Root system of seabuckthorn (*Hippophaë rhamnoides* L.)

Morphology, metabolism and gene expression

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Cover: Top image, cluster root formed by seabuckthorn genotype Pk with an illustration of primary metabolism shown on right corner and exudates on left corner. Bottom left, image of multi-lobed symbiotic N_2-fixing root nodule and bottom right, image of seedling of seabuckthorn cv. Sunny with shoots formed from root. (photos by S. Shah)

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Abstract

Nutrient availability is one of the limiting factors for plant growth and development, and the nutrients on Earth are unevenly distributed. To overcome this, plants adapt by modifying their morphology and physiology, especially of the root system. Seabuckthorn is a small tree growing in temperate regions of Europe and Asia. It performs symbiotic N_2 fixation and has high adaptability to environmental constraints.

This thesis examined factors shaping the root system of seabuckthorn. It was found that seabuckthorn has the ability to produce structures with dense lateral roots called cluster roots (CRs). Different patterns of root system were found at genotype level; Pk, a wild accession of *H. rhamnoides* ssp. *turkestanica* originating from unfertile soils, produced more CRs than cultivars BHi10726 and Sunny with a breeding history in fertile soils. Reduced availability of phosphorus (P), nitrogen or iron affected root morphology by increased lateral roots and CR formation, which may explain the competitive and invasive nature of this plant on nutrient-deficient soils.

Abundant compounds in CRs found by metabolite analysis using ^{1}H-NMR spectroscopy were asparagine, glycine and malate, an organic acid involved in mobilization of P from soil. In Pk as compared to cv. Sunny, the concentration of root metabolites was higher. In roots under low P, RNA sequencing revealed transcripts involved in primary root metabolism, P homeostasis and metabolism consistent with a P-deficient response. To assess functions of CRs which have a determinate growth pattern, analysis of metabolites and transcripts could display changes towards a P-deficient metabolism as well as anaerobic metabolism at later developmental stages of CR. As an enzyme central to organic acid metabolism in plants, the gene family encoding phospho*enol*pyruvate carboxylase (*HrPPC*) was characterised. Both plant-type and bacterial-type isoforms were found with tissue-specific expression patterns and with higher expression of *HrPPC2* under low P. *In vitro* studies showed that auxin stimulated formation of lateral roots at low P levels, while high P gave high formation of shoots from roots, another trait of the seabuckthorn root system.

This study provides a basis to understand functions and roles of CRs in seabuckthorn as an actinorhizal representative among the three groups of CR-forming plants.

Keywords: seabuckthorn, nutrients, cluster root, root-suckers, phosphorus, iron, auxin

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Dedication

To my parents and family…

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Syed Rehmat Ullah Shah, Peter Agbäck and Per-Olof Lundquist (2015). Cluster roots of seabuckthorn (*Hippophaë rhamnoides* L.) in response to nitrogen, phosphorus and iron deficiency. *Submitted for publication*.

II Syed Rehmat Ullah Shah, Johan Meijer and Per-Olof Lundquist (2015). Analysis of plant-type and bacterial-type phospho*enol*pyruvate carboxylase genes in seabuckthorn (*Hippophaë rhamnoides* L.) reveals tissue-specific expression of gene family members. *Submitted for publication*.

III Syed Rehmat Ullah Shah, Johan Meijer, Peter Agbäck and Per-Olof Lundquist (2015). Responses of the seabuckthorn (*Hippophaë rhamnoides* L.) root system to phosphorus availability: Analysis of cluster root formation, metabolite and transcript patterns. *Manuscript*.

IV Syed Rehmat Ullah Shah, Tatiana Plaksina, Sridevy Sriskandarajah and Per-Olof Lundquist (2015). *In vitro* shoot organogenesis from roots of seabuckthorn (*Hippophaë rhamnoides* L.). Structure, initiation and effects of phosphorus and auxin. *Submitted for publication*.

The contribution of Syed Rehmat Ullah Shah to the papers included in this thesis was as follows:

I Participated in planning the project. Conducted the experiments and prepared the fluorescent microscopic graphs. Prepared the samples for metabolomics. Participated in analysing the data and writing the manuscript.

II Participated in planning the project. Carried out the experiments and laboratory work. Participated in analysing the data, preparing diagrams and writing the manuscript.

III Participated in planning the project. Conducted the experiments, carried out laboratory work, prepared the samples for metabolomics and RNA sequencing. Participated in analysing the data, preparing diagrams and writing the manuscript.

IV Participated in planning the project and conducting the experiments. Prepared the fluorescence micrographs and prepared the samples and micrographs for SEM. Participated in analysing the data, preparing figures and writing the manuscript.

Abbreviations

BTPC	bacterial-type phospho*enol*pyruvate carboxylase
CR	cluster root
DAPI	4',6-diamidino-2-phenylindole
Fe	iron
FPKM	fragments (paired-end reads) per kilobase per million fragments mapped
IAA	indole-3-acetic acid
LR	lateral root
MCR	mature cluster root
MDH	malate dehydrogenase
N	nitrogen
NMR	nuclear magnetic resonance
OCR	Old cluster root
P	phosphorus
PAP	purple acid phosphatase
PEPC	phospho*enol*pyruvate carboxylase
PMCR	pre-mature cluster root
PPC	phospho*enol*pyruvate carboxylase gene
PR	primary root
PTPC	plant-type phospho*enol*pyruvate carboxylase
SfR	shoot-from-root
SLR	secondary LR including root meristem, elongation, differentiation and pre-emergence CR zones
WPM	woody plant medium

1 Introduction

1.1 Seabuckthorn

Seabuckthorn (*Hippophaë rhamnoides*) belongs to the family Elaeagnaceae and has a natural distribution in temperate areas of Asia and Europe (Rousi, 1971). In Pakistan, there are natural populations of seabuckthorn in northern areas with a huge diversity in morphology and biochemical constituents (Shah *et al.*, 2007; Sabir *et al.*, 2005). During the last few decades, seabuckthorn has received increasing attention for its nutritional value and the medicinal importance of its berries. It is a small plant that produces nutritious berries and seed rich in bioactive compounds such as antioxidants (vitamin C, carotenes), unsaturated fatty acids, sterols and organic acids (Kallio *et al*., 2002). The berry pulp is used in a range of food commodities and the seed oil is used in phytopharmaceuticals and cosmetics as a UV skin protectant due to its light absorption and emollient properties (Kallio *et al.*, 2002; Gao *et al.*, 2000; Beveridge *et al.*, 1999; Zhang *et al.*, 1990).

Seabuckthorn is an early succession plant (Bartish *et al.*, 1999) and has physiological mechanisms to grow under environmental stresses such as drought, water-logging, salinity, alkalinity and cold (Chen *et al.*, 2009; Yao & Tigerstedt, 1995; Singh & Gupta, 1990). Seabuckthorn has a rapidly growing extensive root system, the ability to form root suckers (shoots from roots, SfR) and can function to conserve soil by preventing soil erosion (Li & Schroeder, 1996; Cireasa, 1986). Seabuckthorn has symbiotic relationships with the actinomycete *Frankia* to fix atmospheric nitrogen (N_2) in root nodules and with fungi of Glomermycota that form arbuscular mycorrhiza (Gardner *et al.*, 1984). It also has tolerance to exist in different types of soils and stress conditions, such as unfertile to barren land (Li & Schroeder, 1996).

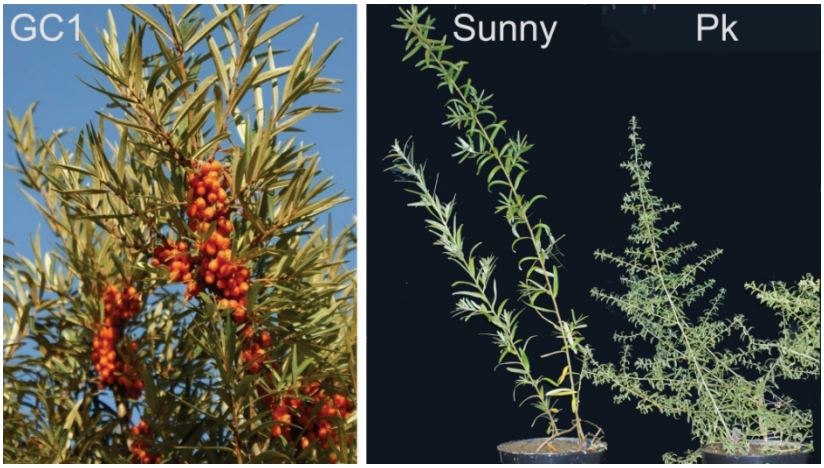

Figure 1. Seabuckthorn plants. Left, genotype GC1 (*H.r.* ssp. *rhamnoides* L.) with berries grown outside and right cv. Sunny and genotype Pk (*H.r.* ssp. *turkestanica* L.) grown under greenhouse conditions.

1.2 Root traits and their role in nutrient acquisition

Adaptation of plants to nutrient availability is necessary for their survival in malnourished stressful rhizosphere conditions. Under such conditions, plant growth and development is totally dependent on the root system, which controls the capacity of the plant to acquire nutrients and water and to establish a connection between plant and soil ecosystem. To manage their nutrient requirements, plants activate different morphological and physiological adaptations and different species may have different mechanisms to adapt to and overcome nutritional stress (Pearse *et al.*, 2007). These adaptations include remobilisation of biomass to the roots (Hermans *et al.*, 2006; Cairns *et al.*, 1997; Canham *et al.*, 1996), development of morphological architectures including lateral roots (LRs) and, in certain plants, formation of cluster roots (CRs) (Shane & Lambers, 2005; López-Bucio *et al.*, 2003), activation of nutrient transporters (Chiou *et al.*, 2001; Gansel *et al.*, 2001), and synthesis and secretion of root exudates that enhance mineral acquisition (Lambers *et al.*, 2008; Neumann *et al.*, 2000). Plant roots also act as hosts for beneficial symbiotic and associative microorganisms Thus, plants have a range of different adaptive strategies to maintain their growth under various environmental constraints.

1.2.1 Cluster roots (CRs)

The cluster root (CR) is a specialised structure formed in some plants in various families to provide the plant with improved root surface area and

exudation (Watt & Evans, 1999). Researchers have defined CRs in different ways, based on their appearance in different plant species. A CR is a portion of a secondary lateral root (SLR) containing bottle brush-like clusters, with a density of 10 or more CR rootlets per cm (Sas *et al.*, 2002; Johnson *et al.*, 1996b). It can be "a single cluster of rootlets" (Skene, 1998) or "an entire root from any species that forms one or more clusters along its length" (Watt & Evans, 1999). Cluster roots are "composed of a number of tightly grouped determinate rootlets that undergo initiation, growth and arrest in a synchronized manner" and should have the components radial, longitudinal and cluster pattern (Skene, 2001). Cluster rootlets continuously emerge in a row (Johnson *et al.*, 1996b; Dinkelaker *et al.*, 1995; Purnell, 1960). The absence of subsequent LR primordia on the CR rootlet is the main difference between CR rootlets and LRs. In addition, Skene (2001) listed the following four key characteristics that define a CR: production of a CR rootlet opposite each xylem pole, determinate-type development, capability for an exudative burst, and enhanced uptake of nutrients. These characteristics may vary among plant species.

Cluster roots were first found and studied in species within the family Proteaceae (Purnell, 1960), *e.g*. *Hakea* sp., *Banksia* sp., *Grevillea* sp. and *Macadamia* sp., and were initially called proteoid roots (Shane & Lambers, 2005; Skene *et al.*, 1996). They were also found among a few genera in the family Leguminoseae, *e.g*. *Lupinus* sp., *Aspalathus* sp. and *Viminaria juncea* (Gardner *et al.*, 1981; Lamont, 1972). Among the actinorhizal plants, CRs have been reported in *Alnus* species in the Betulaceae (Hurd & Schwintzer, 1996), *Casuarina* sp. in the Casuarinaceae and *Morella* (formely *Myrica*) in the Myricaceae (Lambers *et al.*, 2006; Skene, 1998).

An interesting question is why actinorhizal and some legume species of various orders and families have the capability to produce CRs (Shane & Lambers, 2005). High demand for P for symbiotic N_2 fixation (Gentili & Huss-Danell, 2003) could be one of the reasons for actinorhizal plants to produce CRs. Another may be that certain compounds exuded from CRs also work as signals for symbionts. In general, species within the Proteaceae and *Lupinus* sp. possessing the CR trait all lack the ability to form functional arbuscular mycorrhiza symbiosis (Shane & Lambers, 2005), whereas some actinorhizal species, *i.e. Casuarina* and seabuckthorn, have arbuscular mycorrhiza symbiosis (Skene, 1998; Gardner *et al.*, 1984).

The occurrence of CRs on seabuckthorn, as a representative of the Eleagnaceae, was first cited as an unpublished observation in a review by Skene (1998), but to the best of my knowledge there has been no previous systematic experimental study on CR formation by seabuckthorn.

1.2.2 Symbiotic N_2-fixing root nodules

The Elaeagnaceae are one of eight plant families, together with the Betulaceae, Casuarinaceae, Myricaceae, Rosaceae, Elaeagnaceae, Rhamnaceae, Datiscaceae and Coriariaceae, participating in actinorhizal symbiosis to form N_2-fixing root nodules (Santi *et al.*, 2013; Pawlowski, 2009; Wall & Berry, 2008), with the actinomycete *Frankia*. Actinorhizal plants are competitive in N-deficient soils and play important roles in land reclamation and remediation.

Actinorhizal root nodules can fix a substantial proportion of the total amount of N needed by the plant (Busse, 2000) depending on the N content in the soil. *Frankia*-induced root nodules in seabuckthorn are indeterminate-type, with multi-lobe structures (Figure 2). Isolation and *in vitro* culture studies of *Frankia* strains were first reported in 1978 (Benson & Silvester, 1993) and the best characterised strain compatible with seabuckthorn is EAN1pec (Normand *et al.*, 2007; Lalonde *et al.*, 1981).

Different plant species have differing efficiency and compatibility with different *Frankia* strains (Wall, 2000). In the studies on which this thesis is based, after inoculation of *Frankia* strain EAN1pec on seabuckthorn genotype BHi10726, visible nodules at single-lobed stage could be seen within 35 days, while in the seabuckthorn genotype Pk nodulation took 60 days.

In a study of nodulation in alder (*Alnus incana*) affected by P and N, nodule initiation in the form of cortical cell divisions were observed after only two days of inoculation and nodules had emerged through the root surface 14 days after inoculation (Gentili *et al.*, 2006). The number of N_2-fixing nodules and nodule biomass in the seabuckthorn root system is reported to be stimulated by P supply (Gentili & Huss-Danell, 2003). Hence, P is not only necessary as an important cellular component, but is also important to promote nodulation and subsequent plant growth.

1.2.3 Root microbial associations and their roles in nutrient acquisition

Plant roots are always in contact with diverse and complex rhizosphere microbial communities through secreted compounds serving as attractants or repellents. Root-microbe associations can either be direct, *e.g.* with symbionts or pathogens (Walker *et al.*, 2003), or indirect, where microbes depend on rhizodeposition by plants (Hartmann *et al.*, 2009) and, in return, mobilise nutrients, *e.g.* the phosphate-solubilising bacteria (Rodríguez & Fraga, 1999) or zinc-solubilising bacteria (Goteti *et al.*, 2013). Some microbes also produce antibiotics that protect the plants against disease (Burges, 1981).

The specific structure and metabolism of CRs can also affect the rhizosphere microbial community. In CRs of white lupin (*Lupinus albus*), the most abundant organisms are *Burkholderia* species, with a significant increase

in numbers towards maturity as the mature stage provides an acidic environment for root colonisation by *Burkholderia.* In return, the bacteria produce siderophores to solubilise iron (Fe), in addition to P solubilisation for the plants (Weisskopf *et al.*, 2011). Recently, presence of certain bacteria has been shown as one factor regulating formation of CRs by *Hakea* sp. (Proteaceae) and *Viminaria* sp. (Leguminoseae) under certain nutrient conditions and the mechanism is suggested to be through production of indole-3-acetic acid (IAA) (Lamont *et al.*, 2014).

In other recent studies, P-solubilising bacteria with several growth-promoting traits were isolated from the rhizosphere and endosphere of seabuckthorn from cold desert Trans-Himalayan regions (Kumar *et al.*, 2015). Different plants have different effects on rhizosphere communities. A recent study on mixed and monospecific plantation of seabuckthorn reported a synergistic effect of a mixed population on microbial community and various nutritional and texture properties of rhizosphere soil (Yu *et al.*, 2015).

1.2.4 Shoots from roots (root suckers)

Plant root suckers are shoots that emerge from roots. This is a method that higher plants can use to propagate vegetatively and is especially important under conditions where sexual reproduction is not functioning. Four basic types of morphologies of adventitious shoots have been described: shoots from the base of the trunk, shoots from underground stems (*e.g*. rhizomes in grasses), shoots from layered branches, and shoots from roots (root suckers) (Del Tredici, 2001).

Seabuckthorn has the ability to form shoots-from-roots (SfR) in natural populations. Induction of SfR formation has also been observed during cultivation of seabuckthorn seedlings *in vitro* (Sriskandarajah & Lundquist, 2009) and in some other plant species such as passion fruit (*Passiflora edulis*) (Rocha *et al.*, 2012) and *Arabidopsis* (Atta *et al.*, 2009).

1.3 Nutrients and their effect on root architecture

Nutrients act as direct signals for regulation of different cellular and molecular processes involved in the morphology and development of the root system, or indirectly through hormonal regulation of plant growth and morphogenesis, which may coordinate with nutrients and enhance or suppress the role of nutrients (Lin *et al.*, 2013; López-Bucio *et al.*, 2003). Plants take up nutrients from the soil in the form of inorganic ions. Effective absorption of inorganic nutrients depends on the total root surface area of the plant, with LRs and root length being two components contributing to total root biomass and root

surface area. These two traits are mostly affected by nutrients, but through different mechanisms and by different nutritional deficiencies. Under P and Fe deficiency, plants are known to produce more LRs of shorter length, while under low N conditions plants produce longer roots (Gruber *et al.*, 2013). Nutrient deficiency can also induce root hair formation in epidermal cells and low P has been found to produce longer and more dense root hairs (Ma *et al.*, 2001; Bates & Lynch, 1996).

1.3.1 Phosphorus, P

Phosphorus in the form of phosphate is an important component of several compounds in plant metabolism, cell membranes, DNA and RNA (Taiz & Zeiger, 2010). Inorganic P (P_i) depletion of the environment is inevitable. Inorganic phosphate (PO_4^{3-}) is a dwindling resource on a global scale (Van Vuuren *et al.*, 2010; Vance *et al.*, 2003). There is a potential crisis coming, with estimates indicating that in the period 2040-2060, global P reserves will be halved (Vaccari, 2009; Vance *et al.*, 2003). Through artificial fertilisation, the P level in agricultural land in the form of sorbed P is increasing. Plants can take up orthophosphate in the form of $H_2PO_4^-$ and HPO_4^{2-} (Hinsinger, 2001). P availability depends on diffusion towards roots, but the diffusion coefficient for P is very low compared with that for other nutrients (Clarkson, 1981). Anionic forms of P are very insoluble in soil due to attraction of P to cations such as Mg^{2+}, Ca^{2+} or Al^{3+} (López-Bucio *et al.*, 2003; Oenema & Roest, 1998), which makes them unavailable to plants. They must therefore be mobilised first to make them available (Figure 2). In soil, 20-80% of total P is in organic form, with phytic acid (inositol hexaphosphate) being the major component (Richardson *et al.*, 1994). Organic forms of P are also unavailable to plants (Schachtman *et al.*, 1998), and must be mineralised to become available (Horst *et al.*, 2001). Use of rock phosphate as a source of P fertiliser is also remobilising heavy metals in soil, thereby increasing exposure of agricultural land and water bodies to contaminating heavy metals and radionuclides. To maximise P utilisation by plants and minimise run-off losses, more attention should be paid to developing environmentally friendly approaches such as morpho-physiological adaptive strategies to mobilise the anionic, sorbed and organic forms of P (Gupta *et al.*, 2014). Lambers (2006) reviewed different ways used by plants to improve P acquisition, specifying the role of root traits in this regard.

Phosphorus is a key factor affecting plant growth and root architecture, especially CR formation, and P deficiency stimulates plant tissue sensitivity to auxin to promote LR formation (Pérez-Torres *et al.*, 2008). Phosphorus starvation responses are reported to be regulated by the endogenous P status of

the plant controlling root morphology and, especially, CR formation (Cheng *et al.*, 2011; Neumann *et al.*, 2000). Increased P concentration has been found to decrease the number of CRs in bog myrtle (*Myrica gale*) in hydroponic culture (Crocker & Schwintzer, 1993) and in white lupin (Johnson *et al.*, 1996a).

1.3.2 Nitrogen, N

Nitrogen is a major element required for plant growth and development and N limitation or excess can modify the morphological and physiological characteristics of plants. These modifications can be directed by phytohormones, especially abscisic acid, auxin and cytokinins, through local or long distance signalling pathways (Kiba *et al.*, 2011). In white lupin, high N inhibits CR formation, while a low dose of N together with P deficiency can stimulate CR formation (Sas *et al.*, 2002; Dinkelaker *et al.*, 1995). High N improves non-CR growth, but not CR growth (Lamont, 1972). The source of N affects the function of CRs. In one study, early emergence and higher number of CRs were observed in bog myrtle fertilised with urea compared with nitrate (Crocker & Schwintzer, 1993). In another study, CR growth was enhanced and proton excretion was higher when plants were treated with $(NH_4)SO_4$ than with any other source of N (Sas *et al.*, 2002).

1.3.3 Iron, Fe

In addition to P and N, Fe has also been found to alter root morphology. Iron plays an important role in the synthesis of chlorophyll-protein complexes in the chloroplast, which is why Fe deficiency causes chlorosis of younger leaves, leading to stunted growth of plants. It is also an important constituent of enzymes which function in transfer of electrons, *e.g.* cytochromes (Taiz & Zeiger, 2010). In swamp oak (*Casuarina glauca*) and other *Casuarina* species, Fe deficiency rather than P deficiency leads to formation of CRs under hydroponic culture conditions (Zaïd *et al.*, 2003). As both Fe and P stress can induce CR formation, they may share some common pathways controlling CR formation (Skene & James, 2000). Moreover, Fe and P stress have the same effect on root hair development, but are controlled by different signalling pathways (Schmidt & Schikora, 2001). In “strategy I plants”, Fe deficiency enhances Fe-(III) reductase production and exudation of metal-chelating carboxylates and reductants (Marschner & Römheld, 1994). In strategy II plants, Fe deficiency stimulates exudation of phyto-siderophores as chelating agents (Römheld & Marschner, 1986).

1.3.4 Relationships among N, P and Fe

Primary root length shows contrasting responses to P and N availability, while LR length shows similar responses. Nitrate (NO_3^-) supply (Linkohr *et al.*, 2002) and low P (Ward *et al.*, 2008) inhibit primary root elongation through reducing cell elongation, while they increase LR density and growth through a reduction in cell elongation and increases in LR density and growth. It has also been reported that inhibition of primary root elongation could be due to Fe toxicity, which could be recovered by reducing the Fe supply without any change in P availability (Linkohr *et al.*, 2002). In contrast, elongation of LRs is reduced by both high nitrate and high P availability (Linkohr *et al.*, 2002). On the other hand, P deficiency can also inhibit nitrate uptake in white lupin (Neumann *et al.*, 2000), which may result in nitrate deficiency. Nitrogen uptake and metabolism has been shown to be affected by P availability and increased amino acid concentrations in root and shoot have been reported. This could be due to either degradation of proteins (Pant *et al.*, 2015) or negative N feedback (Hogh-jensen *et al.*, 2002; Almeida *et al.*, 2000) due to slow growth and lack of a sink in meristematic parts of the plant.

1.4 Plant growth hormones and their effect on root architecture and CR formation

Plant growth hormones are small molecules derived from metabolic pathways that are present in small concentrations to regulate plant growth and development in response to various environmental factors through a range of complex interactions with other metabolic compounds (Garay-Arroyo *et al.*, 2012). So far, at least nine types of plant hormones have been discovered: auxins, cytokinins, ethylene, gibberellins, abscisic acid, brassinosteroids, jasmonic acids, salicylic acids and strigolactones (Kästner *et al.*, 2014; Santner *et al.*, 2009; Santner & Estelle, 2009; Wolters & Jürgens, 2009). Plant hormones play a major role in nutrient sensing and signal transduction, leading to developmental alterations in root development (Krouk *et al.*, 2011; Rubio *et al.*, 2009; Zhang *et al.*, 2007; López-Bucio *et al.*, 2002; Lynch & Brown, 1997). Interactions and appropriate balanced concentrations of plant hormones regulate root morphology and development (Tanimoto, 2005; Werner *et al.*, 2001; Torrey, 1976). It has been found that LR formation is negatively regulated by cytokinin and abscisic acid, whereas brassinosteroids serve as positive regulators of LR formation (Fukaki & Tasaka, 2009).

Auxin is a well-known plant hormone involved in LR formation. Local auxin gradients are associated with meristematic growth, cell proliferation, elongation and differentiation leading to LR formation (Garay-Arroyo *et al.*,

2012; Van den Berg *et al.*, 1997; van den Berg *et al.*, 1995; Dolan *et al.*, 1993). Ethylene is known to stimulate the biosynthesis and basipetal transport of auxin to activate a local auxin response, leading to inhibition of cell elongation in primary roots (Ruzicka *et al.*, 2007) and stimulation of LR formation (Peret *et al.*, 2013). In the past decade, strigolactones have been reported as novel phytohormones regulating root development under N and P deficiency (Sun *et al.*, 2014; Koltai, 2011).

Similarly to LR formation, CRs are also regulated by endogenous phytohormones. Application of the auxin indole-3-acetic acid application to the growth medium of P sufficient plants increases CR formation, while kinetin inhibits CR formation under P limitation (Neumann *et al.*, 2000). Exogenous application of the synthetic auxin naphthalene acetic acid (NAA) to leaves increases the number of CRs, while the auxin transport inhibitors 2,3,5-triiodobenzoic acid (TIBA) and naphthylphthalamic acid (NPA) reduces the number of CRs in low P treated plants. Although low P increases the ethylene concentrations in the roots, the ethylene inhibitors aminoethoxyvinvylglycine (AVG) and silver thiosulphate do not reduce CR numbers (Gilbert *et al.*, 2000), it was suggested that ethylene does not stimulate CR formation in white lupin. Recently, interaction of gibberellic acid with P deficiency-induced CR formation was shown in white lupin (O'Rourke *et al.*, 2013).

1.5 Function and metabolism of CRs

The function of CRs under nutrient deficiency conditions appears to be to increase the root surface area in order to exploit available nutrients. In white lupin, CRs have been found to be more efficient in uptake of P_i than non-CRs (Skene, 2001; Vorster & Jooste, 1986; Green, 1976; Malajczuk & Bowen, 1974; Jeffrey, 1967). To mobilise the P from organic (Gerke, 1992) and inorganic sources (Hoffland *et al.*, 1989), CRs are known to exude carboxylates such as pyruvate, citrate and malate (Lambers *et al.*, 2006; Shane *et al.*, 2004; Gardner *et al.*, 1983), and phosphatases (Gilbert *et al.*, 1999; Tadano *et al.*, 1993). Organic acids play a key role in intracellular ionic balance in different tissues and, when exuded, are responsible for rhizosphere adaptation in various stress conditions (Chen *et al.*, 2009; Smith & Raven, 1979; Hellebusi, 1976). Aluminium (Al), P and acidic soils interact to regulate malate exudation in soybean (*Glycine max*) (Liang *et al.*, 2013). Secreted organic acids not only mobilise P, either by anion exchange or chelating metal ions from immobile complexes with P (Vance *et al.*, 2003; Lipton *et al.*, 1987), but also detoxify Al by disassociating it from plant roots or making Al-organic acid complexes (Ma, 2000).

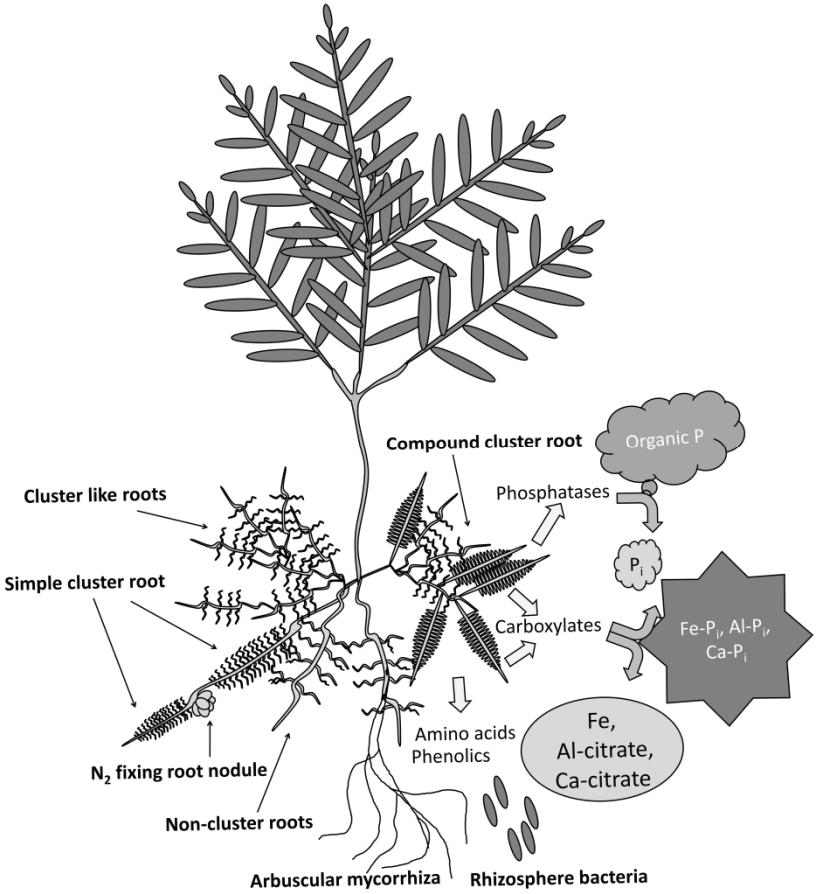

Figure 2. Schematic diagram of the seabuckthorn root system and possible rhizosphere interactions.

To keep the cellular processes running under certain levels of P limitation, plants adapt inorganic pyrophosphate (PP_i)-dependent metabolic flexibility. To conserve ATP and P_i under such conditions, plants activate bypass enzymes, *i.e.* PP_i-dependent phosphofructokinase, pyruvate P_i dikinase and tonoplast inorganic pyrophosphate-dependent H^+-pump, to recycle intracellular P_i (Plaxton & Tran, 2011). In previous CR studies, the activity of enzymes involved in malate and citrate metabolism, such as citrate synthase, malate dehydrogenase (MDH) and phosphoenolpyruvate carboxylase (PEPC), was also found to be higher in CR than non-CR (Massonneau *et al.*, 2001; Neumann *et al.*, 1999; Johnson *et al.*, 1994). Moreover, different

developmental stages of CRs were found to differ in terms of morphology and physiology (Shane *et al.*, 2004; Neumann *et al.*, 2000).

1.5.1 Developmental physiology of CRs

Cluster roots change their metabolism during development from initiation of CR rootlets to maturity. In harsh hakea (*Hakea prostrata*), concentrations of citrate and malate in CR have been shown to increase towards maturity of the CR until after day 12, when a maximum exudation of citrate and malate was observed The concentrations decline by day 20, which is the senescing stage of CR in this plant (Shane *et al.*, 2004).

In white lupin, the citrate concentration shows a similar trend as in harsh hakea, while the malate concentration shows a contrasting trend with higher level, and with higher exudation in the pre-mature stage of CRs than in mature clusters (Neumann *et al.*, 2000). In this plant, different stages of CR are reported to have no differences in P_i uptake, but compared to non-CRs, CRs had higher uptake of P_i (Neumann *et al.*, 2000; Neumann *et al.*, 1999). In view of the studies with these two plants, it is clear that CRs are senesced after exudation of citrate. An unanswered question is whether there is any citrate transporter involved in citrate exudation, or whether exudation is the result of leakage of senescing CRs. Given that after exudation CRs start to senesce, this implies that the same root cannot take up P_i after the exudative burst and that P_i is then taken up by other, younger CRs.

Regarding root physiology in seabuckthorn, the total root system has been investigated under salt and alkali stress, but there are no existing data about CRs, their physiology and exudation, and their role in seabuckthorn survival under nutrient deficiency. Thus this area needs further study.

1.6 Gene expression under P stress and regulation of CR formation

Many gene expression studies have been conducted on various plants to assess responses to nutrients. In an expression profile of *Arabidopsis* with Affymetrix ATH1 geneChips, it was found that P_i deficiency enhanced the expression of 570 ATH1 probe sets and decreased the expression of 488 probe sets (Morcuende *et al.*, 2007). Among the CR-producing species, most of the gene expression studies to date have been conducted on white lupin. In this species, 80 root-specific cDNA clones have been investigated and have homologies with genes encoding enzymes involved in cell modification, carbohydrate and phenolics metabolism, protein kinases and phosphatases, membrane transport proteins, pathogen-related proteins and transcription factors (Neumann *et al.*,

2000). In another study on white lupin, it was found that P supply differentially expressed transcripts related to carbon flux (glyceraldehyde 3-phosphate dehydrogenase), glycolytic bypass (phospho*enol*pyruvate carboxylase) and P recycling (sulpholipid synthase) (Peñaloza *et al.*, 2002b). Nylon filter arrays have revealed 35 genes showing higher expression in CRs than in +P non-CRs, which included genes responsible for carbon metabolism, secondary metabolism, P uptake and remobilisation, and plant hormone signalling (Uhde-Stone *et al.*, 2003). Recently, transcriptome analysis of CRs in white lupin provided new insights into P deprivation responses and hormone signalling. It revealed 2128 differentially expressed genes with a two-fold expression difference in response to P supply (O'Rourke *et al.*, 2013). As a result of consistent differential expression of 12 sequences shown in white lupin, *Arabidopsis* and potato, they are thought to be suitable as a set of marker genes to assess the P status of plants (O'Rourke *et al.*, 2013; Hammond *et al.*, 2011; Thibaud *et al.*, 2010; Morcuende *et al.*, 2007; Misson *et al.*, 2005). In white lupin, three genes containing the SPX domain, which regulates P_i homeostasis (Secco *et al.*, 2012), have been shown to be expressed in both leaves and roots under P_i deficiency. In addition, P_i deficiency in white lupin up-regulates major transcription factors related to general stress and hormonal pathways, phosphate transporters, ABC transporters, genes involved in oxidative stress responses, and root genes involved in exudation of acid phosphatases and organic acids (O'Rourke *et al.*, 2013). In a continuation of RNA sequencing (RNA-seq) analysis of different developmental stages of CRs of white lupin, it was found that among the hormone-related transcripts, auxin- and brassinosteroid-related genes were up-regulated in the initial stages of CR development, while transcripts related to jasmonic acid and abscisic acid and transcripts related to remobilisation of organic P were up-regulated in mature CRs (Wang *et al.*, 2014b).

1.6.1 Role of phospho*enol*pyruvate carboxylase genes (PPC) in organic acid metabolism

The enzyme phospho*enol*pyruvate carboxylase (EC 4.1.1.31) plays a vital role in many metabolic processes in plants, such as in photosynthesis by C4 and CAM plants and maintenance of cellular pH, carbon sources for root nodules, and many different catalytic and regulatory properties of C3 plants (Izui *et al.*, 2004; Latzko & Kelly, 1983). PEPC catalyses bicarbonate (HCO_3^-) fixation through carboxylation of phosphoenolpyruvate (PEP) to yield oxaloacetate, which is further reduced to malate (Plaxton & Carswell, 1999; Theodorou & Plaxton, 1993). Malate can be converted into 2-oxoglutarate, producing the substrate for NH_4^+ assimilation to asparagine and glutamine (Masumoto *et al.*,

2010), or can be exuded directly from roots, especially under conditions of P deficiency in plants (Peñaloza *et al.*, 2005; Peñaloza *et al.*, 2002a; Johnson *et al.*, 1994; Hoffland *et al.*, 1992; Duff *et al.*, 1989). Higher levels of PPC mRNA, PEPC activity and PEPC protein in CRs than in normal roots (Johnson et al., 1996b) suggest diversified roles in different root tissues. Phospho*enol*pyruvate carboxylase genes (PPC) are potential candidate genes involved in various metabolic processes in different tissues of seabuckthorn. Plants have PPC families with at least two plant-type PPC (PTPC) genes and at least one bacterial-type PPC (BTPC) (Brendan *et al.*, 2011; Mamedov *et al.*, 2005; Sánchez & Cejudo, 2003). Studies of BTPC in *Arabidopsis* and rice (Sánchez & Cejudo, 2003) have revealed that *Arabidopsis* has three additional PPC genes, *Atppc1*, *Atppc2* and *Atppc3*, with 10 exons, while the *Atppc4* gene, containing 20 exons, shows higher similarity with *Escherichia coli* than PTPCs.

1.6.2 Role of acid phosphatases in P metabolism

Under P deficiency, plants are known to secrete acid phosphatases to hydrolyse organic phosphate from the rhizosphere (Tran *et al.*, 2010; Tadano *et al.*, 1993; Tadano & Sakai, 1991) and to activate intra-cellular acid phosphatases to recycle the organic P from senescing tissues (Shane *et al.*, 2014; Duff *et al.*, 1994). Acid phosphatases are the enzymes involved in catalysis of phosphoric acid mono- or di-esters (Wahnon *et al.*, 1995; Godavari, 1962). Plants have two types: high molecular weight acid phosphatases with a functional unit of homodimeric form and low molecular weight acid phosphatases containing only a metallo-phosphoesterase motif in monomeric form (Klabunde *et al.*, 1996). Purple acid phosphatases (PAP) form a distinct group, with purple colour in aqueous solution (Vogel *et al.*, 2001). Several isoforms of acid phosphatases have been characterised in various plant species, such as *Arabidopsis* (Wang *et al.*, 2014a; Zhang *et al.*, 2014; Kuang *et al.*, 2009) and soybean (Kong *et al.*, 2014) and the CR-forming species white lupin and harsh hakea (Shane *et al.*, 2014; Wasaki *et al.*, 2000).

Exploring the genes encoding isozymes of intracellular acid phosphatases involved in recycling of P and secretion of purple acid phosphatases, which are up-regulated under P deficiency, may help to improve strategies for engineering P_i-efficient and low P tolerating crops, in order to minimise the P losses from P_i fertilisation in the agriculture sector (Veneklaas *et al.*, 2012; Tran *et al.*, 2010).

2 Aims of the thesis

Considering the limited availability of nutrients and their importance for plant growth and development, it is important to gain knowledge about the diversity of strategies, including morphological and physiological mechanisms, used by plants to overcome this limitation. The overall aim of this thesis was to examine adaptive mechanisms used by roots in response to nutrient-deficient conditions that may play a role in the distribution of species and that may provide concepts and tools for the improvement of plants.

In the rhizosphere, P and Fe are often not readily available to plants. In addition, on a global scale, rock P to be used as fertilizer is becoming a depleted natural resource. Based on these aspects it is important to explore efficient mechanisms used by plants to acquire nutrients.

Seabuckthorn is a N_2-fixing actinorhizal plant found in early succession and disturbed habitats. It was selected for this study as it is a perennial plant that colonises low fertility soils through both seedling growth and clonal growth by formation of shoots from roots (root suckers).

Specific aims were to:

1. Investigate the capacity of seabuckthorn to form CRs and characterise root development.
2. Compare different accessions of seabuckthorn of different geographically and evolutionary origins in terms of how they respond to nutrient deficiency.
3. Investigate changes in primary metabolism and transcript levels of genes involved in primary metabolism, during CR development, and in response to P availability.
4. Characterise the gene family encoding PEPC and elucidate expression patterns to propose possible roles in plant metabolism.
5. Evaluate how P and auxin interact in the formation of LRs as well as shoots from roots.

3 Materials and methods

3.1 Plant material (Papers I-IV)

In order to characterise the CR and evaluate the response to N, P and Fe, plants of two subspecies of *H. rhamnoides L.* were used. One plant source was *H. rhamnoides* cv. BHi10726 cultivated in Öland, Sweden. Cv. BHi10726 originates directly from cv. Omskaya-27 obtained in a breeding programme at All-Union Scientific Research Institute of Horticulture, Michurinsk, Russia, under the leadership of Drs G.A. Lobanov and V.T. Kondrashov (Eydelnant 1998; Kondrashov, 1976). That major breeding programme was based on *H. rhamnoides* ssp. *mongolica* obtained from Altai, Russia. The selection was carried out in agricultural field soil in the central black earth (chernozem) Region of Russia. Chernozem is a very rich soil with high contents of organic matter and nutrients (Chendev *et al.*, 2015; Nikiforova *et al.*, 2012). The other plant source was a seed accession from a natural population of *H. rhamnoides* ssp. *turkestanica* in the mountainous region of Gilgit-Baltistan in northern Pakistan, where this subspecies is common and grows in soils with a low content of organic matter (Ali *et al.*, 2013).

To characterise the PPC gene family, clones and seedlings of ssp. *turkestanica* (Pk) and ssp. *rhamnoides* (GC1) were used. To assess the response to P (Paper III), ssp. *turkestanica* was compared with a cloned seedling obtained from field-grown cv. Sunny with the male cv. Lord, originating from a breeding programme involving Russian and Baltic germplasm, including *H.r.* ssp. *mongolica* (Bruvelis, 2003). Seedlings of cv. Sunny were also used together with other genotypes of ssp. *rhamnoides* and ssp. *sinensis* for the *in vitro* study (Paper IV).

3.2 Anatomical studies (Papers I, III and IV)

3.2.1 Fluorescence microscopy

For visualisation of meristematic development, root tissues were treated with 0.1 μg mL^{-1} of 4′,6-diamidino-2-phenylindole (DAPI) solution for more than 1 h (Zhou *et al.*, 2008). To visualise the endodermis (Paper IV), samples were treated with fluorol yellow solution followed by safranin (Lux *et al.*, 2005). The whole-mount samples and cross-sections were observed using fluorescence microscopy.

3.2.2 Scanning electron microscopy

For morphological studies of SfR initiation and development, different stages of SfRs were fixed in 2.5% glutaraldehyde in phosphate-buffered saline (pH 7). After dehydration in an ethanol series and acetone, dried samples were mounted on tape fixed on an aluminium stub, coated with gold particles and scanned using an electron microscope.

3.2.3 Visualisation of acid phosphatase activity

Activity of secretory acid phosphatase was visualised in CRs and non-CRs of seabuckthorn grown under low P using the 1-naphthyl phosphate method (Dinkelaker & Marschner, 1992).

3.3 Gene expression analysis (Papers II and III)

3.3.1 RNA extraction, cDNA synthesis and qPCR analysis

Frozen samples were ground in liquid N and RNA was extracted from different tissues using the Spectrum RNA extraction kit (Sigma-Aldrich). For transcriptome analysis, the extracted RNA was further purified using the PureLink minikit (Ambion). To avoid degradation of RNA, the RNAse inhibitor Ribolock was added to the final samples. RNA quality was measured using an Agilent Bioanalyzer. cDNA for RACE was synthesized using the qScript Flex cDNA kit (Quanta Biosciences Inc.) and for real-time quantitative polymerase chain reaction (RT-qPCR) using the Fermentas Maxima First Strand cDNA synthesis kit (Thermo Fisher Scientific) or the Superscript cDNA synthesis kit (Invitrogen). Specific primer pairs for qPCR were designed for all PPC genes and cross amplification by each primer pair was tested using standard dilution series of pure cDNA of each gene.

3.3.2 RNA sequencing

Transcriptome libraries for sequencing were constructed according to the Illumina HT TruSeq RNA sample preparation protocol (www.Illumina.com). The libraries were sequenced on the Illumina HiSeq 2000 platform as paired-end 90 bp reads following the manufacturer's recommendations at BGI, Shenzhen, China. The RNA-seq data were assembled as *de novo* transcriptomes without reference genome using Trinity (Grabherr *et al.*, 2011) and clustered using TGICL (Pertea *et al.*, 2003). Contigs and unigenes were annotated using Blast (www.ncbi.nlm.nih.gov). Expression levels for each transcript were calculated as number of fragments (paired-end reads) per kilobase per million fragments (FPKM) mapped per contig. Of the homologous contigs of genes involved in glycolysis, tricarboxylic acid cycle (TCA cycle), P transporters and acid phosphatases, contigs with higher FPKM were selected for hierarchical cluster analysis and heat maps. Differentially expressed transcripts related to root exudates were shown as heat maps in the CLC Main Workbench 7 (CLC bio, Denmark) and the JMP statistical software.

3.3.3 Cloning and analysis of the phospho*enol*pyruvate carboxylase (PEPC) gene family in seabuckthorn

The polymerase chain reaction (PCR) products amplified from root and meristem cDNA and genomic DNA using PTPC and BTPC primers (Paper II) were fractionated on 1.5% agarose gels and then the band of expected size was excised and purified (GeneJET gel extraction kit, Fermentas). Purified products were cloned using a TOPO-TA cloning kit (Life Technologies Corporation) according to the manufacturer's instructions. Cloned PCR products were sent to Macrogen for sequencing. PPC sequences were analysed in CLC Main Workbench 7 (CLC bio, Denmark) and compared with genomic and transcript sequences of PPC.

3.4 Root metabolite analysis through proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy (Paper III)

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique for identifying organic compounds. It is based on the abilities of certain compound nuclei to resonate with radio waves when in strong magnetic fields. NMR spectroscopy is a non-targeted method with no requirement for derivatisation of the sample prior to analysis, which makes it a suitable technique for analysing uncharacterised samples in metabolomic analysis. It is non-destructive, but has the requirement that protons are present on the molecule under study at the pH of the sample. This makes it possible to identify the

structures based on chemical shifts and coupling information and the resonance strength can be used for quantification. In Paper I and III, the metabolite composition of root extracts and root exudates was analysed using ^{1}H-NMR spectroscopy on a Bruker Avance II 600 MHz spectrometer (Bruker) equipped with a QCI-P cryoprobe. The 1D spectra were manually phased and baseline corrected. TSP was used as a concentration standard and as reference for chemical shift at 0.00 ppm. The spectra were analysed and the concentrations of the compounds identified were measured using the Chenomx profiler.

4 Results and discussion

4.1 CR morphology and development in seabuckthorn (I, III)

The results in Papers I and III confirmed that seabuckthorn has the ability to produce CRs. Genotype Pk produces typical CRs in most conditions and thus was used as a model plant to characterise the morphology and metabolite profile of CRs. Cv. BHi10726 could not form CRs in sand-vermiculite cultivation, but could form some CR-like roots. In contrast, in hydroponic cultivation cv. BHi10726 was able to form CRs under P and Fe deficiency (Paper I). Differences in the root system of the two genotypes revealed their different responses to nutrients. The smaller number of CRs observed in cv. BHi10726 could be due to its adaptation to nutrient-rich soils, in contrast to genotype Pk which originates from low fertile soil with a low amount of organic matter (Ali *et al.*, 2013).

Seabuckthorn has both types of CRs (simple and compound) (Figure 3A), while white lupin mostly forms the simple type and plants of the Proteaceae have the compound type of clusters with a difference of multiple rows of CR rootlets (Lambers *et al.*, 2006). In seabuckthorn, CRs are mostly found around the crown or the mature part of the root, while the LRs found at the base of the primary and secondary roots are longer, with active meristem and with continuous growth compared with CR rootlets (Figure 3A,C). Similarly, in white lupin it has been observed that CRs proliferate in the upper layer of the soil that contains more organic matter and that localised application of organic matter containing N and P can stimulate CR formation in P-stressed plants compared with plants with a high content of P in leaves. It is reported that slow release of N and P from organic matter allows CR formation (Li *et al.*, 2010).

Similar to CRs of white lupin, seabuckthorn CRs consist of two rows of cluster rootlets (Paper I). The average number of rootlets per cm on the main

root/CR-bearing root varies between species. In Paper I the number was 10 per cm of main root, as described by Johnson *et al*. (1996) for white lupin.

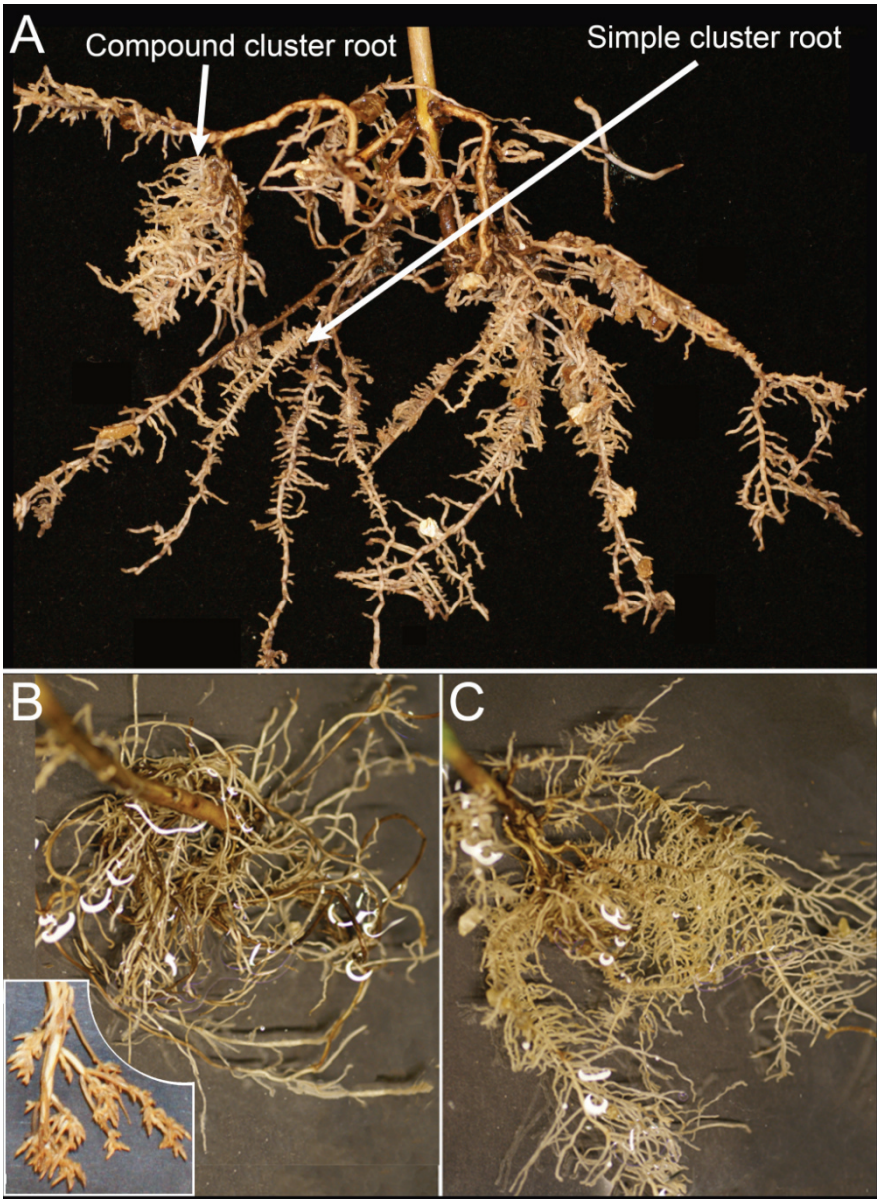

Figure 3. Cluster root (CR) system of seabuckthorn. (A) Simple and compound CR of genotype Pk. (B) Root of cv. BHi10726 grown under low P in sand-vermiculite and (insert) in hydroponics under Fe deficiency. (C) Root of genotype Pk grown under low P in sand-vermiculite.

In seabuckthorn, non-cluster LRs are usually longer than CR rootlets and the length of CR rootlets varies on a spatio-temporal basis from 2-8 mm. Cluster rootlets are usually shorter towards the tip of the CR and the length increases towards the base of the cluster. The length of the CR rootlets also depends on the intensity and type of nutrient stress. The CR rootlets produced on cv. BHi10726 under hydroponic conditions (Figure 3) appeared shorter under Fe deficiency than CR rootlets induced by P deficiency (Paper I, Figure 2DE). These results are consistent with P and Fe deficiency effects on white lupin (Hagstrom *et al*., *2001*).

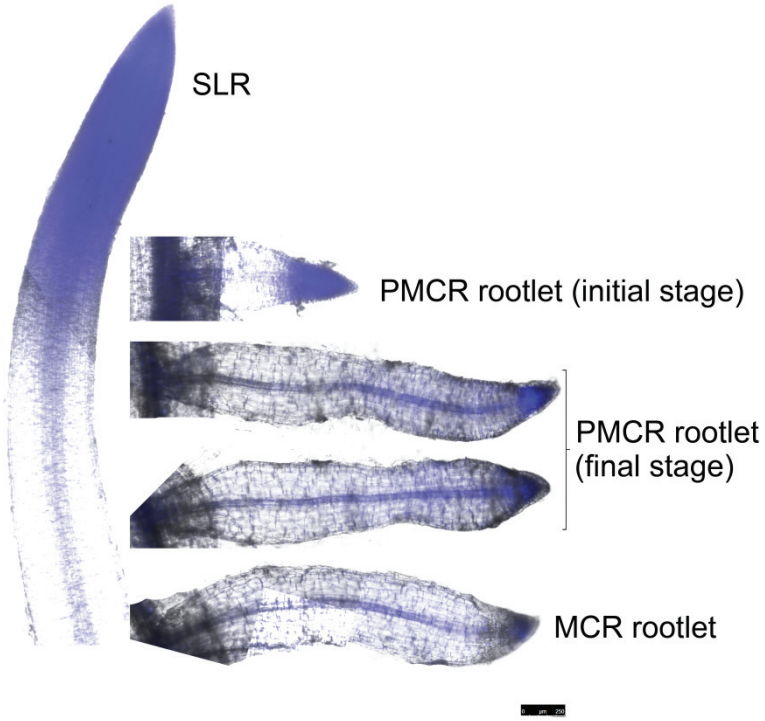

Figure 4. Developmental stages of cluster root (CR) rootlets of genotype Pk of seabuckthorn, with secondary lateral root (SLR) and rootlets of premature cluster root (PMCR) and mature cluster root (MCR). Regions with a high density of small cells, indicating meristem in growing rootlets, are stained blue with 4',6-diamidino-2-phenylindole (DAPI). Bar = 250 µm.

Cluster root rootlets appeared to initiate in the same way as non-cluster LRs, but are formed at higher frequencies and have a typical determinate type of growth (Figure 4), as previously observed in other CR-bearing species. Another difference between LRs and CR rootlets is that LRs can produce sub-

lateral roots, but this characteristic is absent in CR rootlets. At maturity, CR rootlets stop their growth and go into senescence, with a shrunken tip and reduced meristem size, as seen in DAPI-stained roots in Figure 4. Disappearance of meristem from mature CR rootlets is described as one of the traits of CR rootlets (Watt & Evans, 1999). The determinate type of root growth has been reported in non-CR producing plants such as *Arabidopsis* (Sanchez-Calderon *et al.*, 2005) under low P and also in maize (McCully, 1999; Varney & McCully, 1991), but these structures do not display the typical CR-type pattern with compact CR rootlets in rows.

The number of CRs per plant varied among the seabuckthorn genotypes studied. Genotype Pk produced significantly higher numbers of CRs than cvs. BHi10726 and Sunny. The number of CRs increased under Fe deficiency in genotype Pk, while cv. BHi10726 did not show any significant effect. In Paper III, the Pk and Sunny genotypes were grown under three different levels of P and it was found that low levels of P supply increased CR formation in cv. Sunny, but not in genotype Pk. Comparison of total plant biomass produced under three different levels of P supply showed clearly different responses of two genotypes to P levels; increased P supply caused an increase in biomass for cv. Sunny, but a decrease for genotype Pk. Increasing the P supply from 1 to 10 mM increased the P content in leaves in cv. Sunny, but not in genotype Pk, which indicates different responses of the two genotypes to P levels (Paper III). It can also be speculated that the lowest amount of P was not sufficient to cause deficiency for genotype Pk (affecting the number of CRs) or that genotype Pk is not sensitive to P supply and is adapted to low levels of P with evolved characteristics of CR formation.

4.2 Metabolism in CRs in seabuckthorn (III)

4.2.1 Root metabolism in response to P deficiency (III)

Under P deficiency conditions, plants are reported to exude carboxylates, mainly citrate and malate (Lambers *et al.*, 2006). Some other species, such as *Banksia*, *Hakea* and *Dryandra* species in the Proteaceae, have been found to exude different relative proportions of organic acids, especially citrate, lactate and malonate.

In seabuckthorn, metabolite analysis of root extracts revealed a significantly higher level of sucrose in genotype Pk compared with cv. Sunny, which could be an indicator of a higher carbon pool for downstream respiration and organic acid metabolism in genotype Pk roots, reflected as total amount of organic acids and amino acids in these roots. Of the organic acids detected in plants treated with high and low P, the concentration of succinate increased

under low P supply in genotype Pk, but not in cv. Sunny while concentration of fumarate increased at xP. Higher sucrose concentration and enhanced succinate under low P supply in roots of genotype Pk indicated enhanced TCA metabolism to alleviate P stress conditions. This trait is similar to that in other CR-producing plants such as harsh hakea and white lupin (Shane *et al.*, 2004; Neumann *et al.*, 1999).

4.2.2 Functional role of root exudates (III)

Plants can exude over 200 carbon-containing compounds as rhizo-deposits (Curl & Truelove, 1986) the expense of up to 40% of the net carbon fixed during photosynthesis (Dennis *et al.*, 2010). To elucidate a function of CRs, root exudates extracted from rhizo-deposits around CRs were analysed in Paper III. Malate was the third most dominant compound found in the rhizo-deposits after glucose and sucrose. Root exudates in rhizo-deposits may also contain compounds released through leakage because of mechanical damage to roots during harvest (Personeni *et al.*, 2007; Jones, 1998). In comparison, in a previous study on seabuckthorn root metabolism under salt and alkali stress, it was observed that comparatively lower amounts of malate and citrate accumulated in root, while acetate accumulation was dominant under strong alkali stress (Chen *et al.*, 2009).

4.2.3 CR metabolism (III)

Analysis of CR metabolites revealed that malate was the most abundant organic acid in cluster root extracts of genotype Pk (Figure 5).

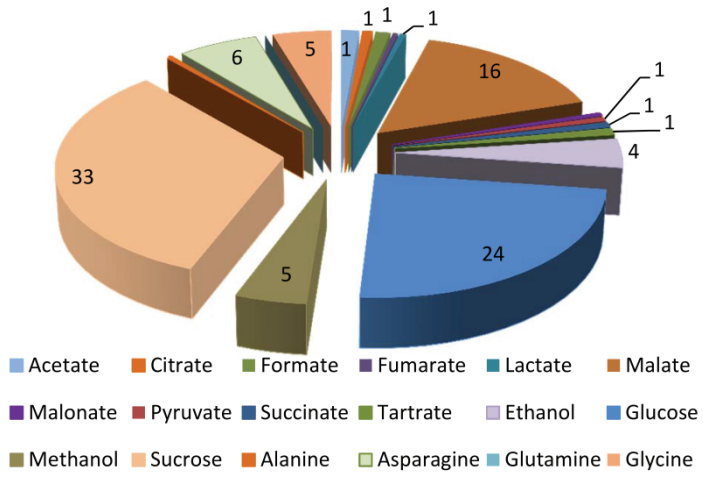

Figure 5. Relative abundance of metabolites in cluster root (CR) extracts of genotype Pk of seabuckthorn.

Malate was the dominant organic acid in all stages of CR, while citrate was only detected in SLR and PMCR. Internal concentration of metabolites in CRs of white lupin and hakea was found altered during development from initiation to maturity of CR rootlets (Shane *et al.*, 2004; Neumann *et al.*, 2000; Neumann *et al.*, 1999). Plasticity of metabolic flux to citrate or malate could be a spatiotemporal and adaptive behaviour of sea-buckthorn to nutrients. There were also CRs stage-specific differences in concentration of some other metabolites such as acetate, succinate, tartrate, sucrose and ethanol.

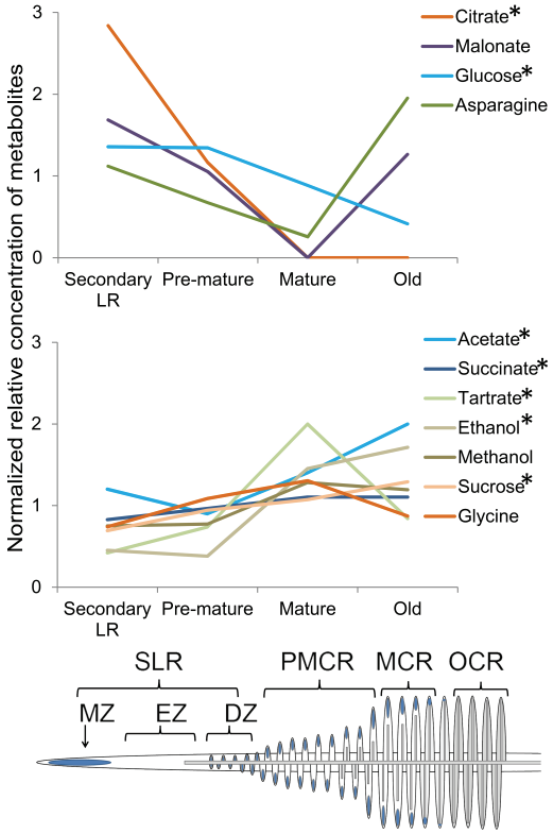

Figure 6. Normalised relative concentration of metabolites (a.u.) in extracts from four stages of seabuckthorn CR development. The model illustrates the CR stages taken for ^{1}H-NMR analysis. Secondary lateral root (SLR) includes meristematic zone (MZ), elongation zone (EZ) and differentiation zone (DZ). Premature CR (PMCR), mature CR (MCR) and old CR (OCR) are the developmental stages of CR rootlets.

Of the sugars detected in CRs of seabuckthorn, the concentration of glucose decreased while the concentration of sucrose and ethanol increased towards

maturity (Figure 6). Accumulation of sucrose in mature CR could be due to decreasing rate of consumption under downstream glycolysis metabolism towards maturity of CRs. Similar decreases in concentration of citrate and higher concentrations of ethanol and succinate (Figure 6) towards maturity is also an indicator of anaerobic metabolism resulting in diversion of carbon into ethanol formation (Bailey-Serres *et al.*, 2012).

The compounds only found in premature stages of CRs were aromatic compounds involved in root development, while other compounds involved in root exudate metabolism were detected in all stages of CR development. As the pre-mature stage of CRs contains actively growing meristem (Figure 4), three aromatic compounds, phenylalanine, tyrosine and tryptophan, were detected (Paper III). Aromatic amino acids are not only essential components in protein synthesis, but also serve as precursors for a wide range of secondary metabolites important for plant growth and development (Tzin & Galili, 2010). Tryptophan is a precursor for the essential growth hormone IAA, serving as an auxin (Muday *et al.*, 2012; Bartel, 1997), which is probably produced in developing root tips of premature CR rootlets. Developing root tips are one of several auxin sources identified in plants (Ljung *et al.*, 2005; Bhalerao *et al.*, 2002; Ljung *et al.*, 2001). Phenylalanine and tyrosine could also be involved as amino-group donors in transamination of tryptophan to yield indole-3-pyruvate in the indolepyruvic acid pathway (Schneider & Wightman, 1974; Sheldrake, 1973). Previous studies (Chen *et al.*, 2009) and the results in Paper III reveal that seabuckthorn roots have high metabolic flexibility to cope with different rhizosphere conditions as well as with different stages of CRs.

4.3 Gene expression patterns under low P and CR development (II, III)

In previous studies, the transcriptomic response of plants to P deficiency have been addressed through various means, from microarrays to RNA-seq analysis. Similarly to the morpho-physiological responses, the transcriptomic responses to P stress also vary among plant species. Papers II and III examined the transcripts involved in root exudate metabolism, including sugars, organic acids and related to P metabolism including acid phosphatases and P transporters.

In glycolysis, transcripts of sucrose synthase were lower in genotype Pk than in cv. Sunny, which could result in sucrose accumulation in genotype Pk. As regards the role of sucrose in genotype Pk, it could either be a pool for carbon source for down-stream reactions or secreted into the rhizosphere to attract a microbial community that in return produces siderophores (Weisskopf

et al., 2011), organic acids and other P-solubilising compounds for plants (Goteti *et al.*, 2013; Rodríguez & Fraga, 1999).

Under certain conditions such as anoxia, when mitochondrial metabolism is not very active, decarboxylation of pyruvate results in biosynthesis of acetaldehyde and then ethanol formation. In CR development, the higher transcript levels of pyruvate decarboxylase in the mature stage are compatible with the significantly higher levels of ethanol found in old Cluster Roots (OCRs).

Acid phosphatases hydrolyse P from various phospho-monoesters within the plant and in the rhizosphere. The *Arabidopsis* genome has 29 genes encoding purple acid phosphatases that are regulated by various environmental factors (Wang *et al.*, 2014a; Tran *et al.*, 2010). Of the transcripts of acid phosphatases in seabuckthorn, a homolog of *PAP26* was the most abundant *PAP* in roots and has also been reported as one of the major genes encoding "secreted acid phosphatase" in *Arabidopsis* (Wang *et al.*, 2014a; Tran *et al.*, 2010). *PAP26* and *PAP3* were the two major PAPs with high transcript levels**,** but the reduced P supply did not show any effect on their levels. In contrast, P deficiency increased the transcript levels of *PAP22* and *PAP15*, which might play a role in P deficiency metabolism in roots of seabuckthorn.

In CR developmental stages, transcript homologs to genes encoding acid phosphatases, *i.e*. *PAP3*, *PAP22* and the P transporters *PhT1-4*, *1-9*, *1-7 PhT1-5*, showed up-regulation towards maturity of CR, explaining their involvement in CR metabolism. After the mature stage of CR, the CR starts senescing and degradation of nucleic acids, lipids and proteins begins (Thomas, 2013). Recycling of nutrients, especially P, from senescing CRs to the growing part of the plant requires acid phosphatases (Wang *et al.*, 2014b). In mature stages of CRs in harsh hakea, remobilisation of P has been observed during senescence as part of this plant's efficient P cycling strategy for growth on nutrient-impoverished soils (Shane *et al.*, 2004). Senescence in CRs of harsh hakea and senescing leaves of *Arabidopsis* enhances the activity of both intra-cellular and cell wall targeted acid phosphatase and the *AtPAP26* mutant shows a drastic decline in cell wall acid phosphatase activity (Shane *et al.*, 2014). However, in contrast to *AtPAP26,* the homolog of the PAP26 transcript in seabuckthorn did not display a significant increase towards maturity of CR, although its involvement in P metabolism cannot be ruled out as it was the dominant transcript of the PAPs in seabuckthorn root.

4.4 Analysis of the phospho*enol*pyruvate carboxylase gene family (II)

Phospho*enol*pyruvate carboxylase catalyses fixation of bicarbonate (HCO_3^-) via carboxylation of PEP to yield oxaloacetate (OAA) and releases phosphate (P_i). Paper II characterised the PPC gene family in seabuckthorn and its expression patterns in different tissues and under P stress (Paper III). Analysis of sequences obtained through RNA-seq and RACE-PCR revealed that PEPC is encoded by a family of six members in total (Figure 7), including five PTPCs (*HrPPC1*, *HrPPC2*, *HrPPC3*, *HrPPC5* and *HrPPC6*) and one BTPC (*HrPPC4*). These results indicate that PTPCs are not restricted to three, as previously reported for *Arabidopsis* and rice (Sánchez & Cejudo, 2003). In later studies, rice has been shown to contain five PTPCs, four cytosolic including one BTPC and one chloroplastic(Muramatsu *et al.*, 2014; Masumoto *et al.*, 2010), while four PTPCs have been reported for white lupin to date (Peñaloza *et al.*, 2005).

Analysis of RNA-seq data for PPC transcripts showed higher transcript levels of all *HrPPC* genes except for *HrPPC2* in three stages of CR, whereas *HrPPC2* showed higher transcript levels in root nodules. Expression analysis of different tissues through qPCR also revealed higher expression of *HrPPC2* in roots followed by root nodules, which indicates its dual function in both root metabolism and root nodules, where PEPC facilitates oxaloacetate for amino acid metabolism as well as providing malate. In root nodules of the legume alfalfa exposed to increased CO_2 levels, the N_2-fixing activity was found to increase, which was interpreted as an important role of PEPC to provide malate for N_2-fixing bacteroids and provide oxaloacetate for NH_4^+ assimilation (Fischinger *et al.*, 2010). Further expression analysis of PPC genes revealed up-regulation of the *HrPPC2* gene under P deficiency in genotype Pk, which confirms its involvement in root metabolism. This could be a strategy of the plant to release P_i and avoid P_i - and ADP-consuming steps as in pyruvate biosynthesis through pyruvate kinase and to produce malate for exudation in the following step through cytosolic MDH (Plaxton & Tran, 2011).

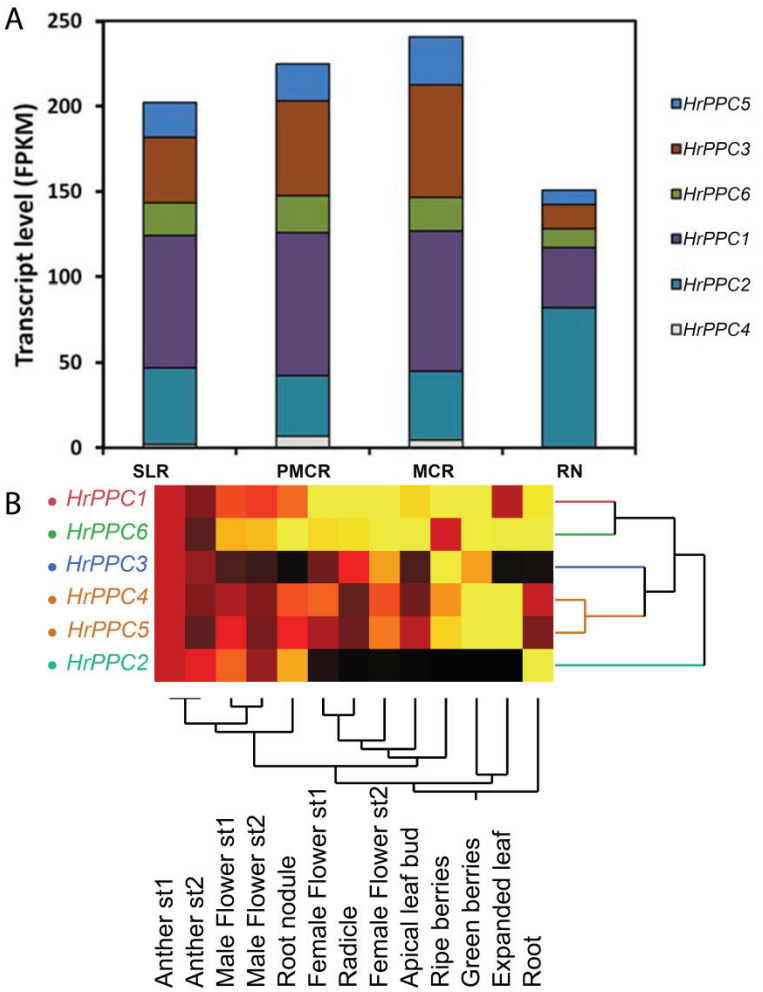

*Figure 7.*Expression of *HrPPCs*. (A) Relative levels (FPKM) of *HrPPC* transcripts in CR development stages of genotype Pk and (B) normalised fold expression of *HrPPC* genes in different tissues of seabuckthorn in female and male plants of genotype GC1. Expression in various tissues was normalised with expression of anther stage (st1). Yellow colour indicates high expression, red intermediate expression and black low expression of *HrPPC* genes. Similar colour of clusters indicates co-expression of the genes.

Screening of the expression pattern in different plant tissues revealed expression of the *HrPPC2* gene in male flowers (Figure 7). Higher expression of *HrPPC1* in flowers and berries suggests a possible function in male gametophyte. In contrast, the BTPC gene *HrPPC4* and the PTPC gene *HrPPC5* had higher expression in green berries and expanded leaves, which could be due to a role in organic acid metabolism in green tissues. In endosperm of developing castor oil plant (*Ricinus communis)* seed, the BTPC

RcPPC4 and the PTPC *RcPPC3* firmly interact to form the Class-2 PEPC which is a hetero-octamer of BTPC and PTPC subunits complex (O'Leary *et al.*, 2011a; Uhrig *et al.*, 2008; Gennidakis *et al.*, 2007). In seabuckthorn, the co-expression of *HrPPC4* and *HrPPC5* (Figure 7B) may suggest that these two genes encode the subunits of the Class-2 PEPC protein, although the *RcPPC3* of castor oil is more similar to the *HrPPC1*, *HrPPC2* and *HrPPC3* than to *HrPPC5*. Expression patterns from the castor oil plant *PPC* genes also showed that the *RcPpc4* and the *RcPpc3* had fairly similar patterns and it was stated that their pattern of co-expression may be significant their participation in Class-2 PEPC (O'Leary *et al.*, 2011a)

Higher expression of *HrPPC6* in most tissues except two stages of anthers and ripe berries revealed its role in developing tissues, where PEPC may play a role in carboxylic acids (oxaloacetate and malate) needed to enhance TCA and glyoxylate cycle activity in developing tissues, or a role as the carbon source of building blocks for organogenesis (O'Leary *et al*., 2011b; Sangwan *et al*., 1992).

4.5 Phosphorus and auxin interaction of formation of lateral root and shoot-from-root (IV)

Shoot-from-root formation in seabuckthorn was observed to emerge from mature parts of the roots under *in vivo* and *in vitro* conditions (Figure 8D,E,F). In greenhouse-cultivated plants of cv. Sunny found to be dormant during the winter, SfRs were seen to emerge (Figure 8E) when axillary buds were sprouted.. Apical dominance of the apical shoots inhibits SfR initiation in poplar (Eliasson, 1971).

The observations made in Paper IV revealed that after winter, because of absence of an apical dominance effect when the auxiliary buds sprout, the SfRs also initiate at the same time. Similarly, under *in vitro* conditions apical dominance was prevented through hormonal regulation. To localise the position of initiation of SfRs under *in vitro* conditions, initial cell division was visualised in cross-sections of roots from W4 medium by staining with DAPI followed by staining with fluorol yellow and safranin (Lux *et al*., 2005) to visualise the vascular region and endodermis. This showed that early cell division (Figure 8A) occurred around the pericycle between the endodermis and vascular region, which is the site for LR initiation (Casimiro *et al*., 2001; Dubrovsky *et al*., 2000). This means that the stem cells between the endodermis and vascular region in roots differentiate into LRs or SfRs.

To understand the development and to localise the emergence of SfRs under *in vitro* conditions, different developmental stages of SfRs were studied

through scanning electron microscopy (SEM). It was observed that initial cell division and protrusion of SfRs were localised at the base of the LR and existing LRs senesced with the development of SfRs (Figure 8C). In later stages, the whole root was covered with multiple SfRs (Figure 8D).

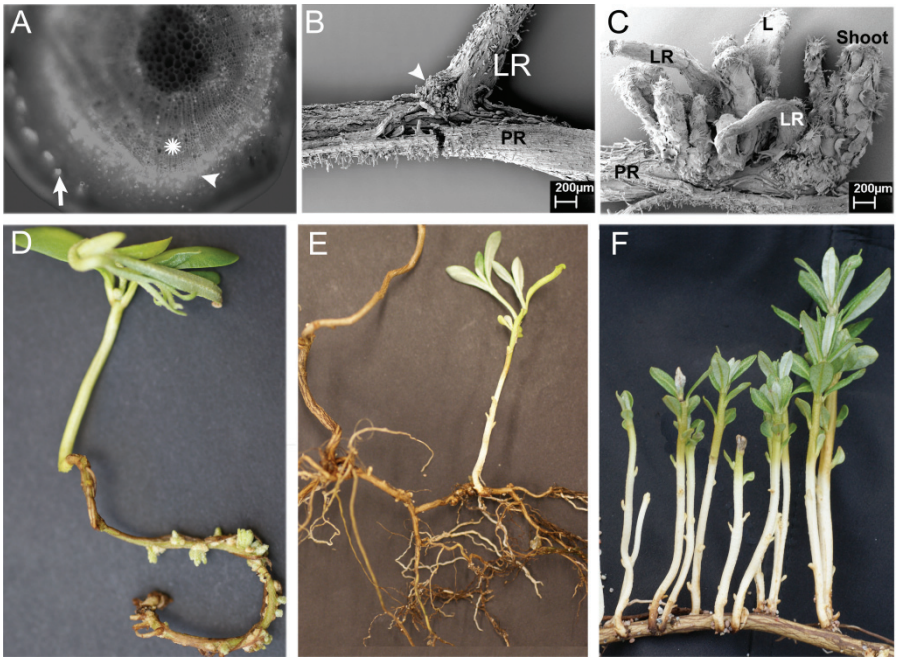

Figure 8. Shoots-from-root (SfR) formation in seabuckthorn. (A) Fluorescence micrograph of a hand-cut cross-section of root from W4 medium. Staining with 4',6-diamidino-2-phenylindole followed by safranin showed a dense layer of cells (arrow head) between the vascular region (asterisk) and suberised cell layer of endodermis (arrow with tail). (B) Pre-emergence stage of shoot initiation in the form of protrusion of 'callus-like' cells at the base of the lateral root (LR). (C) Shoot initiation in the form of young leaves (L) and lateral roots can be seen, together with SfRs around the lateral roots on primary roots (PR). (D) *in vitro* seedling from W4 medium with multiple SfR emerging. (E) Overwintering SfR formation in pot culture. (F) SfR in wild seabuckthorn in nature.

As auxin is a well-known plant hormone to activate LR primordia, the hypothesis tested in Paper IV was that the interaction of auxin and P on shoots and roots can be studied in such a system where both LRs and shoots from roots are initiated from the same region.

To investigate the P-auxin interaction and its impact on shoots and roots, seedlings were treated with three levels of P, with and without auxin, in woody plant medium (WPM) prior to transferring the seedlings to WPM or W4 medium for development of roots or SfRs, respectively.

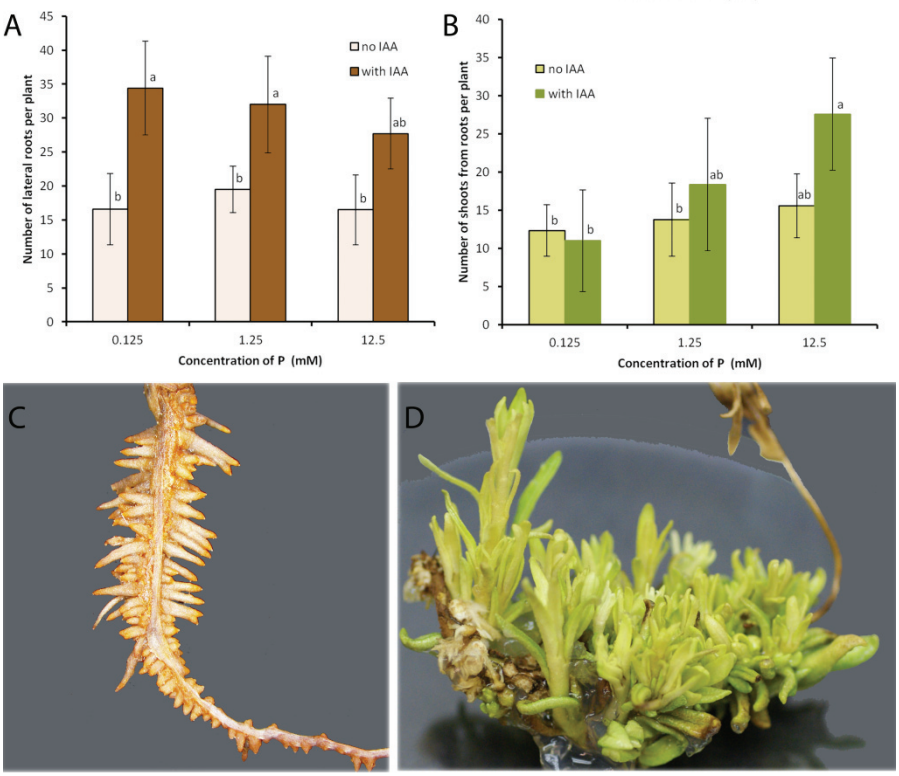

Figure 9. Effect of auxin and P on lateral roots and shoots from roots (SfRs). (A) Number of lateral roots per plant affected by auxin and P. (B) Number of SfRs per plant affected by auxin and P. (C) lateral roots on root of seedling from WPM after treatment with P (0.125 mM) and auxin as pre-treatment. (D) SfRs produced in seedlings W4 medium after treated with P (12.5mM) and auxin.

The results revealed that in interaction with auxin, high P supply increased the number of SfR (Figure 9B,D), while two lower levels of P supply enhanced the number of LRs in seabuckthorn and without auxin the three levels of P showed non-significant effects on LRs and SfRs (Figure 9A,B). These results are in agreement with previous studies on *Arabidopsis* showing that P deficiency makes the root sensitive to auxin (Pérez-Torres *et al.*, 2008).

5 Conclusions

The studies of the root system of seabuckthorn performed in this thesis unravelled a complex organisation with multiple components, including CRs and SfRs, in addition to the N_2-fixing symbiotic root nodules and mycorrhizal symbiosis.

Morphologically, CRs in seabuckthorn are similar to CRs in white lupin. P and Fe deficiency alters root morphology by increasing the number of CRs and root branching. The results indicate that CR formation is not limited to P deficiency and that CRs may play a role that extends further than to P acquisition. This issue needs to be considered in broader terms in root system physiology

Historical selection in different geographical, breeding and growth conditions appears to have resulted in clear differences in root morphology. A highly pronounced ability to form CRs and a highly branched root system, as in genotype Pk, was clearly absent from cvs. BHi10726 and Sunny, the breeding history of which took place on highly fertile soils.

The PPC gene family in seabuckthorn consists of five PTPC genes in two distinct groups and one BTPC gene. Differential expression of the *HrPPC* genes in root development and in a range of plant tissues suggests tissue-specific roles. Co-expression patterns of *HrPPC4* (the BTPC) and *HrPPC5* suggest that they encode the Class-2 PEPC subunits.

HrPPC2 was discovered to have enhanced expression in roots and root nodules especially under conditions of low P. This indicates a role of *HrPPC2* in low P metabolism in roots.

Phosphorus deficiency up-regulated certain transcripts involved in organic acid metabolism, acid phosphatases, P transporters and P homeostasis, which may indicate their importance in seabuckthorn adaptation to low P availability.

Some transcripts encoding acid phosphatases and P transporters showed different patterns of expression in CR development. During CR development,

up-regulation of transcripts homologous to *PAP3*, *PAP22*, *PhT1-4*, *1-9*, *1-7* and *PhT1-5* occurred towards maturity, suggesting their involvement in CR metabolism.

Shoots-from-roots were induced *in vitro* at the same position (groove of LR) as in naturally growing plants and appeared to originate from the pericycle. Phosphorus and auxin were found to interact, but at different levels, in shoot formation and LR formation.

6 Future perspectives

The results presented in this thesis improve the current understanding of several aspects concerning seabuckthorn root dynamics and also open up new areas of research, some of which are discussed below.

One unanswered question concerns what regulates CR formation and why this trait is restricted to certain species. Identification of transcription factors and other regulatory genes, *e.g.* those involved in hormone metabolism in CRs, together with overexpression and mutant studies could prove useful in answering this question.

Several interesting transcript patterns appeared that were related to P deficiency metabolism and to different stages of CRs. Further studies of enzyme activities, proteomics and localisation of cell-specific expression of these genes are needed to confirm their roles in root metabolism under nutrient stress and CR stages.

Systemic regulation of P deficiency in plants is known to occur through microRNAs, *e.g*. miRNA399. Studies using deep RNA sequencing from shoots and roots under different levels of P supply would allow identification of small-RNAs and thus further explain regulation of P responses in seabuckthorn.

To understand why the genotypes showed different responses to P supply, it is important to investigate P storage, recycling, transport and metabolism in these genotypes, together with localisation of transcripts involved in P metabolism.

For nutrient acquisition, CRs may have an independent and alternative role, CRs may also have an additional or supportive role for the two microbial symbioses. Further investigations about CR formation in seabuckthorn could include its relations to mycorrhizal symbiosis that will help to provide knowledge about possible shared signalling pathways.

Since genotype Pk roots are rich in organic acids and sugars, further studies on interactions with symbionts and pathogens would provide additional information about the role of CRs in different nutritional regimes.

Tissue-specific expression of *HrPPC* gene family members revealed a potential area for further exploration. Knockdown studies are needed to identify the functions of these genes in different plant organs.

The pluripotency of the pericycle for the production of LRs, CRs and shoots from roots should be investigated further to identify genes regulating morphogenesis in seabuckthorn LR emergence. Such investigations could be performed through laser capture of pericycle cells to be used in transcriptome analysis.

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