Root exudation pattern of sugar beet (*Beta vulgaris* L.) as influenced by light intensity and P deficiency

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List of Abbreviations

Al	aluminium
AMF	arbuscular mycorrhizal fungi
ANOVA	analysis of variance
Ca	calcium
Cu	copper
Cp	P concentration in nutrient solution
DAT	days after transplanting
ESI	electrospray ionization
Fe	iron
HPLC	high performance liquid chromatography
K	potassium
Mg	magnesium
Mn	manganese
MS	mass spectrometry
m/z	mass-to-charge ratio
OA	organic acid
P	phosphorus
Pi	P concentration in soil solution
PS	phytosiderophores
RL	root length
RP	rock phosphate
SRL	specific root length (length per unit root mass)
Zn	zinc

Chapter 1: General introduction

1.1 Phosphorus

Phosphorus (P) is an essential mineral nutrient required for plant growth. It plays a crucial role in energy metabolism, biosynthesis of nucleic acids and membranes, photosynthesis, respiration, glycolysis, enzyme regulation and redox reactions (Raghothama 1999; Vance et al. 2003). Crop production is limited by low P availability on more than 40% of the world's arable land. Although total P is quite abundant in many soil conditions, only a small proportion is immediately available for plant uptake. Given the current reserved estimates for phosphate deposits, an exhaustion of global reserve is likely to occur in 300 years. However, these estimates are subjected to a significant degree of uncertainty, e.g. exploration efforts, technology development, population dynamics (Scholz and Wellmer 2013). However, even with physical abundance, there is still potential threat of the global phosphate supply to cause problems due to factors such as the skewed geographical distribution of global reserve, especially the potential political instability of those countries (e.g. Jordan, Syria; Heckenmüller et al. 2014). Despite not being a scarce element in a geochemical sense, P has been considered as one of the most crucial inputs for modern agriculture and a main driver behind last century's Green Revolution (Ashley et al. 2011).

1.2 Soil phosphorus and its availability to plants

Low phosphorus bioavailability strongly limits crop production in a wide range of soils across the world (Gaume et al. 2001). This low availability occurs mainly due to its low mobility to plants in most soil conditions compared to the other major nutrients (Hinsinger 2001). The poor mobility of soil inorganic P owes to the large reactivity of phosphate ions

related to numerous soil constituents and to the consequent strong retention of most soil P onto those. Therefore, only a marginal proportion of soil P is presented as P ions in the soil solution. A scheme of the different sources of P in soil and their interrelations is presented in Figure 1.1.

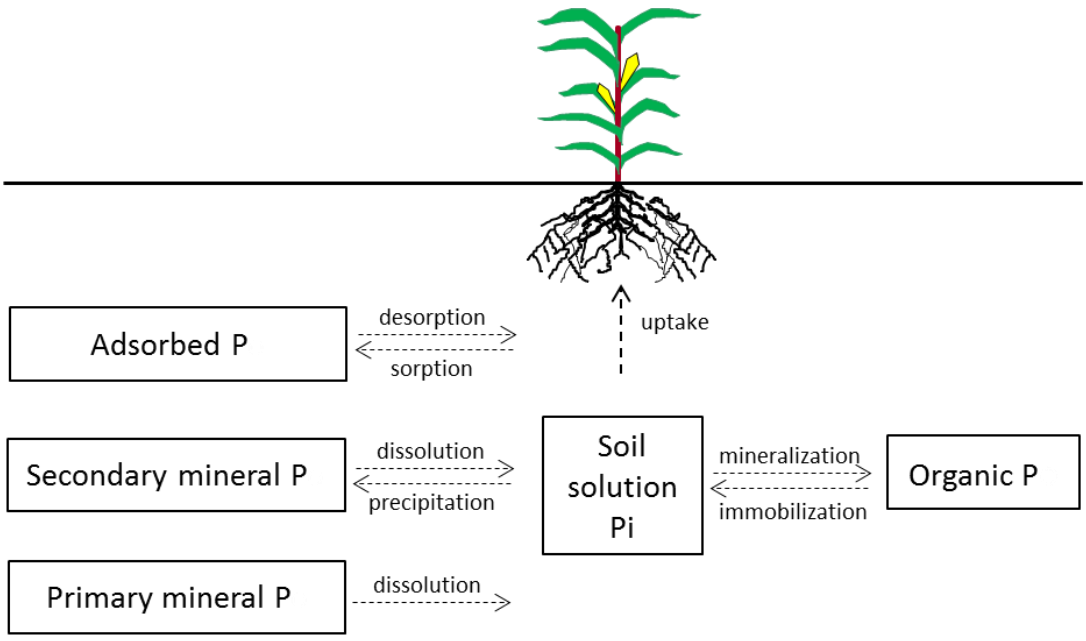

Figure 1.1. Simplified scheme of interactions between different forms of phosphorus in soil and inorganic phosphate in solution which can be taken up by plant roots (modified after Geelhoed 1998).

1.2.1 Phosphorus in soil solution

Plant roots absorb P as phosphate ions from the soil solution. However, the concentration of this part of P in soil is only in micromolar range, from 0.1 to 10 µM (Hinsinger 2001). In tropical soil, the concentration of P in soil solution is normally below 0.2 µM, whereas it is also possible to find relatively high P concentration (32 µM) in the soil solution of fertilized soil (Gillman and Bell 1978; Brady and Weil 1996). However, Föhse et al. (1988) reported that plant species showed large differences in respect to their external P requirements, i.e. the minimum level of P concentration in solution that is adequate for achieving optimal growth:

ranging from 1.5 to 7 μM for most plant species such as rye grass, wheat, rape, tomato, spinach, bean and onion.

The forms of inorganic P present in soil solution are largely depending on the soil pH value, i.e. dissociation of orthophosphoric acid is controlled by pH (Figure 1.2). In most soil (pH 3 to 8), $H_2PO_4^-$ and HPO_4^{2-} are the dominant orthophosphate ions, which can be taken by plant roots. In acid to neutral soils (pH 4.0 to 7.2), the monovalent anion ($H_2PO_4^-$) is the major species, and the divalent anion (HPO_4^{2-}) is the predominant species at pH above 7.2 (Lindsay 1979).

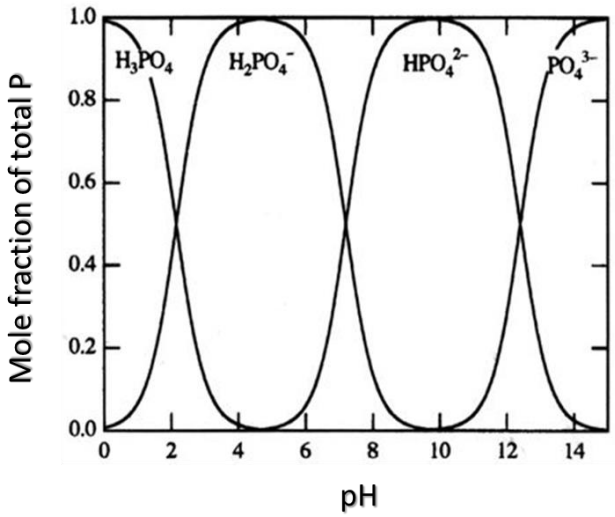

Figure 1.2. Effect of pH on speciation of orthophosphate ions (expressed as mole fraction of total P) in solution (modified after Hinsinger 2001).

1.2.2 Phosphorus in soil

Soil P exists in various chemical forms including organic P and inorganic P. Organic P may account for at least 30% and up to 80% of the total P in soils (Harrison 1987). Soil organic P comprises inositol phosphates (phytate), representing up to 50% of the total organic P, and other organic P fractions are phospholipids, sugar phosphates and nucleic acids (Dalal 1977; Anderson 1980). The bioavailability of phytate is generally low due to the precipitation of Ca/Mg phytate and the strong sorption of phytate on clay minerals and metal (hydr)oxides

(Anderson et al. 1974). The organic P can be released through mineralization processes mediated by soil microorganisms or root-born phosphatase (Anderson 1980).

The inorganic P usually accounts for 35 to 70% of total P in soil (Harrison 1987). This fraction of soil P includes primary P minerals (e.g. apatites), secondary P minerals (e.g. Ca/Mg phosphates) and adsorbed P (e.g. Fe/Al oxides phosphates).

Normally, primary P minerals are quite stable, and the release of available P from them by climate weathering is too slow to satisfy the crop demand (Shen et al. 2011). P ions can precipitate with metal cations, forming a range of precipitated P, i.e. secondary P minerals. The type of secondary P minerals is firstly determined by the soil pH: in acidic soil, Fe and Al phosphates are prevailing, whereas in neutral to alkaline soil Ca phosphates are dominant.

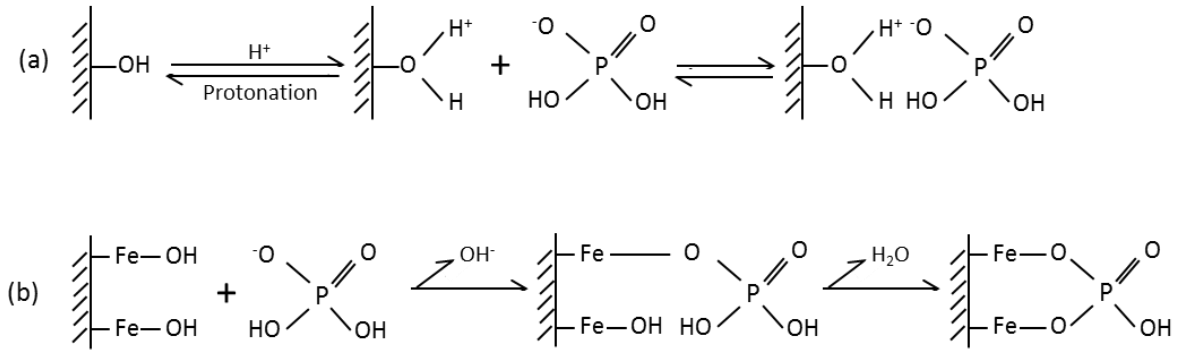

Figure 1.3. Scheme of non-specific and specific adsorption reaction of orthophosphate ions with Fe hydroxides.

Most P ions present in soil solution are negatively charged, therefore they are easily adsorbed by clay minerals and/or Fe/Al oxides which bear positive charges (Hinsinger 2001; Oburger et al. 2011). The adsorption of P ions is done via non-specific adsorption and/or specific adsorption (ligand exchange). Non-specific adsorption may occur due to electrostatic attraction (Figure 1.3a), i.e. in acidic soil, the negatively charged objects (e.g. P ions) are attracted to positively charged objects (e.g. the protonated hydroxides minerals). Fe/Al oxides are capable of adsorbing P ions specifically, i.e. P ions can enter 6 folds coordination with

Fe^{3+} or Al^{3+} ions and replace OH^- ions on hydroxides surfaces (Figure 1.3b). This exchange is called ligand exchange. Ligand exchange is different from non-specific adsorption in the aspect that non-specific adsorption can occur only in positively charged surfaces, while specific adsorption (ligand exchange) can occur on surfaces with negative, positive or neutral charge initially.

1.2.3 Phosphorus availability

All these abovementioned P forms exist in complex equilibria with each other, representing from very stable (primary P minerals), sparingly available (secondary P minerals and adsorbed P), to plant-available P (P ions in soil solution) pools. As plant roots uptake, P ions is rapidly depleted from soil solution pool and will be replenished readily from the other P pools in soil (Figure 1.1). The concentration of P ions in the soil solution is controlled by both precipitation-dissolution and adsorption-desorption equilibria. In other words, dissolution of precipitated P and desorption of adsorbed P are prerequisites for increasing P availability.

Precipitation-dissolution equilibria are described by the following equation that using hydroxyapatite as example (Eq. 1.1):

Eq. 1. 1. $$Ca_5(PO_4)_3OH + 7H_3O^+ \leftrightarrow 3H_2PO_4^- + 5Ca^{2+} + 8H_2O$$

The dissolution of the hydroxyapatite ($Ca_5(PO_4)_3OH$), i.e. the equilibrium of Eq. 1.1 shift to the right, can be enhanced by supply of proton (decreasing pH) or removal of P or Ca ions from soil solution. P ions can be removed by adsorption of other soil constituents or uptake by plants and Ca ions can be complexed by organic ligand such as citrate or oxalate (Hinsinger 2001). The solubility of Fe and Al phosphates increases with increasing soil pH, whilst Ca phosphates have decreasing solubility, except for pH values above eight.

Besides precipitation-dissolution equilibria, the major processes that control the concentration of P ions in soil solution are adsorption-desorption. As abovementioned, most P ions present

in soil solution bear negative charges (e.g. $H_2PO_4^-$ and HPO_4^{2-}), those positively charged soil constituents can work as P sorbents. Those P sorbents comprise various variable charge compounds that contain hydroxyl (Fe/Al oxides), carboxyl (organic matter) or silanol (clays) groups. The surface charge of metal (hydr)oxides can be positive or negative and depends on the pH and composition of the electrolyte solution (Geelhoed 1998). However, because of their rather high isoelectric point (being generally between pH 7 and 10), metal oxides are positively charged over the whole pH range usually faced in soil (i.e. pH 3-8; Hinsinger 2001). Metal oxides play a prominent role in the adsorption of P ions in most soil: not only in ferralsols which are largely influenced by Fe and Al oxides, but also in calcareous soil which at alkaline pH (Matar et al. 1992; Samadi and Gilkes 1998). The sorption of phosphate in soil is influenced by the total phosphate concentrations, the amount of adsorbing surface area, and the pH value (Geelhoed 1998). The capacity of minerals to adsorb anions such as P ions will increase with decreasing pH, because such minerals have an increase in positive charge as a consequence of their larger protonation at low pH (Barrow 1984; Strauss et al. 1997). Therefore, when only considering Fe and Al oxides adsorb P ions, decreasing the pH should result a stronger retention and hence in a decreased mobility of inorganic P. Desorption of adsorbed P occurs either by ligand exchange (Eq. 1.2) or ligand-promoted dissolution of Fe and Al oxides (Eq. 1.3):

Eq. 1.2. $$Fe/Al\ oxide - P + L \rightarrow Fe/Al\ oxide - L + P_i$$

or

Eq. 1.3. $$Fe/Al\ oxide - P + L \rightarrow Fe/Al\ oxide <^{P}_{L}\rightarrow Fe^{3+}/Al^{3+} - L + P_i$$

L: the competing ligand which includes inorganic ligands (e.g. sulphate, bicarbonate) or organic ligands (e.g. organic acid anions).

P_i: P ions in the soil solution.

A decrease in the concentration of P ions in the soil solution and an increase in the concentration of competing anions will both shift the adsorption-desorption equilibrium towards enhanced desorption. However, numerous works have reported that metal oxide surfaces and other soil sorbents, i.e. clay minerals have a stronger affinity for P ions than for most other competing inorganic ligands. Therefore, for both inorganic/organic competing ligands, large concentration (e.g. above 10 μmol g^{-1} soil for citrate or oxalate) must occur for desorbing P ions to any significant extent (Kafkafi et al. 1988; Staunton and Leprince 1996).

1.3 Phosphorus efficiency mechanisms

Enhancing plants P efficiency can be achieved through improving P acquisition, utilization, or both. P acquisition efficiency (PAE) refers to the ability of plants to mobilize phosphorus from poorly soluble sources or take up the soluble P in soil solution (Vance et al. 2003). It is widely believed that the efficiency of uptake is of minor importance for P acquisition from soil because availability of P ions to root surface rather than its uptake is the limiting factor (Barber 1995).

1.3.1 Acquisition of phosphorus by plants

Due to the low concentration and mobility of P in soil solution, the acquisition of P is a problem for plants. The P uptake from soil by plants depends on the ability of root system to intercept new sources of P and the rate of diffusion of orthophosphate through soil towards root surface, unlike nitrogen which is more mobile and transported to plant roots by mass flow (Jungk and Claassen 1997). Roots rapidly deplete P in soil solution and the concentration of P at root surface is estimated in the range of 0.05-0.2 μM (Barber 1995). Although there is a P diffusion gradient from bulk soil through rhizosphere to root surface, the low rate of P diffusion through soil to root surface (about 0.13 mm day^{-1}) is generally insufficient to match the uptake rates occurring at the root surface, unless roots can grow into

and extract P from unexploited P fixing soil. Generally, plants activate three broad categories of efficiency strategies to increase P availability: (i) alternation of the morphological characteristics of root system; (ii) association with microorganisms; (iii) exudation of chemical compounds into the rhizosphere (Raghothama 1999; Hinsinger et al. 2003; Richardson et al. 2009).

1.3.2 Morphological adjustment of root characteristics

Most plant species growing in P-deficient soil allocate a higher proportion of assimilation to root growth. Richardson et al. (2009) summarized the characteristic of roots that benefit the exploration of soil and P acquisition include: high root/shoot dry weight ratio, high specific root length (SRL) (length per unit root mass), and long dense root hairs, these characteristics can greatly increase the soil volume contact with root surface. In the model plant *Arabidopsis*, root hair density was increased 29% under low P conditions, in the meantime, root hair length increased 3 times after 16 days of P starvation (Bates and Lynch 1996). P deficiency also alters the distribution among various root types (Hodge 2009). In various rape cultivars, a highly branched root system with reduced production of primary root and increased number and length of lateral roots was reported when plants were grown with low P supply (Akhtar et al. 2008). In addition, some plant species develop special root structures (e.g. cluster roots) to cope with P deficiency. Cluster roots, also known as proteoid roots, comprise dense numbers of closely spaced, short-lived, determinate lateral roots (rootlets). Of the species forming cluster roots, white lupin (*Lupinus albus* L.) is considered as a model plant for understanding plant adaption to low P supply (see below; Gardner et al. 1981; Dinkelaker et al. 1995; Keerthisinghe et al. 1998; Neumann et al. 1999). Formation of cluster roots can represent a significant proportion of the plant's investment in biomass (30-80% of the total root biomass; Keerthisinghe et al. 1998; Skene 2000; Hocking and Jeffery 2004). The formation of cluster roots by white lupin is regulated by shoot P status and external P

supply (Shane et al. 2003; Shen et al. 2011). Formation of cluster roots in plants growing in low P not only increases the contact surface area between root system and soil, but also increases the release of organic anions (see below), which is supposed to mobilize fixed P (Hocking and Jeffery 2004). Therefore, formation of cluster roots is considered as an effective morphological and physiological response of plants to P deficiency.

1.3.3 Association with mycorrhizae

Association of plants with mycorrhizal fungi can also enhance the availability of P through extension of the plant root system with mycorrhiza hyphae (Bucher 2007). Most species of plants (70-90%) form mutualistic associations with mycorrhizal fungi, with arbuscular mycorrhizal fungi (AMF) important for many crops and ectomycorrhizal fungi for shrubs and other woody species (Parniske 2008; Smith and Read 2010). In the symbioses, P is transferred by AMF via their extensive mycorrhizal mycelium to plants while the fungi receive carbon from the plant. Besides the symbiosis, in some cases, mycorrhizas also enhance the utilization of soil organic P and increase the exploitation of nutrient-rich regions of soil. However, the P pools which mycorrhizal fungi access are those pools readily available for plant uptake (Bolan 1991; Tarafdar and Marschner 1994; Joner et al. 2000; Feng et al. 2003; Gavito and Olsson 2003).

1.3.4 Exudation of P-mobilizing compounds

In soil, the major limiting steps in P acquisition by plants are the mobilization and diffusion of P to the roots (Barber et al. 1963; Ernst et al. 1989). On one hand, root hairs and mycorrhizae increase the volume of soil explored, improve contact between root and soil, thus increase the effective absorbing area of the root system. On the other hand, how to increase P availability, i.e. the mobilization of P, is an urgent problem required to be addressed. Root-induced chemical and biological changes in the rhizosphere play a vital role

in enhancing soil P availability (Hinsinger 2001). The release of root exudates into rhizosphere either directly, or indirectly through promoting the growth of rhizosphere microbial which may assist in both mobilizing and mineralizing, affects the availability of soil P to plants (Randall et al. 2001).

The mechanisms of root exudates directly influence P availability in the rhizosphere include: (i) changing the pH of soil solution, thus promoting the dissolution of sparingly soluble P minerals; (ii) altering surface characteristic of soil particles; (iii) competing with phosphate ions for sorption sites (ligand exchange and ligand promoted dissolution); (iv) complexing and chelating cations which are bound to P; (v) enzyme-catalyzed hydrolysis of organic P (Bar-Yosef 1991; Jones 1998). The importance of each mechanism depends on the soil type, the form of P in the soil, and other factors. For instance, acidification the rhizosphere (pH decreased) would increase solution P by dissolution of Ca-phosphate in neutral to alkaline soil.

1.3.4.1 Effects of root exudates on rhizosphere pH

Root-induced acidification can reduce rhizosphere pH by 2 to 3 units compared to the bulk soil. Rhizosphere pH change is largely determined by cation/anion uptake ratios and nitrogen assimilation. Normally, plants uptake uneven quantities of nutrient cations (Ca^{2+}, Mg^{2+}, Na^{+}, K^{+}, NH_4^{+}) and anions ($H_2PO_4^{-}$, SO_4^{2-}, Cl^{-}, NO_3^{-}), and by excretion of H^{+} (when excess cations uptake) or OH^{-}/HCO_3^{-} (when excess anions uptake) to maintain internal charge balance. Compared to other nutrients, plants take up greater amounts of N. Therefore, the form of available N (NO_3^{-} or NH_4^{+}) to plants affects the pH of rhizosphere, i.e. with NO_3^{-} nutrition resulting in an increase in pH while with NH_4^{+} nutrition leading to a decrease of pH. N-fixing legumes take excess cations over anions, resulting in rhizosphere acidification (Jarvis and Robson 1983; McLay et al. 1997). In addition, it is well-documented that P deficiency also induced the net extrusion of protons from roots of white lupin, tomato, and chickpea (Imas et

al. 1997; Neumann and Römheld 1999). Sas et al. (2001) showed that proton extrusion in P-deficient plants was 2 to 3 fold greater than organic acid exudation on an equimolar basis, however, different mechanisms are involved in proton release and organic acid anions exudation. Generally, proton release results from the activity of a plasma membrane H^+-ATPase, and this enzyme uses ATP to pump protons out of the cell. However, some plant species display completely different proton release pattern as response to P deficiency. For example, under P deficiency, wheat showed no proton extrusion (Neumann and Römheld 1999), and soybean plants even decreased proton release (Tang et al. 2009).

Rhizosphere pH has a strong influence on the bioavailability of soil P and this effect depends on soil properties. Gahoonia et al. (1992) reported that using NH_4^+ as N source rhizosphere pH of ryegrass decreased and this resulted in increased P mobilization from a luvisol contained calcium phosphate which can be dissolved by acidification, but had no effect in an oxisol; on the contrary, using NO_3^- as N source increased rhizosphere pH, and this increased P mobilization in the oxisol, but had no effect on P in the luvisol. Numerous studies with rock phosphate as a P source confirmed that the release of protons by plant roots resulted in increased bioavailability of P, most probably due to an increase of Ca phosphates dissolution rate with decreasing pH (see Eq. 1.1).

1.3.4.2 Release of organic acids and mobilization of soil P

The most investigated compounds exuded from roots into the rhizosphere in terms of the P nutrition are likely to be low-molecular-weight organic acids (Marschner et al. 1986; Dinkelaker et al. 1989). Actually, organic acids are exuded as anions accompanied by H^+ extrusion. Due to their low p*K* value, organic acids are predicted to exist in the cytoplasm (pH 7.1-7.4) in a fully dissociated state (e.g. $citrate^{3-}$ and $malate^{2-}$) rather than in acid form (e.g. H_3·$citrate^0$, H_2·$malate^0$; Ryan et al. 2003). The effectiveness of organic acid to mobilize P largely relies on its capacity to complex metal cations, e.g. Al^{3+}, Fe^{3+} and Ca^{2+} and to

displace P from charged surfaces. The number and arrangement of its carboxyl and hydroxyl groups determine the stability of the ligand: metal complexes. Generally, the tricarboxylic acids (citrate) decrease the adsorption of P in soils more strongly than dicarboxylic acids (malate, oxalate and malonate), and monocarboxylic acids (succinate, fumarate and acetate) are the weakest (Bar-Yosef 1991; Bolan et al. 1994). Numerous studies reported that citrate is particularly effective at mobilizing P from Fe/Al-P complexes in acid soils (Hue et al. 1986; Bar-Yosef 1991; Bolan et al. 1994), and acid-soluble Ca-P in calcareous soils, or from rock phosphate fertilizer, by the decrease in rhizosphere pH through the accompanying H^+ extrusion (Dinkelaker et al. 1989).

The importance of organic acid exudation from roots in the acquisition of soil and P fertilizer by plants is well-documented. Organic acids, particularly citrate and oxalate, added into the soils can mobilize significant quantities of P and reduce the sorption of fertilizer P (Traina et al. 1986; Bar-Yosef 1991; Bolan et al. 1994; Jones and Darrah 1994). A number of plant species are known to increase exudation of organic acids anions in response to P deficiency, e.g. white lupin (*Lupinus alba* L.) (Dinkelaker et al. 1989), rape (*Brassica napus* L.) (Hoffland et al. 1992), alfalfa (*Medicago sativa* L.) (Lipton et al. 1987), and sugar beet (*Beta vulgaris* L.) (Beissner and Römer 1998; Khorassani et al. 2011) and it appears that this mechanism assists these plant species under P-limiting conditions. Among species examined for organic acid production in response to P stress, white lupin is often used as a model plant due to its capability to release huge amounts of organic acids (Dinkelaker et al. 1989; Keerthisinghe et al. 1998; Wang et al. 2006). In response to P deficiency, white lupin developed specialized root structures (cluster roots) which can strongly acidify and also easily exude massive quantities of organic acid anions in the rhizosphere soil (Neumann et al. 1999; Zhu et al. 2005; Lambers et al. 2006). It has been reported that the exudation of citrate and malate from P-deficient white lupin cluster roots was about 20 to 40 times higher than P

sufficient root (Vance et al. 2003). The amount of carbon exuded in citrate and malate can range from 10% to greater than 25% of the total plant dry weight, and the concentration of citrate ranging from 50 to 90 μmol g^{-1} soil have been detected in the rhizosphere of white lupin cluster roots (Dinkelaker et al. 1989; Gerke et al. 1994). Surprisingly, the large amount of organic acids induced by P deficiency does not seem to negatively affect either dry matter or N fixation until the reproductive stage of growth (Dinkelaker et al. 1989; Keerthisinghe et al. 1998). The rate of anion exudation in the range of 0.6-1.4 μmol m^{-1} root length h^{-1} has been reported for active cluster roots of white lupin (Neumann et al. 1999; Watt and Evans 1999). In addition, as we mentioned above, the special structure of cluster root is beneficial for accumulating high amount of organic acid and for inhibiting the microbial degradation by acidification of rhizosphere. However, these high exudation rates and amounts are only found in few extreme cases. Whilst the exudation of organic acids is enhanced from most plant roots under P deficiency, in many cases, the exudation rate is much lower, therefore, their effect on enhancing P availability remains controversial (Drever and Stillings 1997; Ström et al. 2002). A number of studies have indicated that P release only occurs at relatively high organic acids concentrations with the critical threshold for P release found to range from 8.5 to 33 mM, i.e. 2.5 to 10 μmol carboxylate g^{-1} soil (Gerke et al. 2000; Wouterlood et al. 2004; Oburger et al. 2009). Typically, concentrations of organic acids have been detected in the bulk soil solution range between 0-0.1 mM and less than 1 mM in the rhizosphere of most non-cluster root plants (Jones et al. 1996; Raghothama 1999) but are estimated to exceed 50 mM in the rhizosphere of cluster roots (Lipton et al. 1987; Johnson et al. 1996). In addition, as organic acids are excellent substrates for microbial growth, under non-sterile conditions, they are rapidly uptake and biodegraded by rhizosphere microbial (half-life in soil solution range between 0.5 and 12 h; Jones and Darrah 1994; van Hees et al. 2002). In addition, in many soils, particularly in the tropics, there are large amounts of Fe/Al oxides, offering an

enormous domain of anionic binding sites, organic acid anions can also be absorbed onto those soil constituents, in a similar way as P ions, although with a lower affinity. Their adsorption may result in desorption of P ions via a ligand exchange reaction and eventually in an increased bioavailability of soil inorganic P (Geelhoed et al. 1999). However, the strong adsorption of organic anions on these soil constituents can conversely limit their diffusion away from the roots and confine their zone of influence to the immediate vicinity of the root surface (Kirk et al. 1999). Nevertheless, the presence of organic acids in soil solution still can reduce the probability of P ions adsorbed by those sorbents.

Exudation of organic acids as response to P deficiency varies greatly between different plant species (Table 1.1). Citrate, malate and oxalate are the well-documented organic acids when plants subjected to P deficiency. For instance, citrate has been observed as the dominant organic acid exuded by species such as white lupin and alfalfa. In other plant species such as maize and rape, malate are the dominate one. Oxalate appears to be of major organic acid in sugar beet. Besides citrate, malate and oxalate, other organic acids anions in root exudate which might also be involved in P mobilizing was detected in recent years. Ae et al. (1990) first time found piscidate exuded from pigeonpea roots could release P from $FePO_4$ by chelating Fe. Shen et al. (2001) reported that the exudation of glutarate is a response specific to P deficiency in elephantgrass and constitutes a mechanism of tolerance to low P stress. In soybean, malonate was found the highest amount in the root exudates (Tang et al. 2009). Khorassani et al. (2011) detected salicylate and citramalate in root exudates of sugar beet and noted both of them can increase P availability.

1.3.4.3 Release of phosphatase and phytase

Plants can release phosphatase to mobilize organic P by enzyme-catalyzed hydrolysis. The activities of phosphatases are up-regulated under P deficiency (Vance et al. 2003; Radersma and Grierson 2004). Phosphatases are not effective in mineralizing phytate (inositol

hexaphosphate), the major form of organic P in many soil, however, phytase released by microorganisms can work as an alternative approach for improving the ability of plants to acquire P directly from phytate (Richardson et al. 2009).

Table 1.1. Plant species with phosphorus-deficiency induced exudation of organic acid anions by roots.

Plant species	Organic acid anion	References
Alfalfa (*Medicago sativa*)	citrate	Lipton et al. (1987)
Arabidopsis thaliana	citrate, malate	Narang et al. (2000)
Bean (*Phaseolus vulgaris*)	citrate, tartrate, acetate	Shen et al. (2002)
Cabbage (*Brassica oleracea*)	citrate	Dechassa and Schenk (2004)
Chickpea (*Cicer arietinum*)	malate, citrate, malonate	Neumann and Römheld (1999)
Cowpea (*Vigna unguiculata*)	citrate	Jemo et al. (2007)
Elephantgrass (*pennisetum purpureum*)	glutarate	Shen et al. (2001)
Maize (*Zea mays*)	malate, citrate, trans-aconitate	Hinsinger (2001); Li et al. (2008)
Pigeonpea (*Cajanus cajan*)	citrate, piscidate	Otani et al. (1996)
Potato (*Solanum tuberosum*)	succinate	Dechassa and Schenk (2004)
Radish (*Raphanus sativus*)	tartrate, malate, succinate	Zhang et al. (1997)
Rape (*Brassica napus*)	malate, citrate, succinate, acetate, tartate	Hoffland et al. (1989); Zhang et al. (1997)
Rice (*Oryza sativa*)	citrate,oxalate	Hoffland et al. (2006); Kirk et al. (1999)
Soybean (*Glycine max*)	malate, oxalate	Dong et al. (2004)
Sudangrass (*Sorghum vulgare*)	succinate, cis-aconitate, iso-citrate, fumarate, trans-aconitate, citrate	Schwab et al. (1983)
Sugar beet (*Beta vulgaris*)	oxalate, salicylate, citramalate	Beissner and Römer (1998); Khorassani et al. (2011)
Tea (*Camellia sinensis*)	malate, citrate	Lin et al. (2011)
White lupin (*Lupinus albus*)	citrate, malate	Hocking and Jeffery (2004); Keerthisinghe et al. (1998); Neumann et al. (1999)

1.4 Root exudate - a short overview

1.4.1 Introduction

The rhizosphere is defined as the zone of soil surrounding living roots, which is influenced by root activity (Hiltner 1904). In this critical area, plants perceive and respond to their environment. Plants can dramatically modify their rhizosphere through releasing carbon compounds from living roots. The carbon release in the rhizosphere leads to chemical, physical and biological characteristics that differ from those of the bulk soil (Barber and Martin 1976). It has been estimated that nearly 5-21% of photosynthetically fixed carbon is eventually transferred to the rhizosphere in the form of root exudates (Whipps and Lynch 1990; Nguyen 2003; Derrien et al. 2004).

The most common definition of the term "root exudate" is the substances which are released into the surrounding medium by healthy and intact plant roots (Rovira 1969) and is the definition used in this study. Root exudates are often divided two classes of compounds: (i) high-molecular weight compounds, such as mucilage and ectoenzymes (e.g. phosphatase) and (ii) low-molecular weight fraction such as organic acid, amino acids, sugars, phenolic, phytosiderophores (PS) and other secondary metabolites, which account for much of the root exudate diversity (Badri and Vivanco 2009). Among known root exudates, low-molecular weight compounds have drawn considerable interest due to their potential to stimulate microorganism growth, detoxify potentially toxic metals (e.g. Al^{3+}), mobilize poorly soluble nutrients (e.g. P, Fe and Zn) and accelerate mineral weathering (Jones 1998; Neumann and Römheld 1999; Ryan et al. 2001; Dakora and Phillips 2002).

1.4.2 Factors affecting root exudation

The root exudation pattern, i.e. the quantity and quality of root exudates is affected by many factors, including plant species as well as genotypes, plant age, environmental conditions

(e.g. light intensity and temperature), and nutritional status of plants (Hinsinger 2001; Jones et al. 2004).

1.4.2.1 Plant species

Plant species as well as genotypes of a given species vary greatly in their root exudation pattern. Large differences in the capacity for PS secretion occur not only between plant species (barley > wheat > oat > rye > maize > sorghum > rice), but also in cultivars within each single plant species (Kawai et al. 1988; Brown et al. 1991). Low-P tolerant maize genotype was characterized by high organic acid content in roots meanwhile with high organic acid exudation, while low-P susceptible genotype only accumulated organic acid in root (Gaume et al. 2001). Exudation quality, quantity and trends of individual organic acids present in the exudates showed significant differences among rice cultivars (Aulakh et al. 2001).

1.4.2.2 Plant age

Plant age and development stage significantly influence the qualitative and quantitative nature of plant root exudates (Hamlen et al. 1972). Number of experiments using pulse labelling experiments report that plant age significantly affects C partitioning of assimilation between plant-soil compartments (Kuzyakov and Domanski 2000; Narang et al. 2000). As the plant gets older, less carbon allocated to belowground. After 4 weeks and 24 weeks of growing, *Lolium perenne* plants translocated 67% and 14% of assimilates into the soil, respectively (Meharg and Killham 1990). Gransee and Wittenmayer (2000) observed that younger maize plants exuded considerably higher amounts of ^{14}C labelled organic substrates per g root dry matter than older ones, and the relative amount of sugars decreased at the expense of carboxylic acids during plant development.

1.4.2.3 Temperature

Temperature has profound effects on the quality and quantity of root exudates, because it affects the processes of photosynthesis, translocation and respiration in plants (Hale et al. 1971; Hale and Moore 1979). Increase in exudation at high temperature has been reported for many crops, e.g. the stimulation of root exudation in tomato and clover at high temperatures (mean min./max. temperature: 21/31°C; Rovira 1959). Pramanik et al. (2000) reported that the rate of root exudation in vegetative and reproductive stages of cucumber plants for organic acids increased with the elevation of temperature (from 25/20°C to 30/25°C, day/night). In other hand, as microbial activity generally increases with temperature, the biodegradation of root exudates may be faster with high temperature.

1.4.2.4 Light condition

Like temperature, light intensity is also involved in processes of plant photosynthesis, translocation, and respiration (Hale et al. 1971; Hale and Moore 1979; Cheng et al. 2014). Since a large proportion of the organic carbon released into the rhizosphere is derived from photosynthesis, changes in light intensity are likely to modify the intensity of root exudation. Rovira (1959) observed the quantity and quality of amino acids in exudates of tomato and clover changed with decreasing light intensity. Increasing light intensity greatly enhanced PS release of Fe- and Zn-deficient bread wheat and barley cultivars (Cakmak et al. 1998). The exudation of catechin by *Centaurea stoebe* also increased many folds when light levels are high (Tharayil and Triebwasser 2010). The production of secondary metabolites can also be affected by variation in light intensity with the photosynthetic spectrum and also higher wavelengths (Lavola et al. 1997; Koricheva et al. 1998).

In addition, exudation is often found to follow a diurnal pattern (Gessler et al. 2002; Reichman and Parker 2007; Oburger et al. 2011). It has been reported that the released amounts of root-derived carbon were larger in day-time than in the night (Kuzyakov and

Siniakina 2001; Melnitchouck et al. 2005). Many plant metabolites are subjected to similar diurnal patterns of light intensity (Urbanczyk-Wochniak et al. 2005), which can be linked to the diurnal regulation of photosynthetic carbon metabolism (Geiger and Servaites 1994). Furthermore, Pramanik et al. (2000) reported the rate of root exudation of cucumber plants was increased with the elongation of photoperiod, the mean rate was two or more times higher than the minimum exudation with short photoperiod.

1.4.2.5 Plant nutrition

Root exudation of various chemical molecules into the rhizosphere is largely dependent on the nutritional status of the plant. For example, in graminaceous plant species, release of mugineic acids or phytosiderophores is induced not only by limitation of Fe and Zn (Neumann and Römheld 2007), but also by Mn and Cu deficiency (Treeby et al. 1989). Under K deficiency, the amounts of exudates increased by maize plant, and the proportions of sugars and organic acids are shifted in favour of the organic acids (Kraffczyk et al. 1984). Enhanced root exudation of organic acids is well-documented under P deficiency, as we mentioned in above (in *1.3.4.2*). Many plant species release carboxylates which can complex with Al cation in apical root zones in response to Al toxicity (Kochian 1995; Pellet et al. 1995).

1.5 Methods of collection of plant root exudates

The collection of root exudates from plants is the prerequisite for subsequent analysis. The method used for collection depends on the cultivation of plant and the purpose of exudate use. Generally, three approaches have been used to quantify exudation during the last two decades. Each approach has its advantages and shortcomings.

1.5.1 Considering the whole root system

1.5.1.1 Dipping method

In studies dealing with the root exudation process, cultivation of plants in nutrient solution with subsequent collection of root exudates has been widely used, which can effectively avoid mechanical damage to roots as they are free of solid particles (Jones 1998; Personeni et al. 2007). In this method, plants were grown in pure nutrient solution, and exudates were collected by dipping the whole root system in a trap solution (e.g. distilled water) for a time period ranging between 2 min to 25 d (Vranova et al. 2013). The advantage of this methodological approach is very simple and easy to handle, and it is possible to assess root exudation by repeated non-destructive collections over extended time periods. In addition, this method ensures that the exudation rates not overestimated due to root injuries (very easy and frequent in the case of root removal from soil or sand) and prevents the risk of microbial degradation/contamination caused by the presence of soil particles. Normally, before collection, root system washed three times by distilled water in short time (e.g. 5 min) thus we could assume it as a sterile condition in a short time. A limitation of plant cultivation in nutrient solution is that the plants are morphologically and physiologically different from those cultivated in soil, such as less root hairs growing, no mechanical impedance or water stress, and different O_2/CO_2 status (Oburger et al. 2013). In some researches, simulation of the mechanical forces imposed on roots of soil-grown plants could be achieved by addition of small glass beads (Vranova et al. 2013).

1.5.1.2 Percolation method

Collection of root exudates from plants grown in solid substrates culture (e.g. quartz sand, perlite and vermiculite) may be conducted by percolating the culture media with the trap solutions for a defined time period, finally yielding the products released from the root

system during the preceding culture period by repeated washings (Luster et al. 2006). The advantage of this method is provided a (semi) natural growth condition, therefore achieved a relative natural root proliferation. However, using this method, the exudate concentration potentially altered since the adsorption processes of some exudate compounds to matrix of solid culture media cannot be excluded (Oburger et al. 2013). Nevertheless, Gransee and Wittenmayer (2000) concluded that the dipping method is more suitable for a nearly complete sampling and analysis of root exudates than percolation method.

The most commonly collection medias (trap solution) employed for both methods are nutrient culture solution, distilled water, $CaCl_2$ and $CaSO_4$. Nutrient culture solution created complication in the analyses of organic acids by HPLC due to the interference by its components. When using nutrient solution as collection media, there are large background peaks during organic acids analysis by HPLC which overlapped and masked the peaks for several organic acids (Aulakh et al. 2001). For example, it is not possible to detect early-eluting organic acids due to the presence of early-eluting inorganic ions such as NO_3^- in the nutrient solution (Kirk et al. 1999). Using distilled water as collection media can exclude such interference in analytical analyses but alter the turgor of root cells, especially for a longer collection periods than 2 h, while $CaCl_2$ or $CaSO_4$ solution (0.5-2.5 mM) maintained the osmotic environment for root cells (Schapire et al. 2009). Aulakh et al. (2001) compared those two collection media, they found that 20 to 60% more C released in distilled water than in $CaSO_4$ solution, and there were more sugars but less organic acids in root exudates collected by distilled water. However, the addition of ions to the trap solution (Ca^{2+}) might interfere with the subsequent quantitative analysis of the exudates, which is no good to have a better comprehension of root exudation pattern. Moreover, exudates collected by root washings or percolation method are usually highly diluted (large sampling volume with low exudate concentration); therefore subsequent concentration steps are required, which can be

performed by vacuum evaporation (if the interesting compounds are not heat-labile), lyophilization, or solid-phase extraction techniques (Luster et al. 2006). This concentration step can one side favour the detection of the exudates but on the other side, it might easily lead to very high salt concentrations, which may interfere with subsequent analysis or may even cause irreversible precipitation of certain exudate compounds (e.g. Ca-citrate; Neumann and Römheld 2007). Therefore, the use of distilled water as a trap solution should thus be recommended, especially for short collection time.

The time period of exudate collection is another aspect should be paid more attention on. Due to the majority of organic compounds present in exudates are easily decomposable by microbial, prolonged collection times may result in the loss of easily degradable C, leading to an under-estimation of C released by plant roots. Kirk et al. (1999) observed rapid degradation of citrate in the soil suspensions and estimated a half-life less than 5 h for citrate. In addition, the incorporation of ^{14}C-labelled exuded substrate into microbial biomass was highest after 3 h, and then declined (Rattray et al. 1995). Sas et al. (2001) conducted recovery experiments of citrate after each time collection of root exudates, they found that 13-23% of citric acid released from white lupin roots could have been break down and/or was taken up by the roots during the collection period of 2 h, and after 2 h, the decomposition rate of citrate was higher. To limit the microbial degradation processes, bacteriostatic agents (e.g. Micropur) added into the trap solution. However, there are several evidences in literature indicating a negative effect of the presence of Micropur in the trap solutions, no matter how high the Micropur concentration was used (Neumann and Römheld 2007; Valentinuzzi et al. 2015). Therefore, a short period of time, i.e. 2 h, is recommended for collecting root exudates, by which can avoid underestimation of components exuded by plant roots.

1.5.2 Localized sampling methods

In many cases, root exudation is not homogenously distributed along the roots and considerable longitudinal gradients or hot spots of exudation can exist in different root zones. For example, in oilseed rape, young regions of the root exude more organic acids than older parts (Hoffland et al. 1989); adaptive response of root exudation involved in nutrient mobilization or detoxification of toxic elements are frequently restricted to special root structures (e.g. apical root zones, root hairs, cluster roots). The localized and concentrated release enables an accumulation of root exudates in the rhizosphere above the threshold levels required for the specific functions (Neumann and Römheld 2007). Thus, collection techniques based on dipping method or percolation with trap solutions, integrating root exudation over the whole root system, can only give limited information, and methodological methods can detect spatial variations in rhizosphere chemistry along single roots are required to have an understanding of rhizosphere processes.

In hydroponics, small containers filled with trap solution or sorption media such as filter paper, resin foil and agar sheet have been used to collect root exudates from single root segment. In soil culture, localized sampling techniques comprise the use of sorption media placed onto the surface of the respective root zones of plant grown in rhizotrons. Also the insertion of micro-suction-cups has been reported to collect rhizosphere soil solution of plant grown in rhizotrons or rhizoboxes (Oburger et al. 2013). The major problem of these techniques arise from limited and variable recovery of exudate compounds due to rapid microbial degradation in the soil solution, selective and rapid adsorption of certain compounds at the soil matrix and at the root surface. In addition, using these methods the sampling volume is very small, therefore long collection periods required for these method which will increase the risk of microbial degradation (Neumann et al. 2009).

1.6 Analysis of root exudates samples

Analysis of plant root exudates typically has involved chromatographic methods that rely on a priori knowledge of which compounds might be present. The understanding of mechanisms controlling nutrient availability in the soil requires a comprehensive knowledge of the qualitative and quantitative composition of root exudates. It is not sufficient to have only information about specific substances. High-performance liquid chromatography (HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS) is the most often and widely used method for profiling the wide array of different compounds which can present in root exudates. The application of HPLC can separate compounds with medium polarity, highly polar, thermo-labile and high molecular weight molecules, this molecular fractionation prior to mass spectrometric analysis is very necessary. Mass spectrometry is an analytical technique which can provide both qualitative (structure) and quantitative (molecular mass or concentration) information on analyte molecules after their conversion to ions. The molecules of interest are introduced into the ionization source of the mass spectrometer, where they are first ionized to acquire positive or negative charges. The ions then travel through the mass analyser and arrive at different parts of the detector according to their mass/charge (m/z) ratio. After the ions make contact with the detector, useable signals are generated and recorded by a computer system. The computer displays the signals graphically as a mass spectrum showing the relative abundance of the signals based on their m/z ratio. Non-targeted profiling analysis performed by HPLC-MS can provide information of all chromatographic peaks which can be characterized by their mass spectral patterns and HPLC retention time, and this information is helpful for us to compare the metabolic alternation between low P and high P supply.

1.7 Aim of this work

Sugar beet is known as a P uptake efficient plant species with high ability to mobilize P in low P soil. And this mobilization is caused by chemical modification of the rhizosphere through releasing root exudates. However, the known compounds are not evident to explain the mobilization occurred with non-cluster root plants due to their low releasing amount in the rhizosphere. The first part of this work was to develop a simple approach that can better simulates soil conditions for P by using rock phosphate with a low equilibrium concentration in nutrient solution (Chapter 2). The second aim of this study was to use HPLC-ESI-MS to identify compounds of exudates of sugar beet roots that might increase P availability in the soil which have not been in focus yet (Chapter 3). Furthermore, most of previous investigation related to root exudates achieved from experiment conducted in growth chambers with relative low light intensity and might not be representative for field conditions. Hence, the second part of this work addressed to evaluate the impact of light intensity on root exudation pattern of sugar beet under P deficiency (Chapter 4).

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Chapter 2: A simple approach for controlling low phosphorus concentration in nutrient solutions

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2.1 Abstract

A simple approach, employed 3 g Rock Phosphate in teabags, was developed to offer a continually low phosphorus (P) concentration in nutrient solutions. We conducted a P experiment to compare the differences between this new and the classical approach aiming at a 2 µM supply using inorganic phosphorus. The results indicate that this new approach is well suited for P deficiency studies.

Key words: P deficiency, constant phosphorus concentration, rock phosphate, sugar beet

2.2 Introduction

Phosphorus (P) is a major limiting factor of plant growth due to its low mobility in soil. In most types of soil total amounts of P is quite abundant, but a large proportion is bound to different soil constituents, forming complexes of limited bioavailability resulting in P concentrations in soil solution between 0.1 and 10 µM, which can be insufficient to satisfy plant requirements (Hinsinger 2001).

For phosphorus deficiency experiments, it is a typical approach to offer these low concentrations in hydroponic culture. The major drawback of this classical approach is the lack of nutrient buffering capacity in solutions. Due to nutrient uptake, a low P concentration is rapidly decreased to levels close to nil or the minimum concentration of uptake (C_{Lmin}). Depending on the rhythm of solution changes, this leads to a switch on/off supply and P

deficiency is rather due to a restriction in the total amount of P. However, in soils, plants typically face a very low but steady P concentration in soil solution, whereas the total amount of the P in soil is often not limited. Hence, in a natural situation, deficiency is due to a reduced uptake rate at very low concentrations. Plants may show different physiological reactions to these different conditions of deficiency. This is one reason why results obtained in solution experiments cannot easily be transferred to soil conditions.

Theoretically, this problem can be overcame by frequently and often replacing the nutrient solution or by a constant automated addition of small amounts of the respective nutrient. However, these approaches are either plant stressing and laborious or need accurate prior knowledge of plant growth rates and nutrient uptake rates (Asher and Blarney 1987). Besides, these approaches sometimes are not very promising, especially for nutrients with a very low minimum concentration close to the detection limit like P ($C_{Lmin} < 0.2$ µM, Barber 1995). The aim of this study was to develop a simple approach that better simulates soil conditions for P by using rock phosphate with a low equilibrium concentration in nutrient solution.

2.3 Materials and Methods

Seeds of sugar beet (cv. FINOLA KWS) were germinated in moist paper rolls. Seedlings were transferred to pots containing 60 L aerated nutrient solutions 4 days after germination, and cultivated for 7 days in half-strength nutrient solution as follows. After that, the seedlings were selected for uniformity and transplanted to pots containing 3 L of aerated nutrient solution (four seedlings per pot). The experiment was conducted in a greenhouse. The nutrient solution was composed of (µM): $Ca(NO_3)_2 \cdot 4H_2O$ (2500), KCl (1000), K_2SO_4 (1000), $MgSO_4 \cdot 7H_2O$ (750), H_3BO_3 (30), $MnSO_4 \cdot H_2O$ (2.5), $ZnSO_4 \cdot 7H_2O$ (1), $CuSO_4 \cdot 5H_2O$ (1), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.3), and Fe-EDDHA (50). After transplanting into 3 L pots, three different P treatments were applied: (i) 500 µM $NaH_2PO_4 \cdot 2H_2O$ as sufficient control; (ii) 2 µM $NaH_2PO_4 \cdot 2H_2O$ as a typical deficiency treatment in a switch on/off mode; (iii) 3 g rock phosphate (DoloPhos) per pot, i.e. the buffered deficiency treatment. The rock phosphate was kept in teabags to avoid direct contact and adhesion of rock phosphate to the roots. Before use, the filled teabags were washed in pure water for 1 day to remove highly soluble impurities. Each teabag was kept in its pot until nutrient solution changes. Solutions were changed every seven days. Each treatment was conducted with nine replicates.

We measured the pH value and collected nutrient solution daily after the first change of nutrient solution (7 days after onset, DAO). The P concentration in nutrient solution was analyzed colorimetrically according to (Murphy and Riley 1962). The plants were harvested at 29 DAO. The dry weight of root and shoot were recorded separately and the P concentration in plant material was determined by the molybdate-vanadate method (Scheffer and Pajenkamp 1952).

2.4 Results and Discussion

In the P sufficient treatment (P500), P concentration in nutrient solution (Cp) decreased from 500 µM to about 200 µM within 7 days between solution changes due to plant uptake (Figure 2.1). The final 200 µM are still high enough to assure maximum uptake rates (Claassen 1990), i.e. the concentration decrease in the P500 treatment reflects the P-demand of the plants. Cp of the 2 µM treatment (P2) was close to zero all the time. The first solution sample was taken 1 h after nutrient solution change. This indicates that plants had taken up all P in the first hour after solution change and grew without any P supply for the remaining 7 days until next solution change. Even a higher starting concentration than 2 µM would not solve this problem as could be seen by the rapid concentration decrease in the P500 control.

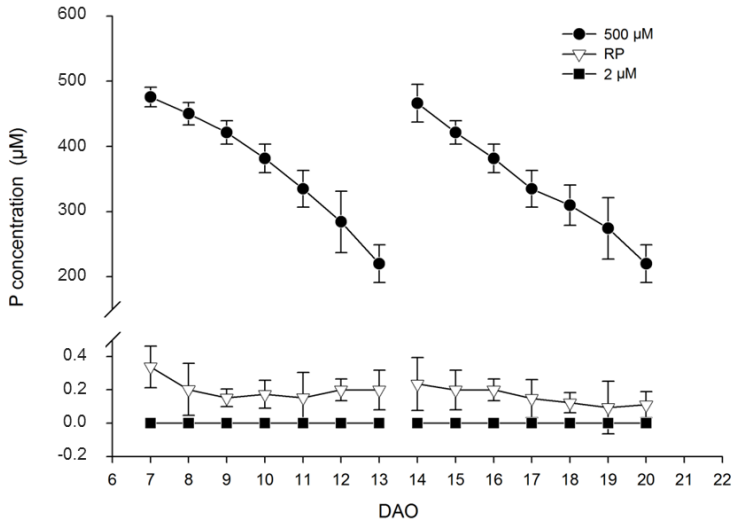

Figure 2.1. Phosphorus concentration (µM) changes in nutrient solutions at 500 µM (high P), 2 µM (typical method, low P) and RP (new method, low P) treatments during 14 days (DAO: Day after onset of P treatments). Values on 7 and 14 DAO represent the P concentration in nutrient solution 1 h after changing nutrient solution. Error bars indicate means ± SE (n=3).

Many previous works which studied the response of plant roots to P deficiency have been conducted at these rather extreme situations where P-deficient plants were not supplied with any P for a longer time, hence a switch on/off situation of P supply (e.g. Khorassani et al. 2011).

In contrast to the P2 treatment, Cp of the RP treatment was kept in the range of 0.2-0.4 µM and 0.1-0.2 µM in the first and second solution change cycle, respectively. However, Cp was not constant over the whole time period 7-20 DAO. This might be due to different reasons. The slightly higher concentrations in the fresh solutions could have been due to fertilizer impurities with better soluble P compounds than rock phosphate. The tea bags had been washed for 1 day to avoid this, but were not checked before use. Another reason could have been a slightly reduced plant uptake rate due to the disturbances during solution changes. The lower concentrations in the second cycle compared to the first one might be caused by higher P uptake rates of the older and larger plants. The steady-state situation of solving rock phosphate and uptake into the plant might have established at a lower concentration. Furthermore, variations in concentration were influenced by solution pH, which decreased from 6.1 to 5.6 due to plant activity.

Table 2.1. Dry weight (g pot^{-1}) and P concentration (%) in different plant parts of sugar beet grown at 500 µM (high P), RP (new method, low P) and 2 µM (typical method, low P). Within each column, significant differences are indicated by different characters ($p < 0.05$).

Treatments	Dry weight (g pot^{-1})		P concentration (%)	
	shoot	root	shoot	root
500 µM	11.02 a	1.34 a	0.56 a	0.43 a
RP	2.15 b	0.59 b	0.21 b	0.38 b
2 µM	0.75 c	0.25 c	0.06 c	0.16 b

Soil solution concentration is usually very low (0.1-10 µM), but the total amount of P in soil is quite high (Hinsinger 2001). In the RP treatment, it was possible to establish a concentration in the lower range of typical soil solution concentrations by also having a nearly unlimited source of P. P concentration was low enough to assure that plants suffered from P deficiency (Table 2.1; Figure 2.2). Both, plants grown with RP and P2 showed clear P-deficiency symptoms at 29 DAO. However, P deficiency symptoms were expressed stronger in P2. Shoot dry weight in RP was clearly higher than that in P2, though in both treatments biomass was significantly lower than in P500 and a similar trend was observed for plant root dry weight. Compared with P2, shoot P concentration in RP was significantly higher, although in both treatments they were below the range considered P sufficiency (0.35-1.1%; Reuter et al. 1997). However, more important than the different levels of P deficiency between the RP and P2 is the fact that the conditions in RP were rather comparable to the situation in soil.

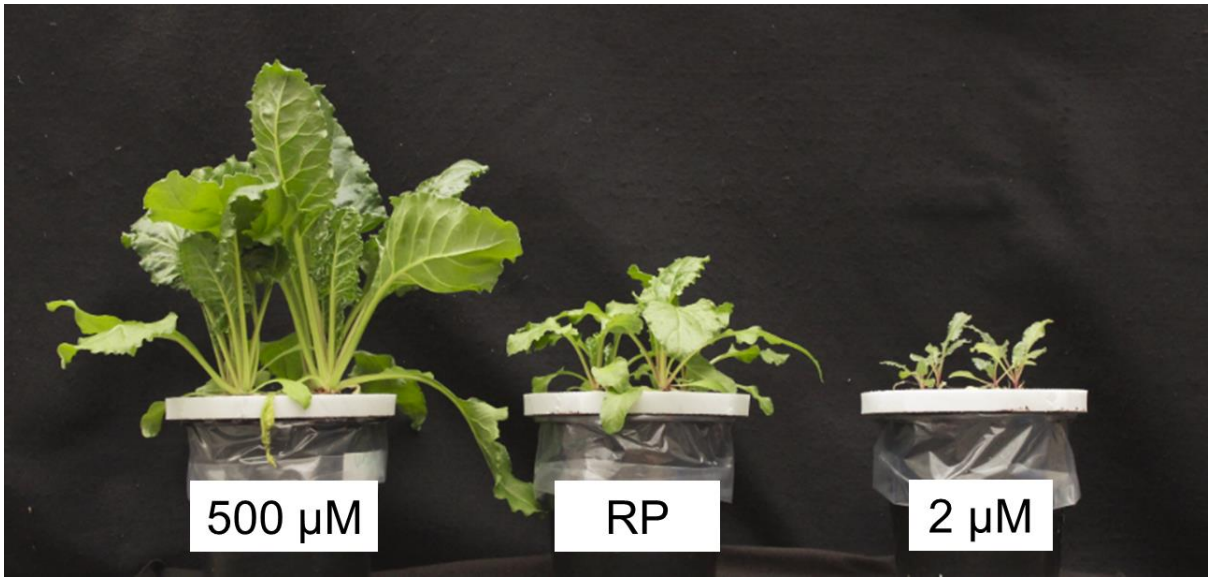

Figure 2.2. A photograph of 29-DAO-old (DAO: Day after onset of P treatments) sugar beet grown in 500 µM phosphorus (left), Rock Phosphate (middle) and 2 µM phosphorus (right) nutrient solution.

Another experiment was performed to achieve even lower P concentrations in solution. For this, a second teabag containing 5 g $CaCO_3$ was added to each pot. Consequently, the solution pH increased to 7.5. However, this pH was too high and the P solution concentration was reduced below the detection limit. Plant growth nearly ceased after transferring the plants into the final solution and plants started to die (Table 2.2). However, this experiment indicates that the steady state concentration can be influenced by the pH value of the solution.

Table 2.2. Effect of adding $CaCO_3$ in Rock Phosphate treatment (RP) on plant growth and P concentration in plant tissue. Within each column, significant differences are indicated by different characters ($p < 0.05$).

Treatments	Dry weight (g pot^{-1})			P concentration (%)		
	shoot	root	beet	shoot	root	beet
RP	22.56 a	3.02 ab	6.47 a	0.26 a	1.22 a	0.16 a
RP+ $Ca(NO_3)_2$	12.04 b	3.22 a	3.59 b	0.08 b	0.58 b	0.07 b

2.5 Conclusions

It can be concluded that the described teabag method is well suited to provide a constantly low P concentration in hydroponic culture for plant growth for periods of at least up to 7 days in P deficiency studies.

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Chapter 3: Exudation pattern of sugar beet (*Beta vulgaris*) as affected by phosphorus deficiency

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3.1 Abstract

Background and aims

Sugar beet (*Beta vulgaris*) is able to acclimate to phosphorus (P) deficiency by releasing organic compounds (mainly organic acids), resulting in the mobilization of sparingly soluble soil phosphate in the rhizosphere. Except citric, malic and oxalic acids, some plant species can exude particular organic acids as a result of long-term adaptation to P limited soils. However, it remains largely unknown whether some specific metabolites exist in root exudate of sugar beet plants which are responsible for P mobilization. The aim of this work was to identify those potential metabolites.

Methods

In the current study, sugar beet plants were cultivated in nutrient solution with either a sufficient (500 μM P) or a deficient P (Rock Phosphate; RP) supply. The root exudates were collected by dipping method and were profiled by high-performance liquid chromatography (HPLC) coupled to electrospray ionization (ESI) in mass spectrometry (MS).

Results

Compared to P sufficient plants, P deficient plants had higher root and lower shoot dry weight. The rate of exudation was about 2-5 fold higher in P-deficient plants (RP treatment) than in P-sufficient plants (P500 treatment). Metabolic signals with intensities differing

between P-deficient and well-supplied plants were identified. Sixty-nine signals enhanced more than 5 fold under P deficiency. Sixteen putative metabolites correlated to those signals were achieved from databases. However, none of them has been definitively confirmed to date.

Keywords: root exudate, phosphorus deficiency, sugar beet, HPLC-ESI-MS, metabolic profiling

3.2 Introduction

Phosphorus (P) deficiency limits crop production in 40% of the world's arable land (Vance 2001; Wang et al. 2010; Chen et al. 2013). In most soil, total amount of P is quite abundant, but a large proportion is bound to different soil constituents, forming complexes of limited bioavailability (e.g. Ca-P, Fe-P). Thus P concentration in soil solution (Pi) is in micromolar range, which is insufficient to satisfy plant needs (Schachtman et al. 1998; Kochian et al. 2004). To cope with limited Pi supply, plants have developed a range of strategies, and one of these strategies is the secretion of P-mobilizing root exudates (mainly organic acid anions) (Schachtman et al. 1998; Raghothama 1999; Vance et al. 2003). Exudation of organic acid anions into rhizosphere can increase P availability by solubilizing P in the soil which is bound to Fe, Al or Ca through forming soluble metal-chelate complexes with P (Kirk 1999; Ryan et al. 2001) or displacing P from adsorption sites (Dinkelaker et al. 1989; Geelhoed et al. 1998).

Several plant species are known to increase exudation of organic acid anions in response to P deficiency, e.g. white lupin (*Lupinus alba* L.; Dinkelaker et al. 1989), rape (*Brassica napus* L.; Hoffland et al. 1992), alfalfa (*Medicago sativa* L.; Lipton et al. 1987) and sugar beet (*Beta vulgaris* L.; Beissner and Römer 1998; Khorassani et al. 2011). Among species examined for organic acids production in response to P deficiency, white lupin is often used as a model plant for root exudation studies due to its capability to release huge amount of organic acids. In response to P deficiency, white lupin developed specialized root structures (cluster roots) which can strongly acidify and also easily exude massive quantities of organic acid anions in rhizosphere (Neumann et al. 1999; Zhu et al. 2005; Lambers et al. 2006). The concentration of organic acid exuded by white lupin is remarkable, e.g. citrate concentrations can exceed 50 mM in rhizosphere solution of cluster roots (Johnson et al. 1996; Dessureault-Rompre et al. 2008). However, concentration of organic acid has been detected in range between 0-0.1 mM

in the bulk soil solution and less than 1 mM in rhizosphere of most non-cluster root plants (Jones et al. 1996; Raghothama 1999). A number of studies indicated that P release only presented at relatively high organic acid concentrations with the critical threshold, range from 8.5 to 33 mM, i.e. 2.5 to 10 μmol carboxylate g^{-1} soil (Gerke et al. 2000; Wouterlood et al. 2004; Oburger et al. 2009). Therefore, for most plants, the effect of such a low concentration of organic acid on increasing P availability is generally considered negligible or small (Drever and Stillings 1997; Ström et al. 2002). In addition, under non-sterile conditions, microbial uptake and biodegradation of organic acid anions may further lower their effective soil solution concentration, particularly this process is known to be rapid (half-life in soil solution typically between 0.5 and 12 h; Jones and Darrah 1994; van Hees et al. 2002). As we summarized above, the ecological importance of organic acid exudation in response to P deficiency is still questioned, but there were still certain amount of studies proposing that the organic acid exudation is an effective strategy to increase P availability. Rape (*Brassica napus* L.) plants have been ascribed to excretion of organic acid during P deficiency as efficient users of rock phosphate (Hoffland et al. 1992). P-efficient genotype of common bean was exuding more organic acids (Shen et al. 2002). Sugar beet was also proposed to achieve high P influx by increasing exudation of organic acids. Beissner and Römer (1998) observed an increased exudation of sugar beet in low P soils and Gerke et al. (2000) reported that the calculated influx of P mobilized by oxalate is 1.5 to 6 times higher than the inflow without mobilization. Based on the literatures described above, we suppose that in low P supply, the contribution of P mobilization by organic acid exuded by roots to P uptake is substantial, at least in sugar beet.

The exudation of organic acid anions induced by P deficiency is, however, subject to large variations, both at quantitative and qualitative aspects, depending on plant species and severity of P deficiency. Some organic acids such as citrate, malate and oxalate seem to be the

most common and effective composition of root exudates in mobilizing P and have therefore been well studied (Rengel 2002; Aoki et al. 2012). For instance, citric acid has been reported as the dominant organic acid released by white lupin (Dinkelaker et al. 1989), alfalfa (Lipton et al. 1987), rape (Hoffland et al. 1989) and chickpea (Ohwaki and Sugahara 1997); malic acid has often been found as dominant organic acid in maize (Jones and Darrah 1994), rape (Hoffland et al. 1992) and tomato (Imas et al. 1997), whilst oxalic acid appeared to be more important for plants such as sugar beet (Beissner and Römer 1998; Gerke et al. 2000). However, in recent years, more studies detected other organic acids in root exudate which might also be involved in P mobilization. Ae et al. (1990) found piscidic acid exuded from pigeonpea roots could release P from $FePO_4$ by chelating Fe. Shen et al. (2001) reported that the exudation of pentanedioic acid is a specific response to P deficiency in elephantgrass and constitutes a mechanism of tolerance to low P stress. In soybean, malonate was the dominant organic acid detected in root exudates (Tang et al. 2009). Khorassani et al. (2011) detected salicylic acid and citramalic acid in root exudates of sugar beet and noted both of them can increase P availability. In addition, Oburger et al. (2009) suggested that the effects of individual organic acids should not be studied isolated, and there might be some interactions between organic acids, since which are released as a cocktail by plants. Prior to the current study, most work observed the influence of P deficiency on root exudates addressing one or some specific organic acids, such as citric, malic and oxalic acid. To our knowledge, it is insufficient only to have information about those specific compounds. We expect that root exudate of sugar beet probably includes other uncommon compounds which could increase P availability more efficiently than those identified and analyzed so far. Here, the "uncommon compound" denotes: (i) compound involved in P mobilization but never reported in the given species (in this study refers to sugar beet); (ii) compound might be capable mobilizing P which was not reported in previous studies. The aim of this study was to get a comprehensive

overview of alternation of root exudate metabolic profiles in sugar beet between P-deficient and well-supplied conditions and to identify metabolite contributing to P mobilization, using HPLC-ESI-MS for analysis.

3.3 Materials and methods

3.3.1 Plant cultivation

Seeds of sugar beet (cv. FINOLA KWS) were germinated on paper rolls. Three days after germination, seedlings were transferred to a 60 L container and cultivated for 3 days and another 4 days in a one-quarter strength nutrient solution (see below), and a one-half strength solution, respectively. After that, the seedlings were selected for uniformity and transplanted to pots containing 3 L of full strength aerated nutrient solution at a density of four plants per pot. The full strength nutrient solution had the following composition (µM): $Ca(NO_3)_2 \cdot 4H_2O$ (2500), KCl (1000), K_2SO_4 (1000), $MgSO_4 \cdot 7H_2O$ (750), H_3BO_3 (30), $MnSO_4 \cdot H_2O$ (2.5), $ZnSO_4 \cdot 7H_2O$ (1), $CuSO_4 \cdot 5H_2O$ (1), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.3), and Fe-EDDHA (50). The nutrient solution was renewed every 7 days. The phosphate sufficient and phosphate deficient media contained 500 µM $NaH_2PO_4 \cdot 2H_2O$ (P500) and 3 g Rock phosphate (Dolo phos) kept in teabags (RP), respectively. Previous studies had shown that RP can provide a low (0.1- 0.4 µM) but relatively constant P concentration despite P uptake by plants. This 'buffered' situation is more comparable to soil conditions (Raghothama 1999) than just offering a low P concentration which is rapidly depleted close to nil by plant uptake. Plant culture was conducted in a greenhouse with a light period of 10 h. The P treatments started at DAT 7 (day after transplanting). Each treatment had six replicates.

3.3.2 Collection of root exudates

Root exudates were collected at 35 and 42 DAT by dipping method (Neumann and Römheld 2007). Prior to collection, the plants were pre-cultured in 500 µM Ca $(NO_3)_2$ solution for 12h. Afterwards, the whole root system was carefully rinsed three times with distilled water to remove ions from the root surface, and then submerged in trap solution (distilled water) for 2h (the volume (100-400 ml) of the trap solution depends on the size of root system). Control

pots were treated similarly without sugar beet plants. The collection of root exudates always began at 10 am after plants had been exposed to light for 2 h to avoid possible variation of exudation release due to a diurnal rhythm (Watt and Evans 1999). Immediately after collection, each sample of exudate solution was filtered through filter paper (MN615 1/4 Ø90mm, MACHEREY-NAGEL GmbH, Düren, Germany) and frozen in liquid nitrogen to minimize microbial degradation of organic compounds. Frozen solutions was lyophilized and dissolved by methanol: water (50:50 v/v) for 1 h, after that evaporated to dryness by use of rotational vacuum concentrator (RVC 2-25 CD plus; Christ GmbH, Germany). The sample weight was recorded, and stored at -20°C until HPLC-MS analysis.

3.3.3 Metabolic profiling by HPLC-MS

After collection of root exudates the dried residue were re-dissolved in 1 mL methanol: water (50:50 v/v) for 1 h at room temperature. Samples were vigorously shaken and centrifuged at 4800 rpm for 10 min, and then the supernatant were centrifuged at 14000 rpm for 10 min.

High performance liquid chromatography was carried out on a binary pump system (Prostar 210, Varian, Darmstadt, Germany) using a reversed-phase column Polaris C18-Ether (100×2mm, 3 µm particle size; Varian) coupled with a C18 security guard cartridge maintained at 40°C. The mobile phase consisted of (A) bi-distilled water: acetonitrile (95:5) and (B) methanol, both containing 7 mM acetic acid. The binary gradient was as follows: 0.0-1.0 min 10% B; 1.0-30.0 min from 10% to 98% B; 30.0-50.0 min 98% B; 50.0-51.0 min from 98% to 10% B; 51.0-70.0 min 10% B. The flow rate was 0.2 mL min^{-1}. Ten µL of the sample was injected for analysis.

HPLC was coupled with electrospray ionization and mass spectrometry detection using an ion trap 500MS (Varian, Darmstadt, Germany). Ionization was done in positive and negative mode with the following parameters (negative/positive): needle voltage -4500 V/+5000 V, shield voltage -600 V/+600V, capillary voltage -/+80 V, drying gas (nitrogen) 15 psi at

350°C, and nebulizing gas (air/nitrogen) 25 psi. In positive mode, ions with a mass-to-charge ratio (m/z) 50 to 1000 were collected in a single run, while in negative mode ranges of m/z 50 to 400 and m/z 400 to 1000 were scanned separately. The scan speed was set to 15000 Da/s. MS workstation/MS Data Review 6.9.1 (Varian, Darmstadt, Germany) was used for data acquisition.

The Component Detection Algorithm (CODA, Windig et al. 1996) including smoothing, baseline correction, and peak picking was carried out for mass spectrometric data analysis using ACD/MS Manager Version 12.0 (Advanced Chemistry Development, Toronto, Canada). Ion chromatograms with a mass quality index (MCQ) of at least 0.8 at a smoothing window width of three scans were considered. Baseline correction that means to remove high level background from the data set was applied to mass chromatograms with box half width of 10 scans and noise factor of 1. Peak tables were created that contained the m/z value, retention time (Rt), peak area in counts (negative: >1000; positive: >200000), MCQ value (>0.8) and the S/N value (>100). Normalization and peak alignment across all samples were carried out using user-written Perl script (Karlovsky, unpublished). After peak alignment, signals occurring in plant-free controls and signals detected in fewer than five of six replicates were discarded.

For identification of compounds of interest mass, information (e.g. m/z, retention time) were used for search in databases (more details in results section). The putative identity of the metabolites of interest was tested by co-elution with pure standards, while retention time, mass of the precursor and fragmentation pattern were used as criteria for identity confirmation.

3.3.4 Plant harvests

The plants were harvested after the last collection of root exudates. All plant tissues were dried at 60-65°C for 24 h and then at 105°C until a constant weight, and dry weights of plant

parts were recorded. The shoot P concentration was determined: 0.3 g of plant material was digested with 4 mL HNO_3 (65%) and 2 mL H_2O_2 in microwave oven. Phosphorus was determined by the molybdate-vanadate method (Scheffer and Pajenkamp 1952).

3.3.5 Root length imaging

For calculating exudation rates, a non-destructive screening procedure was developed to estimate total root length (RL) without destroying the plant. For this, 36 pots of sugar beet plant were grown together with those of the main experiment. Plants were photographed at least twice a week from a defined distance and angle in front of a black background using a digital single-lens reflex camera (Canon EOS 600D, Canon Inc., Japan). The area of pixels in each picture was calculated using Image J software (Rasband 1997). After the imaging procedure, plants of two pots were harvested and RL per pot was determined by the line intersection method of Tennant (1975). Measured RL of the harvested pots was plotted against the respective area of green pixels and a power trendline was fitted (Figure 3.1).

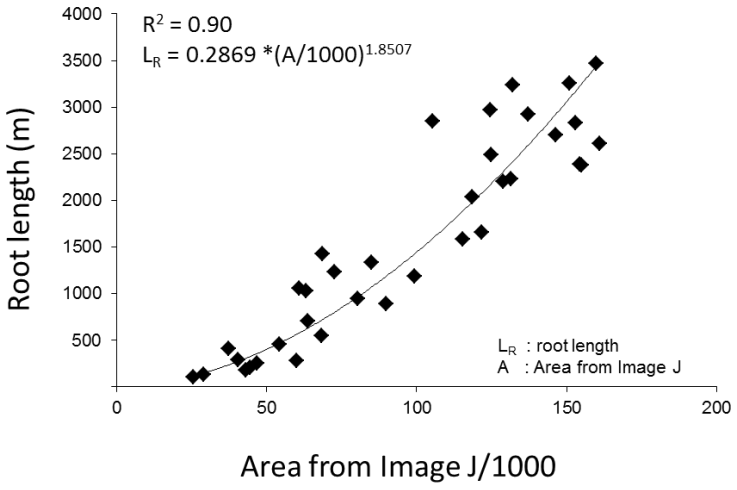

Figure 3.1. Relationship between root length measured by the line intersection method and the respective area obtained by ImageJ software.

The parameters of the power were used for calculating RL per pot from plant images only. The equation is:

$$L_R = 0.2869*(A/1000)^{1.8507}$$

Where, L_R is the RL per pot, A is the area from ImageJ, r^2= 0.90

3.3.6 Statistical analysis

Statistical analysis was carried out with SPSS analytical software (SPSS Inc., Chicago, IL, USA; version 19). Difference between treatments was evaluated for significance by LSD multiple range tests ($p < 0.05$). Data are presented as mean ± S.E.

3.4 Results

3.4.1 Plant growth

The biomass production was evidently inhibited by low P supply. The shoot dry weight of P deficient plants (RP) was 56% lower than well-supplied plants (P500), whereas there is no significant difference in root dry weight (Table 3.1). The effect of P on beet production followed the same pattern as shoot, though more pronounced. The root/shoot dry weight ratio was increased when plants were grown at low P supply, which is a typical response to P deficiency.

Table 3.1. Effect of P supply on the dry weight (SDW), root/shoot dry weight ratio, P concentration and root length by calculation. Plants were grown in nutrient solution at two levels of P supply (P500 and RP), and harvested after 42 d transplant (DAT 42). Results are means ± SE (n=6). Results with different letters are significantly different ($p < 0.05$).

P supply	Dry weight (g pot^{-1})			Root/shoot dry weight	P concentration (%)		Root length (m pot^{-1})	
	Shoot	Root	Beet		Shoot	Root	DAT35	DAT42
P500	24.3±0.2 a	2.12±0.04 a	13.04±0.47 a	0.087±0.002 b	0.64±0.02 a	0.55±0.01 a	2282±98 a	3393±80 a
RP	10.6±0.4 b	2.18±0.06 a	3.75± 0.31 b	0.206±0.009 a	0.18±0.02 b	0.43±0.04 b	1152±76 b	2287±72 b

Increasing P supply clearly increased P concentration in shoot and root (Table 3.1). Shoot P concentration was decreased in plants supplied with RP, but sufficient with P500 treatment. Reuter et al. (1997) defined the threshold for P deficiency in shoot as being 0.35% (3.5 mg g^{-1}), and thus the P deficient and P sufficient treatments produced plants below and above the P-deficiency threshold, respectively (Table 3.1).

3.4.2 Root exudation rate

In the present study, the amount of root exudate was represented by the weight of the collected and lyophilized solution (details described in Materials and Methods section). The

release of root exudate was highly dependent on P supply, i.e. the production of root exudate whether expressed by per plant or per unit of root length was significantly increased with low P supply (Figure 3.2). The amount of root exudate increased with plant age, deficient plants release the double amount of root exudate than that of sufficient plants, and 7 days later, the amount increased even 3 folds higher. However, as P deficiency became severer, the rate of root exudation decreased, whereas in P sufficient plants the release remained at a low level.

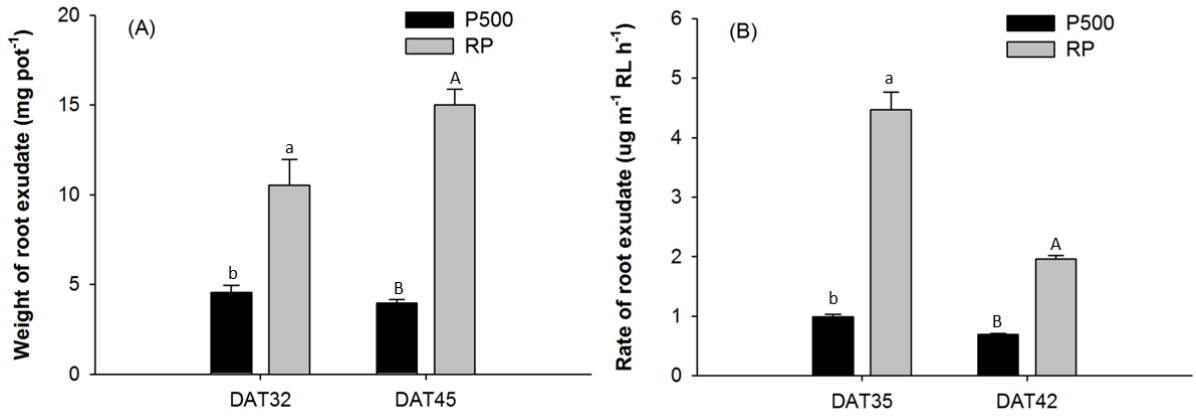

Figure 3.2. Effect of P supply on weight of root exudates (A) and rate of root exudate (B). Plants were grown in nutrient solution at two levels of P supply (P500 and RP). Bars represent means ± SE (n=6). Data with different letters are significantly different ($p < 0.05$).

Since the amount of substances secreted would be directly proportional to the root-size and the P-deficiency effected the formation of root, the exudation was normalized to per unit of root length. This parameter (root exudation rate) reflects the release ability more precisely. Rate of root exudation expressed as per unit root length of P-deficient plants was markedly faster than that of P-sufficient plants, which is a typical response to low P availability. Roots of plants with a low P supply had 2-5 times higher exudation rate compared to those with an

adequate P supply (Figure 3.2). There was a decline of root exudation rate at DAT 42, this decline may be caused by severity of P deficiency and the increased plant root size.

3.4.3 Metabolic profiling of root exudates

To assess the influence of P supply on the quality of root exudates of sugar beet, metabolic profiling (also known as untargeted analysis, metabolite profiling or metabolomics) was conducted by HPLC-EIS-MS as described in Materials and Methods. Profiles of the root exudates collected from low and high P treatments were compared and signals (peaks) were selected according to following criterion: (i) the signal was absence in water control; (ii) the signal collected present in at least 5 of 6 replicates of root exudate samples; (iii) signal intensity must be at least 5 fold higher with low P supply plants compared to well-supply; (iv) standard deviation (SD) should be lower than 100% of corresponding mean value. After primary data processing, we need to manually recheck the signal in the original chromatograph, after which some of signals were excluded. For example, m/z 117 and 145 in negative mode presented at DAT 42, and their chromatographs were given in Figure 3.3. Signal m/z 117 showed a clear peak at 1.8 min, however, signal m/z 145 at 2.6 min included several peaks, thus we assumed that m/z 145 at retention time 2.6 was not a real peak (the peak quality was quite low). The eligible signals were summarized in Table 3.2 to 3.5.

In general, the profile of root exudates presented great difference between two harvests (DAT 35 and 42). Out of few signals, presented at both harvests, e.g. m/z 329, 103 and 115, the other signals induced by P deficiency only occurred at one harvest. Claassen (1990) observed that P deficiency significant decreased sugar beet plant development at early growing stage and after late June or early July stage there was no much different between P-deficient and well-supplied plants. We proposed that this observation was caused by changing exudation pattern which can improve P acquisition efficiency. Therefore, besides those presented at both harvests, signals presented at DAT 42 induced by P deficiency were more interesting for

further investigations. Afterwards, 13 and 17 signals at DAT 42 in negative and positive mode, respectively, were selected for identification process (Table 3.3 and 3.5). While, at DAT 35, only m/z 329 (negative) and m/z 335, 423 and 429 (positive) were chosen for further investigations because those four signals presented not only at DAT 35 but also at DAT 42 (Table 3.2 and 3.6).

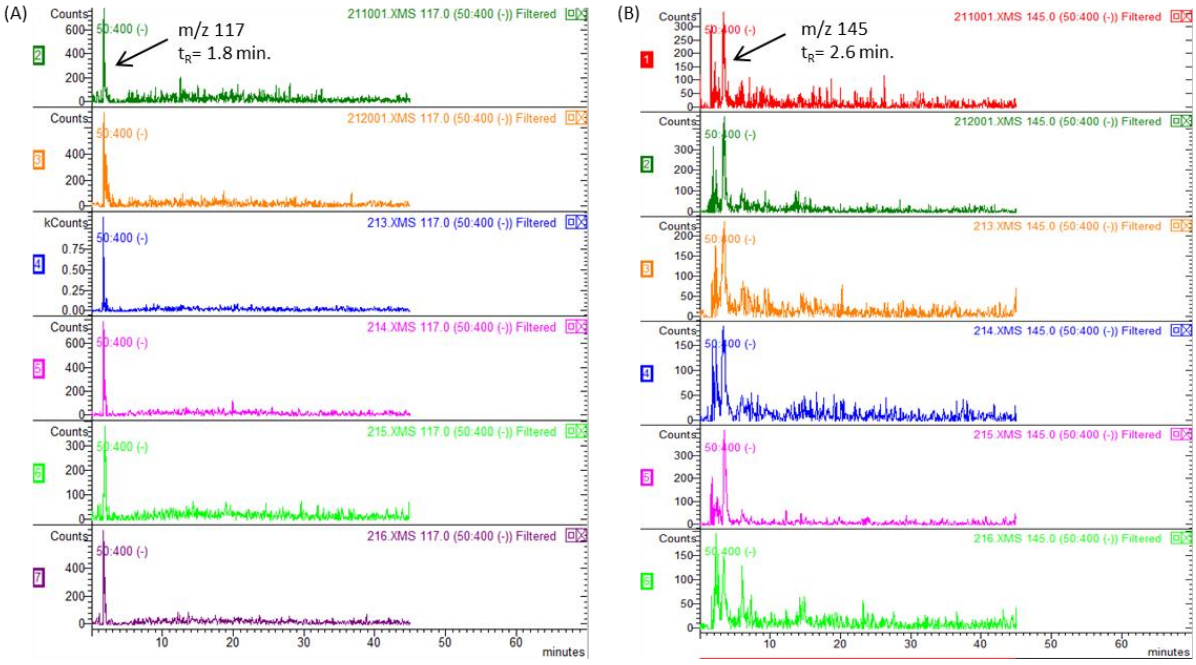

Figure 3.3. Chromatograph of m/z 117 (A) and 145 (B) in negative mode of root exudates collected from P deficient plants at DAT 42.

Table 3.2. Ratio of signal intensity (given as peak area) detected in root exudates under low P (RP) to high P (P500) supply: eligible signal detected at DAT 35 in negative ESI mode.

Ionization mode	Retention time	m/z	RP/P500 Signal intensity	
			DAT 35	DAT 42
–	2.1	60*	6	nd
–	16.9	259*	12	nd
–	31	270	6	nd
–	28	275	5	nd
–	30	329	6	4
–	22	363	x	nd
–	22	495	x	nd
–	23	553	106	nd
–	24	611	7	nd
–	2	659	7	nd
–	2	697*	x	nd
–	26	727	7	nd

* indicates standard deviation ≤ 75% of mean value, nd indicates not detected, x indicates only detected in RP treatment.

Table 3.3. Ratio of signal intensity (given as peak area) detected in root exudates under low P (RP) to high P (P500) supply: eligible signal detected at DAT 42 in negative ESI mode.

Ionization mode	Retention time	m/z	RP/P500 Signal intensity	
			DAT 35	DAT 42
–	2.1	103	2	9
–	6.4	115*	3	5
–	1.8	117*	nd	25
–	31.5	301	nd	5
–	1.6	326	nd	9
–	1.5	636	nd	x
–	1.5	696	nd	x
–	1.5	718*	nd	20
–	30.1	763	0.4	8
–	1.6	779	nd	14
–	1.6	800*	nd	14
–	1.5	802*	x	20
–	1.5	859	nd	10

* indicates standard deviation ≤ 75% of mean value, nd indicates not detected, x indicates only detected in RP treatment.

Table 3.4. Ratio of signal intensity (given as peak area) detected in root exudates under low P (RP) to high P (P500) supply: eligible signal detected at DAT 35 in positive ESI mode.

Ionization mode	Retention time	m/z	RP/P500 Signal intensity	
			DAT 35	DAT 42
+	12	335*	x	0.6
+	13	359	11	nd
+	19	398*	5	nd
+	14	423*	x	0.2
+	20	425	6	nd
+	14	429*	7	0.4
+	14	438*	x	nd
+	20.1	447	x	nd
+	13.9	451	x	nd
+	15	494*	x	nd
+	16	496*	7	nd
+	21	500	x	nd
+	17	508	5	nd
+	20	553	x	nd
+	22	563	x	nd
+	20	571	9	nd
+	25.1	588	x	nd
+	21	627	x	nd
+	2	645*	x	nd
+	2	827	x	nd
+	17	830	17	nd
+	2	846	20	nd
+	17	909*	x	nd
+	20	958*	x	nd
+	17.2	962*	x	nd
+	19.8	979*	x	nd
+	17.8	984	x	nd

* indicates standard deviation ≤ 75% of mean value, nd indicates not detected, x indicates only detected in RP treatment.

Table 3.5. Ratio of signal intensity (given as peak area) detected in root exudates under low P (RP) to high P (P500) supply: eligible signal detected at DAT 42 in positive ESI mode.

Ionization mode	Retention time	m/z	RP/P500 Signal intensity	
			DAT 35	DAT 42
+	1.7	104	0.3	6
+	1.6	377*	3.7	54
+	7.3	453	nd	x
+	8.2	497*	nd	14
+	8.9	542	nd	x
+	9.7	585	nd	13
+	10.3	629*	nd	11
+	11.4	674*	nd	x
+	11.4	717*	nd	18
+	12.9	761*	nd	12
+	11.9	762*	nd	114
+	12.8	790*	nd	7
+	12.4	805*	nd	58
+	13.1	806*	nd	x
+	12.4	807	nd	x
+	12	850*	nd	11
+	13.2	877*	nd	12
+	12.9	893*	nd	29
+	11.8	894*	nd	13
+	13.6	937*	nd	64
+	13.3	981*	nd	50

a: * indicates standard deviation ≤ 75% of mean value, nd indicates not detected, x indicates only detected in RP treatment.

b: m/z 762, 806 and 807 are isotope of m/z 805; m/z 894 is isotope of m/z 893.

3.4.4 Identification of metabolites in root exudates of sugar beet

Searching metabolites in databases according to their masses is often the first step in metabolite identification for a mass spectrometry-based untargeted metabolomics study. The databases used in this study included KEGG (Kyoto Encyclopedia of Genes and Genomes), PMN (Plant Metabolic Network), METLIN and KNApSAcK. The identification process was given in Figure 3.4.

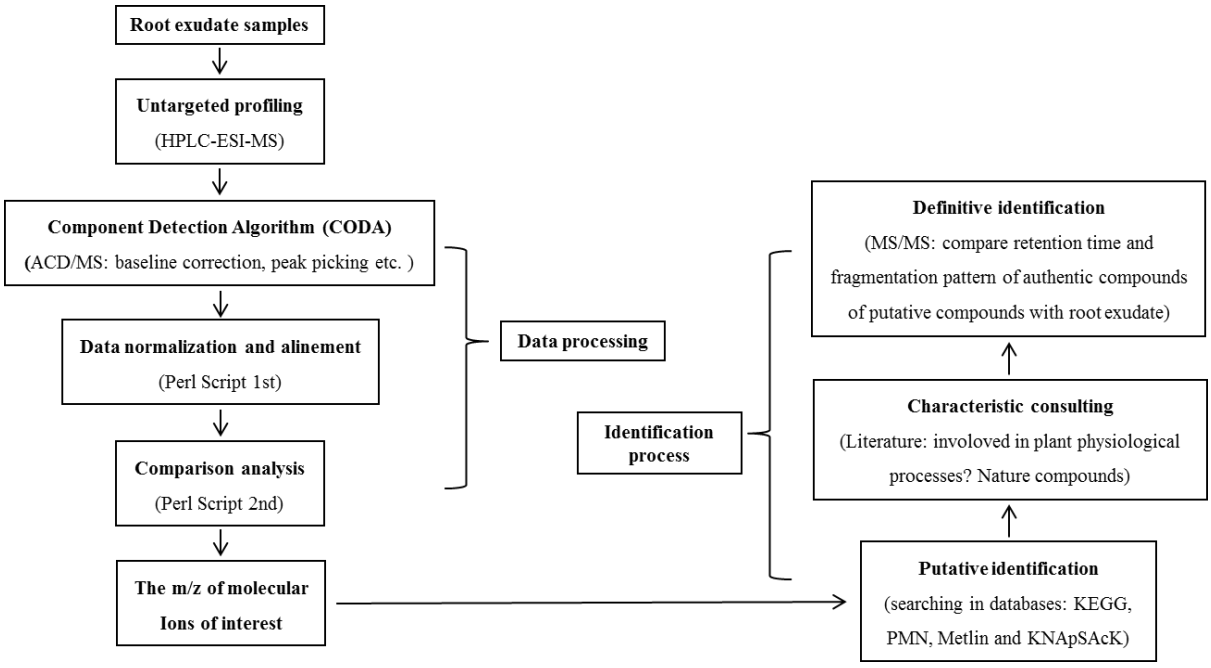

Figure 3.4. Flowchart of root exudate analysis, data processing and compounds identification process.

After searching databases and consulting whether the metabolite participates in plant physiological processes, 16 potential metabolites were chosen depending on their functional groups, i.e. carboxyl. Definitive identification firstly requires comparison of retention time between an authentic chemical standard and the metabolite of interest under identical analysis condition. In addition, to achieve confident identification, the fragmentation mass spectrum of putative metabolites need to be compared with mass spectra acquired from commercially accessible authentic chemical standards or freely available mass spectral libraries. Table 3.6 showed all the potential metabolites selected from databases but only italic ones can get

authentic chemical standards for the following investigation. The chemical structure for the potential metabolites presented was in Figure 3.5.

Table 3.6. Summary of putative metabolites achieved from databases.

Ionization mode	DAT	$[M-H]^-$ m/z	$[M+H]^+$ m/z	[M]	Metabolites	Molecular Formula
–	35	329		330	Pyrrolo-quinoline quinone (PQQ)	$C_{14}H_6N_2O_8$
–	42	103		104	*Malonic acid*	$C_3H_4O_4$
				104	*Choline*	$C_5H_{14}NO$
–	42	115		116	*Fumaric acid*	$C_4H_4O_4$
				116	*Maleic acid*	$C_4H_4O_4$
				116	Hexanoic acid	$C_6H_{12}O_2$
–	42	117		118	*Succinic acid*	$C_4H_6O_4$
				118	*Methylmalonic acid*	$C_4H_6O_4$
–	42	301		302	Abietic acid	$C_{20}H_{30}O_2$
–	42	636		637	Delphinidin 3-O-3",6"-O-dimalonylglucoside	$C_{27}H_{25}O_{18}$
+	42		104	103	*3-Aminobutanoic acid (BABA)*	$C_4H_9NO_2$
				103	*γ-Aminobutanoic acid (GABA)*	$C_4H_9NO_2$
				103	N,N-Dimethylglycine	$C_4H_9NO_2$
+	42		377	376	Loganic acid	$C_{16}H_{24}O_{10}$
+	42		497	496	Polypodine B	$C_{27}H_{44}O_8$

Italic indicates the metabolites tested by co-elution root exudates with pure standards.

Based on the databases, the potential metabolite of signal m/z 103 (negative) was malonic acid, and of signal m/z 115 (negative) was fumaric acid, maleic acid and choline, however, the retention time of those four compounds were different from the corresponding signals in root exudates. The retention time of m/z 104 was quite similar with GABA and BABA standards, but the fragments are different, i.e. m/z 104 > 60 in root exudates, m/z 104 > 87 in standards. Unfortunately, after comparison of the retention time and fragmentation patterns, none of potential metabolites can generate the same signals with which originated from the root exudates.

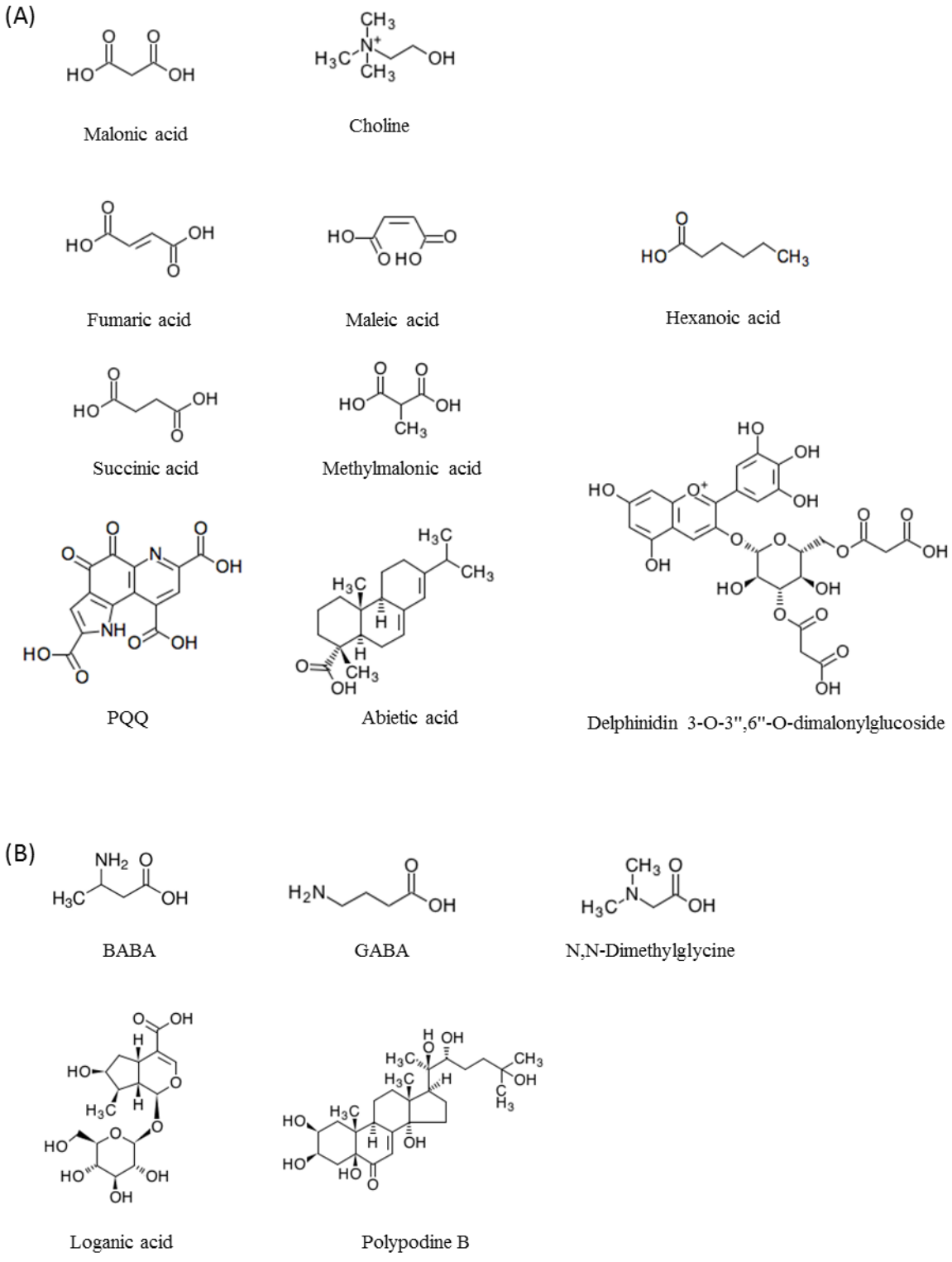

Figure 3.5. Molecular structure of putative metabolites in negative (A) and positive (B) ESI mode.

3.5 Discussion

Compared with former studies on the response of plant to P deficiency, the present work focused on more realistic levels of P concentration, i.e. concentration in a stable micromolar range, which are closer to those that plant roots may experience in soil (Raghothama 1999; Hinsinger 2001). Our previous study (chapter 2) showed that due to plant uptake, supply P in classical way (normally 2 μM inorganic P), the P concentration in nutrient solution (C_P) rapidly decreased close to nil or the minimum concentration of uptake (C_{Lmin}) in one hour after nutrient solution exchange. As we are well aware that soil invariably provides some P, the present study provided a more realistic level of P concentration by using RP treatment, i.e. C_P around 0.4 μM due to the onset of steady state equilibrium between plants uptake and solving of RP. The P situation provided by RP treatment avoided those extreme situations where P-deficient plants did not receive any P at all for days or weeks.

3.5.1 Influence of P supply on plant growth

Root growth is important for acquisition of P which is immobile in soil. In the present study, the total plant growth reduced but the root dry matter increased, leading to a higher root:shoot dry weight ratio, which is the typical response of plant to P deficiency (Hermans et al. 2006; Hammond and White 2008). However, unlike other plant species (e.g. wheat), sugar beet plants did not form a large root system when considering the root length, which is obviously an advantage for exploring a large volume of soil in order to absorb more P (Table 3.1). Therefore, we proposed that the mechanism of sugar beet plants which enhanced their P availability was not to change their root morphological traits but alter physiological processes involved in P mobilization such as increasing exudation of organic compounds (Bhadoria et al. 2002).

3.5.2 Influence of P supply on exudation pattern

It is universally accepted that secretion of organic acid is an important mechanism in sugar beet plants to increase P availability in the rhizosphere (Beissner and Römer 1998; Gerke et al. 2000; Hinsinger 2001; Khorassani et al. 2011). However, some researchers still suspect the significance of organic acids in P mobilization. One of their counter arguments is that except in some rather extreme cases (i.e. white lupin), the concentration of organic acids determined in the rhizosphere or soil solution is quite low to meet the requirement concentration to mobilize soil P. In addition, most of the knowledge about P-deficiency induced organic acids secretion is based on hydroponic culture system, which may distinct differ from soil conditions, e.g. the lack of root hairs, microbial degradation and P concentration in grown condition, hence, it is doubtful whether these results can be regarded as representative of natural growth conditions in soil. However, a comprehensive knowledge of the quantity and quality of root exudates is of great importance for understanding P chemical mobilization process. Gransee and Wittenmayer (2000) compared different methods with respect to their suitability to collect and characterize root exudates and reported that only the dipping method is suitable for a nearly complete sampling and analysis of root exudates. Therefore, in the present study, we used hydroponic culture.

The present results consistently demonstrated P-deficient sugar beet plants induce their root exudation production as a response to P deficiency, no matter expressed as the total release amount or the rate of exudation (Figure 3.2). Compared to the exudation rate of well-supplied plants (about 1 $\mu g\ m^{-1}\ h^{-1}$), P deficiency induced exudation rate range from 2 to 4.5 $\mu g\ m^{-1}\ h^{-1}$ (decreased with plant age). However, the root exudation rates measured in the present study were at least 4 fold lower than the previous estimates by Khorassani et al. (2011), which reported that sugar beet plants maintained a stable exudation rate over the whole growing period observed (DAT 14 to 42). Although the experimental conditions were not identical but

still comparable, one possible reason may be the existing variability between genotypes of sugar beet, but another very important explanation might be the distinct P situation supplied in nutrient solution. Khorassani et al. (2011) used the classical way to establish P deficiency in hydroponic experiments, i.e. they offered inorganic phosphorus at a low concentration (2 µM) at the beginning of a nutrient change cycle without further addition of P. In contrast, the rock phosphate (RP) treatment used in this study provided a relatively constant P concentration which resulted in less severe and more natural P deficiency situation. This might influence exudation pattern as which was demonstrated by Keerthisinghe et al. (1998) who observed that exudation rate of proteoid roots of white lupin was only significantly increased when plants grown without P in solution, even at 1 µM P citrate exudation rate was not significantly different from a well-supplied control.

Comparing the metabolic profiles of root exudates, large variations were observed between DAT 35 and 42, i.e. only few signals occurred at both harvest times, and there were no signals induced more than 5 times by P deficiency at both harvests (Table 3.2 to 3.5). Many previous studies reported that plant age and developmental stage greatly influenced the quality of root exudates (Rovira 1969; Hamlen et al. 1972; Gransee and Wittenmayer 2000). Khorassani et al. (2011) found large differences in the composition of root exudates of P-deficient and well-supplied sugar beet plants at different harvest times. They reported about 65 signals that were at least 5 times higher under deficiency compared to the control, but most of them occurred only at one single harvest, and only 8 signals presented at least two harvests. In addition, these great differences of root exudate composition exist between two harvest times indicating that sugar beet plants change their root exudation pattern, both qualitatively and quantitatively, to cope with aggravated degrees of P deficiency.

In discovery-based investigations, applying untargeted analytical method, prior knowledge of the metabolite classes of interest should be clear. In the present study, we are interested in the

metabolites that favor the desorption of adsorbed P, which will occur mostly via a ligand exchange reaction. A decrease in the concentration of P ions in the soil solution and an increase in the concentration of competing anions will both shift the adsorption-desorption equilibrium towards enhanced desorption. The metabolites of interest in the present study can be categorized into three classes: (i) P sorbents, which can decrease the P ions concentration in soil solution; (ii) inorganic competing ligands, e.g. sulphate and bicarbonate; (iii) organic competing ligands, e.g. carboxylic anions. The molecular structure of putative metabolites selected from databases was given in Figure 3.5. Most of them are organic acids which comprise a wide variety of simple molecules that bear one or more carboxylic groups. The number of carboxyl and their arrangement relative to other carboxyl and hydroxyl groups determine the stability of the ligand: metal complexes (Ryan et al. 2001). Generally, the chelate ability of organic acid decreased in the following order: tricarboxylic acid > dicarboxylic acid > monocarboxylic acid. However, the efficiency of organic acids in mobilizing soil P also may be very context specific with both soil types and the individual organic acid controlling the amount of P released into the soil solution. Ström et al. (2002) tested the P mobilization ability of two most common P mobilizing organic acids (citrate and oxalate) found in root exudates. They observed that 1 mM oxalate (dicarboxylate) addition to the rhizosphere significantly increased P uptake, while citrate (tricarboxylate) had no significant impact on plant P uptake even at high concentration (10 mM). In addition, different organic acids proposed to use different modes of P mobilization. For example, oxalate may primarily release P held in Ca-P minerals through the formation and precipitation of Ca-oxalate. In contrast, citrate which has a poor affinity for Ca^{2+}, but a greater affinity for Fe^{3+} and Al^{3+}, may release P predominantly held in Fe-P and Al-P minerals (Ström et al. 2001).

As dicarboxylic acid, malonic, fumaric, maleic and succinic acids were often reported in root cells or root exudates. For instance, fumaric acid is an intermediate in Krebs Cycle and was supposed to accumulate in root exudates when plants suffering from nutrient starvation, e.g. P (Ohwaki and Hirata 1992). For tomato, at low P supply, fumarate was one of the predominant exudates (Imas et al. 1997). Shane et al. (2008) detected only trace amount of fumaric acid in root exudates of *Lupinus albus L.* grown with phosphorus mineral source (Al-P or Fe-P). Johnson et al. (1994) observed that fumaric acid concentration in root tissue did not differ between high and low P supply. Small amounts of maleic acid were detected in root exudates of white lupin under P deficiency (Shu et al. 2007). Young (2012) compared root exudates of 8 cowpea lines and suggested that maleic acid is the most likely candidates of an organic acid physiological response to low soil P conditions. However, in the present study, none of them occurred in root exudates.

Although the chelate ability of monocarboxylic acid is relativly weak, several monocarboxylic acids were proposed to be involved in the P mobilization process. Khorassani et al. (2011) detected enhanced salicylic acids amounts in root exudates of sugar beet under P deficiency and confirmed that it can solubilize soil P. In the present study, we selected five monocarboxylic acids. Among them, GABA (γ-aminobutyric acid) was more interesting, which is a non-protein amino acid that accumulates rapidly in plant tissues in response to biotic and abiotic stress. Over half a century ago, a report in *Science* disclosed that GABA had been identified in potato tubers (Steward et al. 1949). The function of GABA in plants has been suggested including acting as a buffering mechanism in C and N metabolism, cytosolic pH regulation, protection against oxidative stress and defence against herbivorous pests. Lots of reports showed that high levels of GABA accumulate rapidly in plant tissues when exposed to a variety of different stresses, e.g. mechanical damage (Ramputh and Bown 1996), cold (Cholewa et al. 1997), and drought (Thompson et al. 1966).

It is worth mentioning that Carvalhais et al. (2011) detected higher concentrations of GABA in exudates collected from P-deficient plants.

Unfortunately, none of the metabolites of interest has been definitively confirmed to date, one possible reason is that none of the existing databases guarantees a complete coverage of the metabolome, there is still a significant chance of missing the real one. Actually, the process of metabolite identification in untargeted metabolomics studies is a significant bottleneck in MS-focused metabolomics studies. Several problems exist in untargeted metabolomics studies (root exudate sampling as example): (i) samples of root exudates are complex and can contain hundreds or thousands of chemical species; (ii) most of the metabolites are at low concentration (micromolar or lower); (iii) this kind of discovery-studies is highly depend on databases, since it is often not known which metabolites should be occur in a sample. It is possible that the databases contain large lists of the expected metabolites, but they are far from entirety. In current study, further identification of those interesting signals could not be done because the amount of root exudates was not sufficient for the NMR-spectroscopy. Although the current method (HPLC-ESI-MS) alone is inadequate to identify a metabolite, it gives us much information that there are some strong signals in root exudates of sugar beet induced by P deficiency.

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Chapter 4: The effect of light intensity on the root exudation of sugar beet (*Beta vulgaris* L.) under phosphorus deficiency

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4.1 Abstract

Background and aims

P-deficiency induced release of root exudates is influenced by several factors. As light intensity affects photosynthetic carbon (C) fixation, which is the major C source of root exudates, it is necessary to have a thoroughly understanding on the role of light intensity involved in root exudation process. The aim of the present study was to assess the influence of light intensity on the pattern, i.e. quantity and quality of root exudates of sugar beet under P deficiency.

Methods

Sugar beet plants were grown hydroponically with either high light intensity (without shading) or low light intensity (with shading) and a sufficient (500 μM P) or deficient (Rock Phosphate) P supply. The root exudates were collected by dipping method and the composition was analyzed by HPLC-MS (non-targeted metabolic profiling).

Results

Light intensity positively influenced plant biomass production and root/shoot dry weight ratio, particularly under P-deficient conditions. The release of root exudates was stimulated

by both low P supply and increasing light intensity, whereas the effect of light intensity on root exudation was more pronounced with P-deficient plants. A comparison of signals presented in root exudates from different light intensities with a given P supply level showed that less than 40% of signals occurred at both light intensities. Hence, not only the quantity but also the quality of exudates was strongly influenced by light conditions.

Conclusions

These observations suggest that light intensity is involved in root exudation process and this critical role should not be ignored. It is suggested that in the studies concerning the function of root exudates in plants physiology, a special attention should be given to the light conditions.

Keywords: light intensity, phosphorus deficiency, sugar beet, metabolic profiling of root exudate

4.2 Introduction

The root exudation pattern, i.e. the quantity and quality of root exudates is influenced by several plant and environmental factors (Hinsinger 2001; Jones et al. 2004). Plant species as well as genotypes of a given species vary greatly in their root exudation pattern, especially when submitted to various nutrient deficiencies (Gaume et al. 2001; Bais et al. 2006; Lesuffleur et al. 2007). Increasing evidence has been found that root exudates play an important role in processes of element acquisition, e.g. P, Fe, Zn and Al. When plants are suffering Al toxicity, low Fe availability or P limitation, the synthesis and exudation of several organic acid anions are enhanced (Zhang et al. 1997; Jones 1998; Ligaba et al. 2006). As a response to P deficiency, both citrate and malate are the major organic acid anions exuded by white lupin (*Lupinus albus* L.) (Dinkelaker et al. 1989; Neumann and Römheld 1999), while malate is frequently reported as the dominant organic acid in root exudates of maize, oilseed rape and wheat (Zhang et al. 1997; Neumann and Römheld 1999; Carvalhais et al. 2011). The exudation of piscidate by pigeonpea, which is widely cultivated on Indian semi-arid tropical soils, could increase P availability from sparingly soluble Fe-P (Ae et al. 1990). Khorassani et al. (2011) observed the enhanced exudation of citramalic acid and salicylic acid by sugar beet roots under P deficiency. Several studies stated that enhanced exudation of malate is a response of Zn-efficient rice genotypes to Zn deficiency (Hoffland et al. 2006; Rose et al. 2011). Comparison of root exudates profile of maize plants exposed to N, K, P, or Fe deficiency showed that different nutrient status of plant strongly influences composition of root exudates (Carvalhais et al. 2011).

Exudation is often found to follow a diurnal pattern (Gessler et al. 2002; Reichman and Parker 2007; Oburger et al. 2011). It has been reported that the released amounts of root-derived carbon were larger in day-time than in the night (Kuzyakov and Siniakina 2001; Melnitchouck et al. 2005). Many plant metabolites are subjected to similar diurnal patterns of

light intensity (Urbanczyk-Wochniak et al. 2005), which can be linked to the diurnal regulation of photosynthetic carbon metabolism (Geiger and Servaites 1994). The diurnal patterns in phytosiderophores (PS) release have been well-documented under Fe deficiency (Reichman and Parker 2007). Also, Zn-deficient plants release Zn mobilizing root exudates in a distinct diurnal rhythm with a maximum between 2 and 8 h after the onset of light, which is similar to the release of PS by Fe-deficient barley (Zhang et al. 1989). Tharayil and Triebwasser (2010) demonstrated that catechin exudation also exhibited a possible diurnal rhythm, with the highest concentration 6h after exposure to sunlight.

In addition, light intensity also influences the release of root exudates. Light intensity is involved in processes of plant photosynthesis, translocation, and respiration (Hale et al. 1971; Hale and Moore 1979; Cheng et al. 2014). A large proportion of carbon fixed during photosynthesis by higher plants (20-60%) is translocated belowground (Kuzyakov et al. 2000), and up to 30% of photosynthetic carbon can be exuded into rhizosphere (Marschner 2011). Under P deficiency, the amount of carbon exuded by white lupin as citrate and malate can range from 10% to more than 25% of the net fixed carbon (Dinkelaker et al. 1989; Jones 1998). Increasing light intensity greatly enhanced PS release of Zn-deficient bread wheat and a short-term exposure (48 h) of Fe-deficient barley plants from low to high light intensity or in reverse direction caused around 7 times increasing or decreasing release rate of PS (Cakmak et al. 1998). The exudation of catechin by *Centaurea stoebe*, though at very low concentrations, also increased many folds when light levels are high (Tharayil and Triebwasser 2010). For white lupin, Cheng et al. (2014) showed that increasing light intensity enhanced the increase in cluster root formation, citrate exudation, and P uptake capacity induced by P deficiency. Samal (2007) observed that the exudation rate of sugar beet was several times higher when grown in a screen house (natural light conditions) compared to a growth chamber (low light conditions) under both low and high K supply. The production of

secondary metabolites can also be affected by variation in light intensity with the photosynthetic spectrum and also at higher wavelengths (Lavola et al. 1997; Koricheva et al. 1998).

Previous studies on root exudates have mainly been conducted in growth chambers, with light intensity variation in between different authors. For example, a large number of experiments were carried out with relatively low light intensities from 200 to 250 µmol m^{-2} s^{-1} (Zhang et al. 1989; Neumann and Römheld 1999; Erro et al. 2010; Khorassani et al. 2011). Whereas, Zhu et al. (2005) collected root exudates of white lupin grown at 400 µmol m^{-2} s^{-1} and Duffner et al. (2012) performed a rhizobox experiment even at 525 µmol m^{-2} s^{-1}. Knowing that light intensity influences root exudation pattern, at least the amounts of exudates, makes it difficult to compare these results and to assess their relevance for field grown plants. Furthermore, knowledge about the influence of light intensity on the exudate composition (exudate quality) is scarce. Hence, the objective of this work is to determine root exudation pattern of sugar beet grown in a greenhouse under different light regimes and different P supply levels.

4.3 Materials and methods

4.3.1 Plant cultivation

Seeds of sugar beet (cv. FINOLA KWS) were germinated on paper rolls. Three days after germination, seedlings were transferred to a 60 L container and cultivated for 3 days and another 4 days in a one-quarter strength nutrient solution (see below), and a one-half strength solution, respectively. After that, the seedlings were selected for uniformity and transplanted to pots containing 3 L of full strength aerated nutrient solution at a density of four plants per pot. The full strength nutrient solution had the following composition (μM): $Ca(NO_3)_2 \cdot 4H_2O$ (2500), KCl (1000), K_2SO_4 (1000), $MgSO_4 \cdot 7H_2O$ (750), H_3BO_3 (30), $MnSO_4 \cdot H_2O$ (2.5), $ZnSO_4 \cdot 7H_2O$ (1), $CuSO_4 \cdot 5H_2O$ (1), $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ (0.3), and Fe-EDDHA (50). The nutrient solution was renewed every 7 days. The experiment consisted two P levels and two light intensity levels. The phosphate sufficient and phosphate deficient media contained 500 μM $NaH_2PO_4 \cdot 2H_2O$ (P500) and 3 g Rock phosphate (Dolo phos) kept in teabags (RP), respectively. Previous studies had shown that RP can provide a low (0.1-0.4 μM) but relatively constant P concentration despite P uptake by plants. This 'buffered' situation is more comparable to soil conditions (Raghothama 1999) than just offering a low P concentration which is rapidly depleted close to nil by plant uptake. Plants were placed during month in a greenhouse with additional artificial light for 10 hours per day. Hence, light intensity was not constant but reached about 400 μmol m^{-2} s^{-1} during daytime (high light intensity, HL). The low light intensity treatment (LL) plants were shaded by green plastic screens, which reduced light intensity to about 100 μmol m^{-2} s^{-1}. The P treatments started at DAT 7 (day after transplanting) and the light intensity treatments at DAT 21, respectively. Each treatment had six replicates.

4.3.2 Collection of root exudates

Root exudates were collected at 35 and 42 DAT by dipping method (Neumann and Römheld 2007). Prior to collection, the plants were transferred into 500 µM Ca $(NO_3)_2$ solution for 12h. Afterwards, the whole root system was carefully rinsed three times with deionized water to remove ions from the root surface, and then submerged in trap solution (deionized water) for two hours (the volume (100-400 ml) of the trap solution depends on the size of root system). Control pots were treated similarly without sugar beet plants. The collection of root exudates always began at 10:00 am after plants had been exposed to light for 2h to avoid possible variation of exudation release due to a diurnal rhythm (Watt and Evans 1999). Immediately after collection, each sample of exudate solution was filtered through filter paper (MN615 1/4 Ø90mm, MACHEREY-NAGEL GmbH, Düren, Germany) and frozen in liquid nitrogen to minimize microbial degradation. Frozen solutions were lyophilized and dissolved by methanol: water (50:50 v/v) for 1h, after that evaporated to dryness by use of Rotational vacuum concentrator (RVC 2-25 CD plus; Christ GmbH, Germany). The sample weight was recorded, and stored at -20°C until HPLC-MS analysis.

4.3.3 Metabolic profiling by HPLC-MS

The dried residue was re-dissolved in 1 mL methanol: water (50:50 v/v) for 1h. For removing insoluble compounds, samples were vigorously shaken and centrifuged first at 4800 rpm for 10 min, then the supernatant were centrifuged at 14000 rpm for 10 min. Thereafter, 10 µL of the supernatant of each sample were injected for analysis by HPLC-MS.

Metabolite separation was carried out by high performance liquid chromatography (Prostar 210, Varian, Darmstadt, Germany) onto a reversed-phase column Polaris C18-Ether (100×2mm, 3 µm particle size; Varian) coupled with a C18 security guard cartridge maintained at 40°C. The mobile phase consisted of (A) bi-distilled water: acetonitrile (95:5) and (B) methanol, both containing 7 mM acetic acid. The binary gradient was as follows: 0.0-

1.0 min 10% B; 1.0-30.0 min from 10% to 98% B; 30-50 min 98% B; 50-51 min from 98% to 10% B; 51-70 min 10% B. The flow rate was 0.2 mL min^{-1}.

HPLC was coupled with electrospray ionization and mass spectrometry detection using an ion trap 500MS (Varian, Darmstadt, Germany). Ionization was done in positive and negative mode with the following parameters (negative/positive): needle voltage -4500 V/+5000 V, shield voltage -600 V/+600 V, capillary voltage -/+80 V, drying gas (nitrogen) 15 psi at 350°C, and nebulizing gas (air/nitrogen) 25 psi. In positive mode, ions with a mass-to-charge ratio (m/z) 50 to 1000 were collected in a single run, while in negative mode ranges of m/z 50 to 400 and m/z 400 to 1000 were scanned separately. The scan speed was 15000 Da/s. For data acquisition, MS workstation/MS Data Review 6.9.1 (Varian) was used.

Mass spectrometric data analysis was processed with the Component Detection Algorithm (CODA, Windig et al., 1996) including smoothing, baseline correction, and peak picking using ACD/MS Manager Version 12.0 (Advanced Chemistry Development, Toronto, Canada). The CODA algorithm calculates the similarity index "mass chromatogram quality" between the length-scaled and the smoothed standardized mass chromatogram (Windig et al. 1996). MCQ values above 0.8 with a smoothing window width of 3 scans were used. Baseline correction that means to remove high level background from the data set was applied to mass chromatograms with box half width of 10 scans and noise factor of 1. Peak tables were created that contained the m/z value, retention time (Rt), peak area in counts (negative: >1000; positive: >200000), MCQ value (>0.8) and the S/N value (>100). Normalization and peak alignment across all samples were carried out using user-written Perl script (Karlovsky, unpublished). Signals occurring in controls were excluded from further data analysis and only signals which occurred in at least 4 of 6 replicates were taken into account.

4.3.4 Plant harvests

The plants were harvested after the last collection of root exudates. All plant tissues were dried at 60-65°C for 24h and then at 105 °C until a constant weight, and dry weights of plant parts were recorded. The shoot P concentration was determined: 0.3 g of plant material was digested with 4 mL HNO_3 (65%) and 2 mL H_2O_2 in microwave oven. Phosphorus was determined by the molybdate-vanadate method (Scheffer and Pajenkamp 1952).

4.3.5 Root length imaging

For calculating exudation rates, a non-destructive screening procedure was developed to estimate total root length (RL) without destroying the plant. For this, 36 pots of sugar beet plant were grown together with those of the main experiment. Plants were photographed at least twice a week from a defined distance and angle in front of a black background using a digital single-lens reflex camera (Canon EOS 600D, Canon Inc., Japan). The area of pixels in each picture was calculated using Image J software (Rasband 1997). After the imaging procedure, plants of two pots were harvested and RL per pot was determined by the line intersection method of Tennant (1975). Measured RL of the harvested pots was plotted against the respective area of green pixels and a power trendline was fitted. The parameters of the power were used for calculating RL per pot from plant images only. The equation is

$$L_R = 0.2869*(A/1000)^{1.8507}$$

Where, L_R is the RL per pot, A is the area from ImageJ, r^2= 0.90

4.3.6 Determination of photosynthetic efficiency

Rate of CO_2 assimilation (A) was measured on the youngest fully expanded leaves by using GFS-3000 (Hein Walz GmbH, Effeltrich, Germany) photosynthesis systems during 10:00 and 16:00 after each collection of root exudates. Measured chamber was set as follows:

photosynthetic photon flux density (PPFD) of 1000 μmol m^{-2} s^{-1}, CO_2 concentration at 380 ppm, 55% relative humidity and a temperature of 22°C.

4.3.7 Statistical analysis

Statistical analysis was carried out with SPSS analytical software (SPSS Inc., Chicago, IL, USA; version 19). Difference between treatments was evaluated for significance by LSD multiple range tests ($p < 0.05$). Data are presented as mean ± S.E.

4.4 Results

4.4.1 Plant growth

Shoot biomass production was affected by both phosphorus supply and light intensity. Plants grown under low light intensity produced less shoot dry matter, and this negative effect was even more pronounced for P-deficient plants (Table 4.1). The same tendency was found in the root biomass production however the effect of P supply on the root dry weight was lower than on shoot dry weight whereas that of light intensity was markedly larger (data not shown). Shoot P concentration was decreased in plants supplied with RP. Reuter et al. (1997) defined the threshold for P deficiency in shoots as being 0.35% (3.5 mg g^{-1}), i.e. RP-plants were P deficient whereas P500-plants were well supplied. Increasing light intensity decreased shoot P concentration in plants, but the effect was only significant in P-sufficient plants. This is most probably due to a dilution effect because total P acquisition was significantly increased at high light intensity when plants were grown at adequate P supply (data not shown).

Table 4.1. Effect of light intensity and P supply on the shoot dry weight (SDW), root/shoot of dry weight ratio, shoot P concentration and root length by calculation. Plants were grown in nutrient solution at two levels of P supply (P500 and RP) and two levels of light intensity (high and low light), and harvested after 42 d transplanting (DAT 42). Results are means ± SE (n=6). Results with different letters are significantly different ($p < 0.05$).

Light intensity	Phosphorus treatment	Shoot dry weight (g pot^{-1})	Root/shoot dry weight	Shoot P concentration (%)	Root length (m pot^{-1})	
					DAT35	DAT42
High	P500	24.3 ± 0.2 a	0.087 ± 0.002 c	0.64 ± 0.02 b	2282 ± 98 a	3393 ± 80 a
	RP	10.6 ± 0.4 c	0.206 ± 0.009 a	0.18 ± 0.02 c	1152 ± 76 b	2287 ± 72 b
Low	P500	17.7 ± 0.4 b	0.073 ± 0.002 c	0.74 ± 0.02 a	1252 ± 64 b	1973 ± 55 c
	RP	5.9 ± 0.4 d	0.148 ± 0.011 b	0.21 ± 0.01 c	865 ± 67 c	1241 ± 49 d

The root/shoot dry weight ratio was greater in P-deficient plants. Also, high light intensity increased root/shoot ratio, but this effect was only significant for P-deficient plants. Total root length was decreased by low P supply as well as low light intensity (Table 4.1)

4.4.2 Photosynthetic efficiency

Assimilation did not differ between plants supplied with P500 and RP (Figure 4.1). However, the assimilation increased with increasing light intensity, but this effect was only significant in the earlier stage (DAT 35).

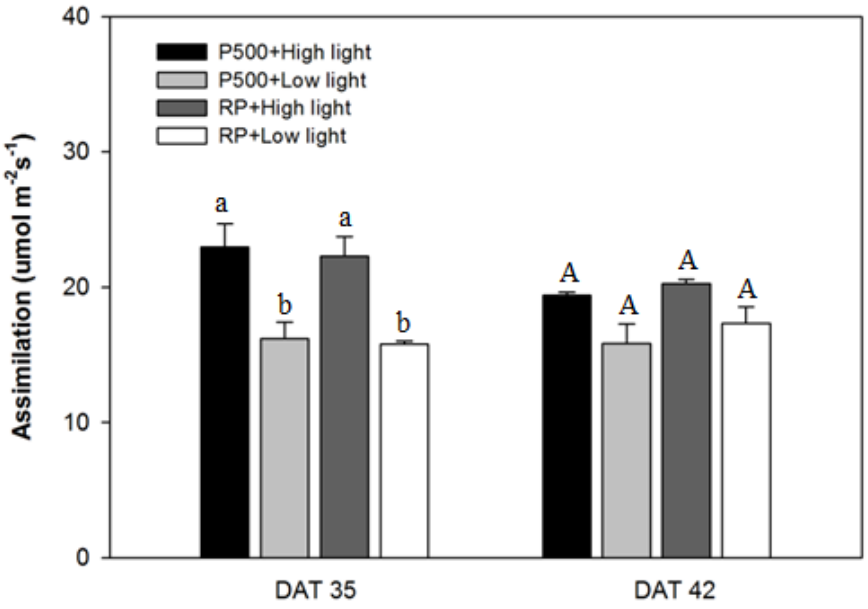

Figure 4.1. Effect of P supply and light intensity on photosynthetic efficiency. Plants were grown in nutrient solution at two levels of P supply (P500 and RP) and two levels of light intensity (high and low light intensity). Bars represent means ± SE (n=6). Data with different letters are significantly different ($p < 0.05$).

4.4.3 Root exudation release

Grown under P deficiency, sugar beet released more root exudates than P-sufficient plants (Figure 4.2). The amount of root exudates increased also with light intensity (Figure 4.2). Taking both effects together, P deficiency results in 2-3 times higher release of exudates than sufficiency only at high light conditions. At low light conditions, deficient plants also tried to increase exudation but with less success. With extending cultivation period, the amount of root exudates decreased slightly, except for P-deficient plants grown at high light intensity.

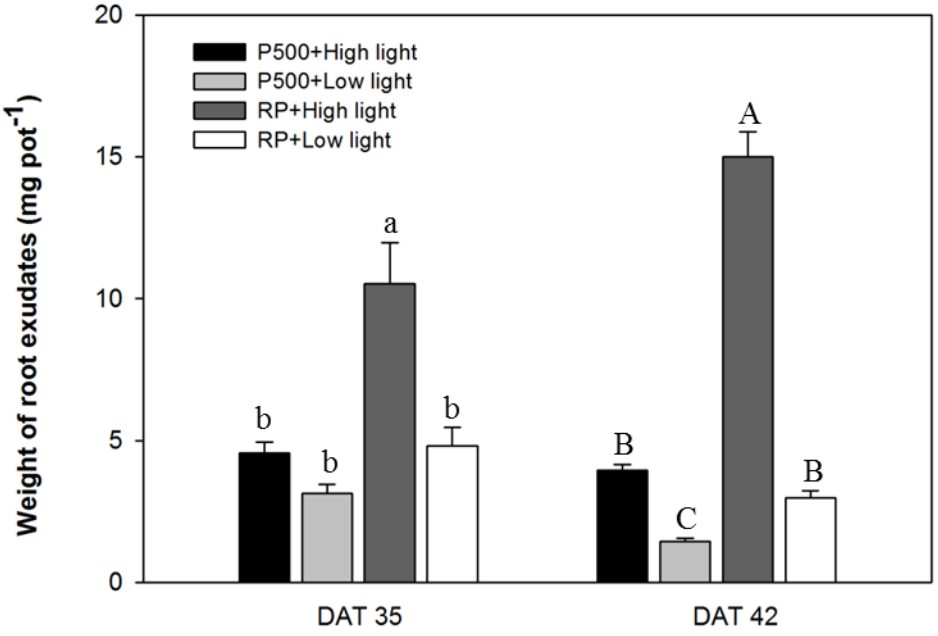

Figure 4.2. Effect of P supply and light intensity on weight of root exudates. Plants were grown in nutrient solution at two levels of P supply (P500 and RP) and two levels of light intensity (high and low light intensity). Bars represent means ± SE (n=6). Data with different letters are significantly different ($p < 0.05$).

Since the amount of compounds exuded is directly depending on the root-size, the exudation rate is a better measure for describing physiological activity. Irrespective of light intensity, exudation rate of P-deficient plants was markedly higher than that of P-sufficient plants, which is a typical response to low P availability (Figure 4.3). Roots of plants with a low P

supply had 2-5 times higher exudation rate compared to those with an adequate P supply. Like for the total amount of root exudates, high light intensity increased exudation rate at P deficiency whereas at a sufficient supply influence of light intensity was much lower. The slightly lower amount of root exudates at low light compared to high light in the P500 treatment (Figure 4.2) was rather due to the lower root length than to a lower exudation rate. There was a decline of exudation rate at DAT 42.

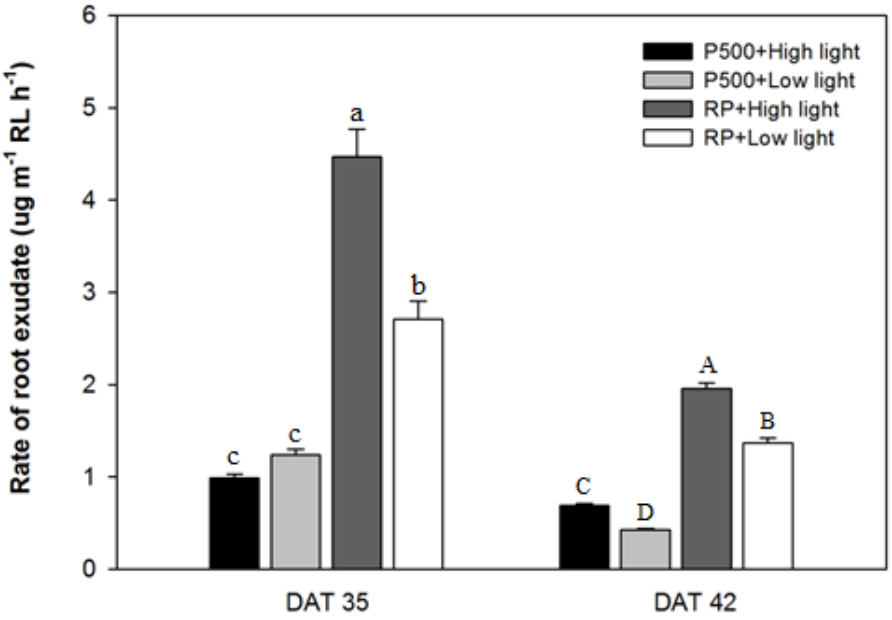

Figure 4.3. Effect of P supply and light intensity on rate of root exudation. Plants were grown in nutrient solution at two levels of P supply (P500 and RP) and two levels of light intensity (high and low light). Bars represent means ± SE (n=6). Data with different letters are significantly different ($p < 0.05$).

4.4.4 Metabolic profiling of root exudates

To assess the impact of light intensity on quality of root exudates of sugar beet under P deficiency, a non-targeted metabolic profiling was conducted by HPLC-ESI-MS. The signals taken into account should have been present in at least 4 of 6 replicates of root exudate sample. P deficiency increased the number of signals at 35 DAT indicating a larger number of different compounds in the exudates (Figure 4.4). This was less pronounced at 42 DAT. Light

intensity also greatly affected the composition of root exudates. Regardless of P supply, metabolic profile varied widely between high and low light intensities. At DAT 35, only 29% and 35% of the signals occurred at both light treatments for high P and low P, respectively. This indicates that less than 40% of the different compounds were exuded regardless of light conditions, whereas the more than 60% was exuded either under low or high light conditions. Hence, quality pattern of root exudates was strongly influenced by light intensity. At DAT 42, the total number of signals did not differ much between the P treatments, however the number of signals occurring at both light intensities (high P: 29%; low P: 27%) differ, i.e. the pattern of exudates from P deficient plants was changed compared to P sufficient plants and was again influenced by light intensity.

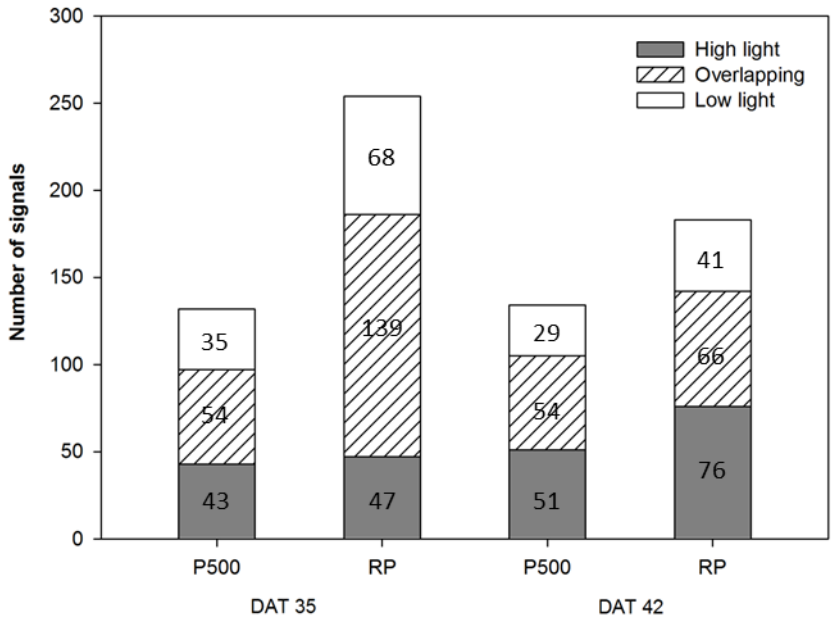

Figure 4.4. Effect of P supply and light intensity on composition of root exudate. Plants were grown in nutrient solution at two levels of P supply (P500 and RP) and two levels of light intensity (high and low light). The digits in shaded area of each column represent the number of signal detected only at high light intensity; the digits in slash area represent signals at both light intensity conditions; the digits in blank area represent signals at low light intensity.

4.5 Discussion

4.5.1 Influence of P supply on exudation pattern

A typical reaction of P-deficient plants is a relatively higher allocation of assimilates into the root system compared to well-supplied plants. This leads to a reduced shoot growth and a higher root/shoot dry weight ratio (Hermans et al. 2006; Hammond and White 2008). The increased import of carbohydrates into the root system is supposed to be used either for morphological reactions, e.g. increasing root growth, enhancing lateral root formation, increasing length and number of root hairs or for physiological reaction such as an increased exudation of organic compounds into the soil which might influence P availability (Vance et al. 2003). Plant species differ in their P efficiency mechanisms, e.g. wheat increased root/shoot ratio under P deficiency on basis of an already large root system whereas sugar beet at the same growing condition did not change root/shoot ratio much but was able to increase P availability in the soil chemically (Bhadoria et al. 2002). The results of this study (Table 4.1, Figure 4.2 and 4.3) are in accordance to this. Root length of sugar beet was reduced due to P deficiency but root/shoot ratio was about doubled indicating a relatively higher allocation of assimilates into the root system. However, exudation rate was even increased by factors of two to four leading to a much larger amount of exudates released especially under high light conditions where assimilations was not (or less) restricted.

It is well-documented that low P supply triggers many P-efficient plant species to increase exudation rate, e.g. white lupin, oilseed rape, sugar beet (Hoffland 1992; Beissner and Römer 1998; Neumann and Römheld 1999; Khorassani et al. 2011). In the present study, exudation rate of well-supplied plants was about 1 µg m^{-1} h^{-1} and P deficiency increased this to 1.5 to 4.5 µg m^{-1} h^{-1}, at all exudation rates decreased with plant age. Under comparable growing and experimental conditions, Khorassani et al. (2011) determined slightly higher exudation rates

in the control but also a stronger increase at P deficiency (about 5-5.5 folds) and no changes with plant age. Besides the fact that the experimental conditions were comparable but not identical, these different results might be due to different conditions of P deficiency. Khorassani et al. (2011) followed a "classical" setup to establish P deficiency in solution experiment, i.e. they offered a low concentration (2 µM) at the beginning of a nutrient change cycle without further adding of P. Due to plant uptake P concentration in solution (C_P) can be rapidly decreased close to nil or the minimum concentration of uptake (C_{Lmin}) for uptake (this took about 1 hours in our experiments, unpublished but paper submitted). The rock phosphate (RP) treatment in this study led to a relatively constant P concentration in solution of about 0.4 µM due to the onset of steady state equilibrium between plant uptake and solving of RP, i.e. the deficiency situation is less severe. This might influence exudation pattern as was shown by Keerthisinghe et al. (1998) who observed that exudation rate of proteoid roots of white lupin was only significantly increased for plants grown without P in solution, even at 1 µM P citrate exudation rate was not significantly different to a well-supplied control. However, a constant low P concentration is rather comparable to soil conditions than a sequential change between a low and a nil concentration.

4.5.2 Influence of light intensity on exudation pattern

At a high P level, there was no influence of light intensity on exudation rate at 32 DAT and only a limited influence for older plants (Figure 4.3). This situation was changed under P deficiency condition: exudation rate was much higher at both light intensity conditions, but even more increased at high light conditions. This indicates a shortage of assimilates for exudation in the shaded plants, partly due to smaller shoot size with less leaf area, partly due to a lower assimilation rate per unit leaf area (Figure 4.1). Cheng et al. (2014) measured the sucrose transport into the root system of white lupin and found similar result: root sucrose

concentration was increased by P deficiency and this was more expressed under high light compared to low light conditions.

Exudation rate decreased also with plant age. It is well-documented that exudation rate of P-deficient plant roots starts with the onset of P deficiency, reaches a maximum level, and declines as P deficiency becomes increasingly severe with plant age (Zhang et al. 1997; Aulakh et al. 2001; Shen et al. 2002). Similar result has been obtained of the release rate of phytosiderophore response to Fe deficiency (Cakmak et al. 1994). In contrast, Khorassani et al. (2011) reported no influence of plant age on exudation rate of sugar beet in the range of 14-42 DAT. However, they grew the plants constantly under low light conditions (about 200 µmol m^{-2} s^{-1}), whereas in our experiment low light treatment started at 21 DAT. The reduced exudation rate with plant age, averaged over the whole root system, might also be an artifact. Exudation rate of root tips and young roots is usually higher than old roots. An older root system has a higher percentage of old roots, i.e. without changes in the exudation activity of young roots and root tips the calculated average exudation rate for the whole root system is lower.

Light intensity also influenced root size, leading to an increased root length and root/shoot ratio with light intensity. Low light reduces photosynthetic activity and less assimilation are available for root growth, this usually shifts growth activity towards the shoot (Buttery and Stone 1988; Hébert et al. 2001; Nagel et al. 2006). The total amount or weight of released root exudates (Figure 4.2) depends on both exudation rate and root system size. The general tendency is the same as exudation rates, but the increase in exudates weight due to light intensity is given now for both P supply levels. A similar influence of light on root exudates has been described by Cakmak et al. (1998), Tharayil and Triebwasser (2010) or Cheng et al. (2014).

Of more interest than the influence of light intensity on the amount of exudates is probably the influence on exudate composition. Most of previous studies paid attention to the effect of light on specific compounds like citrate, phytosiderophore and catechin (Cheng et al. 2014; Cakmak et al. 1998; Chen et al. 2012). Oburger et al. (2014) could even show a diurnal rhythm of phytosiderophore exudation with a maximum release rate around noon. But to our knowledge, the influence of light on the total composition of root exudates is not investigated yet. As a first attempt, Figure 4.4 showed the result of a non-targeted metabolic profiling, i.e. differences in the signal pattern of different exudates as measured in HPLC-MS with positive and negative ionization without defining the signal compound behind the signal. Number of signals roughly reflects the number of different compounds in the exudate and was between 100 and 200. Especially in the first harvest, P deficiency increased the number of exuded compounds from about 100 to nearly 200 under high light conditions. Khorassani et al. (2011) also found large differences in the composition of root exudates of P-deficient and well-supplied sugar beet plants. They reported about 65 signals that were at least 5 times higher under deficiency compared to the control. Besides P, also light intensity had strong influences on exudate quality. Only 27 to 35% of signals occurred under both light regimes. Depending on plant age and P supply, about 43 to 76 signals occurred exclusively under high light, that are about 25 to 54% of all compounds in the high-light exudate. On the other hand, 29 to 68 signals (33 to 39%) occurred only in the low-light exudates.

These results indicate that light intensity and assimilation rate not only influences carbohydrate transport into the root system, but also changes root physiology to a large extent. Cheng et al. (2014) hypothesized that a high sucrose concentration in the root is not only a source for carbon but also a signal which influence physiological as well as morphological reactions. They could show that cluster root development of white lupin was increased at high light even at sufficient P supply. Furthermore, they could show that a high

sucrose concentration in the roots increased the expression of *LaPEPC3*, which might be involved in organic acid synthesis (Peñaloza et al. 2005). This triggered citrate exudation at high light even at a high P supply, whereas at low light and high P citrate exudation was nil. These results make clear that light conditions are an important factor when root exudation pattern are determined in context with P shortage and P efficiency mechanisms. Different light intensity might also be one reason for the difficulties to transfer results obtained in low light growth chamber to the field.

4.5.3 Conclusion

This study revealed a significant impact of light intensity on root exudation patterns of sugar beet, i.e. the amount of exudates but particularly on exudate composition of exudate. Usually, root exudates collected in greenhouses or growth chambers, where ambient light is often substantially lower than in the field condition, which makes the transfer of results difficult. Therefore, we suggest that light intensity should get more attention when conducting experiments related to root exudation.

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Chapter 5 General discussion

Low phosphorus (P) availability is a principal constraint to crop production worldwide. However, this low P availability is not caused by low total P content but by easily and strongly adsorption to soil organic matter or metal oxides, and therefore the P concentration in soil solution is very low, mostly below 1μM. In soil, the major limiting steps in P acquisition by plants are the mobilization and diffusion of P to roots (Barber et al. 1963; Ernst et al. 1989). Organic compounds, particularly organic acids released from plant root system have been hypothesized to be involved in the mobilization and solubilization of those poorly soluble P (Hinsinger 2001; Ryan et al. 2003). Actually, the ecological significance of organic acids in P mobilization remains controversial, as the detected concentration of organic acids in soil solution is too low to obtain successful mobilization of soil P. One exception is in *Lupinus albus* and all the members of the Proteaceae family where there is clear evidence that root exuded organic acids are capable of mobilizing P. For example, Gerke et al. (1994) reported that the concentration of soluble phosphate (Pi) in the rhizosphere of cluster roots of white lupin grown was twice than that in the bulk soil despite depletion due to Pi uptake by the plant, and this was attributed to citrate exudation by cluster roots. As we known, the concentration of organic acids in the rhizosphere are determined by exudation and reabsorption by plant, desorption/adsorption to soil, and microbial degradation (Jones and Farrar 1999). In white lupin, organic acid anions are released from those specialized root structures (cluster roots) in very large concentration, i.e. release rate of citric acid in the range of 1.1-2.4 μmol g^{-1} FW h^{-1} has been reported for cluster roots of white lupin (Keerthisinghe et al. 1998; Neumann et al. 1999; Peñaloza et al. 2005). In addition, combining the structure characteristic of cluster roots (closely arranged) and their strongly rhizosphere acidify ability resulting in a suppression of microbial degradation, the released citric acid can easily

accumulate to a high concentration (e.g. 40-85 μmol g soil^{-1}; Dinkelaker et al. 1989; Gerke et al. 1994; Li et al. 1997). This concentration is sufficient to release P from sparingly soluble forms (Fe, Al and Ca-P; Gerke et al. 1994). However, concentrations of organic acids have been detected in the bulk soil solution range between 0-0.1 mM and less than 1 mM in the rhizosphere of most non-cluster root plants but are estimated to exceed 50 mM in the rhizosphere of cluster-root plants (Jones et al. 1996a; Raghothama 1999). Gerke et al. (2000) presented that P mobilization was negligible or small below an oxalate or citrate concentration of 10 μmol g^{-1} soil (corresponds to an initial carboxylate solution concentration of 33 mM). In addition, when presented at low concentration organic acids are rapidly mineralized by the soil microbial community with a mean residence time in soil between 0.5 and 12 h (Jones and Darrah 1994; Van Hees et al. 2003). In contrast, when releasing high concentrations of organic acids into the soil it is hypothesized that saturation of microbial transport systems occurs at which point they can be expected to have a long residence time in soil and be involved in P mobilization (Jones and Darrah 1994; van Hees et al. 2002). Further, rapid sorption to the solid phase in soil may also significantly lower the concentration (Ryan et al. 2003). However, certain plant species have been reported indeed increased P availability by releasing root exudates. Rape (*Brassica napus* L.) plants efficiently utilize rock phosphate by releasing organic acid during P deficiency (Hoffland et al. 1992). P-efficient common bean genotypes exuded more organic acids response to P deficiency in comparison with P-inefficient one (Shen et al. 2002). The question arises as to why organic acids occurred at quite low concentrations but still can mobilize sparingly soluble P. Several explanations may account for this question. Firstly, sampling technique limits the accurate quantification of organic acids in soil. In many cases, exudation is not uniformly distributed along plant roots and hot spots of exudation can exist in different root zones (e.g. the apical root zone). As the diffusion coefficients of most organic acids (e.g.

citrate, oxalate) are extremely low, the size of these hot spots may be only a few μm in diameter if release occurs from the apical part of root hair (Darrah 1991). Thus, Jones et al. (2003) hypothesized that using current extraction technique, even though soil solution microsampling techniques (e.g. micro suction cups; Göttlein et al. 1996) have already been developed at the millimeter scale, soil solution organic acid concentrations still vastly underestimated by maybe up to several orders of magnitude within soil microsites.

A second possible explanation is that most previous studies working on organic acids related to P mobilization only focused on those organic acids which are commonly identified in root exudates, i.e. citric, malic and oxalic acid (Rengel 2002; Aoki et al. 2012). In recent years, more studies detected uncommon organic acids in root exudate, which might mobilize P more efficiently. For instance, piscidic acid exuded from the roots of pigeonpea could facilitate P release from Fe-P by chelating Fe (Ae et al. 1990); Shen et al. (2001) showed that the exudation of pentanedioic acid is a specific response to P deficiency in elephant grass; malonate was found the highest amount in the root exudates of soybean (Tang et al. 2009). In addition, besides organic acids, phenolic detected in root exudates can also solubilize P from unavailable sources (e.g. ferric phosphate) for uptake by plants (Masaoka et al. 1993; Tomasi et al. 2008).

The P-mobilization efficiency of organic acids was determined in following. Firstly, organic acid anions can solubilize P from mineral surfaces mostly via a ligand exchange reaction. Bolan et al. (1994) indicated that ability of organic acids to form metal complexes are highly correlated with the amount of OH and COOH groups and their relative positions on the main carbon chain. Generally, the sorption affinity of organic acid is tricarboxylic acid, dicarboxylic acid, monocarboxylic acid in descending order. However, Ström et al. (2002) compared P mobilization ability of citrate and oxalate and observed that 1 mM oxalate (dicarboxylate) addition to the rhizosphere significantly increased P uptake, while citrate

(tricarboxylate) had no significant impact on plant P uptake even at high concentration (10 mM). In addition, different organic acids were proposed to use different P mobilization modes. For example, oxalate may primarily release P held in Ca-P minerals through the formation and precipitation of Ca-oxalate. In contrast, citrate which has a poor affinity for Ca^{2+}, but a greater affinity for Fe^{3+} and Al^{3+}, may release P predominantly held in Fe-P and Al-P minerals (Ström et al. 2001). Thus, the P-mobilization efficiency of organic acids also may be very context specific with both soil types and the individual organic acid controlling the amount of P released into soil solution (Oburger et al. 2011). Secondly, under non-sterile conditions, the concentration of organic acids in soil solution was also influenced by microbial uptake and biodegradation. Individual organic acids degrade differently by microbial in rhizosphere, i.e. some organic acids are less preferential. Jones et al. (1996b) found that malate degradation in acid soils could be rapid (half-life approximately 1.7 h), which is much faster than citrate (Shane et al. 2008). When carboxylates were precipitated with metals (i.e. Ca-oxalate) or fixed to the soil's exchange phase, the decomposition has become extremely slow (Ström et al. 1994; Jones et al. 2003). Moreover, organic acids are typically studied is isolatedly, however, roots simultaneously exuded organic acids and other substances in a mixture, and it has been speculated that their combined impact on rhizosphere processes may be quite different. For example, some exudates may play secondary roles, e.g. inhibitors of microbial activity, blockage of sorption sites, i.e. suppress re-adsorption and precipitation of inorganic P, which might enhance the longevity and nutrient-mobilization capacity of others. Li and Copeland (2000) reported that malonate is an inhibitor of succinate dehydrogenase, and therefore toxic for microbial. As such, the high concentration of toxic malonate might protect other organic compounds, which might contribute to P mobilization, from the degradation in the rhizosphere (Oburger et al. 2009).

Sugar beet (*Beta vulgaris*) were shown to be a P-efficient species due to its ability to mobilize P chemically in the rhizosphere (Bhadoria et al. 2002; Khorassani et al. 2011). Beissner and Römer (1998) observed an increased exudation of sugar beet in low P soils and Gerke et al. (2000) reported that the calculated influx of P mobilized by oxalate is 1.5 to 6 times higher than the inflow without mobilization. Khorassani et al. (2011) detected salicylic acid and citramalic acid in root exudates of sugar beet and noted both of them can increase P availability. In addition, Claassen (1990) found that P deficiency significant decreased sugar beet plant development at early growing stage and after late June or early July stage there was no much different between P deficient and well-supplied plants. Based on those literatures, we suppose that chemical mobilization of P by organic compounds can strongly improve P acquisition of sugar beet plants grown in low P availability soil and there might be a shift of root exudation pattern with the development of sugar beet. Therefore, it is necessary to have a comprehensive knowledge of the exudation pattern of sugar beet, i.e. on both qualitative and quantitative aspects, in response to P deficiency. Furthermore, we expect to identify organic compounds that are natural products, but have not been considered in relation to the P mobilization in root exudate.

5.1 Effect of P supply on root exudation pattern

P deficiency resulted in decreased dry matter production, while assimilation allocation to root system increased, which led to an increase of root/shoot dry weight ratio (Table 3.1). Generally, this response appeared to be related to an increased investment of plant resources in development of a more extensive root system (e.g. wheat), however, in this case, the root length reduced under P deficiency. Therefore we proposed this increased investment of assimilation was applied to enhance release of root exudate (Figure 3.2).

Low P supply triggered enhancement in root exudation of sugar beet grown in nutrient solution, whether expressed on the entire root system or root length basis. Growing plants with low P supply resulted in a higher rate of root exudation, i.e. 2 to 5 fold greater than in well-supplied treatment, and the root exudation rate decreased with plant age (Figure 3.2 and 3.3; Hoffland 1992; Neumann and Römheld 1999).

In an earlier study, Khorassani et al. (2011) also reported that sugar beet plants increased root exudation rate in response to P deficiency, which was in agreement with present study. In contrast with the present results, the exudation rates measured in this former study were constant with plant age and at least 4 fold greater compared to ours. Dinkelaker et al. (1995) showed that the amount of citrate released by cluster roots of white lupin changed with plant age and severity of P deficiency. Wouterlood et al. (2004) reported similar results that plant age affected total carboxylate concentration in the rhizosphere. At a young age, plant may grow faster, and not leave much carbon to release as exudates. Actually, besides DAT 35 and 42 (Figure 3.2 and 3.3), we also collected root exudate at DAT 27. Compared with DAT 27, sugar beet plants released more root exudates at DAT 35, with the severity of P deficiency, the exudation rate decreased at DAT 42 (Figure 5.1). In addition to the discrepancy in quantitative characteristic, the compounds they identified contributed to chemical P mobilization, i.e. salicylic and citramalic acid corresponding to m/z 137 and 147 (in negative ESI mode) respectively were absent in present study. However, both of studies found large differences in the metabolite profiles of root exudates collected from different plant stages as response to P deficiency.

Reasons for such discrepancies between previous study and ours are unknown but probably related to the following. Firstly, different cultivars used in various studies might display different responses in root exudation. Secondly, the severity of P deficiency differed between the studies. Khorassani et al. (2011) used the classical way to establish P deficiency in

hydroponic experiment, i.e. they offered inorganic phosphorus at a low concentration (2 µM) at the beginning of a nutrient change cycle without further adding of P. On the contrary, the rock phosphate (RP) treatment used in this study provided a relatively constant P concentration. Root exudation process is regulated by the internal shoot P status (Shane et al. 2008) and also external P supply (Shen et al. 2005; Palomo et al. 2006). In chapter 2, our results clearly showed that the plant development (Figure 2.2) reflects the differences caused by those two contrasting experimental systems, i.e. with "classical P deficiency" treatment, plants were severely P deficient and development was seriously suppressed, while with "teabag method" treatment, the deficiency situation is less severe. Plants grown under "classical P deficiency" condition, the P concentration in solution (C_P) can rapidly decreased close to nil or the minimum concentration of uptake (C_{Lmin}). Consequently, P-deficient plants were not supplied with any P at all for days or weeks. Plants grown in switch on/off P supply situation (2 µM) released more root exudates compared to the constant low P external supply (RP) (Figure 5.2). Several studies reported that carboxylate exudation from certain plant species root system (e.g. white lupin, rape and chickpea; Hoffland et al. 1992; Keerthisinghe et al. 1998; Neumann and Römheld 1999) increased when P was absent in the nutrient solution, even at 1 µM P citrate exudation rate was not significantly different from a well-supplied control. Moreover, chronic P deficiency in plants implicitly causes a significant loss in membrane integrity, which propose that enhanced organic acid exudation under severe P deficiency simply reflects an increase in the permeability of the plasma membrane. In addition, the experimental light conditions differed. In the former study, sugar beet plant grown in climate chamber, where the light intensity is 41 W m^{-2} PAR (approximately 200 µmol m^{-2} s^{-1}), while experiment in the present study was conducted in green house where light intensity is much higher, i.e. 400 µmol m^{-2} s^{-1}. Besides light intensity, the exudation rate was also influenced by the duration of photoperiod, i.e. the root exudation rate increased with

the elongation of photoperiod (Pramanik et al. 2000). In former study, plants grown with 16 h photoperiod, while in the present study the photoperiod was only 10 h. Despite the rate of exudation increased at higher light intensity (chapter 4), the shorter photoperiod might be the reason to explain why our root exudation rate is 4 times lower than Khorassani et al. (2011) measured.

5.2 Effect of light intensity on root exudation pattern

As photosynthetic carbon is the major source of root exudate, any alternation in the environmental factors affecting photo-assimilation may be expected to affect exudate release (Hodge et al. 1997; Kuzyakov et al. 2000; Marschner 2011). Samal (2007) observed that the exudation rate of wheat and sugar beet plants was several times, ranging from 2 to 100, higher when plants grown in screen house compared to growth chamber, depending on plant age and K supply level. The major difference between the screen house and the growth chamber conditions was light intensity, i.e. the light intensity in growth chamber is around 200 µmol m^{-2} s^{-1}, which is typical for the cultivation of plants in the laboratory, but it is much lower than natural light intensity found in screen house or field conditions. Actually, light intensity has rarely been considered as an important influencing factor for root exudation process. We summarized the literatures and found that large light intensity variation exists among different investigations. For example, a large number of experiments on root exudate were carried out in growth chamber with relatively low light intensity from 200 to 250 µmol m^{-2} s^{-1} (Zhang et al. 1989; Neumann and Römheld 1999; Erro et al. 2010; Khorassani et al. 2011). Whereas, Zhu et al. (2005) collected root exudates of white lupin grown at 400 µmol m^{-2} s^{-1} and Duffner et al. (2012) performed a rhizobox experiment even at 525 µmol m^{-2} s^{-1}. Based on several reports (Cakmak et al. 1998; Cheng et al. 2014), we knew that light intensity influences the amounts of exudates, but the knowledge about the impact of light

intensity on the exudate composition (exudate quality) is scarce. Hence, another objective of this PhD study is to estimate to what extent light intensity affected root exudation pattern, particularly in exudate composition aspect, and this information is important to our basic understanding of the role of root exudates involved in P mobilization. Therefore, we conducted a greenhouse experiment in which we grew sugar beet under different light regime and different P supply levels.

At high P level, the root exudation rate was low and independent of light intensity, especially at DAT 35 (Figure 4.3). However, under P deficiency conditions, the exudation rate was much higher at both light intensity levels, but even more increased at high light conditions. This indicates the release of root exudate as response to nutrient deficiency was highly dependent on light intensity, confirming former observations, i.e. the difference of exudation rates measured in screen house and growth chamber, which was speculated to be caused by light intensity, was much severer under K deficiency (Samal 2007). As light intensity influenced photosynthetic carbon (C) fixation and the supply of C to root, high light intensity is possibly required for providing more assimilation to meet the enhancement of exudation caused by P deficiency. A shortage of assimilation for exudation in the shaded plants, partly due to smaller shoot size with less leaf number and area (data not shown), partly due to a lower assimilation rate per unit leaf area (Figure 4.1). Cakmak et al. (1998) reported that under low light conditions soluble sugar concentration in root tips (the hot spot for exudation) of wheat was extremely low or no measurable, which limited the biosynthesis of phytosiderophores. Similar result was observed by Cheng et al. (2014): increases in root sucrose concentration in white lupin under P deficient supply and this was expressed more evidently under high light compared low light conditions.

In contrast to all previous work that studied the influence of light condition on root exudation (e.g. Cheng et al. 2014; Cakmak et al. 1998; Chen et al. 2012), this study is the first attempt

to investigate whether metabolite profiles of root exudate alternated by light intensity. As a first attempt, it was not necessary to have a deep insight of the metabolites behind each signal, we used the number of signals roughly reflects the number of different metabolites in the exudate. Generally, high light intensity increased the number of metabolites in the root exudates. Irrespective P supply level, large variation existed in the composition of root exudates collected from different light intensity conditions (Figure 4.4). Only 27 to 35% of signals occurred under both light regimes. Depending on plant age and P supply, about 43 to 76 signals occurred exclusively under high light, that are about 25 to 54% of all compounds in the high-light exudate. On the other hand, 29 to 68 signals (33 to 39%) occurred only in the low-light exudates. Wouterlood et al. (2004) reported that plant size affected total carboxylate concentration in rhizosphere, as well as the composition of these carboxylates. In addition, P deficiency increased the number of exuded compounds from about 100 to nearly 200 under high light conditions, especially at DAT 35. Khorassani et al. (2011) also found large differences in the composition of root exudates of P-deficient and well-supplied sugar beet plants. They reported about 65 signals that were at least 5 times higher under deficiency compared to the control.

Light intensity not only affects assimilation transport from leaves into the roots resulting in the alternation of exudation amount but also alters root physiology to a large extent. Cheng et al. (2014) hypothesized that a high sucrose concentration in the root is not only a source for carbon but also a signal which influences both physiological and morphological reactions. Their results clearly showed that cluster root development of white lupin was increased at high light even at sufficient P supply and a high sucrose concentration in the roots increased the expression of *LaPEPC3*, which might be involved in organic acid synthesis (Peñaloza et al. 2005). This triggered citrate exudation at high light even at a sufficient P supply, whereas at low light and high P citrate exudation was nil.

These results make clear that light conditions are an important factor when root exudation pattern are determined in context with P shortage and P efficiency mechanisms. Different light intensity might also be one reason for the difficulties to transfer results obtained in low light growth chamber to the field condition. Without correct organic compounds, it will be impossible to understand how plants cope with P deficiency by releasing exudates.

5.3 Challenge in root exudate metabolite investigation

Study on root exudate metabolite is challenging regarding both sampling and analysis processes. A completing collection of root exudates is a prerequisite for the present study to gain a comprehensive understanding of the effect of P deficiency and light intensity on root exudation pattern. Firstly, the planting set-ups and sampling technique should ensure the alteration of exudates concentration and composition as slight as possible, e.g. (i) increment due to root damage; (ii) loss due to adsorption on the soil or other culture media (e.g. glass beads and quartz sand); (iii) loss due to microbial biodegradation. Secondly, due to the big sample volume we need the following analysis, localized collection method is not suitable in present study, though Beißner (1997) reported that sugar beet plants excrete oxalate mainly in the region 1.5 cm behind the root tip.

Considering above-mentioned reasons, sugar beet plants grown in hydroponic solution and the dipping method were employed to collect root exudates. Oburger et al. (2013) compared a range of commonly used root exudate sampling techniques using hydroponic as well as soil-grown maize plants. They concluded that although qualitative and quantitative differences existed between methods, all exudation rates were in the same order of magnitude, and suggested that hydroponic techniques may still be suitable for screening general plant root metabolic reaction to nutrient deficiency. Concerning the analysis, complex matrix such as salts originating from nutrient solutions have to be taken into account, thus we used distilled

water as trap solution. In addition, before each collection, we pre-cultured the plants in 500 μM Ca $(NO_3)_2$ solution for 12 h to reduce the interfering ions. For longer time (12 h), low concentrations of Ca could be helpful to limit osmotic stress and possible passive leakage and/or diffusion. Valentinuzzi et al. (2015) stated that in work aimed at studying root exudation processes, distilled water is the most suitable trap solution to collect root exudates like organic acids and flavonoids, especially in short time (e.g. 2 h). Moreover, in order to minimize microbial degradation of organic compounds in the root exudates, a short collection period, i.e. 2 h, is recommended for collecting root exudates (Sas et al. 2001).

As present study is a discovery-based study, metabolic profiling (untargeted analysis of metabolite) is an appropriate study method. Metabolic profiling studies typically apply mass spectrometry coupled to a range of diverse chromatographic platforms, e.g. GC, HPLC and UHPLC, because natural extracts (e.g. root exudate) may contain complex compositions, the chromatographic separation is required before detection. Since the early 1980s, HPLC has been recognized as the universal technique for the efficient and direct separation of natural products from crude cocktail without complex ample preparation step (Kingston 1979). The workflow of metabolite identification was presented in Figure 3.3. The major challenge in present study is how to identify the metabolite based on the information acquired from MS analysis. A successful metabolite identification was largely based on the existence of databases performed in study. However, none of the existing databases guarantees a complete coverage of the metabolome, there are still strong possibility losing the real one. Although to date present study cannot confidently identify the compounds involved in P mobilization, it gives much information that there are some strong signals in root exudates of sugar beet which was induced by P deficiency. Actually, when analyzing unknown compounds, nuclear magnetic resonance (NMR) remains the technique of choice for structural identification. NMR is a mandatory complement to MS-based de-replication strategies when an unknown

metabolite must be identified. However, in current study, further identification of those interesting signals could not be done because the amount of root exudates was not sufficient for the NMR-spectroscopy.

In conclusion, the present results indicate that root exudation pattern, both quantity and composition, is strongly influenced by the experimental condition, at least the way of P supply and light intensity. However, much of current knowledge for the role of organic compounds in P mobilization comes from in vitro experiments performed under conditions which are vastly different from those that exist in vivo. Therefore, special caution should be taken into account when comparing and interpreting data from exudation studies using different experimental conditions.

5.4 Appendix

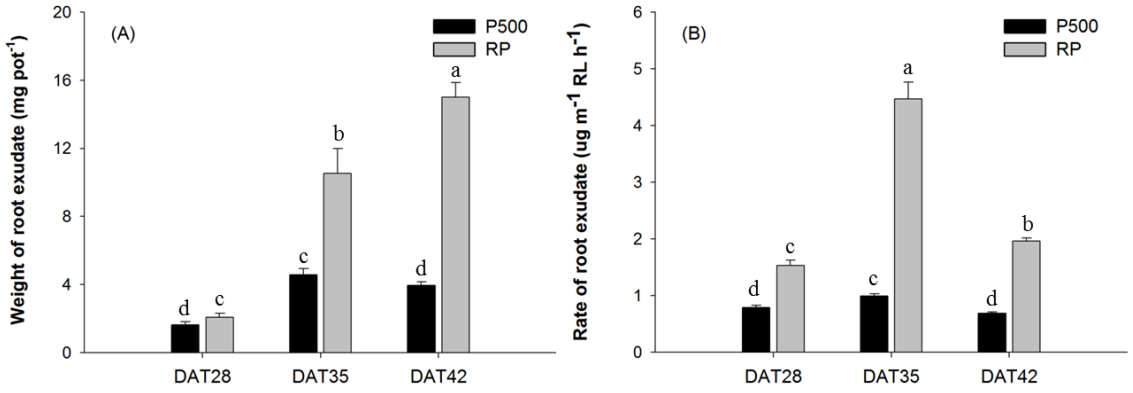

Figure 5.1. Effect of P supply on weight of root exudates (A) and rate of root exudation (B) at three harvests (DAT 28, 35 and 42). Plants were grown in nutrient solution at two levels of P supply (P500 and RP). Bars represent means ± SE (n=6). Data with different letters are significantly different ($p < 0.05$).

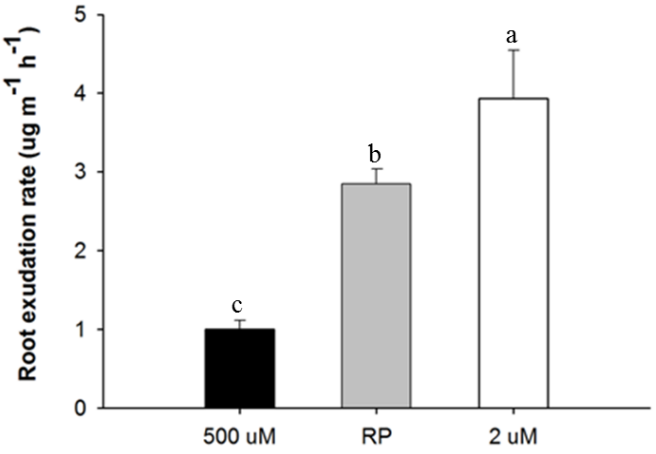

Figure 5.2. Effect of P supply on rate of root exudation. Plants were grown in nutrient solution at three levels of P supply: 500 µM (high P), RP (new method, low P) and 2 µM (typical method, low P), and root exudates harvest at DAO 29. Bars represent means ± SE (n=9). Data with different letters are significantly different ($p < 0.05$).

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Summary

Sugar beet is known as a P uptake efficient plant species due to its ability for increasing P availability in soil. This is done by changing rhizosphere chemistry through exudation of e.g. citric, malic, oxalic, citramalic and/or salicylic acids. All of these acids are able to improve P availability, but unfortunately, exuded amounts are far too low to explain the P efficiency of sugar beet quantitatively. The aim of this PhD study was to analyze root exudates of P-deficient sugar beet plants for compounds which haven't been in focus, yet, and which might take part in chemical mobilization of soil P. Furthermore, most of previous published experiments have been performed in growth chambers with relative low light intensity and might not be representative for field conditions. Hence, a second topic was to evaluate the impact of light intensity on root exudation pattern of sugar beet under P deficiency.

Sugar beet plants were grown in hydroponic culture in green house with two P levels (high and low) and with either high light intensity (without shading) or low light intensity (with shading). Root exudates were collected by dipping method at different plant growth stage. The results showed a critical role of light intensity on both quantity and quality of root exudates. The root exudation rate was increased by enhancing light intensity, regardless of P supply. Moreover, the profile of root exudates was significantly changed by light intensity alteration. It is suggested that in the studies concerning the function of root exudates in plants physiology, a special attention should be given to the light conditions.

The major objective of the work was the identification of organic acids different from the common ones (e.g. citric, malic or oxalic acid) in sugar beet root exudates which may be responsible for mobilizing P. Root exudation rate was roughly 4-times higher under low P compared to high P supply. To get a comprehensive knowledge of the composition of root exudates, a full-scan (non-targeted) metabolic profiling based on HPLC-ESI-MS was used in

this study. Root exudates collected from high and low P conditions were compared and signals that meet following criteria were screened (i) the signal was not detected in water control; (ii) the signal collected present in at least 5 of 6 replicates of root exudate samples; (iii) signal intensity must be at least 5 folds higher with low P supply plants compared to well-supply; (iv) standard deviation (SD) should be lower than 100% of corresponding mean value. After data processing, 69 signals were selected for further investigations. Among these signals, 16 putative metabolites were achieved from databases depending on their functional groups, i.e. carboxyl. Seven of putative metabolites were tested by co-elution root exudates with pure standards, however, none of them has been definitively confirmed to date. Malonic acid ($C_3H_4O_4$) corresponded to m/z 103; fumaric acid ($C_4H_4O_4$), maleic acid ($C_4H_4O_4$) and choline ($C_5H_{14}NO$) corresponded to m/z 115; succinic acid ($C_4H_6O_4$) corresponded to m/z 117; 4-aminobutanoic acid (GABA; $C_4H_9NO_2$) and 3-aminobutanoic acid (BABA; $C_4H_9NO_2$) corresponded to m/z 104 were shown to be involved in plant physiological processes and pure standard compounds could be used for following confirmation by co-elution with root exudates. Other signals were so far omitted from further investigations because they are not mentioned in KEGG or other databases, they play unknown role in plant physiology or they cannot achieved as a pure compound for further investigations. In addition, the amount of collected root exudates was not ample for structural elucidation by nuclear magnetic resonance spectroscopy (NMR- spectroscopy).

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1. **Luojin Yang**, Bernd Steingrobe, Klaus Dittert 2015. Exudation of organic acids by sugar beet and implications on phosphorus. In: Boden, Nährstoffe, Wasser-Forschung für die nachhaltige und effiziente Nutzung von Ressourcen,117-118. Jahrestagung der Deutschen Gesellschaft für Pflanzenernährung e.V. Göttingen, September 17-18, 2015 (Post presentations).

2. **Luojin Yang**, Bernd Steingrobe, Klaus Dittert 2014. A simple approach for controlling low phosphorus concentration in nutrient solution experiments. In: Plant Nutrition 2014 From Basic Understanding To Better Crops, 142. International Conference of the German Society of Plant Nutrition e.V. Halle (Saale), September 10-12, 2014 (Post presentations).

3. **L.J., Yang**, H.B., Tao, P., Wang 2012. Effects of Planting Density on the Plant Growth and Root Morphology of Maize. Chinese Journal of Applied & Environmental Biology, 18 (06): 1009-1013.

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