with high amounts of lignin in the sclerenchyma, cutin in thick cuticles, and/or epidermal cell walls rich in silica (Beadle, 1966; Dillon, 2002; Jordan *et al*., 2005). The evolution of scleromorphy still remains controversial and it has been considered to be an adaptation to seasonal water deficits, especially common in temperate, dry summer climates (Edwards *at al*., 2000).

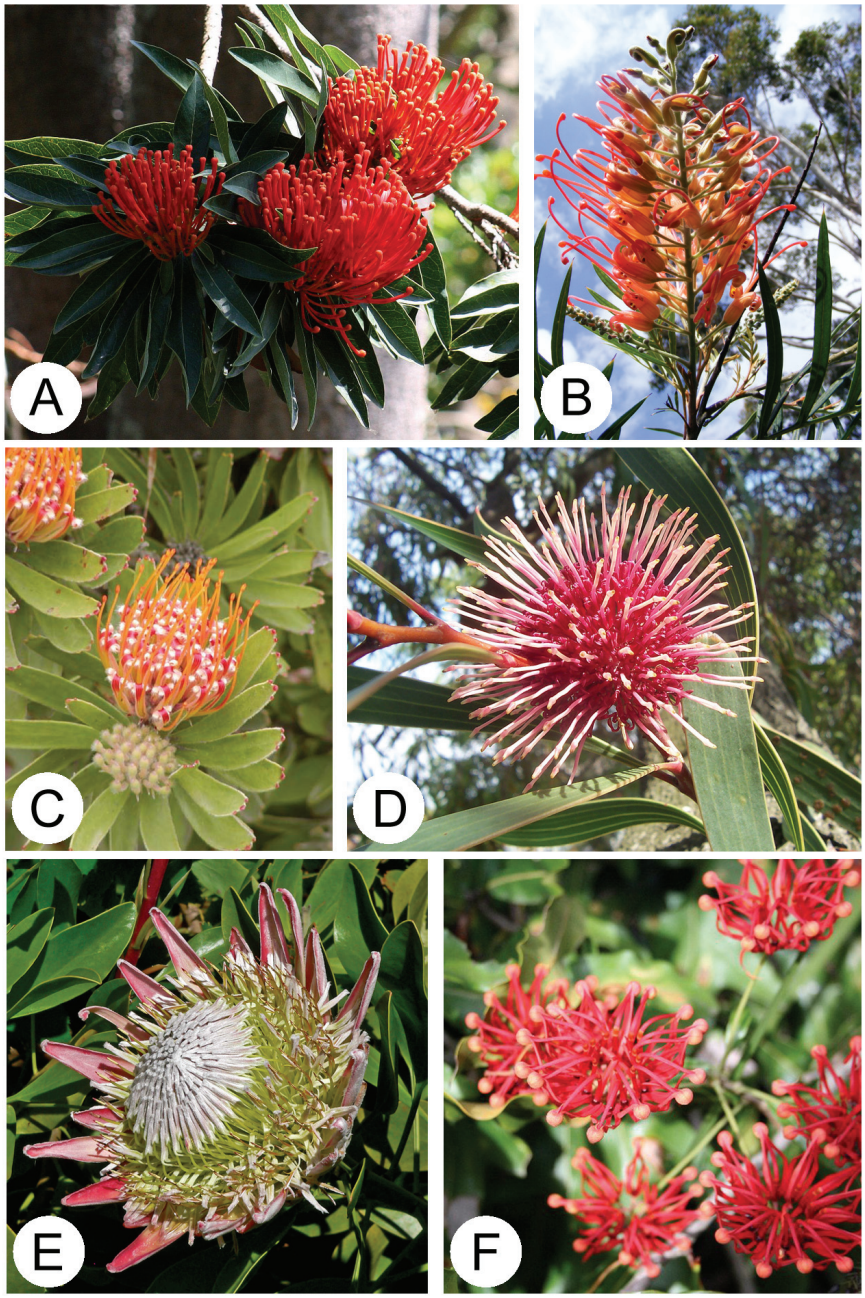

Figure 1.1 Proteaceae diversity.

(A) *Alloxylon flammeum* is one of the most spectacular Australian native plants. This tall tree can reach up to 30 m high in its native habitat, the rainforests of Australia. (B) *Grevillea banksii* is widely cultivated in its shrubby form, although it can be found in the "tree form" in the wild. (C) *Leucospermum erubescens* is native to Zimbabwe and South Africa, occupying a variety of habitats, including scrub, forest, and mountain slopes. (D) *Hakea laurina* is found mostly in sandplains or sandy-clay of south-western Australia and is frost tolerant. (E) *Protea cynaroides*, also known as King Protea, is the National Flower of South Africa, and it grows in the southern part of the country. Their unusual flowers are becoming very popular for flower arrangements due to their long vase life, making also excellent dried flowers. (F) *Stenocarpus sinuatus* occurs in rainforests of north-eastern Australia and Papua New Guinea. It prefers fairly rich, loamy soils but is tolerant of most well drained soils.

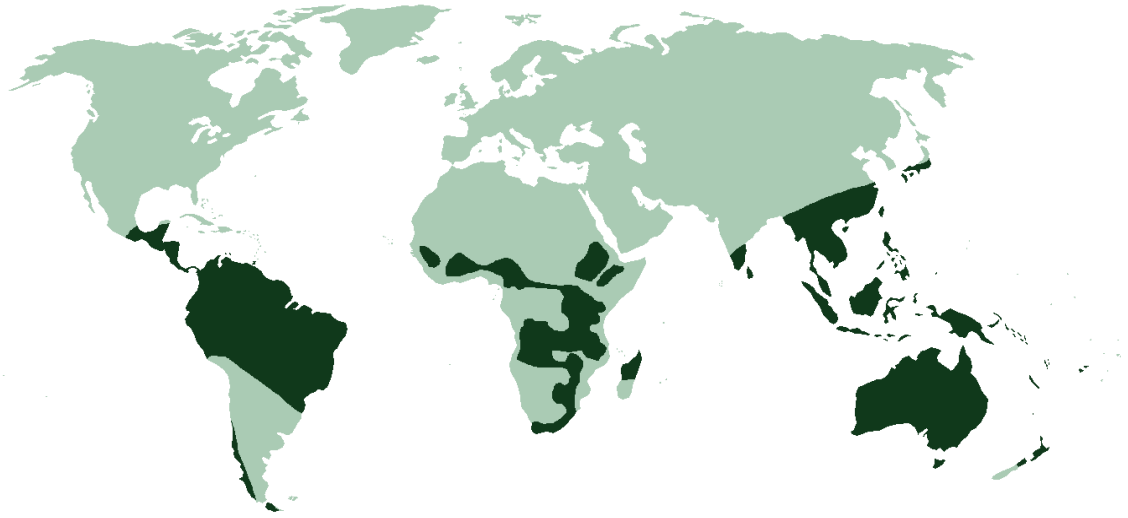

Figure 1.2 Native distribution of Proteaceae (adapted from Weston, 2006).

Although scleromorphy was suggested to increase drought tolerance by increasing the ability of leaves to tolerate low water potentials, this is poorly supported by experimental evidence (Salleo and Nardini, 2000). In fact, cuticle thickness is poorly related to resistance to diffusion of water vapour (Kerstiens, 1996; Riederer and Schreiber, 2001). Alternatively, low nutrient availability appears to have been more important than aridity in the evolution of scleromorphy in many Southern hemisphere groups (Hill, 1998). Indeed, diverse scleromorphic proteaceous fossil leaves occurred in the Paleogene, long before the drying of Australia (Carpenter *et al.*, 1994; Carpenter and Jordan, 1997; Jordan *et al.*, 1998). The evolution of scleromorphy in response to low nutrient environments can be explained by two different models. Loveless (1962) proposed that phosphate limitation may originate photosynthates that cannot be used metabolically, and thus, would be accumulated as nonfunctional sclerenchyma or cuticle. This model would predict nonspecific distribution of sclerenchyma in leaves, perhaps concentrated near the site of production, the mesophyll. A more recent model proposes that scleromorphy may protect long-lived leaves in low productivity environments from different types of damage and stress (Turner, 1994). This model could explain scleromorphy not only in low nutrient environments, but also in dry and cold ones.

The range of specialized scleromorphic leaf anatomical structures in Proteaceae is remarkably wide and includes structures associated with the vascular bundles, leaf margins, and mesophyll (Dillon, 2002). Some are also associated with the leaf surface exposed to radiation and include cuticles up to 35 mm thick and five anatomically distinct kinds of lignified layers between the epidermis and the mesophyll. Thereby, the solar radiation that reaches the mesophyll is attenuated as the amount of tissue and number of surfaces that radiation must pass through is largely increased (Jordan *et al*., 2005). Other scleromorphic structures such as osteosclereids, thick mesophyll cell walls and bundle sheath extensions prevent leaf collapse (Dillon, 2002) and should also increase resistance to piercing and shearing, such as those caused by many herbivores (Read *et al*., 2000). In addition, scleromorphic structures increase leaf carbon to nitrogen ratios, which may reduce herbivory (Coley *et al*., 1985; Coley, 1988). Besides scleromorphy as an adaptation to poor-nutrient soils, all Proteaceae, except the Persoonioideae and Symphionematoideae, rely on the formation of proteoid roots for the uptake of inorganic nutrients (Purnell, 1960; Jeffrey, 1967; Lamont, 1972; Malajczuk and Bowen, 1974; Pate, 1994; Jeschke and Pate, 1995 and Pate *et al*., 1998).

Seeds of most Proteaceae species do not store starch. Instead, they tend to be provisioned with resources that establishing seedlings will find scarce in their habitat. Usually, seeds from species endemic to rainforests tend to be larger and have a very high ratio of lipid/protein (Grundon, 1972; Kuo *et al*., 1982). These energy reserves seem to have high survival value to seedlings under rainforest canopies, where light may be in short supply (Pate *et al*., 1986). Conversely, Proteaceae occurring in infertile soils typically have smaller seeds with very high amounts of phosphorus, mostly in the form of phytate, which functions as *foci* for the accumulation of a range of cationic reserves, including calcium, magnesium, potassium and a number of micronutrients (Pate *et al*., 1986; Stock *et al*., 1989; Lamont and Groom, 1998).

Survival of seedlings can be enhanced if germination occurs during the periods when the resources for establishment and growth are high and the abundance of potential predators and pathogenic organisms are low (Myerscough

et al., 2001). Many Proteaceae species endemic to fire-prone and infertile habitats may control the timing of germination in three different ways. Mature, non-dormant seeds may be retained on parental plants in fairly tough and woody fruits that open and disperse the seeds usually following a fire or plant exposure to extremely hot weather, such as in *Hakea sericea* and *Banksia serrata* (Whelan *et al*., 1998; Brown and Whelan, 1999). Seeds in a dormant state may be shed onto the soil, with their dormancy being subsequently broken by the passage of a fire (dormant soil seed bank), as in *Grevillea rivularis* (Pickup *et al*., 2003). Finally, after surviving a fire, Proteaceae species such as *Lomatia silaifolia* and *Telopea speciosissima* are stimulated to flower and produce non-dormant seeds that are shed on maturity (Pyke, 1983; Bradstock, 1995; Denham and Auld, 1999; Denham and Whelan, 2000). Although this mode of germination seems to be the rarest, it is widespread along many species of monocotyledons (Keith, 1996).

Dormancy of seeds is common among many Proteaceae growing in fire-prone habitats. For example, the released mature fruits of *Persoonia* maintain seeds in a dormant state, which is hard to break artificially (Wrigley and Fagg, 1989; Ketelhohn *et al*., 1996). Also, the dormancy of seeds from *Conospermum* can be broken with the exposure to smoke (Dixon *et al.,* 1995; Roche *et al*., 1997). Proteaceae species endemic to regions where wildfires are common are often serotinous, with mature seeds stored in robust fruits, ranging from leathery to massive woody structures (Lamont *et al*., 1991; Read and Stokes, 2006). Although protection of seeds from heat is related to wall thickness and fruit size (Bradstock *et al*., 1994), woody fruits also confer protection against insect larvae and seed-eating birds (Groom and Lamont, 1997). Another adaptation developed by some Proteaceae to cope with extreme temperatures is the ability to resprout from a lignotuber and/or epicormic shoots following a fire (Zammit, 2006).

Taking into account these abilities, many Proteaceae members tend to fall into the category of stress-tolerators as described by Grime (1974), in which plants are able to inhabit environments where at least one resource for growth is in low supply.

1.1.2 Hakea sericea *Schrad.*

Hakea Schrader & J.C.Wendl is one of the 80 genera found in the Proteaceae, named after Baron Christian Ludwig von Hake (1745–1818), a German patron of botany. The genus includes 149 species, most native of Western Australia, with around 40 species found in the eastern states (Barker *et al*, 1999). It is accepted that the genus *Grevillea* is paraphyletic with respect to *Hakea*, being expected that *Hakea* will soon be transferred into *Grevillea* (Briggs, 1998).

Hakea sericea (needlebush or silky hakea), formerly known as *H. acicularis* or *H. tenuifolia*, is native of the dry sclerophyll forests and heaths on sandstone soils and shales of the Australian states of New South Wales, Victoria and Tasmania. It can also be found naturalized elsewhere in Australia, southern Europe, South Africa and New Zealand (Weber, 2003).

H. sericea grows into erect, branched, evergreen woody shrubs that can reach up to 5 m high and 3 m across (Fig 1.3.A). It presents xeromorphic adaptations in the morphology and anatomy of mature leaves (Fig 1.3.B). The acicular rigid leaves (terete), 2-7 cm long with diameter of 1 mm, are protected by a thick cuticle, ranging between 12-18 µm in young leaves and 20-30 µm in older leaves (Teixeira *et al*., 2008). Leaves are amphistomatous and present sunken stomata below the epidermal surface. Mesophyll is characterized by a dual layer palisade with increased thickness (Groom *et al*., 1997). Osteosclereids, bone shaped sclereids with columnar middles and small enlargements at both ends are inserted between the mesophyll cells. Their presence is required for support, since turgor pressure cannot be maintained at high levels within the tissues. The leaf core contains vascular bundles randomly scattered and a well-developed sclenchymatous bundle sheath surrounding each vein.

Hermaphrodite white or pink flowers occur in axillary clusters (Fig 1.3.C) from winter to early spring – June to September in the Southern hemisphere - and fruit development usually begins in October. As in all *Hakea* species, fruits consist of persistent non-fleshy (woody) follicles comprising two dehiscent valves, each valve containing one black, compressed, winged seed (Fig 1.3.D). *H. sericea* seeds contain considerable amounts of phosphorus, most in the form

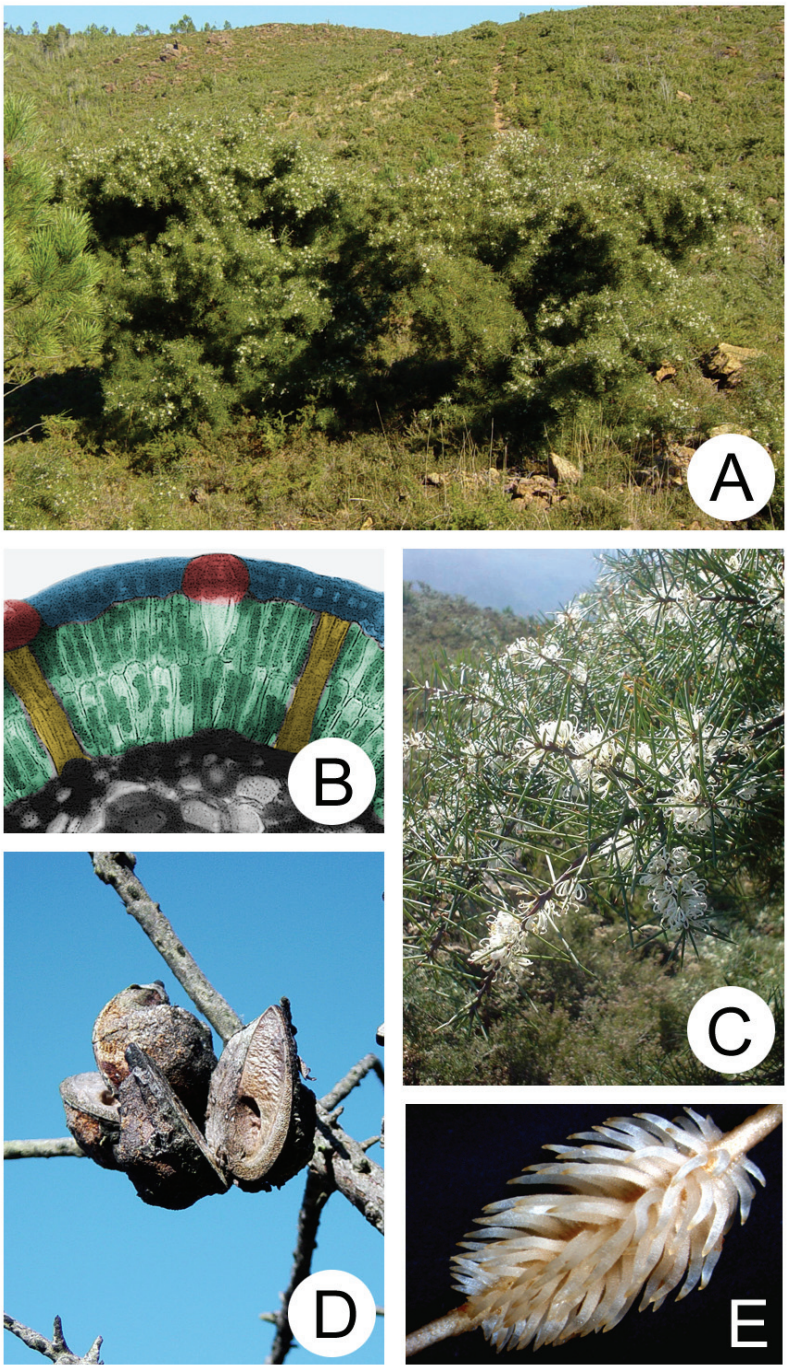

Figure 1.3 Morphological aspects of *Hakea sericea*.

(A) Adult bushes, which can reach up to 5 m high and 3 m across. (B) Transversal cross-section of acicular leaves (1 mm diameter) after being coloured to highlight the xeromorphic adaptations: epidermis with thick cuticle (in blue), sunken stomata below the epidermal surface (in red), osteosclereids (in yellow) and two layers of palisade mesophyll cells (in green). (C) White flowers occurring in axillary clusters. (D) Fruits consist of persistent woody follicles exhibiting two dehiscent valves that open when the plant dies. (E) Cluster of rootlets of a young proteoid root.

of potassium, sodium, calcium salts and ferric phytate (Mitchell and Allsopp, 1984). Ranging from 2-3 cm in length (from the point of the seed to the end of the wing), seeds are produced annually, stored in the canopy and only released after fire or when the plant dies (Bradstock, 1991). While most seeds fall near the parent forming dense stands, some are dispersed primarily by wind and also by surface water flow (Whelan, 1986). They germinate rapidly following release and new viable seeds are produced after 2 years (Dyer and Richardson, 1992). Besides seeds, *H. sericea* may also regenerate from rootstocks after fire exposure.

H. sericea, as most Proteaceae, is able to develop proteoid roots in response to mineral scarcity (Fig 1.3.E). This adaptive advantage grants *H. sericea* the ability to propagate in poor-nutrient soils, where survival for most plant species seems difficult. Due to its great importance in the establishment of *H. sericea* stands outside its natural range, this feature will be dealt in more detail in subsequent sections.

1.2 THE ROLE OF PROTEOID ROOTS IN PHOSPHORUS NUTRITION

1.2.1 Phosphorus importance and availability

Phosphorus (P) is one of the 17 essential elements required for plant growth (Bieleski, 1973; reviewed by Raghothama, 1999), making up 0.05 to 0.50% of the plant dry weight (Schachtman *et al*, 1998; Vance *et al*., 2003). This element is a component of many key molecules such as nucleic acids, phospholipids, and ATP. It also plays an important role in an array of major biochemical processes that includes energy generation, photosynthesis, glycolysis, respiration, membrane synthesis, enzyme activation/inactivation, redox reactions and nitrogen or sulphur assimilation (Theodorou and Plaxton, 1993; reviewed by Vance *et al*., 2003). Moreover, phosphorylation/dephosphorylation of proteins is crucial for signal-transduction pathways in plants, and phosphate homeostasis in chloroplasts regulates the transport of phosphorylated sugars

across the membrane and the synthesis of starch (Raghothama and Karthikeyan, 2005).

Although phosphorus is abundant in the lithosphere, its physicochemical properties and soil chemistry make it, after N, the most frequent limiting macronutrient for plant growth (Schachtman *et al*, 1998; Ticconi and Abel, 2004). In fact, soil concentrations of soluble P are often up to a 1000-fold lower than those of other required ions (Vance *et al*., 2003). The level of available P in soil solution is mainly regulated by its interaction with organic or inorganic surfaces in the soil. Organic P (Po) represents 20 to 80% of total P in the soil, of which phytic acid is usually a major component (Richardson, 1994). The remainder P in soil is found in the inorganic fraction, which contains about 170 mineral forms of P (Holford, 1997). A large portion of inorganic phosphate (Pi) is however unavailable to plants. Insoluble complexes with cations, such as aluminum and iron predominate in acidic soils, while calcium complexes are usually found in alkaline soil types. Regarding Po, it has to be mineralized to the inorganic form before it becomes available to plants. Many soil microorganisms release these immobile forms of P to the soil solution, but at the same time they compete with plants to obtain the newly available P (Raghothama, 1999).

The form of P most readily accessed by plants is phosphate. If in fertile soils phosphate availability can reach up to 10 µM, in many soils Pi seldom exceeds 1 µM (Bieleski, 1973; Schachtman *et al*., 1998). Phosphorus is mainly moved by diffusion contrarily to other soluble minerals such as K, which also moves through the soil via bulk flow into the sites where uptake by root cells occurs. The relative slow rate of Pi diffusion (10^{-12} to 10^{-15} m^2 s^{-1}) turns the rhizosphere depleted of Pi. In order to survive, plants developed strategies for improving phosphate uptake (1) aiming the reduction of Pi depletion zone, while at the same time (2) increasing the concentration of soluble phosphate at the sites of phosphate uptake in roots (Smith, 2002).

1.2.2 Proteoid root geometry and morphology

The low availability of Pi in many soils forced plants to evolve mechanisms to scavenge for this nutrient. Plant root geometry and morphology directly affect

the efficiency of Pi uptake, since root systems with higher ratios of surface area can explore a larger volume of soil (Lynch, 1995). Following this principle, some species have developed proteoid roots, a type of root system specialized in nutrient acquisition, particularly phosphorus.

Proteoid roots were first observed on *Banksia* spp. growing in the botanical gardens in Leipzig, Germany, and were described as roots *very much branched* (Engler, 1894). Nowadays, it is known that proteoid roots are almost ubiquitous amongst Proteaceae [from which the term *proteoid* comes from (Purnell, 1960)], and can also be found in some members of Betulaceae (Hurd and Schwintzer, 1996), Casuarinaceae (Zaïd *et al*., 2003), Curcurbitaceae (Waters and Blevins, 2000), Eleagnaceae (Skene, 1998), Fabaceae (Gardner *et al*., 1982), Moraceae (Rosenfield *et al*., 1991) and Myricaceae (Hurd and Schwintzer, 1997). Proteoid roots, also known as cluster roots, can be defined as roots that develop one or more discrete clusters of closely spaced rootlets along its length (Watt and Evans, 1999). The rootlets are covered with root hairs and arise in contiguous rows, opposite to every protoxylem pole. Consequently, species with more protoxylem poles present a greater rootlet density (Lambers *et al*., 2003). In *Lupinus albus* (Fabaceae) cluster roots usually display 1 to 4 rootlets mm^{-1} root axis (Johnson *et al*., 1994), while in *Hakea prostrate* they are composed by 100 rootlets mm^{-1} root axis (Lamont, 1972). Proteoid roots can be divided in two types: *simple*, as in *Hakea* and *Lupinus* and *compound* as in *Banksia* and *Dryandra* species (Shane and Lambers, 2005). While simple cluster roots display bottlebrush-like morphology, compound cluster roots are an assemblage of many simple cluster roots, exhibiting a Christmas-tree-like morphology.

The proliferation of root clusters greatly increases the root surface area in contact with the soil, thus enhancing nutrient uptake. In *Hakea obliqua*, the surface area of a mature proteoid root cluster is increased 25-fold taking into account the equivalent mass of axial root (Dell *et al*., 1980). In *Leucadendron laureolum* the soil volume explored within the boundaries of these rootlets is 288-fold higher than the equivalent length of non-proteoid root (Lamont, 1983). During growing season, root clusters constantly explore new soil regions (Lamont, 2003), thus minimising the path length for Pi and NH_4, nutrients whose

uptake is only dependent on diffusion (Voorster and Jooste, 1986). It also maximises the soil-root water potential gradient pathways for nutrients such as Ca, Mg, S and NO_3, whose uptake is largely controlled by bulk flow (Gould, 1998).

1.2.3 Proteoid root exudates

Coupled with increased ratios of surface area to volume, proteoid roots are also able to chemically modify the surrounding soil (Neumann and Martinoia, 2002). According to Grierson and Attiwill (1989), the proteoid root mat of *Banksia integrifolia* enhances the uptake of nutrients by promoting the chemical modification of soil environment. By exuding carboxylate organic anions, acid phosphatases, phenolics, mucilages and water, many soil nutrients that were previously inaccessible to the plant become available for uptake (Fig 1.4) (Lambers *et al*., 2006).

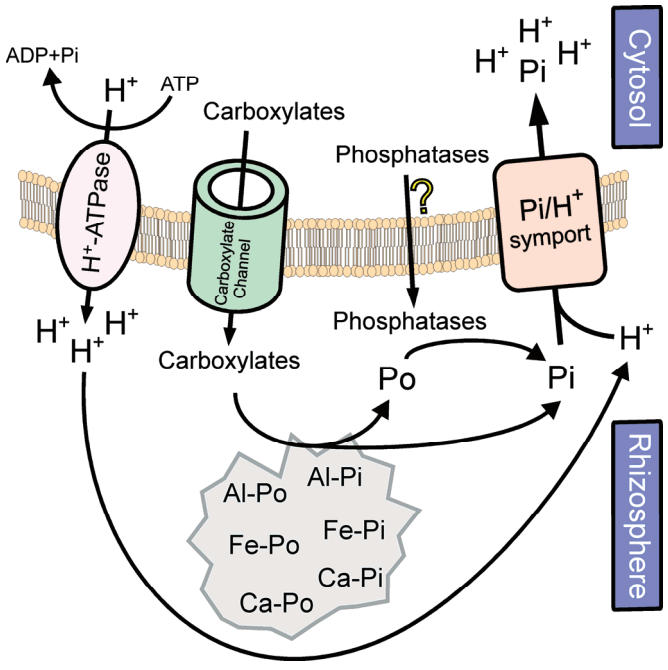

Figure 1.4 Model for root-induced inorganic phosphate (Pi) and organic phosphate (Po) mobilization in the rhizosphere by exudation of carboxylates, protons and acid phosphatases.

Carboxylates may be released via anion channel, but the mechanism used for phosphatases release is unknown. Carboxylates mobilize both Pi and Po, while phosphatases hydrolyze Po compounds into Pi. Protons are released in the rhizosphere through H^+-ATPase pumps, at the expense of ATP. Available Pi is transported from the rhizosphere to the cytoplasm through Pi transporters in symport with protons (adapted from Lambers *et al*., 2006).

Many plant species export organic anions into the soil in response to P privation, since organic anions can mobilize P by chelating soil minerals that are bound to this nutrient (Shane and Lambers, 2005). The carboxylates export rates from roots of proteoid species are usually several times higher than those described for non-proteoid species (Jones, 1998). In fact, proteoid species are able to export amounts of carbon that range from 11% (Gardner *et al*., 1983) to greater than 23% (Dinkelaker *et al*., 1989) of the total plant dry weight. The organic anions pattern in root exudates varies among species. The release of citrate, as well as small amounts of malate and succinate, by proteoid roots of *Lupinus albus* mobilizes precipitates of Pi, both in acidic (Gardner *et al*., 1982) and calcareous soils (Dinkelaker *et al*., 1989). Conversely, exudates of *Hakea undulata* are mostly composed of malate and fumarate, while citrate is poorly represented (Dinkelaker *et al*., 1997). Interestingly, plants seem to perceive the chemistry of the soil where they grow. For example, the production of proteoid roots in seedlings of both *Telopea speciosissima* and *Banksia ericifolia* was found to be directly dependent on the level of phosphate supplied (Grose, 1989; Handreck, 1991). Also, the pattern of carboxylate exudation in *Banksia grandis* is affected by the form of phosphate added to the soil (Lambers *et al*., 2002). Tri- and dicarboxylates (citrate, 60%; malate, 25%; trans-aconitate, 14%) are the major organic anions in root exudates when P is supplied as Al-phosphate. In contrast, when P is supplied as Fe-phosphate, besides the same tri- and dicarboxylates (31, 14 and 12%, respectively), plants also release monocarboxylates (lactate, 30%; acetate, 12%).

Before becoming available to plants, soil Po must be first hydrolyzed to Pi (George *et al*., 2002). Acid phosphatases can hydrolyse a wide range of Po compounds (Tarafdar and Claassen, 2001), including phosphate mono- and di-esters, both common Po forms in soils (Sumann *et al*., 1998). The release of acid phosphatases to the rhizosphere in response to P low availability is a universal response by plants (Duff *et al*., 1994). In accordance, proteoid-forming species such as *Lupinus albus* (Gilbert *et al*., 1999; Wasaki *et al*., 2003) and *Hakea undulata* (Dinkelaker *et al*., 1997) can exude large amounts of acid phosphatases to the rhizosphere in response to P scarcity, during several hours

or even days (Miller *et al*., 2001). In *Lupinus albus*, a novel acid phosphatase is specifically induced as a response to low internal P concentration (Gilbert *et al*., 1999; Miller *et al*., 2001).

The efflux of large amounts of carboxylates is linked to a modified organic anion metabolism, in which both enhancement and reduction of the specific activity of several key enzymes occur (Neumann and Martinoia, 2002). The carbon required for exudation is derived from both photosynthetic (65%) and dark CO_2 (35%) fixation (Johnson *et al*., 1994). Dark CO_2 fixation occurring in roots is mediated by several enzymes including carbamoyl phosphate synthase (EC 6.3.4.16) (McClure *et al*., 1983) and phosphoribosylaminoimidazole (EC 4.1.1.21) (Boland and Schubert, 1982). The anaplerotic pathway seems to contribute mostly to this phenomenon, in which phosphoenolpyruvate carboxylase (EC 4.1.1.31; PEPC) catalyzes the carboxylation of phosphoenolpyruvate (PEP) to form oxaloacetate (Vance *et al*., 1983; Johnson *et al*., 1994). Studies in *Lupinus albus* (Uhde-Stone *et al*., 2003), *Brassica napus* (Moraes and Plaxton, 2000) and *Sesbania rostrata* (Aono *et al*., 2001) have shown that enhanced CO_2 fixation in roots is coupled with an increased specific activity of PEPC, as well as malate dehydrogenase (EC 1.1.1.37; MDH), and citrate synthase (EC 2.3.3.1; CS), which are responsible for the synthesis of carboxylate intermediates in the tricarboxylic acid cycle (TCA) (Fig 1.5). Moreover, reduced degradation or utilization of citrate is also involved in carboxylates exudation. In cluster roots of *Lupinus albus* the activity of aconitase (EC 4.2.1.3; Aco), an enzyme that catalyzes the conversion of citrate to isocitrate, is reduced (Neumann *et al*., 1999; Kihara *et al*., 2003), thus resulting in more citrate available for exudation (Fig 1.5). The reduced activity of Aco also leads to lower respiration rates, which are an indirect measure of flux through the TCA cycle (Johnson *et al*., 1994; Neumann *et al*., 1999). Despite the large quantities of organic acids produced and exuded by proteoid roots, the mechanism by which exudation actually occurs is not yet fully understood. Efflux of carboxylates may be mediated by anion channels such as chloride channels and MATE (Multidrug And Toxin Extrusion) (Johnson *et al*., 1996;

Vance *et al*., 2003). Alternatively, carboxylates could be packaged in vesicles and released into the rhizosphere by exocytosis (Watt and Evans, 1999).

The export of organic acid anions coincides with a marked acidification of the rhizosphere due to the release of protons via plasma membrane ATPase (Fig 1.4) (Dinkelaker *et al*., 1995; Neumann and Martinoia, 2002). This phenomenon is mainly attributed to the fact that Pi can only be acquired by roots through an energy-mediated co-transport process driven by protons (Ullrich-Eberius *et al*., 1984; Sakano *et al*., 1992). However, the extrusion of protons along with carboxylates into the rhizosphere may also be triggered to prevent the inhibiting effect of cytosolic acidification (Sakano, 2001). Plant glycolysis only occurs when the cytoplasm is sufficiently alkaline to stimulate PEPC activity. Since the synthesis of organic anions generates an excess of protons, their extrusion would prevent glycolysis inhibition, ultimately resulting in the production of more organic anions. The resulting acidification of the rhizosphere may also enhance the hydrolysis of organic phosphate by acid phosphatases (Braum and Helmke, 1995).

Other soil chemical modifications promoted by proteoid roots include the production of anionic mucopolysaccharides and phenolics by root cap cells that act in the same way as carboxylates, although to a much lesser extent (Dell *et al*., 1980; Neumann and Römheld, 2001; Guppy *et al*., 2005). Exudation of these compounds is also enhanced under P deficiency (Grimal *et al*., 2001; Neumann and Römheld, 2001; Juszczuk *et al*., 2004), contributing for the enhanced Pi availability in soil. Furthermore, the distance that nutrients must diffuse to reach the root surface is reduced by mucilage, since it contributes to the binding of soil particles, thus improving the soil root contact area (Watt *et al*., 1994). Although phenolics can also contribute to P mobilization, their ecophysiological role seems to be the prevention of microbial breakdown of exuded carboxylates, since phenolics slow down fungal metabolism by stimulating sporulation (Neumann and Römheld, 2001; Weisskopf *et al*., 2006). Examples of phenolics that are exuded into the rhizosphere are piscidic acid from roots of *Cajanus cajan* (Ae *et al*., 1990), alfafuran from *Medicago sativa*

roots (Masaoka *et al*., 1993), and isoflavonoids from cluster roots of white lupin (Neumann *et al*., 2000).

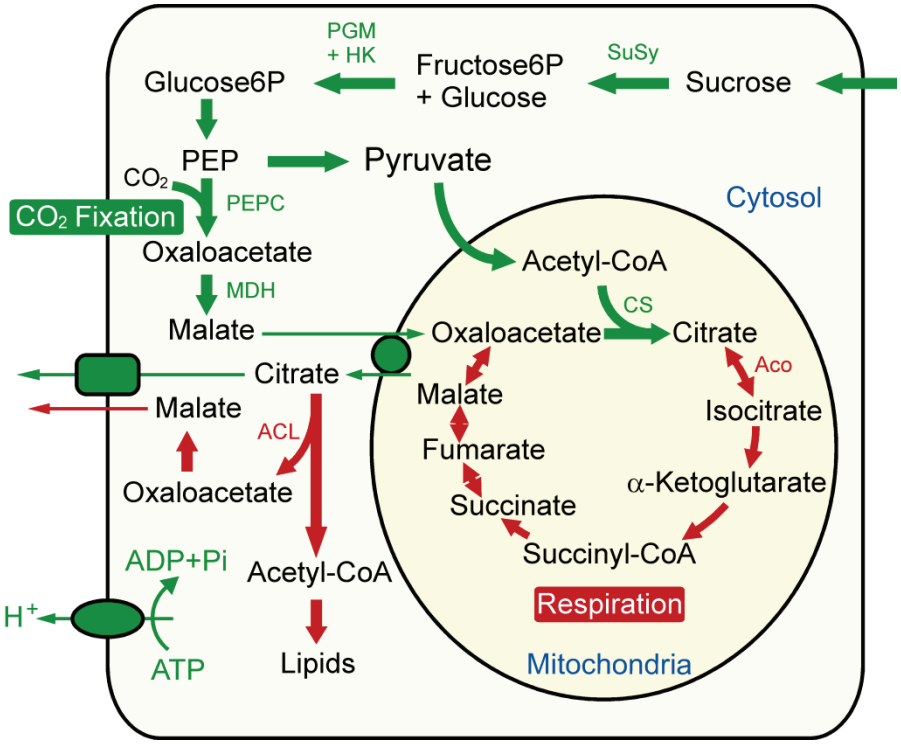

Figure 1.5 Model for modified anion metabolism leading to the carboxylates efflux in cluster roots of *Lupinus albus*.

The stimulated metabolic reactions and sequences are depicted in green and those inhibited are depicted in red. Organic acid extrusion requires additional carbon. CO_2 fixation is achieved by the increase of PEPC activity. The enhancement of MDH and CS activities coupled with a decrease on Aco and ACL activities lead to an accumulation of citrate. Citrate is exported along with protons to the rhizosphere, enhancing Pi availability. Abbreviations: ACL, ATP-citrate lyase; Aco, aconitase; CS, citrate synthase; HK, hexokinase; MDH, malate dehydrogenase; PEP, phosphoenol pyruvate; PEPC, phosphoenolpyruvate carboxylase; PGM, phosphoglucomutase; SuSy, sucrose synthase (adapted from Neumann and Martinoia, 2002).

Many plant species release a wide range of compounds as a response to nutrient scarcity, but only proteoid-forming species seem to achieve it through exudate bursts (Lambers *et al*., 2006). Young cluster roots only release small amounts or even no material into the rhizosphere. However, when rootlets reach maturity they export large quantities of carboxylates for a short period of 2-3 days, followed by an almost absence of exudation. After uptaking the newly available Pi, proteoid rootlets become unnecessary and become senescent (Dinkelaker *et al.,* 1995). This process is continuously repeated and can be

explained by two main reasons: (1) since P has a low diffusion coefficient, a rapid and concentrated burst in newly explored soil areas would benefit more the plant than a long-term, continuous but lower level of exudation, and (2) it may prevent soil bacteria from completely metabolizing the exudates before they enhanced P uptake (Skene, 1998).

When a plant has some roots in contact with moist soils and others with dry soil, water may flow from moist to dry patches (Lambers *et al*., 2006). Plants can deliberately release water into superficial dry soil horizons to enhance the mobility of Pi mobilized by other exudates, in a process named hydraulic redistribution (Burgess *et al*., 1998, 2000). Furthermore, the hydration of the proteoid rhizosphere also seems to increase the longevity of rootlets in a dry environment (Pate and Dawson, 1999).

1.2.4 Association between proteoid roots and microorganisms

More than 90% of land plants form symbiotic associations with mycorrhizal fungi. While plants provide organic C compounds to the fungi, the mycelium is responsible for increasing the soil volume explored by the roots, playing an important role in plant mineral nutrition (Bolan, 1991; Smith and Read, 1997). Within the same plant species, uptake of P in roots colonized by mycorrhizal fungi can be several times higher than in nonmycorrhizal roots (Smith and Read, 1997). Both mycorrhizas and cluster roots seem to have similar functions, increasing the area for absorption of water and inorganic nutrients from the soil (Malajczuk and Bowen, 1974; Lamont, 1982, 1984). Although fungi may be found in association with roots from Proteaceae species, no symbiotic mycorrhizas appear to be formed (Reddell *et al*., 1997).

Bacteria may also be found associated with roots from Proteaceae. The possible enhancement of nutrient uptake resulting from this interaction has been studied by Wenzel *et al.,* (1994). Bacteria isolated from proteoid and non-proteoid roots of *Telopea speciosissima* enhanced the solubilisation of calcium phosphates in culture. However, the ecophysiological importance of this phenomenon is still unknown, since it was not shown that bacteria render insoluble soil phosphates available to plant uptake.

The role of microorganisms in cluster root initiation has also been studied. Lamont and McComb (1974) showed that formation of cluster roots in *Hakea prostrata* growing in autoclaved sand was only achieved after the addition of non-autoclaved soil extract. Malajczuk and Bowen (1974) also demonstrated that *Banksia grandis* grown in two γ-irradiated soil types only produced proteoid roots after inoculation with a proteoid root extract. At present, proteoid root formation is suggested to be triggered by auxins released by free-living rhizosphere bacteria (Glick *et al*., 1994). The application of exogenous synthetic auxins to *Lupinus albus* induced proteoid root formation, even when plants were grown with high P supply (Gilbert *et al*., 2000; Skene and James, 2000). In contrast, inhibitors of auxin transport suppressed cluster root growth in P-deficient *Lupinus albus*. The soil layers rich in decomposing organic matter and presenting an active nutrient release usually have higher bacteria content. Being induced by bacteria, proteoid roots would be preferentially developed in soil portions where nutrient concentration is potentially higher. Thus, the exploration of soil regions completely depleted from Pi will be limited even for proteoid-forming species.

1.3 *HAKEA SERICEA* AS AN INVADER OF NATURAL HABITATS

1.3.1 Biotic invasions

According to Elton (1958), a biotic invader can be defined as an organism that, after arriving to a new habitat, proliferates, spreads and persists over time. Biotic invasions can occur naturally, but in the last 500 years their frequency and number of species involved have dramatically increased, as a result of human activity, such as migrations and commerce (Wells *et al*., 1986; di Castri, 1989). As a consequence, at the present it is difficult to find habitats on earth free of species introduced by humans (Mack *et al*., 2000). Fortunately, not every species introduced in a new habitat become invaders. Most are quickly eliminated by physical and biotic agents present at the arriving site (Kruger *et al*., 1986; Mack, 1995). Some survive and reproduce, but their descendants proliferate only for a few generations before becoming extinct. Consequently,

only a very small number of species eventually persist and adapt to the new habitat, becoming naturalized. Finally, from this group of survivalists, only a few will become invaders and even those may take decades before becoming abundant and widespread. One example is the Brazilian pepper (*Schinus terebinthifolius*), a bush that grows in dense stands excluding the growth of any other vegetation. Although introduced to Florida in the 19th century, only in the early 1960s it became invasive (Schmitz *et al*., 1997). Different theories have been proposed to explain this lag phase: (1) some populations are so small and isolated that even in exponential growth phase they may not be detected - this could be wrongly perceived as a lag phase (Crooks and Soule, 1996); (2) genetic variation along generations may favour new genotypes allowing species to better cope with environmental factors in the new habitat – the lag phase would reflect the period needed for emergence of these new genotypes (Baker, 1974; Crooks and Soule, 1996) and finally, (3) environmental conditions may alter during time, triggering the spreading of naturalized species (Simberloff, 1988).

Concerning the threat to global biodiversity, the alteration of the earth biota caused by invasive species is the second most important factor following the direct destruction of habitat (Walker and Steffen, 1997). The ecological alterations include changing the role of autochthon species in communities, disrupting evolutionary processes, and causing changes in relative abundances, ultimately leading to extinctions (Cronk and Fuller, 1995; Rhymer and Simberloff, 1996). In many cases, the consequence of a biotic invasion is the complete replacement of native species by the invader. Examples are found in northern Australia, where 80.000 ha of heterogeneous tropical wetland habitat were transformed into a uniform *Mimosa pigra* shrubland (Braithwaite *et al*., 1989). Also, in Florida, USA, the Australian paperbark tree (*Melaleuca quinquenervia*) has claimed 160.000 ha, replacing cypress and sawgrass amongst other native species. In Cape Province, South Africa, eucalypts, pines, *Acacia*, and *Hakea* spp. have threatened the survival of many endemic species by removing enormous amounts of water from soil (van Wilgen *et al*., 1996).

Invasive plants have developed diverse features that confer them adaptive advantages over natives. Many limit the light, water or minerals available to the native plants; some release phytotoxic agents into the soil; while others are just able to grow faster or stronger following a natural disaster. For example, *Carpobrotus edulis*, a succulent mat-former introduced to California, usurps the water in the soil, making it unavailable for native species (D'Antonio and Mahall, 1991).

When plant invasions are detected early, it is possible to eradicate them (Simberloff, 1997). In many cases, however, early eradication is not followed because regulatory agencies feel that control is neither worth the trouble nor the expense. Often, agencies tend to act only when the alien species becomes completely widespread and invasive. Unfortunately, in most cases, once the invasion is fully established, it is extremely difficult to control (Simberloff, 1997).

1.3.2 Hakea sericea *as a biotic invader*

Native from south-eastern Australia, *Hakea sericea* was introduced to South Africa during the 1930s as a hedge plant (Neser and Fugler, 1978). However, its copious seed production and serotiny, associated with high seed longevity in the canopy and efficient seed dispersal, allowed *H. sericea* to rapidly proliferate and disperse throughout the new habitat, invading thousands of hectares of fynbos in South Africa (Richardson *et al.,* 1989). Moreover, the woody follicles produced by *H. sericea* only release the trapped winged seeds after fires or upon the death of plants (Richardson and van Wilgen, 1986; Bradstock, 1991). Since the fynbos ecosystem is fire-prone, meaning that the fynbos vegetation relies on periodic fires for its own regeneration, the invasion by *H. sericea* has been strongly intensified (van Wilgen, 1981; van Wilgen and Richardson, 1985). The presence of *H. sericea* by itself promotes fires, since it increases fuel loads, while at the same time decreases herbaceous fuel moisture (Versfeld and van Wilgen, 1986). By forming dense and impenetrable stands of shrubs that put in jeopardy the autochthon fynbos vegetation, *H. sericea* shortly became a major problem in most costal mountain ranges and catchments of the Western Cape Province (Richardson *et al*., 1989; Gordon, 1999).

In 1970, a biological control programme against *H. sericea* focusing the use of insects was initiated (Neser and Annecke, 1973; Gordon, 1999). The programme consisted in the introduction of non-autochthon species such as *Carposina autologa*, a moth whose larvae feed on *H. sericea* seeds, and *Erytenna consputa*, a weevil that destroys the developing fruits of *H. sericea*. Mechanical methods (felling and fire) were also tested and cleared large invaded areas. However, problems occurred with high intensity burning due to excessive fuel loads, which led to damage of the soil, seedbanks and soil microbial populations (Cilliers *et al.,* 2004). At present, control of *H. sericea* in South Africa is still dependant on seed-feeding insects, although their impact is severely constrained by numerous factors, notably fires.

H. sericea was also introduced to Portugal as a hedge plant to form fence lines or windbreaks, due to its dense and impenetrable cover. It is currently considered by regulatory agencies one of the most invasive plant species established in Portuguese territory (Diário da República, Decreto-Lei nº 565/99, 21 de Dezembro). The same morphological and physiological traits that made it invasive in South Africa allowed *H. sericea* to spread in Portugal, mostly throughout the north-west region (Fig 1.6).

Figure 1.6 Proliferation and spreading of *H. sericea* standings throughout "Serra de Arga" in northern Portugal.

Until now, no large scale attempts have been made to control *H. sericea* spreading, although mechanical control methods have been tested with relative success in the military field of Stª Margarida (south of Tagus river, Santarém district) (Espírito-Santo and Arsénio, 1999).

The aim of the present study is (1) to identify a pathogenic agent able to infect *H. sericea*, which could be used as a biological control agent of the invader, as well as (2) to better understand the ecophysiological features underlying the ability of *H. sericea* to invade new territories, specifically, its capability to survive in poor nutrient soils.

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CHAPTER 2

FIRST REPORT OF HAKEA SERICEA *LEAF INFECTION CAUSED BY* PESTALOTIOPSIS FUNEREA *IN PORTUGAL*

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ABSTRACT

Hakea sericea (Proteaceae) is native to south-eastern Australia and has been considered as an invader of natural habitats. In northern Portugal, dense stands are rapidly spreading after forest fire. Unusual leaf spots were observed on naturally growing plants. Infected plants exhibited reddish leaves bearing black, 1-3 mm circular lesions. Leaf sections containing necrotic lesions were plated onto potato dextrose agar (PDA) and eight fungal isolates were obtained. Pure cultures exhibited pinkish mycelium, bearing compact acervuli that contained black slimy spore masses. Microscopic observation revealed typical *Pestalotiopsis* sp. 5-celled spores (3 coloured median and 2 hyaline end cells) with 3-4 apical and 1 basal appendages. Genomic DNA from fungi isolates was purified and used in thermocyclic amplifications with *ITS5* and *ITS4* internal transcribed spacer (ITS) universal primers. The amplified sequences from all fungal isolates were identical to each other and only four base pair different (99.3% similarity) from *Pestalotiopsis funerea*. To confirm the pathogenicity of *P. funerea* on *H. sericea*, leaf-wounded and non-wounded 6 week-old *in vitro* plants were sprayed with a suspension containing 10^5 spores per mL or with sterile water. After 6 days, only leaf-wounded plants infected by *P. funerea* exhibited lesions identical to those observed in field plants, bearing fungus spores identical to those from original isolates. Members of *Pestalotiopsis* genus have been described as pathogenic for Proteaceae species. In South Africa where *H. sericea* invasion has become a problem, its biological control has been successfully achieved using *Colletotrichum gloeosporioides*. The identification of naturally occurring pathogenic fungi in portuguese *H. sericea* could allow the design of an integrated control strategy for this invader.

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2.1 INTRODUCTION

Biotic invaders can be defined as species that outside their natural range are still able to proliferate, spread, and persist to the detriment of the environment (Mack *et al*., 2000). Invasive exotic species threaten native biodiversity (Wilcove *et al*., 1998) and alter fundamental ecological properties. They can change the abundance and distribution of species in a community and modify ecosystem physical features, such as nutrient cycling and plant productivity (Bertness, 1984; Vitousek, 1990). Moreover, invasive species have been identified as the second greatest threat to biodiversity after habitat loss (Mack *et al*., 2000) and annually cost up to $120 billion in the United States alone (Pimentel *et al*., 2005).

Control of invasive exotic species is usually achieved through three main approaches applied singly or in combination: chemical, mechanical, and biological control. Introduced about 1895 (Hansen, 1921), chemical control probably remains the chief tool in combating invasive species (Mack *et al*., 2000). Herbicides usually inhibit the growth and establishment of plants by interfering with various physiological and biochemical pathways (Inderjit, 2004). Chemical control, however, has been too often related with health issues for humans and non-target species (Acquavella *et al*., 1999). Moreover, if the goal is to control an invasive species in a vast natural area, the cost of chemical methods alone can be prohibitive. Finally, the frequent evolution of plant resistance and the necessity of repeated applications often make continued chemical control impossible (Mack *et al*., 2000). Mechanical methods for controlling invasive species have shown to be quite effective (Roberts *et al*., 1996; Alemán, 2001) and encompass an array of tools ranging from harrows to brushes and hoes to disrupt weed growth (Tillett, 1999). However, equipment expenses, rough landscapes, and the geographic scale of some weed infestations frequently render mechanical control impossible (Mack *et al*., 2000). The perception of high economic, health and environmental costs of both chemical and mechanical weed control has stimulated interest in biological

control (Louda *et al*., 1997; Zhou *et al*., 2005). This type of control is seen as an especially attractive option for large natural areas, such as parks, reserves, national forests, and open rangelands (Center *et al*., 1995). Biological control methods include crop competition, allelopathy, and more frequently, the introduction of specific insect predators, herbivores and plant pathogens (Inderjit, 2004). Some biological control projects have succeeded in restraining very widespread, damaging infestations at acceptable levels with minimal costs (Westbrooks, 1998; Britton *et al*., 2002).

Hakea sericea Schrad. (Proteaceae) is native of south-eastern Australia and has been considered as an invader of natural habitats. It grows into large woody and dense shrubs, with 6 cm-long rigid leaves. These dense stands of *H. sericea* transform the natural landscape, thus modifying many ecosystem processes and reducing the diversity of indigenous species (Richardson *et al*., 1989). In Portugal, it was first introduced as a hedge plant to form fence lines or windbreaks, because of its dense and impenetrable cover, but became an ecological problem due to its high adaptability to climatic conditions and capacity to survive to frequent forest fires. Actually, aspects of *H. sericea* reproduction and regeneration turn this species as one of the most dangerous and aggressive invader in Portugal. Plants are able to regenerate not only from seeds but also from rootstocks. Winged seeds are produced in large number and held in woody follicles until the parent plant dies. When parent shrubs are killed by fire, the release of the large accumulated seed store is strongly promoted. Most seeds fall near the parent forming dense stands, but some are dispersed by wind. Seeds germinate rapidly and viable seeds are produced after 2 years upon germination (Dyer and Richardson, 1992). Mechanical control methods have been tested with some success in the military field of Stª Margarida (south of Tagus river, Santarém district), being, to our knowledge, the only form of control practiced in Portugal (Espírito-Santo and Arsénio, 1999). Despite the promising results, this control method cannot be applied in the northern region of Portugal, where the landscape is much rougher. The aim of our work is to identify a pathogenic agent able to infect *H. sericea*, which could, in a nearby future, be used for the biological control of the invader.

2.2 MATERIAL AND METHODS

2.2.1 Plant material

Mature follicles (>2 years old) from *Hakea sericea* adult plants were collected in "Serra de Arga" mountains (430-450 m elevation), northern Portugal, centered at 8º 45' 06" W, and 41º 51' 20" N. After opening the follicles at 125ºC for 1 h, the recovered seeds were water-embedded overnight. Seed surface was disinfected by 35% (v/v) H_2O_2 immersion for 15 min, followed by thorough rinsing using sterile water. To break dormancy, seeds were placed for one week at 4ºC in the dark. Sterile *H. sericea* seedlings were obtained by germinating seeds in MS medium (0.185 $g.L^{-1}$ $MgSO_4.7H_2O$, 0.22 $g.L^{-1}$ $CaCl_2.2H_2O$, 0.95 $g.L^{-1}$ KNO_3, 0.825 $g.L^{-1}$ NH_4NO_3, 0.085 $g.L^{-1}$ KH_2PO_4, 16.9 $mg.L^{-1}$ $MnSO_4.H_2O$, 0.83 $mg.L^{-1}$ KI, 0.025 $mg.L^{-1}$ $CoCl_2.6H_2O$, 8.6 $mg.L^{-1}$ $ZnSO_4.7H_2O$, 0.025 $mg.L^{-1}$ $CuSO_4.5H_2O$, 6.2 $mg.L^{-1}$ H_3BO_3, 0.25 $mg.L^{-1}$ $Na_2MoO_4.2H_2O$, 27.8 $mg.L^{-1}$ $FeSO_4.7H_2O$, 41.3 $mg.L^{-1}$ $Na_2EDTA.2H_2O$, 20 $g.L^{-1}$ sucrose and 7 $g.L^{-1}$ agar, pH 6.0), using a 16/8 h photoperiod under cool white fluorescent light (150 $\mu W.cm^{-2}$), at 25ºC in a growth chamber. To prevent dehydration of the medium, cotton corks were partially flamed and double capped with aluminum foil. Seedlings were allowed to develop for 6 weeks.

2.2.2 Fungal material

Fungal isolates were obtained from circular lesions on the mature leaves of diseased plants of *H. sericea* grown in "Serra de Arga". Leaf sections containing necrotic lesions were plated onto PDA and mycelium was allowed to grow in the dark at 25ºC, for 20 days. Following acervuli formation, spores were harvested with a sterile needle and plated onto new sterile plates of PDA. The process was systematically repeated (up to 6 times) until pure cultures were obtained. Spores were observed under a light microscope for genus identification.

2.2.3 Inoculation

Spore suspensions were obtained from 7-day-old Petri dish pure cultures by washing the mycelium with sterile deionised water. Spore concentrations were adjusted by dilution after counting with a haemocytometer. Leaves of *in vitro* grown 6-weeks-old plants, either intact or induced-wounded by a bisturi, were sprayed with 1 mL of inoculum containing 10^5 spores. Incubation proceeded as used for growing (16/8 h photoperiod, at 25ºC) for 6 days. Control plants (intact or induced-wounded) were treated with sterile deionised water.

2.2.4 Extraction of fungal DNA

Fungal mycelium was obtained from 200 mL cultures (0.4 $g.L^{-1}$ sucrose, 0.04 $g.L^{-1}$ yeast extract, 0.04 $g.L^{-1}$ peptone, 0.1 $g.L^{-1}$ $MgSO_4$, 0.108 $g.L^{-1}$ KH_2PO_4) (Mathur *et al*., 1950), incubated three days at 28ºC, with shaking (140 rpm). After collecting on Scott paper, the mycelium was ground to a fine powder in liquid N_2 and stored at -20ºC. Fungal gDNA was extracted using the *DNeasy® Plant Mini Kit* (Qiagen) according to the supplier's instructions. Briefly, after cell lysis and protein and polysaccharide precipitation, the lysate containing DNA was applied to a *QIAshredder™ Mini Spin Column* (Qiagen). DNA was washed using a kit provided washing buffer and 100 µL of Buffer AE (10 mM Tris-Cl, 0.5 mM EDTA pH 9.0) were used for eluting the DNA. After incubating the *DNeasy®* membrane for 5 min at 25ºC, DNA was recovered by centrifugation for 1 min at 6000 *g*.

2.2.5 Spectrophotometric quantification of DNA

Quantification of DNA was performed spectrophotometrically by measuring the A_{260} of sample solution on a *Cary 1E UV-Vis Spectrophotometer* (Varian). For estimation of DNA concentration, it was considered that 1 A_{260} = 50 $ng.\mu L^{-1}$ DNA. Purity was evaluated after determining the values of A_{230} and A_{280} (Sambrook *et al*., 1989).

2.2.6 rDNA amplification and sequencing

Thermocyclic amplification of the ITS and 5.8S rRNA subunit regions of the fungal rDNA was carried out using *ITS4* and *ITS5* universal primers (White *et al*., 1990). Polymerase chain reaction (PCR) was performed using the *PureTaq™ Ready-to-Go™ PCR Beads* (GE Healthcare), according to the supplier's instructions. For each PCR, 25 pmol of *ITS4*, 25 pmol of *ITS5* and 50 ng of fungal gDNA were used. Sterile high-quality water was added to each bead to a final volume of 25 µL. The final concentration of each dNTP was 200 µM in a buffer containing 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM $MgCl_2$. After gently flicking the tube with a finger, and bringing the components back to the bottom of the tube with a few seconds centrifugation, the beads were placed on a *Mastercycler Gradient* (Eppendorf). PCR steps were as follows: (1) denaturation for 5 min at 95°C; (2) 25 cycles of annealing for 30 s at 50.4ºC, 55.1ºC or 60.1ºC, polymerization for 1 min at 72ºC, denaturation for 30 s at 95ºC; (3) extension for 10 min at 72°C.

DNA fragments were resolved by electrophoretic separation using horizontal slab gel apparatus. According to the expected length of the amplified fragments, an agarose concentration of 1.2% (w/v) was used. Gel was made by melting agarose in 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA), also used as running buffer. DNA samples, including molecular weight marker (*1 kb DNA Ladder*, Invitrogen), were mixed with 0.25 vol. of loading buffer [glycerol 30% (w/v), 0.1 M EDTA and bromophenol blue 0.25% (w/v)] and 1 µL of 1 $mg.mL^{-1}$ EtBr. Electrophoresis was carried out at 80 V, until the bromophenol blue dye had reached two thirds the length of the gel. The agarose gel was visualized under long wave UV light (*Transilluminator 2020E*, Stratagene) and analyzed using the *Eagle Eye® II Still Video System* (Stratagene) through corresponding software (*EagleSight™* 3.2, Stratagene).

DNA fragments used for sequencing were directly purified from the *PureTaq™ Ready-to-Go™ PCR Beads* solution, after running a PCR using 60ºC as annealing temperature. For DNA purification, *GFX PCR DNA and Gel Band Purification Kit* (Amersham) was used. Briefly, the DNA solution was transferred

to a *GFX Column*, from which salt contaminants, primers and free nucleotides were washed away using a kit provided washing buffer. After applying 50 µL of sterile ultrapure water and incubating for 1 min, DNA was recovered by centrifugation for 1 min at 8000 *g*.

DNA fragments were sequenced in both directions using *ITS4* and *ITS5* primers by BigDye™ Terminator Chemistry (ABI Prism®; Stabvida sequencing services).

2.2.7 Sequence analysis

Nucleotide editing and analysis was performed using the sequence analysis software from *DNASTAR* (Lasergene). Within the software package, *EditSeq* was used to edit sequences and multiple sequence alignments were generated with *MegAlign*, using the ClustalW algorithm (Jeanmougin *et al*., 1998).

The unrooted phylogenetic tree, based on the alignment of fungal ITS sequences, was constructed using the *DrawTree,* PHYLIP software suit (Felsenstein, 1989). The maximum likelihood method (Doyle and Gaut, 2000) was used to resolve the phylogenetic relationship between the isolated fungus and the remaining selected species.

2.3 RESULTS

As referred to in the Introduction (section 2.1), the Proteaceae *Hakea sericea* has proven to be invasive in some regions outside its natural range. In Portugal, it was first introduced as a hedge plant because of its dense and impenetrable cover, but became an ecological problem due to its high adaptability to climatic conditions and capacity to survive to frequent forest fires. Hard and woody follicles accumulate for years and massively open upon plant dehydration, either when the plant dies or is burnt. Two large winged seeds are then released per follicle. As a consequence, vast areas of pine and oak forests have been replaced by *H. sericea*, like those occurring in "Serra de Arga" mountain, northern Portugal (Fig 2.1).

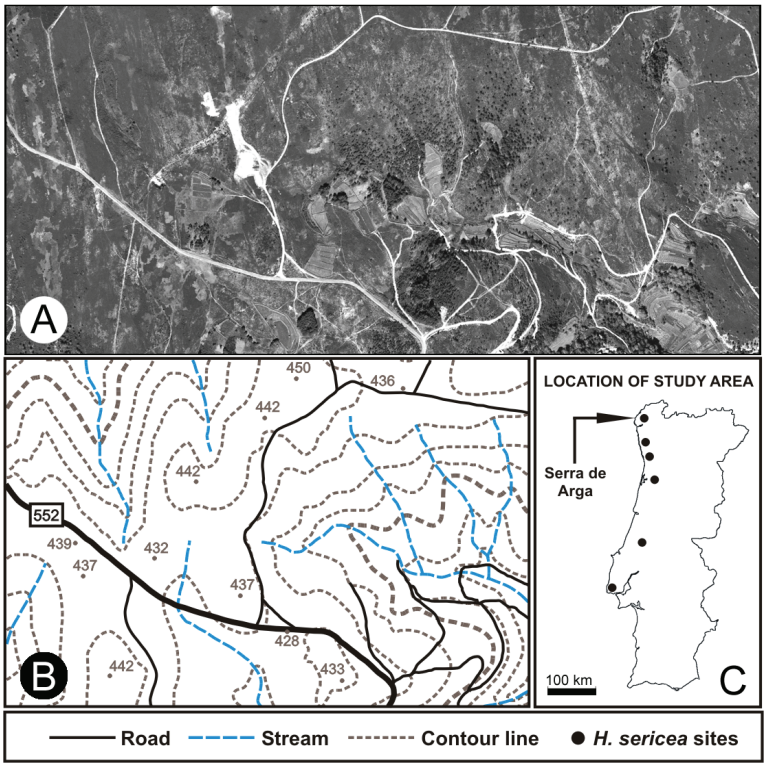

Figure 2.1 *Hakea sericea* harvesting region located in "Serra de Arga" mountain, northern Portugal, centred at 8º 45' 06" W, and 41º 51' 20" N.

The region is shown both as a digital orthophoto (A) and as a topographic map (B). Elevation is in meters. The distribution of *H. sericea* throughout continental Portugal is also displayed in the minimap (C).

When scouting the “Serra de Arga” area, it was noticed that some *H. sericea* specimens presented a slight reddish colour with chlorotic leaf spots. The apparently diseased specimens were harvested and observed under stereo microscopy in the lab (Fig 2.2). Unusual black 1-3 mm circular lesions were observed, suggesting leaf infection by some sort of pathogenic fungus. After plating leaf sections containing necrotic lesions onto PDA for 20 days, eight fungal isolates were obtained.

Figure 2.2 Chlorotic leaf spots on *H. sericea* infected leaves

(stereo microscope, bars = 2 mm).

Pure fungal cultures exhibited a pinkish mycelium upon 1 week of culture (Fig 2.3.A). Compact acervuli containing black slimy spore masses were visible after 4 weeks of culture (Fig 2.3.B). Microscopic observation revealed typical *Pestalotiopsis* sp. five-celled spores (three coloured median and two hyaline end cells) with three apical and one basal appendages (Fig 2.3.C).

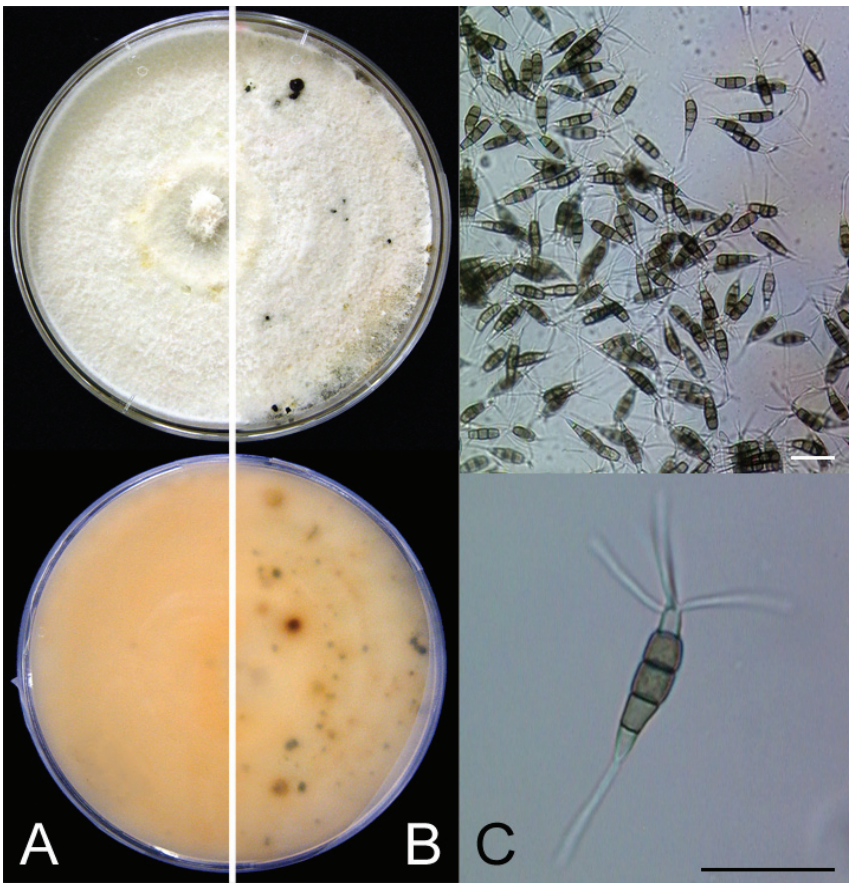

Figure 2.3 *Pestalotiopsis* isolate collected from infected *Hakea sericea* leaves.

Mycelium growth on PDA, 1 (A) and 4 (B) weeks after inoculation and corresponding conidia [(C), light microscopy, bar = 20 µm].

The genus *Pestalotiopsis* is a heterogeneous group of coelomycetous fungi where species are defined primarily based on conidial characteristics or conidiogenesis (Jeewon *et al*., 2003). In order to further identify the *Pestalotiopsis* sp. isolated from infected *H. sericea*, the ITS region of the fungal rDNA was amplified, using *ITS4* and *ITS5* universal primers (White *et al*., 1990). The electrophoretic analysis of the obtained PCR products showed the

amplification of a 599 bp fragment, regardless the annealing temperature used (50.4ºC, 55.1ºC or 60.1ºC) (Fig 2.4). The fragment obtained with the highest stringent condition (60ºC) was selected for sequencing, since the probability of unspecific contaminant amplifications would be much lower. This newly obtained ITS sequence was then compared with orthologs from the Amphisphaeriaceae family, where the *Pestalotiopsis* genus is included. The multiple alignment was performed using the ClustalW method and the unrooted tree was plotted using the maximum-likelihood algorithm (Figs 2.5 and 2.6). This analysis indicated that the isolated fungus is very similar to *Pestalotiopsis funerea* (99.3% similarity). To our knowledge, this was the first report of a naturally occurring pathogenic fungus in portuguese *H. sericea* stands.

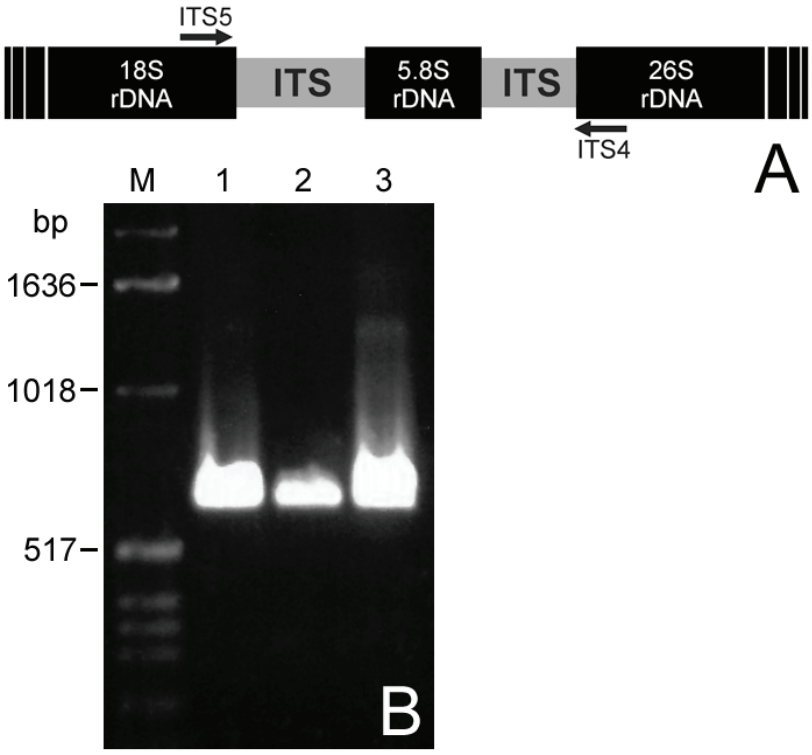

Figure 2.4 Amplification of ITS regions from *Pestalotiopsis* genome.

Position of the universal primers *ITS4* and *ITS5* in fungal nuclear rDNA, with arrowheads representing the 3' end of each primer (A). Electrophoretic analysis of PCR amplification products from *Pestalotiopsis* genome using, *ITS4*/*ITS5* universal primers and annealing temperatures of 50°C (1); 55°C (2) and 60°C (3). (M) Molecular weight marker *1kb DNA Ladder* (Invitrogen) (B).

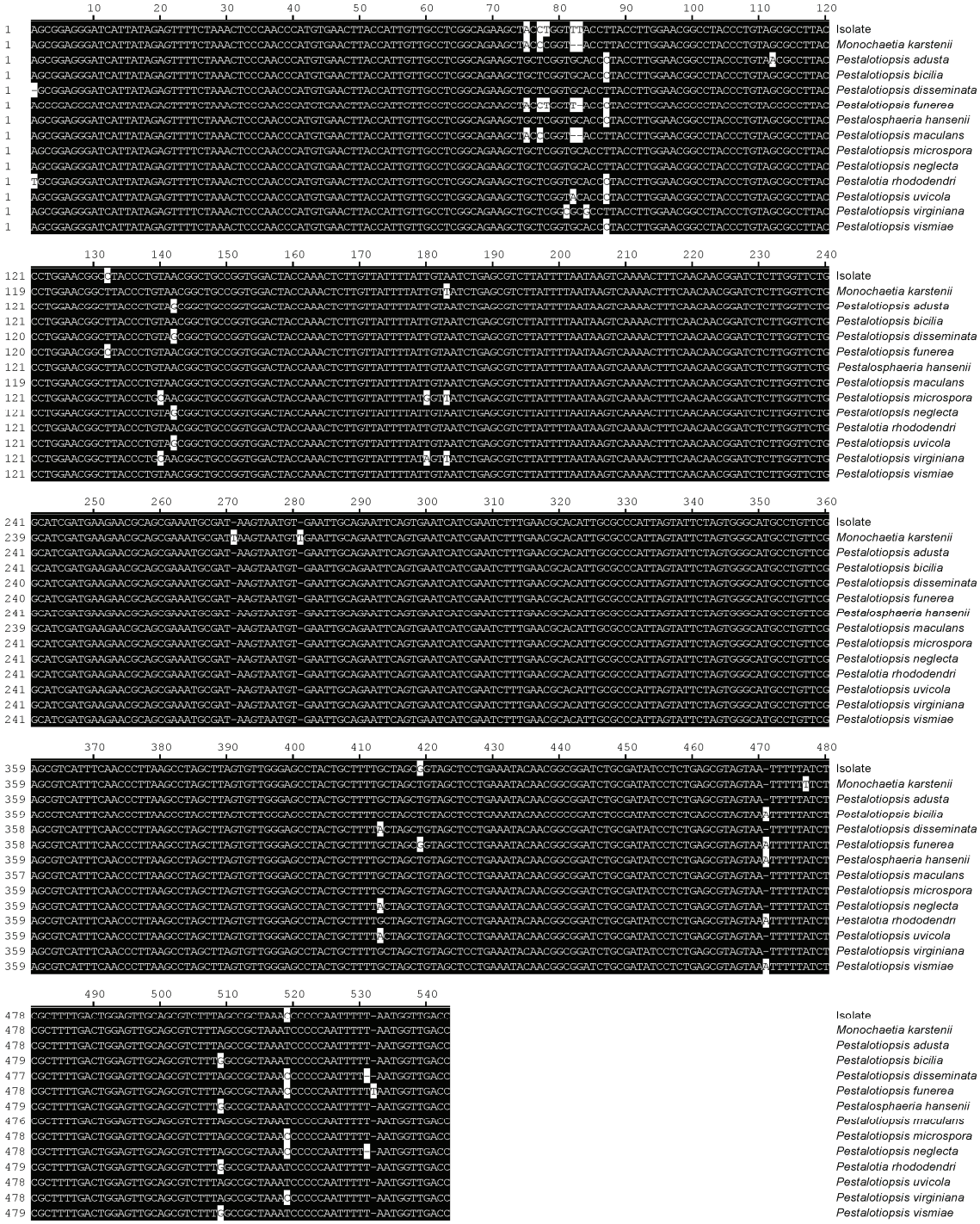

Figure 2.5 DNA sequence analysis of isolated *Pestalotiopsis* ITS region with other fungal corresponding sequences.

Multiple alignment was performed using the ClustalW method. (*Monochaetia karstenii* – acc. no. AF405300; *Pestalosphaeria hansenii* – acc. no. AF377290; *Pestalotia rhododendri* – acc. no. AF377294; *Pestalotiopsis adusta* – acc. no. AF409955; *Pestalotiopsis bicilia* – acc. no. AF409973; *Pestalotiopsis disseminata* – acc. no. AB251918; *Pestalotiopsis funerea* – acc. no. AF405299; *Pestalotiopsis microspora* – acc. no. AF409958; *Pestalotiopsis maculans* – acc. no. AF405296; *Pestalotiopsis neglecta* – acc. no. AF409975; *Pestalotiopsis virginiana* – acc. no. AF409959; *Pestalotiopsis uvicola* – acc. no. AF409974; *Pestalotiopsis vismiae* – acc. no. EF451801). Conserved nucleotides are shadowed in black.

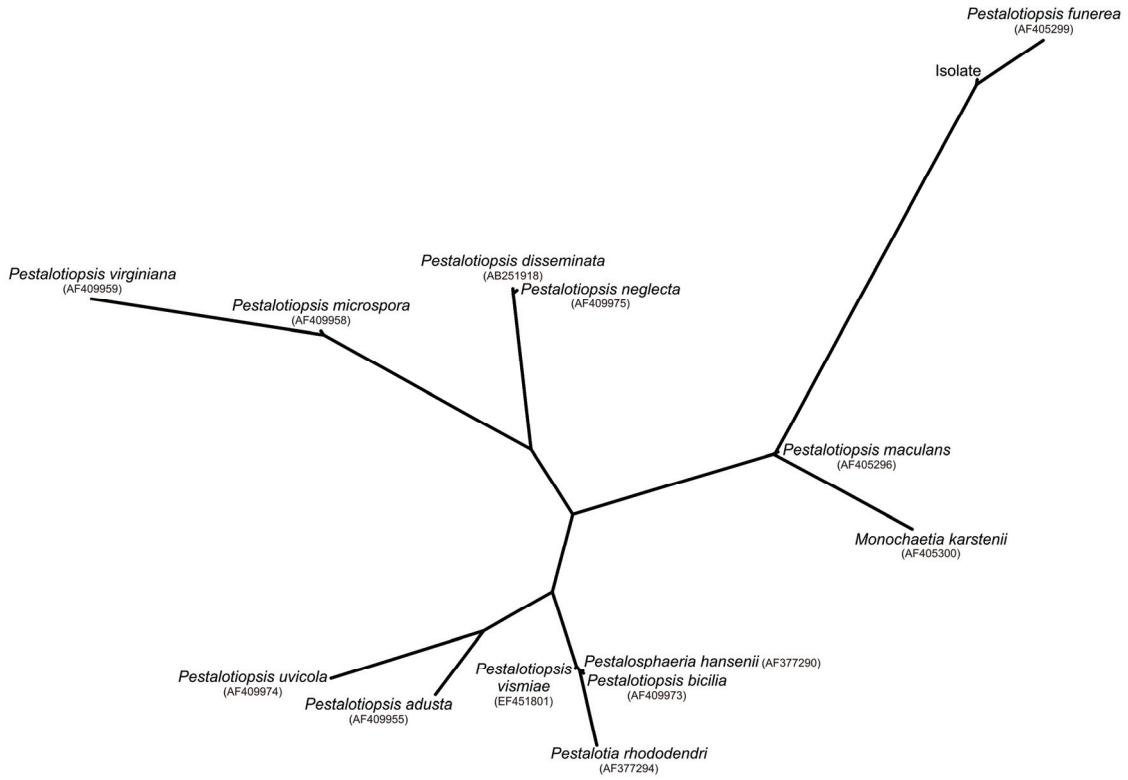

Figure 2.6 Unrooted tree representing the phylogenetic relationship between the isolated *Pestalotiopsis* and other species of the Amphisphaeriaceae family.

Coding sequences were analyzed using ClustalW and maximum-likelihood algorithms. The tree was plotted using PHYLIP's DrawTree software. GenBank accession numbers are indicated for all entries.

To confirm *P. funerea* pathogenicity, sterile seedlings of *H. sericea* were exposed to the newly isolated *P. funerea* spores and the symptoms were compared to those displayed by the naturally infected plants. After harvesting follicles in "Serra de Arga", and dehydrating them at 125ºC for 1 hour, released seeds were disinfected with H_2O_2. Germination occurred in MS medium and seedlings were allowed to develop for 6 weeks (Fig 2.7). Leaf-wounded and non-wounded *in vitro* plants were sprayed with a suspension containing 10^5 spores per mL or with sterile water. After 6 days, only leaf-wounded plants infected with *P. funerea* exhibited similar necrotic lesions and fruiting bodies as displayed by naturally infected plants occurring in "Serra de Arga" (Fig 2.8). Microscopic observation revealed typical *P. funerea* spores (Fig 2.8, insert).

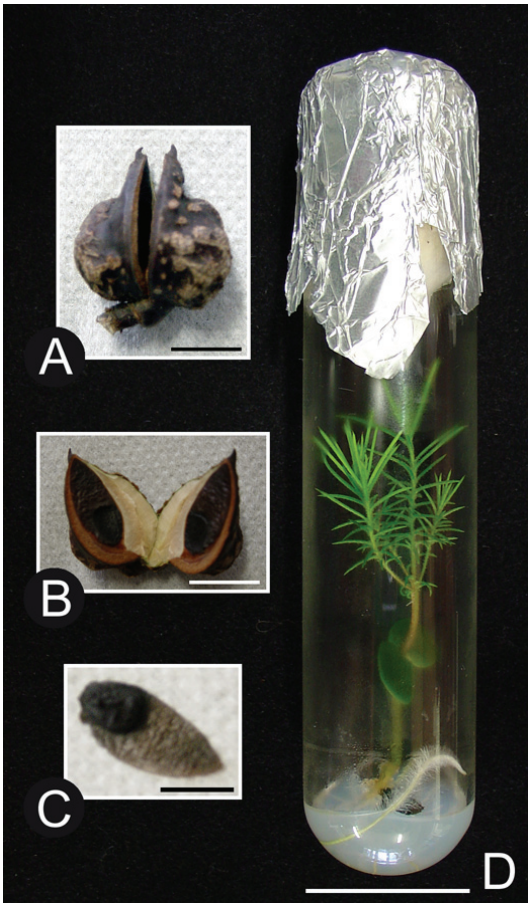

Figure 2.7 Production of *Hakea sericea* plants upon in vitro-seed germination.

Follicles were harvested in "Serra de Arga" [(A), bar = 2.5 cm] and dehydrated to promote opening [(B), bar = 2 cm] and releasing of seeds [(C), bar = 1 cm]. In vitro-germination was promoted in MS medium [(D), bar = 5 cm].

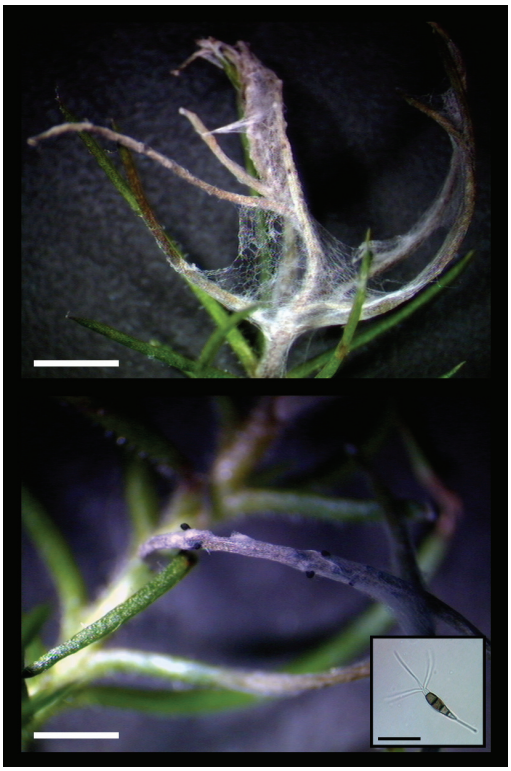

Figure 2.8 In vitro-infected *Hakea sericea* plants with isolated Pestalotiopsis sp. spores.

Infected leaves exhibit similar necrotic lesions and fruiting bodies as displayed by naturally infected plants occurring in "Serra de Arga" (bar = 1 cm). Conidia collected from *in vitro*-infected *H. sericea* specimens were similar to *P. funerea* spores [(in insert), light microscopy, bar = 20 µm].

2.4 DISCUSSION

Vast areas of pine and oak forests are being replaced by *Hakea sericea* in northern Portugal. *H. sericea* spreading compromises natural regeneration of the main forest species, and has a profound negative impact on animal biodiversity. Introduced to South Africa from Australia during the 1930s as a hedge plant, *H. sericea* has become a major problem in nearly all of the coastal mountain ranges and catchments of the Western Cape Province (Neser and Fugler 1978; Richardson and van Wilgen, 1986). At the time, control of *H. sericea* involved felling the shrubs and leaving them *in situ* for releasing canopy-stored seeds. The area was then burnt once the seeds had germinated to kill the seedlings (Fenn, 1980; Richardson and van Wilgen, 1986). There are, however, several issues associated with mechanical control combined with controlled burning. In very dense stands fires may not spread through the crowns of *Hakea* shrubs. In opposition, the additional fuel load of dead *H. sericea* plants can result in exceptionally intense fires which may compromise the recovery of autochthon elements (Richardson and Manders, 1985). Also, the follow-up time-consuming operations required to eradicate resultant seedlings can be hampered by the standing dead shrubs, making such operations prohibitively expensive.

Biological control of *H. sericea* in South Africa was initiated in 1970 and focussed on the release of insects that reduce seed production and seedling development (Neser and Annecke, 1973; Gordon, 1999). The larvae of a fruit weevil, *Erytenna consputa*, from Australia, destroy the young seeds and stunt the development of the fruit capsule. Also from Australia, the larvae of a seed moth, *Carposina autologa*, destroy any mature seeds that have been produced. The shoot-boring weevil *Cydmaea binotata* Lea was released in 1979 to suppress seedling regeneration. However, in opposition to *E. consputa* and *C. autologa*, this insect has been mostly ineffective at the few sites where it became established (Kluge and Neser, 1991). Nevertheless, the interaction

between these agents and Portuguese autochthon *H. sericea* has never been studied, making them unsuitable for biological control, at least for the moment.

Active human dispersal of spores of the virulent fungal pathogen *Colletotrichum gloeosporioides* was also tested in South Africa (Richardson and Manders, 1985; Morris, 1991). The analysis of the symptoms progression of the disease over 21 months, allowed the prediction of about 82% mortality after 10 years. Despite these promising results, the use of *C. gloeosporioides* as a biological control agent in Portugal seems unlikely, since several autochthon species of economic interest can also be infected by this pathogen. For example, in olives (*Olea europaea*), *C. gloeosporioides* can cause major yield losses and poor oil quality (Talhinhas *et al*., 2005). Several subtropical and temperate fruits, such as grapevine, nectarine, apple and peach can also be affected by *C. gloeosporioides* (Freeman and Shabi, 1996; Ramos *et al.*, 2006).

In Portugal, to our knowledge, no large scale control measures have been taken so far. Since mechanical or chemical control seem unfit to apply in the rough northern Portugal landscape, it seems imperative to find alternative biological agents that can be used in *H. sericea* control.

Based on this assumption, apparently diseased specimens bearing necrotic lesions were harvested from their natural environment in “Serra de Arga” from which several fungal isolates were obtained. Microscopic observation revealed typical *Pestalotiopsis* spp. five-celled spores with three apical and one basal appendages. This result was in agreement with the detection of black 1-3 mm circular lesions observed on infected leaves, corresponding to the typical leaf spots caused by this agent (Keith *et al*., 2006).

Pestalotiopsis Steyaert consists of approximately 205 described species broadly distributed in the world (Wang *et al*., 2005). Most species are plant pathogens, causing disease in plants such as *Camellia sinensis* (Wei *et al*., 2007), *Phoenix roebelenii* (Ushida, 2004), and Proteaceae *Protea* and *Telopea* (Crous *et al*., 2000), while others are saprobes in soil and in plant debris (reviewed by Hu *et al.*, 2007). Espinosa-Garcia and Langenheim (1990) first reported *Pestalotiopsis funerea* as an ecologically important endophyte from

Sequoia sempervirens. Several *Pestalotiopsis* species have since been isolated as endophytes (reviewed by Hu *et al.*, 2007), some of which have the ability to produce taxol, which had been reported to be effective against cancer (Qiu *et al.*, 1994; Strobel *et al.,* 1996).

The identification of *Pestalotiopsis* is primarily based on morphologic unique characters, mainly by the presence of a fusiform conidia formed within compact acervuli. The conidia are usually five-celled, with three pigmented median cells and hyaline end cells (Griffiths and Swart, 1974; Jeewon *et al*., 2003; Keith *et al.*, 2006). The pigmentation of the median cells ranging from light brown and dark brown to varying shades of olive green, results from the deposition of melanin granules within the cell matrix (Keith *et al*., 2006). The presence of two to four apical appendages arising from the apical cell and a centric basal appendage is also typical for *Pestalotiopsis* conidia.

Although *Pestalotiopsis* genus is easily distinguishable with cultural and morphological characterization of conidia, the proper identification at the species level is problematic because there are few valid morphological characters available and they frequently overlap. For example, the morphological difference among *P. disseminata*, *P. neglecta* and *P. uvicola* is obscure and species designation appears to be based on personal opinions (Hu *et al.*, 2007). The identification based on cultural and morphologic characters is even more problematic due the variation occurring between subcultures of the same isolate (Hu *et al.*, 2007). When strains were cultured in the same media and under the same environmental conditions, conidial length, conidial width and length of the three median pigmented cells remained similar even after several subcultures. However, the length of apical and basal appendages can be variable, possibly being affected due to growth in the artificial media. When a *P. funerea* strain was first isolated, the average length of apical appendages was 12.5-22.5 µm, being reduced to 5-15 µm long after being subcultured twice (Hu *et al.*, 2007). Since most species in this genus have few morphologic characters that overlap in many aspects and vary upon subculturing, the taxonomy of *Pestalotiopsis* has been equivocal and resolved differently by various authors (Steyeart, 1949; Guba, 1961; Sutton, 1980; Nag Rag, 1993).

Comparative studies of the nucleotide sequences of rRNA and/or β-tubulin genes have been conducted as a means for analysing phylogenetic relationships between *Pestalotiopsis* and other closely related genera species (Jeewon *et al.*, 2003; Keith *et al.*, 2006; Hu *et al*., 2007). The molecular data obtained do not tend always to reveal a close relationship among species with similar conidia, revealing once more the complex nature of *Pestalotiopsis* genus and the difficulty in classifying it at species level (Jeewon *et al.*, 2003; Keith *et al.*, 2006). Therefore, the use of the recent molecular approaches provides a means to overcome the difficulties of more traditional methods. In this work, sequencing of internal transcribed spacer (ITS) of rRNA genes was used for identifying fungal isolates obtained from *H. sericea* deseased plants. The ITS region and intergenic spacer of the nuclear rRNA repeat units have evolved so fast that may vary among species within a genus or even among populations (White *et al*., 1990). The amplification and analysis of the ITS region of the isolated fungi indicated 99.3% similarity to the same sequence of *Pestalotiopsis funerea*. In the unrooted tree representing the phylogenetic relation between *Pestalotiopsis* species, *P. maculans* and *P. karstenii* are placed in the same branch as *P. funerea*. Accordingly, these three species share many morphological aspects such as the presence of three to four pigmented median cells, two to four apical appendages arising as tubular extensions and only one unbranched and central basal appendage (Jeewon *et al.*, 2003).

The fungal pathogenic *P. funerea* occurs worldwide and it has been described has a primary pathogen or as an opportunistic invader of conifers such as *Cupressus arizonica* (Bajo *et al*., 2008), *Pinus radiata* (Gadgil, 2005) and *Taxus baccata* (Thomas and Polwart, 2003), causing necrosis on infected tissues, and sometimes death of plants. To our knowledge, in this work was reported for the first time the infection of a Proteacea species by *P. funerea.*

When the Koch's postulates were tested by subjecting *H. sericea* sterile seedlings to the isolated *P. funerea* spores, it was observed that wounding prior to inoculation was necessary for disease development. Similar results were obtained with other species of *Pestalotiopsis* when infecting plants such as guava, rambutan, tea, or *Miltonia* orchids (Hopkins and McQuilken, 2000;

Rivera and Wright, 2000; Keith *et al.*, 2006). Also, plant wounding enhanced symptom development, as was seen for ginger and lychee. Humidity and temperature also seem to influence symptom development. While hot and dry conditions normally prevent dissemination of *Pestalotiopsis* (Tuset *et al*., 1999), a humid atmosphere (Kaushik *et al*., 1972) or unusual climatic conditions including heavy rainfall are favourable for disease development (Tuset *et al*., 1999). Altogether, these results suggest that *Pestalotiopsis* behaves as an opportunistic pathogen that affects stressed plants. Being native from southern Australia, and thus more adapted to hot and dry conditions, infected specimens of *H. sericea* found in "Serra de Arga" may suffer from cold injury during winter, becoming more susceptible to *P. funerea* attack. The requirement for stress imposition could make *P. funerea* a useful biological control agent, in association with mechanical or chemical methods. Although a control strategy based on this assumption could leave non-wounded autochthon species safe from infection, more knowledge should be gained about *P. funerea* pathogenicity potential against other hosts. Therefore, many questions arise when considering its use for biological control. Firstly, would the costs of production and dissemination of the fungal agent be affordable? And more important, how would *P. funerea* interact with the Portuguese autochthon species? Taking these into account, this paper should be understood as a basis for further studies aiming the biological control of *H. sericea* using *P. funerea* as pathogenic agent.

2.5 REFERENCES

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CHAPTER 3

PHOSPHATE TRANSPORT BY PROTEOID ROOTS OF HAKEA SERICEA

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ABSTRACT

Up to now the higher capacity of proteoid roots to absorb inorganic phosphate from Pi-poor soils has been related mainly to their increased root surface area and higher exudation of organic acids and phosphatases, while much less attention has been directed to their mechanisms of Pi uptake. Here we report a characterization of the Pi uptake kinetics of the proteoid root-forming species *Hakea sericea* Schrad. This Proteaceae is an Australian native, which is disseminating very fast through forests of the European south. Dense mats of proteoid roots were observed in the upper soil layers of the invaded area in Portugal where availability of P and N was shown to be very low. Plants grown hydroponically under low-Pi also developed proteoid roots, and the proteoid clusters presented a major role in Pi absorption in comparison to the non-proteoid portions of the root system as revealed by their higher ^{32}Pi labeling. The ^{32}Pi uptake by proteoid roots was dependent on H^+ gradient and yielded a biphasic kinetics, suggesting the involvement of H^+/Pi co-transport systems with K_m values of 0.225 and 40.8 μM Pi. The analogs phosphite (Phi) and arsenate, but not vanadate, inhibited competitively the Pi absorption. Such biphasic Pi uptake pattern with the highest affinity at submicromolar range is likely to be of critical importance for the capacity of this plant species to invade and proliferate throughout vast areas of nutrient-deprived soils.

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3.1 INTRODUCTION

Phosphorus (P) is an important plant macronutrient, making up about 0.2% of plant dry weight. P deficiency limits plant growth more frequently than any other nutrients except nitrogen (Schachtman *et al*., 1998; Vance *et al*., 2003; Raghothama and Karthikeyan, 2005). Although total soil P content typically varies from 500 to 2000 $mg.kg^{-1}$, total bioavailable P may be only a few $mg.kg^{-1}$ (Vance *et al*., 2003). The form of P most readily accessed by plants is Pi, the concentration of which rarely exceeds 10 mM in soil solutions (Bieleski, 1973). The Pi level in soil solution is regulated mainly by its interaction with organic or inorganic surfaces in the soil. Aluminium and iron ions in acid soils, and calcium ions in alkaline soils, interact strongly with Pi and render it unavailable to plants. Consequently, plants have developed numerous morphological, physiological, biochemical and molecular adaptations to acquire Pi (Vance *et al*., 2003; Raghothama and Karthikeyan, 2005).

Proteoid roots (or cluster roots) are considered, along with mycorrhizas and nitrogen-fixing nodules, to be one of the major adaptations to enhance nutrient acquisition (Skene, 2000). Along a proteoid root, discrete clusters of closely spaced rootlets develop. The rootlets emerge in continuous rows from the cortex and are covered with root hairs, increasing the absorption surface area (Watt and Evans, 1999). Species with proteoid roots can grow in soils with poorly available nutrients, and their induction has been reported mainly in response to low availability of P and Fe (Shane and Lambers, 2005). Until now the ability of proteoid roots in improving Pi mobilization from soil has been related mainly to the increase of root surface area and exudation of carboxylic acids, acid phosphatases, phenolics, mucilages, and water (Watt and Evans, 1999; Neumann and Martinoia, 2002; Lambers *et al*., 2006).

Hakea sericea Schrad. is a Proteaceae native in south-eastern Australia and its ability to produce proteoid roots, together with the production of large number of seeds protected by woody follicles, efficient dispersal of seeds and rapid germination, have enabled the species to become well-adapted to cope with

fires and Pi-poor soils. These advantages have contributed to make *H. sericea* an aggressive invader of natural vegetation in the Mediterranean basin, similarly to what has been reported in New Zealand and South Africa (Dyer and Richardson, 1992). It has been postulated that Proteaceae species could present enhanced Pi absorption capacity. This feature, in association with a deficiency for down-regulating their Pi transport, may result in Pi toxicity symptoms (Shane *et al*., 2003; Shane *et al*., 2004). Nevertheless, up to now, detailed data on kinetics and energetics of Pi uptake by proteoid roots of *Hakea* spp. are still lacking, the only approach published so far showing transport depending linearly on the external Pi concentration (Roelofs *et al*., 2001). The present work reports a kinetic characterization of the Pi uptake system of the *H. sericea* proteoid roots and its relationship with the adaptive aspects of this invasive species in nutrient-poor soils.

3.2 MATERIAL AND METHODS

3.2.1 Sampling area

Proteoid roots and follicles from wild *H. sericea* plants, as well as soil samples, were collected in "Serra de Arga" mountains (430–450 m elevation), northern Portugal, centred at 8º45'06" W, and 41º51'20"N.

3.2.2 Soil analysis

Samples were collected in 10–40 cm of depth in the soil, airdried and passed through a 2 mm sieve prior to analysis. Extractable P content was determined in the modified Egner–Riehm extract (Egner *et al*., 1960); Pi was measured colorimetrically by the ascorbic acid method (Murphy and Riley, 1962). Total P was extracted with *aqua regia* ($HCl:HNO_3$, 3:1) and measured colorimetrically. Mineral N content was determined in an aqueous extract of the soil (1:5) after reduction with $FeSO_4$ and Ag^+ followed by alkalinization. NH_3 was separated by distillation and titrated. Total N was determined by the Kjeldhal method (Bremner and Mulvaney, 1982).

3.2.3 Hydroponic culture of H. sericea

To obtain hydroponically-grown plants, follicles were opened at 120ºC for 1 h and seed dormancy was broken in the dark, at 4ºC for 1 week. Seeds were embedded for 24 h, and germination occurred in quartz sand. One-month seedlings were transferred to vessels containing 8 L mineral medium with 200 µM $Ca(NO_3)_2$, 100 µM $MgSO_4$, 330 µM KNO_3, 50 µM NH_4NO_3, 18µM H_3BO_3, 8 µM $MnSO_4$, 0.16 µM $CuSO_4$, 0.32 µM $ZnSO_4$, 0.4 µM Na_2MoO_4, 20 µM FeEDTA, and 1, 100 or 1000 µM NaH_2PO_4 (depending on the experiment), at pH 5.8. This nutrient solution was aerated and changed biweekly. The hydroponic system was kept in a green-house with a photoperiod of 16 h and a quantum irradiance of 200 µmol m^{-2} s^{-1}, at 25ºC. After 6 weeks, cotyledons were removed. To evaluate the effect of Pi availability on the induction of proteoid roots, two sets of seedlings were grown in different nutrient solutions containing

1 µM or 100 µM of NaH_2PO_4. To study the correlation between plant P status and Pi uptake, plants grown in the 1 µM Pi nutrient solution were transferred to a 1 mM Pi nutrient solution for 2 days prior to uptake experiments.

3.2.4 Measurement of ^{32}Pi uptake by proteoid roots

Radioactive phosphate (^{32}Pi) uptake experiments were carried out according to a modified protocol of Sentenac and Grignon (1985). The assays were performed in fresh proteoid root segments (excised proteoid roots) collected from hydroponically-grown *H. sericea* plants. After sampling, proteoid roots were carefully washed with deionised water and immediately used in ^{32}Pi uptake experiments. In order to correlate the results obtained using roots from hydroponically-grown plants with the Pi uptake phenomena occurring in plants growing in their natural environment, proteoid roots excised from wild-grown plants were also used in ^{32}Pi uptake experiments. However, technical issues related with detachment of soil particles from the roots and extensive washing, together with the heterogeneity exhibited by the plant material, impaired detailed analysis of ^{32}Pi uptake.

In order to estimate initial ^{32}Pi uptake rates, 50–70 mg FW of root segments were incubated in 20 mL of transport solution (10 mM $CaCl_2$, 20 mM MES and 0.1–100 µM H_3PO_4 labeled with ^{32}Pi), adjusted to pH 4.5, 5.5, 6.0, or 6.5 to impose transmembrane proton gradients, at 25ºC. ^{32}Pi uptake was also measured in root segments pre-incubated with 100 µM of the protonophore CCCP and 100 µM TPP^+ for 10 min to evaluate the dependence on proton electrochemical gradient. After shaking for 2 min, a period that ensures linearity of uptake, the reaction was stopped by washing the root segments with ice-cold 2 mM $CaCl_2$ under vacuum. Root segments were transferred to 50 mL Falcon tubes containing 5 mL of 2% (w/v) Triton X-100 and incubated for 12 h. Aliquots of extracts (0.5–2 mL) were transferred to vials containing scintillation fluid (*OptiPhase HiSafe II*; LKB Scintillation Products). The radioactivity was measured in a *Packard Tri-Carb 2200 CA* liquid scintillation counter (Packard Instruments Co. Inc., Rockville, MD). Non-specific binding of labeled Pi to the filters and/or root cells was determined after root segments had been incubated

with 2% (w/v) Triton X-100 for 12 h prior to uptake experiments and these results were used to correct Pi uptake data.

3.2.5 Effect of Pi analogs and mercurial reagents on ^{32}Pi uptake

Inhibition of Pi transport by Phi, arsenate, or vanadate was assayed by adding simultaneously 2 µM ^{32}Pi and 1 mM of these compounds. The transport reaction was stopped as described previously after 2 min of incubation. Competition between Pi and the analogs Phi and arsenate was tested by running competitive uptake kinetics. The transport reaction was started after the addition of the unlabeled substrates (250 µM). ^{32}Pi transport was also measured in the presence of 100 µM $HgCl_2$ or 250 µM mersalyl (mersalic acid), after proteoid roots had been pre-incubated during 10 min with the mercurial reagents.

3.2.6 Calculation of kinetic parameters

The data of initial Pi uptake rates were analysed by a nonlinear regression analysis (Graph-Pad Prism, version 4.0, Graph-Pad software, San Diego, CA, USA). By this method, the transport kinetics best fitting the experimental initial uptake rates were determined, and estimates for the kinetic parameters were obtained. Substrate uptake is presented as mean ± SE, and *n* denotes the number of independent experiments.

3.2.7 Autoradiography of ^{32}Pi labeled roots

Root segments were incubated in 20 mL of 1 µM ^{32}Pi transport solution and, at selected times, the reaction was stopped by washing the root segments with ice-cold 2 mM $CaCl_2$ under vacuum, as described previously for ^{32}Pi uptake experiments. Root segments were dried with paper and protected with a cellophane sheet. The labeling was visualized using a *Bio-Rad Phosphorimager system* (Molecular Imager® FX).

3.2.8 Rhizosphere acidification by proteoid roots

The exudating activity of proteoid roots was determined according to Yan *et al*. (2002). Intact roots of 10-weeks-old plants were carefully washed with

deionised water, spread on an agar sheet containing 0.75% (w/v) agar, 0.006% (w/v) bromocresol purple, 2.5 mM K_2SO_4, and 1 mM $CaSO_4$, at pH 6.0, and were slightly pressed into the agar avoiding any damage to the roots. Rhizosphere acidification was conducted under light for 5 h. Vanadate sensitivity of rhizosphere acidification by proteoid roots was determined by performing the same experiment in an agar sheet with the same composition as the one mentioned above, supplemented by 1 mM Na_3VO_4.

3.2.9 Phosphate content determination

Phosphate content was determined by sampling roots, stems and leaves of *H. sericea* plants grown (a) in the wild, (b) in a 1 µM Pi nutrient solution, and (c) in a 1 µM Pi nutrient solution followed by 2-days-incubation in a 1 mM Pi nutrient solution. The excised tissues were rinsed in distilled water, oven-dried at 70ºC, for 36 h, and ashed in a muffle furnace, at 550ºC, for 3 h. Ashed plant samples were digested with 10 mL of 1.4% (v/v) HNO_3 solution. The digested samples were filtered and analysed by inductively coupled plasma atomic emission spectrometry (ICP).

3.3 RESULTS

3.3.1 Kinetics of Pi uptake by H. sericea *proteoid roots*

Proteoid roots from wild *H. sericea* plants are similar to those of other *Hakea* species, showing dense clusters of rootlets covered with root hairs (Fig 3.1.A) at intervals along lateral secondary roots. Dense mats of proteoid roots were observed in upper soil layers of the sampling area, where P and N availability was very low, as follows: total P, 232 mg kg^{-1}; Pi, 3.1 mg kg^{-1}; total N, 0.07% (w/w); mineral N, 14 mg kg^{-1}. When grown in hydroponics in a 1 µM Pi nutrient solution, plants developed an average of 20 rootlet clusters throughout the root system within 8–10 weeks (Fig 3.1.B). Rootlet clusters ranged from juvenile (8–12 days; white-coloured) to mature (14–18 days; light-brown-coloured). On the other hand, plants grown in a 100 µM Pi nutrient solution failed to develop proteoid roots.

The contribution to the Pi uptake performed by root clusters in proteoid root-forming species is still a matter of controversy. The large differences in the morphology and density of proteoid and non-proteoid root portions of the root system impair an accurate comparison of transport capacities when initial velocities are expressed per unit of root dry or fresh weight. To circumvent this problem, the comparison of Pi uptake in both root types was performed directly by autoradiography using the root system of *H. sericea* plants grown hydroponically in a 1 µM Pi nutrient solution. As can be seen in Fig 3.2.A the proteoid portions of the root system displayed a high labelling intensity suggesting a major role on Pi uptake. As shown in Fig 3.2.B, intense acidification of rhizosphere was observed in the vicinity of the proteoid portions of *H. sericea* root system, most likely due to H^+ pumping activity mediated by plasma membrane ATPase, since it was prevented by vanadate. Taking these results into account, all the subsequent transport experiments were performed in proteoid portions of the *H. sericea* root system.

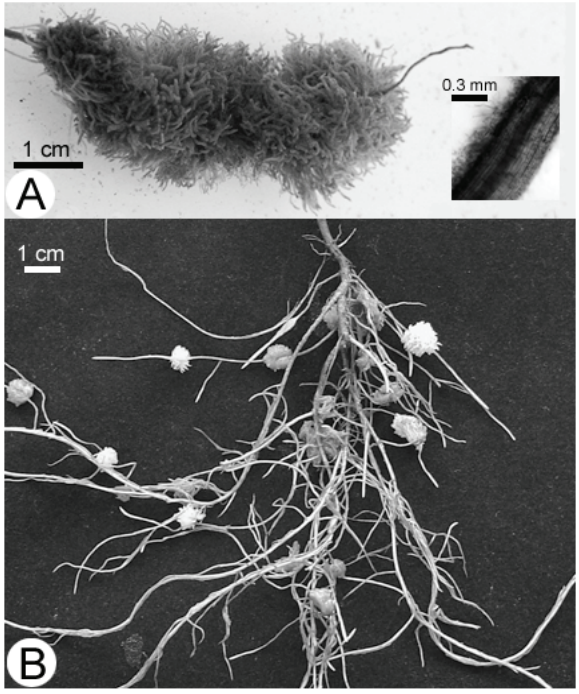

Figure 3.1 External structure of proteoid roots in *H. sericea*.

Excised proteoid root collected from wild-grown *H. sericea* with densely spaced rootlets and root hairs observed under light microscope (A). Root system of *H. sericea* grown in hydroponic culture in 1 µM Pi nutrient mineral solution for 10 weeks (B).

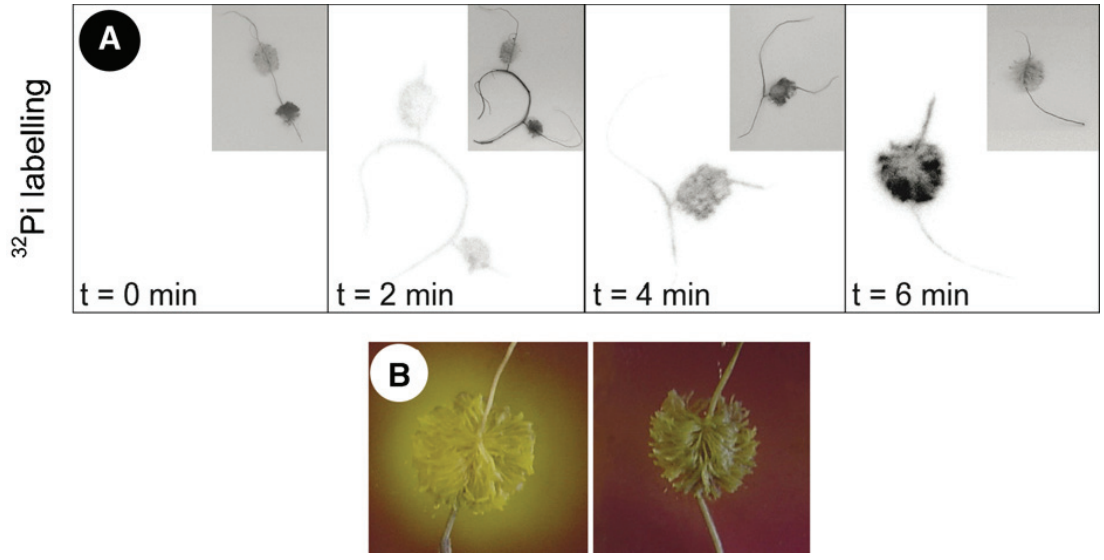

Figure 3.2 Qualititive experiments on Pi uptake by proteoid roots of *H. sericea.*

Autoradiographs of the root system (proteoid and non-proteoid portions) of hydroponically-grown *H. sericea* after incubation with 1 µM ^{32}Pi where the grey intensity is proportional to ^{32}Pi accumulation (A) (pictures of corresponding root systems are presented as inserts) and acidification of rhizosphere by proteoid roots (B) in the absence (picture on the left) and in the presence of 1 mM vanadate (picture on the right).

Juvenile proteoid roots excised from *H. sericea* grown hydroponically in a 1 µM Pi nutrient solution were used for determining the time course of 1 µM ^{32}Pi uptake (initial concentration), at pH 6.0 (Fig 3.3.A). ^{32}Pi transport appeared to be linear as a function of time up to 6 min. A time period of incubation of 2 min was subsequently used to estimate the initial uptake rates of 0.1–150 µM ^{32}Pi (initial concentrations) (Fig 3.3.B). The dependence of the initial velocities of Pi uptake on the concentration of external Pi was analysed by GraphPad Prism, version 3.0 (GraphPad Software, San Diego, CA) using the following kinetic models:

$$v = \frac{v_{max} S}{K_m + S} \qquad \text{(1 saturating component)} \qquad (1)$$

$$v = \frac{v^1_{max} S}{K^1_m + S} + \frac{v^2_{max} S}{K^2_m + S} \qquad \text{(2 saturating component)} \qquad (2)$$

where S is the substrate concentration (Pi), v the initial uptake rate, v_{max} the maximal velocity and K_m is the Michaelis–Menten constant. The transport kinetics best fit was determined, and estimates for the parameters v_{max} and K_m were obtained. The application of the computer-assisted non-linear regression analysis showed that experimental data displayed a better fit to the Eq. (2), indicating the involvement of a biphasic Pi uptake model, as suggested from the graphical analysis of the Eadie–Hofstee plot. Besides the low-affinity (K_m, 40.8 µM Pi; v_{max}, 3.5 µmol Pi h^{-1} g^{-1} FW) Pi transport component commonly found in plants, another Pi transport system operating in the submicromolar range (K_m, 225 nM Pi; v_{max}, 0.22 µmol Pi h^{-1} g^{-1} FW) also became apparent. Similar results were obtained in mature proteoid roots (not shown) suggesting that Pi uptake characteristics do not change significantly with the root development. When *H. sericea* plants grown hydroponically in a 1 µM Pi nutrient solution were transferred to a 1 mM Pi nutrient solution for 2 days, a biphasic kinetics was also revealed but the v_{max} of Pi transport in the low-affinity range decreased by 50% (Fig 3.3.C). Pi transport experiments were also performed in proteoid roots from wild-grown *H. sericea*. The initial velocities of 0.2, 4 and 20 µMPi were 0.10, 1.04 and 1.97 µmol Pi h^{-1} g^{-1} FW, respectively,

suggesting that Pi uptake characteristics did not differ too much from those of hydroponically-grown plants.

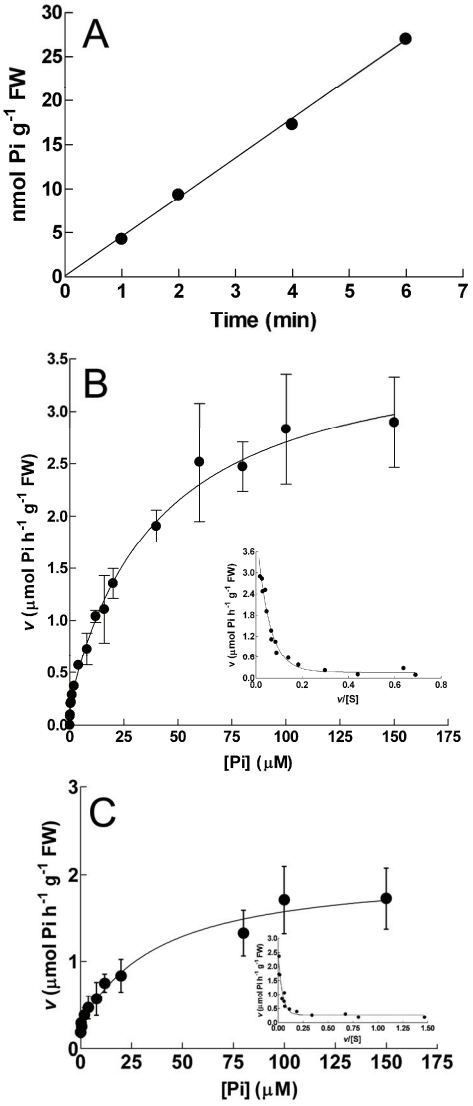

Figure 3.3 Transport of ^{32}Pi, at pH 6.0, by proteoid roots of hydroponically-grown *H. sericea*.

(A) Time course of 1 µM ^{32}Pi uptake and (B and C) initial uptake rates of 0.1–150 µM ^{32}Pi. Plants were grown in a 1 µMPi nutrient solution during 10 weeks (A and B) or in a 1 µM Pi nutrient solution for a period of 10 weeks followed by incubation in a 1 mM Pi nutrient solution for 2 days (C). Experimental data of initial uptake rates fitted to the equation $v = v^1_{max} S/(K^1_m + S) + v^2_{max} S/(K^2_m + S)$ (2 saturating components) by a computer-assisted non-linear regression analysis (r^2 = 0.94). Vertical bars denote SE, *n* = 3. Inserts, Eadie–Hofstee plots of the initial uptake rates.

3.3.2 Effect of proton electrochemical gradient and mercuric reagents on Pi uptake

Under normal physiological conditions there is a requirement for the energized transport of Pi across the plasma membrane from the soil to the plant because of the very high concentration of Pi in the cytoplasm compared with soil solution, and of the negative membrane potential that is characteristic of plant cells. Results showed that the protonophore CCCP strongly inhibited the initial uptake rates of 0.1–150 μM ^{32}Pi, as shown in Table 3.1 for 2 μM ^{32}Pi, suggesting the involvement of energy mediated co-transport systems driven by a proton gradient. Fig 3.4.A shows the effect of external pH on the initial uptake rates of 2 μM ^{32}Pi by excised proteoid roots. Similar results were obtained with 0.3 and 20 μM ^{32}Pi (not shown). To achieve the same concentration of the transported form throughout the pH range 4.5–6.5, total Pi concentrations were adjusted according to Henderson–Hasselbach equation. Results showed that from pH 5.5 to 6.5 there was a reduction of about 50% on Pi transport capacity corroborating the involvement of H^+-dependent transport. To determine which Pi form is preferentially transported by proteoid roots of *H. sericea*, uptake experiments depicted in Fig 3.3.B at pH 6.0 were also performed at pH 4.5, 5.5, and 6.5 and the new kinetic parameters evaluated. Because K_m variation was lower when Pi concentration was expressed as [$H_2PO_4^-$] through this pH range, the results suggest that this anionic form is the substrate for Pi transport systems of *H. sericea*, as it has been described for other plant species (Vance *et al*., 2003; Raghothama and Karthikeyan, 2005). To evaluate the effect of membrane potential on Pi transport, the lipophilic and highly permeant cation TPP^+ was used to dissipate electrical current across biological membrane. TPP^+ inhibited Pi transport by 40% (Table 3.1), suggesting a net influx of positive charges into the cells, implying that more than one proton is co-transported per $H_2PO_4^-$ molecule, at 2 μM Pi and pH 6.0. In fact, the stoichiometry of Pi uptake is not yet resolved, but calculations and experimental data suggest that two to four protons are co-transported with each $H_2PO_4^-$ (Rausch and Bucher, 2002).

Table 3.1 Effect of uncouplers, mercurial reagents and phosphate analogs on 2 µM Pi uptake, at pH 6.0, by proteoid roots of hydroponically-grown *H. sericea*.

Inhibitor	Inhibition (%)
CCCP (0.1mM)	96
TPP^+ (0.1 mM)	40
$HgCl_2$ (0.1 mM)	67
Mersalyl (0.25 mM)	30
Phosphite (0.1 mM)	85
Arsenate (0.1 mM)	89
Vanadate (0.1 mM)	1

Pi transport of *H. sericea* was also sensitive to the inorganic mercuric compound $HgCl_2$ and to mersalyl, an organomercurial that modifies cysteine residues (Table 3.1). Fig 3.4.B shows the dose-response effect of mersalyl on Pi uptake. Maximal inhibition capacity was obtained with 250 µM mersalyl, a reduction by 30% on initial uptake rates of 2 µM Pi being achieved.

3.3.3 Effect of the Pi analogs phosphite, arsenic and vanadate on Pi uptake

Phosphite (Phi) has been described as an analog of Pi. However, there is no evidence suggesting that plants can utilize Phi as a sole source of P (Varadarajan *et al*., 2002). Arsenic is a non-essential element for plants whose inorganic species, such as arsenate and arsenite, are generally highly phytotoxic (Wang *et al*., 2002). As shown in Table 3.1, Phi and arsenate inhibited ^{32}Pi uptake in *H. sericea*. In addition, competition experiments showed that both compounds can be absorbed via Pi transporters in *H. sericea* proteoid roots. Fig 3.4.C shows the effect of 250 µM arsenate and Phi on 1–20 µM ^{32}Pi uptake. Arsenate has been described as a strong competitive physiological analog of phosphate in higher plants (Ullrich-Eberius *et al*., 1989). Vanadate did not inhibit Pi transport in *H. sericea* (Table 3.1). However, the Pi analog

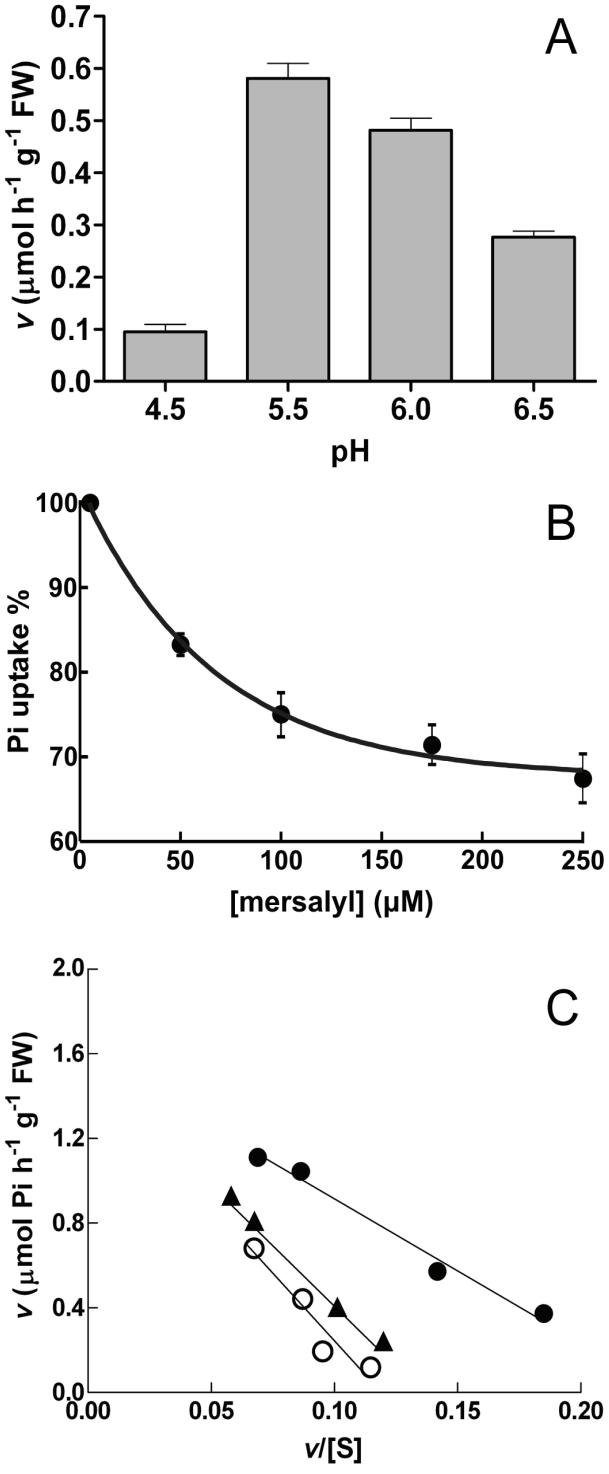

Figure 3.4 Effect of pH and inhibitors on Pi uptake.

Effect of pH (A) and dose-response effect of mersalyl (B), at pH 6.0, on the initial velocity of 2 µM ^{32}Pi uptake. Eadie–Hofstee plots of the initial uptake rates of 1–20 µM ^{32}Pi, at pH 6.0, in the absence (●) or in the presence of 250 µM Phi (○) and 250 µM arsenate (▲) (C). Values are mean ± SE, *n* = 3.

severely inhibited ATPase, probably as a result of its capacity to bind tightly to the phosphorylation site of ATPase (Bowman, 1983), preventing the acidification of rhizosphere, as shown above. Therefore, an alternative mechanism for vanadate uptake must exist in the plasma membrane for explaining the inhibition of proton pumping in *H. sericea*. Since Pi uptake experiments were performed under an artificially imposed transmembrane pH gradient, inhibition of proton pumping activity mediated by vanadate did not impair ^{32}Pi transport. In other plant species it was also shown that phosphate transport is insensitive to vanadate and that other transporters are involved on its uptake (Ullrich-Eberius *et al*., 1989; Poder and Penot, 1992). In *Lemna gibba* vanadate uptake is mediated by a H^+ co-transport mechanism and does not interfere with phosphate uptake (Ullrich-Eberius *et al*., 1989).

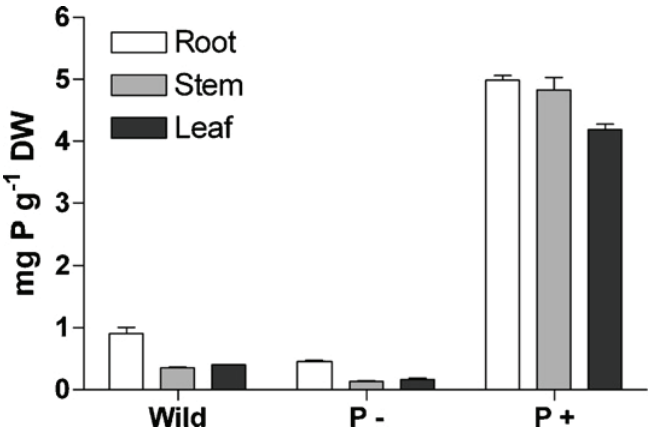

Figure 3.5 Concentration of P in tissues of *H. sericea*

Phosphorus concentration in roots, stems and leaves of *H. sericea* plants grown in the wild, in a 1 µM Pi nutrient solution during 10 weeks (P^-) and 1 µM Pi nutrient solution for a period of 10 weeks followed by incubation in a 1 µM Pi nutrient-poor solution for 2 days (P+). Values are mean ± SE, *n* = 3.

3.3.4 Phosphorus concentration in leaves, roots and stems of H. sericea

P concentration in leaves, roots and stems of hydroponically-grown plants is shown in Fig 3.5, compared to the values found in wild-grown plants. As expected, given the residual amounts of Pi in the soil of the sampling area, P status of roots, stems and leaves of wild-grown plants were similar to those

found in hydroponically-grown plants in a 1 µM Pi nutrient solution. In both cases, roots behaved as the preferential site for P storage. In contrast, 48 h after the transfer of hydroponically-grown plants to a 1 mM Pi nutrient solution, plant P status increased dramatically. Roots capacity for P storage appears to have been exceeded since the concentration of P in leaves increased dramatically to values where toxicity symptoms started to appear (not shown). This increase in the leaf P status may also be due to a limitation of the recirculation of shoot P via phloem.

3.4 DISCUSSION

It has been described that low-Pi status in the soil is a major cause of proteoid root formation (Neumann and Martinoia, 2002) that can be enhanced by N deficiency (Dinkelaker *et al*., 1995; Sas *et al*., 2002). Accordingly, hydroponically-grown *H. sericea* developed proteoid roots with low-P, but not in P-rich media. Also, P and N availability was shown to be very low in the upper soil layers of the sampling area where proteoid roots from wild-plants were collected.

It has been difficult to demonstrate that the proteoid portion of the roots system is able to uptake Pi in a much higher extent than the non-proteoid portion in proteoid root-forming species. Roelofs *et al.,* (2001) compared the uptake of Pi by proteoid and non-proteoid roots of *H. prostrata* by measuring the depletion of 63, 124 and 151 μM KH_2PO_4 (initial concentrations) over 45 min by a colorimetric method. Pi transport depended linearly on the external Pi concentration, and uptake rates were of the same order of magnitude for both cluster and non-cluster roots, the latter being twice as high. Authors concluded that results must be interpreted with caution due, for instance, to differences in the morphology of both root types. Furthermore, Keerthisinghe *et al.,* (1998) showed for *Lupinus albus* that not only the rate of Pi uptake was similar for both cluster and non-cluster roots but also that the results may depend on the method and the manner of expression of the uptake rate. Nevertheless, Neumann *et al.,* (1999, 2000) reported that ^{33}Pi uptake per unit root fresh weight or per root surface area in P-deficient white lupin was approximately 2-fold higher in root clusters compared to the apices of non-proteoid roots. In the present work, autoradiographs with ^{32}Pi of root system portions demonstrated that the proteoid portions of the root system are the sites of a more effective Pi uptake in *H. sericea*. Although qualitative, this approach allowed the direct comparison of Pi uptake by both root types. In addition, the intense acidification observed in the vicinity of proteoid roots mediated by the plasma membrane H^+-ATPase suggests an increased capacity to mobilize Pi from the rhizosphere

by decreasing its interaction with inorganic surfaces in the soil and to energize Pi transport systems. Both the increased Pi uptake and rhizosphere acidification around proteoid roots can result from the activity of a higher number of transporter proteins due to the higher surface to volume ratio of proteoid roots when compared to non-proteoid portions of the root system. Therefore, the systems responsible for Pi uptake by cluster roots may be similar to those used in non-cluster roots, as already described for other species (Lambers *at al*., 2003), being the observed differences simply due to root morphology.

Pi uptake capacity did not change considerably along with proteoid root development of hydroponically-grown *H. sericea*. Juvenile and mature proteoid roots exhibit similar Pi uptake characteristics. In contrast, it has been described that the exudation of compounds, such as organic acids and ectoenzymes, that chemically modify the surrounding soil in order to facilitate the mobilization of Pi, is a transient phenomenon (Watt and Evans, 1999; Neumann and Martinoia, 2002; Lambers *et al*., 2003). Maintaining Pi uptake in older proteoid roots after the pulse of root exudation could be a pre-requisite for uptake of mobilized Pi and thus for the function of cluster roots and has been similarly reported for *L. albus* (Neumann *et al*., 1999).

Pi uptake by proteoid roots from hydroponically-grown *H. sericea* was shown to be dependent on H^+ gradient and yielded a biphasic kinetics suggesting the involvement of H^+/Pi co-transport systems with distinct substrate affinities (K_m 225 nM and 40.8 µM). The establishment of the transmembrane proton electrochemical potential is mediated by the plasma membrane H^+-ATPase (Raghothama, 1999) whose activity can be modulated by P starvation (Shen *et al*., 2006). In several different tissues and plant species estimates of the K_m for high-affinity uptake range from 3 to 7 µM, whereas for low-affinity transporters the K_m estimates are more variable, from 50 to 330 µM (Clarkson, 1984; Ullrich-Eberius *et al*., 1984; McPharlin and Bieleski, 1987; Furihata *et al*., 1992). The existence of biphasic high- and low-affinity Pi transport systems has been shown by molecular and/or biochemical methods not only for plant cells, but also for bacteria (Rao and Torriani, 1990), mammals (Olah *et al*., 1994), yeast (Borst-Pauwels, 1981) and other fungi, including germ tubes of the

endomycorrhizas (Thomson *et al*., 1990) and intact ectomycorrhizas (Beever and Burns, 1980; Colpaert *et al*., 1999). However, for mycorrhizas, Pi affinity appears to span a range of K_m values often quite small compared to that of non-mycorrhizal roots, varying from K_m of 1.6 (Cress *et al*., 1979) to 0.01 µM (Straker and Mitchell, 1987). Therefore, the very high affinity Pi transport component found in *H. sericea* (K_m 0.225 µM) suggests that proteoid roots can express a specific Pi transport system with the highest affinity characterized to date in non-mycorrhizal roots. This Pi transport system operating in the submicromolar range is likely a key element for plant survival in soils with low-Pi levels.

In terms of transport capacity (v_{max}), Pi uptake rates in *H. sericea* proteoid roots are similar to those measured in other plant species, including the proteoid roots of *H. prostrata* (Roelofs *et al*., 2001) and *L. albus* (Keerthisinghe *et al*., 1998; Neumann *et al*., 1999), the dauciform roots of *Schoenus unispiculatus* (Shane *et al*., 2005), the non-specialized roots of *Zea mays* (Sentenac and Grignon, 1985; Façanha and Okorokova-Façanha, 2002) and *L. gibba* (Ullrich-Eberius *et al*., 1981). However, it is difficult to compare results obtained from different plant species under different culture conditions using various experimental approaches.

In spite of all published data, the mechanisms regulating and coordinating the expression and activity of plant solute transporters are far from being well understood. The same protein can mediate dual-affinity uptake regulated post transcriptionally by phosphorylation/dephosphorylation, such as the case of the nitrate transporter CHL1, with a K_m of 50 µM for the high affinity phase of uptake and 4 mM for the low-affinity phase (Liu *et al*., 1999; Liu and Tsay, 2003), or exhibit dual functions with spatially distinct domains for each function, such as the case of TRP family of ion channels that uses an N-terminal domain for its channel activity and a C-terminal domain for enzyme activity (Cahalan, 2001; Perraud *et al*., 2001). Also, the potassium channel gene *AKT1* isolated in *Arabidopsis* was found to be responsible for high affinity potassium uptake (Hirsch *et al*., 1998) and the potassium transporter AtKUP1 also functions as a dual-affinity transporter (Fu and Luan, 1999). The question of the existence of

several Pi transporters with different functional characteristics in plant cell membranes or only one transporter with characteristics that vary with internal P status or external concentration has been addressed using kinetic analysis of uptake (Schachtman *et al*., 1998). However, recent advances in the molecular biology of putative plasma membrane and tonoplast Pi transporters confirm that plants have multiple transporters for Pi. In *Arabidopsis thaliana*, nine high-affinity Pi transporter genes have been identified and characterized (Raghothama and Karthikeyan, 2005). At least three of these genes are expressed in roots and are up-regulated by Pi starvation. Similarly, in potato one gene was specifically induced in roots and stolons by starving the plants of Pi, whereas a second gene was expressed throughout the plant under conditions of high or low phosphate (Schachtman *et al*., 1998; Raghothama, 1999).

To study regulation of P-uptake in *H. prostrate*, Shane *et al.,* (2003) cultivated plants with distinct P regimes and obtained an estimate of P uptake (by dividing total plant P by the mass of the root) as dependent on external P concentration (P uptake / [P]). In view of the fact that this value was about 3.5-fold higher when roots received 1 µM Pi than in plants with one root half immersed in a medium with 1 µM Pi, and the other half immersed in 75 µM Pi, the authors concluded that in *H. prostrata* Pi is able to down-regulate the P-uptake system. In the present work, we studied ^{32}Pi uptake by proteoid roots of *in vitro H. sericea* plants grown under Pi deficiency and in plants subject to high Pi concentration for 48 h, the former exhibiting low-P status and the latter displaying high P concentration in roots, stems and leaves. As described for *H. prostrata*, it appears that P concentration affects Pi transport negatively. However, Pi uptake in the high affinity range was apparently not sensitive to fluctuations on both external Pi availability and P status. This is in contrast to the most common interpretation of the kinetic and molecular data available in plants suggesting that the activity of the high affinity Pi transport system is either increased or de-repressed by Pi starvation and the activity of the lower affinity transport system is constitutive (Raghothama and Karthikeyan, 2005). In spite of the low-affinity Pi transport component had been reduced by 50% in

H. sericea plants subject to high Pi, the concentration of P in plant tissues increased dramatically and plants started to exhibit foliar symptoms of P toxicity. However, since P in soil is more often in short supply than in excess, the invasiveness capacity of *H. sericea* may be enhanced by the production of proteoid roots displaying high efficient Pi transport, although poorly down-regulated by P availability.

Both P status and Pi intake characteristics of wild-grown *H. sericea* plants did not differ too much from the values found in hydroponically-grown plants cultivated in a medium with low-Pi, which correlates with the residual amounts of P found in the sampling area. Besides the substantial mobilization of Pi within the activity period of typical mature proteoid roots (*e.g*. 10–50 μmol g^{-1} soil as reported by Dinkelaker *et al.,* (1989), associated with the transient release of carboxylates, pH changes and acid phosphatases, here we have shown that *H. sericea* proteoid roots are also specialized sites for Pi uptake, a feature that must confer adaptive advantages to the plant to invade Pi-impoverished soils.

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CHAPTER 4

MOLECULAR CHARACTERIZATION OF HAKEA SERICEA *PHOSPHATE TRANSPORTER ENCODING GENES*

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ABSTRACT

In plants, different members of the phosphate transporters (PhT) family are responsible for the uptake, translocation through tissues and interorganellar allocation of phosphate (Pi). Here we report the identification of four *PhT* genes (*PiT2*, *PiT6*, *PH5* and *PH7*) from *Hakea sericea* Schrad., an Australian Proteaceae invader of natural habitats with enhanced ability to uptake Pi. The phylogenetic analysis and transmembrane domain (TMD) prediction of the partial sequences of all identified genes place them as members of PhT1 family. These high- and low-affinity H^+/Pi symporters, containing 12 TMDs in plasma membranes, are involved in Pi uptake into the cell. Phylogenetic analysis of 55 PhTs from 13 dicot and monocot species confirms the high identity between all PhT1 proteins, placing PhT2 members - H^+/Pi symporters expressed in the chloroplast - in a different cluster. Although Na^+/Pi symport mechanisms have been almost exclusively described in bacteria, fungi and animals, we also report the possible existence of a 10 TMD chloroplastidial Na^+/Pi symporter family in plants. Through phylogenetic analysis it was also demonstrated that the expression site of each *PhT1* cannot be correlated with its deduced amino acid sequence and therefore be inferred based on its structural basis only.

To better understand the Pi transport regulation mechanism of *H. sericea* it would be necessary not only to complete the sequencing of *PiT2*, *PiT6*, *PH5* and *PH7*, but also to study the expression patterns of these genes in plants under high- or low-Pi availability.

Manuscript in preparation.

4.1 INTRODUCTION

The phosphate transporters (PhT) belong to the major facilitator superfamily (MFS) that is present in all biological cells. This large and diverse family of proteins is involved in the transport of sugars, antibiotics, ions and amino acids (Pao *et al*., 1998; Law *et al*., 2008). PhT comprise different subfamilies that are responsible not only for the uptake of phosphate (Pi) from soil to root, but also for its translocation to other parts of the plant or its interorganellar allocation (Rausch and Bucher, 2002). The PhT1 proteins are mainly found in plasma membranes, suggesting an important role in Pi uptake into the cell. However, besides being uptaken into the cytoplasm, Pi must also be transported between organelles (Mimura, 1999). For example, Pi involvement in photophosphorylation as well as in the partitioning of triose phosphates renders essential its presence in the chloroplast (Versaw and Harrison, 2002). Pi import into the chloroplast may occur via triose-phosphate/phosphate translocator (TPT), in a counter-exchange of stromal triose phosphates generated from CO_2 fixation with cytosolic Pi (Walters *et al*., 2004). However, due to the stoichiometric ratio of this process, plants must rely on alternative mechanisms for concentrating Pi in the chloroplast. This role is played by PhT2 protein transporters. Although several works performed with green algae reported that Na^+ could have a stimulatory effect on Pi uptake (Ullrich and Glaser, 1982; Raven, 1984), until the beginning of this decade it was widely accepted that Na^+/Pi symporters were exclusively found in bacteria, fungi and animals (Mann *et al*., 1989; Bun-Ya *et al*., 1991; Martinez and Persson, 1998; Werner and Kinne, 2001). Reid *et al*. (2000) elegantly showed that the green algae *Chara corallina* also relies on a high-affinity Na^+/Pi symporter for Pi uptake. In addition, Rubio *et al*. (2005) proposed the presence of a Na^+/Pi symporter in *Zostera marina*, a submerged aquatic angiosperm able to uptake Pi both by leaves and roots (Pérez-Lloréns and Niell, 1995). According to the authors a high-affinity Na^+/Pi coupled transport system operating in the epidermal root cells of *Z. marina* is induced upon 8 days following Pi starvation. More recently, Gao

et al. (2006) have identified a Na^+/Pi transporter gene from *Thellungiella halophila*, whose protein seems to have a long N-terminal extension that functions as a chloroplastidial transit peptide.

PhT1 proteins contains between 518 and 587 amino acid residues, which are arranged in two groups of six transmembranar domains that are connected by a large hydrophilic charged region containing 60 amino acids (Fig 4.1) (Schachtman *et al*., 1998; Smith *et al*., 2000; Vance *et al*., 2003; Raghothama and Karthikeyan, 2005). The similarity between the first and the second half of the protein suggests that Pi transporters have evolved by a tandem intragenic duplication of the original structural unit of a six membrane-spanning protein (Pao *et al*., 1998; Raghothama and Karthikeyan, 2005).

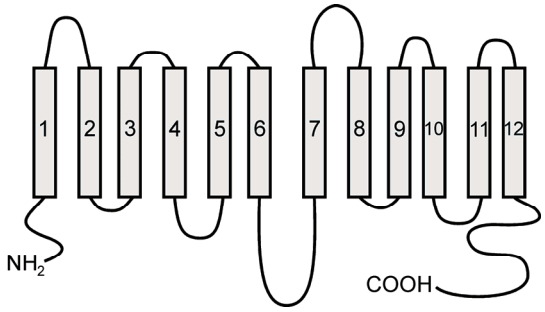

Figure 4.1 Diagram of the typical structure of a PhT1 protein.

Twelve membrane spanning regions are separated into two groups of six by a large hydrophilic charged region.

The importance of Pi in so many cellular processes requires a highly regulated Pi uptake into the cell and interorganellar transport (Ticconi and Abel, 2004). Pi flux between chloroplasts, mitochondria, vacuoles and cytoplasm is primarily mediated by continuously expressed low-affinity PhTs (Mimura, 1999; Rausch and Bucher, 2002). However, the mobilization of this nutrient within plants under Pi deficiency mainly relies on induced high-affinity PhTs (Karthikeyan *et al*., 2002; Mudge *et al*., 2002). Physiological studies regarding Pi uptake from soil to roots have suggested a dual uptake model involving both the high- and low-affinity systems (Ullrich-Eberius *et al*., 1984; Sentenac and Grignon, 1985; Amijee *et al*., 1991). These results were corroborated by the presence of transcripts and proteins of both systems in root epidermal and root hair cells (Daram *et al*., 1998; Liu *et al*., 1998; Chiou *et al*., 2001).

The identification and characterization of 9 high-affinity PhTs in *Arabidopsis thaliana* and 13 high- and low-affinity PhTs in *Oryza sativa* suggests the presence of a small family of high-affinity PhTs in plants (Karthikeyan *et al*., 2002; Mudge *et al*., 2002). Many of the corresponding genes are mainly expressed in roots, only under low internal Pi status, reflecting the attempt of plants to counteract Pi deficiency. In contrast, when internal Pi status is high, the expression of these genes is low as well as the amount of the resultant PhT protein (Muchhal *et al*., 1996; Smith *et al*., 1997; Dong *et al*., 1999). When growing under Pi-deficiency, cluster roots forming species display a reduction on the apparent K_m for Pi uptake. For example, in *Lupinus albus* the K_m decreases from 30.7 µM to 8.6 µM, whereas uptake of Pi on a gram fresh weight basis is greatly increased (Keerthisinghe *et al*., 1998; Neumann *et al*., 1999). This enhancement of Pi uptake was suggested to be related with the expression of a novel high-affinity Pi uptake system, LaPT1 (Liu *et al*., 2001).

Hakea sericea Schrad., an Australian Proteaceae, also relies on proteoid roots for enhanced Pi uptake. Under low Pi availability, the K_m for the high-affinity Pi uptake system can reach the sub-micromolar range (225 nM Pi) (Sousa *et al*., 2007). However, in contrast to what has been described for *A. thaliana*, *H. sericea* seems unable to consistently down-regulate its high-affinity Pi uptake system under Pi excess. Consequently, Pi levels within stems and leaves of plants growing in these conditions increased more than 10-fold and phosphate toxicity symptoms were observed in leaves (Sousa *et al*., 2007; refer to Chapter 3).

At present, studies regarding the characterization of PhT coding genes, not only for *H. sericea* but also for all *Hakea* genus, are inexistent. The aim of our work is to find and characterize PhT coding genes in *H. sericea* and correlate them with PhT proteins from other plant species. The expression analysis of the identified genes would allow to understand in detail the Pi transport regulation in this plant species. The expression analysis of the identified genes would allow the understanding the Pi transport regulation in this plant species, which is able to cope with nutrient-poor soils.

4.2 MATERIAL AND METHODS

4.2.1 Plant material

Hakea sericea plants were grown hydroponically in vessels containing 8 L of mineral medium with 200 µM $Ca(NO_3)_2$, 100 µM $MgSO_4$, 330 µM KNO_3, 50 µM NH_4NO_3, 18 µM H_3BO_3, 8 µM $MnSO_4$, 0.16 µM $CuSO_4$, 0.32 µM $ZnSO_4$, 0.4 µM Na_2MoO_4, 20 µM FeEDTA, 100 µM NaH_2PO_4, at pH 5.8. Up to 8 plants shared the same 8-L container and nutrient solution was biweekly replaced. The hydroponic system was kept under a photoperiod of 16 h, at 25ºC, with a quantum irradiance of 200 µmol m^{-2} s^{-1}.

4.2.2 Genomic DNA extraction

Genomic DNA (gDNA) purification was performed using a modification of the method described by Mason and Schmidt (2002).

H. sericea leaves from 6-weeks-old plants were collected, frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle. Approximately 2 g of fresh weigh was added to 10 mL of preheated (60ºC) extraction buffer [2% (w/v) SDS, 2.5% (w/v) polyvinylpyrrolidone-40 (PVP-40), 0.2 M Tris , 25 mM EDTA, 0.5 M NaCl, 2% (v/v) β-mercaptoethanol, pH 8.0]. The mixture was incubated at 60ºC for 30 min, with occasional swirling. One volume of chloroform-isoamyl alcohol [24:1 (v/v)] was added to the solution, mixed for 5 min and centrifuged at 7500 *g* for 10 min, at 10°C. For carbohydrates removal, 0.25 volumes of pure ethanol were added to the aqueous layer, and the solution was extracted twice in one volume of chloroform-isoamyl alcohol [24:1 (v/v)]. Total nucleic acids were precipitated with two volumes of ethanol and collected without centrifugation by carefully pouring off the supernatant. After transferring to a 1.5 mL microtube, nucleic acids were pelleted at 14000 *g* for 10 min, at 4°C, and resuspended in 200 µL of water. Total RNA was digested by incubation with 50 µg RNase A, at 37°C, for 1.5 h. RNase A and other contaminating proteins were removed by addition of one volume of chloroform-isoamyl alcohol [24:1 (v/v)], mixing for 5 min, and centrifugation at

14000 *g* for 5 min. The aqueous phase was retained, and genomic DNA was precipitated by adding two volumes of ethanol and 0.1 volumes of 3 M sodium acetate (pH 5.2) followed by overnight incubation at 4ºC. The sample was centrifuged for 10 min at 14000 *g* and 4ºC. The pellet was briefly washed with 70% (v/v) ethanol and air-dried for 5 min. After resuspension in 50 µL TE buffer [10 mM Tris (pH 8.0), 1 mM EDTA], gDNA was stored at 4ºC. Quantification and purity evaluation of gDNA solution was determined as described in Annex I.

4.2.3 Amplification of phosphate transporter genes by PCR

Thermocyclic amplification of phosphate transporter genes present of *H. sericea* genome was carried out using degenerated primers (*PiF1* and *PiR1*). *PiF1* and *PiR1* were designed based on conserved regions of plant phosphate transporter genes, as determined by the multiple sequence alignment of homologous genes. *PiF1* and *PiR1* design followed several parameters as suggested by Griffin and Griffin (1994). Both primers were 20 bases long, the G/C content was maintained between 45%-55%, the melting temperatures (Tm) were as similar as possible, and the corresponding annealing sites were separated by 437 bp. The redundancy of the degenerated primers, referred to as the possible combinations of synthesized oligonucleotides sequences, was kept low (128×) and synthesis was performed by MWG. Polymerase chain reaction (PCR) was performed using the *PureTaq™ Ready-to-Go™ PCR Beads* (GE Healthcare), according to the supplier's instructions. For each PCR, 25 pmol of each primer (*PiF1* and *PiR1*) and 50 ng of gDNA were used. The final concentration of each dNTP was 200 µM in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM $MgCl_2$. Sterile ultrapure water was added up to a final volume of 25 µL. Tubes were gently flicked with a finger, and components were brought back to the bottom of the tube with a few seconds centrifugation. PCR amplification was carried out on a *Mastercycler Gradient* (Eppendorf) with an annealing temperature gradient. PCR steps were as follows: (1) denaturation for 5 min at 94°C; (2) 35 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 40.0ºC, 45.2ºC, 50.5ºC or 55.8ºC, polymerization for 1 min at 72°C;

(3) extension for 10 min at 72°C. PCR products were resolved by agarose electrophoresis as described in Annex I.

4.2.4 Cloning of PCR fragments into pPCR-Script Amp SK(+) vector

DNA fragments used for cloning were recovered from agarose gels using the *GFX PCR DNA and Gel Band Purification Kit* (Amersham). A maximum of 300 mg of agarose gel, containing the DNA fragment of interest, were sliced in small pieces and placed in a microcentrifuge tube. An equal volume (1 mg = 1 µL) of capture buffer was added. After vortexing vigorously, the mixture was incubated at 60ºC until the agarose gel was completely dissolved (~10 min). The sample was loaded onto a *GFX* column previously placed in a collection tube and incubated at room temperature for 1 min. Following a centrifugation at 8000 *g* for 30 s, the flow-through was discarded. The *GFX* column was washed by loading 500 µL of washing buffer, followed by a centrifugation at 8000 *g* for 30 s. DNA elution was promoted by applying 50 µL of sterile ultra pure water, followed by incubation at room temperature for 1 min and centrifugation at 8000 *g* for 1 min.

DNA fragments were cloned onto the *pPCR-Script Amp SK(+)* cloning vector (Stratagene), an expression vector suited to efficiently clone blunt-ended PCR products. As *Taq* DNA polymerase amplifies fragments leaving A (adenine) overhangs, a polishing reaction was set up using 10 µL of the purified PCR product, 1 µL of 10 mM dNTP mix (2.5 mM each), 1.3 µL of 10× polishing buffer and 1 µL of cloned *Pfu* DNA polymerase (0.5 U) (Stratagene). The polishing reaction was mixed gently and incubated at 72ºC for 30 min. The ligation reaction was set up using 1 µL of the *pPCR-Script Amp SK(+)* cloning vector (10 ng/µL), 4 µL of the blunt-ended PCR product, 1 µL of *PCR-Script* 10× reaction buffer, 0.5 µL of 10 mM ATP, 1 µL of *Srf* I restriction enzyme (5 U/µL) (Roche), 1 µL of T4 DNA ligase (4 U/µL) and sterile ultrapure water up to a final volume of 10 µL. The ligation reaction was mixed gently and allowed to proceed at room temperature for 1 h before heat treating at 65°C for 10 min. An aliquot of the ligation reaction (2 µL) was used to transform *E. coli* JM109 cells followed by cell plating onto selective LB-Amp plates [1% (w/v) bacto-tryptone,

0.5% (w/v) yeast extract, 1% (w/v) NaCl, 100 µg.mL^{-1} ampicillin, pH 7.0] containing X-gal (40 µg.mL^{-1}) and IPTG (40 µg.mL^{-1}) for recombinant selection.

4.2.5 gDNA library preparation

H. sericea gDNA library preparation was performed according to standard procedures (Ausubel *et al*., 1996), following the instructions provided with the *Lambda DASH® II/BamH I Vector Kit* (Stratagene).

Preparation of genomic fragments to be cloned

Preparation of the gDNA library requires 20 kb DNA fragments. Pilot digestions (15 µL each) of gDNA were performed with 9 different concentrations of *Sau*3A I (Roche), ranging from 0.025 to 1x10^{-5} U.µg^{-1} of gDNA, for 1 h at 37ºC (refer to Annex 1). DNA fragments resulting from each reaction were analyzed by electrophoretic separation [0.5% (w/v) agarose, refer to Annex 1]. After determining the *Sau*3A I concentration at which 20 kb DNA fragments were predominantly obtained, 5 large scale digestions (500 µL each) were carried out using the optimized conditions and 100 µg of gDNA each. Digested DNA fragments were extracted twice in one volume of chloroform-isoamyl alcohol [24:1 (v/v)] and precipitated with two volumes of ethanol at -20ºC during overnight. After centrifugation at 14000 *g* for 10 min at 4°C, DNA was resuspended in 200 µL of water. DNA fragments were subsequently fractioned by centrifugation in continuous sucrose gradient. Sucrose gradient was produced in 30 mL tubes with a gradient generator, using 10% (w/v) and 40% (w/v) sucrose solutions prepared in gradient buffer [10 mM Tris-HCl (pH 8.0), 1 M NaCl and 5 mM EDTA]. After being incubated at 65ºC for 5 min, DNA samples were applied to the top of the gradient and centrifuged at 12000 *g* for 20 h at 4ºC. The gradient was fractionated in 1 mL samples, collected from the bottom. After electrophoretic analysis [0.5% (w/v) agarose, refer to Annex 1], fractions containing 20 kb DNA fragments were precipitated with two volumes of ethanol at -20ºC, during overnight. DNA was recovered by centrifugation at 14000 *g* for 10 min at 4°C and resuspended in 100 µL of

ultrapure water. Quantification and purity evaluation of DNA solution was determined as described in Annex I.

Cloning of genomic fragments and library packaging

The ligation of inserts to *Lambda DASH® II* vector (Stratagene) was performed using 0.4 µg of *H. sericea* gDNA fragments, 1 µg of lambda vector, 0.5 µL of 10× ligase buffer [500 mM Tris-HCl (pH 7.5), 70 mM $MgCl_2$, 10 mM DTT], 0.5 µL of 10 mM ATP and 2 U of T4 DNA ligase (Roche) up to a final volume of 5 µL. The mixture was incubated at 4ºC, during overnight.

The lambda packaging extracts (*Gigapack® III Packaging Extract*, Stratagene) were removed from -80ºC and quickly thawed. The volume of ligation reaction mixture, corresponding to 1.0 µg of DNA, was immediately added to the packaging extract and gently mixed. The packaging mixture was then incubated at 22ºC for 2 h, followed by addition of 500 µL of SM buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgSO_4.7H_2O$; 0.01% (w/v) gelatine] and 20 µL of chloroform. The suspension was mixed and briefly centrifuged to sediment debris.

Titering the genomic library

A single colony of *E. coli* XL1-Blue MRF' strain (Jerpseth *et al*., 1992) was used to inoculate 500 mL of LB [1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0], supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose. Growth was promoted by incubation at 30ºC with shaking (200 rpm), during overnight. The bacteria were sedimented by centrifugation at 500 *g* for 10 min, and resuspended in half the original volume with sterile 10 mM $MgSO_4$. Cells were further diluted to an A_{600} = 0.5 with sterile 10 mM $MgSO_4$.

For titering the phage solution, 10-fold dilutions (10^{-1} to 10^{-3}) of the packaged reaction were prepared in SM buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgSO_4.7H_2O$; 0.01% (w/v) gelatine], and 1 µL of each dilution was added to 200 µL of XL1-Blue MRF' host cells. After incubation at 37°C for 15 min, 3 mL of melted Top Agarose NZY medium [0.5% (w/v) yeast extract, 1% (w/v) NZ amine (casein hydrolysate), 0.5% (w/v) NaCl, 0.2% (w/v)

$MgSO_4.7H_2O$, 0.7% (w/v) agarose, pH 7.5] were added to the phage-cell suspension. After mixing, the suspension was quickly poured onto the NZY agar plates [0.5% (w/v) yeast extract, 1% (w/v) NZ amine (casein hydrolysate), 0.5% (w/v) NaCl, 0.2% (w/v) $MgSO_4.7H_2O$, 1.5% (w/v) agar, pH 7.5] and incubated at 37°C for 6 h. The titer was determined by counting the number of phage plaques.

Amplification of the genomic library

The gDNA library was amplified by separately combine 20 aliquots of the packaged library suspension, each containing $5x10^4$ pfu, with 600 µL of XL1-Blue MRF' cells prepared as previously described. After incubation at 37ºC for 15 min, 6.5 mL of melted Top Agarose NZY medium [0.5% (w/v) yeast extract, 1% (w/v) NZ amine (casein hydrolysate), 0.5% (w/v) NaCl, 0.2% (w/v) $MgSO_4.7H_2O$, 0.7% (w/v) agarose, pH 7.5] were added. The mixture was quickly poured onto the NZY agar medium [0.5% (w/v) yeast extract, 1% (w/v) NZ amine (casein hydrolysate), 0.5% (w/v) NaCl, 0.2% (w/v) $MgSO_4.7H_2O$, 1.5% (w/v) agar, pH 7.5] (150-mm plates) and incubated at 37°C for 6 h. Plates were overlaid with 8 mL of SM buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgSO_4.7H_2O$; 0.01% (w/v) gelatine]. For promoting the diffusion of phage into the SM buffer, an overnight incubation with gentle agitation was performed at 4ºC. The bacteriophage suspension recovered from all the plates was pooled together and chloroform to a 5% (v/v) final concentration was added. The cell debris was removed by centrifugation for 10 min at 500 *g*. After recovering the supernatant, chloroform was added to a 0.3% (v/v) final concentration and the suspension was stored at 4ºC. Long term storage was made at -80ºC using aliquots of the amplified library in 7% (v/v) DMSO. Before using the genomic library, the titer was determined using 10-fold serial dilutions and XL1-Blue MRF' host cells, as previously described.

4.2.6 gDNA library screening

Amplified *H. sericea* DNA fragments coding for phosphate transporters (section 4.2.3) were used as homologous probes for the screening of *H. sericea* gDNA library.

The gDNA library was plated onto 150-mm NZY agar plates, as described in section 4.2.5 (*Amplification of genomic library*), but using a density of 25000 plaques per plate. Inoculated plates were incubated at 37°C for 12 h, followed by further incubation at 4ºC for 2 h. Phage particles were transferred from plaques to duplicate nylon filter discs (*Hybond-N+*; Amersham). The first disc was placed onto each NZY agar plate for 2 min, while the replica disc was placed for 4 min, to allow the transfer of phage particles to the membrane. Release of DNA from the phage particle was promoted by incubating the membrane in denaturation solution (1.5 M NaCl, 0.5 M NaOH) for 2 min. Plaque lifts were then transferred to the neutralization solution [0.5 M Tris-HCl (pH 8.0), 1.5 M NaCl] for 5 min, rinsed in washing solution [0.2 M Tris-HCl (pH 7.5); 30 mM Na citrate (pH 7.0); 0.3 M NaCl] for 30 seconds and air dried on *Whatman® 3MM paper*. DNA was crosslinked to the membranes by UV light in a *Stratalinker® UV crosslinker* (Model 1800, Stratagene) using the autocrosslink setting (1200 mJ). The hybridization proceeded as described in Annex 1.

The phage plaques responsible for coincident positive signals in both lifts were removed from the NZY agar plate and resuspended in 500 µL of SM buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgSO_4.7H_2O$; 0.01% (w/v) gelatine] containing 20 µL of chloroform. After vortexing, lambda phages were re-plated at low density in NZY agar medium, using the same conditions as previously referred (section 4.2.5, *Titering the genomic library*), but incubating the plates at 37°C for 12 h, followed by further incubation at 4ºC for 2 h. For the second screening, *Hybond-N* (Amersham) nylon filter discs were used, but no duplicates were performed. Positive individualized clones were recovered from the agar plates, resuspended in 500 µL of SM buffer [50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM $MgSO_4.7H_2O$; 0.01% (w/v) gelatine] containing 20 µL of chloroform and vortexed.

4.2.7 DNA recovery from lambda phages

DNA recovery from lambda phages was performed using the *Lambda Kit* (Qiagen). Briefly, 250 µL of the isolated single-clone lambda phage suspension (section 4.2.6) were combined with 200 µL of XL1-Blue MRF' cells, prepared as referred in section 4.2.5 (*Titering the genomic library*), and incubated at 37°C for 15 min. After incubation at 37°C for 15 min, 3 mL of NZY medium [0.5% (w/v) yeast extract, 1% (w/v) NZ amine (casein hydrolysate), 0.5% (w/v) NaCl, 0.2% (w/v) $MgSO_4.7H_2O$, pH 7.5] were added and the suspension was incubated at 37ºC, during overnight, with agitation. Following the addition of 2% (v/v) chloroform, the incubation at 37°C was allowed to proceed for further 15 min to enhance lysis efficiency. The phage suspension was centrifuged at 12000 *g* for 10 min to remove bacterial debris. After adding 10 µL of Buffer L1 [300 mM NaCl, 100 mM Tris-HCl (pH 7.5), 10 mM EDTA, 0.2 $mg.mL^{-1}$ BSA, 20 $mg.mL^{-1}$ RNase A, 6 $mg.mL^{-1}$ DNase I] to 3 mL of the supernatant, an incubation at 37ºC for 30 min was performed for bacterial DNA and RNA removal. Following the addition of 700 µL of ice-cold buffer L2 [30% (w/v) polyethylene glycol (PEG 6000), 3 M NaCl], the solution was incubated on ice for 60 min to promote phage precipitation. After centrifugation at 12000 *g* for 10 min, the pellet was resuspended in 1 mL of buffer L3 [100 mM NaCl, 100 mM Tris-HCl (pH 7.5), 25 mM EDTA].

To allow denaturation of phage proteins and release of DNA, phage suspension was gently mixed with 1 mL of buffer L4 [4% (w/v) SDS] and incubated at 70ºC for 10 min. The DNA solution was recovered after centrifugation at 15000 *g* for 10 min, at 4ºC. A Qiagen-tip column was equilibrated by applying 1 mL of Buffer QBT [750 mM NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) isopropanol, 0.15% (w/v) Triton X-100], using the gravity flow. After applying the DNA solution, the column was washed with 2 mL of Buffer QC [1.0 M NaCl, 50 mM MOPS (pH 7.0), 15% (v/v) isopropanol]. DNA was eluted with 1.5 mL of Buffer QF [1.25 M NaCl, 50 mM Tris-HCl (pH 8.5)] and precipitated by adding 1 mL of isopropanol. The solution was mixed and centrifuged at 15000 *g* for 30 min at 4°C. The precipitated DNA was washed with 1 mL 70% (v/v) ethanol, air-dried

and dissolved in 50 µL of ultrapure water. Quantification and purity evaluation of gDNA solution was determined as described in Annex I.

4.2.8 Sequence search and analysis

DNA fragments were sequenced by *BigDye™ Terminator Chemistry* (ABI Prism®; Stabvida sequencing services). Database search for nucleotide and protein sequences was performed at the NCBI website using *Entrez* (http://www.ncbi.nlm.nih.gov/Entrez/index.html). The database search for highly similar sequences was carried out using the BLAST algorithm (Altschul *et al*., 1997; http://www.ncbi.nlm.nih.gov/BLAST). Nucleotide editing and analysis was performed using the sequence analysis software from *DNASTAR* (Lasergene). Within the software package, *EditSeq* was used to edit sequences and multiple sequence alignments were generated with *MegAlign*, using the ClustalW algorithm (Jeanmougin *et al*., 1998). The unrooted phylogenetic tree, based on the alignment of plant Pi transporter genes, was constructed using FigTree 1.2.2 (freeware). The maximum likelihood method (Doyle and Gaut, 2000) was used to resolve the phylogenetic relationship between the newly identified *H. sericea* Pi transporter genes and those from other plant species.

The analysis of *Arabidopsis thaliana* Pi transporter genes expression was performed using eFP Browser (Winter *et al*., 2007; http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi).

Prediction of transmembrane domains was carried out using the TMHMM server version 2.0 (Sonnhammer *et al*., 1998; http://www.cbs.dtu.dk/services/TMHMM). All predictions were performed using standard settings.

4.3 RESULTS

4.3.1 Search for genes encoding PhTs in Hakea sericea genome

In order to identify genes encoding PhTs in *H. sericea*, two different approaches were attempted. Using *H. sericea* genomic DNA as template, DNA fragments from *H. sericea PhT* genes were amplified using degenerated primers. The amplified fragments were subsequently used as probes for screening other *PhT* genes in a gDNA library of *H. sericea*.

The amplification of *PhT* gene fragments in *H. sericea* genome was carried out using degenerated primers (*PiF1* and *PiR1*). For their design, the conserved regions of higher plant H^+/Pi symporters encoding genes were used, as determined by the multiple sequence alignment of homologous genes (Fig 4.2) The electrophoretic analysis of the reaction products showed the amplification of fragments with the expected molecular weight of 437 bp in all tested annealing temperatures (Fig 4.3.A). When performing the amplification using the annealing temperature of 40ºC, besides the 437 bp fragments, other unspecific amplified products with different molecular weights were observed. However, subsequent Southern blot analysis using as [α-^{32}P]dCTP labeled probe a *LaPT1* gene fragment from *Lupinus albus* showed that only the 437 bp fragments hybridized with *LaPT1* (Fig 4.3.B). Therefore, these fragments will be further referred to as *PiT* fragments.

After being purified from the gel, the *PiT* fragments from the PCR amplification using 55°C as annealing temperature were cloned into the pPCR-Script Amp SK(+) vector. Since fragments from different *PhT* genes could have been simultaneously amplified, six recombinant plasmids were isolated, purified and digested with *Eco*RI/*Sac*I. The restriction fragments were analyzed by electrophoresis and Southern blot analysis (Fig 4.4). Two different restriction patterns were detected, indicating the amplification of fragments from at least two different *PhT* genes during PCR [(*PiT2*, *PiT4* and *PiT5*) and (*PiT1*, *PiT3* and *PiT6*)]. *PiT2* and *PiT6* fragments were then completely sequenced (Fig 4.5).

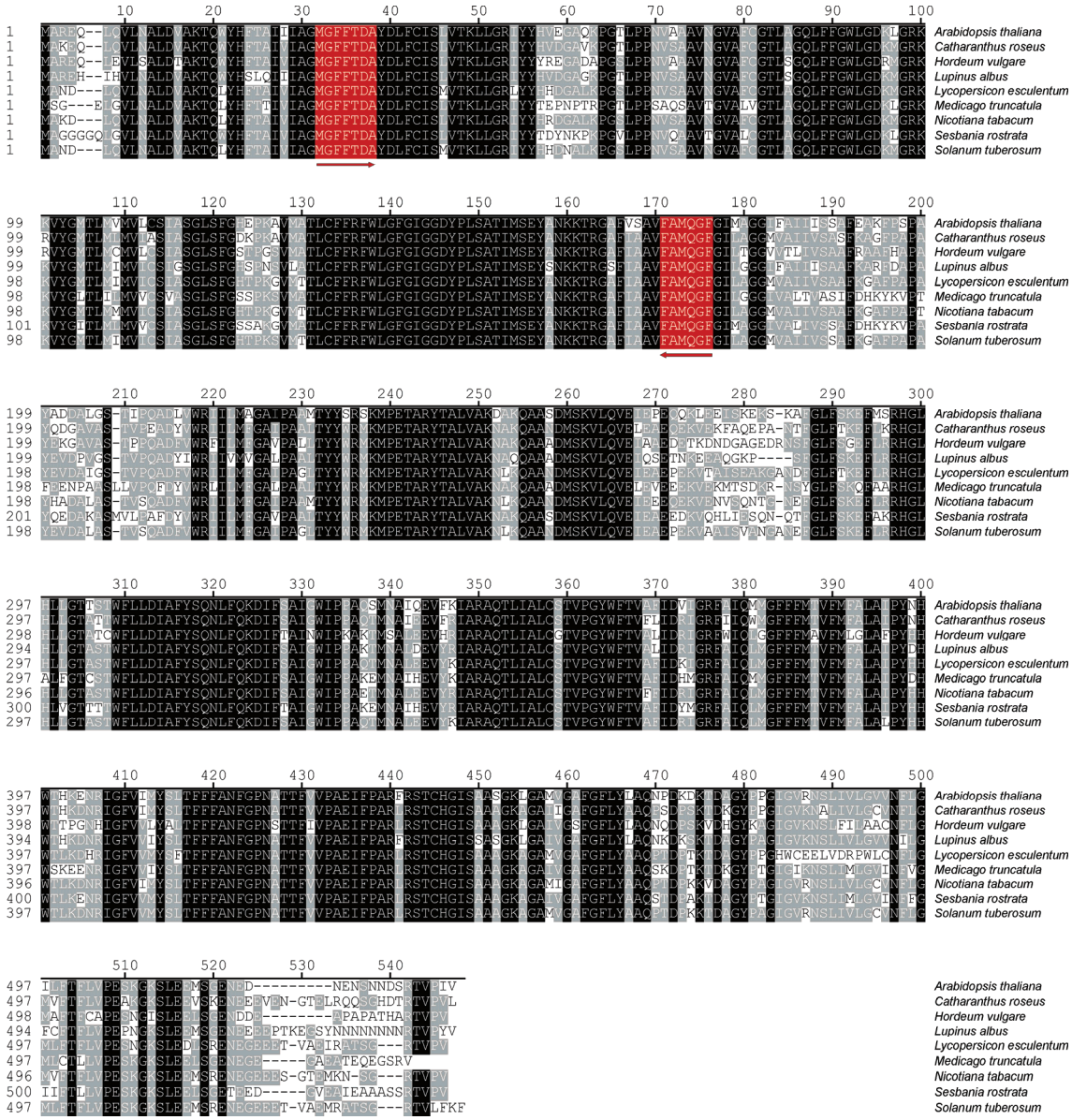

Figure 4.2 Amino acid sequence alignment of higher plant H^+/Pi symporters.

The residues conserved among the majority of the sequences are shadowed in gray, and those conserved in all sequences are shadowed in black. The sequences used for degenerated primer design are highlighted in red. [*Arabidopsis thaliana* – acc. no. NP_181428; *Catharanthus roseus* – acc. no. BAA20522; *Hordeum vulgare* – acc. no. AAO72437; *Lupinus albus* – acc. no. AAK01938; *Lycopersicon esculentum* – acc. no. AAB82146; *Medicago truncatula* – acc. no. ABM69111; *Nicotiana tabacum* – acc. no. AAF74025; *Sesbania rostrata* – acc. no. CAC28219; *Solanum tuberosum* – acc. no. AAD38859].

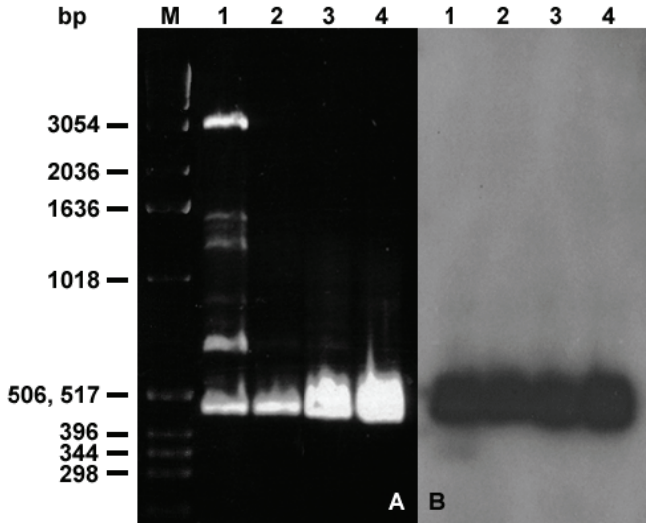

Figure 4.3 Southern analysis of PCR amplification products of *H. sericea* gDNA using degenerated primers for the conserved regions of *PiT* genes from higher plants.

For the amplification, annealing temperatures of 40°C (1); 45°C (2); 50°C (3) and 55°C (4) were used. (A) Electrophoretic analysis [1.2% (w/v) agarose gel]; (B) Southern blot analysis performed using the [α-^{32}P]dCTP labeled *Lupinus albus LaPT1* gene as probe. (M) Molecular weight marker *1 kb DNA ladder* (Invitrogen).

The search for other genes encoding PhTs in the *H. sericea* genome proceeded by the screening of a gDNA library of *H. sericea*. This library was constructed using gDNA extracted from young leaves of hydroponically-grown *H. sericea* plants (Fig 4.6.A). After optimization of DNA/restriction enzyme (*Sau*3A I) ratio to be used in gDNA digestion (Fig 4.6.B), DNA fragments were fractionated on a continuous gradient of sucrose (Fig 4.6.C), from which 20 kb fragments were collected and cloned into *Lambda DASH® II* vector (Stratagene). After packaging and amplification, the library presented a final titre of 2.28×10^5 pfu.µL^{-1}.

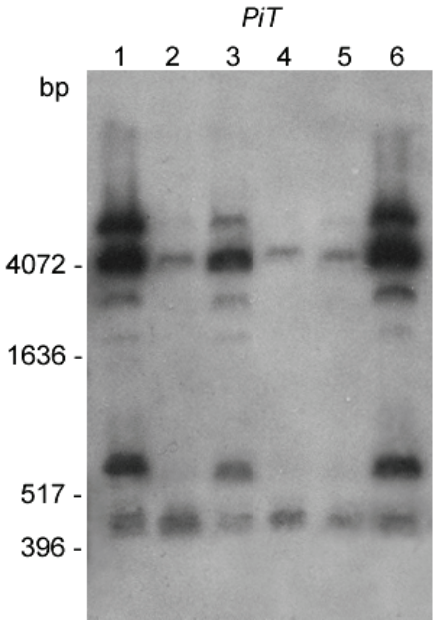

Figure 4.4 Restriction pattern (*Eco*RI/*Sac*I) of different recombinant plasmids containing *PiT* fragments (*PiT1-6*).

Following electrophoretic analysis [1.2% (w/v) agarose gel], the Southern blot analysis was performed using as [α-32P]dCTP labeled probe a LaPT1 gene fragment from Lupinus albus. Molecular weight marker 1 kb DNA ladder (Invitrogen).

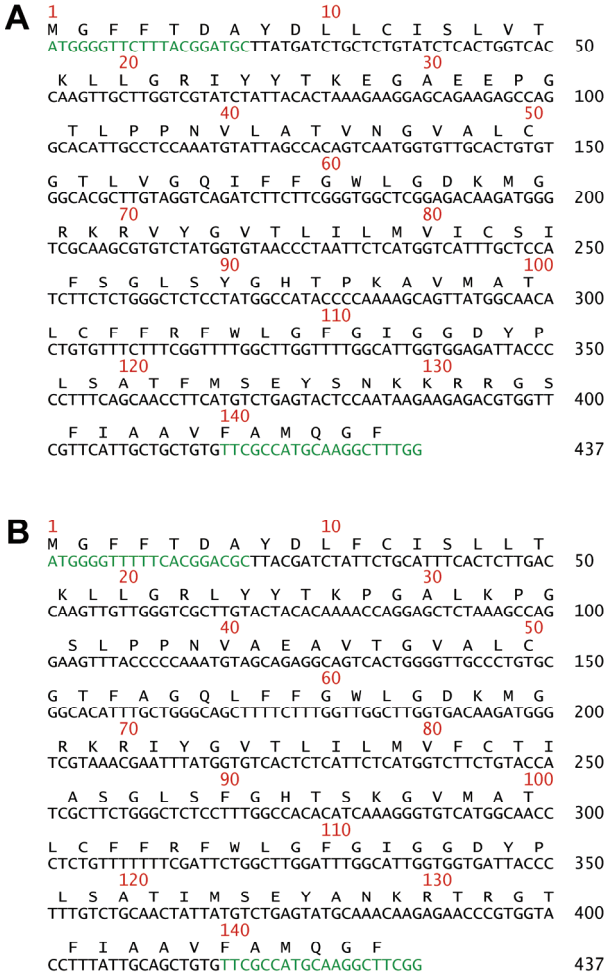

Figure 4.5 Nucleotide and deduced amino acid sequences of (A) *PiT2* and (B) *PiT6* fragments from *H.sericea*.

The deduced amino acid sequence is represented above the nucleotide sequence, in the one letter code. The numbers on the right correspond to the nucleotides and the numbers above (in red) to the amino acids. The sequences corresponding to the primers used in the amplification are represented in green.

For the screening of the gDNA library, a mixture of the previously identified *PiT2* and *PiT6* fragments labeled with [α-^{32}P]dCTP was used as probe. Several clones hybridizing with the labeled *PiT2* and *PiT6* probes were identified (Fig 4.7). Fifteen lambda clones from different intensity spots were isolated, purified and digested with *Eco*RI, and the corresponding restriction patterns were obtained by electrophoresis (Fig 4.8). All the analysed clones presented a different restriction pattern, suggesting that all of them were independent clones from *H. sericea* genomic library. Therefore, the recombinant DNA from all the

clones was used as template in a PCR amplification with *PiF1* and *PiR1* as degenerated primers. The amplified fragments were purified from the gel and completely sequenced. Two new *PhT* genes from *H. sericea* (*PH5* and *PH7*) were then identified (Fig 4.9).

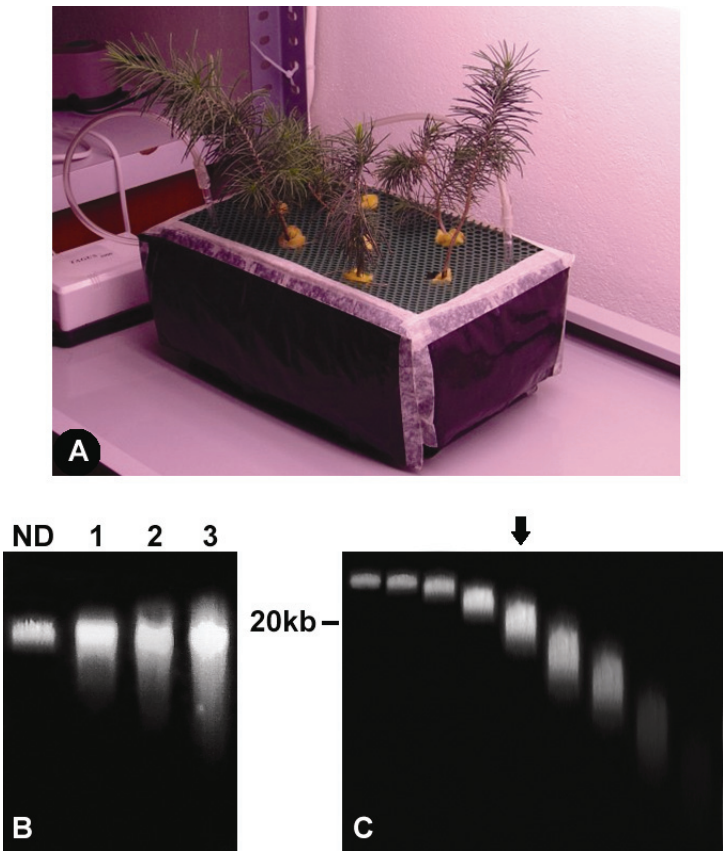

Figure 4.6 Construction of a *Hakea sericea* gDNA library.

(A) Genomic DNA was extracted from young leaves of hydroponically-grown plants. (B) Electrophoretic analysis [0.5% (w/v) agarose gel] of DNA fragments after digestion with Sau3A I (Roche), for 1h at 37ºC. For digestion optimization, 9 concentrations of Sau3A I were used, including 1.9x10-4 U.µg-1 of gDNA (1), 7.8x10-4 U.µg-1 of gDNA (2) and 3.1x10-3 U.µg-1 of gDNA (3). Non-digested gDNA (ND) was used as control. (C) Electrophoretic analysis [0.5% (w/v) agarose gel] of DNA fragments fractioned by centrifugation on continuous sucrose gradient. The arrow points to the chosen fraction containing fragments around 20 kb.

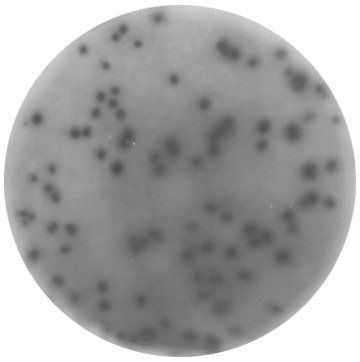

Figure 4.7 Autoradiogram corresponding to the screening of the *H. sericea* gDNA library with a mixture of the *H. sericea PiT2* and *PiT6* fragments labeled with [α-^{32}P]dCTP.

Each dark spot corresponds to a clone that hybridizes with *PiT2*/*PiT6* probe.

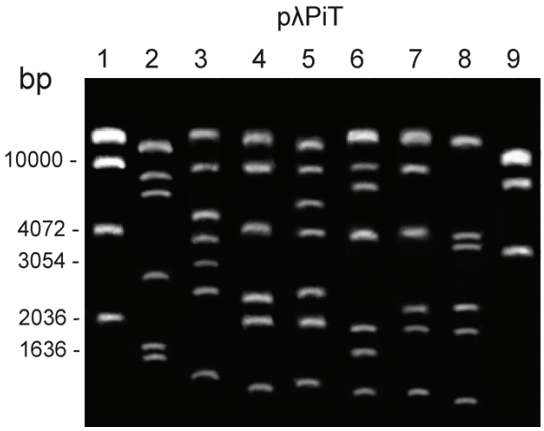

Figure 4.8 Restriction pattern (*Eco*RI) of different clones containing fragments that hybridized with *PiT2*/*PiT6* probe.

A different restriction pattern was observed for all the recombinant clones analysed. Electrophoresis was performed in a 1.0% (w/v) agarose gel. Molecular weight marker *1 kb DNA ladder* (Invitrogen)

A

```
1                                 10
 Y  D  L  F  C  I  S  L  V  T  K  L  L  G  R  I  Y
CTATGATCTCTTCTGTATCTCCCTTGTCACCAAATTGCTTGGCCGTATCT   50
         20                                30
  Y  T  K  A  G  S  S  T  P  G  T  L  P  P  T  V
ATTATACCAAAGCTGGATCATCCACTCCAGGTACATTACCACCCACAGTA  100
               40                                50
N  S  A  I  N  G  V  A  F  C  G  T  L  A  G  Q  L
AACTCAGCCATCAATGGTGTTGCCTTCTGTGGAACCCTTGCTGGGCAATT  150
                        60
 F  F  G  W  L  G  D  K  M  G  R  K  R  V  Y  G  L
GTTCTTTGGATGGCTCGGAGACAAGATGGGCAGGAAGCGTGTTTACGGTC  200
         70                                80
  T  L  V  L  M  V  V  C  S  V  A  S  G  L  S  F
TCACGCTTGTGCTCATGGTTGTGTGCTCTGTTGCCTCTGGTCTATCCTTT  250
               90                                100
G  H  S  A  K  G  V  M  A  T  L  C  F  F  R  F  W
GGTCACTCAGCCAAGGGAGTCATGGCCACCTTATGTTTCTTCAGGTTTTG  300
                        110
 L  G  F  G  I  G  G  D  Y  P  L  S  A  T  I  M  S
GTTAGGTTTTGGCATTGGTGGTGACTACCCATTATCAGCAACCATTATGT  350
         120                               130
  E  Y  A  N  K  K  T  R  G  A  F  I  A  A  V  F
CTGAGTATGCCAACAAGAAAACAAGAGGTGCCTTCATTGCTGCAGTGTTT  400

A  M  Q  G  F
GCTATGCAAGGTTTTGG                                   417
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B

```
1                                 10
 Y  D  L  F  C  V  S  L  V  T  K  L  I  G  R  I  Y
CTATGACCTCTTCTGTGTTTCTCTTGTCACCAAGTTGATAGGTCGCATCT   50
         20                                30
  Y  Y  S  P  G  S  P  N  P  G  S  L  P  P  S  A
ATTACTATAGTCCTGGATCACCCAACCCTGGTTCATTACCCCCTAGTGCA  100
               40                                50
A  S  A  I  N  G  V  A  L  C  G  T  L  A  G  Q  L
GCCTCTGCCATCAATGGTGTTGCCCTTTGTGGCACCCTTGCTGGACAACT  150
                        60
 F  F  G  W  L  G  D  K  M  G  R  K  R  V  Y  G  V
ATTCTTCGGATGGCTCGGAGATAAGATGGGTCGAAAGCGTGTGTATGGTG  200
         70                                80
  T  L  M  L  M  V  V  C  S  I  A  S  G  L  S  F
TTACACTTATGCTTATGGTTGTTTGCTCTATTGCTTCTGGTCTATCCTTT  250
               90                                100
G  S  T  A  K  G  V  V  A  T  L  C  F  F  R  F  W
GGCTCCACAGCAAAAGGAGTAGTGGCCACATTGTGTTTCTTCAGGTTTTG  300
                        110
 L  G  F  G  I  G  G  D  Y  P  L  S  A  T  I  M  S
GTTAGGTTTTGGCATTGGAGGTGATTACCCATTATCAGCTACCATTATGT  350
         120                               130
  E  Y  A  N  K  K  T  R  G  A  F  I  A  A  V  F
CTGAGTATGCTAACAAGAAGACCAGAGGTGCCTTCATTGCTGCAGTTTTT  400

A  M  Q  G  F
GCCATGCAAGGATTTGG                                   417
```

Figure 4.9 Partial nucleotide and deduced amino acid sequences of (A) PH5 and (B) PH7 identified in H. 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 (in red) are related with the amino acids.

4.3.2 PhTs in plants: phylogenetic analysis and domain prediction

The phylogenetic analysis of 55 PhTs found in mono- and dicotyledons was performed. The multiple alignment of aminoacidic sequences was performed using the ClustalW method and an unrooted tree was plotted using the maximum-likelihood algorithm (Fig 4.10). Analysed proteins belong to three distinct subfamilies of Pi transporters: PhT1, which includes high- and low-affinity H^+/Pi symporters presumed to be targeted to the plasma membrane; PhT2, a family of low-affinity H^+/Pi symporters expressed in chloroplasts; and a hypothetical Na^+/Pi symporters family. Moreover, the PhT1 family was further subdivided in two major clusters, comprising monocot or dicot proteins. The organ in which PhT proteins are mainly expressed is also represented in the phylogenetic tree (Fig 4.10).

The phylogenetic relations between PiT2, PiT6, PH5 and PH7 were also analysed (Fig 4.11). As their incomplete deduced aminoacid sequences impaired direct comparison with PhTs from other species, the 55 PhT sequences were trimmed before alignment to include only the same region as *H. sericea* sequences. As in Fig 4.10, results primarily showed an out-group composed by PhT2 members. The hypothetical Na^+/Pi symporters also clustered together, but shared their branch with some PhT1 members. Regarding *H. sericea* PhTs, PiT2 clustered with a Pi transporter expressed mainly in flowers and pollen of *Arabidopsis thaliana* (At5G43340). PiT6 was included in the cluster in which the Na^+/Pi symporters were included, together with other dicot PhT1 members: two *A. thaliana* proteins (At1G20860 and At1G76430) and a PhT1 member expressed in the roots of *Medicago truncatula* (MtPT1;4). Finally, PH5 and PH7 clustered together with PhT1 members from the dicots *A. thaliana*, *M. truncatula* and *L. albus*, which are mostly expressed in roots (At5G43350, At5G43360, At5G43370 and LaPT1;2), senescent leaves (At2G32830) or in a location yet to study (MtPT1;6). Also included in this cluster is the PhT1 proteins from the monocot *Oryza sativa* (OsPT1;4 and OsPT1;5), for which the expression site is currently unknown.

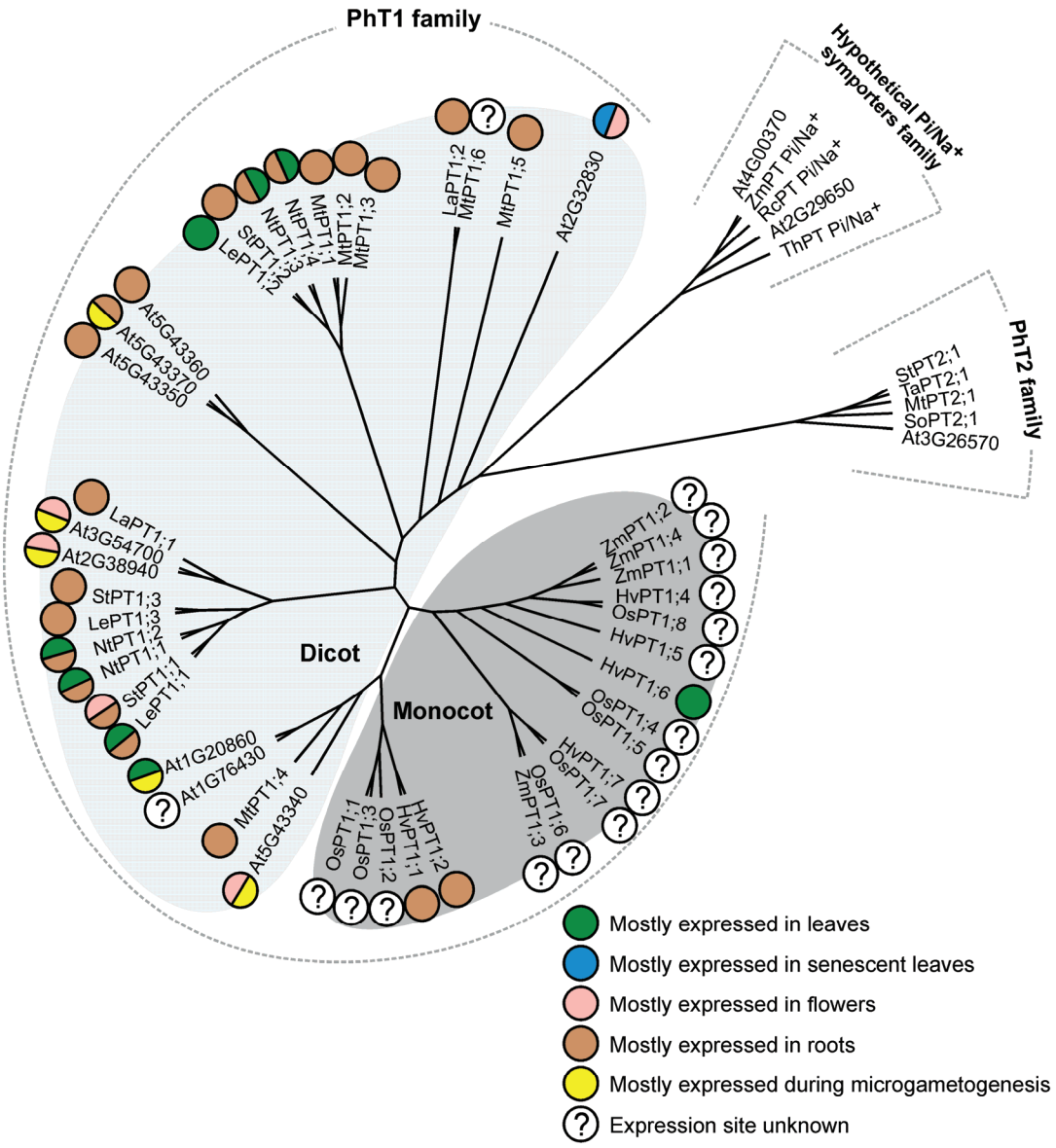

Figure 4.10 Phylogenetic tree of plant PhTs and corresponding expression sites.

Multiple sequence alignments were generated with *MegAlign* (DNASTAR, Lasergene), using the ClustalW algorithm (Jeanmougin *et al.*, 1998). The unrooted phylogenetic tree was constructed using the FigTree v1.2.2 (freeware). The maximum likelihood method (Doyle and Gaut, 2000) was used to resolve the phylogenetic relationship between phosphate transporters. The organs in which expression analysis of PhT gene/protein is known are displayed by coloured circles. The analysis of *Arabidopsis thaliana* (At) gene expression was performed using eFP Browser. Other PhT protein/gene expression information was obtained from the literature [*Hordeum vulgare* (HvPT) in Rae *et al.* (2003); *Lupinus albus* (LaPT) in Liu *et al.* (2001); *Lycopersicon esculentum* (LePT) in Liu *et al.* (1998a); Nagy *et al.* (2005); Xu *et al.* (2007); *Medicago truncatula* (MtPT) in Liu *et al*. (1998b); Harrison *et al*. (2002); Liu *et al*. (2008); *Nicotiana tabacum* (NtPT) in Kai *et al*. (2002); *Solanum tuberosum* (StPT) in Leggewie *et al*. (1997); Rausch *et al*. (2001)]. Several identified PhT proteins displayed in the phylogenetic tree do not have yet information about their expression, namely proteins/genes from *Oryza sativa* (OsPT) *Ricinus communis* (RcPT), *Spinacia oleracea* (SoPT), *Triticum aestivum* (TaPT), *Thellungiella halophila* (ThPT), *Zea mays* (ZmPT).

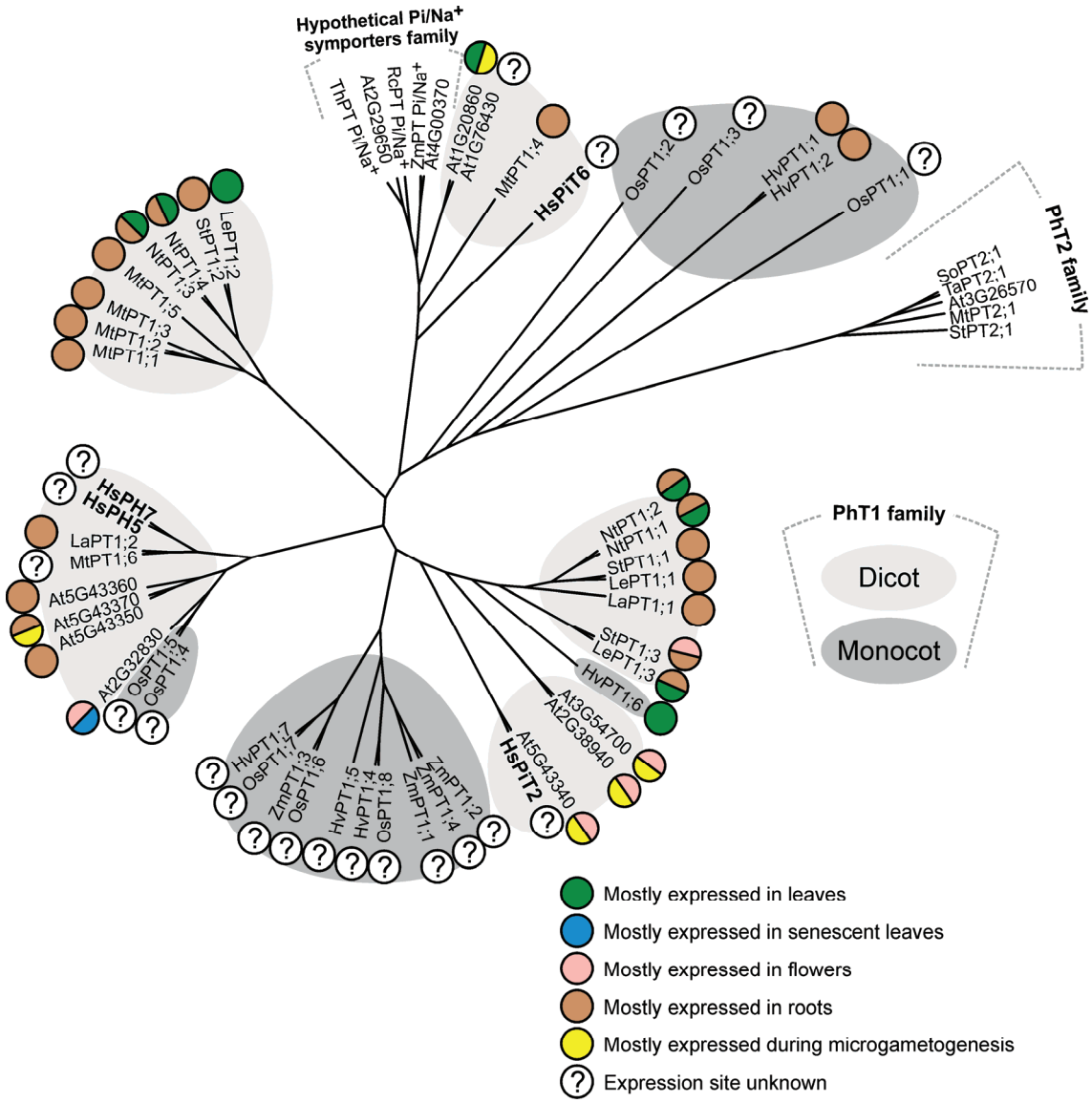

Figure 4.11 Phylogenetic relation between *H. sericea* PhTs and orthologs from other plant species.

All the sequences were trimmed before alignment to include only the same region of the *H. sericea* incomplete sequences. Multiple sequence alignments were generated with *MegAlign* (DNASTAR, Lasergene), using the ClustalW algorithm (Jeanmougin *et al*., 1998). The unrooted phylogenetic tree was constructed using the FigTree v1.2.2 (freeware). The maximum likelihood method (Doyle and Gaut, 2000) was used to resolve the phylogenetic relationship between phosphate transporters. The organs in which expression analysis of PhT gene/protein is known are displayed by coloured circles. The analysis of *Arabidopsis thaliana* (At) gene expression was performed using eFP Browser. Other PhT protein/gene expression information was obtained from the literature [*Hordeum vulgare* (HvPT) in Rae *et al.* (2003); *Lupinus albus* (LaPT) in Liu *et al.* (2001); *Lycopersicon esculentum* (LePT) in Liu *et al.* (1998a); Nagy *et al.* (2005); Xu *et al.* (2007); *Medicago truncatula* (MtPT) in Liu *et al*. (1998b); Harrison *et al*. (2002); Liu *et al*. (2008); *Nicotiana tabacum* (NtPT) in Kai *et al*. (2002); *Solanum tuberosum* (StPT) in Leggewie *et al*. (1997); Rausch *et al*. (2001)]. Several identified PhT proteins displayed in the phylogenetic tree do not have yet information about their expression, namely proteins/genes from *Oryza sativa* (OsPT) *Ricinus communis* (RcPT), *Spinacia oleracea* (SoPT), *Triticum aestivum* (TaPT), *Thellungiella halophila* (ThPT), *Zea mays* (ZmPT).

The transmembrane domains (TMD) of representatives from the three analysed PhT subfamilies were predicted with the TMHMM v2 algorithm (Fig 4.12). PhT2 members presented typical 12 membrane spanning domains, separated by a hydrophilic loop between TMD8 and TMD9 (Fig 4.12.A). It also presents a long extracellular hydrophilic N terminus. For the hypothetical Na^+/Pi symporters, TMHMM v2 algorithm predicted 10 membrane spanning domains and a long hydrophilic N terminus (Fig 4.12.B). PhT1 members, regardless of their main expression site, typically present 12 membrane spanning domains as PhT2 proteins, but being divided into two groups of 6 each (Fig 4.12.C). Both groups are connected by a large hydrophilic charged region, between TMD6 and TMD7, containing 60 amino acids. For the incomplete deduced aminoacid sequences of *PiT2*, *PiT6*, *PH5* and *PH7*, the TMHMM v2 algorithm predicted three transmembrane domains that overlap TMD2, TMD3 and TMD4 from PhT1 (Fig 4.12.D).

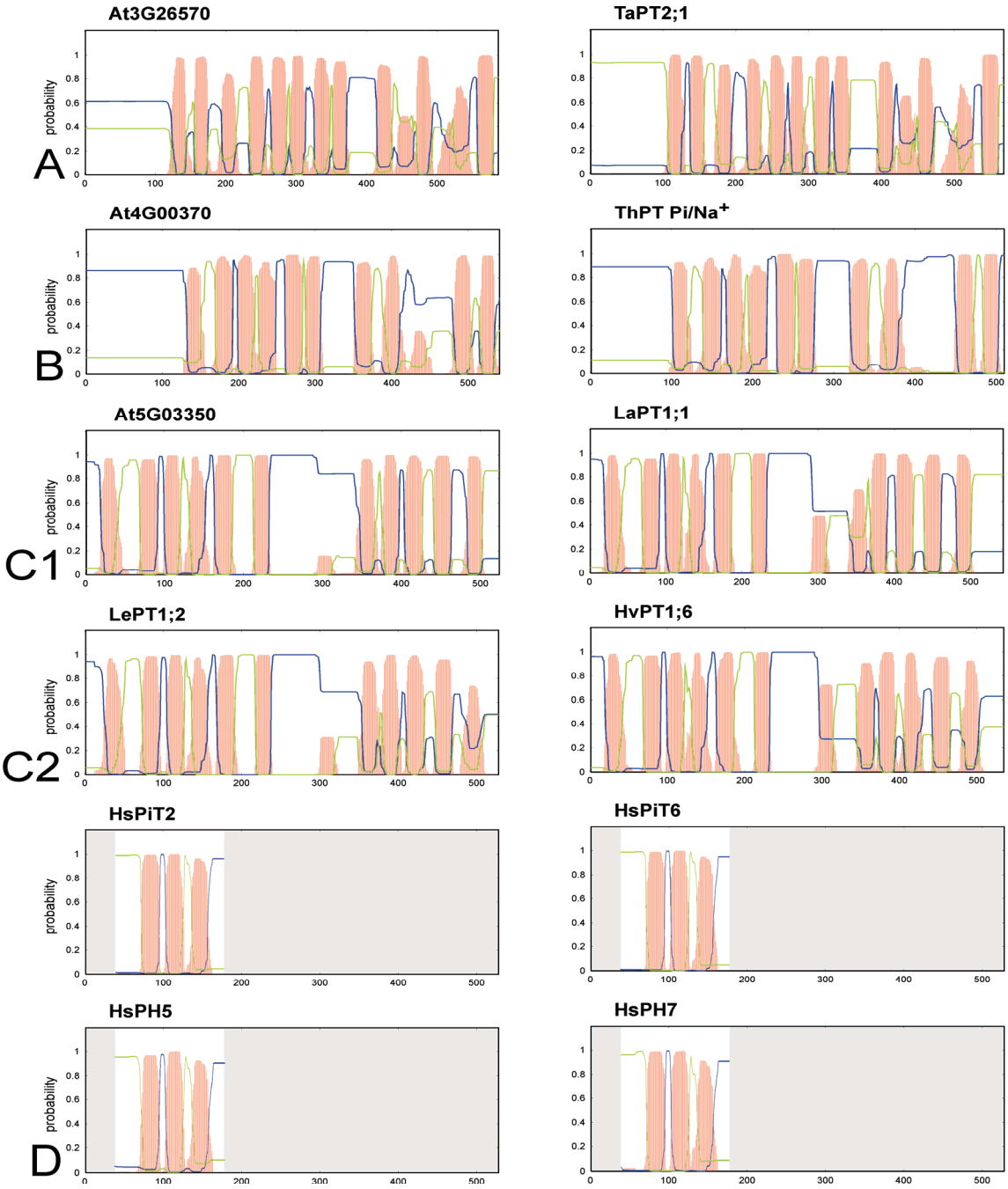

Figure 4.12 Predicted transmembrane domains (TMD) of members from the three analysed PhT subfamilies and identified H^+/Pi symporters from *H. sericea*.

(A) PhT2 family of chloroplastidial H^+/Pi symporters; (B) hypothetical Na^+/Pi symporters family; (C) PhT1 family of H^+/Pi symporters, including members mainly expressed in roots (C1) and mainly expressed in leaves (C2); (D) Pi transporter fragments identified in *H. sericea*. Transmembranar domains are depicted in red. Those protein regions external to the membrane surface are highlighted in green and those on the cytosolic surface are highlighted in blue. The prediction was performed with TMHMM v2 algorithm (Sonnhammer *et al*., 1998). (At proteins are from *Arabidopsis thaliana*; HvPT is from *Hordeum vulgare*; LaPT is from *Lupinus albus*; LePT is from *Lycopersicon esculentum*; TaPT is from *Triticum aestivum*; ThPT is from *Thellungiella halophila*).

4.4 DISCUSSION

Phosphate is one of the most important macronutrients for plant growth and development. It is generally acquired by epidermal cells at the root-soil interface and distributed to the aerial parts of the plant through its secretion into the xylem sap. During Pi shortage or senescence, this nutrient can eventually be cycled through the plant via the phloem (Daram, 1999; Mimura, 1999; Raghothama, 1999). It is commonly accepted that both processes occur at the plasma membrane through the activity of high- and low-affinity H^+/Pi symporters from the PhT1 subfamily (Lefebvre and Clarkson, 1984; Ullrich-Eberius *et al*., 1984; Mimura, 1999; Rausch and Bucher, 2002). The computational modelling of PhT1 proteins secondary structure predicted a six-loop-six structure, containing 12 TMDs with a large hydrophilic loop between TMD6 and TMD7. Also PhT2 transporters contain 12 TMDs, but in opposition to PhT1 members, these Pi transporters present a large hydrophilic loop between TMD8 and TMD9 and a long N-terminal extension that functions as a transit peptide, directing the protein to the chloroplast (Walters *et al*., 2004). The hypothetical Na^+/Pi transporters have 10 TMDs and a long N-terminal chloroplastidial transit peptide (Gao *et al*., 2006).

The phylogenetic analysis of 55 PhTs distributed across 13 plant species showed that all analyzed PhT1 members are included in the same cluster, forming two subgroups composed by either dicot or monocot proteins. The high similarity of PhT1 proteins with each other as well as with the yeast PHO84 phosphate transporter (*Saccharomyces cereviseae*) and the fungi GvPT phosphate transporter (*Glomus versiforme*) (Bun-Ya *et al*., 1991; Harrison and van Buuren, 1995; Leggewie *et al*., 1997; Smith *et al*., 1997) suggests that H^+/Pi symport is a highly conserved and widespread mechanism in plants and fungi. Taking into account that PhT1 and other individual MFS families diverged from each other long before eukarya and archaea diverged from bacteria, it is expected that members of this subfamily should be present in other taxa besides those from plants or fungi (Pao *et al*., 1998). In what concerns the PhT2

members, all have clustered together and do not share homology with the PhT1 family. In fact, plant PhT2 members share more identity with Pi transporters from bacteria, *Caenorhabditis elegans* and the Na^+/Pi symporters from both mammals and fungi than with plant PhT1 members (Mann *et al*., 1989; Martinez and Persson, 1998; Rausch and Bucher, 2002). Still, functional analysis indicates that PhT2 are low-affinity H^+/Pi symporters (Daram *et al*., 1999; Rausch and Bucher, 2002). The phylogenetic analysis also suggests the existence of a new chloroplastidial Na^+/Pi transporter family with orthologs in both dicot (*A. thaliana*) and monocot (*Oryza sativa*). The identification of this new putative PhT family suggests that plants also rely on Na^+/Pi transporters. This transport mechanism could be particularly useful in halophytes or in plants living in alkaline media, where high Na^+ concentrations could be used for energization of anion transport (Rausch and Bucher, 2002).

In this work, four *Hakea sericea PhT* genes (*PiT2*, *PiT6*, *PH5* and *PH7*) were identified and partially sequenced. The genomic amplification of *H. sericea* gDNA, using degenerated primers based on the conserved regions of *PhT1* sequences from other plant species, resulted in the identification of *PiT2* and *PiT6* gene sequences. The subsequent screening of the *H. sericea* gDNA library, using *PiT2* and *PiT6* gene fragments as probes, resulted in the additional identification of *PH5* and *PH7* genes. According to the protein sequence alignment of higher plant H^+/Pi symporters, the partial deduced aminoacid sequences of *PiT2*, *PiT6*, *PH5* and *PH7* correspond only to the transmembranar domains TMD2, TMD3 and TMD4 of PhT1 proteins.

The sequences of 55 higher plant H^+/Pi symporters were used for performing the phylogenetic analysis of the newly identified *H. sericea* genes. For performing this, all PhT proteins were trimmed before alignment to include only the coding region of TMD2, TMD3 and TMD4 (TMD2-4). In this analysis, PhT1 from either dicot or monocot do not cluster in separate groups, contrasting with the results previously obtained when considering all the PhT amino acid sequences. Moreover, PhT1s (TMD2-4) by themselves do not cluster together alone, as PhT1 complete proteins do, since the hypothetical Na^+/Pi symporter family is included in their cluster. These results suggest that TMD2-4 region is

highly conserved amongst PhTs from different subfamilies and plant species and alone do not represent the evolutionary pathway of H^+/Pi symporters. To perform a complete phylogenetic study, other regions of PiT2, PiT6, PH5 and PH7 proteins must be sequenced and analysed.

The first expression studies of *PhT* genes in *Arabidopsis thaliana* (Muchhal *et al*., 1996), *Solanum tuberosum* (Leggewie *et al*., 1997) and *Lycopersicon esculentum* (Daram *et al*., 1998) showed high-affinity PhT1 members being expressed mainly in root organs of Pi deficient plants. In contrast, low-affinity PhT members were ubiquitously expressed throughout the whole plant. These findings led to the assumption that high-affinity PhT members should be involved in Pi uptake from soil, whereas the translocation of Pi throughout the plant was carried by low-affinity transporters (Daram *et al*., 1999). Subsequent studies have shown that under Pi deficiency, high-affinity PhT1 members are also expressed in other plant parts besides roots (Karthikeyan *et al*., 2002; Mudge *et al*., 2002). We have attempted to find a possible relation between the amino acid sequence of PhT1 members and their main expression site. However, the preferential expression site of any given *PhT1* is not directly related to its deduced amino acid sequence and cannot be inferred only based on its structure. For example, both LaPT1;1 and LaPT1;2 are expressed in roots of *L. albus* (Liu *et al.*, 2001) but seem fairly unrelated. In contrast, At1G20860 and At5G43340 are structurally alike but are expressed in different *A. thaliana* plant organs.

The expression studies on similar PhTs that group together into small clusters (*e.g.* At5G43350, At5G43350 and At5G43350 or MtPT1;1, MtPT1;2 and MtPT1;3) show that even structurally similar proteins may have different physiological roles. In *Medicago truncatula*, *MtPT1;1* and *MtPT1;2* are almost identical, sharing 91.6% and 98% sequence identity at the nucleotide and amino acid levels, respectively. However, the expression of *MtPT1;1* and *MtPT1;2* genes, evaluated by Northern blot analysis, revealed differences in their expression patterns (Liu *et al.*, 1998b). Although both genes are only expressed in roots, *MtPT1;1* is much less responsive to Pi fluctuations than *MtPT1;2*, which is only induced in Pi deficiency and repressed in Pi sufficiency.

In *S. tuberosum*, StPT1;1 and StPT1;3 share 83,9% amino acid sequence identity. Although *StPT1;1* seems to encode a low-affinity PhT involved in Pi translocation within the plant and is expressed in different plant parts, *StPT1;3* encodes a high-affinity PhT whose expression is strongly induced in roots due to arbuscular mycorrhizal fungal colonisation (Leggewie *et al*., 1997; Rausch *et al*., 2001). Both studies, on *M. truncatula* and *S. tuberosum*, suggest that small differences in the protein amino acid sequence are sufficient to completely alter the kinetic parameters of the PhT proteins.

In order to understand the regulation mechanisms of Pi transport in *H. sericea*, the expression patterns and tissue specifity of the four *PhT* genes in plants grown under either high- or low-Pi availability should be analized.

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ANNEX I. GENERAL MOLECULAR BIOLOGY PROTOCOLS

Spectrophotometric quantification of DNA

Quantification of DNA was performed by spectrophotometry on a *Cary 1E UV-Vis Spectrophotometer* (Varian). The A_{260} of diluted sample solution was determined and DNA concentration was estimated taking into account that 1 A_{260} = 50 ng/µL DNA. Purity was evaluated after determining the values of A_{230} and A_{280} (Sambrook *et al*., 1989).

Nucleic acid electrophoretic separation

DNA fragments were resolved by electrophoresis using a horizontal slab gel apparatus. Gel was made by melting agarose in 0.5× TBE (45 mM Tris; 45 mM boric acid; 1 mM EDTA), also used as running buffer. DNA samples, including molecular weight marker (*1 kb DNA Ladder*, Invitrogen), were mixed with 0.25 vol of loading buffer [glycerol 30% (w/v); 0.1 M EDTA and bromophenol blue 0.25% (w/v)] and 1 µL of 1 $mg.mL^{-1}$ EtBr. Electrophoresis was carried out at 80 V, until the bromophenol blue dye had reached two thirds of the gel length. The agarose gel was visualized under long wave UV light (*Transilluminator 2020E*, Stratagene) and analyzed using the *Eagle Eye® II Still Video System* (Stratagene) through corresponding software (*EagleSight™* 3.2, Stratagene).

Southern blotting

Transfer of DNA from agarose gels to nylon membranes (*Hybond N+*, Amersham) was performed by the capillary transfer method (Ausubel *et al*. 1996). After electrophoresis, DNA denaturation was promoted by rinsing the agarose gel in 0.4 M NaOH for 20 min. A transfer apparatus was set up by placing a platform inside a reservoir that contained transfer solution (0.4 M NaOH). Three sheets of *Whatman® 3MM* paper soaked in transfer solution were laid on the platform and their ends were immersed into the transfer solution.

The agarose gel was placed above the soaked paper. On top of the agarose gel and cut to gel size were successively layered: (1) the nylon membrane; (2) three sheets of soaked *Whatman® 3MM paper*; (3) a 7 cm stack of paper towels. Finally, a glass plate and a small weight (0.6-1 kg) were placed on top to promote capillary transfer of reservoir liquid to the absorbing paper towels. Capillary transfer was allowed to proceed overnight. Nucleic acids were crosslinked to the membranes with UV light (120 mJ) on the *Stratalinker® UV crosslinker* (Model 1800, Stratagene).

Hybridization with ^{32}P-labelled DNA probes

DNA fragments were ^{32}P-labeled by random oligonucleotide priming using the *Rediprime II DNA labelling system* (Amersham) and [α-^{32}P] dCTP (*Redivue*, Amersham). Briefly, each DNA probe (100-200 ng) was diluted to a final volume of 45 µL in TE buffer [10 mM Tris-HCl (pH 7.6); 1 mM EDTA], denaturated at 95°C for 5 min, and cooled on ice for 5 min. The solution was used to reconstitute the *Rediprime II* labelling mix, after which 5 µL of [α-^{32}P] dCTP (50 µCi) were added. Radioactive nucleotide incorporation was promoted by incubating the reaction mixture at 37°C for 1 h.

Radiolabelled probes were purified from unincorporated nucleotides by gel filtration through a *Sephadex G-50* (Pharmacia) (Sambrook *et al*., 1989). A sterile Pasteur pipette was partially blocked with a glass pearl and filled with *Sephadex G-50* resin equilibrated in TE buffer. The column was washed with 3 mL of TE and the radiolabelled probe loaded. Size separation by gravity flow was promoted by loading into the column one 450 µL TEN fraction [100 mM NaCl in TE buffer] followed by twelve 150 µL TEN fractions. All fractions were separately collected in microtubes and evaluated for radioactivity with a mini-monitor (*Series 900*, Morgan). The first 4-5 fractions to present radioactivity were pooled.

Membrane filters were pre-hybridized in hybridization buffer [50 mM sodium phosphate (pH 7.0); 0.9 M NaCl; 5 mM EDTA; 10× Denhardt reagent [5% (w/v) Ficoll 400; 5% (w/v) PVP 360; 5% (w/v) BSA (fraction V)]; 0.1% SDS, 250 µg.mL^{-1} denatured salmon sperm DNA and 30% (v/v) formamide] at 42°C,

for 3 h, in a hybridization oven (Amersham). The radiolabelled probe was heat denatured for 5 min, cooled on ice and added to the hybridization buffer. Hybridization was allowed to proceed overnight at 42ºC, with shaking. After hybridization, filters were successively washed for 20 min in the following solutions: (1) 2× SSC [30 mM sodium citrate (pH 7.0); 0.3 M NaCl], 0.1% SDS, at 45°C; (2) 2× SSC, 0.1% SDS, at 50°C; (3) 1× SSC, 0.1% SDS, at 50°C. The final washing step was 1× SSC, 0.1% SDS, at 55°C.

Autoradiography

Radioactive membrane filters were wrapped in plastic film and placed into hybridization cassetes (*Hypercassete*, Amersham) containing two intensifying screens (*Hyperscreen*, Amersham). An autoradiographic film (*BioMax MS*, Kodak) was put in contact with the membrane and exposure was performed at –80°C for the appropriated period (overnight to 1 week). The autoradiographic film was developed in a darkroom by its submersion in *X-ray Developer D-19* (Kodak) for up to 5 min. Development was stopped by rinsing the film in 3% (v/v) acetic acid stop solution and fixation was performed by submerging the film in *Rapid Fixer* (Ilford) for 5 min. After rinsing with running water for 5 min, the film was dried at room temperature.

Transformation of **E. coli** *cells*

Preparation and transformation of *E. coli* competent cells was performed using the method described by Inoue *et al*. (1990).

Competent cells were prepared by inoculating 250 mL of SOB medium [2% (w/v) bacto-tryptone; 0.5% (w/v) yeast extract; 2.5 mM KCl; 10 mM NaCl; 10 mM $MgSO_4$; 10 mM $MgCl_2$] with a single colony of *E. coli* JM109 (Yanish-Perron *et al*., 1985). Cells were grown at 18°C with vigorous shaking (200-250 rpm) until A_{600} = 0.6. The culture was placed on ice for 10 min and cells were collected by centrifugation at 2500 *g* for 10 min at 4ºC. The pellet was resuspended in 80 mL of ice-cold TB buffer (10 mM PIPES; 15 mM $CaCl_2$; 250 mM KCl; 55 mM $MnCl_2$), and left on ice for 10 min. Cells were centrifuged at 2500 *g* for 10 min at 4ºC, and gently resuspended in 20 mL of ice-cold TB

buffer. DMSO was carefully added to a final concentration of 7% (v/v). The cell suspension was left on ice for 10 minutes and distributed in 200 µL aliquots, which were immediately placed in liquid nitrogen and stored at -80°C.

Transformation of *E. coli* cells was initiated by thawing competent cells on ice. The DNA sample (1-20 µL) was added to 200 µL of competent cells by gentle mixing, and the mixture was incubated at 4°C for 30 min. Cells were heat-shocked by incubation at 42°C for 30 s with agitation, followed by 10 min on ice. After addition of 0.8 mL of SOC medium [2% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 2.5 mM KCl, 10 mM NaCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, 20 mM glucose], an incubation for 1 h at 37°C with vigorous shaking (200-250 rpm) was performed. Cells were spun down for a 30 s at 10000 *g* and the pellet resuspended in 50 µL of the supernatant. Finally, cells were transferred to LB selective plates [1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0], containing X-gal (40 $\mu g.mL^{-1}$) and IPTG (40 $\mu g.mL^{-1}$), and incubated overnight at 37°C.

Plasmid isolation

The purification of small amounts of DNA to be used in restriction fragment analysis was performed by the boiling method (Holmes and Quigley, 1981). Bacteria were grown overnight in the appropriate liquid culture media. An aliquot of 1.5 mL of the culture was collected and centrifuged at 8000 *g* for 5 min. The pellet was resuspended in 400 µL of STET [10 mM Tris-HCl (pH 8.0); 100 mM NaCl; 1 mM EDTA; 5% (v/v) Triton X-100], supplemented with 25 µL of freshly prepared lysozyme solution [10 mM Tris-HCl (pH 8.0); 10 $mg.mL^{-1}$ lysozyme]. Lysis was promoted by incubation at room temperature for 10 min, followed by incubation at 95ºC for 1 min. Denaturated proteins and chromosomal DNA were removed by centrifugation at 14000 *g* for 15 min. The supernatant was recovered and mixed with 300 µL of isopropanol to precipitate plasmid DNA. After centrifugation under the same conditions, the supernatant was discarded and the plasmid was resuspended in 20-100 µL of TE [10 mM Tris-HCl (pH 8.0); 1 mM EDTA].

Medium scale isolation of high purity plasmid DNA to be used for sequencing and probe isolation was performed using the *Wizard™ Plus Midipreps DNA Purification System* (Promega), according to the supplier's instructions. Briefly, bacteria were grown overnight in the appropriate liquid culture media (100 mL). The culture was centrifuged at 8000 *g* for 5 min. Cells were resuspended in 3 mL of resuspension buffer [50 mM Tris-HCl (pH 7.5); 10 mM EDTA; 100 $\mu g.mL^{-1}$ of RNase A] and an equal volume of lysis buffer [200 mM NaOH, 1% (w/v) SDS] was added. The mixture was gentle mixed by swirling until clearing. The lysate was neutralized by the addition of 3 mL of neutralization buffer [1.32 M KOAc (pH 4.8)], followed by gentle mixing. Cell debris was removed by centrifugation at 14000 *g* for 15 min at 4ºC. The supernatant was combined with 10 mL of the *Wizard® Midiprep DNA Purification Resin* (Promega) and loaded onto the midicolumn. Vacuum was applied to the column to promote packaging of the resin and 2x15 mL of washing buffer [8.3 mM Tris-HCl (pH 7.5); 80 mM KOAc; 40 µM EDTA; 55% (v/v) ethanol] were added. The midicolumn was placed inside a microtube and centrifuged at 8000 *g* for 2 min to remove any residual washing buffer. The plasmid DNA was eluted by loading 300 µL of sterile water at 70ºC. After incubating for 1 min, the column was centrifuged at 8000 *g* for 20 s to collect the eluted plasmid. The plasmid DNA solution was further centrifuged at 8000 *g* for 5 min to precipitate column debris and the supernatant was stored at -20ºC.

Digestion with endonucleases

The digestion of DNA with restriction endonucleases was performed according to standard procedures (Sambrook *et al*. 1989; Ausubel *et al*. 1996), following the supplier's instructions. Acetylated BSA was used to stabilize endonuclease activity. Each reaction contained 1-15.5 µL of DNA, 2 µL enzyme buffer (10×, Roche), 2 µL BSA (5 mg/mL, Stratagene), 0.5 µL restriction enzyme and ultrapure water up to a final volume of 20 µL. Reactions were performed at 37°C, for 1.5 h to overnight.

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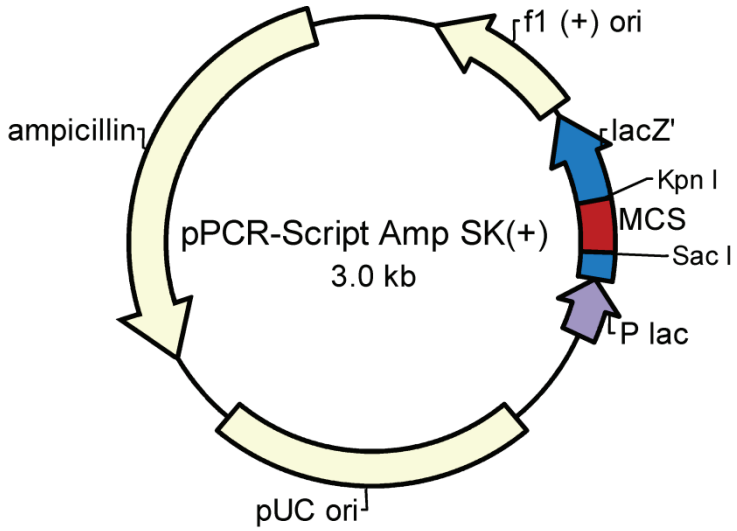
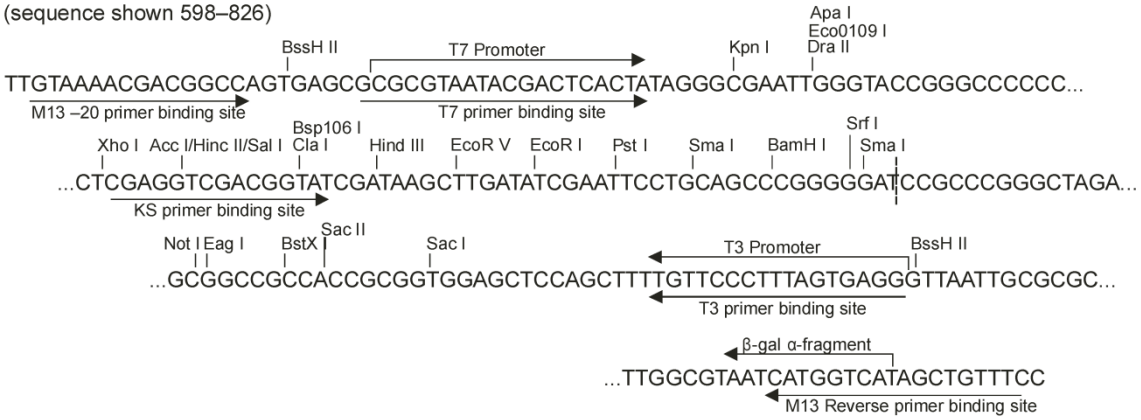
ANNEX II. PPCR-SCRIPT™ AMP SK(+) VECTOR MAP



pPCR-Script Amp SK(+) Multiple Cloning Site Region
(sequence shown 598–826)



Feature	Nucleotide Position
f1 (+) origin of ss-DNA replication	135–441
β-galactosidase α-fragment coding sequence (lacZ')	460–816
multiple cloning site	653–760
T7 promoter transcription initiation site	643
T3 promoter transcription initiation site	774
lac promoter	817–938
pUC origin of replication	1158–1825
ampicillin resistance (*bla*) ORF	1976–2833

CHAPTER 5

CONCLUSION

5.1 FINAL CONSIDERATIONS

The invasion of habitats by alien species is a constant and fairly common process. Still, only a few species are able to establish and spread in the new territory. Human activity, however, is driving a dramatic increase on the frequency and number of invasions, unbalancing and endangering many ecosystems (Wells *et al*., 1986; di Castri, 1989; Cronk and Fuller, 1995).

The migration of *Hakea sericea* from south-eastern Australia to countries such as South Africa, New Zealand or Portugal (Neser and Fugler, 1978; Weber, 2003) is a perfect example of this reckless human activity. Initially introduced as a hedge plant to form fence lines or windbreaks, this Proteaceae species soon relied on its xeromorphic adaptations (Groom *et al*., 1997), high production, longevity and dispersion of seeds (Richardson *et al*. 1989), fire resistance from both seeds and rootstocks (Bradstock, 1991), and enhancement of nutrient uptake by proteoid roots (Shane and Lambers, 2005) to spread and overcome autochthon populations. Currently, *H. sericeae* has been reported as an aggressive invader in all of these new habitats.

The north-western part of Portugal has been severely affected by the invasion of *H. sericea*, mainly triggered by the disruption of natural ecosystems by frequent forest fires. Taking this into account, one of the aims of the present work was to study possible approaches for controlling this invader. As necrotic lesions of unknown origin were detected in the leaves of wild shrubs of *H. sericea* growing in northern Portugal ("Serra de Arga"), the identification of a pathogenic agent able to infect *H. sericea* was carried out. Injured tissues were incubated on PDA, from which a fungal species was isolated. Based on morphological characters such as conidia form, cell number, pigmentation and number of appendages, the pathogenic agent was identified as belonging to the *Pestalotiopsis* genus. Since most species in this genus share the same morphological characters (Hu *et al.*, 2007), classification at the species level was accomplished through the internal transcribed spacer (ITS) of rRNA genes molecular marker. The amplification and sequencing of ITS allowed the identification of the pathogen as *Pestalotiopsis funerea*. This ubiquitous species

has been described has pathogenic for many plants, causing necrosis on infected tissues, that can ultimately lead to plant death. However, several studies regarding the influence of plant health (Keith *et al*., 2006) and environmental conditions (Tuset *et al*., 1999) on the success of *P. funerea* invasiveness have shown that this fungal agent behaves as an opportunistic pathogen mostly affecting stressed plants. This feature could make *P. funerea* a useful biological control tool in association with mechanical or chemical methods, since non-wounded authocton species would be safe from infection. Another main advantage of using *P. funerea* as a biological agent in northern Portugal relies on the fact that the pathogen was isolated from the invaded area by itself. Consequently, it would not be introduced into the ecosystem for the first time, thus reducing the probability of collateral damage in autochthon species.

Soils from the mountain areas of northern Portugal, likewise many found in Australia and in Cape region in South Africa (Lambers *et al*., 2006), tend to have a bellow average concentration of important mineral nutrients, particularly N and P. This would have a negative impact on the growth and development of most plant species. Conversely, *H. sericea* seems to be perfectly fit to cope with such harsh environmental conditions. Therefore, another aim of this work was to further understand the ecophysiological mechanisms underlying the ability of *H. sericea* to successfully establish in such Pi-poor soils. For studying Pi uptake by roots of *H. sericea*, an efficient hydroponic system was firstly developed. The growth of plants in different media showed that proteoid roots are only induced in solutions with low Pi concentrations – similarly to those found in the soils of "Serra de Arga", in north-western Portugal. Through autoradiography of root fragments incubated in ^{32}Pi, it was shown that proteoid roots present a higher effectiveness in uptaking Pi than non-proteoid roots. Pi uptake by itself seems to be dependent on proton gradient and yielded a biphasic kinetics, suggesting the involvement of H^+/Pi co-transport systems with distinct substrate affinities (K_m 225 nM and 40.8 µM Pi). Moreover, the high-affinity Pi transport component described in this work has the highest Pi affinity characterized to date in plant roots, matching the values described for Pi transport even in mycorrhiza. We suggest that the presence of a Pi uptake system with such high-affinity to Pi

may confer an adaptive advantage for *H. sericea* over most plants in Pi-poor soils.

Phosphate transport from soil to plant and across plant tissues and organelles occurs through the activity of phosphate transporters (PhT family). Members of PhT1 subfamily are involved in the uptake of Pi into the cell, thus being located in the plasma membrane. Transmembrane domain (TMD) prediction showed that all PhT1 members must have 12 TMD separated in two groups of six by a large hydrophilic charged loop. Phylogenetic analysis of 55 PhTs distributed across 13 plant species clustered PhT1 members altogether, forming two subgroups composed by either dicot or monocot proteins. Prediction of transmembrane domains in PhT2, a family of chloroplastidial H^+/Pi symporters, suggests all members must also have 12 TMD. However, in opposition to PhT1 members, the large hydrophilic loop is located between TMD8 and TMD9 and the N-terminal contains an extension that functions as a transit peptide, directing the protein to the chloroplast. The phylogenetic analysis shows that all analyzed PhT2 members cluster together and do not share homology with PhT1. Finally, the phylogenetic analysis suggests the existence of a new chloroplastidial Na^+/Pi transporter family, with 10 predicted TMD and a long N-terminal chloroplastidial transit peptide. The existence of such family may be particularly relevant in halophytes or in plants living in alkaline media. In this work, four PhT members were found in *H. sericea* (*PiT2*, *PiT6*, *PH5* and *PH7*). Transmembrane domain prediction of the deduced amino acid sequence suggests all four members belong to the PhT1 subfamily. However, since the complete nucleotide sequences of these *H. sericea* genes were not achieved, an accurate phylogenetic analysis including *PiT2*, *PiT6*, *PH5* and *PH7* was impaired.

The possible relation between the amino acid sequence of PhT1 members and their main expression site was studied. Apparently the preferential expression site of any given *PhT1* may not be inferred based only on its deduced amino acid sequenced. While some fairly unrelated proteins may be expressed in the same tissue (*e.g.* LaPT1 and LaPT2 in roots), structurally alike PhTs may be expressed in different plant organs (*e.g.* At1G20860 and At5G43340).

In *Arabidopsis thaliana*, genes enconding high-affinity PhTs are repressed in the presence of high concentrations of Pi (Dong *et al*., 1999). Conversely, when grown under high Pi concentration, *H. sericea* accumulates abnormal amounts of Pi in the root, stem and leaves that ultimately lead to foliar Pi toxicity symptoms. These results suggest that *H. sericea* may be unable to down-regulate the expression of high-affinity *PhT* genes in such experimental conditions. Since *H. sericea* inhabits Pi impoverished soils in its native habitat, an efficient mechanism for preventing excessive Pi uptake may have never been developed, or alternatively, could have been lost during evolution.

5.2 FUTURE PERSPECTIVES

Following the work described in this thesis, several research lines can be suggested:

a) Regarding the control of *H. sericea* with *P. funerea*, further studies are required before its environmental application. Since infection was only tested in six-weeks-old plants, an infection of adult specimens should be attempted. It would also be interesting to infect plants exposed to different stresses, such as drought, cold, mechanical injuries or excess Pi to predict in which conditions infection by *P. funerea* would be favoured. Collateral damage on autochthon populations should also be anticipated, by testing at lab scale the effect of *P. funerea* on these species.

b) In what concerns Pi homeostasis, it would be imperative to complete the sequencing of the four genes identified in *H. sericea* genome. The expression analysis of these genes and their tissue specifity in plants under high- or low-Pi availability would allow the understanding of Pi transport regulation in this plant species.

c) The functional characterization of the identified Pi transporters genes through heterologous expression (in *Saccharomyces cerevisiae*) would allow a more detailed study on their role, kinetic properties, energetics and specificity.

d) Finally, since P is frequently a limiting nutrient for plant growth, it would be interesting to assess if the over-expression of these high-affinity Pi transporters in other plant species would improve their ability to cope with Pi-poor soils.

The work presented in this dissertation contributed to better understand the ecophysiological mechanisms underlying the ability of *H. sericea* to invade new habitats. A new pathogenic agent, *P. funerea*, was also described as harmful for this Australian Proteaceae. It is hoped that these studies can be a starting point for the development of integrated control strategies against this aggressive invader in Portuguese territory.

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