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Phosphorus form-related metabolic responses in roots of *Triticum aestivum* and the impact of beneficial soil microorganisms

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Abstract

Phosphate fertilizers are a finite resource, thus sustainable crop production will most likely depend on the utilization of P sources naturally found in soil, in particular predominant organic P forms. To date we know little of the biochemical sensing and adaptation of crops to different organic and inorganic P that potentially affects its assimilation. Furthermore, the use of inoculants of beneficial soil microorganisms has become of increasing interest due to their ability to mobilize P from organic and inorganic sources naturally occurring in soil. However, many factors including nutrient type and status, plant species and the presence of other microbes have a positive or detrimental affect on microbial fitness and activity and as a result on crop P uptake and growth. Therefore, the aim of this PhD thesis was to improve our understanding of the biochemistry of phosphorus sensing, mobilization and uptake in wheat from various sources, and subsequently address the impact of mycorrhizal fungi as well as plant growth promoting rhizobacteria on these processes.

Mesocosm studies provided detailed evidence that wheat root metabolism and the secretion of root exudates are sensitive to organic and inorganic P forms. In addition and with respect to P uptake and growth, wheat responsiveness to mycorrhizal fungi and plant growth promoting rhizobacteria colonization was highly dependent on the present P form. However, long-term changes in root metabolism were mainly driven by the P source.

Based on these results, it is still necessary to ascertain if these metabolic changes are general responses of wheat or cultivar specific. Further, it is essential to link these responses to P uptake mechanisms and determine their effect on rhizosphere microorganisms in order to develop cultivars that not only have enhanced soil P exploitation and utilization capacities, but also positively respond to beneficial rhizomicroorganisms.

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Chapter 1: General Introduction

1.1 Introduction

The United Nations (2011) projected that the world population is going to rise to over 9 billion in 2050. In order to fulfil the rising demand for food, food production has to increase by 70% by 2050 (FAO, 2009). Therefore, farmers need to enhance the annual cereal production from 2.1 billion tonnes today to 3 billion tonnes, which mostly has to come from an increase in productivity than arable land expansion (FAO, 2009). As a result, agricultural production will heavily rely on fertilizer applications, in particular phosphorus, which is derived from phosphate rock. In 2009, the average phosphate EU wide fertilizer consumption was 6 kg of P per hectare (EUROSTAT, 2011). The demand for phosphorus fertilizers is on the rise; however the resources are finite and estimated to be exhausted within the next 30-300 years (Cordell et al., 2009; Van Vuuren et al., 2010; Tiessen et al., 2011; Cordell et al., 2013). Thus, sustainable P management strategies of agro-ecosystems are essential for future food security.

In the UK, the total utilized agricultural area comprises 17.1 million hectares of which 6.1 million hectares are attributed to croppable land (DEFRA, 2011a). Increasing crop prices (between 2005 and 2010 prices for wheat and barley increased up to 90% and 63%, respectively) resulted in a shift towards enhanced cereal growing (Angus et al., 2009; DEFRA, 2011b). About 50% of croppable land is now used for cereal growing, especially wheat and barley (DEFRA, 2011a).

Sustainable P management strategies not only have to reduce mineral fertilizer dependency in cereal production, but also need to secure and increase crop yields. At present, breeding programmes select wheat cultivars that are highly dependent on mineral fertilizers to receive enough P through their root systems for optimal growth (Zhu et al., 2001). Thus, a reduction in fertilizer applications would result in drastic yield losses. However, it has been shown that certain wheat varieties successfully acquired sufficient amounts of P through the hyphal network of root inhabiting arbuscular mycorrhizal fungi

(AMF) at low soil P levels, which resulted in increased growth rates (e.g. Hetrick et al., 1992 and 1996). Hence, AMF seems to have the potential to allow fertilizer reduction in cereal production without any yield losses. But there are variations in P uptake strategies and efficiencies between different AMF species and genotypes and the mechanisms behind it are not still understood (Jansa et al. 2005; Munkvoldt et al., 2004). Further, despite receiving P from the fungus not every cereal type or cultivar show a positive growth response to mycorrhizal infection and it is still not known why (e.g. Hetrick et al., 1996; Bryla and Duniway 1997; Grace et al., 2009).

There are indications that AMF can mineralize P from organic compounds through enzyme release, hence accessing a P pool normally unavailable to cereals (Tarafdar and Marschner, 1994; Koide and Kabir, 2000). There is, however, still a debate about the magnitude and importance of this process. It has been argued that other soil microorganisms than AMF are responsible for the solubilisation and mineralization of organic P and that P assimilation by AMF occurs after P mineralization (Joner and Jakobsen, 1995; Joner and Johansen, 2000). Therefore it is essential to improve our understanding of the mycorrhizal P uptake pathway. Which P pools can be exploited and how is P assimilation regulated? Do other soil microorganism facilitate mycorrhizal P acquisition? Are AMF dependent on phosphorus solubilizing microorganisms to access organic P compounds? How is plant responsiveness to AMF infection regulated? It is inevitable to answer these questions in order to consider AMF as a reliable alternative to fertilizer use.

1.2 Phosphorus: an essential plant nutrient and its soil availability

Phosphorus is one of the major macronutrients essential for plant growth and reproduction. It makes up about 0.2% of plant dry weight (e.g. Smith et al., 2011) and is a structural element in nucleic acids, phospholipids, several enzymes and co-enzymes (e.g. Karandashov and Bucher, 2005). The involvement of P in processes such as energy

metabolism, activation of metabolic intermediates, signal transduction cascades, ion transport through membranes and enzyme regulations shows the need of a sufficient supply in order to achieve maximum growth (e.g. Karandashov and Bucher, 2005; Fageria, 2009).

Phosphorus deficiency can cause a reduction in shoot growth and root development (Baligar et al., 1998). Several studies have reported a decrease in leaf areas, numbers and leaf expansion due to an insufficient supply of P (Lynch et al., 1991; Qui and Israel, 1992). Mengel et al. (2001) observed a depression in seed and fruit development in plants exposed to P stress. Furthermore, an insufficient supply of P can reduce carbon fixation and assimilation due to limited triose phosphate translocator function (Heber and Heldt, 1981) or a decline in ribulose biphosphate regeneration (Fredeen et al., 1990).

1.2.1 Inorganic soil P forms

Phosphorus is difficult to acquire by plants. In the soil, it might be present in sufficient amounts, but the directly accessible P pool is very limited. Only 1% of the total soil P can be utilised by plants (Blake et al., 2000; Quiquampoix and Mousain, 2005). This is in the form of negatively charged orthophosphate (P_i) in soil solution ($H_2PO_4^-$, HPO_4^{2-}) (e.g. Rausch and Bucher, 2002; Smith et al., 2011).

Concentrations of P_i in soil solution are driven by the three major processes of the soil P cycle:

1. Dissolution and precipitation
2. Adsorption and desorption
3. Mineralization and immobilization

The amount of P_i in soil solution is very low (<0.01 to 1 mg/L in very fertile soil), since most of the P_i is fixed and immobilized through sorption and precipitation processes (Jones and Oburger, 2011; Doolette and Smernik, 2011). Orthophosphate is highly reactive to iron, aluminium and calcium ions or minerals. Depending on the ambient soil pH they

form different crystalline or amorphous structures that have a low solubility (Fageria, 2009; Jones and Oburger, 2011). In acidic soils, fixed inorganic P occurs as Al and Fe phosphates or is bound to amorphous forms such as Fe and Al (hydr)oxides through chemical sorption. In contrast, neutral to alkaline soils contain Ca phosphates, mainly as forms of apatite (Sims and Pierzynski, 2005; Fageria, 2009).

1.2.2 Organic soil P forms

Soil phosphorus is also present in several organic forms (P_o), which can account for 20 to 80% of the total P in the upper soil horizon (Dalal, 1977). In soils with very high organic matter content it can approach up to 90% (Jones and Oburger, 2011). However, the large range in reported P_o concentrations cannot only be attributed to variations in soil organic matter contents, but also to the limitations of analytical methods. Soil organic phosphorus cannot be measured directly in-situ. Thus, contents are determined by colometric analysis of soil extracts. Current methods only extract both organic and inorganic P. Therefore; the amount of P_o needs to be determined by establishing the difference between total and inorganic P concentrations. Soil extraction usually involves soil treatments with strong acidic or alkaline solutions (Turner et al., 2005). This can cause the hydrolysis of labile organic P forms and, in the case of acidic treatments, the release of inorganic phosphate. As a result, determined concentrations are likely to over- or underestimate soil P_o contents (Turner et al., 2005). Hence, it is important to develop methods that allow direct analysis of P_o without prior extraction.

The main identified organic P forms are inositol phosphate, phospholipids and nucleic acids (Turner et al., 2002a and 2002b; Quiquampoix and Mousain, 2005). Inositol phosphate is the most common organic compound representing ≥80% of the total P_o (Dalal, 1977). It occurs in various stereoisomeric forms (*myo, scyllo, neo,* D-*chiro*) (Dalal, 1977; Cosgrove, 1980; Celi and Barberis, 2005) with different stabilities, which are closely linked to the number of phosphate groups (Quiquampoix and Mousain, 2005). As a

consequence, the most dominant form in soils is *myo*-inositol hexakisphosphate. In general, inositol phosphates are more resistant to mineralization processes than other P_o compounds. They can form complexes or precipitates with clay minerals, fulvic acids, proteins, lipids as well as certain metals such as copper, zinc, nickel, cobalt, manganese, iron and calcium (Anderson and Alridge, 1962; Rojo et al., 1990; Celi and Barberis, 2005). The stability and solubility of these complexes is a function of pH (Martin and Evans, 1987). Insoluble complexes with proteins and lipids are present in acidic soils, whereas insoluble metal complexes mostly occur in soils with mid-range pH values (Harrison, 1987; Martin and Evans, 1987). The complexation and precipitation of inositol phosphate as well as its general resistance to mineralization makes it poorly accessible to plants.

Phospholipids represent 0.5 to 7% of the total P_o, with phosphoglycerides as the predominant form (Dalal, 1977). Soil phospholipids derive from microorganisms, plant material and animals. Usually, their synthesis and degradation are rapid in the soil (Quiquampoix and Mousain, 2005).

Nucleic acids and their derivatives comprise less than 3% of total P_o and originate from the decomposition of living organisms (Dalal, 1977). Like phospholipids, nucleic acids are mineralized, re-synthesized and combined with other soil constituents or incorporated into microbial biomass within a very short period of time (Anderson and Malcolm, 1974).

In addition to the three groups of organic P, other less abundant forms occur. These include sugar phosphates, monophosphorylated carboxylic acids (Anderson and Malcolm, 1974) and teichoic acid, which is a major cell wall component of Gram-positive bacteria (Zhang et al., 1998) and can act as P reserves for them under P limited conditions (Grant, 1979; Gaechter and Meyer, 1993).

Beside teichoic acid, soil microorganisms incorporate soil P in the form of nucleic acids, phospholipids, cytoplasmic inorganic and organic P as well as polyphosphates, thereby competing with plants for P (Oberson and Joner, 2005; Buenemann et al., 2011).

Several studies reported an increase in microbial P uptake when soluble C was added to the soil, thereby indicating an indirect link between soluble C concetrations and microbial P mineralization (Oehl et al., 2001; Buenemann et al., 2004; Olander and Vitousek, 2004). Microorganisms can easily mineralize soluble C. Thus, elevated concentrations of this readily available C pool promote microbial growth and activity and indirectly increase microbial P assimilation. However, Buenemann et al. (2004) pointed out that this effect seems to be relatively short-lived. In their experiments, microbial populations rapidly collapsed after two days when P uptake reached its maximum, re-releasing P incorporated in microbial biomass into the soil. In addition, all of these studies have been performed in the laboratory under controlled conditions. Hence, it is still unclear whether this indirect interaction can be observed in the field where other factors such as different plant communities, soil chemistry and composition, temperature as well as drought and flooding events potentially affect soil microbial activity and soluble C pools.

Soil microorganisms are also able to solubilize and mineralize P fixed in inorganic and organic compounds, hence, making it available for plants to assimilate. Both, the uptake and subsequent release as well as the mobilization of P from soil minerals reflect the strong influence of the soil microbial community on the major processes that drive the soil P cycle.

1.3 Phosphorus solubilisation by soil microorganisms

Certain groups of soil bacteria, fungi and actinomycetes are able to solubilize and mineralize P from organic compounds and soil minerals by altering the chemical environment through the release of specific organic and inorganic ions and molecules (Jones and Oburger, 2011). If this microbial P mobilization outweighs microbial uptake and incorporation of P as well as mineral sorption, concentrations of P_i in soil solution increase and consequently P_i availability to plants (Jones and Oburger, 2011). About 1-50% of soil bacteria and 0.1-0.5% of fungi can be attributed to these so-called phosphorus

solubilizing microorganisms (PSM) (Kucey et al., 1989; Gyaneshwar et al., 2002). Microbial processes that are potentially involved in the mobilization of P are (McGill and Cole, 1981; Jones and Oburger, 2011):

- The release of protons or hydroxide ions that alters the soil pH and results in mineral dissolution
- The exudation of organic acid anions that either complex or dissolve soil minerals
- The mineralization of organic P by the use of either cell bound or extracellular enzymes.

1.3.1 Alteration of the soil pH

Microorganisms can change the ambient soil pH via the release of protons or hydroxide ions. The stability of most P containing minerals is a factor of the prevailing pH. Thus, a change in the soil pH can cause destabilization and subsequent dissolution. Microbial mediated P mobilization through soil acidification has been documented in several in vitro studies (Illmer and Shinner, 1992; Gyaneshwar et al., 1999; Ben Farhat et al., 2009). In these studies, calcium phosphates, in particular, showed a high susceptibility to this process. Furthermore, it has been reported that the supply of nitrogen might have an impact on P_i mobilization by acidification, since some microorganisms solely use NH_4^+ to facilitate P_i solubilization (Asea et al., 1988). Depending on the form and amount of N supplied it can either enhance or decrease the efficiency of this mechanism (Roos and Luckner, 1984; Illmer et al., 1995; Reyes et al., 1999; Sharan et al., 2008).

Since all studies have been accomplished in vitro on pure cultures, it should be carefully considered whether these results are applicable to natural soils, under field conditions. In the field, a deficient supply of nitrogen and carbon due to a lack of availability and competition with other soil microbes can limit microbial proliferation and activity, which in turn reduces soil acidification (Jones and Oburger, 2011). Moreover,

some soils, especially calcareous ones, have a pH buffer capacity that could limit microbial acidification (Jones and Oburger, 2011).

1.3.2 Organic acid anions

Besides microbial induced soil acidification, P_i solubilisation can also be facilitated through the microbial exudation of low-molecular weight organic acid anions (carboxylates) (Illmer et al., 1995; Reyes et al., 1999; Patel et al., 2008). Such organic acid anions include gluconic, 2-ketogluconic, citric, malic, malonic, oxalic, succinic, lactic, tartaric and glycolic acids (Kucey et al., 1989; Gyanshwar et al., 2002). They are released in their negatively charged, dissociated form unable to release another proton that would cause soil acidification (Jones and Oburger, 2011).

Phosphorus mobilization is caused by the negative charge or the metal complexation properties of the anions (Jones and Oburger, 2011). Mineral dissolution via ligand exchange seems to be the main P_i mobilizing mechanism (Gerke et al., 2000; Stroem et al., 2005). However, the release of P_i through adsorption of organic acid anions to metal oxide surfaces has also been observed (Filius et al., 1997). In addition to the dissolution of inorganic P, it has been reported that some organic acid anions are able to increase the solubility of organic P-compounds, thus, making them more susceptible to mineralization via enzymatic hydrolysis (Otani and Ae, 1999; Tang et al., 2006).

As in the case of microbial soil acidification, it is still uncertain to what extend microbial exudation of organic acid anions contributes to P_i mobilization. Sorption and microbial uptake can drastically reduce organic acid anion availability in soil solution (Stroem et al., 2005; Oburger et al., 2009; Oburger and Jones, 2009). Hence, soil properties such as pH, mineral composition as well as carbon availability that affect these processes can have a strong impact on the functional efficiency of organic acid anions in P_i mobilization processes (van Hees et al., 2003; Oburger et al., 2009; Oburger and Jones, 2009).

1.3.3 Enzymatic mineralization of P

Phosphatases or phosphohydrolases are enzymes that can mineralize organic P by catalyzing the hydrolysis of both the esters and anhydrides of H_3PO_4 (Tabatabai, 1994; Quiquampoix and Mousain, 2005; Nannipieri et al., 2011). These enzymes can either be bound to cell walls or released into the soil. Phosphomonoesterases are the most abundant group of enzymes that are released by PSM and their pH optima for hydrolysis varies between 2.5 and 8.5 (Kim et al., 1998; Quiquampoix and Mousain, 2005; Jorquera et al., 2008).

Phosphatase activity can be inhibited by increasing concentrations of orthophosphate (P_i) and other polyvalent anions (e.g. MoO_4^{2-}), high amounts of metals such as Hg, Cu, Mn (II) and Fe (II) and enzyme sorption to mineral surfaces (Huang et al., 2005; Quiquampoix and Mousain, 2005). In contrast to that, several divalent cations (e.g. Ca, Mg, Zn, Co) are known to be cofactors that are essential for the activation of enzyme activity (Quiquampoix and Mousain, 2005; Jones and Oburger, 2011). For example, von Tigerstrom and Stelmaschuk found that the divalent cations Zn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+} and Mn^{2+} reactivated the activitiy of the alkaline phosphatase of *Lysobacter enzymogenes* after inihibition with EDTA (a universal chelator of divalent metal cations). Similarly, AgpP glucose-1-phosphatase activity of the soil bacterium *Pantoea sp.* Strain 3.5.1 was stimulated by the presence of the same cations as well as Fe^{2+} and Cu^{2+} (Suleimanova et al., 2015).

The strong interactions of phosphatases with metal ions as well as soil minerals makes it very difficult to determine how much of total soil P_o mineralization was actually derived from enzyme activity. Studies investigating the correlation between phosphatase activity and P_i concentrations reported contradictory results. Some of them found a positive correlation (Rojo et al., 1990; George et al., 2002a) whereas others observed a negative or no correlation at all (Criquet et al., 2002, 2004; Olander and Vitousek, 2000; Ali et al., 2009).

Overall, it is very difficult to assess to what extent microbial phosphorus solubilisation can actually contribute to P mineralization in soils, since the impact of biological, chemical and biochemical processes on PSM efficiency is very complex and therefore hard to predict. Nevertheless, it should not be disregarded that a combination of PSM and AMF activity could potentially increase plant P availability. Several studies reported an increase in P uptake and growth in wheat cultivars grown in soil inoculated with PSM and AMF (Kucey, 1987; Omar, 1998; Babana and Antoun, 2006). However, Wu et al. (2005) observed an inhibiting effect of AMF on PSM activity. Thus, further research is necessary on the interaction between AMF and PSM and whether a co-inoculation can promote growth in other cereals than wheat.

1.4 Phosphorus uptake by plants and arbuscular-mycorrhizal fungi: The function of phosphate transporters

Phosphorus availability to plants can be very restricted, since it was thought that plants could only directly absorb P_i from soil solution and as a consequence relied on microbial mediated P mineralization in order to acquire phosphorus from other P sources. However, Paungfoo-Lonhienne et al. (2010) showed that Arabidopsis roots and root hairs assimilated P in form of DNA and that the exogenous DNA increased lateral root branching and root hair length. The mechanisms behind DNA assimilation and root growth promotion are still unknown (Fig. 1.1b). Moreover, it needs to be seen whether other plants are able to absorb and utilize exogenous DNA as phosphorus source as well. Nevertheless, these findings will fuel the debate on whether organic P compounds can substantially contribute to plant P supply beside P_i.

1.4.1 P uptake by plant roots

Still being considered as the major P source, plants can assimilate P_i via two different pathways (Fig. 1.1a). The first pathway is the direct uptake of P_i through root

cells. In the second pathway, plants indirectly acquire P_i by forming a symbiotic relationship with mycorrhizal fungi.

Plants directly absorb P_i as negatively charged molecules from soil solution through their root epidermis (Fig. 1.1b). In doing so, they have to overcome a negative membrane potential and a steep concentration gradient (i.e. phosphate concentrations are about 1000 fold higher within the cell compared to the external environment) (Bieleski and Ferguson, 1983; Karandashov et al., 2005). This requires metabolic energy and involves high-affinity cell membrane-spanning transporter proteins that have specific binding sites and include phosphate transporters and a proton extrusion pump (Smith, 2002; Rausch and Bucher, 2002; Karandashov et al., 2005, Smith et al., 2011).

Several studies identified an H^+/P_i co-transport that is coupled to the activity of an H^+-extrusion pump, which maintains a proton gradient across the membrane (Bieleski and Ferguson, 1983; Ullrich-Eberius et al., 1981, 1984; Smith, 2002). Sakano (1990) estimated that this high affinity P_i acquisition through the membrane requires two to four protons for each phosphate molecule transported through the membrane. In addition to the H^+/P_i co-transport, a second transport system has been observed in some fungal species and in green algae where Na^+ is used as the co-transport cation (Versaw and Metzenberg, 1995; Martinez and Persson, 1998; Persson et al., 1998; Reid et al., 2000; Zvyagilskaya et al., 2001). Mimura et al. (2002) showed that the capacity of Na^+/P_i co-transport activity in the freshwater alga *Chara corallina* was dependent on external Na^+ concentrations. Both co-transport systems exhibit different pH optimums. While H^+/P_i co-transport showed the highest activity in yeast and *N. crassa* at pH values between 4.5 and 6.0, Na^+/P_i co-transport had an optimum at pH values between 8.0 and 10.0 (Reid et al., 2000; Versaw and Metzenberg, 1995; Martinez and Persson, 1998; Zvyagilskaya et al., 2001). The existence of different transport systems with different pH optimum in certain organisms indicates a high flexibility to changes in environmental conditions (Smith et al., 2011). Furthermore, Na^+/P_i co-transport systems might play an important role in plants and

other microorganisms when growing in alkaline soil or adjusting to salt stress. This however, needs further investigation.

First molecular investigations of phosphate transporter genes in an *Arabidopsis* clone revealed that plant phosphate transporters have similarities with the ones identified in yeast and fungi (Muchhal et al., 1996; Smith et al., 1997). Transporter genes now have been successfully isolated from tomato (Daram et al., 1998; Liu et al., 1998a), potato (Leggewie et al., 1997), *Medicago* (Liu et al., 1998b) as well as barley (Smith et al., 1999), and all of them are members of the fast growing high-affinity transporter family *Pht1*. A strong accumulation of P_i transporters and the expression of their gene transcripts have been observed primarily in rhizodermal cells directly behind the root apex as well as in root hairs and root cap cells, along with the respective proteins in the plasma membrane (Daram et al., 1998; Muchal and Raghothama, 1999; Smith et al., 2011). This indicates an active role of *Pht1* transporters in P_i acquisition at the root-soil interface, and that the uptake is most efficient at and directly behind the root apex. Liu et al. (1998a) as well as Muchal and Raghothama (1999) suggested a transcriptional regulation of the activity of P_i transporters since they observed an increase in the transcript abundance under P_i deprivation conditions, which declined again after a subsequent supply of P_i.

Phosphate absorption by root cells is much faster than phosphate diffusion through the soil, (Rausch and Bucher, 2002). As a consequence a depletion zone establishes in the rhizosphere that drastically reduces the P_i supply (e.g. Rausch and Bucher, 2002; Karandashov and Bucher, 2005; Smith et al., 2011). In order to compensate for the restricted P availability and to improve soil P exploitation, plants developed various P acquisition strategies such as changes in root morphology and architecture (Rausch and Bucher, 2002), an increase in absorptive surface area via root hairs (Marschner, 1995; Bates and Lynch, 1996; Jungk, 2001), secretion of phosphomonoesterases and organic acids into the rhizosphere (Raghothama, 1999) and the formation of cluster roots (Neumann and Martinoia, 2002). However, the most

common strategy that allows plants to exploit larger soil volumes and possibly organic P forms is the formation of a symbiotic relationship with AMF (e.g. Smith and Read, 2008).

1.4.2 P uptake by AMF

AMF can provide a very effective indirect pathway for plants to acquire phosphorus. The formation of an extensive hyphal network allows them to penetrate smaller soil pores and enter areas outside the rhizosphere (Fig. 1.1a) (Pearson and Jakobsen, 1993; Drew et al., 2003; Smith and Read, 2008; Schnepf et al., 2011). Thus, AMF can exploit larger soil volumes for P than the plant itself. It is still unclear whether AMF can utilize P from organic compounds (P_o) (Fig. 1.1c). It has been reported that AMF are able to access and mineralize P from organic components such as lecithin, RNA and sodium phytate through the release of phosphatases (Tarafdar and Marschner, 1994; Feng et al., 2003). However, it has been argued that soil organic P is mineralized by phosphatases released by PSM prior to AMF assimilation (Joner et al., 1995 and Joner and Johansen, 2000). Furthermore, Joner et al. (2000) also stated that the contribution of AMF assimilated organic P to the P supply of the plant is minimal. Therefore, more investigations on organic P uptake by AMF and the involvement of PSM are necessary to solve this debate.

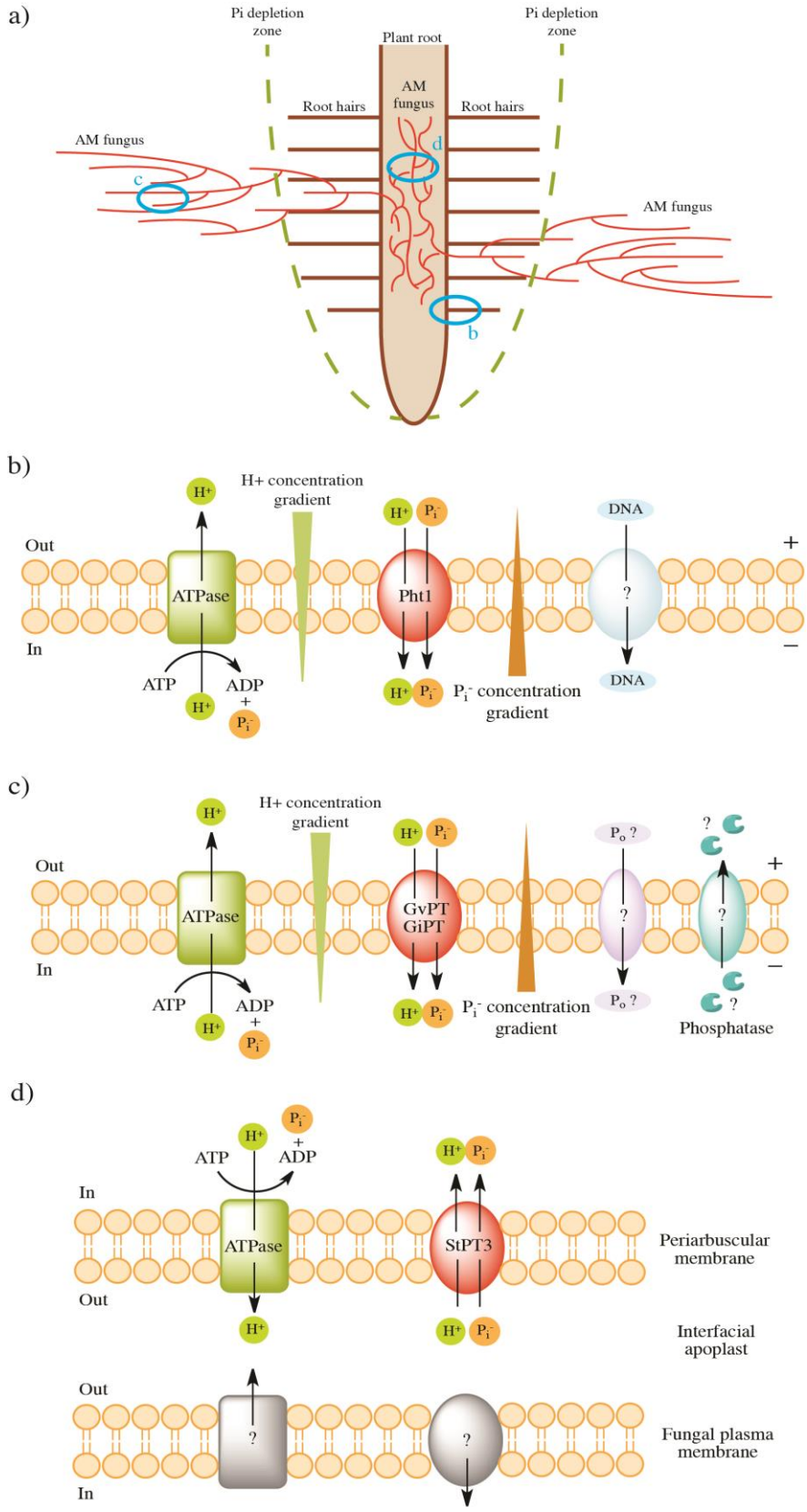


Fig. 1.1: Root and AM fungal interfaces involved in P uptake and transfer. A) General sketch of an AMF infected root. Different areas of P uptake are highlighted. Root induced P_i depletion zone is also indicated. Additionally, schematic views of P uptake along the rhizodermal cell membrane and root hairs (b) and in AM hyphae (c) as well as P transfer at the plant interface (d) are shown.

Phosphorus uptake along the soil hyphae interface occurs through a P_i transporter system, which is similar to that found in rhizodermal cells (Fig. 1.1c). Thomson et al. (1990) observed in *Gigaspora margarita* a high-affinity $2H^+/H_2PO_4^-$ co-transporter system that was driven by an H^+-translocating ATPase. In addition, two P_i transporters, *GvPT* and *GiPT*, have been identified in the extraradical hyphae in *Glomus versiforme* and *Glomus intraradices* that are related to members of the plant *Pht1* transporter family (Harrison and Van Buuren, 1995; Maldonado-Mendoza et al., 2001). Their presence in the mycelium indicates their involvement in the initial acquisition of P_i from the soil. Further, Maldonado-Mendoza et al. (2001) observed that the gene expression of *GiPT* was influenced by ambient P_i concentrations.

It is still unclear which mechanisms are responsible for the translocation of P_i within the hyphal network from the uptake site to the fungus-root interface. Phosphorus translocation seems to be rapid and likely in the form of polyphosphate (Smith et al., 2011). Moreover, Cooper and Tinker (1981) proposed that the involved processes are based on mass flow and cytoplasmic streaming.

Phosphorus transfer from fungus to plant takes place along a specialised interface (Fig. 1.1d) (Rausch and Bucher, 2002; Smith and Read 2008). Root colonization by AMF occurs behind the root apex in older sections. Within the root cortical cells AMF form arbuscules, which are surrounded by the plant cell plasma membrane called periarbuscular membrane. Both fungal and plant cell membrane are separated by a narrow apoplastic region (Harrison, 1999; Smith et al., 2011). The three layers together constitute the fungal-plant interface along which the P_i transfer takes place. The transfer involves two transport steps (e.g. Rausch and Bucher, 2002):

1. The release of P_i from the fungus into the interfacial apoplast
2. The uptake of P_i from the apoplastic interlayer into the root cortical cells.

The underlying mechanisms and molecular components involved in the first transport step are still unknown (Smith and Read, 2008). In the case of the second

transport step, there were indications for an active co-transport system within the periarbuscular membrane, which is energized by the activity of H^+-ATPase (Gianinazzi-Pearson et al., 2000; Smith and Read, 2008). Eventually, Rausch et al. (2001) identified the novel P_i transporter *StPT3* in the periarbuscular membrane of potato. *StPT3* is, as the P_i transporter found in the fungal mycelium, related to the high affinity transporter family *Pht1* in plants.

1.4.3 Interactions and signalling between both P uptake pathways

Investigations on P uptake in plants through both pathways showed that there is a contribution of the AMF pathway to the total plant P (Pearson and Jakobsen, 1993; Jakobsen, 1999; Smith et al., 2004). Furthermore, it can outweigh or even completely inhibit the direct uptake via root cells. The extent of inhibition however seemed to depend on the fungi and rate of colonization (Smith et al., 2004, 2011; Schnepf et al., 2008, Nagy et al., 2009). It is still not clear how AMF colonization exactly reduces the direct P_i uptake pathway. So far research suggests the involvement of signalling events between the fungus and the host plant that subsequently result in the down-regulation of P_i transporters in the root epidermis (Smith et al., 2011; Smith and Smith, 2012). However, it may also be possible that competition between the fungus and plant for P_i in the rhizosphere limits direct root uptake (Smith and Smith, 2011; Smith and Smith, 2012).

Investigations on P_i transporter regulation are an on-going area of research. Recent studies showed that under P_i starvation conditions transporter regulation is driven by a complex cascade of gene signals, which is coupled to a subsequent release of phytohormones and sugars (Rouached et al., 2010; Chiou and Lin, 2011; Lei et al., 2011). During gene signalling the transcription factor PHR1 induces a number of genes including the microRNA miR399. MiR399 inhibits the activity of the enzyme PHO2. This enzyme normally suppresses P_i transporter gene expression. Its inhibition results in an increase in transport gene expression that might be followed by enhanced P_i transporter activity of

the direct P uptake pathway. It should be noted that most studies on gene signalling have been made on AMF non-host plants. Thus, it is still questionable how far this is applicable to AMF host plants. However, similar elements of the gene signal network have been found in AMF infected roots that were connected to fungal infection and P_i transporter activity (Branscheid et al., 2010; Gu et al., 2010; Chen et al., 2011). This suggests that gene-signalling events on P_i transporter regulation might be similar in plants establishing arbuscular mycorrhizal (AM) symbioses. Grace et al. (2009) also hypothesized a post-transcriptional or post-translational control of the direct P_i uptake pathway in AM plants. This is because they did not detect a decrease in the expression of epidermal P_i transporters even though the direct P_i pathway was down regulated.

Phytohormones in roots are involved in signalling events that can either indirectly or directly affect P uptake. For example, auxin, ethylene as well as strigolactones are phytohormones that are linked to lateral root and root hair development, which is in turn affected by P_i availability (Rubio et al., 2009; Ruyter-Spira et al., 2011). Strigolactones are also involved in early signalling events between AMF and the host plant forming a symbiotic relationship. Akiyama et al. (2005) reported that strigolactones exudation by roots initiated hyphal branching. In addition to that, Zhang et al. (2010) also suggested an involvement of strigolactones in arbuscule formation. Moreover, Balzergue et al. (2011) observed an absence of strigolactones and reduction of AMF colonization in roots at high phosphate concentrations. However, they also have pointed out that strigolactones cannot be the sole regulation factor of AM symbiosis responding to phosphate levels. Hause et al. (2002) reported increased levels of jasmonates after a fully established AM symbiosis in barley roots. They propose that these lipid-based hormonal signals are involved in the regulation of the carbon transfer from plant to fungus. This in turn might indirectly affect the P_i transfer from fungus to plant.

Like phytohormones, sugar signalling is linked to P_i starvation responses. High root-shoot ratios of sucrose in connection with an increased transport of sucrose to the

roots for growth as well as the overexpression of sucrose transporter genes has been observed under P limited conditions (Hammond and White, 2008; Liu et al., 2010; Lei et al., 2011). Liu et al. (2010) proposed that in addition to root-based signalling events, long-distance signalling from the shoots are involved in P_i starvation responses. Furthermore, Hammond and White (2008) also indicated that sugar signalling is likely responsible for enhanced P_i transporter expression and activity. The reported observations were made in AMF non-host or uninfected plants. Thus, it still needs to be seen whether these findings can be applied to AM plants. In AM plants, increased carbon relocation to the roots might rather benefit the fungal association as carbon source instead of promoting root growth. This in turn, could indirectly support P_i uptake via the hyphal network than the roots.

Changes in secondary metabolites have been detected as a response to AMF root colonization and phosphate availability. Maier et al. (1995) found that concentration levels of terpenoid glycoside (blumenin, a secondary root metabolite) were directly correlated to the grade of AMF colonization in roots of different cereals. Recently, Laparre et al. (2011) established metabolic profiles of pea roots exposed to either low or high phosphate levels. Through statistical analyses they discovered that 34 ions distinguished the two phosphate conditions. These findings further support Balzergue et al. (2011) suggestion that there are more regulation factors involved than currently identified.

Overall, there are similar and shared components involved in response signalling to AM symbiosis and P_i starvation in roots. This, as Smith et al. (2011) pointed out, potentially enables a cross talk between both P uptake pathways. However, the possibly involved regulatory elements are still unknown.

1.5 The effect of AMF symbiosis on P uptake in wheat

Mycorrhizal fungi colonization has been found in many crop plants including wheat. But AMF infection is not always beneficial. As a result of fungal functional diversity and plant compatibility, different AM fungi or AMF associations can entail different growth

responses in the same plant and the same AM fungi or fungal associations do not necessarily cause the same growth response in other plant species or even varieties (Baon et al., 1993a, b; Ravnskov and Jakobsen, 1995; Klironomos, 2003; Munkvold et al., 2004; Smith et al., 2004).

Numerous studies have been undertaken on the impact of AMF on P uptake and subsequent growth in wheat. The results, however, show a lot of variation. Growth responses in wheat can range from positive (e.g. Kucey, 1987; Hetrick et al., 1996; Hu et al., 2010) to negative (e.g. Bryla and Duniway 1997; Hetrick et al., 1996; Li et al., 2006) or non-responsive (Hetrick et al., 1996; Bryla and Duniway 1997; Li et al., 2006). Nevertheless, it can be concluded that certain factors have an impact on the efficiency of the AM symbiosis with respect to P uptake.

High soil P_i concentrations generally impede AMF colonization (Graham and Menge, 1982; Hetrick et al., 1996; Li et al., 2005, 2006; Smith and Read, 2008). This usually results in plant growth depression (Chen et al., 2005). Bryla and Duniway (1997) reported that AMF infected wheat had a lower P content and shoot dry weight under water stress compared to non-infected plants. Under drought stress P content as well as shoot dry weight were similar in AMF and non-AMF plants. Thompson et al. (1990) and Pearson et al. (1991) suggested that factors like soil temperature, light intensity, root mass as well as the AMF infection rate affect AM symbiosis and resulting plant response. In the case of the infection rate, the opinions are divided. Hetrick et al. (1996) and Hu et al. (2010) found a positive correlation between plant growth response and AMF colonization in wheat cultivars. In contrast, Clarke and Mosse (1981) as well as Grace et al. (2009) stated that growth responses could not be related to the level of colonization. Inoculations of phosphorus solubilising bacteria improved P_i uptake via the AMF pathway in wheat, which also resulted in a positive growth response (Kucey, 1987; Omar, 1998; Tarafdar and Marschner, 1995).

Interestingly, wheat cultivars that responded negatively or not at all to AMF infection still received significant amounts of P_i from the fungus (Hetrick et al., 1996; Li et al, 2006). It is still under debate what causes the AMF induced growth depression. Negative growth responses have often been explained by an excessive C supply to the fungus. However, several studies suggest that the growth repression is rather linked to the inability of the AMF uptake pathway to outbalance the reduced direct uptake activity (Li et al., 2008; Smith et al., 2009; Smith and Smith, 2011).

1.6 Aims and Hypotheses

The objective of this thesis is to improve our understanding of P uptake in wheat and the potential role of AMF and plant growth promoting rhizobacteria (PGPR) in improving P uptake efficiency of wheat. Firstly, it will be investigated how wheat root metabolism responds to the exposure to different P sources. Can wheat sense different P compounds and as a result activate different metabolic pathways? Are these differences in metabolic pathway activation connected to P uptake or other processes such as the release of root exudates?

In addition, it will be tested if AMF and/or PGPR can mobilise and transfer P from different organic and inorganic sources to wheat roots, thus enhancing P-uptake in wheat compared to non-colonized plants. Furthermore, it will test if inoculations of phosphorus solubilising microorganisms or their exudates can increase AMF mediated P supply. The following hypotheses are proposed:

1. Wheat roots can recognize different P forms and alter their metabolism accordingly. Metabolic changes will occur along pathways sensitive to P stress, such as glycolysis, the TCA cycle, pentose phosphate pathway and plant hormone biosynthesis.

2. Different P sources induce the release of different root exudates. The excretion of organic acids and secondary metabolites such as plant hormones and phenylpropanoids will most likely be affected.
3. AMF and PGPR improve P uptake from organic and bound inorganic P sources.
4. The extent of AMF and PGPR colonization depends on the present P form, but is not necessarily correlated to their P mobilization and transfer efficiency.
5. Wheat root metabolite profiles vary with P and microbial treatment. The formation of plant hormones and other secondary metabolites are mostly affected.

Chapter 2: Metabolic responses in roots of *Triticum aestivum* exposed to organic and inorganic phosphorus sources

2.1 Introduction

One of the major issues in agriculture today is an unsustainable and depleting phosphorus (P) supply. Phosphorus is one of the major macronutrients indispensable for plant growth and development (e.g. Schachtman et al., 1998; Raghothama, 1999; Abel et al., 2002; Abel, 2011). It is an essential element in many cellular molecules including sugars, lipids, ATP and nucleic acids, and is thus a critical component for plant metabolism. However, it is also one of the most limiting factors in crop growth (Schachtman et al., 1998; Abel, 2011) because plant roots can only take up a limited range of chemical forms (e.g. Hinsinger, 2001, Rausch and Bucher, 2002; Vance et al., 2003; Smith et al., 2011), and for the most part, plants directly assimilate inorganic phosphate (Pi) though recent evidence suggests that plant roots can take up DNA and small peptides (Paungfoo-Lonhienne et al., 2010 and 2012; Soper et al., 2011). The importance of this alternative uptake, however, remains unclear.

Even though P can be abundant in arable soils in a variety of inorganic and organic forms, readily available Pi in the soil solution is often in very low concentrations due to low P mobility as the Pi ion is rapidly immobilized through sorption to clay and metal surfaces (Blake et al., 2000; Quiquampoix and Mousain, 2005). For this reason, crop production currently relies on substantial fertilizer input. For instance in 2009, the average phosphate fertilizer consumption within the EU was 6 kg of P per hectare (EUROSTAT, 2011). Further, the global phosphate fertilizer demand in 2014 was estimated to be 38,060 tons, and the trend is increasing (FAO, 2012). It is of major concern, therefore, that the supply of rock phosphate used for fertilizer production is finite, with estimates of between 30-300 years supply remaining (Cordell et al., 2009; Van Vuuren et al., 2010; Tiessen et al., 2011; Cordell et al., 2013). Hence, it is crucial to fully understand plant P acquisition strategies, particularly in relation to different P chemical

forms in order to develop cereal cultivars that can efficiently exploit P from inorganic and organic sources naturally occurring in arable soils.

In nature, plants can take advantage of a number of mechanisms to solubilize P in the soil, from the exudation of low molecular weight organic acids to liberate sorbed-P from particle surfaces (Jones, 1998; Hinsinger, 2001; Dakora and Phillips, 2002; Bertin et al., 2003) along with production of P-degrading enzymes to mineralize P in the rhizosphere (Tadano and Sakai, 1991; Tadano et al., 1993; Duff et al., 1994; Li et al., 1997; Richardson et al., 2000; George et al., 2002), the zone of soil directly under the influence of plant roots. The rhizosphere bacterial community also plays a role, with so-called P solubilizing bacteria mineralizing P near the roots with some fraction available for plant uptake (Rodriguez and Fraga, 1999; Wu et al., 2005; Chen et al.; 2006; Richardson and Simpson, 2011). However, despite the critical nature of P for plant growth, the physiological basis underpinning how plants sense and respond to differing forms of soil P is poorly understood.

One technique that has the capacity to 'lift the lid' on this black box is metabolomics. Over the last two decades metabolomics have become increasingly important not only to illustrate secondary metabolite expression in plant tissue, but also to link genotypic to phenotypic changes through specific metabolic marker patterns and to identify plant responses and adaptations to environmental influences such as climate change and various biotic (e.g. pathogens) and abiotic (e.g. nutrient deficiency, drought, salinity) stresses.

More recently, cereal metabolomics have become an established tool in cereal cultivar improvement. Analyzing differences and changes in metabolic patterns allow breeders to evaluate the ability of cultivars to respond and adapt to difficult environmental conditions such as drought, flooding, parasites, disease or nutrient deficiency (Fernie and Schauer, 2009; Khakimov et al., 2014). It enables breeders to select cultivars suitable for specific local growth conditions and therefore helps to overcome

challenges that have emerged from climate change, increase in food demand and crop yield enhancement under more sustainable agricultural approaches such as reduced input of fertilizers, pesticides and water.

Information relating to adaptations of plant metabolic pathways to P availability is still limited, especially for cereals. So far, most research has been focused on metabolic responses to Pi limited conditions. Under Pi deficiency, distinctive changes in certain groups of metabolites affecting carbon, nitrogen and lipid metabolism and enabling Pi remobilization have been reported, such as a general reduction of P-containing intermediates such as nucleotides (Zrenner et al., 2006), the substitution of phospholipids with galactolipids (e.g. Dormann and Benning, 2002) and reduced levels of RNA (Hewitt et al., 2005).

Even though it is important to understand metabolic responses to Pi in order to find more sustainable methods for a secure crop P supply, critically Pi only comprises the smallest portion of soil P. A substantial amount of P naturally occurs in organic forms such as DNA or phytic acid, which are thought to be largely inaccessible to crops. However, it is highly likely that sustainable methods will rely on exploiting these P sources since they represent the major stocks of naturally occurring P in soil. Therefore, it is essential to understand metabolic responses of crops to organic P. With this in mind, this study analyses metabolite profiles of roots of the most common cereal grown in the UK, *Triticum aestivum* that have been either exposed to different organic P compounds or readily available Pi. Through this, the study tests the hypothesis that wheat roots are able to recognize different P forms and alter their metabolism accordingly. It is assumed that pathways sensitive to P stress, such as glycolysis, the TCA cycle, pentose phosphate pathway and plant hormone biosynthesis, are also responsive to different P forms and thus, show distinctions in their metabolite abundances.

2.2 Materials and Methods

2.2.1 Phosphorus treatments

Three different phosphorus compounds were used: di-sodium hydrogen orthophosphate (Na_2HPO_4, Fisher Scientific), phytic acid dodecasodium salt from rice (Sigma-Aldrich) and DNA sodium salt from herring testes (Sigma-Aldrich). According to the purity batch analysis by the maunfacturer phytic acid dodecasodium salt had an inorganic phosphorus impurity of ≤ 0.1%. Di-sodium hydrogen orthophosphate was chosen to represent inorganic phosphate readily available to plant roots for uptake. Phytic acid and DNA are complex organic P forms that are predominant in soils.

2.2.2 Experimental set-up and plant growth

Seeds of *Triticum aestivum* L. cv. Cadenza were surface sterilized in 3% calcium hypochlorite for 10 min and subsequently rinsed with sterile distilled water. The seeds were then germinated on sterile moist Whatman No 1 filter paper at 18°C in a temperature controlled greenhouse chamber for 7 days.

For metabolite profiling and total P determination, square petri dishes (10x10x2cm) were pierced with a lateral groove on one side with sterile forceps in a laminar flow cabinet to provide an outlet for the wheat shoot. A 0.8% agar medium was prepared containing the following nutrients per litre: 147.33 mg $MgSO_4 \cdot 7H_2O$, 212.1 mg $(NH_4)_2SO_4$, 217.12 mg $KHSO_4$, 235.2 mg $CaCl_2 \cdot 2H_2O$, 13.6 mg FeNa-EDTA, 0.9 mg $MnSO_4 \cdot 4H_2O$, 0.117 mg $ZnSO_4 \cdot 7H_2O$, 0.103 mg $CuSO_4 \cdot 5H_2O$, 1.24 mg H_3BO_3 and 0.052 mg $NaMoO_4 \cdot 2H_2O$, 2.36 mg NaCl. The agar medium was adjusted to pH 6 with 1M NaOH and sterilized at 126°C for 11 minutes. 100ml of the sterile medium was then poured into each petri dish in the flow cabinet. After cooling all dishes were sealed with parafilm, stored for 7 days and then checked for any contamination. One wheat seedling was transferred to each square petri dish in the laminar flow cabinet (Fig. 2.1) with the shoot protruding from the lateral groove. Afterwards, all petri dishes were re-sealed with sterile lanolin at the lateral groove

and parafilm, then subsequently wrapped in aluminium foil in order to keep the roots in the dark. The seedlings were grown for another 7 days in a temperature and light controlled growth chamber at 18°C and 16 hours of daylight until phosphorus application, which resulted in a total growth period of 14 days prior to the P applications. A longer growth period was not possible due to difficulties keeping the mesocosms sterile as a result of spontaneous microbial infection.

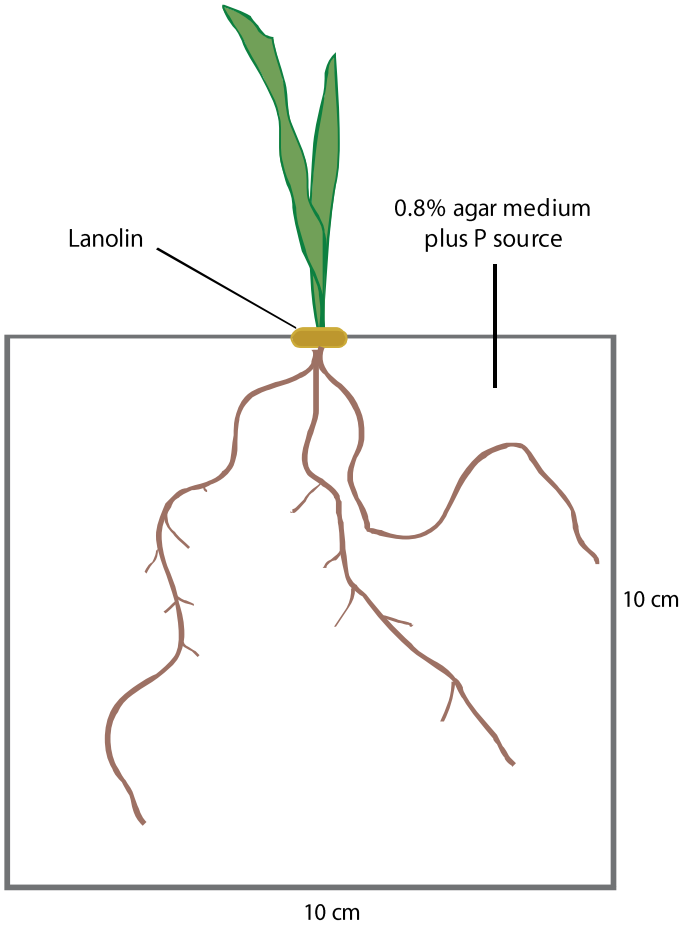


Fig 2.1: Diagram of the axenic mesocosms. The petri dishes were sealed with sterile lanolin (yellow oval) and parafilm to avoid contamination.

2.2.3 Phosphorus application

Each P source was administered in solution having been dissolved in sterile de-ionized water with a final P concentration of 100 µg/ml. Prior to applying the P sources; the petri dishes were checked for any sign of contamination. In a flow cabinet, 10 ml of the

different P solutions were spread evenly onto the agar plates, applying a total P content of 1mg/ml. In the case of phytic acid, up to 1 µg of the 1mg P is inorganic P due to the product impurity of ≤ 0.1%. The plates were incubated for 5, 24, 48 and 72 hours, respectively. For subsequent metabolite profiling six replicates of each P source and time point had been prepared. Furthermore, for each of these time points, six control plates were prepared that did not receive any P.

For tissue biomass and P concentration determination, another set of agar plates with five replicates for each P source and no P treatment were also incubated for 72 hours after P administration.

2.2.4 Harvest and sample storage

The square petri dishes were opened and rinsed with de-ionized water in order to remove the residual P solution. Plates that showed any sign of contamination after the incubation period were discarded. For metabolite analyses, three subsamples (10-20 mg) of root tissue from different sections and one sample of leaf tissue (50-100 mg) were cut off, weighed and flash frozen in liquid nitrogen. All samples were subsequently stored at -80°C. For total tissue P determination, root and shoot tissues were separated, frozen at -20°C and subsequently freeze-dried. The samples were then stored in a desiccator until further processing.

2.2.5 Metabolite extraction

Non-targeted metabolite profiling enables to detect overall changes in the metabolome to different treatments, in this case the P form. However, it needs to be considered that this analysis only shows a snapshot of the metabolic fingerprint at the time of extraction. In addition, it only detects changes in metabolic responses that last longer than a few seconds. Many metabolites are quite labile and highly reactive; thus their turnover can occur within nanoseconds. Tissue sampling and extraction methods are

too slow to capture these metabolic changes. In order to minimize this problem, tissue samples were shock frozen (see section 2.24) to stop metabolic reactions, and extractions were performed under sub-zero conditions.

Metabolites were extracted using a standard bi-phasic chloroform-methanol-water extraction (Overy et al., 2005). Root samples were ground to a fine powder with a pestle and 100 µl of a methanol/chloroform/de-ionized water mixture (2.5:1:1, v/v/v, pre-chilled at -20°C) added. All sample tubes were vortexed for 5 seconds and incubated for 5 minutes on a cooling block that was pre-cooled at -20°C. The tubes were then centrifuged for 2 minutes at 14000 rpm and 4°C and the supernatant transferred to a new tube. The remaining tissue pellets were re-extracted with 50 µl of methanol/chloroform (1:1, v/v, pre-chilled at -20°C), vortexed for 5 seconds, incubated for 5 minutes on the cooling block and centrifuged for 2 minutes at 14000 rpm and 4°C. Afterwards, the supernatant was added to the tube containing the first supernatant. In order to separate the polar (aqueous) phase from the organic (chloroform) phase, 25 µl of sterile de-ionized water was added. The tubes were vortexed for a few seconds and centrifuged for 2 minutes at 14000 rpm and 4°C. Each phase was carefully transferred to separate tubes. All extracts were stored at -80°C until analysis.

2.2.6 Direct injection time of flight electrospray mass spectrometry analysis

Non-targeted direct injection time of flight electrospray mass spectrometry analysis (DIMS) was chosen to investigate P from related variations in wheat root metabolite profiles. This analysis is a robust method that allows a high sample throughput with high reproducibility and stable instrumentation characteristics that enhances sample comparability (Viant and Sommer, 2013). There are two major limitations of DIMS, which are ion suppression and non-separation of isobaric compounds and stereoisomers. High concentrations of metabolites that are easily ionisable can outcompete compounds of lower abundance for the electronic charge and thus, prevent their ionization, known as

charge theft (Viant and Sommer, 2013). However, this ion suppression can be reduced by sample dilution as there is often a non-linear relationship between the concentration of chemical agent responsible for charge theft and the degree of ion suppression. In this study, a 1:10 dilution of the root extracts resulted in the best ionization of metabolites of lower abundance. In the case of the non-separation of isobaric compounds and steroisomers, this study took into account and listed all potential compounds with the same monoisotopic mass for each discriminatory mass bin during subsequent putative metabolite identification (see section 2.3.2).

Polar phases of metabolite extracts were analysed on a Waters/Micromass LCT-ES-MS in both electrospray ion modes (ESI). The capillary voltage was 2.8 kV in the ESI- mode and 3.2 kV in the ESI+ mode, with a cone voltage of 15 V and 10 V, respectively. Nitrogen was used as desolvation gas with a flow rate of 400 l/hour in both ion modes. No cone gas flow was used. Desolvation and source temperature were 120°C and 110°C in ESI- mode and 150°C and 100°C in ESI+ mode. The acquisition method was set to a mass scan range of 50-800 *m/z* and a scan time of 0.5 seconds. The mass error between the measured and accurate ion mass is 5 ppm (0.0005%).

For analysis, replicate extractions were combined and as mentioned above diluted 1:10 with a methanol/de-ionized water mixture (50:50). The diluted extracts were then injected into the LCT-ES-MS via auto sampler of the Waters Alliance 2695 HPLC unit. The injection volume was 200 µl and the mobile phase methanol/de-ionized water (50:50) containing 0.1% formic acid with a flow rate of 0.04 ml/min. Each run lasted 10 minutes. In order to correct for mass shifts the LCT-MS was connected to a lock spray using a standard solution of sulphadimethoxine (10 µg/ml) and a flow rate of 0.02 ml/min.

2.2.7 Phosphorus analysis

For total P determination, the freeze-dried root and leaf samples were homogenized by grinding to a fine powder and digested using the Kjeldahl method (Allen,

1989). All samples were digested and analysed in triplicates using 50 mg of sample each digested in 1 ml of a salycilic acid/sulphuric acid with a lithium sulphate/copper sulphate catalyst. Samples were digested at 370°C for approximately 4 hours until the digests were clear. After cooling to room temperature the samples were diluted to 25 ml with distilled water.

Total P concentrations were determined by colorimetric analysis using the ammonium molybdate antimony potassium tartrate method adapted from Murphy and Riley (1962) and John (1970). An aliquot of 0.5 ml of each sample was transferred to 4 ml cuvettes. To each aliquot 0.2 ml of 0.1M L-ascorbic acid and 0.5 ml of the colour developing ammonium molybdate and antimony potassium tartrate solution were added and a final volume of 3.8 ml were made up with dH_2O. 500 ml of the colour developing solution contained 2M sulphuric acid, 4.8 g of ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$) and 0.1 g of antimony potassium tartrate ($C_6H_4O_7SbK$). The samples were left for 45 minutes for colour development and absorbance subsequently measured on a spectrophotometer (Cecil CE 1020, Spectronic, Leeds, UK) at 882 nm. P concentrations were calculated by using a P standard curve with P concentrations between 0 and 5 ppm of sodium di-hydrogen orthophosphate ($NaH_2PO_4.H_2O$).

2.2.8 MS data processing and statistics

The impact of the different P sources on tissue dry weights and P concentrations were assessed by one-way ANOVA followed by TukeyHSD tests using the R statistical package (R Development Core Team, 2011).

Raw MS data were converted to centroid data using MassLynx 4.0 (Waters Ltd., Watford, Herefordshire, UK). Subsequent noise reduction and mass binning was performed using an in-house macro run in Excel 2007as defined by Overy et al. (2005). The bin size was set to 0.2 atomic mass units (amu). This means that, for example, mass bin 130 contains masses ranging from 129.9 to 130.1 amu. Principal component analysis

(PCA) followed by orthogonal partial least square discriminant analysis (OPLS-DA) was used to investigate differences in metabolite data between the various P treatments. PCA is an unsupervised, multivariate technique commonly used in metabolite data analyses in order to identify general differences and similarities among groups and/or variables (e.g. Khakimov et al., 2014). The analysis has been conducted on the average intensities (ion counts) per mg root tissue of the mass bins detected in either positive or negative ion mode. Average intensities normalized to one mg root tissue have been chosen, since, even though they don't give absolute concentrations, they can be used as a measure of abundance of one mass relative to another mass in the same sample or relative to the same mass in another sample.

OPLS-DA is a supervised, multivariate analysis that uses a linear regression model to correlate X data matrix with a Y data matrix in order to identify and highlight relationships among the observed data (Bylesjo et al., 2006; Sugimoto et al., 2012; Khakimov et al., 2014). Further, it reduces noise seen in PCA plots, which makes it easier to identify relationships between variables. Here, OPLS-DA was used to identify mass bins responsible for metabolite profile discriminations seen in the PCA plots. However, using OPLS-DA on such large datasets can cause random associations. Therefore, subsequent one-way ANOVA followed by TukeyHSD test (R statistical package) has been conducted using average intensities (per mg root tissue) of each selected mass bin in order to check if there really are significant differences between P treatments at one or more time points. In cases where data did not fulfil ANOVA's assumptions, average intensities were log transformed.

Mass bins showing most significant discriminations after OPLS-DA were selected and putatively identified using an in house database as well as online resources such as Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/), GOLM Metabolome Database (http://gmd.mpimp-golm.mpg.de/) and Metacyc (http://metacyc.org/).

2.3 Results and Discussion

2.3.1 Dry weights, phosphorus concentrations and root-to-shoot ratios

There were no significant differences in plant tissue dry weights and P concentrations between the different P treatments (Fig. 2.2). However, there seemed to be a trend towards lower tissue biomass in control plants as well as seedlings incubated with phytic acid. No significant increase in P concentrations compared to the control plants has been observed. Each P source has been administered in solution. The solution was partially absorbed by the agar, and in combination with the small sizes of the roots at that growth stage, P uptake might have been too slow to detect higher P concentrations than the control plants after 72 hours of incubation.

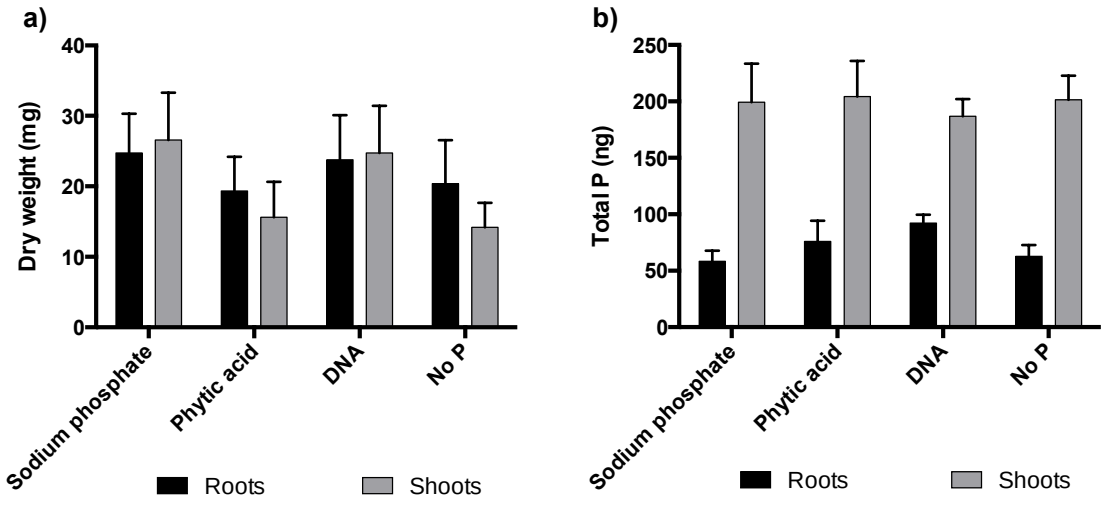


Fig. 2.2: Root and shoot dry weights (a) as well as total P contents (b) of wheat seedlings after 14 days of growth and an additional incubation period of 72 hours. Error bars represent the standard error of the mean. No statistically significant differences were found (n=5, p>0.05, one-way ANOVA, TukeyHSD post-test).

Another reason for the absent differences in total P contents among the treatments could be that the wheat seedlings still acquire P from the aleurone and scutellum reserves where phytate can account for 75±5c% of the total seed P content (Bartnik and Szafranska, 1987; Brinch-Pedersen et al., 2002; Lott et al., 2000). Phosphorus from seed phytate is remobilised by endogenous seed phyatases. Azeke et al. (2011) reported

highest phytase activity in wheat seeds in combination with a reduction of phytate concentrations by 86% after eight to ten days of germination. These results suggest that phytate reserves in wheat seeds may have been already exhausted after 14 days of growth. However, seed phytate contents and phytase activity vary in different wheat cultivars (Barrier-Guillot et al., 1996), which in turn can reduce or prolong seed phytate utilization. Hence, it cannot be excluded that, in this study, wheat seedlings were still using seed-borne phytate as an additional P source after 14 days of growth.

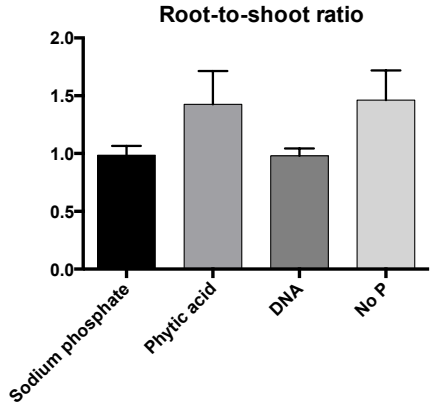


Fig. 2.3: Root-to-shoot ratio of wheat seedlings after 14 days of growth and an incubation period of 72 hours. Error bars represent the standard error of the mean. No statistical significant differences were found (n=5, p>0.05, one-way ANOVA, TukeyHSD post-test).

Root-to-shoot ratios seemed to be higher in seedlings that received either phytic acid or no P (Fig. 2.3). While, this trend was not statistically significant, an increase in root-to-shoot ratios is thought to be a common P stress response in order to improve soil P exploration and exploitation (Hermans et al., 2006; Zhang et al., 2014). For instance, Arabidopsis (Lopez-Bucio et al., 2003; Sanchez-Calderon et al., 2006) as well as beans (Cakmak et al., 1994; Lynch and Brown, 2001) showed higher root-to-shoot ratios through changes in root architecture such as lateral root growth and root hair formation. Thus, higher root-to-shoot ratios of wheat seedlings grown with phytic acid seems to indicate that these plants have difficulty accessing this organic P source and therefore suffering from P stress. In contrast, the (non-statistically different) lower root-to-shoot ratios of seedlings grown with DNA suggest seedlings were able to exploit DNA. None-the-less,

since there were no significant differences in P concentrations, longer incubation periods are necessary to confirm this.

2.3.2 Metabolite profiles

According to PCA, differences in metabolite profiles were already apparent after 5 hours between the P treatments and the non-P control (Fig. 2.4) as well as between the inorganic (sodium phosphate) and both organic (DNA and phytic acid) P treatments (Fig. 2.5) in both ESI modes. However, no clear distinction between the two organic treatments was found (Fig. 2.5). These patterns remained mostly constant throughout the 72 hours incubation period except for some variations where discriminations were not as pronounced. The early discrimination between the inorganic and organic P as well as the no P treatment already after five hours of incubation strongly suggests that the changes in root metabolism is likely a response to the ability of wheat roots to sense and distinguish exogenous inorganic and organic P forms rather than to P or growth status of the plant.

Over two hundred mass bins were selected from both ESI modes and putatively identified. In many cases, more than one compound was assigned to one mass bin, since bin sizes were 0.2 amu and differences of mono-isotopic masses between compounds were often much smaller than that. Furthermore, mass bins with masses (*m/z* values) higher than 400 or smaller than 90 were often impossible to identify. This might be due to the possibility that these molecules are fragments of larger molecules that broke apart during metabolite extraction or LCT-MS analysis. Prior to one-way ANOVA, average intensities of mass bins in ESI+ mode that were adducts of the same compound were combined. Tables 2.1 and 2.2 show mass bins that were possible to putatively identify and that had numerous significant differences between P treatments. Most frequently represented compound groups were carboxylic acids, sugars and sugar phosphates, amino acids, plant hormones and phenylpropanoids. These suggest that metabolic pathways that might have been affected by the different P treatments were glycolysis, the pentose

phosphate pathway, shikimic acid pathway and phenylalanine biosynthesis as well as phenylpropanoid and plant hormone biosynthesis.

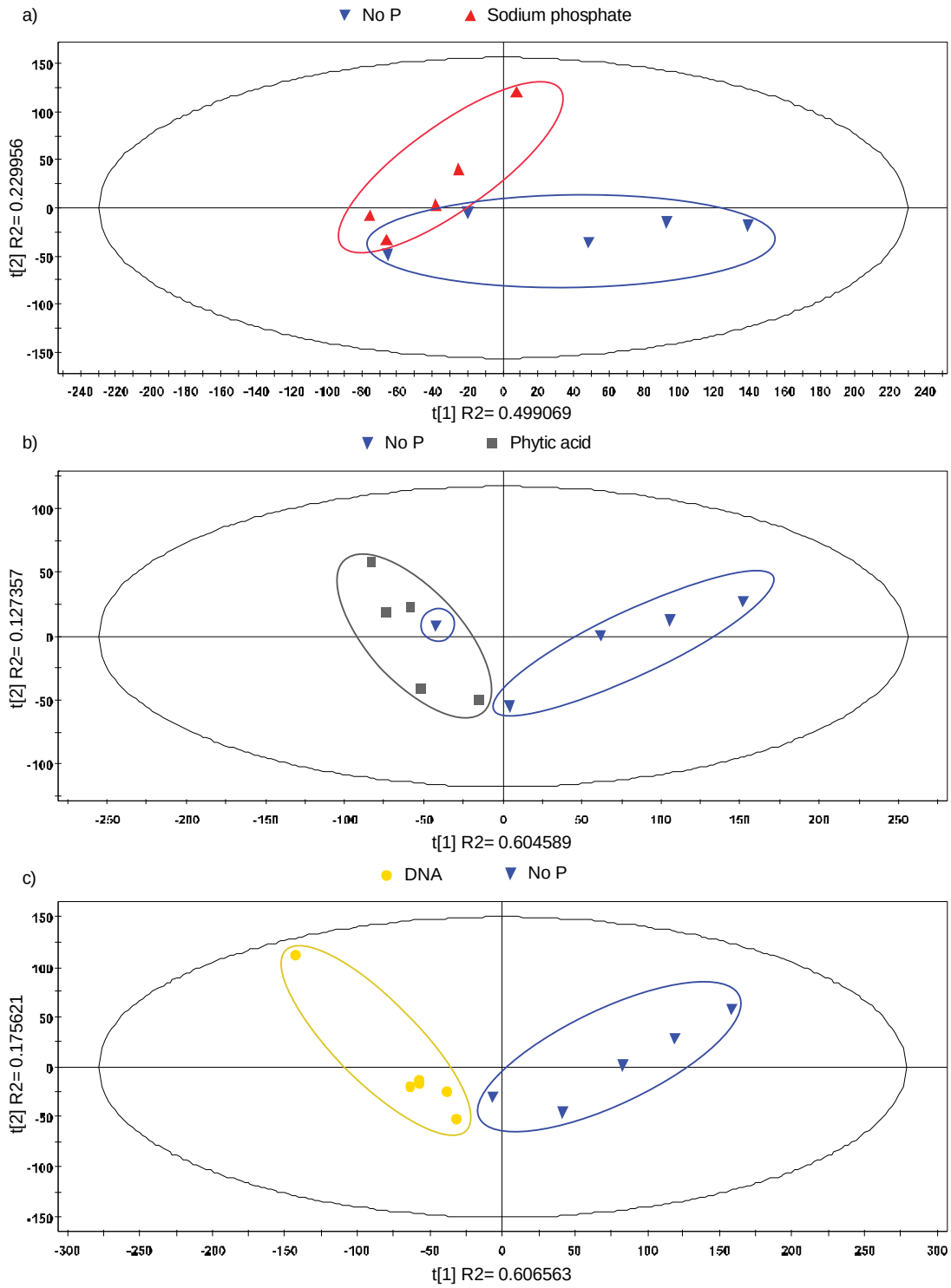


Fig. 2.4: PCA plots showing differences in metabolite profiles between the control plants and P treatments. Profiles were detected after 5 hours of incubation and analysed in ESI+ mode. (a) No P versus sodium phosphate, (b) no P versus phytic acid, (c) no P versus DNA.

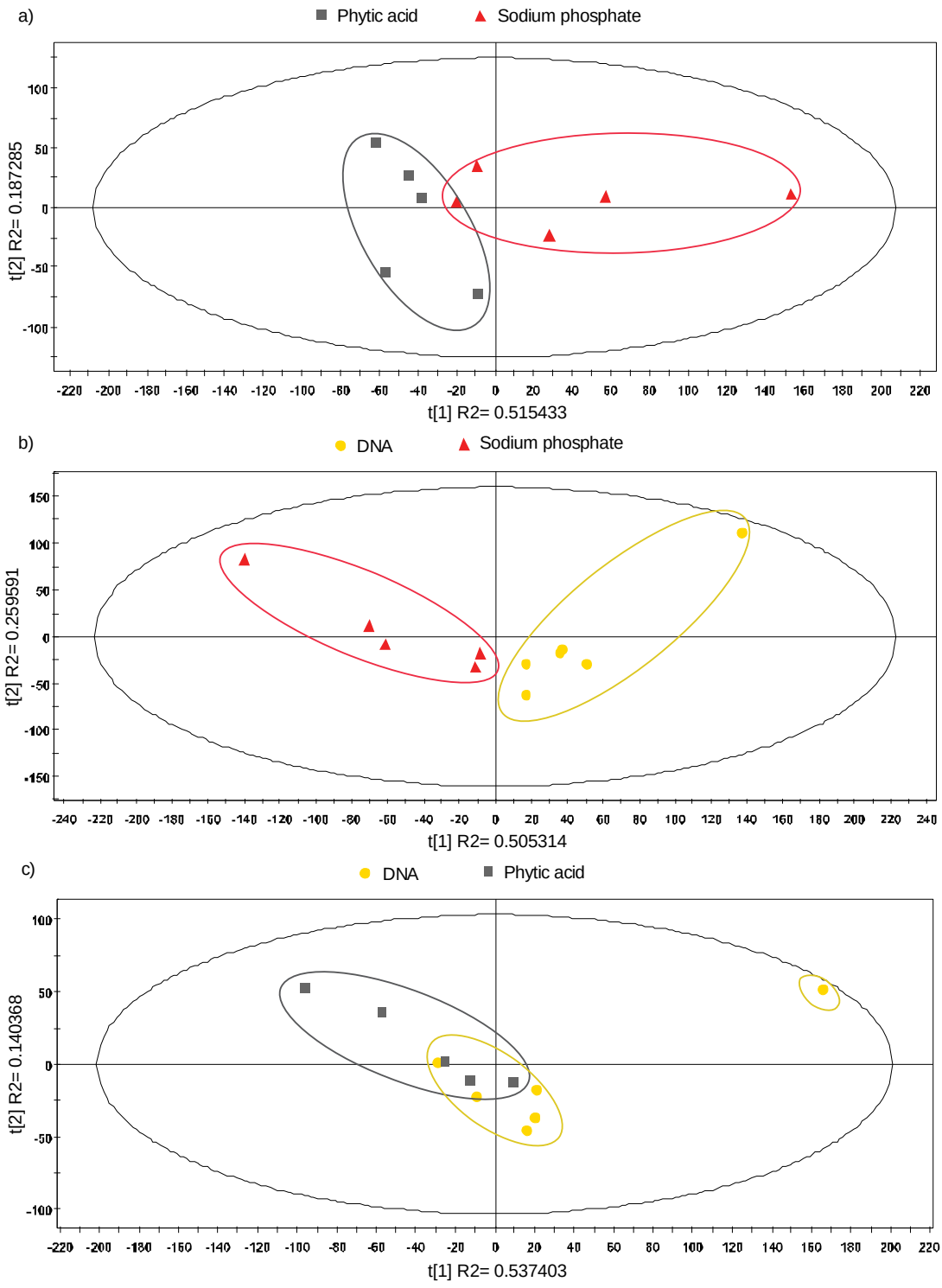


Fig. 2.5: PCA plots showing discriminations of metabolite profiles between the different P treatments. Profiles were detected after 5 hours of incubation and analysed in ESI+ mode. (a) Phytic acid versus sodium phosphate, (b) DNA versus sodium phosphate, (c) phytic acid versus DNA.

Table 2.1: ESI- discriminatory mass bins with detected (without an H^+ ion, *m/z* value 1.008) and accurate *m/z* values, putative compound identifications and statistically significant differences of ion count intensities per mg root tissue between P treatments (SP= sodium phosphate, PA= phytic acid, no P= control plants that did not receive any P) after 5, 24, 48 and 72 hours of incubation. *= p< 0.05; **= p< 0.01; *** = p< 0.001; ns= no significant differences (n=5, one-way ANOVA, post Tukey HSD). Variations between the accurate and detected *m/z* values are within the 5ppm mass error.

m/z-	Detected *m/z*	Accurate *m/z*	Putative compound	Compound group	5 hours	24 hours	48 hours	72 hours
	131.0344	132.0423	Glutaric acid	Carboxylic acid				
	130.9981	132.0059	Oxalacetic acid	Carboxylic acid			SP-DNA **	
	131.0344	132.0423	2-acetolactate	Fatty acid			SP-no P **	
131	131.0344	132.0423	3-hydroxy-3-methyl-2-oxobutanoate	Carboxylic acid	ns	SP-no P *	SP-PA **	ns
							SP-DNA ***	
							SP-no P **	
132	132.0297	133.0375	L-aspartic acid	Amino acid	ns	SP-PA *	SP-PA ***	ns
	133.0137	134.0215	Malic acid	Carboxylic acid				
	133.0501	134.0579	2,3-dihydroxy-3-methylbutanoate	Carboxylic acid		PA-no P *		
133	133.0501	134.0579	Deoxyribose	Monosaccharide	SP-DNA *	SP-PA **	ns	ns
					No P-DNA ***			
					SP-DNA **			
	145.0137	146.0215	2-oxoglutarate	Carboxylic acid	PA-no P ***			
145	145.0501	146.0579	2-dehydropantoate	Carboxylic acid	SP-PA *	SP-no P *	ns	ns
							SP-DNA ***	SP-DNA *
							SP-no P ***	SP-no P *
157	157.0501	158.0579	2-isopropylmaleate	Carboxylic acid	ns	ns	SP-PA ***	SP-PA *
					No P-DNA **			
					SP-DNA **			
					PA-no P ***	PA-no P *		No P-DNA **
164	164.0704	165.079	Phenylalanine	Amino acid	SP-PA ***	SP-PA *	ns	PA-no P *
							SP-DNA ***	
							SP-no P ***	SP-DNA *
165	165.0552	166.063	Phenyllactate	Carboxylic acid	SP-PA *	ns	SP-PA ***	SP-PA *
	166.9746	167.9824	Phosphoenolpyruvic acid (PEP)	Carboxylic acid			SP-DNA ***	SP-DNA *
	167.0821	168.0899	Pyridoxamine	Vitamin			SP-no P***	SP-no P ***
167	167.0344	168.0423	Vanillic acid	Phenolic acid	ns	SP-no P **	SP-PA ***	SP-PA *
							SP-DNA **	SP-DNA *
	168.0062	169.014	3-Amino-2-oxopropyl phosphate	Organic phosphate			SP-no P *	SP-no P **
168	168.0661	169.0739	Pyridoxine	Vitamin	ns	ns	SP-PA ***	SP-PA **
							SP-DNA **	SP-DNA ***
	173.0449	174.0528	(2S)-2-isopropyl-3-oxosuccinate	Carboxylic acid			SP-no P *	SP-no P *
173	173.0449	174.0528	Shikimic acid	Carboxylic acid	ns	SP-PA *	SP-PA **	SP-PA *

m/z-	Detected *m/z*	Accurate *m/z*	Putative compound	Compound group	5 hours	24 hours	48 hours	72 hours
174	174.0555	175.0633	Indole-3-acetic acid	Plant hormone (Auxin)	ns	ns	ns	SP-DNA*
	179.0556	180.0634	alpha-D-glucose	Monosaccharide				
	179.0556	180.0634	beta-D-glucose	Monosaccharide				
	179.0556	180.0634	D-fructose	Monosaccharide				
	179.0556	180.0634	D-galactose	Monosaccharide				
	179.0556	180.0634	D-glucose	Monosaccharide				
	179.0556	180.0634	D-mannose	Monosaccharide				
	179.0344	180.0423	Caffeic acid	Phenylpropanoid (monolignol)	No P-DNA ***	No P-DNA *		No P-DNA **
	179.0708	180.0786	Coniferyl alcohol	Phenylpropanoid (monolignol)	SP-DNA *	PA-no P ***		SP-DNA *
179	179.0556	180.0634	Myo-inositol	Cyclitol	PA-no P **	SP-no P **	SP-PA *	PA-no P *
							SP-DNA ***	No P-DNA **
					No P-DNA ***		PA-no P *	SP-DNA **
					SP-DNA *		SP-no P *	PA-no P *
180	180.0661	181.0739	L-Tyrosine	Amino acid	PA-no P **	PA-no P **	SP-PA ***	SP-PA *
					No P-DNA **			
	181.0712	182.079	Dulcitol	Alditol	SP-DNA **			
	181.0712	182.079	Mannitol	Alditol	PA-no P ***	No P-DNA *		
181	181.0712	182.079	Sorbitol	Alditol	SP-PA **	PA-no P *	ns	SP-DNA *
							SP-DNA *	SP-DNA *
195	195.0505	196.0583	D-gluconic acid	Monosaccharide	ns	ns	SP-PA *	SP-PA *
							SP-no P *	SP-DNA *
197	197.0449	198.0528	Syringic acid	Phenolic acid	ns	ns	SP-PA *	SP-no P *
					SP-DNA *			No P-DNA **
214	214.0117	215.0195	O-Phospho-4-hydroxy-L-threonine	Modified amino acid	SP-PA **	SP-DNA *	ns	PA-no P *
					No P-DNA ***			
					SP-DNA ***	No P-DNA *		
	215.0321	216.0399	2-C-Methyl-D-erythritol 4-phosphate	Alditol phosphate	PA-no P ***	PA-no P ***		
215	214.9957	216.0035	4-Phospho-D-erythronate	Fatty acid	SP-PA *	SP-no P ***	ns	No P-DNA **
						No P-DNA *		
	225.0399	226.0477	Chorismate	Carboxylic acid	No P-DNA *	PA-no P **		
225	225.0399	226.0477	Prephenate	Caboxylic acid	PA-no P *	SP-no P *	ns	PA-no P *
							SP-DNA *	SP-DNA *
							SP-no P *	SP-no P **
227	227.0708	228.0786	Xanthyletin	Phenylpropanoid (coumarin)	ns	ns	SP-PA **	SP-PA **
	229.0113	230.0192	D-ribose-5-phosphate	Phospho sugar			SP-DNA **	
	229.0113	230.0192	D-ribulose-5-phosphate	Phospho sugar		SP-no P *	SP-no P **	
229	229.0113	230.0192	D-xylulose-5-phosphate	Phospho sugar	ns	SP-PA *	SP-PA **	ns

m/z-	Detected *m/z*	Accurate *m/z*	Putative compound	Compound group	5 hours	24 hours	48 hours	72 hours
					No P-DNA ***			
					SP-DNA *			
	243.0803	244.0882	Biotin	Vitamin	PA-no P **			No P-DNA *
243	243.0617	244.0695	Uridine	Nucleoside	SP-PA *	ns	ns	SP-DNA *
					No P-DNA *			SP-DNA ***
					SP-DNA **			SP-no P ***
255	255.0657	256.0736	Isoliquiritigenin	Phenylpropanoid (flavonoid)	SP-PA *	ns	ns	SP-PA ***
								SP-DNA *
								SP-no P **
257	257.0062	258.0141	D-Glucono-1,5-lactone 6-phosphate	Carbohydrate lactone	ns	ns	ns	SP-PA *
	259.0219	260.0297	1L-myo-inositol-1-phosphate	Organic phosphate				
	259.0219	260.0297	alpha-D-glucose-6-phosphate	Phospho sugar				
	259.0219	260.0297	beta-D-fructose-6-phosphate	Phospho sugar	No P-DNA ***			
	259.0219	260.0297	beta-D-glucose-6-phosphate	Phospho sugar	SP-DNA ***			
	259.0219	260.0297	alpha-D-glucose-1-phosphate	Phospho sugar	PA-no P ***	PA-no P ***		No P-DNA **
259	259.0219	260.0297	fructose-1-phosphate	Phospho sugar	SP-PA **	SP-PA *	SP-PA *	SP-DNA **
								No P-DNA *
								SP-DNA *
	285.0399	286.0477	Kaempferol	Phenylpropanoid (flavonoid)				PA-no P *
285	285.0399	286.0477	Luteolin	Phenylpropanoid (flavonoid)	ns	ns	ns	SP-PA *
							SP-DNA ***	SP-DNA **
	289.0712	290.079	Catechin	Phenylpropanoid (flavonoid)	SP-DNA *		SP-no P ***	SP-no P *
289	289.0325	290.0403	D-sedoheptulose-7-phosphate	Phospho sugar	SP-PA *	ns	SP-PA ***	SP-PA ***
						SP-DNA **	SP-DNA *	
293.2	293.2481	294.2559	Sterculic acid	Fatty acid	SP-PA *	SP-PA ***	SP-PA *	ns
						No P-DNA *		
						SP-no P **		SP-DNA *
300	300.0484	301.0563	N-Acetyl-D-glucosamine 6-phosphate	Phospho sugar	ns	SP-PA *	ns	SP-PA *
							SP-DNA *	
309.2	309.1603	310.1654	Sinapoylcholine	Phenylpropanoid (monolignol)	SP-PA *	SP-PA **	SP-PA *	ns
							SP-DNA **	
							SP-no P *	SP-DNA **
313	313.0437	314.0515	5'-phosphoribosyl-N-formylglycinamide	Phospho sugar	SP-DNA *	ns	SP-PA **	SP-PA **
								SP-DNA **
						SP-DNA *		SP-no P **
331.2	331.1545	332.1624	Gibberellin A4	Plant hormone	ns	SP-PA *	ns	SP-PA **
						SP-DNA *	SP-DNA ***	
						SP-no P *	SP-no P **	SP-DNA *
339.2	339.1092	340.1157	beta-D-Fructose 1,6-bisphosphate	Phospho sugar	PA-DNA *	SP-PA **	SP-PA **	SP-PA *

m/z-	Detected *m/z*	Accurate *m/z*	Putative compound	Compound group	5 hours	24 hours	48 hours	72 hours
	341.1084	342.1162	Cellobiose	Disaccharide				
	341.1084	342.1162	Isomaltose	Disaccharide				
	341.1084	342.1162	Maltose	Disaccharide				
	341.1084	342.1162	Sucrose	Disaccharide	No P-DNA **		SP-no P *	
341.2	341.1084	342.1162	Trehalose	Disaccharide	PA-no P ***	ns	SP-PA *	ns
387.2	387.1444	388.1522	Strigyl acetate	Plant hormone (strigolactone)	No P-DNA *** SP-DNA * PA-no P **	ns	SP-PA *	ns

Table 2.2: ESI+ discriminatory mass bins with detected (adducts with either an H^+ [*m/z*= 1.008] or Na^+ ion [*m/z*= 22.99]) and accurate *m/z* values putative compound identifications and statistically significant differences of ion count intensities per mg root tissue between P treatments (SP= sodium phosphate, PA= phytic acid, no P= control plants that did not receive any P) after 5, 24, 48 and 72 hours of incubation. Mass bins representing adducts of the putative compound have been combined before statistical analyses. *= p< 0.05; **= p< 0.01; *** = p< 0.001; ns= no significant differences (n=5, one-way ANOVA, post Tukey HSD). Variations between the accurate and detected *m/z* values are within the 5ppm mass error.

m/z+	Accurate *m/z*	Putative compounds and detected *m/z* values	Compound group	5 hours	24 hours	48 hours	72 hours
147, 169	146.0691	L-Glutamine (147.0770, 169.0589)	Amino acid	No P-DNA *** SP-DNA *** PA-no P ** SP-PA **	SP-DNA **	SP-DNA **	SP-no P *
156, 178	155.0695	L-histidine (156.0773, 178.0592)	Amino Acid	SP-DNA *	ns	ns	No P-DNA * PA-DNA * SP-no P ** SP-PA **
163, 185	162.0317	Umbelliferone (163.0395, 185.0215)	Phenylpropanoid (coumarin)	No P-DNA *** SP-DNA *** PA-no P ** SP-PA *	SP-no P*	SP-DNA * SP-no P *	SP-no P ** SP-PA *
185.2, 207.2	184.1507	Phosphocholine (185.1598, 207.1418)	Amino alcohol	No P-DNA*	PA-DNA*	ns	ns
198, 220	197.0688	L-dopa (198.0766, 220.0586)	Amino Acid	No P-DNA** PA-no P*	No P-DNA*** PA-no P *** SP-no P ***	ns	No P-DNA ** SP-no P *

m/z+	Accurate *m/z*	Putative compounds and detected *m/z* values	Compound group	5 hours	24 hours	48 hours	72 hours
217.2, 239.2	216.2343	gamma-Glutamyl-gamma -aminobutyraldehyde (217.2438, 239.2258)	Modified amino acid	No P-DNA *	ns	SP-DNA ** SP-no P ** SP-PA*	SP-DNA ** SP-no P ** SP-PA**
221, 243	220.0249 220.0848	Imidazole-acetol phosphate (221.0327, 243.0147) 5-hydroxy-L-tryptophan (221.0926, 243.0746)	Organic aromatic compound Modified amino acid	No P-DNA *** SP-DNA ** PA-no P *	No P-DNA * PA-no P ** SP-no P *	ns	No P-DNA ** SP-no P **
222.2, 244.2	221.259	Dihydrozeatin (222.2688, 244.2508)	Plant hormone (cytokinin)	No P-DNA ** SP-DNA * PA-no P *	No P-DNA * PA-no P ** SP-no P *	ns	No P-DNA * SP-no P *** SP-PA **
228, 250	227.0195 227.0794	alpha-D-glutamyl phosphate (228.0273, 250.0093) L-arogenate (228.0872, 250.0691)	Modified amino acid Modified amino acid	No P-DNA * SP-DNA ** PA-no P ** SP-PA **	PA-no P *	ns	No P-DNA* SP-no P *
230, 252	229.0351	5-phosphoribosylamine (230.0430, 252.0249)	Phospho sugar	SP-no P *	PA-no P **	ns	PA-no P *
243, 265	242.0903	Thymidine (243.0981, 265.0800)	Nucleoside	ns	No P-DNA* PA-DNA* PA-no P *** SP-no P ***	ns	PA-no P ** SP-no P *
257.2, 279.2	256.2402	Palmitic acid (257.2481, 279.2300)	Fatty acid	SP-PA*	No P-DNA * PA-no P * SP-no P *	ns	SP-no P **
260.2, 282.2	259.151	D-glucosamine-6-phosphate (260.1598, 282.1418)	Amino sugar	ns	ns	ns	No P-DNA ** PA-no P ** SP-no P **
287.2, 309.2	286.1764	5'-Phosphoribosylglycinamide (287.1858, 309.1678)	Phospho sugar	No P-DNA*	ns	SP-DNA ** SP-no P ** SP-PA*	SP-DNA * SP-no P* SP-PA*

2.3.3 Effect of exogenous organic and inorganic P sources on metabolic pathways

Metabolites that have been putatively identified (Tables 2.1 and 2.2) are intermediates or end products of several metabolic pathways. In order to visualize P source dependent changes in metabolic pathway fluxes, percentage differences of each mass bin's average intensities between plants that received one of the three P forms and the control plants have been calculated. Fig. 2.6 shows a simplified sketch of glycolysis, the pentose phosphate pathway as well as the shikimic acid pathway together with the percentage differences of detected metabolites. In addition, average intensities of metabolites displayed in Fig. 2.6 can be found in Fig. A2.1 and A2.2 in the appendix.

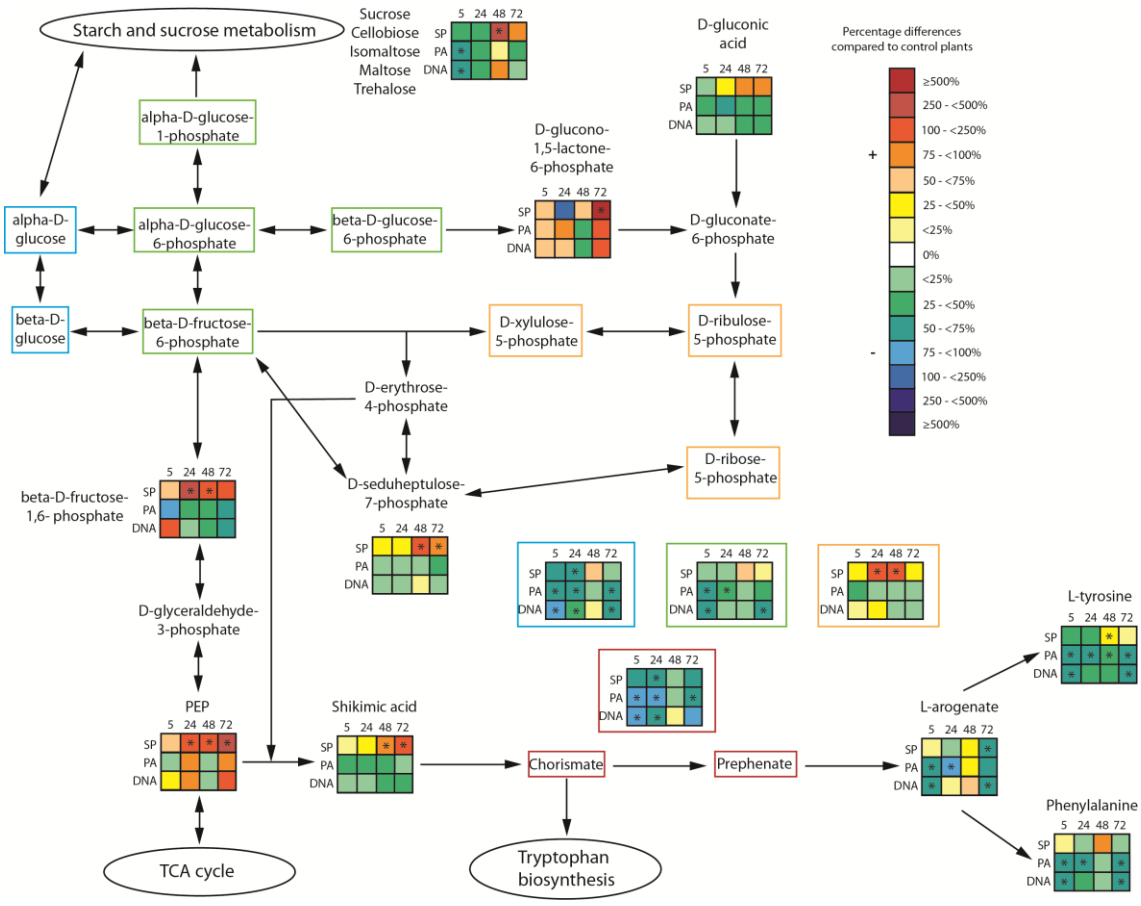


Fig. 2.6: Simplified sketch of glycolysis, the pentose phosphate and shikimic acid pathway. Colour codes represent positive and negative percentage differences in metabolite abundances between the P treated plants and the control plants. Horizontal numbers above grids are hours of P treatment, vertical codes are P compounds supplied (SP, sodium phosphate; PA, phytic acid), * indicates a significant change compared to the no P treatment at $p<0.05$ (n=5, one-way ANOVA, post Tukey HSD).

Differences in abundances of most carboxylic acids, sugars and sugar phosphates were higher between the sodium phosphate treatment and the control plants than the organic P treatments and the control plants, especially after 48 and 72 hours. In the sodium phosphate treatment, increased abundances of metabolic intermediates involved in glycolysis and the pentose phosphate pathway suggest enhanced ATP and NADPH formation. Elevated metabolic abundances along the shikimic acid pathway resulted in higher abundances of L-tyrosine and phenylalanine. However, phenylalanine abundances decreased after 5 hours (Fig. A2.1). This might be an indication for an increased biosynthesis of phenylpropanoids, since phenylalanine is a major precursor of this metabolic group. Furthermore, shikimic acid and chorismate are precursors of tryptophan. In turn, tryptophan is the precursor of indole-3-acetic acid (IAA). Hence, increased metabolic activity along the shikimic acid pathway might have resulted in an increase in IAA.

In control plants, abundances of glucose and sugar phosphates, which are involved in glycolysis, were elevated. Abundances decreased between 5 and 48 hours of incubation, but subsequently increased again (Fig. A2.1 and A2.2). Levels of phosphorylated sugars that are intermediates in the pentose phosphate pathway were significantly lower in controls compared to the sodium phosphate treatment. Reduced levels of various phosphorylated sugars have been observed in Pi starved roots of barley, beans and *Arabidopsis* (Rychter and Randall, 1994; Ciereszko and Barbachowska, 2000; Hernández et al., 2007; Morcuende et al., 2007; Huang et al., 2008; Hammond and White, 2008). These studies concluded that Pi deprived plants salvage P from these small, phosphorylated metabolites in order to use it for more essential functions. Here, this suggests that wheat seedlings reduced pentose phosphate pathway activity to minimize P consumption and at the same time to salvage and remobilize endogenous Pi from already existing sugar phosphates as a result of Pi deprivation. Further, higher abundances of glucose might indicate the onset of glycolysis disruption. However, a concurrent increase in di- and tri-

saccharide abundances that has been observed previously in barley, bean and *Arabidopsis* roots (Rychter and Randall, 1994; Ciereszko and Barbachowska, 2000; Hernández et al., 2007; Morcuende et al., 2007; Huang et al., 2008; Hammond and White, 2008), has not been detected. The reason for this might be that control wheat seedlings were in the early stages of P stress responses and that with progressing Pi deprivation accumulation of di- and tri-saccharides occurs.

Chorismate or prephenate abundances were the highest in control plants. Because phenylalanine and L-tyrosine abundances are similar to the ones observed in seedlings treated with sodium phosphate, elevated chorismate abundances in control plants might indicate a down-regulation of tryptophan and subsequent auxin/IAA biosynthesis. Increased formation of IAA is considered to be a common Pi stress response, since it regulates lateral root and root hair formation (Casimiro et al., 2001; Al-Ghazi et al., 2003; Nacry et al., 2005; Chiou and Lin, 2011; Zhang et al., 2014). However, most studies supporting this have been performed on *Arabidopsis thaliana*. Recently, Li et al. (2012) reported that lateral root growth was inhibited in *Zea mays* under Pi limited conditions, but primary root growth enhanced within first 6 days of Pi deprivation. This suggests that IAA regulated changes in root architecture as a response to Pi deprivation is rather plant specific and does not necessarily lead to the initiation of lateral root formation. This seems to be the case in wheat seedlings, since IAA synthesis biosynthesis appears to be down regulated in control plants.

Exposure to either DNA or phytic acid resulted in low and stable abundances of root metabolites throughout the incubation period (Fig. 2.6). There appeared to be no increase in metabolic activity in contrast to the seedlings that received sodium phosphate, except for PEP and D-glucono-1,5-lactone-6-phosphate. However, the enrichments of both compounds were not statistically significant. Additionally, metabolites along the shikimic acid pathway as well as various sugars and sugar phosphates were depleted compared to the no P treatment (Fig. 2.6). It seems that wheat seedlings were able to detect exogenous

organic P forms and subsequently inhibited any Pi stress response. Further though, due to the fact that P from organic sources is either more difficult to access or completely inaccessible, metabolic activity remains low. Even though it was not statistically significant, the trend to higher root-to-shoot ratios in plants from the phytic acid treatment (Fig. 2.3) might be a first indication that wheat seedlings have greater difficulty in accessing and utilizing P from phytic acid compared to DNA. In order to determine whether wheat seedlings are able to assimilate P from these organic P forms, longer incubation times in combination with ^{33}P pulse chase labelling are necessary to show whether metabolic activity eventually increases as a result of a delayed P supply or whether root metabolism instead changes towards Pi stress responses.

2.3.4 Impact of organic and inorganic P sources on plant hormone abundances

Discriminatory mass bins that show significant differences in relative compound abundances among P treatments at one or more time points have been detected putatively for four plant hormones (Fig. 2.7) (Tables 2.1 and 2.2). The identified plant hormones are dihydrozeatin (cytokinin), IAA (auxin), gibberellins and the strigolactone strigyl acetate.

Most remarkable differences among P treatments were found in cytokinin abundances. Roots of wheat seedlings that were incubated with sodium phosphate had a low abundance of dihydrozeatin after 5 hours. However, over the rest of the incubation period the average intensity steadily increased. Throughout the incubation period, control plants had low average intensities of dihydrozeatin, only showing a slight increase at 48 hours. Plants treated with either DNA or phytic acid exhibited high abundances of dihydrozeatin within the first 24 hours of incubation, which subsequently declined. This was the opposite of what was observed in plants treated with sodium phosphate.

Abundances of IAA remained stable in roots of all P treatments for the whole 72 hours, except for sodium phosphate, which experienced a sharp increase in the average intensity after 48 hours. Elevated abundances of gibberellins were also found in root

metabolite profiles of the sodium phosphate treatment. This trend was only significant at 24 and 72 hours, since variations of the measured intensities in replicates were too high at the other time points. There were no explicit differences in strigolactone abundances. Control plants appeared to have elevated abundances of strigyl acetate, but this was only significant after 5 hours of incubation. Strigyl acetate abundances in plants incubated with sodium phosphate seemed to increase after 24 hours, but this trend was mostly not significant. Strigolactones induce spore germination and hyphal branching of mycorrhiza fungi and mediate root fungal colonization, when released as root exudates (e.g. Balzergue et al., 2011). The establishment of a symbiosis with mycorrhizal fungi is beneficial under Pi limited conditions to improve P supply. Therefore, higher abundances of strygil acetate in roots of control wheat seedlings might indicate an enhanced biosynthesis as a P stress response to induce mycorrhiza fungi colonization.

Antagonistic interactions between cytokinins and auxin/IAA are thought to regulate lateral root initiation and development (Laplaze et al., 2007; Moubayidin et al., 2009; El-Showk et al., 2013) as well as to control and balance cell differentiation and division in root meristems (Dello Ioio et al., 2008; Moubayidin et al., 2009). However, these interactions are highly complex, in which each hormone affects the other's synthesis, transport and degradation, thus controlling their signalling pathways. Induction of lateral root formation relies on the creation of an auxin gradient carried out by PIN transporters (Blilou et al., 2005; Paponov et al., 2005; Laplaze et al., 2007; Moubayidin et al., 2009). Cytokinins are able to inhibit these, thus disrupting auxin transport and as a result inhibiting lateral root development. Furthermore, cytokinins seem to stimulate the production and stability of IAA proteins in *Arabidopsis thaliana* that are involved in auxin degradation (Jones et al., 2010). In addition, cytokinins suppress P starvation response gene up-regulation (Martín et al., 2000; Franco-Zorrilla et al., 2002; Karthikeyan et al., 2002; Franco-Zorrilla et al., 2005). Nordstrom et al. (2004) found that elevated auxin levels caused a decrease in cytokinin biosynthesis within 5 hours in *Arabidopsis* seedlings.

However, increased cytokinin formation did not reduce auxin pools (in this case IAA) or synthesis rate within the first 36 hours. They concluded that while auxin appears to have direct control on cytokinin levels, cytokinins only affect auxin pools in an indirect and less efficient way. Additionally, it has been demonstrated that auxin impacts cytokinin degradation (Werner et al., 2006) and directly regulates cytokinin signaling (Muller and Sheen, 2008).

The presence of sodium phosphate caused a steady increase in root dihydrozeatin abundance throughout the incubation period. Furthermore, there was a concurrent rise in IAA abundance after 48 hours. Since cytokinins do not seem to have a direct impact on auxin biosynthesis (Nordstrom et al., 2004), it was not surprising to see an increase in IAA despite their counteractive relationship. However, elevated IAA abundances unexpectedly did not result in a reduction of dihydrozeatin after 48 hours, even though it has been previously shown that elevated auxin/IAA levels directly lower cytokinin pools (Nordstrom et al., 2004). This suggests that another factor, in this case possibly Pi availability, affects auxin driven cytokinin pool regulation. Here, sodium phosphate is the Pi source readily available for uptake. The amount administered in the experiment was sufficient enough to simulate unlimited Pi supply. In this scenario, it seems that maintaining elevated dihydrozeatin levels in order to prevent P stress response gene up-regulation is more important than IAA induced lateral root formation. This is further supported by the increased abundance of gibberellins, which are known to suppress lateral root formation, but promote primary root growth (Jiang et al., 2007). Therefore, it is likely that under unlimited Pi conditions IAA's main function is to counterbalance cytokinin induced cell differentiation in the root meristem with cell division.

Cytokinins also have different functions in shoot development (Mok and Mok, 2001). Matsumoto-Kitano et al. (2008) demonstrated in a reciprocal grafting experiment the root-to-shoot transport of *trans*zeatin-type cytokinins. In this study, unlimited Pi supply in form of sodium phosphate allows wheat seedlings to invest in shoot rather than

root development. Hence, sustaining increased abundances of dihydrozeatin would enable secure cytokinin supply to the shoot. However, no significant differences in either shoot and root biomass (Fig. 2.2a) or root-to-shoot ratios (Fig. 2.3) compared to the control plants have been detected, although there appeared to be a trend towards lower root-to-shoot ratios.

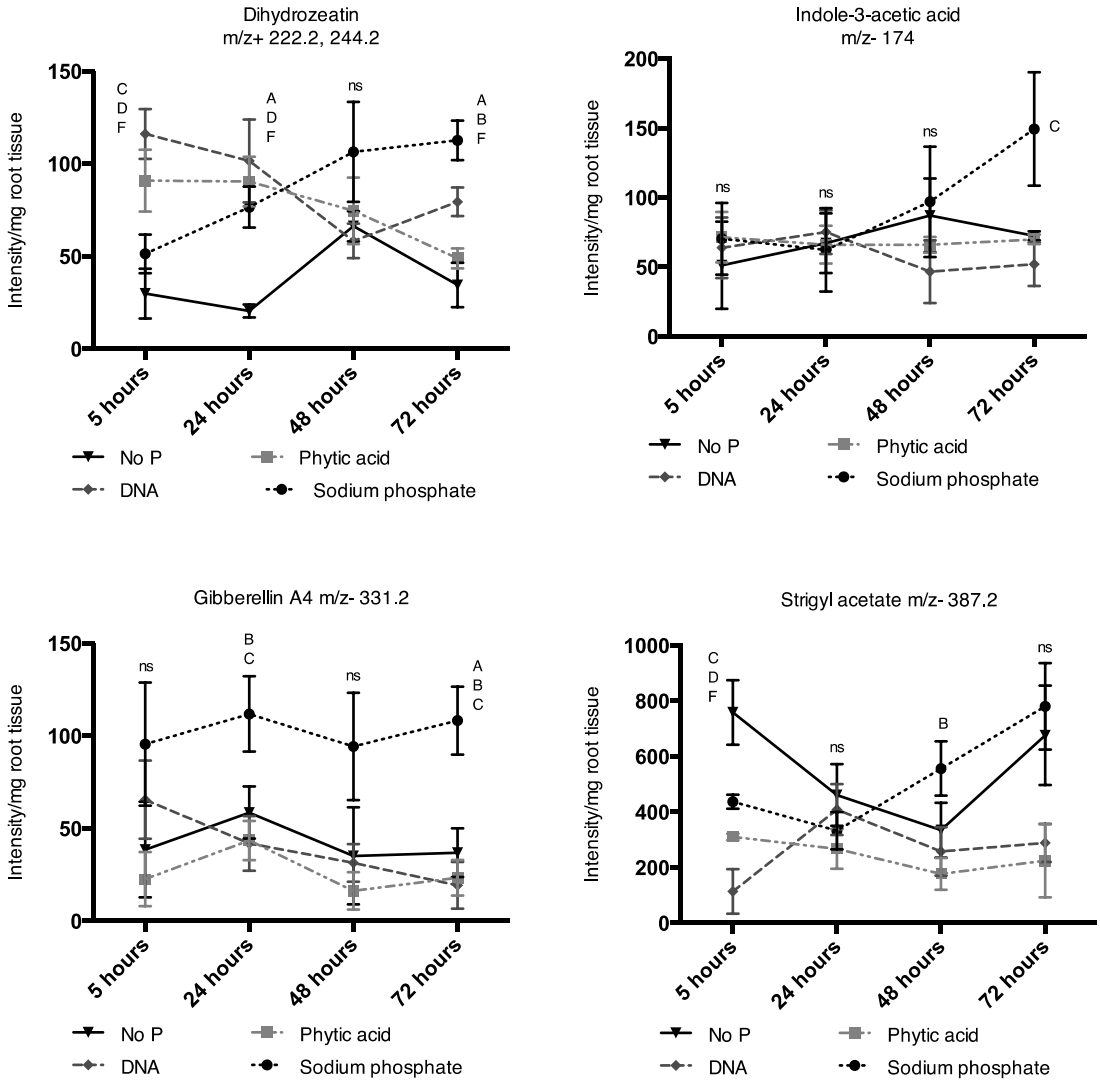


Fig. 2.7: Average intensities (ion counts) per mg root tissue of mass bins of presumed plant hormones after 5, 24, 48 and 72 hours detected in either negative (*m/z-*) or positive (*m/z+*) ion mode. Error bars show the standard error of the mean. All *m/z* values refer to the respective mass bins listed in Tables 2.1 and 2.2. Letters indicate significant differences between the treatments (n=5, $p<0.05$, one-way ANOVA, TukeyHSD post-test): A = Sodium phosphate-no P; B = Sodium phosphate-phytic acid; C = Sodium phosphate-DNA; D = Phytic acid-no P; E = Phytic acid-DNA; F = No P-DNA; ns = not significant.

DNA as well as phytic acid only seemed to have a major impact on dihydrozeatin biosynthesis. Abundances of the other plant hormones remained relatively stable throughout the incubation period. Within the first 24 hours, both organic P sources boosted root dihydrozeatin abundances. These were both significantly higher compared to the control plants, but only in the case of DNA significantly higher than the sodium phosphate treatment. Afterwards, dihydrozeatin levels slowly dropped again. It is unclear why DNA and phytic acid had such a stimulating effect on dihydrozeatin biosynthesis within the first 24 hours of incubation, thereby prompting meristem cell differentiation and suppressing Pi stress response gene expression. None-the-less, it seems to be further indication of organic P recognition by wheat roots. In the case of phytic acid, dihydrozeatin abundances declined to levels found in control plants allowing up-regulation of Pi stress response genes. This decrease seems to be another indication that wheat seedlings had difficulties in accessing P from phytic acid despite being responsive to its presence.

Even though there was a decline, dihydrozeatin abundances in plants that received DNA remained significantly higher than those of the control plants after 72 hours. In combination with the non-significant trend in lower root-to-shoot ratios (Fig. 2.3), this supports the assumption that wheat seedlings are able to utilize P from DNA. As in the case of sodium phosphate, elevated root dihydrozeatin levels might not only inhibit Pi stress response gene expression, but also secure cytokinin supply for shoot growth promotion.

Abundances of IAA were reduced in control wheat seedlings. This is consistent with observed increased abundances of chorismate that suggested a disruption of tryptophan and subsequent IAA synthesis, as discussed in section 2.3.3. Dihydrozeatin abundances were significantly lower than those of P treated plants suggesting up-regulation of Pi stress response genes.

Gibberellins are negative regulators in plant P stress responses. In *Arabidopsis thaliana*, Jiang et al. (2007) demonstrated that bioactive gibberellins promote primary

root growth and suppress lateral root growth. Furthermore, they observed a decline in endogenous gibberellins under Pi starvation. Therefore, it is not surprising that wheat seedlings, which received readily available sodium phosphate as the P source, exhibited elevated levels of gibberellins. A lower abundance of gibberellins in roots of wheat seedlings from the other P treatments is concordant with the observations made by Jiang et al. (2007) in *Arabidopsis thaliana* under Pi stress.

Lower levels of gibberellins as well as cytokinins are supposed to enable plants to reduce primary root growth and induce lateral root formation. Thus, an increase in auxin abundance, which promotes lateral root and root hair formation, would be expected. However, in this study, this is not the case. Intensities of indole-3-acetic- acid remained low in control and organic P plants over the 72 hours incubation period. As discussed in section 2.3.3, promotion of lateral root growth does not seem to be an initial response to Pi stress in wheat seedlings, which is similar to the observations of Li et al. (2012) observed in *Zea mays* roots.

2.3.5 Effect of sodium phosphate on phenylpropanoid abundances

Seven phenylpropanoids, which include monolignols, coumarins as well as flavonoids, were found to exhibit significant differences among P treatments (Fig. 2.8, Tables 2.1 and 2.2). Six of them showed elevated or increasing abundances in roots of wheat seedlings treated with sodium phosphate. Phenylpropanoid abundances remained low in both organic P treatments. Caffeic acid, coniferyl alcohol as well as umbelliferone had elevated abundances after 5 hours in control plants that subsequently declined. Abundances of caffeic acid and coniferyl alcohol should be considered with caution, since it is the same mass bin of alpha- and beta-D-glucose, which are more common metabolites in root metabolism (see section 2.3.3). Thus, it is more likely that average intensities of mass bin 179 rather reflect the abundance of glucose than that of caffeic acid and coniferyl

alcohol. As in the case of sodium phosphate, kaempferol and luteolin increased in control plants after 48 hours.

Phenylpropanoids are involved in plant stress responses in shoot and root tissue to pathogen attacks, wounding, UV radiation, low temperatures as well as nutrient deficiency (Dixon and Paiva, 1995; Solecka, 1997; Bollina and Kushalappa, 2011; Oksinska et al., 2013). Wheat seedlings incubated with sodium phosphate were not exposed to any of these stresses, suggesting a different function of phenylpropanoids. At sufficient Pi supply, symbiosis with soil microorganisms such as mycorrhizal fungi and plant growth promoting bacteria (in order to improve P uptake) is not beneficial. Thus, instead of functioning as pathogen defence, phenylpropanoids might be used to prevent root colonization with symbiotic microorganisms. Weiss et al. (1999) found that phenylpropanoids restricted ectomycorrhizal growth in conifer roots, which seems to support this hypothesis. However, Chabot et al. (1992) demonstrated that depending on the type, phenylpropanoids could have stimulatory as well as inhibitory effects on hyphal growth of arbuscular-mycorrhiza. For instance, kaempferol was one of the flavonoids promoting hyphal growth. In this study, control wheat seedlings grew under non-P conditions. Establishing a symbiotic relationship with mycorrhizal fungi would be beneficial in order to potentially improve ambient P exploitation. If kaempferol not only supports hyphal growth but also the induction of root mycorrhiza colonization, it would explain the increase in kaempferol abundances in control seedlings. Hence, further research is needed on the impact of phenylpropanoids on the establishment of a symbiotic relationship between cereals and beneficial soil microorganisms. Additionally, the impact of different environmental factors, such as nutrient availability, drought, salinity and the presence of pathogens on this effect needs to be addressed.

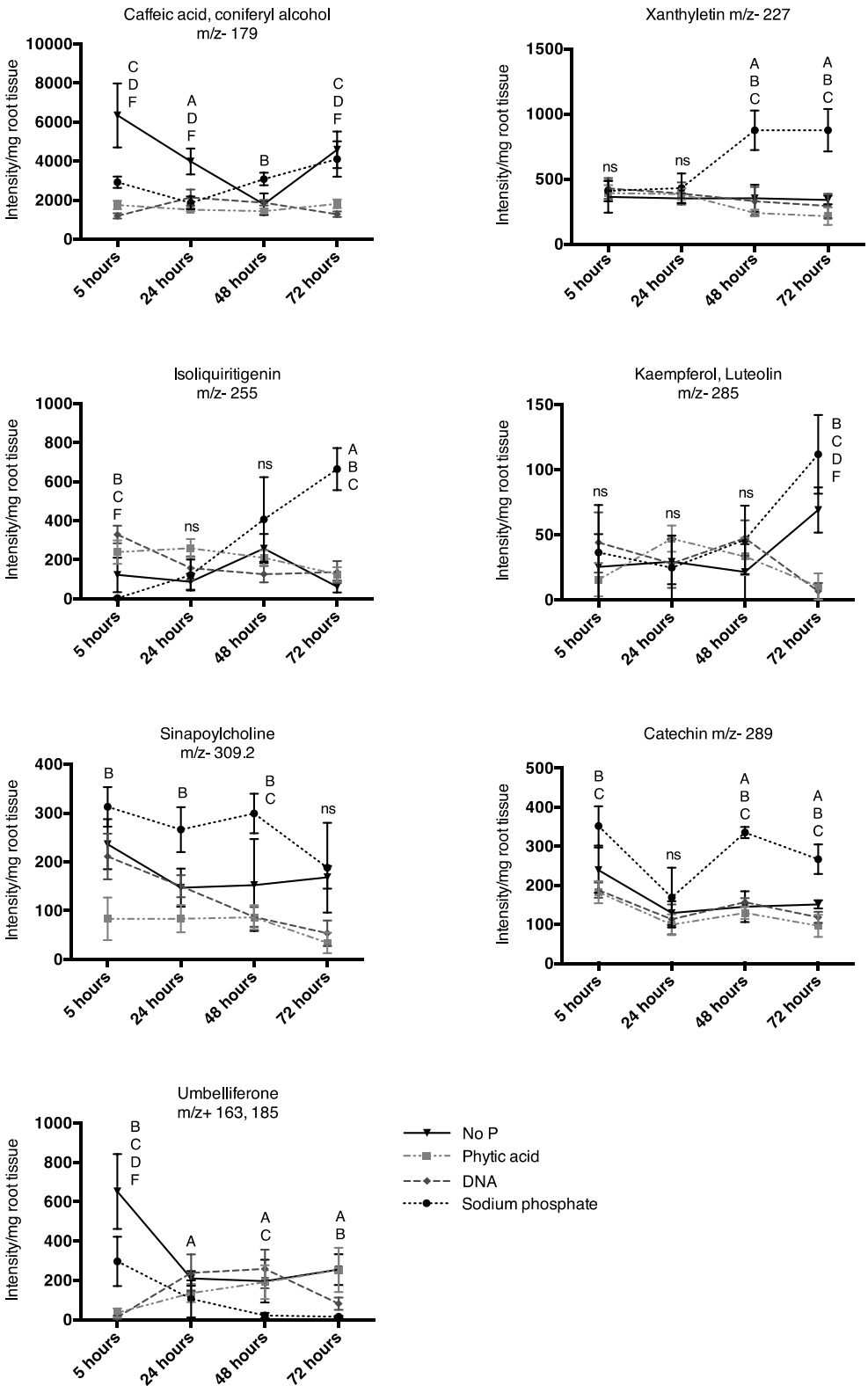


Fig. 2.8: Average intensities (ion counts) per mg root tissue of mass bins of putative phenylpropanoids after 5, 24, 48 and 72 hours detected in either negative (*m/z-*) or positive (*m/z+*) ion mode. Error bars show the standard error of the mean. All *m/z* values refer to the respective mass bins listed in Tables 2.1 and 2.2. Letters indicate significant differences between the treatments (n=5, $p<0.05$, one-way ANOVA, TukeyHSD post-test): A = Sodium phosphate-no P; B = Sodium phosphate-phytic acid; C = Sodium phosphate-DNA; D = Phytic acid-no P; E = Phytic acid-DNA; F = No P-DNA; ns = not significant.

2.4 Conclusion

This study provided evidence that wheat root metabolic responses differ in the presence of inorganic and organic P forms. The early on-set of these metabolic discriminations suggest that they are the response to P form sensing rather than P status. Readily available sodium phosphate enhanced metabolic abundances along the glycolytic, pentose phosphate and shikimic acid pathway. Furthermore, it promotes plant hormone and phenylpropanoid biosynthesis. Within the 72 hours of incubation, organic P forms only had an effect on cytokinin formation. Compared to the sodium phosphate treatment, metabolic activity remained low. Control plants seem to show first signs of Pi deprivation. Interestingly, tryptophan and subsequent IAA biosynthesis appeared to be interrupted.

The role of plant hormones on wheat root and shoot development under Pi sufficient and deficient conditions has not become very clear. Abundance patterns found were different to what has been reported in previous studies, suggesting that plant hormone regulation mechanisms are rather plant-specific. IAA induced lateral root and root hair growth was thought to be a common response to Pi stress. However, results presented in this study imply that lateral root growth is not a prior Pi stress response, at least in the early stages of P deprivation. In addition, concurrent elevated abundances of counteractive cytokinins, IAA and gibberellins under Pi sufficient conditions suggests that the cross talk between them is more complex.

Phenylpropanoids seem to be involved in regulating interactions with beneficial soil microorganisms, thereby potentially acting either as chemoattractant and growth stimulant or repellent (Chabot et al., 1992; Weiss et al., 1999). In this study, the enrichment of kaempferol in wheat roots of the no P treatment may be linked to promote AMF colonization, since it has previously shown that kaempferol promotes hyphal growth (Chabot et al., 1992).

Chapter 3: Root exudate compositions of *Triticum aestivum* exposed to either organic or inorganic phosphorus forms

3.1 Introduction

Modern agriculture is still more selective towards crop cultivars that depend on high soil fertility, therefore relying on high fertilizer input in order to provide sufficient amounts of macronutrients such as phosphorus to achieve optimal crop growth. In the case of P, rock resources used for fertilizer production are limited (for more details see chapter 2.1). Thus, alternative, more sustainable strategies involving cultivars that thrive under low-nutrient conditions and are able to efficiently exploit natural occurring soil P resources are essential in order to secure maximum crop yields in the future.

In order for crop plants to capitalise on naturally occurring P compounds rather than being reliant on exogenous applications of super phosphate fertilisers, crops need to be able to sense different chemical forms of P and adjust their metabolism accordingly to capture P from the soil. In chapter 2, I showed that the roots of wheat (cv. Cadenza) are able to distinguish between sodium phosphate, phytic acid and DNA and modify their root metabolism in response. Specifically, I demonstrated that there was differential up regulation of the glycolytic, pentose phosphate and shikimic acid pathways by the different P sources. Furthermore, inorganic and organic P forms as well as the non-P treatment induced different responses in plant hormone and phenylpropanoid production. The functional consequences of this modulation of root metabolism for P capture, however, remain unclear.

Natural soil P resources have the potential to contribute a substantial amount of P required for optimal crop growth. However, up to or even more than 99% of the soil P is immobilised through precipitation, mineral adsorption and incorporation into organic compounds and as a result difficult to access by plants (Holford, 1997; Blake et al., 2000; Jones and Oburger, 2011). The organic P pool comprises 20 to 80% of immobilized P with phytic acid being its major component (Schachtman et al., 1998; Turner et al., 2000a,

2000b). Therefore, improving the ability of crops to access and mobilize P from this P pool could considerably reduce the current dependence on high fertilizer inputs.

In order to release immobilised P compounds, plants continually release numerous low- and high-molecular weight compounds, ions and gaseous molecules into the rhizosphere. These root exudates not only mediate plant-plant and plant-microbe communication and interaction (Bais et al., 2006; Bertin et al., 2003; Faure et al., 2009), but also promote the acquisition of different nutrients such as N, P, S, Ca, Mg, Fe, Na and Cu (Marschener, 1998; Dakora and Phillips, 2002). Most research on the role of root exudates during nutrient assimilation has been performed under deficiency conditions. In the case of P, several studies detected elevated secretion rates of organic acid anions with a simultaneous release of cations under low P conditions (Zhang et al., 1997; Neumann and Rohmheld, 1999; Penaloza et al., 2002; Dakora and Phillips, 2002). However, the amount released and organic acid composition varied with plant species (Hoffland et al., 1992; Neumann and Rohmheld, 1999; Dakora and Phillips, 2002). Evidence suggests that organic acid anions can adsorb to mineral surfaces, thereby replacing and mobilizing P (Jones, 1998; Hinsinger, 2001; Shen et al., 2002; Bais et al., 2006; Oburger et al., 2011). Moreover, there are indications that the coupled release of organic acid anions and cations alter the ambient soil pH, which can potentially improve P mobilization (Haynes, 1990; Jones, 1998; Hinsinger, 2001; Dakora and Phillips, 2002). Additionally, organic acids are an attractive carbon source for soil microbes. Thus, enhanced secretion of these acids can promote the colonization of phosphorus solubilizing microorganisms of the rhizosphere in order to increase P mobilization (Dakora and Phillips, 2002; Bertin et al., 2003; Faure et al., 2009; Richardson et al., 2011).

In addition to organic acids, the release of phosphatases has been detected under P deficient conditions (Tadano and Sakai, 1991; Gaume et al., 2001; Dakora and Phillips, 2002; Playsted et al., 2006). Phosphatases releases P bound to organic molecules through hydrolysis of these compounds, hence improving P availability for root uptake (George et

al., 2002; Dakora and Phillips, 2002; Nannipieri et al., 2011). Since the release of root exudates is linked to root metabolism, it is likely that the absence of P or presence of different P forms results in distinctive root exudate compositions. Thus, this study examines metabolite profiles of root exudates of wheat seedlings treated with different P forms or no P. Specifically, I hypothesise that root exudate compositions will vary depending on the P treatment and that the release of organic acids, phenylpropanoids and plant hormones will most likely be affected.

3.2 Materials and Methods

3.2.1 Phosphorus treatments

In this study, the same phosphorus compounds (sodium phosphate, DNA and phytic acid) that have been used in chapter 2 (see section 2.2.1) were selected, which represent readily available inorganic phosphate (Pi) as well as complex organic P forms.

3.2.2 Experimental set-up and plant growth

For this study, wheat were grown and exposed to organic and inorganic P forms in sterile mesocosms containing liquid medium (Fig. 3.1). This artificial and aseptic system enables to investigate exclusively the effect of the P form on wheat root exudation. Such single-determinant examinations are essential in order to identify and improve our understanding of factors influencing the secretion of root exudates. However, it needs to be subsequently assessed to what extent potential P form related changes identified under such conditions, can actually be observed in natural ecosystems, since various biotic and abiotic factors such as microbial activity, soil type and chemistry will likely post-alter root exudate composition.

Seeds of *Triticum aestivum* L. cv. Cadenza were sterilized and germinated on filter paper as described in chapter 2 section 2.2.2. After 7 days the seedlings were transferred to sterile 15 ml centrifuge tubes filled with 10 ml of sterile growth medium (Fig. 3.1). The

medium contained the same nutrients as the 0.8% agar medium without the agar (see chapter 2 section 2.2.2). Wheat roots were immersed in the medium with the shoots sticking out of the tubes. Afterwards, all centrifuge tubes were sealed by carefully packing sterile cotton wool around the shoot at the tube opening. All tubes were then wrapped in aluminium foil to keep the roots in the dark. The wheat seedlings were grown for another 7 days in a temperature and light controlled growth chamber at 18°C and 16 hours of daylight.

3.2.3 Phosphorus application

Centrifuge tubes were checked for contamination before P administration. All P sources were dispensed in solution at concentrations of 100 µg P/ml. In a laminar flow cabinet, 1ml of one of the P solutions were added to each tube. As mentioned in chapter 2 section 2.2.3, phytic acid had an inorganic P impurity of ≤0.1%, which means that up to 100 ng of the 100 µg P potentially was inorganic phosphate. The tubes were then resealed with cotton wool and incubated for 72 hours. For each P treatment, six replicate tubes were prepared. In addition, six replicate tubes were used as control plants and did not receive any phosphorus.

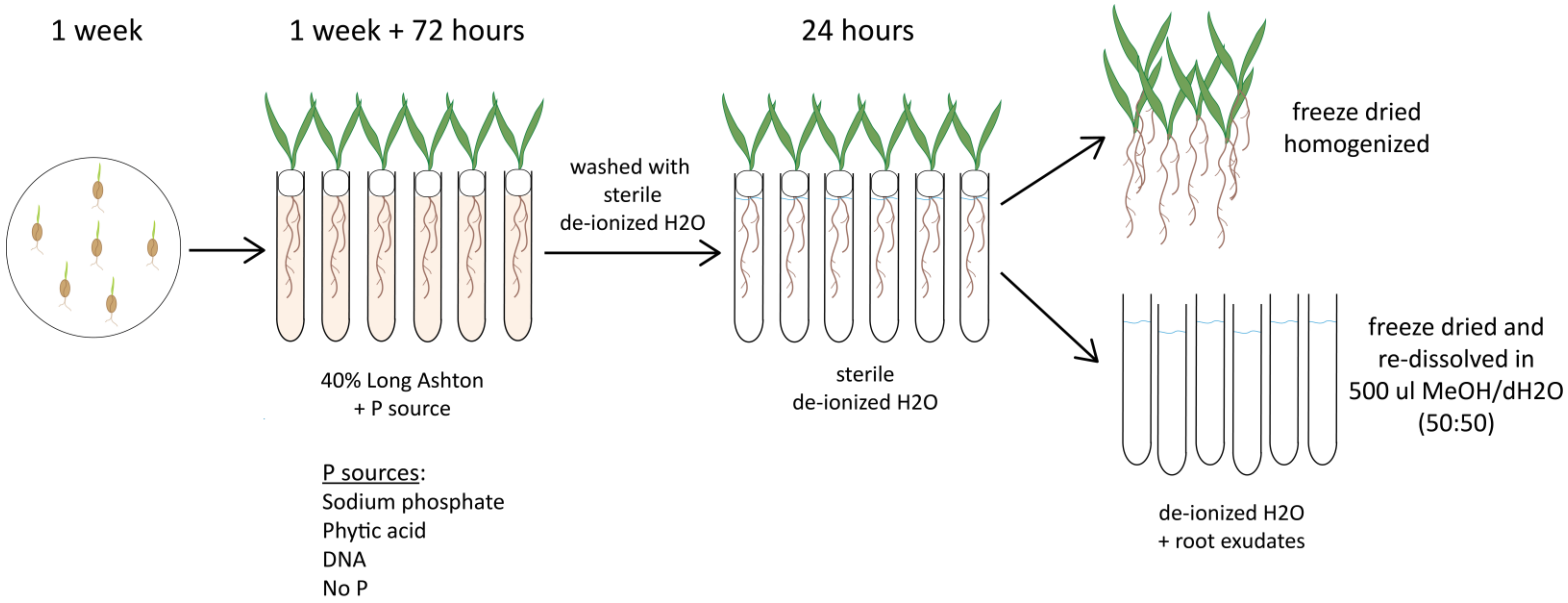


Fig. 3.1: Illustration of the experimental design. Wheat seedlings were incubated in sterile 15 ml falcon tubes. The tubes were sealed with cotton wool and wrapped in aluminium foil in order to keep the roots in the dark.

3.2.4 Root exudates collection and harvest

For root exudate collection, wheat seedlings were carefully removed from the medium containing centrifuge tubes. The roots were then thoroughly rinsed with sterile de-ionized water, and the seedlings subsequently transferred to new 15 ml centrifuge tubes containing 10 ml of sterile de-ionized water (Fig. 3.1). The tubes were sealed with sterile cotton wool and wrapped in aluminium foil to keep the roots in the dark. Root exudates were collected in these tubes for 24 hours.

During harvest, root and shoot tissue were collected separately, frozen at -20°C, freeze-dried and stored in a desiccator until further processing for total P analysis. Centrifuge tubes containing the root exudates were frozen at -80°C and subsequently freeze-dried. Afterwards, root exudates were re-dissolved in 500 µl methanol/de-ionized water (50:50) and stored at -80°C until analysis.

3.2.5 Total phosphorus and root exudates analysis

Total P concentrations of root and shoot tissues were determined as described in chapter 2 section 2.2.6. Root exudates were analysed through direct injection electrospray mass spectrometry on a Waters/Micromass LCT-ES-MS in both electrospray ion modes (ESI) as specified in chapter 2 section 2.2.6 but with no further dilution.

3.2.6 Tandem mass spectrometer analysis (MS-MS)

Molecule fragmentation patterns of masses of interest were analysed on a Qstar Elite (Applied Biosystems) in either positive or negative ESI mode. The Qstar and instrumental parameters were controlled by Applied Biosystems' Analyst software version 2.0. Samples were directly injected with a flow rate of 10 µl/min and nitrogen was used as collision gas. The instrumental settings can be found in Table 3.1. In Analyst, all settings are stated in arbitrary units.

Table 3.1: Instrumental settings of the Qstar Elite.

Negative ESI mode	Positive ESI mode:
Ion source gas: 15	Ion source gas: 27
Curtain gas: 15	Curtain gas: 20
Ion spray voltage: -3500	Ion spray voltage: 3500
Collision energy: 15-40	Collision energy: 15-40
Collision gas: 4	Collision gas: 4-5

For compound identification, mass fragmentation patterns of the samples were compared with fragmentation patterns of standards that were analysed under the same conditions (see example in Fig. 3.2). Depending on the compound, samples and standards (10mg/ml) were diluted between 1:10 and 1:100 to improve ionization and fragmentation. Fragmentation patterns of all identified compounds in the root exudates and of their complementary standards can be found in Figures A3.1-3.8 in the appendix.

3.2.7 Data processing and statistical analysis

Significant differences in tissue dry weights and phosphorus concentrations between the different P treatments were determined by one-way ANOVA followed by TukeyHSD test using the R statistical package software (R Development Core Team, 2011).

MS raw data of root exudates were processed as described in chapter 2 section 2.2.8. Combined principal component analysis (PCA) and orthogonal partial least square discriminant analysis (OPLS-DA) was used to identify differences in root exudates composition between treatments and the mass bins responsible. Putative compound identification was performed using various databases specified in chapter 2 section 2.2.8. As in chapter 2, average intensities of root exudates normalized to one mg root tissue were statistically evaluated in R using one-way ANOVA followed by TukeyHSD test. Intensities were log transformed when they did not fulfil ANOVA's assumptions.

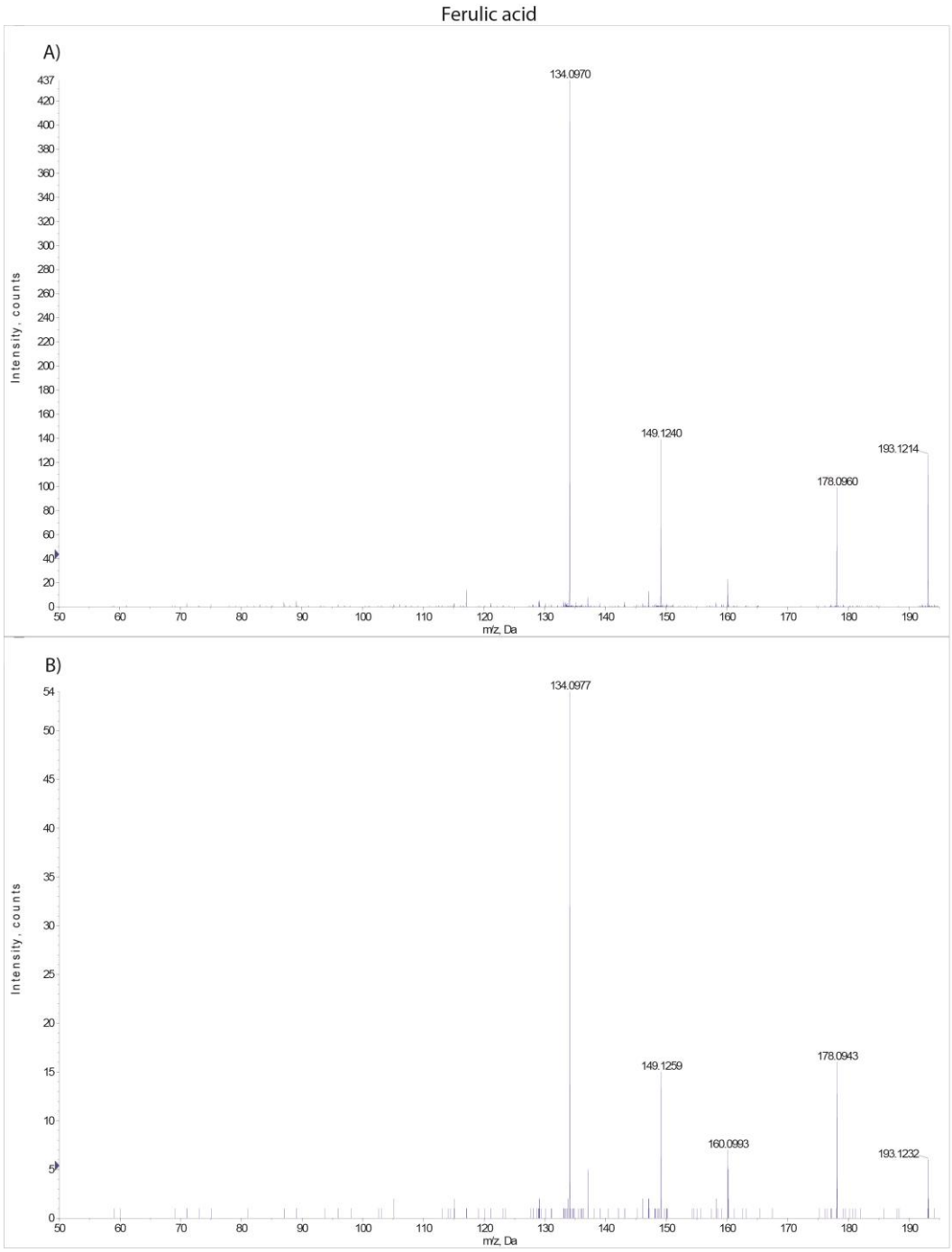


Fig. 3.2: MS-MS fragmentation pattern of ferulic acid (mass bin 193) analysed in negative ESI mode. A) Ferulic acid in standard solution. B) Ferulic acid in root exudates.

3.3 Results

3.3.1 Plant tissue dry weights and total phosphorus concentrations

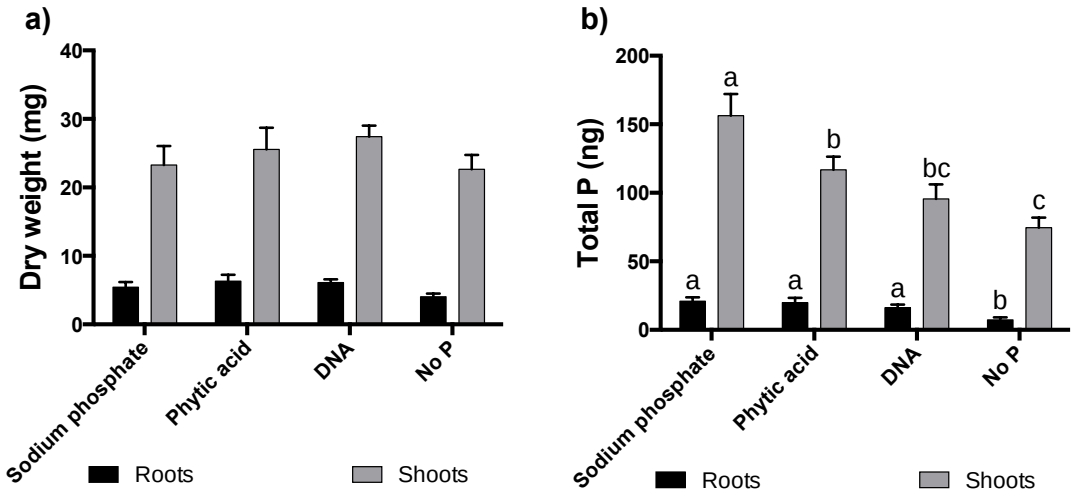


Fig. 3.3: Root and shoot dry weights (a) as well as total P contents (b) after 14 days of growth and a subsequent P incubation and exudate collection period 96 hours in total. Error bars represent the standard error of the mean. a) No statistical significant differences were found in tissue biomass (n=6, $p>0.05$, one-way ANOVA, TukeyHSD post-test). b) Different letters refer to significant differences in either root or shoot P concentrations (n=6, $p<0.05$, one-way ANOVA) between treatments.

There were no significant differences in shoot and root dry weights between the P treatments (Fig. 3.3a). Interestingly, all wheat seedlings that received P in either inorganic or organic form had significantly higher P concentrations than the control plants in their root tissue and, except for the DNA treatment, shoot tissue (Fig. 3.3b). There were no significant differences in total root P concentrations between the organic and inorganic P treatments. In contrast to that, highest shoot P concentrations were found in seedlings that received readily available sodium phosphate. Interestingly, wheat seedlings that were treated with phytic acid had the second highest shoot P concentrations.

3.3.2 Metabolite profiles of root exudates

PCA analyses of root exudate profiles indicated differences between the P treatments, but discriminations were not very obvious. Only root exudates of wheat seedlings that received phytic acid as P source showed clear distinctions from the other treatments in the negative ESI mode (Fig. 3.4). Nevertheless, after OPLS-DA it was possible

to select over 150 discriminating mass bins and individually assess their average intensities for statistically significant differences. As mentioned in chapter 2 section 2.3.2, average intensities of masses can be used as a measure of abundance. Mass bins together with their putative compound identification, which showed significant differences in their abundances, are listed in Table 3.2 and 3.3. Most discriminating mass bins were found in negative ESI mode and only a few in positive ESI mode. Mass bins comprised various compound classes such as organic acids, amino acids, sugar phosphates, phenylpropanoids and other miscellaneous compounds such as phenols and fatty acids as well as nucleosides. Only one plant hormone mass bin (methyl jasmonate) showed differences between the treatments. No discriminations have been found in mass bins of common sugars such as glucose or fructose.

Root exudate profiles of wheat seedlings that were treated with phytic acid showed significant higher abundances in the majority of mass bins found in ESI- mode compared to the sodium phosphate treatment and in several cases to the control plants and DNA treatment (Table 3.2). Discriminations in abundances were most distinctive in mass bins with *m/z* values higher than 240. Unfortunately, several of these bins could not be assigned to a putative compound. This might be due to the fact that these masses only represent fragments of larger molecules that broke apart during analyses. In positive ESI mode, mass bins were mainly significantly different from the control plants with abundances either being reduced or elevated (Table 3.3).

Wheat seedlings that received sodium phosphate showed lower abundances of most mass bins in negative ESI mode. However in positive ion mode, several mass bins had increased average intensities. Root exudate profiles of the control plants as well as the DNA treatment ranged in-between profiles of the sodium phosphate and phytic acid treatment in negative ESI mode. Here, mass bin abundances were often increased compared to the sodium phosphate treatment, but not to the same extent as seen in the

phytic acid treatment. In positive ESI mode, control plants had the highest abundances of mass bins 106 and 158.

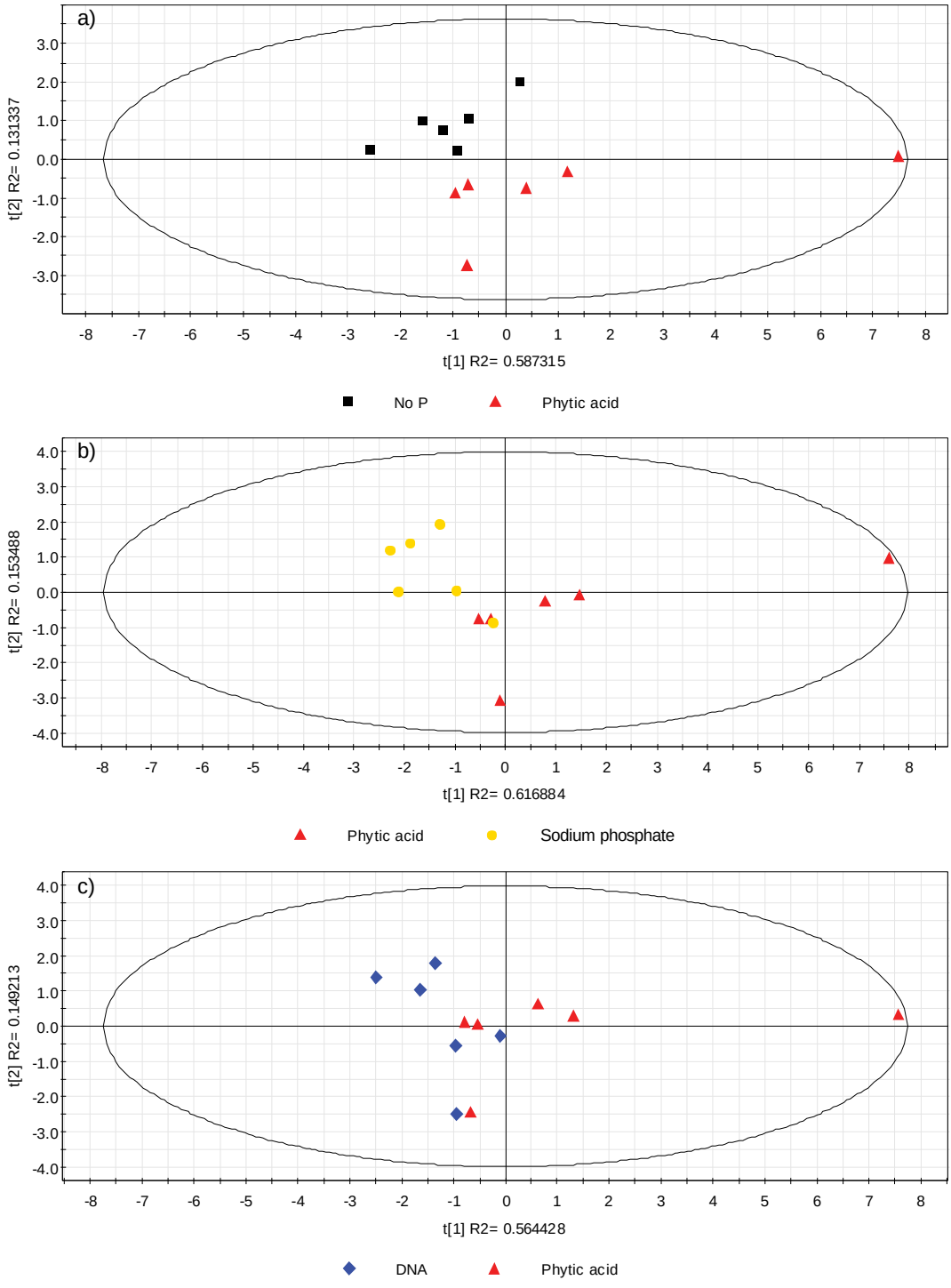


Fig 3.4: PCA plots showing discriminations in root exudates between the phytic acid and other P treatments. Root exudate profiles were analysed in negative ESI mode. (a) Phytic acid versus control (no P), (b) phytic acid versus sodium phosphate, (c) Phytic acid versus DNA.

Mass bins that showed increased average intensities in root exudates of the phytic acid treatment and to some extent in the DNA treatment as well as in the control plants putatively included various organic (e.g. *m/z*- 87, 115, 131, 133 and 191) and amino acids (e.g. *m/z*- 131 and 164, *m/z*+ 106 and 158) and sugar phosphates (*m/z*- 259 and 339). Remarkably, higher abundances were also detected for compounds that have allelopathic properties such as phenylpropanoids (*m/z*- 191, 193 and *m/z*+ 233) and phenols (*m/z*- 153). Additionally, elevated intensities have been found for the plant hormone methyl jasmonate (*m/z*+ 247.2).

Table 3.2: Discriminatory mass bins (*m/z* values) detected in ESI- mode, their putative identification and abundances in root exudates of the different P treatments (SP= sodium phosphate, PA= phytic acid, no P= control plants that did not receive any P). Different letters indicate significant differences in intensities after one-way ANOVA and TukeyHSD post-test (n=6, $p<0.05$).

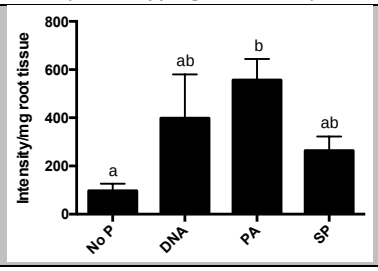
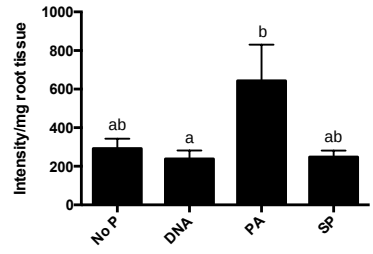
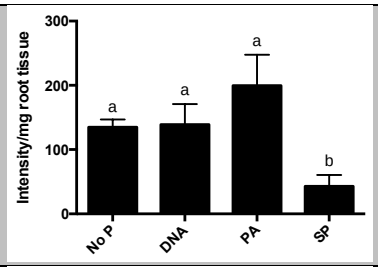
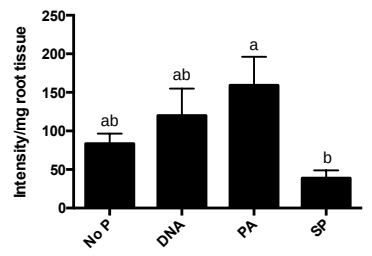
m/z-	**Putative compound**	**Chemical group**	**Mass abundance (Intensity/mg root tissue)**
79	Unknown	Unknown	
80	Unknown	Unknown	
85	Unknown	Unknown	
87	Pyvuric acid Butyric acid Isobutyric acid Putrescine	Organic acid Organic acid Organic acid Peptide	

Table 3.2 cont.

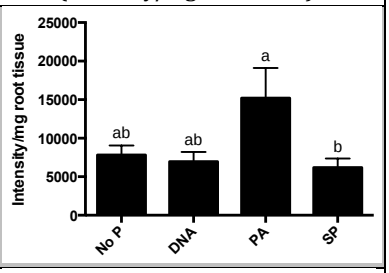
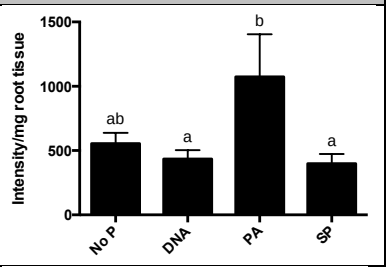
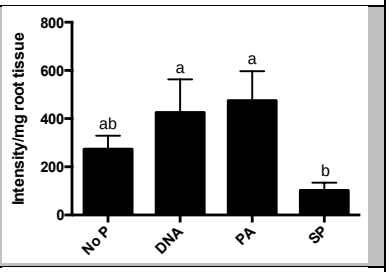
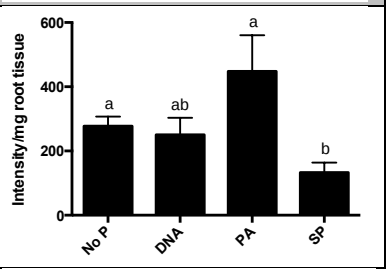
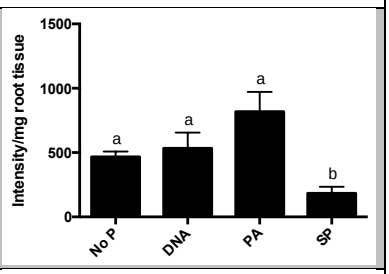
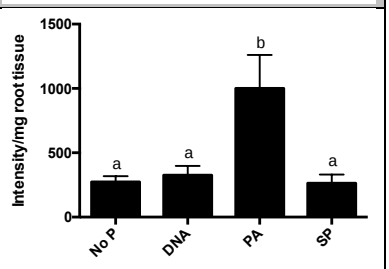
m/z-	Putative compound	Chemical group	Mass abundance (Intensity/mg root tissue)
97	Unknown	Unknown	
99	Unknown	Unknown	
111	Uracil	Nucleobase	
113	Unknown	Unknown	
115	Fumaric acid Succinic acid 2-oxovaleric acid 2-oxoisovaleric acid	Organic acid Organic acid Organic acid Organic acid	
119	3-(methylthio)propionic acid	Organic acid	

Table 3.2 cont.

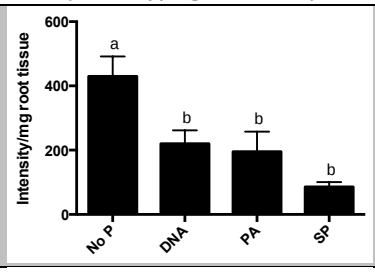
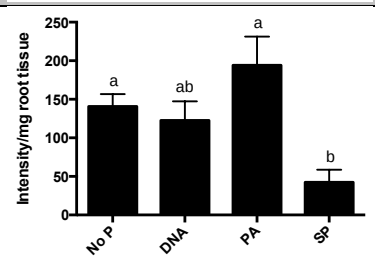
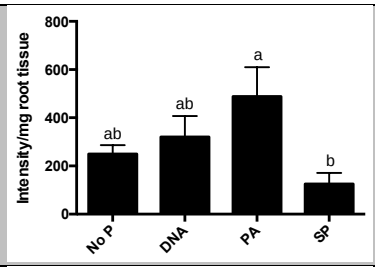
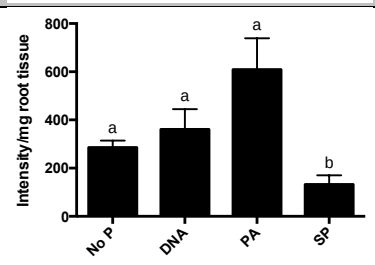
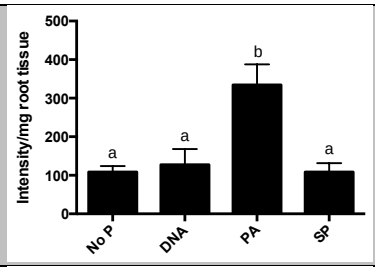
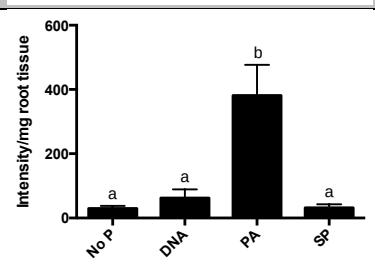
m/z-	Putative compound	Chemical group	Mass abundance (Intensity/mg root tissue)
128	Unknown	Unknown	
129	Itaconate 2-oxoisocaproate	Organic acid Organic acid	
131	Oxalacetic acid Glutaric acid Asparagine Ornithine	Organic acid Organic acid Amino acid Amino acid	
133	Malic acid	Organic acid	
153	Dihydroxybenzoic acid	Phenolic acid	
159	2-oxoadipic acid Pimelic acid	Organic acid Organic acid	

Table 3.2 cont.

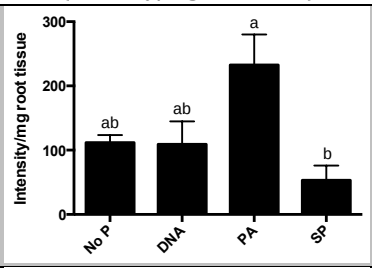
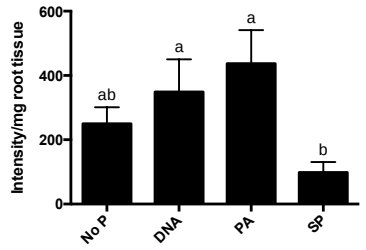
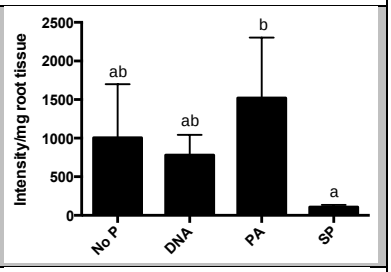
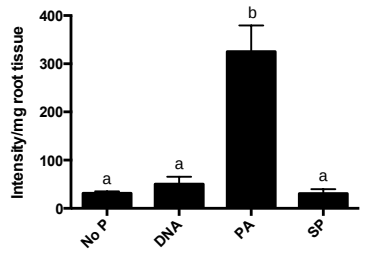
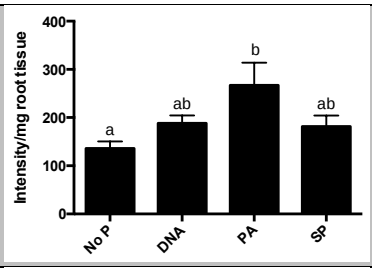
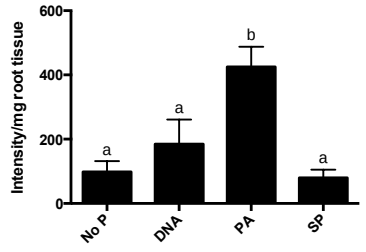
m/z-	Putative compound	Chemical group	Mass abundance (Intensity/mg root tissue)
164	Phenylalanine	Amino acid	
191	Citric acid Isocitric acid Scopoletin	Organic acid Organic acid Phenylpropanoid (coumarin)	
193	Glucuronic acid Ferulic acid L-bornesitol Ononitol Pinitol	Organic acid Phenylpropanoid (monolignol) Cyclitol Cyclitol Cyclitol	
241	Galactosylglycerol Thymidine	Alditol Nucleoside	
255.2	Palmitic acid	Fatty acid	
259	1L-myo-inositol-1-phosphate alpha-D-glucose-6-phosphate alpha-D-mannose-1-phosphate beta-D-fructose-6-phosphate beta-D-glucose-6-phosphate Fructose-6-phosphate Galactose-1-phosphate Glucose-1-phosphate Glucose-6-phosphate Mmannose-6-phosphate Tagatose-6-phosphate Fructose-1-phosphate	Organic phosphate Sugar phosphate Sugar phosphate Sugar phosphate Sugar phosphate Sugar phosphate Sugar phosphate Sugar phosphate Sugar phosphate Sugar phosphate Sugar phosphate Sugar phosphate	

Table 3.2 cont.

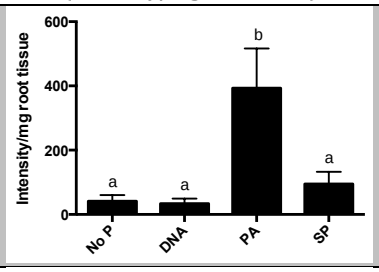
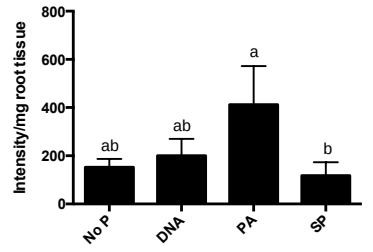
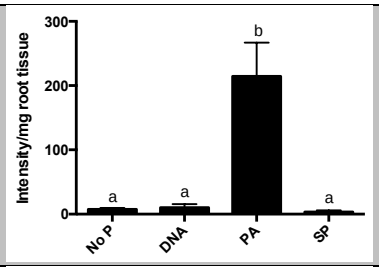
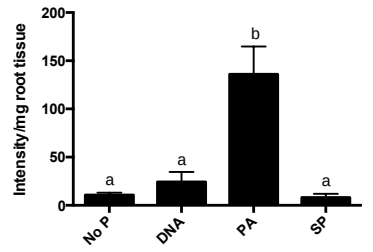
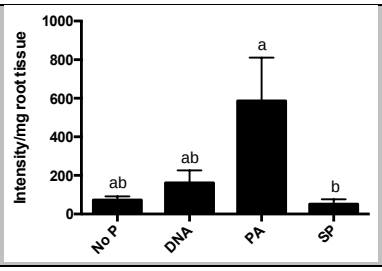
m/z-	Putative compound	Chemical group	Mass abundance (Intensity/mg root tissue)
260.8	Unknown	Unknown	
277	Unknown	Unknown	
321	Unknown	Unknown	
339	beta-D-fructose-1,6-bisphosphate fructose-2,6-bisphosphate glucose-1,6-bisphosphate tagatose-1,6-bisphosphate fructose-1,6-bisphosphate Sinapoyl malate	Sugar biphosphate Sugar biphosphate Sugar biphosphate Sugar biphosphate Sugar biphosphate Carboxylic acid anion	
413.2	Unknown	Unknown	

Table 3.3: Discriminatory mass bins (*m/z* values) detected in ESI+ mode, their putative identification and abundances in root exudates of the different P treatments (SP= sodium phosphate, PA= phytic acid, no P= control plants that did not receive any P). Different letters indicate significant differences in intensities after one-way ANOVA and TukeyHSD post-test (n=6, $p<0.05$).

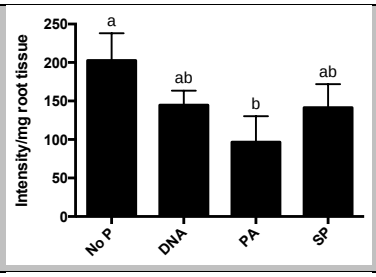
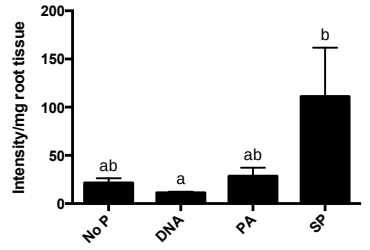
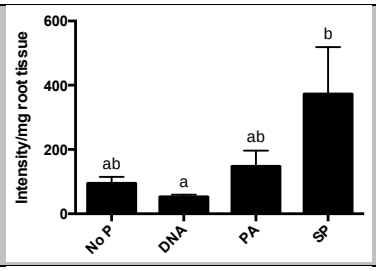
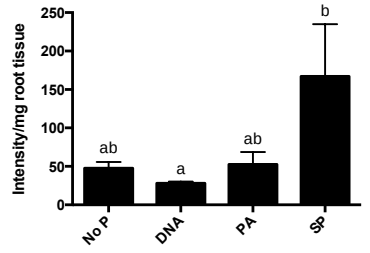
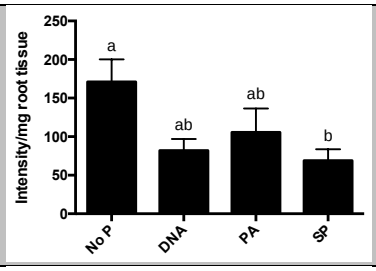
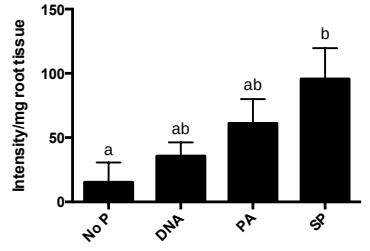
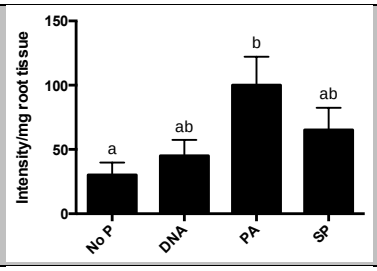
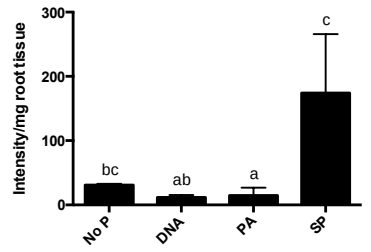
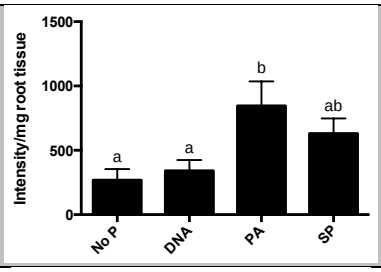
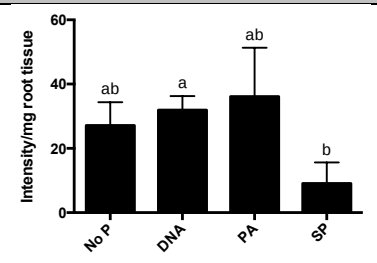
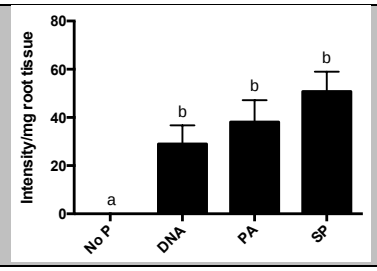
m/z+	Putative compound	Chemical group	Mass abundance (Intensity/mg root tissue)
106	Serine	Amino acid	
113.6	Unknown	Unknown	
120.6	Unknown	Unknown	
125	Acetoacetate Isovaleric acid	Organic acid Organic acid	
158	P-aminobenzoic acid L-Threonine Homoserine Homocysteine Adenine	Amino acid Amino acid Amino acid Amino acid Nucleobase	
233	Saccharic acid Sedoheptulose Sinapyl alcohol	Carboxylic acid Sugar Phenylpropanoid (Monolignol)	

Table 3.3 cont.

m/z+	Putative compound	Chemical group	Mass abundance (Intensity/mg root tissue)
234	Glucosaminic acid	Organic acid	
234.2	Dopamine	Alkaloid	
236	4-phospho-L-aspartate	Modified amino acid	
247.2	Methyl jasmonate	Fatty acyl/plant hormone (Jasmonic acid)	
266	Cytidine	Nucleoside	

3.3.3 MS-MS compound identification

MS-MS analyses could confirm the presence of several organic and amino acids as well as various allelochemicals in wheat root exudates. Out of the 38 mass bins only eight

compounds were possible to identify, which are listed in Table 3.4. This was mostly due to the lack of standards of compounds of interest or fragmentation of the molecule. Fragmentation patterns of all identified compounds in the root exudates and of their complementary standards can be found in Figures A3.1-3.8 in the appendix. In the case of mass bin 131, glutaric acid has been identified. Surprisingly, mass bin 191 was identified as the allelochemical scopoletin and not citric acid. Further identified allelochemicals were dihydroxybenzoic acid and ferulic acid.

Table 3.4: Mass bins from neg. ESI (*m/z*-) mode and their MS-MS compound identification through fragmention pattern comparison between root exudates and selected standards.

Mass bins *m/z-*	**Identified compounds via MS-MS fragmentation**
87	Pyruvic acid
115	Fumaric acid
131	Glutaric acid
133	Malic acid
153	Dihydroxybenzoic acid
164	Phenylalanine
191	Scopoletin
193	Ferulic acid

3.4 Discussion

3.4.1 Plant biomass and P uptake

While there were no differences in plant biomass between the P treatments after the 72 hours incubation period, plant tissue P contents were higher in both the inorganic and organic P treatments compared to the control plants. In the case of both organic P treatments, the significantly higher root P and (only phytic acid) shoot P contents indicate that wheat roots seem to be able to acquire and utilize P from DNA as well as phytic acid. However, the difference in the total plant P content between the phytic acid and no P treatment was less than 100 ng. The phytic acid used in this study potentially had an

inorganic phosphate impurity of up to 100 ng according to the purity batch analysis by the maunfacturer. Therefore, it is possible that the additional detected P content has come from the assimilation of this phosphate impurity and not phytic acid. In conjunction with previous studies, no explicit conclusion can be made on wheat P assimilation efficiency from phytic acid. Richardson et al. (2000) reported poor P uptake from phytic acid, apparently due to only low phytase activity in the roots. In contrast, Sepehr et al. (2012) found highest P concentrations in wheat treated with phytate, but they did not measure phytase activity in either roots and/or root exudates. This study and the other two used different wheat cultivars, suggesting that the extent of phytase activity and P uptake capacity from phytic acid and potentially other organic P sources might be genotype specific. Thus, it is necessary to identify wheat cultivars for breeding programmes that have the enhanced capabilities to utilize organic carbon in order to improve P uptake from naturally occurring soil resources. In the case of phytases, polyclonal antibodies can be used to localize and characterize the expression of specific phytases in root tissue as well as exudates in competitive binding assays (Li et al., 1997). This method allows the screening of a large number of wheat varieties for this trait. Identification and subsequent selection of varieties with high and low root phytase activity can then be used to develop a mapping population of recombinant inbred lines (RILs) derived from parents with opposing phenotype. By defining the quantitative phenotype of large numbers of RILs, quantitative trait loci (QTL) can be statistically defined. These QTL in turn provide insight into candidate genes that could be connected with root phytase formation and secretion. Identification of these genes is essential for developing wheat cultivars that can more efficiently mobilize P from soil phytate.

3.4.2 Phosphorus form-dependent root exudation

Increased secretion of root exudates is a common plant response to low P stress (Zhang et al., 1997; Marschener, 1998; Dakora and Phillips, 2002; Penaloza et al., 2002).

Root exudates can not only directly enhance the dissolution and mineralization of inorganic phosphate (Pi) adsorbed to minerals or incorporated in organic molecules, but also attract soil microorganisms that can facilitate such Pi mobilization (Jones and Darrah, 1994; Bertin et al., 2003; Bais et al., 2006; Faure et al., 2009).

In this study, detected low abundances of root exudates from wheat seedlings that received sodium phosphate were expected. Sodium phosphate is readily available inorganic phosphate that can be directly assimilated by wheat roots. Even though control plants exhibit increased abundances of several root exudates compared to the sodium phosphate treatment, it is surprising that the differences between these two treatments are not more pronounced; as mentioned previously in chapter 2 section 2.3, this might be due to the fact that control wheat seedlings were possibly only in the early stages of P deficiency due to the short growth and incubation time.

The strongly enhanced abundances of root exudates found in the phytic acid treatment and to some smaller extent in the DNA treatment together with the results found in chapter 2 section 2.3 further suggests that wheat exhibit P form specific metabolic response strategies in addition to P stress responses. In this study, root exudates of wheat seedlings seem to be highly receptive to phytic acid.

3.4.3 Organic phosphorus induced secretion of organic acids and its function

Organic acids that had high abundances in root exudates of the phytic acid treatment and that were possible to identify through MS-MS fragmentation were pyruvic, glutaric, fumaric and malic acid. The involvement of organic acids in P mobilization from phytic acid is still not well understood. Phosphorus mobilization usually occurs through the degradation of phytic acid by phytase activity (George et al., 2005; Tang et al., 2006; Giaveno et al., 2010). Root phytase activity and secretion have been detected in *Zea mays* (Hubel and Beck, 1996), tomatoes (Li et al., 1997), clover (Li et al., 1997), rice (Li et al., 1997) and tobacco (Lung et al., 2008). So far, four classes of phytases have been

characterized in plants as well as microorganisms (e.g. Richardson et al., 2001; Konietzny and Greiner, 2002; Zimmermann et al., 2003; Vats and Banerjee, 2004; Lung et al., 2005; Xiao et al., 2005; Tang et al., 2006; Lim et al., 2007). In wheat, phytase activity has mainly been detected in bran (Brinch-Pedersen et al., 2002), where it facilitates P mobilization through degradation of seed phytic acid reserves during germination (Lott et al., 2000; Azeke et al., 2011, also see chapter 2 section 2.3.1). These phytases have been identified as PHY1 and PHY2 and belong to the class of purple acid phosphatases (Tang et al., 2006). In addition, Richardson et al. (2000) detected phytase activity in wheat roots, but not in root exudates thus it is unclear where these enzymes are surface bound (as is the case for many acid phosphatases) or exuded/leached from the root tissues. Moreover, these authors did not characterize the chemical composition and form of these detected phytases. As already discussed in section 3.4.1, the studies of Richardson et al. (2000) and Sepehr et al. (2012) seem to indicate that the capacity and extent of wheat roots to mobilize P from phytic acid varies with genotype. This is likely due to differences in the extent of root phytase formation and possibly excretion. Studies on this topic are still rare, therefore more research is needed on the localization and quantification of phytases in wheat roots and potentially root exudates.

When in solution, phytic acid can be hydrolyzed by phytases and Pi is released (Quiquampoix and Mousain, 2005). However due to its negative charge, phytic acid in soil is mostly precipitated as salts or adsorbed to Al or Fe(III) minerals (Turner et al., 2002b). Tang et al. (2006) showed that phytases were able to release inorganic phosphate (Pi) from phytate salts. But this depended on the prevailing cation. A release of Pi was only detected from Ca-, Mg- and Mn-phytates, but not from Al-, Cu-, Fe- and Zn-salts. Moreover, phytase-driven hydrolysis of phytic acid was drastically reduced or completely absent when phytic acid was adsorbed to Al or Fe(III) minerals indicating substrate inaccessibility (Tang et al., 2006; Giaveno et al., 2010).

Organic acids have been linked to the mobilization of Pi bound to Al, Fe and Ca minerals (Jones, 1998; Hinsinger, 2001; Dakora and Phillips, 2002; Shen et al., 2002; Bais et al., 2006; Oburger et al., 2011). There are indications that organic acids can also solubilize phytic acid from precipitated salts (Tang et al., 2006). However, this highly depended on the secreted acid and cation of the salt. Tang et al. (2006) showed that citric acid was the most potent acid while oxalic and malic acid were hardly effective. Further, by far the highest amount of phytic acid released was from Cu-phytates followed by Zn-, Fe- and Al-salts. In this study, wheat seedlings did not secrete any citric acid, suggesting that they are not capable to acquire sufficient amounts of Pi from phytate salts. However, Tang et al. (2006) also reported a release of phytic acid from Al-oxides, but not Fe(III)-oxides, in the presence of either citric, oxalic or malic acid through potentially ligand exchange or metal complexion. Thus, it is possible that the increase in malic acid exudation of wheat seedlings are supposed to release phytic acid adsorbed to Al-minerals.

Like phytic acid, secreted phytases can get adsorbed to Al- and Fe-minerals. Such immobilization results in a decline in enzyme activity over time (George et al., 2005; Giaveno et al., 2010). The degree of phytase adsorption and activity inhibition depends on many factors such as the isoelectric point (pI) of the enzyme, the type and electric charge of the mineral, the ambient pH as well as the amount of organic matter and phytic acid in the soil, since they compete with the phytases over mineral sorption surfaces (Quiquampoix et al., 1993, 2002; Rao et al., 2000; George et al., 2005; Giaveno et al., 2010). Desorption of acid phosphatases by organic acids has been reported by Huang et al. (2003). Hence, it is possible that organic acids are able to desorb phytases from minerals as well. This, in turn, would also explain the detected higher abundances of organic acids in root exudates of wheat seedlings exposed to phytic acid. However, Tang et al. (2006) also reported that higher concentrations of organic acids inhibited phytase activity, and that the inhibitory effect depended on the acid released. In their studies, citric and oxalic acid impeded phytase activity the most. Nonetheless, it has to be considered that their

experiments have been performed in vitro. Factors that strongly affect the mobilization and immobilization of phytases as well as their activity, such as soil composition, mineralogy and pH are likely to have an impact on the interaction between organic acids and phytases. Thus, further research is needed to address this issue.

Root exudation of organic acids also attracts and promotes soil microbe colonization of the rhizosphere, since these acids are ideal carbon substrates for microbial growth (Dakora and Phillips, 2002; Bertin et al., 2003; Faure et al., 2009; Richardson and Simpson, 2011). Many of these microbes, including rhizobacteria and fungi, can mobilize P from phytic acid through hydrolysis by phytase activity (e.g. Richardson and Hadobas, 1997; Rodriguez et al., 2006; Lim et al., 2007; Patel et al., 2010; Plassard et al., 2011), thus potentially increasing Pi availability. Tarafdar and Marschner (1995) as well as Richardson et al. (2000) reported a drastic increase in P uptake in wheat from phytic acid in the presence of phytate hydrolyzing rhizobacteria and fungi (investigated in detail in chapter 4). In both studies, shoot P concentrations doubled after microbial inoculation. Therefore in this study, the induced increase in organic acid secretion of wheat seedlings exposed to phytic acid might have been rather meant to attract beneficial microorganism to increase Pi availability. Furthermore, in a natural soil environment, microbial degradation possibly lower organic acid concentrations down to levels where they no longer have an effect on phytic acid and phytase adsorption/desorption processes.

3.4.4 Organic phosphorus induced allelopathy of wheat seedlings

Organic phosphorus forms, in particular phytic acid, induced the enhanced wheat root exudation of dihydroxybenzoic acid, scopoletin and ferulic acid (Table 3.2). All three compounds possess allelopathic properties. Allelopathy of wheat is well known and its induction in response to weed and pathogen defense has been studied extensively (Baghestani et al., 1999; Wu et al., 2000, 2001; Huang et al., 2003; Belz et al., 2007). In addition to the presence of weed and pathogens, evidence suggests that different

environmental factors like temperature, irradiation, humidity, soil type as well as nutrients might be involved in the induction and regulation of allelochemical secretion (Gutierrez et al., 1995; Zhao et al., 2005; Dayan, 2006; Belz et al., 2007). In the case of nutrients, Schmid et al. (2014) demonstrated that Fe-deficiency in *Arabidopsis* initiated an increased release of coumarins, especially scopoletin. So far, neither an organic P form sensitive secretion of allelochemicals nor the function of these compounds on organic P assimilation has been reported. Moreover, it has been shown that certain plant hormones such as methyl jasmonate and methyl salicylate can induce allelopathy (Bi et al., 2007). In this study, elevated intensities of mass bin 247.2 (positive ESI mode, Table 3.3), which was putatively assigned to methyl jasmonate, were found in both organic P treatments. Therefore, it cannot be concluded whether the promoted release of allelochemicals is a direct response to organic P form or whether it is an indirect result of enhanced methyl jasmonate secretion.

As described in section 3.4.3, the dissolution and acquisition of Pi from phytic aicd can be a complex process that potentially involves a high expense in carbon through the enhanced secretion of organic acids and other root exudates. Since allelochemicals have a toxic and thus inhibitory effect on weed growth (Wu et al., 2000, 2001; Huang et al., 2003; Belz et al., 2007), the enhanced release of these compounds might mediate elimination of competition for freshly mobilized Pi from phytic acid.

Recently, Zuo et al. (2014) investigated the effect of wheat allelopathy on soil microbe population. They found that microbial abundances increased with the allelopathic potential of different wheat genotypes. Moreover, Cameron et al. (2013) further propose that the release of allelochemicals such as benzoxazinoids is used as signals to recruit beneficial rhizosphere microorganisms that promote plant systemic resistance against pathogens. Hence, it is possible that, similar to organic acids, allelochemicals are meant to attract beneficial microorganisms to improve Pi mobilization from phytic acid as well as DNA. However, there is also evidence that indicates a suppression of microbial growth

through allelochemicals. Kato-Noguchi et al. (2007) reported that rice secretion of momilactone B impeded microbial growth. Wacker et al. (1990) showed that increasing ferulic acid concentrations reduced spore germination, hyphal growth and colonization of arbuscular mycorrhiza fungi in green asparagus. Moreover, Prasad and Devi (2002) found that ferulic acid inhibited acid phosphatase activity and phosphate uptake in maize. The inhibitory effect was pH dependent and highest between 4.5 and 5.5. Thus, further research is needed to fully understand the role of allelochemicals in P utilization from phytic acid and other organic P forms as well as in recruiting beneficial soil microorganisms in order to improve wheat P uptake. Further, it needs to be assessed how biotic and abiotic factors affect or even regulate the impact of allelochemicals on enzyme activity, microbial colonization and P uptake. Moreover, it needs to be examined whether the release of ferulic acid has a self-inhibitory effect on wheat phytase activity, and how it influences microbial phytase activity at the same time.

Also, the allelopathic potential of wheat is not only genotype specific, but also varies with growth stage (Zuo et al., 2014). In this study, root exudates of wheat seedlings in response to different P forms have only been investigated. Therefore, more work is required to determine changes in wheat root exudate composition and allelopathy at different growth stages and to evaluate the impact on P uptake over time.

3.4.5 Phytic acid induced release of putative sugar phosphates

The putative identification of mass bins 259 and 339 of the negative ESI mode (Table 3.2) suggests that phytic acid induced an elevated release of sugar phosphates and bi-phosphates. This seems to be unlikely, since the secretion of sugar phosphates would result in an additional loss of phosphorus, which then has to be sufficiently compensated by P mobilization from phytic acid. However, the comparison of *in silico* simulated fragmentation patterns with the measured fragmentation patterns of mass bins 259 and 339 (data not shown) strongly suggest that these mass bins are indeed sugar phosphates

and bi-phosphates. Nevertheless, it is unclear what the function of these sugar phosphates is. Like organic acids or sugars, they might act as chemoattractant for phosphorus solubilizing microorganisms. If this would be the case, these microorganisms need to be capable in mobilizing enough P from phytic acid in order to outbalance the P loss. Considering the numerous factors that can affect dissolution and hydrolization of phytic acid in natural environments, the use of sugar phosphates to recruit phosphorus-solubilizing microorganisms could be a high P expense that may not pay off. In order to address this issue it firstly needs to be confirmed that wheat really releases sugar phosphates in the presence of phytic acid. If this is the case, it is necessary to monitor and quantify microbial growth and mineralization of phytic acid in the presence of sugar phosphates under various environmental conditions. This can be achieved by using ^{33}P labelled phytate.

3.4.6 Impact of P specific root exudation on root metabolism: A comparison with detected metabolite profiles in chapter 2

Pyruvic, fumaric and malic acid are major metabolites involved in the TCA-cycle. The enhanced release of these acids in the phytic acid treatment and to some smaller extent in the DNA treatment demands a constant and increased replenishment of these acids to sustain this metabolic pathway. Pyruvic acid is the main precursor of the TCA-cycle. Through the activity of pyruvate carboxylase it gets converted into oxalacetic acid the first intermediate in the TCA-cycle and direct precursor of citric acid. Pyruvic acid is produced through the breakdown of phosphoenolpyruvic acid (PEP). PEP is the end product of Glycolysis and also a key precursor of the shikimic acid pathway. The shikimic acid pathway, in turn, feeds both phenol (e.g. dihydroxybenzoic acid) as well as phenylpropanoid (e.g. ferulic acid and scopoletin) biosynthesis via the formation of chorismate and phenylalanine. Thus, elevated abundances of phenylalanine, phenylpropanoids and phenols in root exudates of the phytic acid treatment indicate

enhanced activity along this pathway. The increased activity along the shikimic acid pathway together with the increased secretion of pyruvic and other organic acids requires an enhanced production of PEP through an up-regulation of glycolysis.

In chapter 2, root metabolite profiles of wheat seedlings treated with phytic acid or DNA showed low abundances of metabolites involved in glycolysis and the shikimic acid pathway. It was concluded that this was due to a down-regulation of both pathways. However, the results found in this study rather suggest the opposite. Therefore, it is possible that the depletion of metabolites reflects an imbalance between metabolite supply (Glycolysis) and demand (TCA-cycle and shikimic acid pathway) as a result of limited P availability. In chapter 2, plants were grown on agar and no P uptake has been detected within the 72 hours incubation period. In this study, seedlings were grown in liquid medium. After 72 hours a significant increase in tissue P concentrations has been found in both organic P treatments. Hence, the abundances of metabolites involved in glycolysis and the shikimic acid pathway might be higher in this study compared to chapter 2. More work is needed to address the issue of agar versus liquid medium effects on organic P utilization and concurrent metabolic activity.

3.5 Conclusion

Similar to their root metabolism in chapter 2, wheat seedlings exhibit a P form sensitive release of root exudates. Phytic acid in particular, caused a strong response in root exudate composition, inducing an enhanced release of organic acids, sugar phosphates and certain allelochemicals. However, this study was performed with only one genotype grown in liquid medium under sterile condition. Therefore, it is necessary to determine whether this response in root exudation is common in wheat cultivars or rather genotype specific. Moreover, while the change in and increase of root exudation seem to support P mobilization and uptake from phytic acid in liquid medium under sterile condition, it is unclear how effective this root exudate promoted P mobilization is under

more natural conditions. Thus, the impact of different biotic and abiotic factors such as soil microbial activity, soil type, mineralogy and chemistry, temperature and drought has to be evaluated.

Chapter 4: The effect of *Rhizophagus intraradices* and *Pseudomonas putida* on phosphorus uptake and root metabolism of *Triticum aestivum* exposed to either organic or inorganic phosphorus forms

4.1 Introduction

Improving soil P exploitation from naturally occurring sources in order to reduce fertilizer dependence and concurrently maintain a sufficient P supply for crops has to be a key element in sustainable agriculture procedures. Over the last decade, the use of inoculants of plant-beneficial root colonizing soil microbes such as plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhiza fungi (AMF) has become of increasing interest in order to improve crop nutrient status, growth and health. With respect to P management strategies, these microbes offer the potential to enhance the exploitation of P compounds naturally occurring in the soil, which would reduce fertilizer dependency. These bacteria and fungi are able to mobilize P bound to different organic and inorganic soil matter through the excretion of organic acids and enzymes such as phosphatases and phytases and transfer it to plants in exchange for carbon (Artursson et al., 2006; Patel et al., 2010; Plassard et al., 2011; Richardson and Simpson, 2011). However, numerous biotic and abiotic factors including the host plant species (or cultivar/genotype), the presence of other microbial populations as well as nutrient type and status directly and indirectly influence this relationship and eventually determine whether it is of positive, neutral or negative nature in terms of host plant fitness (Johnson et al., 1997; Johansson et al., 2004; Marschner et al., 2004; Nazir et al., 2010; Owen et al., 2015). As a consequence, it is currently very difficult to predict whether the application of microbial inoculants improves nutrient uptake and growth of crops. More research is needed that simultaneously investigates the effect of multiple variables such as plant species, nutrient type as well as the use of one or more microbial species/genotype assemblages.

Plant-microbe compatibility is an important factor that needs to be considered when selecting microbial inoculants. Plants are highly selective towards their rhizosphere microbial community and symbiotic partner, in particular to different AMF species (Klironomos et al., 2003; Marschner et al., 2004). Responses to different AMF species can vary from symbiotic to parasitic among plant species; for example, *Glomus macrocarpon* is known to be the causal agent of tobacco stunt disease (Eom et al., 2000; Klironomos et al., 2003; Jansa et al., 2005; Hart and Forsythe, 2012). Plant-microbe interactions occur within the rhizosphere, where the release of chemical signals from both plants and microbes establishes and defines the nature of their relationship (Bias et al., 2006; Faure et al., 2009). Plants are able to either attract and recruit or repel specific groups of microorganisms through the release of root exudates such as plant hormones (e.g. strigolactones) and aromatic compounds, due to differences in microbial capacities to digest these metabolites (Harwood et al., 1984; Bais et al., 2004; Neal et al. 2012; Cameron et al., 2013). As a result, plants can actively influence the rhizosphere microbial community structure (Marschner et al., 2004; Kato-Noguchi et al., 2007; Cameron et al., 2013; Zuo et al., 2014).

With respect to AMF, at the early stages of the establishment of a symbiotic relationship, mycorrhizal infection alters root metabolism by triggering a temporary salicylic acid-dependant plant defence response (Kapulnik et al., 1996; Blilou et al., 1999; Zhang and Zhou, 2010; Cameron et al., 2013). This is followed by a local suppression of plant defences through AMF induced host specific changes in plant hormone profiles such as the promotion of abscisic acid production, thus enabling the progression of AMF root colonization (Bothe et al., 1994; Herrera-Medina et al., 2007; Lopez-Raez et al., 2010; Cameron et al., 2013). In addition to this, several studies reported that AMF changed root exudate composition by enhancing the allocation of carbon compounds to roots and increasing secondary metabolite biosynthesis, therefore indirectly affecting microbial

community structure within the mycorhizosphere (Marschner et al., 1997; Sood, 2003; Drigo et al. 2010; Song et al., 2011; Walker et al., 2012).

As demonstrated in chapters 2 and 3, the chemical form of P supplied to roots significantly alters root metabolism and root exudate composition; including changes in plant hormone and phenylpropanoid biosynthesis as well as inducing the excretion of organic acids and allelopathic compounds. It is still unclear how the P form and AMF affect root metabolism and exudation, if and how the AMF and P form interact with each other and whether this influences P uptake and root colonization of AMF and other beneficial soil microorganisms. This study examines the impact of inorganic and organic P form in combination with single- and dual-inoculation of AMF and PGPR on P uptake, plant biomass, extent of microbial root colonization and root metabolism of wheat. We hypothesize that the extent of AMF and PGPR colonization depends on the present P form and that both microorganisms improve P uptake from organic and bound inorganic P sources. Moreover, I propose that wheat root metabolite profiles vary with P and microbial treatment, and that the production of plant hormones and other secondary metabolites are mostly affected.

4.2 Materials and Methods

4.2.1 Phosphorus compounds

In this experiment, two inorganic and organic P forms were used: Sodium phosphate (Sigma-Aldrich), hydroxyapatite (Sigma-Aldrich), DNA sodium salt from herring testes (Sigma-Aldrich), and phytic acid dipotassium salt (Sigma-Aldrich) that had an inorganic phosphate impurity of ≤ 0.1% as stated by the manufacturer.

4.2.2 Bacterial cultivation

For this study, *Pseudomonas putida* was chosen as PGPR, since it is well known for its phosphorus solubilizing capabilities and common occurrence in soil (Richardson and

Hadobas, 1997; Rodriguez and Farga, 1999; Naik et al., 2008; Zabihi et al., 2011). Moreover, Zabihi et al. (2011) showed previously that *P. putida* increased P uptake and grain yield under greenhouse and field conditions.

The green fluorescent protein (GFP)-tagged *Pseudomonas putida* KT2440 derivative strain FBC004 (Neal et al., 2012) used in this experiment was provided by Professor Jurriaan Ton at the University of Sheffield. Even though no transcription for phytases has been found in the genome sequence (Nelson et al., 2002; Weinel et al., 2002), Patel et al. (2010b) detected phytase activity in KT2440 (see section 4.4.2.2). Fresh cultures of FBC004 were started from stocks and grown in M9 minimal medium for 24 hours at 28°C. The M9 minimal medium contained the following ingredients per litre: 33.9 g disodium phosphate, 15 g monopotassium phosphate, 2.5 g sodium chloride, 5 g ammonium chloride, 3.6 g glucose and 0.24 g magnesium sulphate. In order to remove the P containing medium, the freshly grown bacteria were centrifuged at 5000 rpm (revolutions per minute) for three minutes, the supernatant discarded and the cells re-suspended in sterile 10 mM magnesium sulphate heptahydrate. Cell densities were assessed through the absorbance at 600 nm (OD_{600}) on a spectrophotometer. An OD_{600} value of 1.0 equals a density of $1x10^9$ cells per ml. Average OD_{600} value of the prepared cell suspension was 1.27.

4.2.3 In-vitro AMF cultivation and inoculum preparation

In-vitro cultures of *Rhizophagus intraradices* were kindly provided by Professor Toby Kiers' lab at the VU Amsterdam and cultivated as described in Kiers et al. (2011) and Engelmoer et al. (2013). The selection of the AMF species was based on its occurrence in agricultural ecosystems and compatibility with wheat with respect to root colonization. *R. intraradices* was chosen, since it can be an abundant or even the dominating AMF species in agricultural soil (Mathimaran et al., 2005) and is known for colonizing wheat (Li et al., 2005 and 2006; Jaederlund et al., 2008; Daei et al., 2009). Further, its P for C dynamics is

well known due to extensive studies by Kiers et al. (2011). In addition, Kiers et al., (2011) developed qPCR primers for determining abundances of *R. intraradices* in homogenized root systems.

Inoculum of *R. intraradices* isolate 09 (Schenck & Smith; collected from Southwest Spain by Mycovitro S.L. Biotechnología ecológica, Granada, Spain) was grown in Petri dishes on MSR medium (Cranenbrouck et al., 2005) in the presence of Ri T-DNA transformed carrot (*Daucus carota* clone DCI) roots. Cultures were incubated for approximately 16 weeks in the dark at 25°C. Afterwards, the *R. intraradices* root cultures including the medium were transferred to a sterile beaker and blended with a 25 ml of sterile dH_2O. Spore densities of this fungal inoculant were determined using a standard volume and custom-made spore counter. Spore counts resulted in approximately 120 spores per 100 µl.

4.2.4 Experimental set-up

Seeds of *Triticum aestivum* L. cv. Cadenza were germinated in Petri dishes on moist Whatman No1 filter paper for five days at 20°C. The seedlings were then planted into pots containing silica sand that was autoclaved at 124°C for an hour. The seedlings were either grown without any added inoculum or incubated with *R. intraradices* only, *P. putida* only or both. For each P source a set of these four microbial treatments were prepared in replicates of five.

All four P sources were dissolved/suspended in dH_2O. Each pot received 20 ml of one of the P solution/suspension containing a total amount of 10 mg P. This amount was chosen, since P concentration between 5 and 20 mg P per kg soil are most beneficial for mycorrhizal associated nutrient uptake and plant growth (Jakobsen 1995; Ortas et al., 2002 Li et al., 2005; Morgan et al., 2005). After four weeks of wheat growth, another 10 mg P of each P source were added to the pots, which means that all wheat plants received a

total of 20 mg of P throughout the experiment. In the case of the phytic acid treatment, up to 20 µg consist of inorganic phosphate due to the ≤ 0.1% impurity.

For the AMF treatments, roots of wheat seedlings were covered with 2 ml of *R. intraradices* inoculum that had a total spore count of 1200 spores. As a control, wheat seedlings of the non-AMF treatments received 2 ml of the same inoculum after it was autoclaved at 126°C for 20 minutes. Plant pots that were inoculated with *P. putida* received 20 ml of the cell suspension. The other plant pots were treated with 20 ml of sterile 10 mM magnesium sulphate heptahydrate to eliminate any effect on wheat growth and P uptake.

In order to allow *R. intraradices* to colonize the roots and form a hyphal network within the sand, wheat was grown for 10 weeks at 18°C and with 16 hours daylight in a light and temperature controlled growth chamber. Previous experiments in this research group showed (data not shown) that it takes at least 10 weeks to fully establish a hyphal network. However, a growth period longer than 10 weeks were not possible, since wheat plants of the hydroxyapatite treatment showed severe signs of P deprivation and would not have survived much longer. During the growth period the plants were fed with 30 ml of a P-free Long Ashton solution every two weeks that contained the following ingredients per litre: 161.9 mg KNO_3, 256.8 mg anhydrous $Ca(NO_3)_2$, 147.2 mg $MgSO_4 \cdot 7H_2O$, 13.4 mg FeNa-EDTA, 0.892 mg $MnSO_4 \cdot 4H_2O$, 0.116 mg $ZnSO_4 \cdot 7H_2O$, 0.1 mg $CuSO_4 \cdot 5H_2O$, 1.24 mg H_3BO_3 and 0.048 mg $NaMoO_4 \cdot 2H_2O$, 2.34 mg NaCl.

4.2.5 Sample collection

After 10 weeks, all wheat plants were harvested. For tissue dry weights, P content and mycorrhiza colonization determination, roots and shoots were collected separately, frozen at -20°C and subsequently freeze-dried. Prior to this, 1 to 1.5 g of root and adherent sand were taken and suspended in 10 ml of 10 mM magnesium sulphate heptahydrate in order to perform *P. putida* colonization count assays. Another root subsample of 100 mg

were collected, washed, shock frozen in liquid nitrogen and stored at -80°C for metabolite analyses.

4.2.6 Phosphorus content analysis

Phosphorus contents of freeze-dried and homogenized root and shoot samples were determined following the method described in chapter 2 section 2.2.7.

4.2.7 AMF DNA isolation and molecular analysis

DNA isolation and molecular analysis were performed in Professor Toby Kiers' lab at the VU Amsterdam using the method of Kiers et al. (2011), but applying the modifications described in Engelmoer et al. (2013). The fungal DNA was extracted from freeze-dried and homogenized roots following the standard protocol of the Plant DNeasy mini kit (Qiagen) with slight modifications (Engelmoer et al., 2013). After the lysis step, each sample was spiked with 10 µl of an internal standard that had a known fixed copy number. The internal standard was a plasmid that contained a fragment of the cassava mosaic virus. The addition of this standard enables the determination of the actual fraction of fungal DNA extracted from each sample. This fraction varies among samples; therefore the number of copies theoretically found in 100% extracted DNA was calculated to assure a more accurate comparison of treatments.

DNA amplification was performed on a LightCycler (CFX96) with CFX manager analysis software using TaqMan probe-based qPCR (Biorad). Prior to the qPCR analyses, all samples were diluted 1:10 with DNase-free H_2O. For the amplification reaction, the iTaq universal probes supermix (Biorad) was used as well as 0.5 µM of each primer (forward primer: TTTTAGCGATAGCGTAACAGC, reverse primer: TACATCTAGGACAGGGTTTCG) and 0.11 µM of the hydrolysis probe (***FAM***-AAACTGCCAC TCCCTCCATATCCAA-***BHQ1***, ***FAM*** = fluorescein, ***BHQ1*** = fluorescence quencher). The final reaction volume was 20 µl, which included 4 µl of DNA template (i.e. diluted sample). In

total, 40 qPCR reaction cycles were run under the following reaction conditions: denaturation at 95°C for 5 seconds, annealing at 60°C for 30 seconds and amplification at 72°C for 1 seconds. The selected primers and reaction conditions were targeting the mitochondrial large ribosomal subunit (mtLSU) of *R. intraradices* mitochondrial DNA.

Abundances of *R. intraradices* were calculated as described in Engelmoer et al. (2013). A standard curve of Cq values (quantification cycle) was produced by a dilution series of plasmid that contained fragments of the mtLSU of the cassava mosaic virus (internal standard). For this purpose, Cq values of the samples were converted into raw copy numbers using the following equation derived from the standard curve: 10^((Cq value of the sample −41.611)/ −3.4229). DNA isolation efficiency was then determined by dividing the measured copy numbers of the internal standard after the extraction by the initial known copy number at the time of spiking the sample. Finally, the calculated DNA efficiency combined with the raw fungal copy numbers of each sample were used to determine fungal copy numbers at 100% extraction efficiency. These numbers were then normalized to number of copies per gram dry weight root tissue.

4.2.8 Determination of P. putida abundances

Abundances of *P. putida* in the rhizoplane were determined through the number of colony forming units (CFUs). Fresh root material with adherent sand (1-1.5 g) were suspended in 10 ml of sterile 10 mM magnesium sulphate heptahydrate and shaken for 20 minutes at 200 rpm. Afterwards, a dilution series of each suspension was prepared in a 96-well plate with 10 mM magnesium sulphate heptahydrate using dilution factors between 1/10 and 1/10000. Of each dilution 10 µl was transferred to agar plates containing LB medium. All plates were incubated in the dark at 25°C for 36 hours. Green-fluorescent colonies were counted under blue UV light on a transilluminator. Colony

counts were then used to calculate the number of colony forming units per gram of dry weight.

4.2.9 Root metabolite extraction

Root metabolites were extracted using the standard bi-phasic chloroform-methanol-water extraction (Overy et al., 2005) described in chapter 2 section 2.2.5. However, since the amount of root tissue collected in this experiment was ten times higher than that used in chapter 2, the volumes of each extraction solution were adjusted accordingly.

4.2.10 Matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) analysis of root metabolites

The polar phase of root metabolite extracts were analysed on the MALDI-Synapt G2 (Waters) in positive ionization mode. Alpha-Cyano-4-hydroxycinnamic acid (5mg/ml) was used as matrix and sulphadimethoxine (0.1mg/ml) as the lockmass to correct for any mass drift. Samples were analysed in triplicates. Masses were scanned within the range of 50 to 1000 *m/z*. The laser energy was set to 180 with a firing rate of 1000 Hz. Nitrogen was used as carrier gas. The run-time for each sample was 2.5 minutes.

4.2.11 Data processing and statistical analysis

All statistical analyses were performed in R (R Development Core Team, 2011). A linear mixed-effect model (nlme package, Pinheiro et al., 2011) was used to investigate microbial (*R. intraradices* and *P. putida*) and P source related changes in plant biomass and tissue P concentrations and content. It allowed taking into account random tillering of the wheat plants, which had an impact on the plant biomass. Moreover, linear mixed-effect models also assessed the effects of dual-inoculation and different P treaments on root

colonization of *R. intraradices* and *P. putida*. Prior to statistical analyses both microbial abundances were log transformed in order to fulfil the assumption of normality.

Mass peaks were centroided in MassLynx 4.0 (Waters Ltd., Watford, Herefordshire, UK). Peak alignment and binning were performed in R (R Development Core Team, 2011) using the MALDIquant package (Gibb and Strimmer, 2015). During the binning process, the ion counts of each mass peak were also normalised to the total ion count (TIC). Principal component analysis (PCA) followed by orthogonal partial least square discriminant analysis (OPLS-DA) was performed with Simca P (Umetrics) on the normalized ion counts in order to identify masses that show any discriminations between the different P and microbial treatments. The normalized ion counts of the masses were chosen, since they can be used as a measure of abundance of the respective mass. Over 250 mass bins were selected and putatively identified using online databases such as the METLIN Metabolomics Database (https://metlin.scripps.edu/) and Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/). Since metabolite profiles were analysed in positive ionization mode, compound identification of masses was conducted for both hydrogen $[M+H]^+$ and sodium $[M+Na]^+$ adducts. Variations between detected *m/z* values of the repeated measures and experimental replicates ranged between 3 to 40 ppm. Therefore, putative compounds were only selected if the difference between monoisotopic mass and the average detected mass were not higher than 40 ppm.

Three-way ANOVA followed by the TukeyHSD test was used to determine whether the different P forms and microbial treatments had a significant effect on the normalized intensities (ion counts) of selected mass bin. Intensities were log transformed if they did not fulfil the assumption of normality.

In order to better illustrate and assess the effects of both the P and microbial treatments on the putatively identified mass bins, percentage differences were calculated between the different P forms and the sodium phosphate treatment for each microbial

treatment (P form effect) and between the non-microbial treatment and the different microbial treatments for each P form (microbial effect).

4.3 Results

4.3.1 AMF and P. putida root colonization

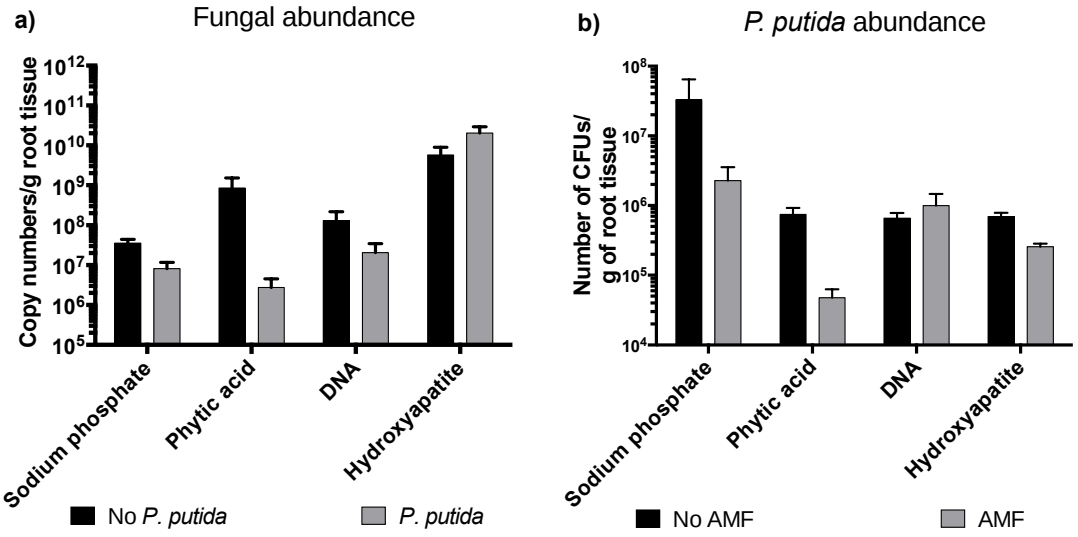


Fig. 4.1: Abundances of a) *R. intraradices* and b) *P. putida* in wheat roots after 10 weeks of incubation. Fungal abundances are shown in copy numbers and *P. putida* abundances in number of colony forming units per gram dry root tissue (see sections 4.2.5 and 4.2.6). Error bars represent the standard error of the mean.

Table 4.1: Statistical results of linear mixed effect models (n=5) on fungal and bacterial abundances. ns= not significant, *= p< 0.05, **= p<0.01

Functional Group	**Source of variation**	
Fungal abundance	P treatment	**
	P. putida	**
	P treatment**P. putida*	ns
P. putida abundance	P treatment	**
	R. intraradices	**
	P treatment**R. intraradices*	*

P treatments as well as the presence of *P. putida* had a significant effect on the fungal abundance in root tissue (Table 4.1). But no statistically significant interactions between P treatment and *P. putida* occurred. Highest fungal abundances were found in

wheat plants treated with hydroxyapatite (Fig. 4.1a). In most cases, the presence of *P. putida* had a negative impact on fungal colonization, which was most strongly pronounced in the phytic acid treatment. Decreases in mean abundances varied between 77 and almost 100%. However, wheat plants that received hydroxyapatite had an increase in fungal abundances of over 250% in the presence of *P. putida*.

Similar to AMF abundances, different P sources and the presence of *Rhizophagus intraradices* had a significant impact on *P. putida* colonization in the rhizoplane (Table 4.1). In addition, there was also a significant interaction between the P treatment and AMF inoculation. In contrast to fungal abundances, highest root colonization of *P. putida* occurred in wheat of the sodium phosphate treatment (Fig. 4.1b). The presence of *R. intraradices* had a negative effect on *P. putida* abundances with reductions in colony forming units between 63 and 94%. Only in plants treated with DNA abundances increased by 51%.

4.3.2 Variations in total P contents

Total root P contents significantly varied among the P treatments (Table 4.2). Highest P contents were found in the sodium phosphate treatment followed by the DNA treatment (Fig. 4.2). Lowest P contents were detected in roots of the hydroxyapatite treatment. Compared to that, root tissue of wheat plants that received phytic acid had slightly higher P contents. Linear mixed effect models showed that the two-way interaction between the P treatment and *P. putida* as well as the three-way interaction between P treatment, *P. putida* and *R. intraradices* had a significant effect on total root P contents. While the presence of *R. intraradices* reduced root P contents by 15.5 and 25% in the sodium phosphate and phytic acid treatment, no effect was detected in the case of DNA and hydroxyapatite. *P. putida* lowered root P contents by more than 35% in almost all P treatments. Only in the case of hydroxyapatite, an increase of 43.2% was detected. Dual inoculation of *P. putida* and *R. intraradices* resulted in lower root P contents in the sodium

phosphate and phytic acid treatment, but to a lesser degree than both single inoculations (Fig. 4.2). In the case of DNA, dual inoculation reduced root P contents by over 50%. Wheat plants that received hydroxyapatite were the only ones that exhibit an increase (19.8%) in root P contents in in the presence of *P. putida* and *R. intraradices*. However, this increase was not as high as the one detected in the single inoculation treatment with *P. putida*.

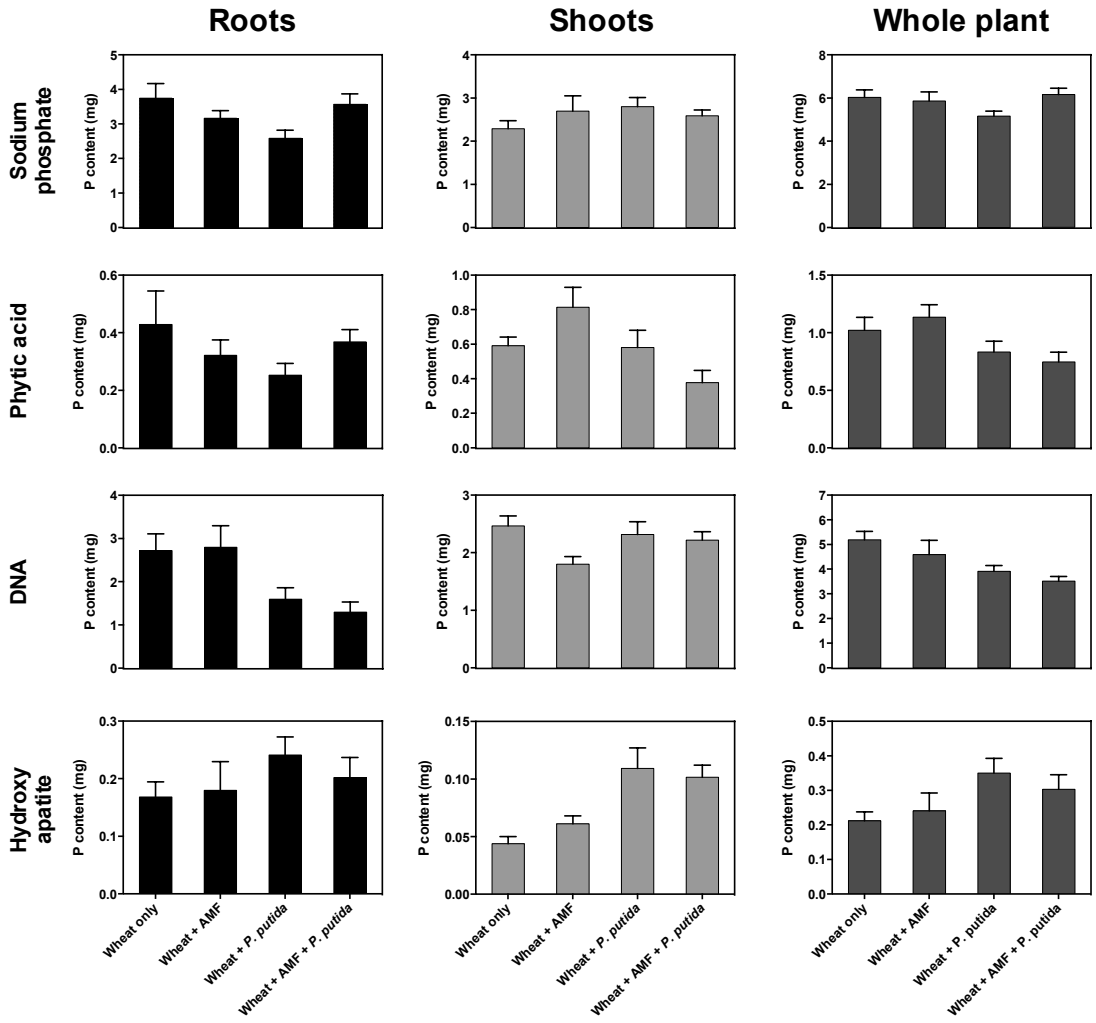


Fig. 4.2: Total P contents detected in wheat roots, shoots and the whole plant of the sodium phosphate, phytic acid, DNA and hydroxyapatite treatment after 10 weeks of growth. The error bars are the standard error of the mean. Note the different scales of the P content in each P treatment.

Table 4.2: Statistical results of linear mixed effect models (n=5) on total root, shoot and whole plant P. ns= not significant, *= p< 0.05, **= p<0.01, ***= p<0.001

Functional group	Source of variation	
Total root P content	P treatment	***
	R. intraradices	ns
	P. putida	***
	P treatment**R. intraradices*	ns
	P treatment**P. putida*	***
	*R. intraradices***P. putida*	ns
	P treatment* *R. intraradices***P. putida*	*
Total shoot P content	P treatment	***
	R. intraradices	**
	P. putida	ns
	P treatment**R. intraradices*	**
	P treatment**P. putida*	*
	*R. intraradices***P. putida*	ns
	P treatment* *R. intraradices***P. putida*	*
Whole plant P content	P treatment	***
	R. intraradices	*
	P. putida	***
	P treatment**R. intraradices*	**
	P treatment**P. putida*	***
	*R. intraradices***P. putida*	ns
	P treatment* *R. intraradices***P. putida*	ns

The total P content in shoot tissue significantly varied with P treatment (Fig. 4.2, Table 4.2). Highest P contents were found in shoot tissue from the sodium phosphate treatment, closely followed by the DNA treatment (Fig. 4.2). Interestingly, wheat plants had the highest shoot P contents in the absence of *R. intraradices* and/or *P. putida* when treated with DNA and not sodium phosphate. Lowest P contents were detected in shoots of the hydroxyapatite treatment (Fig. 4.2). The presence of *R. intraradices* as well as the two-way interaction between the P treatment and *R. intraradices* had a significant impact on shoot P contents. Depending on the P source the presence of *R. intraradices* either increased or decreased shoot P contents (Fig. 4.2). In the case of sodium phosphate, phytic acid and hydroxyapatite, total shoot P contents increased between 17.8 and 39.6%. In contrast to that, shoot P contents of the DNA treatment were reduced by almost 27% in the presence of *R. intraradices*. *P. putida* did not have a significant effect on total shoot P content as a sole factor (Table 4.2). However, there was a significant interaction between

P. putida and the P treatment (Table 4.2). Like *R. intraradices*, *P. putida* either had a positive or negative impact on the shoot P content depending on the P source. In the case of both inorganic P treatments, the presence of *P. putida* increased shoot P contents by 19% (sodium phosphate) and 149.1% (hydroxyapatite), respectively. In contrast to that, shoot P contents were slightly reduced (≤10%) in both organic P treatments. Total shoot P contents were also significantly affected by a three-way interaction between the P treatments, *R. intraradices* and *P. putida* (Table 4.2). In the sodium phosphate treatment, dual inoculation with *R. intraradices* and *P. putida* resulted in an increase in P contents by 12.9%. However, these were still slightly lower than those detected in both single inoculation treatments. In the presence of phytic acid, dual inoculation reduced shoot P contents between 35 and 53.6% compared to the non- and single inoculation treatments. Similar to the single inoculation with either *R. intraradices* or *P. putida*, the presence of both microorganisms lowered shoot P contents in the DNA treatment. Nevertheless, the dual inoculation resulted in higher P contents than the *R. intraradices* only treatment. In the case of the hydroxyapatite treatment, dual inoculation resulted in 131.9% higher total shoot P contents compared to the non-microbial wheat only treatment. Moreover, shoot P contents were also 66.1% higher than those found in wheat treated with *R. intraradices* alone. However, single inoculation with *P. putida* resulted in higher P contents (6.9%) than the dual inoculation.

The available P source as well as both microorganisms significantly affected whole plant P contents. Furthermore, a significant two-way interaction between the P treatment and either microbial inoculum was determined. Within the sodium phosphate treatment, whole plant P contents remained fairly constant. Only the sole presence of *P. putida* reduced total P contents by 14.55%. In both organic P treatments, *P. putida* as well as dual inoculation with *R. intraradices* and *P. putida* reduced between 18.3 and 32.2%. However, single inoculation with *R. intraradices* resulted in slightly higher (11.4%) P contents in the phytic acid treatment, but slightly lower (11.4%) contents in the DNA treatment. Both

microbes increased total P contents in the hydroxyapatite treatment. However, the presence of *P. putida* resulted in a higher and more significant increase (65.1%) than *R. intraradices* (13.5%). Dual inoculation also improved whole plant P contents, but to a lesser extent than single inoculation with *P. putida*.

Variations in P concentrations per mg dry tissue of roots and shoots showed trends similar to the ones found in total P contents (Fig. 4.3). However, only the P source and *P. putida* had a significant effect (Table 4.3).

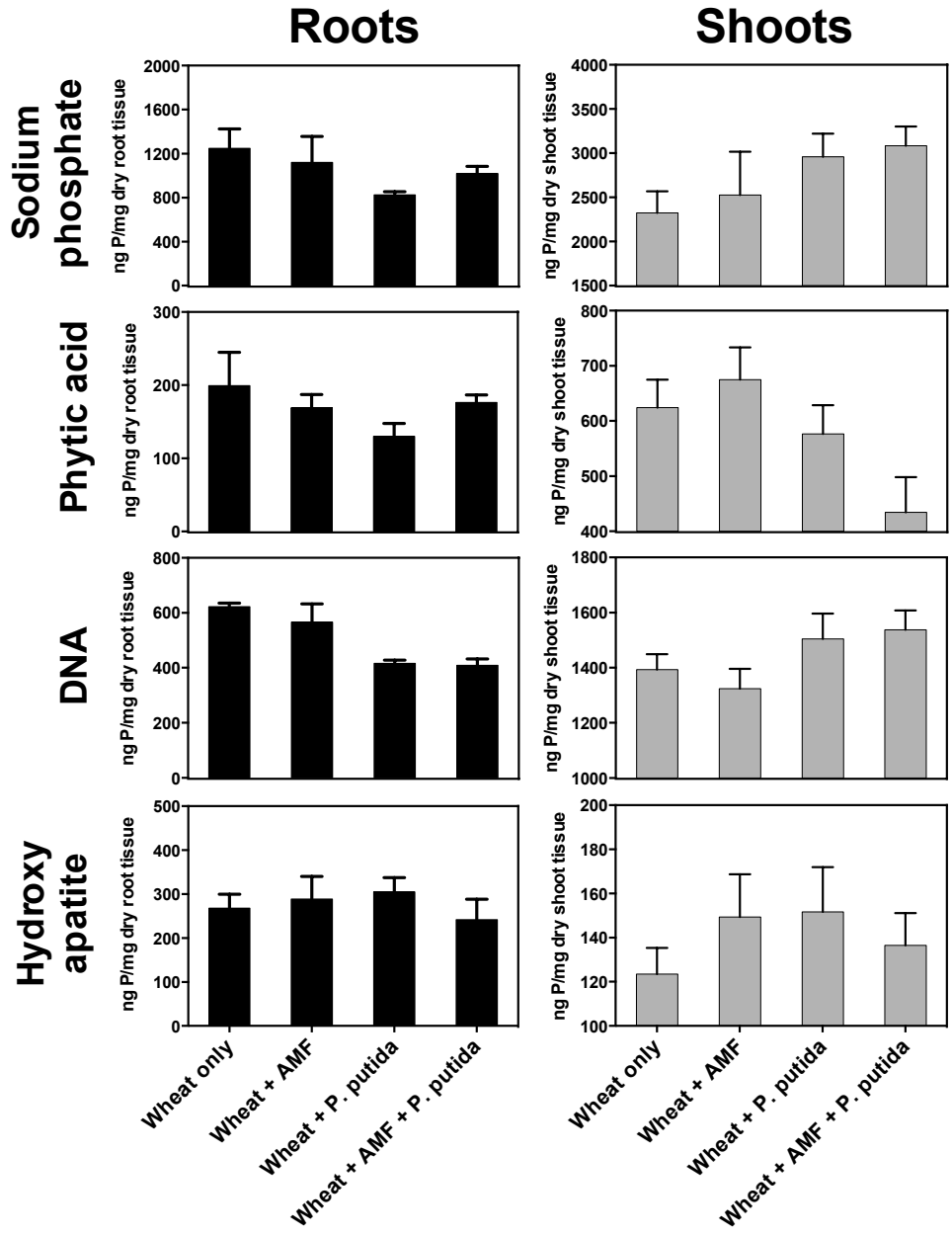


Fig. 4.3: P concentrations per mg dry weight of wheat roots and shoots after 10 weeks of growth. Error bars are the standard error of the mean.

Table 4.3: Statistical results of the effects of P and microbial treatments on root and shoot P concentrations. ns= not significant, *= p< 0.05, **= p<0.01, ***= p<0.001 (linear mixed-effect model, n=5)

Functional group	Source of variation	
Root P concentrations	P treatment	***
	R. intraradices	ns
	P. putida	**
	P treatment**R. intraradices*	ns
	P treatment**P. putida*	ns
	*R. intraradices***P. putida*	ns
	P treatment* *R. intraradices***P. putida*	ns
Shoot P concentrations	P treatment	***
	R. intraradices	ns
	P. putida	ns
	P treatment**R. intraradices*	ns
	P treatment**P. putida*	ns
	*R. intraradices***P. putida*	ns
	P treatment* *R. intraradices***P. putida*	*

4.3.3 Root and shoot biomass

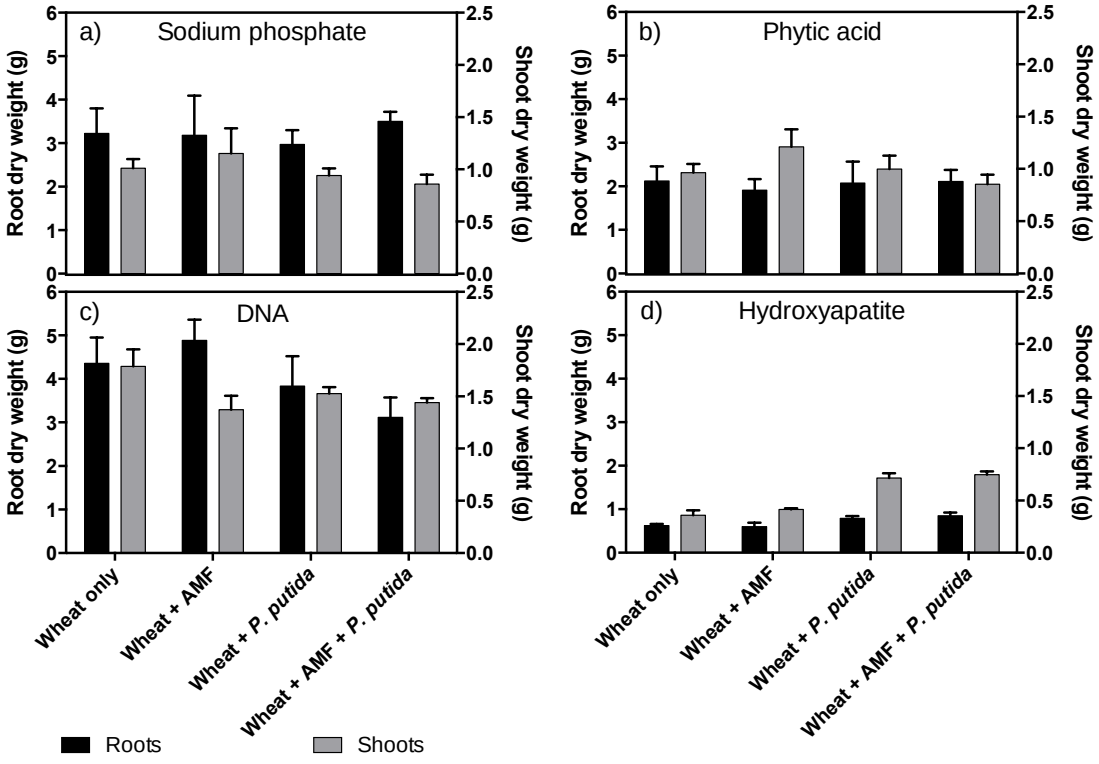


Fig. 4.4: Wheat root (left y-axis) and shoot (right y-axis) dry weights after 10 weeks of growth. Error bars represent the standard error of the mean.

Table 4.4: Statistical results of the effects of P and microbial treatments on root and shoot biomass. ns= not significant, *= p< 0.05, **= p<0.01, ***= p<0.001 (linear mixed-effect model, n=5)

Functional group	Source of variation	
Root dry weight	P treatment	***
	R. intraradices	ns
	P. putida	ns
	P treatment**R. intraradices*	ns
	P treatment**P. putida*	**
	*R. intraradices***P. putida*	ns
	P treatment* *R. intraradices***P. putida*	ns
Shoot dry weight	P treatment	***
	R. intraradices	**
	P. putida	**
	P treatment* *R. intraradices*	**
	P treatment**P. putida*	***
	*R. intraradices***P. putida*	ns
	P treatment* *R. intraradices***P. putida*	ns

While the different P treatments and the interaction between the P treatment and *P. putida* had a significant effect; *P. putida* as a sole factor as well as *R. intraradices* did not have any impact on the mean root biomass (Table 4.4). Unexpectedly, highest root dry weights were found in wheat that received DNA and not readily available sodium phosphate as P source (Fig. 4.4a, c). In addition, wheat plants that were treated with hydroxyapatite exhibit the lowest root dry weights (Fig. 4.4d). Depending on the P source the presence of *P. putida* had either a negative or positive impact on root growth. In the case of sodium phosphate and both organic P treatments, *P. putida* reduced root biomass between 2.2 and 12%. In contrast to that, *P. putida* increased root dry weights by 27.2% in the presence of hydroxyapatite. Nevertheless, it still was by far the lowest dry weights compared to all other P treatments.

Shoot growth was majorly affected by the different P sources as well as the presence of *P. putida* and *R. intraradices* (Table 4.4). Furthermore, there was a significant two-way interaction between the P sources and both microbial treatments; however, no interaction between *P. putida* and *R. intraradices* affected shoot dry weights. Plants treated with DNA had the highest mean shoot biomass, while plants from the hydroxyapatite treatment exhibited the lowest (Fig. 4.4c, d). It is noteworthy that despite the distinctively

lower shoot P contents; shoot dry weights in the phytic acid treatment were very similar to the ones found in the sodium phosphate treatment. The impact of *R. intraradices* on shoot growth highly depended on the respective P source. In the case of DNA, mean shoot biomass was reduced by 23.3%. In all other P treatments, shoot growth was promoted by AMF colonization. The highest increase of 25.9% in mean dry weights was found in the phytic acid treatment. Even though plants of the hydroxyapatite treatment possessed the highest fungal abundances (Fig. 4.4a), the increase in mean shoot biomass was relatively small (15.3%). As in the case of *R. intraradices*, the effect of *P. putida* on shoot growth highly depended on the present P source. In the cases of sodium phosphate and DNA, *P. putida* decreased shoot biomass by 7 and 14.6%, respectively (Fig. 4.4a, c). In the phytic acid treatment, the effect of *P. putida* was minimal positive. Wheat shoot biomass only increased by 3.7%. Despite the fact that *P. putida* abundance was the lowest, shoot dry weights increased by 98.8% in the hydroxyapatite treatment.

4.3.4 PCA of root metabolite profiles

PCA and OPLSA showed clear discriminations of root metabolite profiles between the different P treatments (Fig. 4.5). This P source related distinction was not affected by the different microbial treatments, since similar PCA separation patterns were observed in the present of *R. intraradices* and *P. putida* or both. In contrast to that, microbial treatments hardly affect root metabolite profiles after 10 weeks of growth. A mass discrimination was only found in the hydroxyapatite treatment in the presence of *P. putida* compared to the non-microbial treatment (Fig. 4.6b). Statistical analyses of all selected mass bins supported the results of both PCA and OPLSA. The majority of mass bins only showed significant differences in abundances between P applications. However, *P. putida* had a significant effect on eighteen mass bins, mostly in the hydroxyapatite and, surprisingly, sodium phosphate treatment.

Discriminating masses were found for putative carboxylic acids, sugar and sugar phosphates and amino acids that are involved in or linked to the glycolytic and shikimic acid pathway as well as the TCA cycle (Table A4.1 in the appendix). Furthermore, several discriminating mass bins were secondary metabolites such as phenylpropanoids, flavonoids, terpenoids and alkaloids (Table A4.3 and A4.4 in the appendix). Identified metabolites also included compounds that are known to function as plant hormones or to be involved in their biosynthesis (Table A4.2 in the appendix). Interestingly, numerous discriminating mass bins were also assigned to different di-, tri- and tetrapeptides (Table A4.5 in the appendix).

4.3.5 Abundances of metabolites of the glycolytic, shikimic acid pathway and the TCA cycle

Mass bin abundances of putative metabolites that are intermediates of the glycolytic and shikimic acid pathway, the TCA cycle and the starch and sucrose metabolism were significantly different between the P treatments (Table A4.1). Wheat plants the received either hydroxyapatite or DNA as respective P source had reduced abundances of these metabolites compared to the sodium phosphate treatment (Fig. 4.7). However, sugar phosphates were highly enriched in roots of the DNA treatment. In the phytic acid treatment, abundances of most these metabolites seemed to be slightly elevated in the presence of either *R. intraradices* or *P. putida* or both compared to the sodium phosphate treatment. But this trend was not significant. In contrast to this, sugar phosphates were strongly depleted.

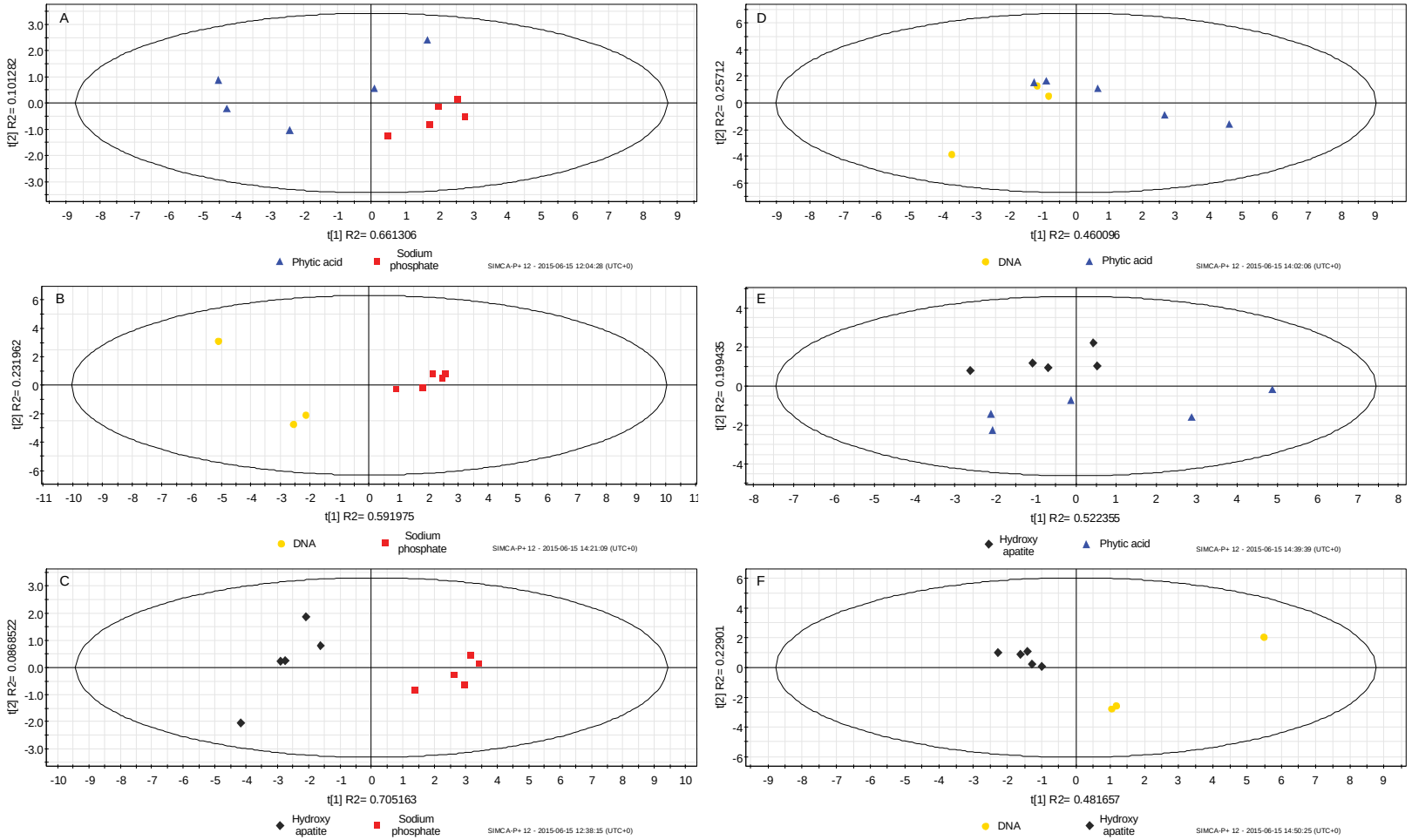


Fig. 4.5: PCA plots showing discrimination of root metabolite profiles between the different P forms of wheat plant that did not receive any microbial treatment. A) phytic acid (blue) versus sodium phosphate (red), B) DNA (yellow) versus sodium phosphate, C) hydroxyapatite (dark grey) versus sodium phosphate, D) DNA versus phytic acid, E) hydroxyapatite versus phytic acid and F) DNA versus hydroxyapatite.

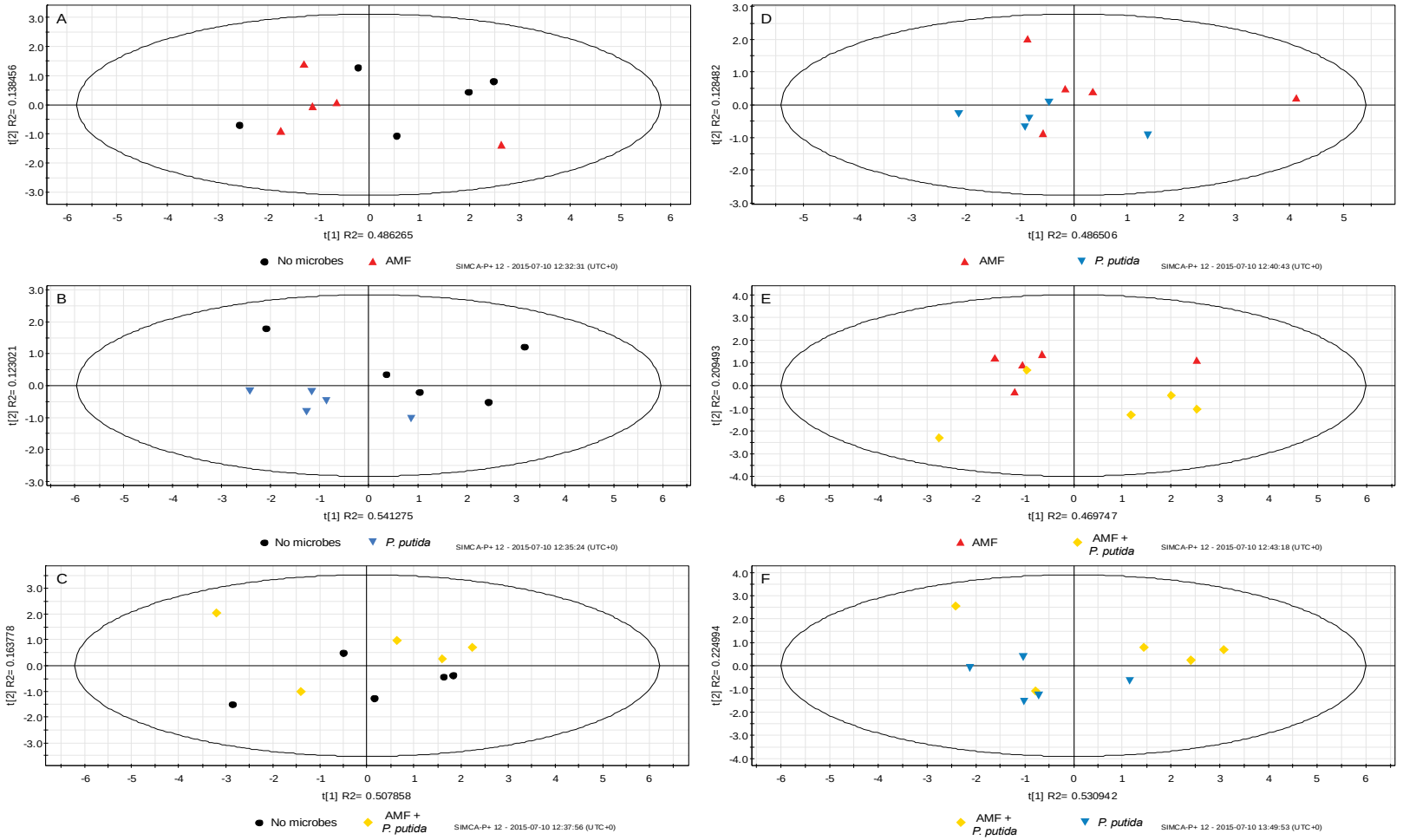


Fig. 4.6: PCA plots showing the effect of *R. intraradices* (AMF) and *P. putida* on root metabolite profiles of wheat plants treated with hydroxyapatite. A) No microbes (black) versus AMF (red), B) no microbes versus *P. putida* (blue), C) no microbes versus dual inoculation (yellow), D) AMF versus *P. putida*, E) AMF versus dual inoculation and F) *P. putida* versus dual inoculation.

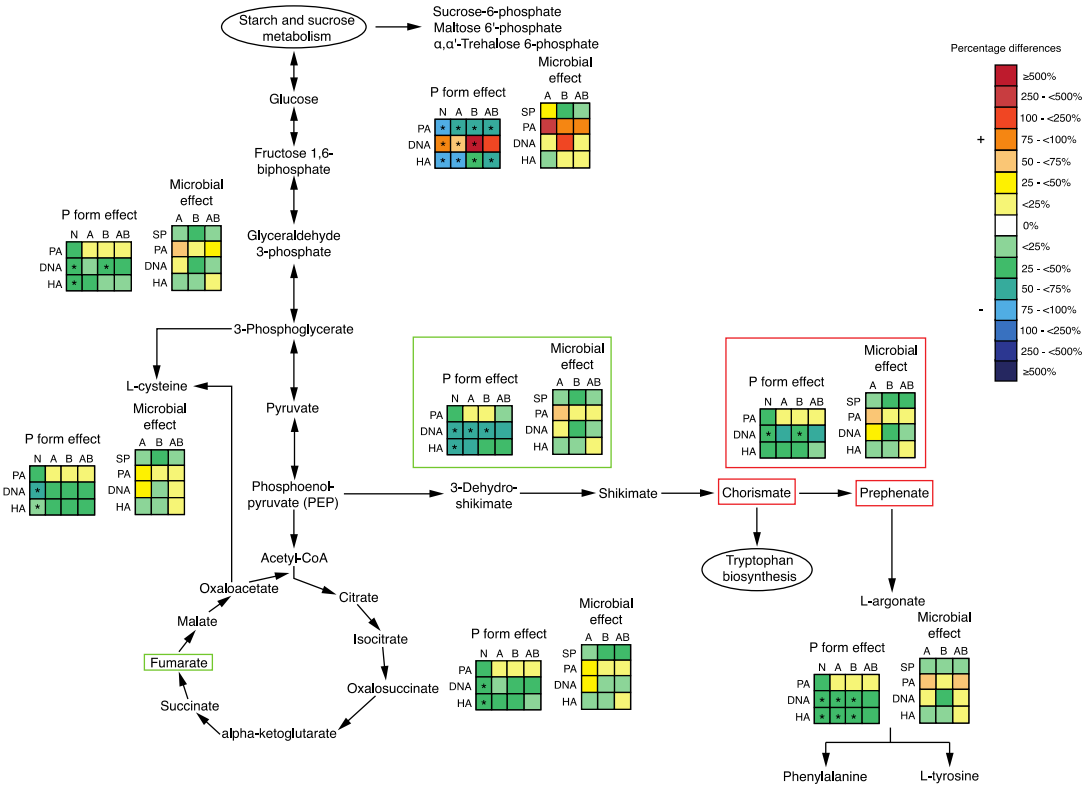


Fig. 4.7: Simplified sketch of glycolytic, shikimic acid pathway and the TCA cycle. The P form effect shows percentage differences in metabolite abundances between the sodium phosphate (SP) and the other P treatments (PA= phytic acid, HA= hydroxyapatite) of each microbial application (N= no microbes, A= *R. intraradices*, B= *P. putida*, AB= *R. intraradices* + *P. putida*). The microbial effect displays the percentage differences between the non-microbial treatment and the two single-inoculations as well as the dual-inoculation of each P treatment. * indicates statistically significant changes in abundances (three-way ANOVA, TukeyHSD post-test, n=5).

4.3.6 Plant hormone abundances

Several plant hormones and metabolites involved in their biosynthesis or degradation showed significant differences in their abundances (Table A4.2). Discriminations were found for jasmonic and traumatic acid, gibberellins and gibberellin glycosides. Abscisic acid was not detected, but its catabolites 8'hydroxyabscisic acid and phaseic acid were resolved however.

Jasmonic and traumatic acid that derive from alpha-linolenic acid metabolism were highly enriched in roots of the phytic acid and hydroxyapatite treatment compared to the sodium phosphate treatment (Fig. 4.8). In contrast to this, these compounds were all either depleted or only slightly enriched in roots of wheat that received DNA as P source. The same was found for the catabolites of abscisic acid (ABA) along the carotenoid pathway (Fig. 4.9). Even though this trend was not significant, *R. intraradices* as well as *P. putida* seemed to reduce abundances of these groups of plant hormones within each P application. However, abundances of 8'hydroxyabscisic acid and phaseic acid appeared to rise in the presence of *R. intraradices, P. putida* or both in the sodium phosphate treatment (Fig. 4.9). Similarly, microbial applications seemed to increase abundances of traumatic and jasmonic acid within the DNA treatment (Fig. 4.9). In the hydroxyapatite treatment, the presence of *P. putida* decreased the abundances of jasmonic acid and 8'hydroxyabscisic acid and/or phaseic acid compared to the non-microbial treatment.

Gibberellins are formed along the diterpenoid pathway. Abundances of either gibberellin A12 or gibberellin A14 or A53 aldehyde were depleted in both organic P and the hydroxyapatite treatment (Fig. 4.9). However, gibberellin A3 or gibberellin A29 and/or A34 catabolite were enriched in the phytic acid and hydroxyapatite treatment, but depleted in the DNA application. Microbial inoculants seemed to slightly increase abundances of gibberellin A12 in the phytic acid and gibberellin A3 in the DNA and hydroxyapatite treatment, but these trends were not statistically significant.

Gibberellin glucosides were depleted in the phytic acid and hydroxyapatite treatment, but highly enriched in wheat roots that received DNA. Microbial inoculants appeared to increase abundances of gibberellin glucosides in particular in the case of phytic acid. However, this trend was not significant.

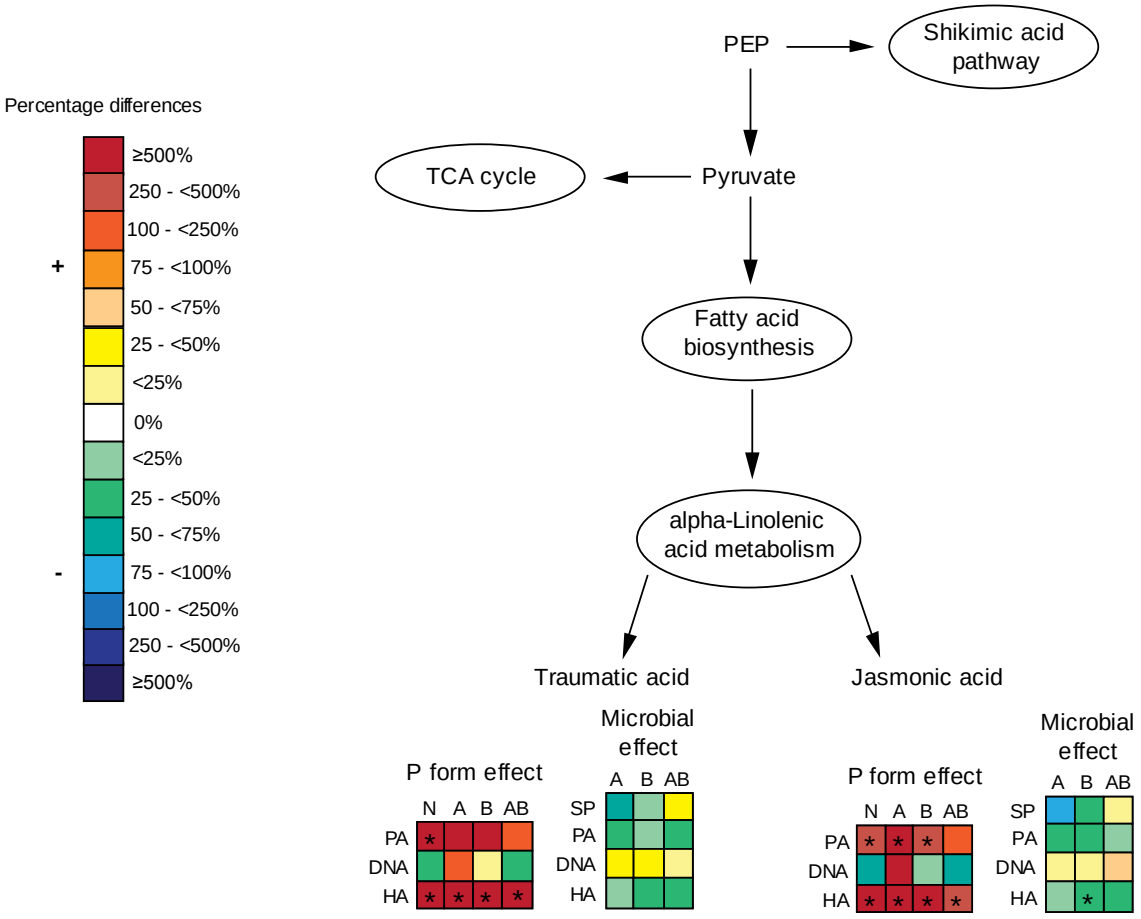


Fig. 4.8: Simplified sketch of the jasmonic and traumatic biosynthesis pathway. The P form effect shows percentage differences in metabolite abundances between the sodium phosphate (SP) and the other P treatments (PA= phytic acid, HA= hydroxyapatite) of each microbial application (N= no microbes, A= *R. intraradices*, B= *P. putida*, AB= *R. intraradices* + *P. putida*). The microbial effect displays the percentage differences between the non-microbial treatment and the two single-inoculations as well as the dual-inoculation of each P treatment. * indicates statistically significant changes in abundances (three-way ANOVA, TukeyHSD post-test, n=5).

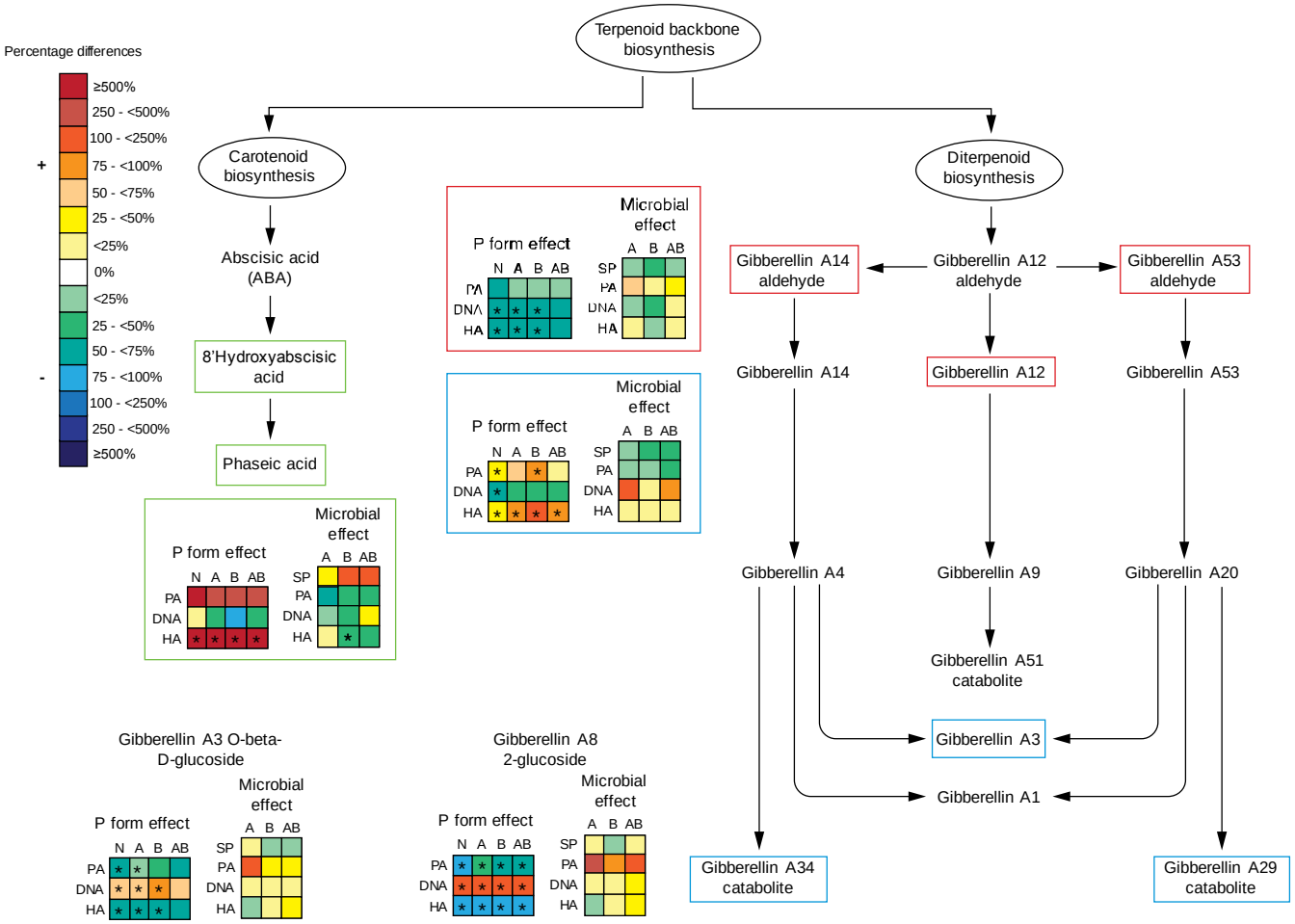


Fig. 4.9: Simplified sketch of the abscisic acid and gibberellins formation and degradation pathways. The P form effect shows percentage differences in metabolite abundances between the sodium phosphate (SP) and the other P treatments (PA= phytic acid, HA= hydroxyapatite) of each microbial application (N= no microbes, A= *R. intraradices*, B= *P. putida*, AB= *R. intraradices* + *P. putida*). The microbial effect displays the percentage differences between the non-microbial treatment and the two single-inoculations as well as the dual-inoculation of each P treatment. * indicates statistically significant changes in abundances (three-way ANOVA, TukeyHSD post-test, n=5).

4.3.7 Abundances of different groups of phenylpropanoids, flavonoids, alkaloids and terpenoids

The different P treatments had a significant effect on abundances of mass bins that were putatively identified as various flavonoids and phenylpropanoids including coumarins, monolignols, hydroxycinnamic acids and lignans (Table A4.3) as well as alkaloids and terpenoids (Table A4.4).

Compared to the sodium phosphate treatment, enrichments of some of phenylpropanoids and flavonoids were mostly found in roots of wheat plants that either received hydroxyapatite or DNA (Fig. 4.10 and 4.11). A strong accumulation of putative idenified ferulic acid was detected in roots of the hydroxyapatite treatment. In addition, abundances of dihydroxysamidin or visnadin (Fig. 4.10), luteolin or kaempferol (Fig. 4.11) and sumatrol or tephrosin were increased (Fig. 4.11). In contrast, coniferin was highly enriched in the DNA treatment (Fig. 4.10). Further, mass bin abundances of the coumarin byakangelicin (Fig. 4.10) as well as the flavonoids artelastchromene, millettone and artelastofuran (Fig. 4.11) were elevated. Interestingly, abundances of phenylpropanoids and flavonoids that contain glucosides were strongly increased in both of these treatments and also, to a smaller extent, in the phytic acid treatment.

Microbial inoculants seemed to have a slight positive effect on phenylpropanoid and flavonoid abundances, in the presence of the more complex P forms (phytic acid, DNA, hydroxyapatite). However, as in the case of the plant hormones, this trend was not significant.

Significant enrichments of some alkaloids were found in the hydroxyapatite and the DNA treatment (Table 4.5). Nevertheless, the majority of alkaloids and terpenoids were depleted compared to the sodium phosphate treatment. In the phytic acid treatment, abundances of most alkaloids and terpenoids seemed to be slightly elevated, in particular in the presence of *R. intraradices* and *P. putida*. However, this trend was not statistically significant.

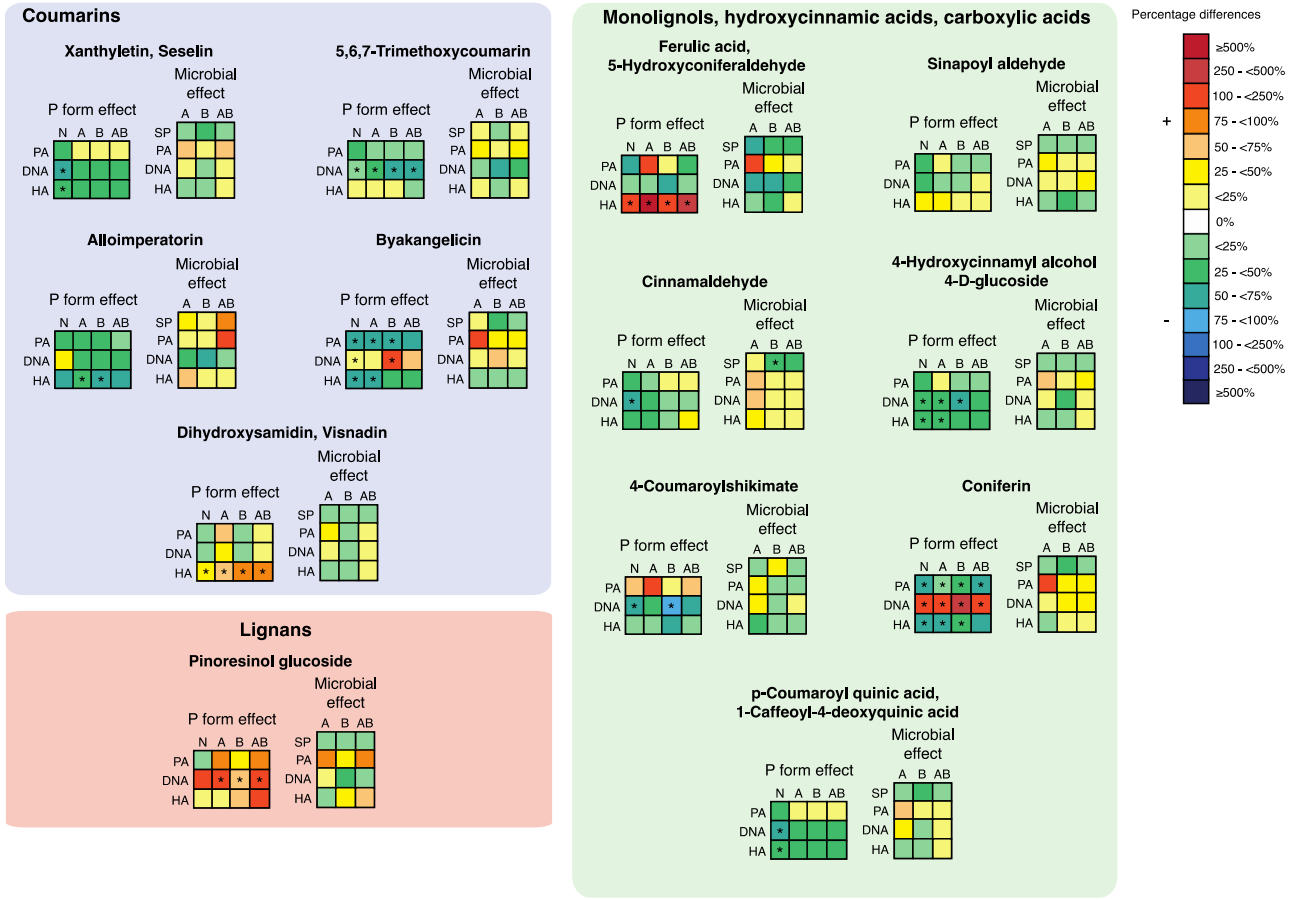


Fig. 4.10: Illustrated enrichment and depletion of phenylpropanoids. The P form effect shows percentage differences in metabolite abundances between the sodium phosphate (SP) and the other P treatments (PA= phytic acid, HA= hydroxyapatite) of each microbial application (N= no microbes, A= *R. intraradices*, B= *P. putida*, AB= *R. intraradices* + *P. putida*). The microbial effect displays the percentage differences between the non-microbial treatment and the two single-inoculations as well as the dual-inoculation of each P treatment. * indicates statistically significant changes in abundances (three-way ANOVA, TukeyHSD post-test, n=5).

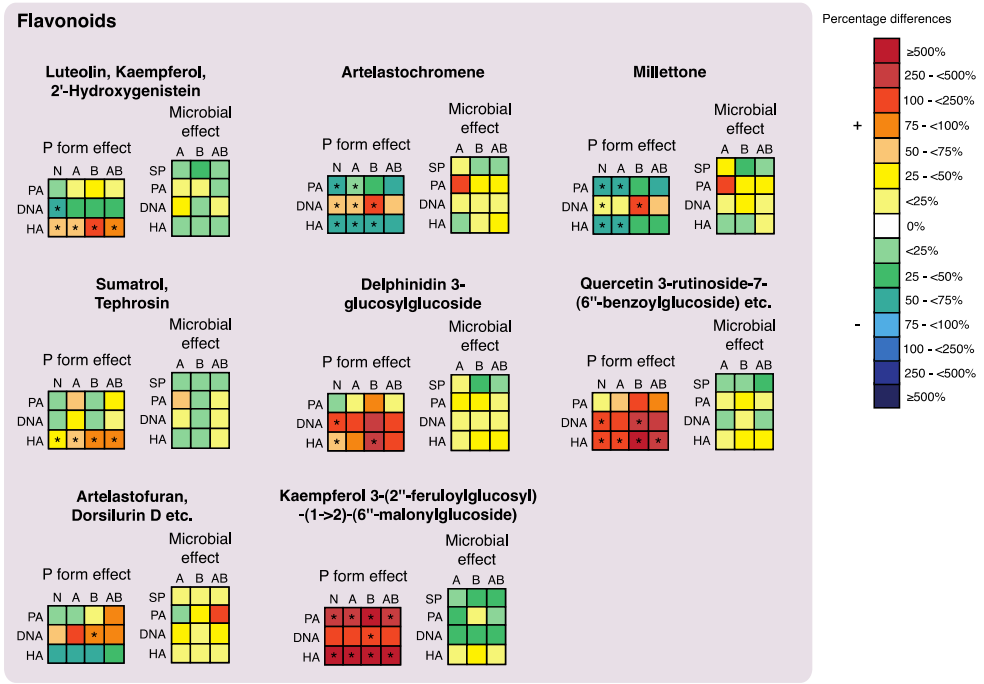


Fig. 4.11: Illustrated enrichment and depletion of flavonoids. The P form effect shows percentage differences in metabolite abundances between the sodium phosphate (SP) and the other P treatments (PA= phytic acid, HA= hydroxyapatite) of each microbial application (N= no microbes, A= *R. intraradices*, B= *P. putida*, AB= *R. intraradices* + *P. putida*). The microbial effect displays the percentage differences between the non-microbial treatment and the two single-inoculations as well as the dual-inoculation of each P treatment. * indicates statistically significant changes in abundances (three-way ANOVA, TukeyHSD post-test, n=5).

Table 4.5: P form effect on abundances of discriminating mass bins of putatively identified alkaloids and terpenoids. Compounds were either detected as hydrogen **[**M+H]+ or sodium adducts [M+NA]+. The P form effect shows percentage differences in metabolite abundances between the sodium phosphate (SP) and the other P treatments (PA= phytic acid, HA= hydroxyapatite) of each microbial application (N= no microbes, A= *R. intraradices*, B= *P. putida*, AB= *R. intraradices* + *P. putida*). * indicates statistically significant changes in abundances (three-way ANOVA, TukeyHSD post-test, n=5).

Mass bin m/z		Name	P form effect											
[M+H]+	[M+Na]+		N PA	A PA	B PA	AB PA	N DNA	A DNA	B DNA	AB DNA	N HA	A HA	B HA	AB HA
140.0357346		6-Hydroxynicotinic acid	-57%	70%	99%	23%	97%*	219%*	369%*	224%*	58%	95%	171%	71%
	148.0406879	3,6-Dihydronicotinic acid	-33%	13%	16%	1%	-54%*	-38%	-50%	-36%	-35%	-37%	-18%	-12%
203.0862282		Vasicinone	-35%	-13%	-44%	0%	-49%*	-41%	-53%	-37%	-31%	-52%	-41%	-14%
	205.0320827	7-Methyluric acid 1-Methyluric acid	-33%	28%	17%	3%	-33%	7%	-7%	13%	-36%*	-32%	-18%	-15%
	219.0498294	3,7-Dimethyluric acid 1,7-Dimethyluric acid	-46%*	-9%	-20%	-26%	-42%*	-19%	-45%	-21%	-44%*	-40%	-38%	-32%
	222.1108149	Tussilagine	42%	54%	439%	88%	-44%	-43%	111%	-15%	105%*	201%*	594%*	304%*
237.1045683		Glycosminine	105%*	81%	479%	85%	-30%	-28%	276%	77%	218%*	413%*	951%*	507%*
	237.1045683	Harmaline												
249.0696888		Tryptanthrine	-47%*	14%	3%	-3%	-52%*	-25%	-33%	-20%	-62%*	-56%*	-41%	-41%
	287.1580746	Dehydrojuvabione	136%	88%	241%	100%	-62%	-58%	-54%	3%	545%*	576%*	580%*	800%*
	291.1069795	Lysergic acid	-34%	16%	13%	-1%	-58%*	-46%	-50%	-43%	-36%	-41%	-28%	-21%
	304.0928009	Annolobine	22%	30%	3%	-43%	-45%	-58%	-56%	-65%	209%*	231%*	139%*	53%
310.1056368		Lunamarine	-32%	31%	5%	7%	-47%*	-21%	-37%	-12%	-39%*	-36%	-23%	-13%
	310.1056368	(S)-Norlaudanosoline												
	336.1118491	Angustine Acetylcaranine	-34%	16%	15%	1%	-58%*	-40%	-46%	-40%	-43%*	-44%	-32%	-26%

Mass bin m/z		Name	P form effect											
[M+H]+	[M+Na]+		N PA	A PA	B PA	AB PA	N DNA	A DNA	B DNA	AB DNA	N HA	A HA	B HA	AB HA
380.1095725	**402.09882**	12-Hydroxydihydrochelirubine	-30% -70%*	4% -52%*	1% -28%*	-4% -78%*	-39%* 26%	-33%* 4%	-46%* 160%	-33% -30%	-38%* -52%*	-41%* -67%*	-33%* -30%	-34% -72%
	380.2253238	Songorine	-43%	-4%	-9%	-12%	-54%*	-51%*	-60%*	-46%	-49%*	-52%*	-41%*	-44%
	381.1170238	10-Deoxygeniposidic acid	-32%	16%	17%	6%	-48%*	-26%	-35%	-25%	-38%*	-34%	-15%	-11%
441.3308		Pfaffic acid	-72%*	-68%*	-49%*	-69%*	-25%	-50%	26%	-28%	-62%*	-81%*	-69%*	-68%*
	691.3232	14-Deacetylnudicauline Lycaconitine	-75%*	-29%*	-52%	-59%	120%	110%*	176%*	113%*	-67%*	-75%*	-54%	-57%

4.3.8 Abundances of peptides

Numerous mass bins that showed significant discriminations between the different P treatments were putatively identified as various di-, tri- and tetra-peptides (Table A4.5). Compared to the sodium phosphate treatment, most peptides were depleted in roots of the other three P applications (Table 4.6). Nevertheless, significant enrichments of mostly tri- and tetra-peptides were found in all three treatments. It is noteworthy that peptides that were enriched in the DNA treatment differed from those accumulated in the phytic acid and hydroxyapatite treatments.

Table 4.6: P form effect on abundances of discriminating mass bins of putatively identified peptides. Compounds were either detected as hydrogen **[**M+H]+ or sodium adducts [M+NA]+. The P form effect shows percentage differences in metabolite abundances between the sodium phosphate (SP) and the other P treatments (PA= phytic acid, HA= hydroxyapatite) of each microbial application (N= no microbes, A= *R. intraradices*, B= *P. putida*, AB= *R. intraradices* + *P. putida*). * indicates statistically significant changes in abundances.

Mass bin m/z		P form effect											
[M+H]+	[M+Na]+	C PA	CA PA	CB PA	CAB PA	C DNA	CA DNA	CB DNA	CAB DNA	C HA	CA HA	CB HA	CAB HA
219.0966014		695%*	445%*	1155%*	612%	-32%	-1%	184%	61%	1066%*	1624%*	2379%*	1786%*
220.0985329		64%*	72%	325%*	74%	-43%	-43%	45%	-18%	144%*	269%*	544%*	295%*
	270.0633913	-78%*	-55%*	-58%*	-61%*	36%*	29%	162%*	86%	-55%*	-68%*	-40%	-40%
310.1056368		-32%	31%	5%	7%	-47%*	-21%	-37%	-12%	-39%*	-36%	-23%	-13%
335.1046854		-32%	6%	-1%	-6%	-43%*	-42%*	-51%*	-41%	-40%*	-48%*	-39%*	-39%
336.1118491		-34%	16%	15%	1%	-58%*	-40%	-46%	-40%	-43%*	-44%	-32%	-26%
337.1223281		-36%	12%	12%	-1%	-60%*	-44%	-49%	-41%	-46%*	-46%	-34%	-28%
	344.0832505	-8%	37%	-81%	11%	-59%*	-36%	-92%*	-41%	29%	53%	-75%	63%
	357.0966335	-74%*	-51%*	-35%*	-54%	21%*	14%	216%*	71%	-52%*	-64%*	-29%	-40%
362.0913942		-38%	13%	10%	-1%	-59%*	-40%	-46%	-38%	-53%*	-52%	-41%	-33%
	366.1232526	-56%*	0%	-16%	-38%	60%*	111%	215%*	142%*	14%	18%	29%	19%
373.1446638		-24%	46%	25%	-19%	-65%	-56%	39%	-27%	-89%*	-76%	-87%	-63%
379.1120258		-20%	23%	33%	18%	-33%	0%	-10%	2%	-22%	-11%	4%	10%
	401.0961417	-73%*	-54%*	-35%	-55%	25%*	3%	159%*	53%	-57%*	-71%*	-39%	-46%
	379.2307438	-40%	6%	6%	-4%	-53%*	-37%*	-44%*	-36%	-47%*	-44%*	-30%	-29%
380.1095725		-30%	4%	1%	-4%	-39%*	-33%*	-46%*	-33%	-38%*	-41%*	-33%*	-34%
	402.09882	-70%*	-52%*	-28%*	-78%*	26%	4%	160%	-30%	-52%*	-67%*	-30%	-72%
	380.1095725	-30%	4%	1%	-4%	-39%*	-33%*	-46%*	-33%	-38%*	-41%*	-33%*	-34%
380.2253238		-43%	-4%	-9%	-12%	-54%*	-51%*	-60%*	-46%	-49%*	-52%*	-41%*	-44%
381.1170238		-32%	16%	17%	6%	-48%*	-26%	-35%	-25%	-38%*	-34%	-15%	-11%

Mass bin m/z		P form effect											
[M+H]+	[M+Na]+	C PA	CA PA	CB PA	CAB PA	C DNA	CA DNA	CB DNA	CAB DNA	C HA	CA HA	CB HA	CAB HA
	383.0901917	-46%*	-47%*	0%	-22%	44%*	26%	204%*	78%	-7%	-19%	80%	18%
389.1754597		4%	-6%	45%	-20%	-10%	-40%	-12%	20%	100%*	38%	104%	37%
	411.1512316	-19%	56%	-1%	17%	-13%	36%	-7%	16%	43%*	55%*	75%*	85%*
	389.1754597	4%	-6%	45%	-20%	-10%	-40%	-12%	20%	100%*	38%	104%	37%
411.1512316		-19%	56%	-1%	17%	-13%	36%	-7%	16%	43%*	55%*	75%*	85%*
	417.0748789	23%	54%*	116%*	80%	-4%	-3%	1%	13%	103%*	101%*	205%*	201%*
	418.0780225	20%	54%	113%*	74%	-2%	1%	7%	15%	103%*	106%*	212%*	199%*
422.1990763		5686%*	827%*	4380%*	1016%	473%	101%	642%	22%	9224%*	3044%*	10362%*	2942%*
424.2156578		1147%*	1073%*	1777%*	889%	-34%	105%	11%	-32%	2217%*	4117%*	4386%*	2671%*
445.3086693		746%	143%	-18%	790%	449%	294%	34%	844%	914%*	472%*	32%	1297%
450.1729452		-38%	21%	20%	2%	-46%	-20%	-46%	-28%	-100%*	-83%*	-63%	-40%
	461.0537418	-83%*	-38%*	-38%	-53%	87%*	101%*	762%*	295%*	-60%*	-61%	-5%	-12%
468.2647834		-30%	3%	7%	-53%	-12%	22%	15%	-5%	345%*	258%*	237%*	93%
498.2239688		-50%	14%	-41%	-36%	-58%	-54%	-63%	-47%	-86%*	-89%*	-72%*	-83%
	512.1481422	258%*	309%*	837%*	3795%	-100%	-37%	34%	597%	408%*	516%*	1184%*	6300%*
	524.1151702	-45%	-23%	-50%	-37%	63%	-2%	-21%	-18%	-73%*	-78%*	-72%*	-82%*
528.2243343		-74%*	-23%*	-48%	-56%	111%*	147%*	223%*	154%*	-69%*	-75%*	-57%	-59%
529.2067731		-2%	-14%	26%	94%	72%	125%	95%*	92%	-51%	-53%	-51%	-48%
543.1990684		-11%	87%	46%	99%	104%*	159%*	57%	140%	8%	2%	64%	111%
568.2062099		-41%	4%	4%	-9%	-60%*	-46%	-45%	-41%	-50%*	-54%	-41%	-35%
	689.3239742	-86%*	-37%*	-68%*	-70%	153%	129%	193%	133%	-83%*	-89%*	-73%*	-73%
690.3270731		-84%*	-36%*	-66%	-70%	150%	128%	196%	131%	-80%*	-87%*	-70%	-70%

4.4 Discussion

4.4.1 The impact of organic and inorganic P on microbial root colonization

Low soil P accessibility and plant P deprivation encourage root colonization of AMF and PGPR (Richardson et al., 2009, Smith and Smith, 2012). However, little is known about whether different inorganic and organic P forms have an impact on root colonization as well. This study demonstrates that the extent of root and rhizoplane colonization of *R. intraradices* and *P. putida* depended on the present P source.

The highest rhizoplane colonization of *P. putida* was found in the presence of sodium phosphate (Fig. 4.1b), thus indicating that the conditions in this treatment were most favourable for this PGPR. Plant growth promoting rhizobacteria including *Pseudomonas* species are attracted by, and thrive on, carbon compounds excreted from the plant roots, which include organic acids, sugars and other secondary metabolites (Bais et al., 2006; Badri et al., 2009; Badri and Vivanco, 2009; Neal et al., 2012). Thus, the high abundance of *P. putida* may be due to enhanced availability of carbon compounds from root exudates in the sodium phosphate treatment. This is supported by the higher metabolic abundances along the glycolytic and shikimic acid pathway as well as the TCA cycle (Fig. 4.7) and increased abundances of most secondary metabolites compared to the other P treatments (Fig. 4.10 and 4.11, Table 4.5). In contrast to this, root colonization of *R. intraradices* was the lowest in the sodium phosphate treatment. Unlike PGPR, mycorrhiza fungi take up plant carbon compounds in the form of sugars within the roots along their intercellular fungal structures through the interfacial apoplast rather than from root exudates through their extracellular hyphae (Shachar-Hill et al., 1995; Schuessler et al., 2006; Smith and Smith, 2012). Therefore, the low abundance of *R. intraradices* may indicate that less carbon was transferred from the plant along this internal pathway relative to the amount released into the rhizosphere. Nevertheless, other aspects of root metabolism such as of plant hormonal balance and the presence and

composition of other secondary metabolites may have contributed to low AMF root colonization, which will be discussed later in this chapter.

Abundances of *P. putida* were similar in the phytic acid, DNA and hydroxyapatite treatments, but distinctively lower in comparison with the sodium phosphate treatment. The reduced abundances may indicate a lower supply of carbon compounds through root exudates, which is concurrent with the detected lower metabolic abundances in these treatments. However, root colonization of *R. intraradices* was higher in these treatments and abundances were increasing from the DNA over phytic acid to the hydroxyapatite treatment. This suggests that the presence of more complex P sources enhances the recruitment and colonization of AMF by wheat plants, possibly through enhanced intercellular carbon transfer. However, P contents of wheat roots and shoots were very low in the phytic acid and hydroxyapatite treatment. Therefore, it needs to be considered that overall plant P deprivation may be the dominating factor for the enhanced AMF recruitment in these two treatments. In addition to a potential increase in plant-to-fungus carbon transfer, wheat roots from both P treatments were strongly enriched in the plant hormone jasmonic acid (Fig 4.8). Moreover, the ABA catabolite phaseic acid was accumulated in these roots indicating increased metabolic abundances along the ABA biosynthetic pathway. Increased levels of jasmonic acid and ABA were linked to root mycorrhization in previous studies (Hause et al., 2007; Herrera-Medina et al., 2007; Hause and Schaarschmidt, 2009). Thus, both hormones may have promoted *R. intraradices* colonization in the phytic acid and hydroxyapatite treatments. The role and function of plant hormones in this study will be further discussed in section 4.4.4.

In most P treatments of this study, dual-inoculation resulted in a decrease in root colonization of both *R. intraradices* and *P. putida*, especially in the phytic acid treatment. But growth of *P. putida* was not supressed in the DNA treatment and abundances of *R. intraradices* did not decrease in roots that received hydroxyapatite. Overall, this indicates a rather hostile relationship between these two microbial species. From the results of this

study, it is difficult to concretely deduce the reasons and processes behind this antagonistic behaviour and whether it is more plant or microbial driven. One possibility for the reciprocal inhibition may be the competition for wheat root-derived carbon compounds. However, as mentioned above, *R. intraradices* receives plant carbon within the roots through their intercellular structures, while *P. putida* acquires carbon from root exudates. Thus, changes in carbon flow within the plant would determine this competition. Even though it is known that AMF can alter root exudate composition (Artursson et al., 2006) and there are also suggestions that AMF may be capable of increasing sugar transport from the plant by activating specific sugar carriers (Smith and Smith, 2012), it is more likely that in the case of the sodium phosphate, DNA and phytic acid treatment, wheat plants controlled the carbon transfer. Otherwise, carbon supply would possibly be more beneficial for *R. intraradices* than *P. putida* and the rate of mycorrhization would not decrease. Further, the prevailing low P conditions in this experiment, even in the sodium phosphate treatment, must have had a limiting effect on the formation of plant carbon compounds. Thus, a division of the available plant carbon pool between two microbes would reduce the carbon supply of each microbe compared to single-inoculation conditions and consequently limiting microbial growth. However, Germida and Walley (1996) found that in spring wheat, under the same growth conditions, the effect of PGPR on AMF colonization, P uptake and plant growth varied from positive to negative depending on the PGPR species. This suggests that more direct and species-specific interactions may be additionally responsible for the suppression.

Studies that investigated interactions between AMF and soil bacteria found that depending on the fungal and bacterial species, microbial exudation of metabolites either had a positive, neutral or negative impact on their colonization of the rhizosphere (Johansson et al., 2004; Artursson et al., 2006; Pivato et al., 2009; Nazir et al., 2010). For example, mycorrhizal fungi can actively change microbial community structure not only through the release of fungal exudates (de Boer et al., 2005; Duponnois et al., 2005) or

alteration of root exudate composition (Artursson et al., 2006), but also through changes of the local pH (Johansson et al., 2004; Singh et al., 2008). Mansfeld-Giese et al. (2002) showed that promotion and inhibition of bacterial growth by *R. intraradices* was species specific in the rhizosphere of *Cucumis satvius*. Moreover, Larsen et al. (2009) found that combined inoculation of *R. intraradices*, *Paenibacillus macerans* and *Paenibacillus polymyxa* resulted in the suppression of all three microorganisms and that of plant growth. However, this effect partially depended on the presence or absence of additive organic matter as well as possible changes in the local pH through increased microbial activity. Numerous PGPR including *P. putida* are able to produce or alter levels of phytohormones such as auxins (Patten and Glick, 1996; Khalid et al., 2004; Richardson et al., 2009), cytokinins (Timmusk et al., 1999; Perrig et al., 2007; Richardson et al., 2009), gibberellins (Gutierrez-Manero et al., 2001; Bottini et al., 2004; Richardson et al., 2009) or ethylene (Glick et al., 1994; Li et al., 2000), which are involved in the regulation of root development and architecture. Moreover, ethylene is an essential compound involved in inducing plant systemic resistance responses (Bleecker and Kende, 2000; Cameron et al., 2013). In addition to this, PGPR are also capable of altering the biosynthesis of phenylpropanoids and flavonoids, which can have allelopathic effects on microbial growth (Lavania et al., 2006; Dardanelli et al., 2008; Cheynier et al., 2013). Thus, it is not farfetched to assume that PGPR may also indirectly affect AMF colonization through changes in plant hormonal, phenylpropanoid and flavonoid levels.

With respect to these previous studies, it is rather unlikely that a potential change in plant-to-microbe carbon transfer is the sole factor that inhibited the growth of *R. intraradices* and *P. putida*. It is more likely that a combination of several factors that further includes direct and indirect microbial interactions through fungal and bacterial exudation, the prevailing P source, changes in soil pH and potentially P source specific alterations in root metabolism contributed to the suppression of *R. intraradices* and *P. putida*. Further research is needed using carbon tracer studies, detailed monitoring of

plant hormone levels as well as analysing microbial exudates in the presence of different P forms and under single- and dual-inoculation conditions in order to identify the processes involved.

In the DNA treatment, abundances of *P. putida* did not decrease in the presence of *R. intraradices*. Considering that colonization of *R. intraradices* was second lowest in the single-inoculation treatment compared to the other P treatments, it is likely that the presence of DNA induced a higher flow of carbon compounds towards root exudates, thus potentially increasing the carbon supply of *P. putida* and restricting it for *R. intraradices*. Moreover, wheat roots that received DNA as P source also showed accumulations of specific metabolites including plant hormones (Fig. 4.9), phenylpropanoids and flavonoids (Fig. 4.10 and 4.11), and sugar phosphates (Fig. 4.7) that were distinctive from the other P treatments. Therefore, it is possible that some of these compounds can only be utilized by *P. putida* and not by *R. intraradices*. For example in terms of plant hormones, possible enrichments of two biologically inactive gibberellin-conjugates gibberellin A8 2-glucoside and gibberellin A3 O-beta-D- glucoside were detected. Cassan et al. (2001) found that strains from the PGPR *Azospirillum brasilense* and *Azospirillum lipoferum* were able to hydrolyse glycosyl-conjugates of gibberellic acid. Hence, the possibility exists that *P. putida* may also be capable of acquiring carbon through hydrolyses of gibberellin-glucosides.

In the hydroxyapatite treatment, *R. intraradices* not only showed highest root colonization in the single-inoculation treatment, but abundance also slightly increased in the presence of *P. putida*. As mentioned previously, colonization of *R. intraradices* was possibly promoted by the accumulation of jasmonic acid and ABA in the roots. However, this was also the case in the phytic acid treatment and *R. intraradices* abundances still decreased in the dual-inoculation treatment. Similar to *P. putida* in the DNA treatment, an enhanced carbon transfer to the fungus may have caused the further increase in fungal abundances. However, it is unclear whether the plant or the fungus instigated this.

Overall, this study provides evidence that the prevailing P form affects the extent of wheat root colonization with *R. intraradices* and *P. putida*. This is possibly linked to P form-induced changes in internal (root-to-fungus) and external (root exudation) carbon transfer and root metabolite composition. Moreover, *R. intraradices* and *P. putida* exhibited a strong antagonistic relationship in most P treatments, suggesting an incompatibility as microbial inoculants. However, it needs to be considered that this study has been performed only once under semi-sterile conditions. Therefore, it is necessary to confirm these results by repeating the experiment. Furthermore, it needs to be assessed whether the interaction between *R. intraradices* and *P. putida* is the same under more natural conditions. The presence of a real soil community would not only increase the competition for plant carbon compounds, but also affect root metabolism and exudation, which in turn may alter colonization and interaction of *R. intraradices* and *P. putida*.

4.4.2 P form dependent impact of R. intraradices and P. putida on P uptake and wheat biomass

The effects of *R. intraradices* and *P. putida* on P uptake and plant biomass of wheat varied from slightly positive to negative. Only in the hydroxyapatite treatment, the presence of *P. putida* resulted in a dramatic increase in P content and root and shoot biomass. Similar variations in wheat responses to AMF colonization have been discussed in previous studies (Kucey, 1987; Hetrick et al., 1992, 1993; Hetrick et al., 1996; Li et al., 2006; Hu et al., 2010); however reported effects of PGPR were mostly positive (Khalid et al., 2004; Hassen and Labuschagne, 2010; Zabihi et al., 2011). It was argued that plant responses to AMF colonization depended on the extent of their mycorrhizal dependency (Hetrick et al., 1992, 1993; Tawaraya, 2003), plant-fungal compatibility (Klironomos, 2003), soil P levels (Hetrick et al., 1996; Li et al., 2005, 2006; Smith and Read, 2008; Lavakush et al., 2014) and, in the case of PGPR, the extent of their auxin formation capabilities (Lavakush et al., 2014; Nadeem et al., 2014). However, this experiment shows

that the response outcome of wheat to AMF and PGPR infection also relied on the prevailing P source.

4.4.2.1 Sodium phosphate

Even though wheat plants of the sodium phosphate treatment had the highest P contents (Fig. 4.2) and increased metabolic abundances along the major metabolic pathways compared to the other treatments, shoot biomasses were low (Fig. 4.4a). In fact, plants of the phytic acid treatment developed almost the same amount of shoot biomass with a fraction of available P. This suggests that despite the readily available P in the sodium phosphate treatment, wheat plants responded with root rather than shoot growth. From the acquired data it is not possible to determine the actual causes. Plant growth is regulated through a crosstalk between plant hormone and sugar-signalling pathways (Paul, 2007; Smeekens et al., 2010). Therefore, an interruption of this crosstalk or the enhanced expression of growth inhibiting proteins within the sugar-signalling pathway may have limited shoot growth. Further research is required in order to solve this issue.

Inoculation with *R. intraradices* seemed to decrease root P and concurrently increase shoot P contents, but did not significantly change total plant P contents compared to the non-microbial treatment. This indicates that the presence of *R. intraradices* rather enhances root-to-shoot P allocation than P uptake from sodium phosphate, but the underlying process is unclear. Root-to-shoot transport of phosphate ions occurs via the xylem (Poirier et al., 1991; Mimura et al., 1995; Schachtman et al., 1998; Hammond et al., 2011). Thus, it may be possible that *R. intraradices* stimulates xylem loading with phosphate ions. Similar to plant growth, root-to-shoot transfer of P along the xylem is also coupled to a cascade of shoot-to-root sugar signals (Poirier et al., 1991; Hammond et al., 2011). Therefore, *R. intraradices* may alternatively increase P root-to-shoot transport by altering sugar signalling along the phloem.

Similar to *R. intraradices*, inoculation with *P. putida* enhanced root-to-shoot P allocation. However, whole plant P contents also decreased indicating a reduction in P uptake (Fig. 4.2). As discussed before, *P. putida* colonizes the root surface and ambient soil, thereby being able to influence the direct P uptake pathway of roots. *P. putida* may have caused the reduction in P uptake by consuming root exudates used by the wheat to facilitate P assimilation. This is supported by the detected high abundance of *P. putida* in the rhizoplane (Fig. 4.1b). Further, even though shoot P contents were similar to the ones found in the AMF treatment, shoot biomass was slightly lower. As discussed in section 4.4.1, PGPR are able to alter the plant hormonal balance (Richardson et al., 2009). Therefore, it is possible that *P. putida*-induced changes in plant hormone levels may have resulted in reduced shoot growth. Dual-inoculation seemed to have almost no impact on P contents (Fig. 4.2). However, it is more likely that P provided by *R. intraradices* compensated for the loss from the direct uptake pathway caused by *P. putida*.

4.4.2.2 Phytic acid

Wheat plants were able to assimilate P from phytic acid, but the amount was distinctively lower compared to the sodium phosphate and DNA treatment. Phosphorus uptake was most likely facilitated by phytic acid specific root exudation (see chapter 3) and phytase activity. But so far, phytase activity in wheat roots has only been reported by Richardson et al. (2000, see also chapter 3 section 3.4.1). Thus, it is necessary to screen a larger number of wheat varieties for root phytase activity in order to confirm Richardson et al. (2000) findings.

Inoculation with *R. intraradices* resulted in the highest AMF related increase in shoot P contents of all P treatments and an increase in shoot growth. At the same time, there was a decrease in root P contents (Fig. 4.2) suggesting an enhanced P allocation from root to shoot. The overall detected increase in whole plant P contents indicates that *R. intraradices* increased P uptake from phytic acid. However, the amount was small (116 µg)

and may not have been significant. Moreover, Tisserant et al. (2012) did not find any phytase, but a wide variety of phosphatase transcripts in the genome of another *R. intraradices* isolate (DAOM 197198). Since this experiment has only been executed once, repitions are essential in order to confim that there is a real increase in plant P uptake from phytic acid in the presence of *R. intraradices*. In previous studies, fungal excretion of phosphatases has been detected that potentially promoted organic P mobilization and utilization (Joner and Johansen, 2000; Koide and Kabir, 2000). However, it has been questioned whether fungal phosphatase activity significantly contributed to plant P assimilation (Joner et al., 2000; Wang et al., 2013). Nevertheless, increased phytase activity, whether derived from the fungus or the plant, has been detected in the presence of AMF (Shu et al., 2014; Zhang et al., 2014). Wang et al. (2013) suggested that AMF increases plant- or bacteria-derived phytase activity by changing the anion/cation balance in the soil and consequently the pH. In their experiment, they demonstrated that AMF altered the soil pH by utilizing either NH_4^+ or NO_3^- ions. In both nitrogen treatments, phosphatase and phytase activity increased, with NH_4^+-related acidification being more efficient. In addition, Bago et al. (1996) also observed changes in ambient pH during nitrate consumption by extraradical hyphae. In this experiment, wheat plants received nitrate every two weeks from the administered Long-Ashton solution. Thus, it is possible that *R. intraradices* increased the activity of wheat root excreted phytases through nitrate degradation and subsequent changes in soil pH.

P. putida reduced root and whole plant P contents in the phytic acid treatment, but shoot P content and biomass were similar to the ones found in the non-microbial treatment. The lower P contents not only suggest that *P. putida* strain KT2440 derivate FBC004 is very limited in or lacks phytase activity, but also indicate that *P. putida* further limits P uptake possibly through the consumption of root exudates involved in P mobilization from phytic acid. As mentioned in section 4.2.2, no transcription for phytases has been found in the genome of KT2440 (Nelson et al., 2002; Weinel et al., 2002).

However, Patel et al. (2010b) reported low activities in KT2440. Furthermore, they also showed that a transgenic derivate of KT2440 transformed with the recombinant construct pCPappA of the *Citrobacter braakii* phytase gene *appA* possessed a phytase activity over a 150 times higher than that of the untransformed KT2440 strain. The presence of this transgenic strain resulted in a significant increase in P uptake of mung beans from Na- and Ca-phytate. Therefore, it would be interesting to test whether this transformed KT2440 strain as well as other rhizobacterial strains, known for their phytase activity such as *Bacillus subtilis* and *B. amyloliquefaciens*, *Citrobacter braakii* and *Enterobacter cloacae* (Kerovuo et al., 1998; Idriss et al., 2002; Herter et al., 2006; Rodriguez et al., 2006; Lim et al., 2007; Patel et al., 2010a, b), colonize the rhizosphere of wheat and improve P uptake from phytic acid.

Dual-inoculation resulted in a decrease in P contents, especially in the shoots (Fig. 4.2, 4.3). Despite the fact that abundances of both microbes were lowest in this treatment indicating strong antagonistic interactions that may have been enhanced by the presence of phytic acid, it is likely that these interactions not only limited P uptake, but also inhibited root-to-shoot P transfer. Though, the underlying mechanisms are unknown.

4.4.2.3 DNA

Wheat plants of the DNA treatment exhibited second highest P contents and produced the highest root and shoot biomass in the absence of both microorganisms. P uptake from nucleic acid utilization has been previously observed in wheat and *Arabidopsis thaliana* (Richardson et al., 2000; Chen et al., 2000). Both studies suggested that the excretion of nucleases was responsible for nucleic acid breakdown. Moreover, Paungfoo-Lonhienne et al. (2000) showed that root hairs of *A. thaliana* directly assimilated short strands of DNA (25 base pairs). In addition, the presence of DNA in the growth medium induced an increase in lateral root and root hair formation. DNA strands administered in this study were longer than 25 base pairs, thus it is likely that the

excretion of DNAses facilitated DNA degradation. Nevertheless, this does not exclude the possibility that, after the breakdown, wheat roots directly absorbed smaller DNA strands.

Inoculation with *R. intraradices* decreased shoot and whole plant P contents as well as shoot biomass (Fig. 4.2, 4.4c). However, root P contents were the same as found in the non-microbial treatment. The limitation in P uptake was possibly caused by a combination of several factors. First, AMF infection and colonization of roots always inhibits the direct P uptake pathway of the roots, even if the colonization rate is low (Grace et al., 2009; Li et al., 2008; Smith and Smith, 2012). Therefore, P assimilation through the root epidermis was automatically restricted compared to the aseptic treatment. Second, *R. intraradices* was not involved in DNA degradation potentially due to the lack of nucleases in their exudates and DNA breakdown was solely accomplished by plant excreted DNAses. Third, as discussed for the phytic acid treatment, *R. intraradices* can alter ambient pH through nitrate utilization, which in turn can inhibit DNAse activity. Reported pH optimums for plant DNAses varied between 6.0 and 7.5 (Wilson, 1968; Brown and Ho, 1986; Dominguez et al., 2004; Syros et al., 2008). Therefore, soil acidification would reduce DNA degradation through DNAse activity. Fourth, the capability of *R. intraradices* to assimilate P from degraded DNA was limited. Plant-derived breakdown of DNA likely resulted in a mix of small stranded DNA fragments and free phosphate ions. While wheat roots were possibly able to take up both, *R. intraradices* may have been restricted to the free phosphate ions. Hence, the amount of P provided by *R. intraradices* did not compensate for the loss of P caused by the down regulation of the direct uptake pathway.

Single-inoculation with *P. putida* and dual-inoculation with both cultures reduced whole plant P contents due to a strong decrease in root P contents. Shoot P contents were similar to the ones found in the non-microbial treatment, however the shoot biomass was lower. Similar to *R. intraradices*, *P. putida* strain FBC004 appears to be unable to degrade DNA through extracellular nuclease activity. As in the case of *R. intraradices*, the release of

bacterial exudates and consumption of root exudates by *P. putida* may have resulted in a change of ambient pH. This in turn may have impeded the activity of root-excreted DNAses. Considering the stronger decrease in P contents, DNAse inhibition by *P. putida* was more pronounced than by *R. intraradices*. More research is needed to investigate the impact of different soil microorganism on soil pH and DNAse activity to confirm this hypothesis. In the dual-inoculation treatment, root P contents were further reduced. This may be due to the combined negative impact of *R. intraradices* and *P. putida* on P uptake.

Even though shoot P contents did not decrease in the presence of *P. putida*, shoot biomass formation was still impeded. As discussed previously for the sodium phosphate treatment, potential *P. putida*-induced changes in plant hormone levels may have caused shoot growth inhibition. Additionally, the low root P contents may have triggered systemic root-to-shoot stress-signals, which resulted in growth limitation (Lin et al., 2013).

4.4.2.4 Hydroxyapatite

In the hydroxyapatite treatment, the extremely low P contents show that wheat plants are very limited in their ability to mobilise P from this source (Fig. 4.2). The presence of *R. intraradices* improved P uptake, which was mainly reflected in higher shoot P contents and biomass. Increased P uptake from rock phosphate in the presence of AMF has previously been observed in various mycorrhizal plants (Kucey et al., 1987; Bolan et al., 1991; Duponnois et al., 2005, Antunes et al., 2007). However, the mechanisms involved in fungal mobilization of P from rock phosphate are still not very well understood. The most widely believed hypothesis is that AMF can promote the release of H^+ ions and/or chelating low molecular weight organic acids, hence establishing acidic conditions that are more favourable for P solubilisation (Smith and Read, 2008). In addition, AMF-induced changes in soil pH can also be the result of the uptake of NH_4^+ or NO_3^- ions, as discussed previously. However, only a few studies analysed changes in soil pH in the presence of AMF and the results are contradictory (Bago et al., 1996; Antunes et al., 2007; Villegas and

Fortin, 2001, 2002). Moreover, it was suggested that the change in pH was rather caused by the presence and interaction of other rhizomicroorganisms. More research is needed to address this issue.

The presence of *P. putida* in the hydroxyapatite treatment increased whole plant P contents by more than 60% and shoot P contents by almost 150% (Fig. 4.2). Furthermore, shoot biomass almost doubled (Fig. 4.4d). This dramatic rise in P uptake and plant growth shows that *P. putida* are distinctively better P mobilisers than *R. intraradices*. Strong phosphate solubilizing abilities of various genera of bacteria including *Pseudomonas* has been reported in numerous studies (Kucey, 1987; Rodriguez and Farga, 1999, Sundara et al., 2002). It has been shown that phosphate-solubilizing bacteria mobilise P from rock phosphate through the release of chelating organic acids and by lowering the ambient pH (Chen et al., 2006). Therefore, it is likely that *P. putida* used these mechanisms to solubilize P from hydroxyapatite. However, considering that wheat plants received a total of 20 mg P in form of hydroxyapatite, P recovery from this source, even in the presence of *P. putida*, is very low. Thus, using rock phosphate as an alternative P source needs to be appraised carefully.

Dual-inoculation with *R. intraradices* and *P. putida* resulted in a similar outcome as seen in the single-inoculation treatment with *P. putida* (Fig. 4.2, 4.4d). Given that *P. putida* is more efficient in P mobilization from hydroxyapatite than *R. intraradices* and that P contents are almost the same in these two treatments, it is easy to conclude that *P. putida* solely provided P to the plant, while *R. intraradices* just received carbon without contributing any P. This assumption is further supported by the detected high abundance of *R. intraradices* (Fig. 4.1). However, as Smith and Smith (2012) argued, the AMF P uptake pathway always provides P to the roots and the occurrence of parasitic relationships with plants is rather rare. Further, AMF infection always down-regulates the direct P uptake pathway independent from the extent of colonization. Therefore, it is more likely that P contents detected in the dual-inoculation treatment are the result of the combination of

decreased P assimilation activity along root epidermal cells, which has been counterbalanced by P from the AMF pathway. Nevertheless, P mobilization most probably occurred by the activity of *P. putida*.

4.4.3 Responses of root metabolism to different P forms and microbial inoculants

After 10 weeks of growth, mostly the prevailing P form and not the microbial inoculants significantly affected wheat root metabolism (Tables A4.1-A4.5). This is contradictory to the report of Schliemann et al. (2008). They found that root metabolite profiles of *Medicago truncatula* were significantly different in the presence of AMF even after eight weeks of growth. This suggests that the extent of root metabolic responses to AMF infection is plant-specific. Moreover, the responsiveness of root metabolism may also influence the overall plant response to AMF colonization. In the case of the hydroxyapatite treatment, it seems surprising that only 18 mass bins were significantly affected by *P. putida* (Tables A4.1-A4.5), despite the increase in shoot biomass of 98.8%. If the increase in shoot biomass is just directly related to the increased amount of available P and not the presence of *P. putida*, *P. putida*-related discrminations in the major root metabolic pathways should not occur. Further, considering that, despite the enhanced P availability, wheat plants were still deprived in P, P-stress would be still the dominant factor. However, if *P. putida* altered the formation of plant growth regulating hormones such as auxin and cytokinins, metabolic turnovers along these pathways would have been too fast to detect in this study, since root samples have been collected just at one time point (Epstein et al., 1980; Mok et al., 2000; Ljung et al., 2002). Only time course experiments using radiotracers and transcriptomic analyses can reveal metabolic changes associated with plant development.

Even though no significant effect of *R. intraradices* and only a very minor of *P. putida* on root metabolism were detected, it seems that, depending on the P source and the metabolite, the presence of either or both microbes had a promoting or inhibitory

impact (Fig. 4.7-4.11). In the early stages of colonization, AMF and PGPR change root metabolism of the host plant in particular plant defence responses and plant hormone signalling in order to facilitate the establishment of a symbiotic relationship (Richardson et al., 2009; Cameron et al., 2013; Fusconi, 2014). It is possible that in wheat the impact of AMF and PGPR on the host's metabolism diminishes with time and other factors like in this case the P form and status is more dominant. As mentioned before, this experiment has only been performed once. Therefore, these findings need to be confirmed through experimental repetition.

Highest root metabolic abundances were found in the sodium phosphate treatment with some exceptions along the plant hormone and secondary metabolites biosynthesis (Fig. 4.7-4.11). Compared to that, metabolic activity was reduced in the phytic acid, hydroxyapatite and even the DNA treatment despite the second highest detected P contents and highest root and shoot biomass. However, several plant hormones (Fig. 4.7, 4.8), secondary metabolites (Fig. 4.9, 4.10, Table 4.5) and peptides (Table 4.6) were enriched in these P treatments. Accumulations of some of these compounds were restricted to one or two P forms, which indicate P form-specific activations of metabolic pathways as well as different functions of the produced compounds. This will be discussed in sections 4.4.4-4.4.6.

4.4.4 P form dependent function of plant hormones

Wheat roots that received DNA as P source were highly enriched in putative gibberellin glucosides, but potentially depleted in the bioactive gibberellins A3 and A12 (Fig. 4.9). Gibberellin glucosides are biologically inactive conjugates of gibberellins. They are considered to be the storage and transport form of this group of plant hormones due to their polarity (Schneider and Schlieman, 1994; Piotrowska and Bajguz, 2011). This suggests that the biosynthesis of gibberellins was enhanced in roots of the DNA treatment, but they were subsequently converted into their inactive form whether for storage and/or

transport is unclear. In their active form, gibberellins promote root elongation, but suppress lateral root and root hair formation (Yaxley et al., 2001; Jiang et al., 2007). Wheat plants of the DNA treatment possessed the highest root biomass with an extensive lateral root system. Paungfoo-Lonhienne et al. (2000) reported increased lateral root growth and root hair formation in the presence of exogenous DNA, but did not provide an explanation about the involved mechanisms. This study suggests that the enhancement of lateral root growth and consequently biomass may be the result of DNA induced conversion of active gibberellins into inactive conjugates. Moreover, Brown and Ho (1986) found that gibberellic acid increased the excretion of nucleases from aleurone tissue of barley, but at the same time reduced nuclease synthesis within the tissue. Therefore, enhanced formation of gibberellin glucosides may be the process of wheat roots to prevent the inhibition of DNAse biosynthesis and to transport gibberellins to root epidermal cells where it promotes DNAse secretion. However, it is still unclear how gibberellin promotes DNAse secretion and whether its promotion is endogenous within root epidermal cells or exogenous through excretion into the rhizosphere.

Mass bin 347.1592883 that was putatively identified as either gibberellin A3 or gibberellin catabolites were enriched in the phytic acid and hydroxyapatite treatments independent of the microbial treatments (Fig. 4.9). As mentioned before, biologically active gibberellin A3 suppresses lateral root and root hair formation, which is a common P stress response (Yaxley et al., 2001; Nacry et al., 2005; Jiang et al., 2007; Zhang et al., 2014). Furthermore, Foo et al. (2013) demonstrated that gibberellin A3 reduced arbuscule development in pea roots. Since wheat plants of both P treatments were P deprived and exhibited the highest abundances of *R. intraradices* in their roots, it is more likely that mass bin 347.1592883 is a gibberellin catabolite indicating an increased degradation of bioactive gibberellins.

In addition to gibberellin catabolites, jasmonic and traumatic acid (Fig. 4.8) as well as 8'-hydroxyabscisic acid and/or phaseic acid (Fig. 4.9) were accumulated in wheat roots

of the phytic acid and hydroxyapatite treatment. The increase in these plant hormones was independent from the microbial applications, thus suggesting that it was induced by the P source or, in this case, P status. Jasmonic acid possesses several regulatory roles in the roots. Chacon-Lopez et al. (2001) found that jasmonic acid in a cross talk with ethylene inhibited primary root growth in P deprived seedlings of *Arabidopsis thaliana* indicating its involvement in P starvation related changes in root architecture. Further, it is involved in regulating PGPR- and AMF-induced priming of systemic plant defence responses (Van der Ent et al., 2009; Cameron et al., 2013). Jasmonic acid also mediates the formation of secondary metabolites, especially flavonoids (Farag et al., 2008; Hause and Schaarschmidt, 2009). Their function will be discussed in section 4.4.5. Moreover, it has been shown that jasmonic acid is an important signal in establishing and promoting root mycorrhization (Hause et al., 2007; Hause and Schaarschmidt, 2009; Cameron et al., 2013). An AMF-induced increase in jasmonate concentrations was detected in roots of *Hordeum vulgare* (Hause et al., 2002), *Cucumis sativus* (Vierheilig and Piche, 2002) and *Medicago truncatula* (Stumpe et al., 2005). In this study however, enhanced abundances of jasmonic acid in the phytic acid and hydroxyapatite treatment were rather caused by P deprivation, since the same enrichment was also found in the non-microbial treatment. Nevertheless, the presence of elevated jasmonic acid levels appeared to have facilitated wheat root mycorrhization, since abundances of *R. intraradices* were highest and second highest in these P treatments.

Traumatic acid showed similar enrichments as jasmonic acid (Fig. 4.8). Both plant hormones are formed via the alpha-linolenic acid pathway. Therefore, their biosynthesis may be connected. Traumatic acid is a wound hormone that mediates cell division near the wounding site (Zimmerman et al., 1979; Wishart et al., 2005). Very little is known about its function as a growth hormone. An early study of Pilet (1965) demonstrated that traumatic acid stimulated the elongation of apical root sections of *Lens culinaris* and further suggested that it has an inhibitory effect on indole-3-acetic acid (IAA) biosynthesis. IAA

promotes lateral root formation under P deprived conditions (Zhang et al, 2014). In this study, the enrichment of traumatic acid potentially suppressed IAA formation. This suggests that either lateral root and root hair formation was inhibited despite the fact that wheat plants were P deprived or that the development of lateral roots and root hairs was rather regulated by the cross talk between jasmonic acid and ethylene than IAA signalling.

Enhanced levels of 8'-hydroxyabscisic acid and/or phaseic acid (Fig. 4.9) in wheat roots of the phytic acid and hydroxyapatite treatment either indicate an elevated metabolic flux along the ABA formation pathway and/or the increased catabolism of ABA. It has been demonstrated that ABA inhibits the expression of P starvation response genes that are involved in the re-allocation of P to the roots to promote lateral root growth (Shin et al., 2006; Zhang et al., 2014) as well as the expression of phosphate transporters (Ribot et al., 2008). Hence, it is more likely that the increased abundances of 8'-hydroxyabscisic acid and/or phaseic acid indicate a down-regulation of ABA activity through degradation in order to prevent the repression of P starvation response genes and phosphate transporters.

4.4.5 P form dependent formation of secondary metabolites

Accumulation of phenylpropanoids, flavonoids, alkaloids and terpenoids was P form specific (Fig. 4.10, 4.11, Table 4.5). The highest number of enriched secondary metabolites was found in the sodium phosphate treatment suggesting overall increased metabolic abundances in this treatment possibly due to better P availability. However, abundances of certain secondary metabolites were elevated in the other P applications compared to the sodium phosphate treatment. This indicates that these more complex P pools seemed to stimulate specific formation pathways of secondary metabolites. The P source dependent discriminations in secondary metabolite expression are consistent with the results found in chapter 2 and 3. However, abundances of compounds that were elevated partially differed from those found in chapter 2 and 3. This implies that metabolic

abundances in wheat roots change at different growth stages, but remain affected by the prevailing P source.

The role and function of different secondary metabolites in root metabolism as well as root exudates were discussed previously in detail in chapter 2.3.5 and 3.4.3. Secondary metabolites are involved in plant responses to different biotic and abiotic stresses including nutrient starvation (Dixon and Paiva, 1995; Solecka, 1997; Bollina and Kushalappa, 2011; Oksinska et al., 2013). For example, Dixon et al. (2002) found that P starvation increased the expression of an isoflavone synthase gene. In this study, accumulation of certain phenylpropanoids, flavonoids and alkaloids were detected in the hydroxyapatite treatment. Due to the low plant P concentrations in this treatment, enrichment of these compounds may be linked to P starvation. However, wheat roots of the phytic acid treatment did not exhibited the same accumulation of secondary metabolites, even though the plants were also P deprived. This suggests that, unlike the biosynthesis of plant hormones, the formation of secondary metabolites was rather a response to the prevailing P form and not P status. For instance, in the case of ferulic acid, high abundances were detected in the hydroxyapatite, but not in the phytic acid treatment. Prasad and Devi (2002) demonstrated that ferulic acid inhibited phosphatase activity in maize. Therefore, wheat may have down-regulated ferulic acid formation in order to prevent inhibition of phytase activity. However, accumualtion of ferulic acid was found in root exudates of wheat seedlings that received phytic acid as sole P source (see chapter 3). Therefore, it is possible that the low abundance of ferulic acid found in wheat roots of the phytic acid treatment is rather the result of increased exudation than of reduced formation. Instead of down-regulating ferulic acid formation, wheat plants may use increased exudation as a mechanism to prevent the inhibition of endogenous root phytase activity by ferulic acid. Furthermore, with respect to mycorrhization, ferulic acid inhibited AMF colonization and hyphal growth in green asparagus (Wacker et al., 1990). In this study, highest root mycorrhization was found in the hydroxyapatite treatment that

had the highest enrichment of ferulic acid. It is possible that the inhibitory effects of ferulic acid are plant and fungal specific. Thus, more research is needed to better understand the role and impact of ferulic acid on P uptake and AMF colonization.

Secondary metabolites increase allelopathic properties of plants, which in turn can protect them from pathogen attacks and when excreted to the rhizosphere inhibit weed growth. As discussed in chapter 3.4.3, the role of wheat allelopathy in pathogen and weed defence has been studied in detail (Baghestani et al., 1999; Wu et al., 2000, 2001; Iqbal et al., 2002; Huang et al., 2003; Belz et al., 2007). Wheat roots of the sodium phosphate treatment had the highest abundances of secondary metabolites and potentially the highest allelopathic properties. Sodium phosphate is a P source easily accessible to plants and microbes. Thus, the competition for this P source is potentially high. In natural environments, the high allelopathy of wheat potentially gives it an advantage over the other competitors. Through the release of its allelopathic compounds into the rhizosphere wheat may be able to reduce the number of microbes and other plant species accessing the same P pool. In the case of the other more complex P forms, the competition for these pools may be limited, since they are more difficult to access. Hence, the increased accumulation of specific secondary metabolites may serve a different purpose.

Secondary metabolites can attract or repel rhizosphere microbes depending on their capability to digest and use these compounds as carbon source. For example, Siquera et al. (1991) found that flavonoids stimulated AMF growth and mycorrhization of white clover. In addition, Chabot et al. (1992) reported that the presence of kaempferol also promoted hyphal growth. Zuo et al. (2014) reported that allelopathy in wheat increased microbial abundances. On the other hand, Kato-Noguchi et al. (2007) showed that momilactone B excreted by rice impeded microbial growth. Therefore, it is possible that the more complex P forms in this study induced the formation of specific secondary metabolites, which were meant to selectively alter the soil microbial community structure so that it promotes P mobilization from these sources. If phenylpropanoids, flavonoids and

alkaloids that accumulated in the DNA treatment were indeed carbon sources accessible to *P. putida* and enhanced its colonization, it did not result in an improved P uptake. Thus, it could be concluded that the P form induced synthesis of secondary metabolites is not a very efficient strategy of wheat to attract microorganism to improve P mobilization and uptake. However, considering the conditions that prevailed in this experiment, which only included two beneficial soil microorganisms, this conclusion would probably be false. More research in a more natural environment is necessary to better determine the role of secondary metabolites in P mobilization strategies of wheat.

4.4.6 P form dependent discriminations in peptide abundances

Wheat roots of the phytic acid and hydroxyapatite treatment were enriched in almost the same tri- and tetra-peptides (Table 4.6). This suggests that, as in the case of plant hormones, the accumulation of these peptides was rather a response to P deprivation than to the P form. It has been shown that CLE-peptides are involved in the regulation of root growth and lateral root development in *A. thaliana* (Chilley et al., 2006; Meng et al., 2012). Not much is known about their regulatory role, but CLE-peptides seemed to inhibit lateral root formation by interfering with the ethylene- and auxin-signalling pathways. Hence, this would suggest a down- rather than up-regulation of these peptides under P starvation conditions. However, it is highly likely that different peptides exhibit different regulatory functions and are involved in different signalling pathways. This is further supported by the accumulation of separate peptides in the DNA treatment indicating that they possess another regulatory role. Therefore, it is still possible that the peptides enriched in the phytic acid and hydroxyapatite treatment are still involved in P starvation responses. More research is needed to better understand the regulatory functions of peptides.

4.5 Conclusion

This study shows that the use of inoculants of beneficial microorgnisms to improve soil P exploitation, mobilization and uptake in agricultural systems needs to be carefully considered. Microbe-microbe interactions as well as the relationship with the host plant is highly affected and determined by the occurring P forms. Therefore, the wrong combination of microbial species and prevailing environmental conditions can result in an antagonistic rather than synergistic effect on crop P uptake and growth.

Chapter 5: General Discussion

Modern agriculture faces the challenges to maximize crop yields to meet increasing food demand and, at the same time, to find more sustainable farming approaches that decreases fertilizer dependency, especially for P, since its resources are finite. This is a difficult task, which not only requires the discovery of more sustainable P sources, but also to develop crop cultivars that are adapted to lower nutrient levels and that are able to access and assimilate P from these alternative pools. Complex organic and immobilised inorganic phosphorus bound to organic matter and mineral surfaces occurring naturally in the soil but have low availability to plants. In spite of this, these soil P pools have become of increasing interest as potential alternative P resources. However, these pools are only suitable if plants can mobilise this recalcitrant P; either directly or by secondary means such as beneficial phosphorus solubilizing rhizosphere microbes including bacteria and fungi.

The successful development of crop cultivars that possess enhanced capabilities of exploiting various soil P resources rely on the thorough understanding of plant sensing and adaptation responses to different P forms, particular within the roots. However, most research has been done on root sensing and responding to low or high levels of readily available inorganic phosphate levels (e.g. Zhang et al., 2014). It is also unclear how root adaptions to organic and inorganic P forms effects and interacts with the recruitment and performance of beneficial microbes. Moreover, information on microbial compatibilities and the factors that determine their relationship are still limited (Marschner et al., 2004, Nazir et al., 2010).

With respect to this, this PhD thesis gives new insights into P form-related responses in root metabolism and root exudation of wheat. It further provides evidence that these root responses in combination with microbial interactions had an impact on growth and performance of two common beneficial rhizosphere microorganisms used as inoculants.

5.1 Organic and inorganic P forms induce long-term changes in wheat root metabolism

Exposure of wheat roots to different organic and inorganic P sources initiated different alterations in root metabolism (see chapter 2). These changes also varied from those found in the control plants, indicating that wheat seedlings responded to the actual P form and not P availability. Similar variations in root metabolism were found in wheat plants after ten weeks of growth (see chapter 4). This suggests that in spite of some slight changes in discriminating mass bin compositions, the presence of a specific P form causes an overall long-term response in root metabolism. However, in the case of the least accessible P sources the response was similar to what has been found in P-deprived plants indicating a mixed effect of P form and status.

Despite metabolic adaptations to the prevailing P form, P uptake from these more complex P forms was not as efficient as it was in the sodium phosphate. Therefore, and in order to improve P uptake efficiency in wheat and other crop plants, more research is needed on how these detected responses are linked to direct (through the root epidermal cells) and indirect (through mycorrhizal fungi) P assimilation pathways and whether they promote or inhibit them. For example, in the case of the DNA treatment (chapter 4) there are indications that the increased formation and accumulation of gibberellin glucosides may be connected to DNAse secretion and activity, although the mechanistic basis for this remains unclear. Thus, identifying the link between the biosynthesis of these compounds and DNAse activity is necessary to confirm this hypothesis.

Biosynthesis of plant hormones and other secondary metabolites such as phenylpropanoids, flavonoids and alkaloids were the main metabolic pathways affected by organic and inorganic P forms. These metabolites are not only involved in the regulation of root development (Chiou and Lin, 2011; Zhang et al., 2014) and P starvation responses (Franco-Zorrila et al., 2005; Nacry et al., 2005; Zhang et al., 2014), but also can affect the attraction and recruitment of beneficial soil microorganisms such as mycorrhizal fungi

and plant growth promoting rhizobacteria (PGPR) in both positive and negative ways (Wacker et al. 1990; Kato-Noguchi et al., 2007). In chapter 4, P form-dependent formation of plant hormones and other secondary metabolites appeared to be linked to either the promotion or inhibition of *R. intraradices* and *P. putida* colonization and as a result their contribution to P mobilization and transfer. Thus and with respect to the development of new cultivars it needs to be carefully deliberated about whether to either improve P assimilation abilities of the plant, but risking a potential restriction in mycorrhizal fungi and PGPR activity or to promote metabolic traits that enhance microbial colonization and their contribution to plant P uptake.

All root metabolic changes to organic and inorganic P sources were investigated in the presence of just one P form at a time. Therefore at this point, it is still unclear how wheat roots respond to more than one P source and whether one of the here detected P form specific metabolic responses would override the others. Further, it is still unknown whether the P form effect is a dominant or inferior factor on root metabolism responses compared to other abiotic and biotic influences such different nutrients and nutrient status and/or soil microorganisms. Root metabolite profiles of the hydroxyapatite and phytic acid treatment in chapter 4 suggest that the low P status of wheat plants also drove metabolic alterations alongside the present P form. This may also indicate that P deprivation responses are stronger than those induced by P forms, possibly because the survival of the plants relies more on them.

Soil type, composition and pH determine which phytic acid salts are present and to which minerals inorganic phosphate is bound (Turner et al. 2002b; Fageria, 2009; Jones and Oburger, 2011). It is possible that different phytic acid salts and phosphate minerals induce different metabolic responses in crop, which in turn would make crop breeding that targets the improvement of soil P exploitation abilities more complex, since it has to consider specific soil conditions.

5.2 Enhancing P form related adjustments in root exudate composition might have the potential to improve soil P exploitation

The results in chapter 3 showed that wheat alters its root exudate composition with respect to the prevailing P source. Phytic acid in particular caused a strong response in root exudation. Inositol phosphates are the most abundant organic soil P form (Dalal, 1977). Therefore, if root exudation of crops can be refined to access and mobilize P from this complex organic soil resource, P fertilizer dependency can be reduced. However in order to achieve this, individual metabolites in the root exudates and their role in P mobilization from phytic acid need to be identified.

The presence of phytic acid induced an increased secretion of organic acids. Organic acid can potentially occupy various direct and indirect functions in P mobilization from phytic acid (see chapter 3.4.3), which include the release of phytic acid from metal salts and oxides, desorption of phytases and attraction of phosphorus-solubilizing microorganism. While numerous studies demonstrated that organic acids attract soil microorganisms including phytate hydrolyzing bacteria and fungi (Tarafdar and Marschner, 1995; Richardson et al., 2000), their effectiveness in P mobilization and phytase desorption is still under debate. There are indications that organic acids can either enhance or inhibit P mobilization and phytase desorption, but this depended on their composition and concentrations (Tang et al., 2006). Moreover, most studies have been performed in vitro; hence it is still unclear how environmental factors such as soil composition, mineralogy, pH and the presence of soil microbes influence organic acid efficacy for these to processes.

Phytic acid increased the release of allelochemicals, but their function in P acquisition is remains unclear. Enhanced allelopathy of arable systems can inhibit the growth of weeds (Wu et al., 2000, 2001; Huang et al., 2003; Belz et al., 2008) and therefore eliminate competitors for freshly mobilized P from phytic acid. Indeed, water-soluble allelopathic compounds, produced either directly by arbuscular mycorrhiza or by

colonised roots, have been hypothesised as the mechanism underpinning mycorrhiza-induced suppression of non-mycorrhizal weeds via inhibition of root hair production (Cameron, 2010). In addition, allelochemicals can attract beneficial soil microbes if they can use these compounds as carbon source. However, studies also found that certain allelochemicals had an inhibitory effect on microbial growth including that of mycorrhizal fungi (Wacker et al. 1990; Kato-Noguchi et al., 2007). Prasad and Devi (2002) further reported that allelochemicals limited phosphatase activity, although this effect was pH dependent. Thus, enhancing allelopathy of crops can potentially lower soil P exploitation capacities. In order to prevent this, more research is needed on the effect of allelochemicals on soil microbial communities as well as phytase activity. Further, it needs to be investigated how the interactions with other factors such as soil type and mineralogy, organic matter content as well as pH influence the effect of allelochemicals.

5.3 Incompatibility between different microbial inoculants and with inorganic or organic P forms can reduce P assimilation

In chapter 4, *R. intraradices* and *P. putida* had an antagonistic relationship in wheat resulting in a decrease of their abundances. Microbial compatibility is determined by numerous factors and their interactions. Factors include plant host characteristics including their nutrient status and root exudate composition, interactions between the microbial inoculants and the indigenous microbial community, soil conditions and management and, as shown chapter 4, the prevailing P form (Marschner et al., 2004, Nazir et al., 2010; Owen et al., 2015). Due to this complexity and the limited number of data, it is currently impossible to predict the outcome of microbial behavior and performance in soil P exploitation and mobilization in agricultural systems, hence making the selection of inoculants difficult. Testing various combinations of microbial inoculants on different crop plants and in different agricultural soil systems together with a range of biochemical and

molecular analyses is necessary to improve our understanding of these intricate interactions.

5.4 Phosphorus form-related root metabolic responses as potential factors indirectly influencing soil health and quality by altering the microbial community in the rhizosphere

Cropping systems are complex ecosystems whose prosperity can be affected by numerous from large-scale to micro-scale abiotic, biotic and biochemical factors, some of which are illustrated in Fig. 5.1 in a simplified way. Optimal plant health is essential to achieve maximum crop/grain yields. The health of crops relies on a sufficient supply of nutrients including phosphorus and minimal spread of disease. Both of these factors are linked to the health and quality of agricultural soil. Under natural conditions, a diverse population of soil microorganisms plays a key role in promoting and maintaining soil health and quality (Doran and Zeiss, 2000; Tilak et al., 2005). These beneficial bacteria and fungi increase soil fertility through organic matter degradation and nutrient cycling, thereby improving nutrient availability of crops. In addition, they can enhance the immune resistance responses of crops towards pathogens, thereby protecting them from disease (Cameron et al., 2013). However, conventional agricultural practices such as ploughing, mono-cropping as well as high fertilizer inputs can have detrimental impacts on soil microbial diversity (Doran and Zeiss, 2000), concurrently increasing the abundance of plant pathogens. This dramatically decreases soil health and quality, and consequently reduces plant fitness. Therefore, a rigorous change in agricultural soil management practises using less invasive methods is necessary in order to reverse these deleterious effects.

With respect to a sufficient but sustainable supply of nutrients, in this case P, the reduction in fertilizer use forces crop plants to gain P from sources naturally occurring in

soil. This, however, depends on their capability to access these more complex P pools either by themselves or through the support of the microbial community within the rhizosphere. Through the secretion of root exudates crop plants can attract and promote the growth of rhizospheric bacterial and fungal populations that are able to mobilize P from inorganic and organic forms. However, certain compounds secreted by the roots can also have inhibiting effects on these beneficial microbes and their relationship to the crop plant. As a consequence, these changes in microbial diversity and structure can potentially reduce soil fertility and quality, and adverse plant-microbe interactions can limit crop growth. Therefore, it is essential to determine and understand all the factors that influence root metabolism and exudation.

This PhD thesis provided evidence that different inorganic and organic P forms induced immediate and long-term changes in root metabolism and exudation of wheat that seemed to be linked to P form sensing and possibly direct (by wheat itself) and indirect (through microorganisms) P mobilization. Moreover, this thesis also demonstrated that depending on the present P source and the associated alteration in root metabolism, root colonization of the mycorrhiza fungi *Rhizophagus intraradices* and the soil bacterium *Pseudomonas putida* was either promoted or limited. In addition, the P form and root metabolism also seemed to determine whether the presence of *Rhizophagus intraradices* and *Pseudomonas putida* resulted in an increase or decrease in plant P content and biomass. Even though the experiments in this thesis have been conducted under fairly unnatural conditions, they still give valuable information about how soil conditions such as different P pools have the capability to change crop root biochemistry and that this change can alter microbial community structure and not necessarily result in an increase, but reduction in plant nutrient status and growth and hence crop yields.

This thesis focused on the impact of inorganic and organic P forms on wheat root biochemistry and plant-microbe interaction. However, it is likely that these effects may

different in other crop plant or even in different wheat varieties. Furthermore, as discussed in the previous chapter other environmental factors such as soil chemistry and mineralogy, organic matter content, pH, temperature and water content affect nutrient uptake, plant-microbe and microbe-microbe interactions. It shows that the successful establishment of sustainable agricultural practices in connection with maximum yields not only relies on less invasive soil management methods and fertilizer input, but also on a thorough understanding of environmental, biological and biochemical processes and their interaction within the soil ecosystem.

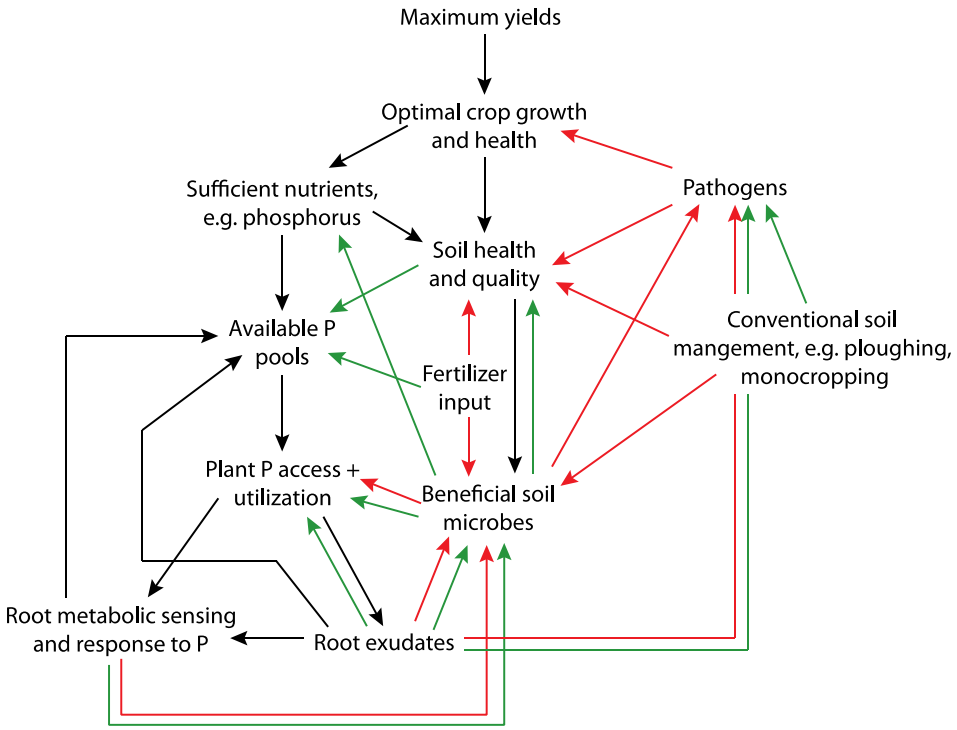


Fig. 5.1: Simplified illustration of biotic, abiotic and biochemical factors and their interactions that affect crop growth and yield. Black arrows indicate a link/dependence between factors. Red arrows indicate negative/reducing effects, while green arrows represent positive/increasing impacts between factors.

5.5 Conclusion

This PhD thesis enhanced our knowledge of the sensing and metabolic adaptations of wheat roots to different organic and inorganic P forms. However, it remains to be

determined whether these metabolic changes are general responses of wheat plants or if they are cultivar specific. Furthermore, it is necessary to link these responses to P uptake mechanisms in order to use this information to develop cultivars with enhanced soil P exploitation and utilization capacities. Moreover, it is essential to fully understand how these improved traits affect microbial communities within the rhizosphere, since it might result in the obstruction of P uptake and growth.

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Appendix

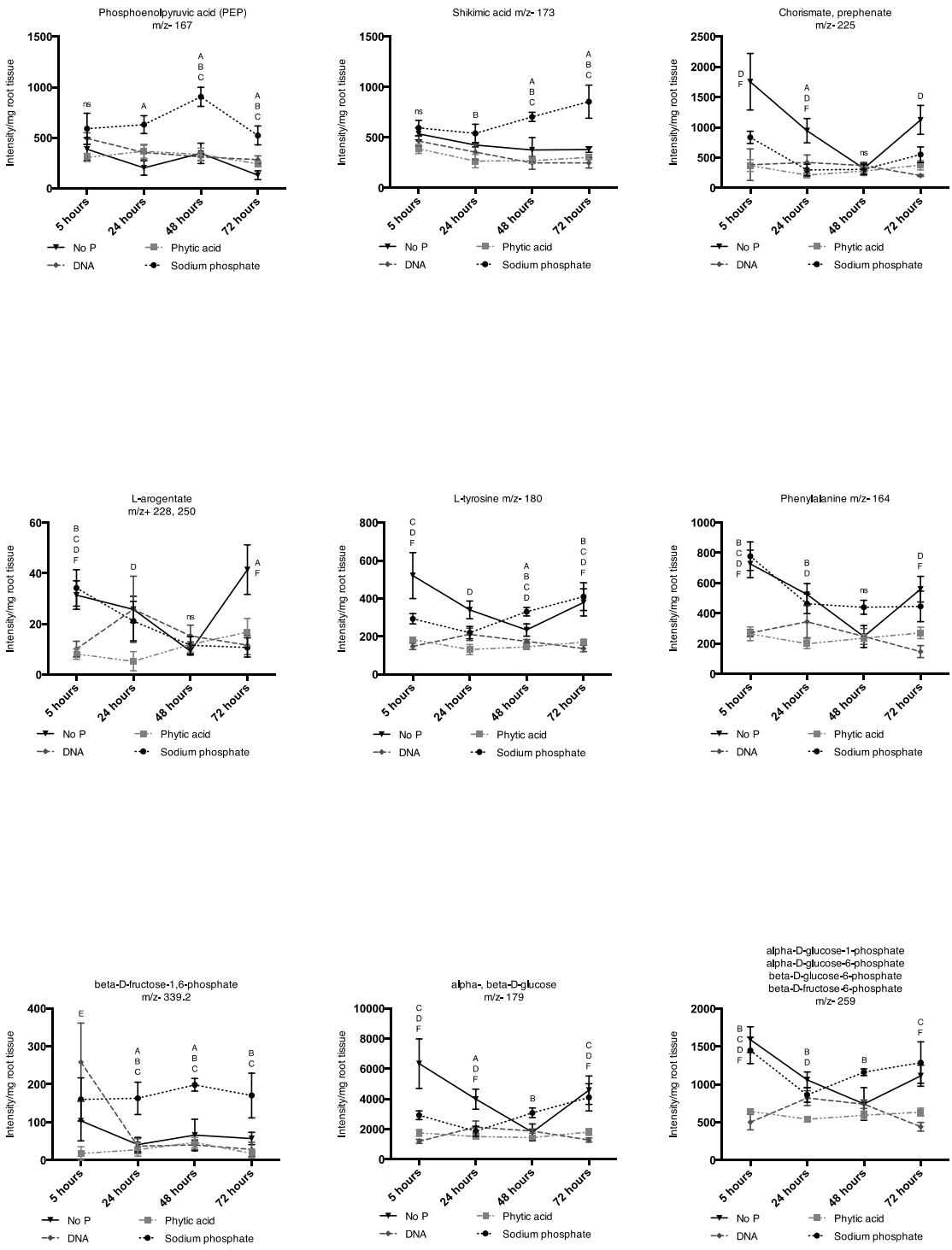

Fig. A2.1: Average intensities of metabolites illustrated in Fig. 2.4 detected in either negative (*m/z-*) or positive (*m/z+*) ion mode. Error bars represent the standard error of the mean. All *m/z* values refer to the respective mass bins listed in Tables 2.1 and 2.2. Letters indicate significant differences between the treatments (n=5, $p<0.05$, one-way ANOVA, TukeyHSD post-test): A = Sodium phosphate-no P; B = Sodium phosphate-phytic acid; C = Sodium phosphate-DNA; D = Phytic acid-no P; E = Phytic acid-DNA; F = No P-DNA.

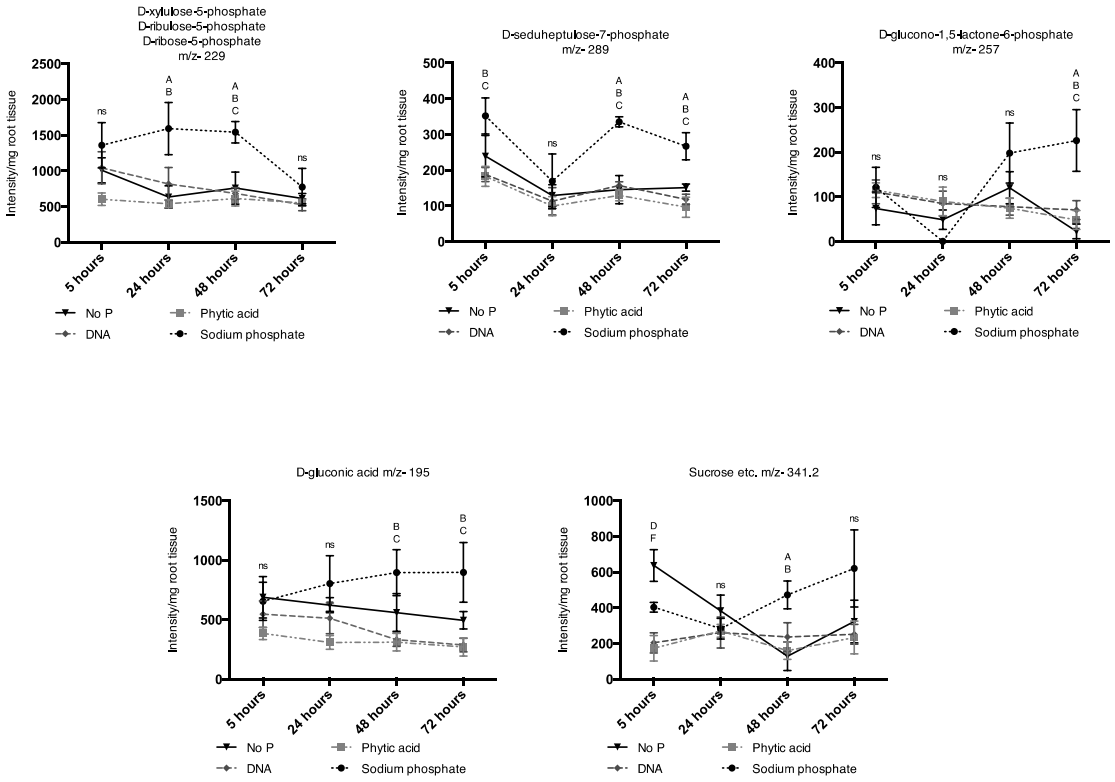

Fig. A2.2: Average intensities of remaining metabolites illustrated in Fig. 2.4 detected in either negative (*m/z*-) or positive (*m/z*+) ion mode. Error bar show the standard error of the mean. All *m/z* values refer to the respective mass bins listed in Tables 2.1 and 2.2. Letters indicate significant differences between the treatments (n=5, $p<0.05$, one-way ANOVA, TukeyHSD post-test): A = Sodium phosphate-no P; B = Sodium phosphate-phytic acid; C = Sodium phosphate-DNA; D = Phytic acid-no P; E = Phytic acid-DNA; F = No P-DNA.

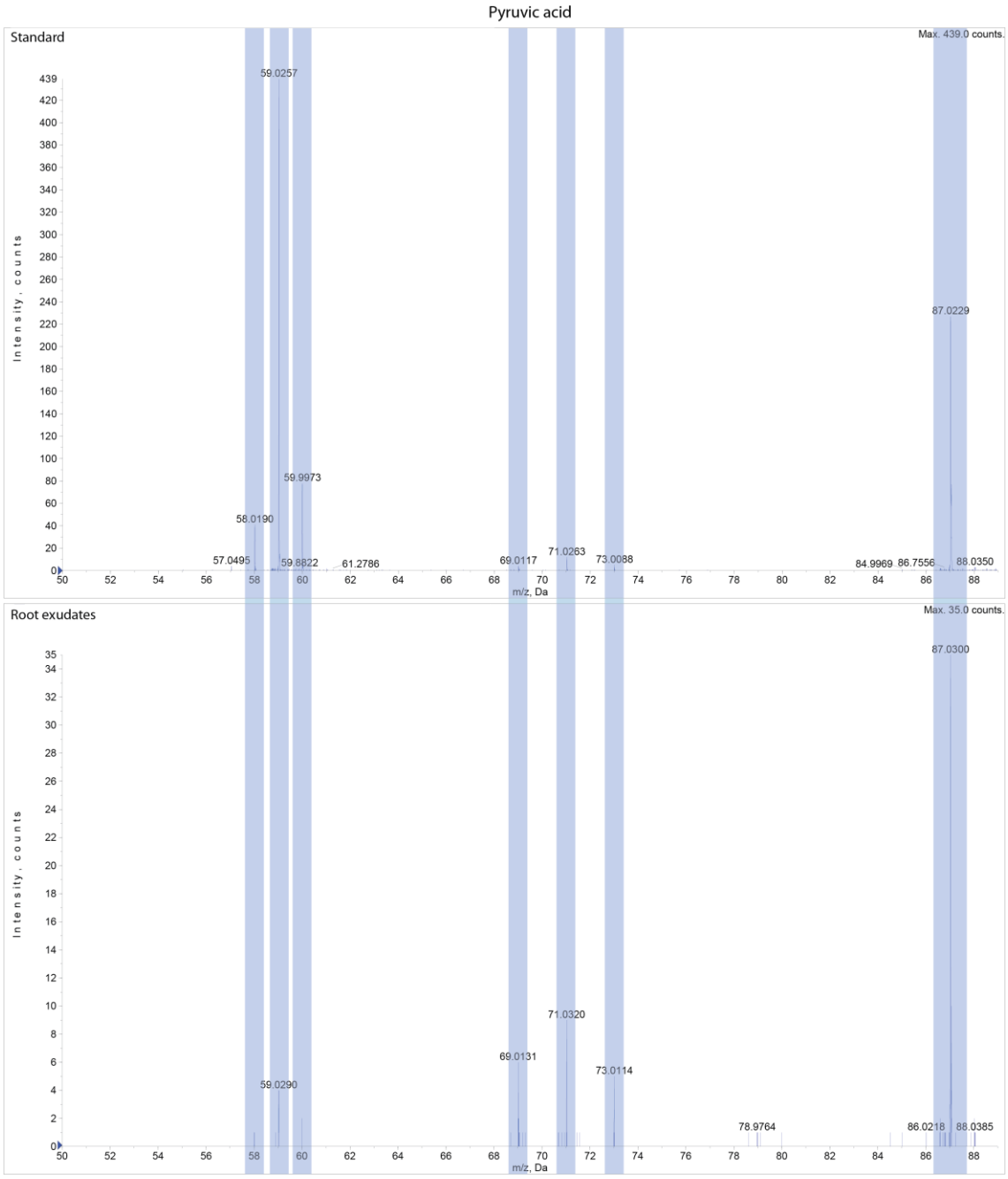

Fig. A3.1: MS-MS ion fragmentation patterns of pyruvic acid in standard solution (top) and in collected root exudates (bottom).

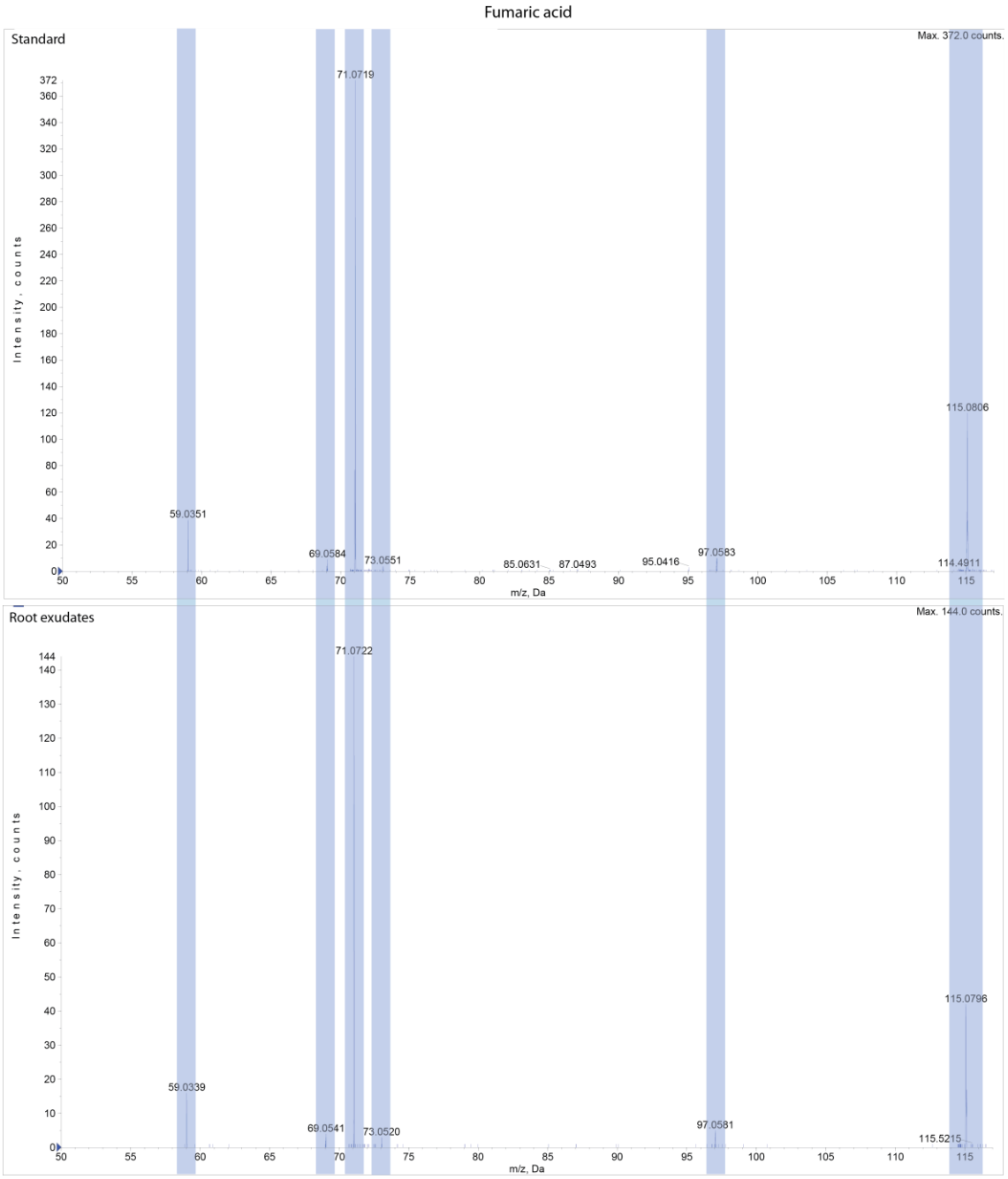

Fig. A3.2: MS-MS ion fragmentation patterns of fumaric acid in standard solution (top) and in collected root exudates (bottom).

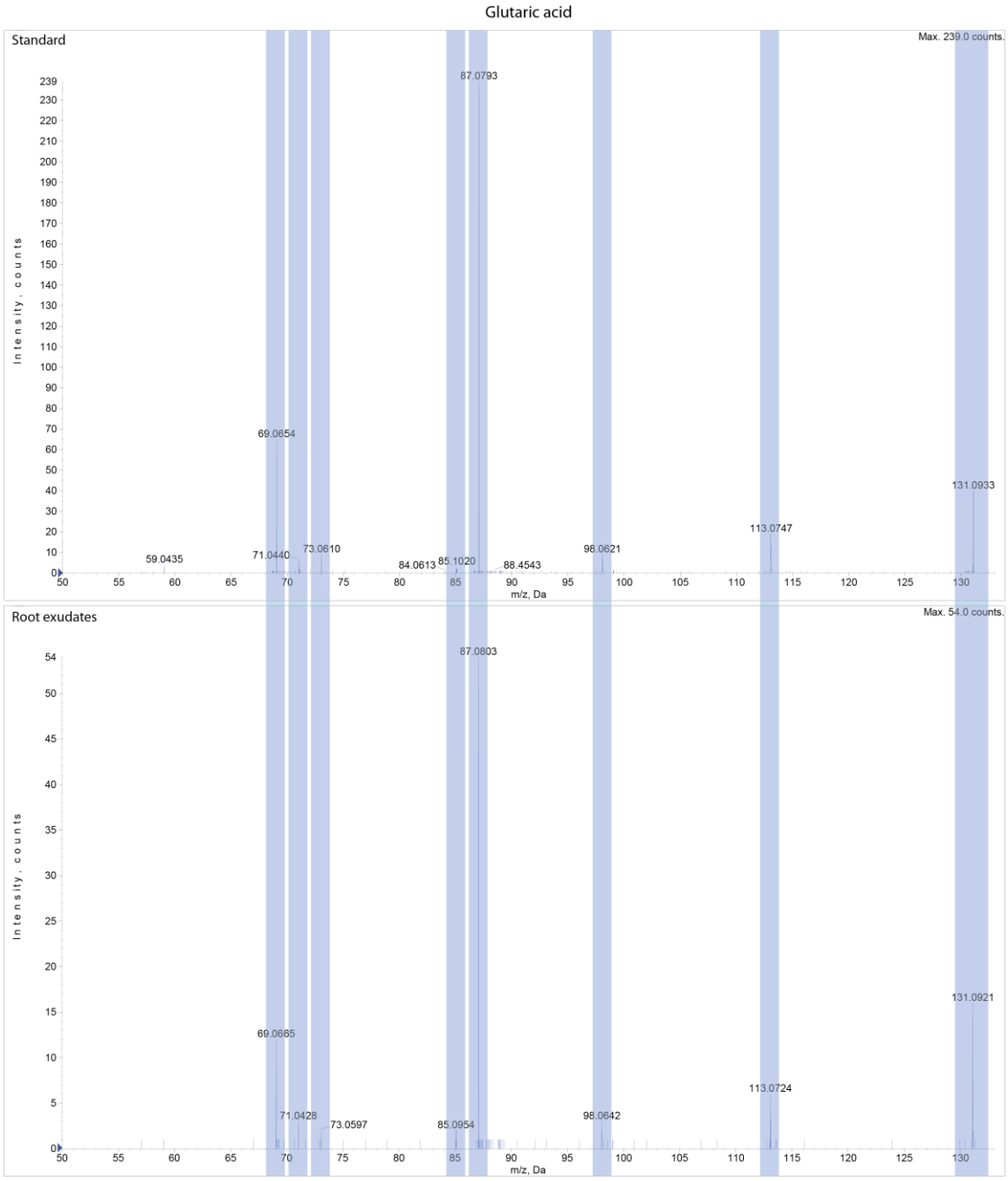

Fig. A3.3: MS-MS ion fragmentation patterns of glutaric acid in standard solution (top) and in collected root exudates (bottom).

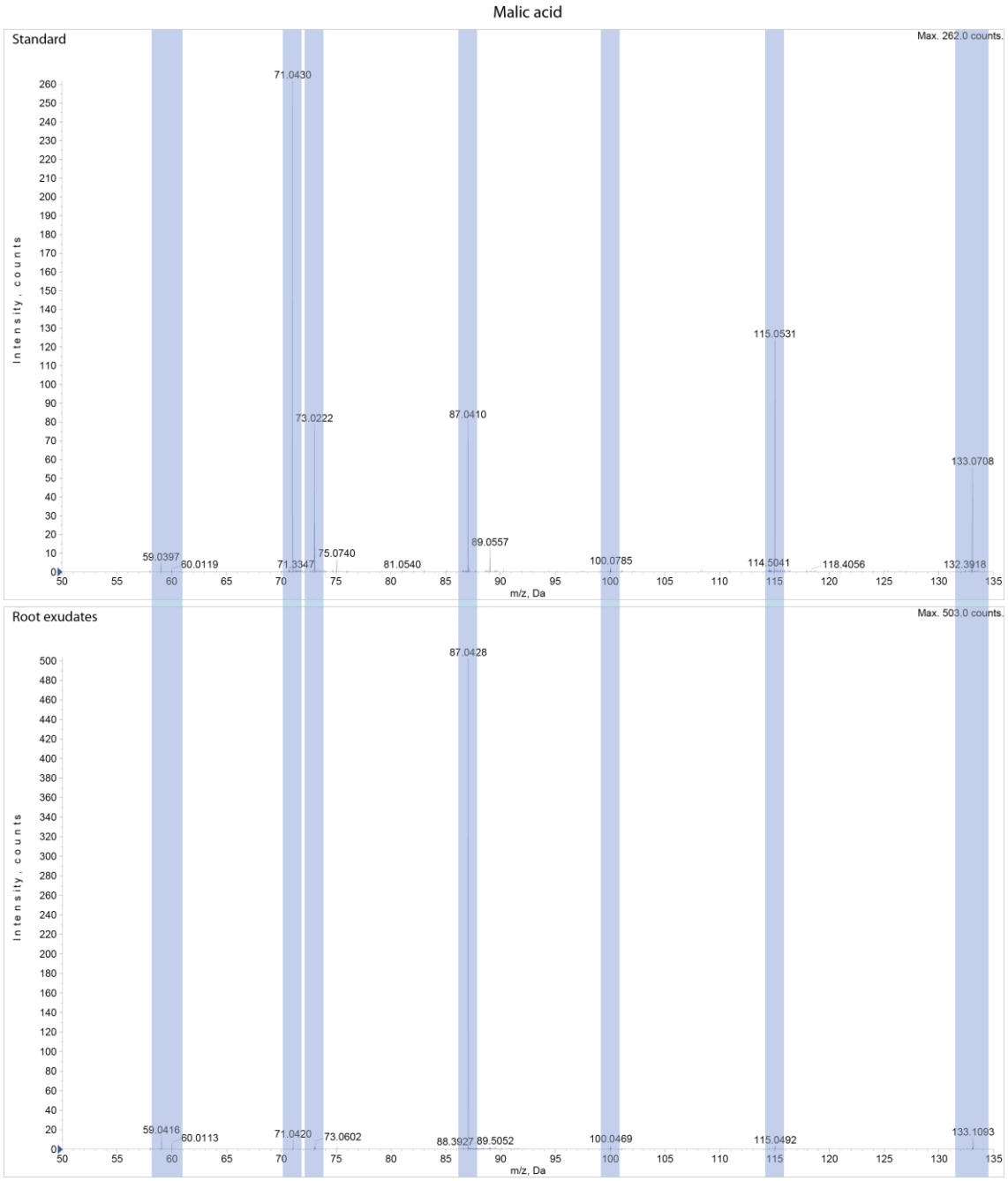

Fig. A3.4: MS-MS ion fragmentation patterns of malic acid in standard solution (top) and in collected root exudates (bottom).

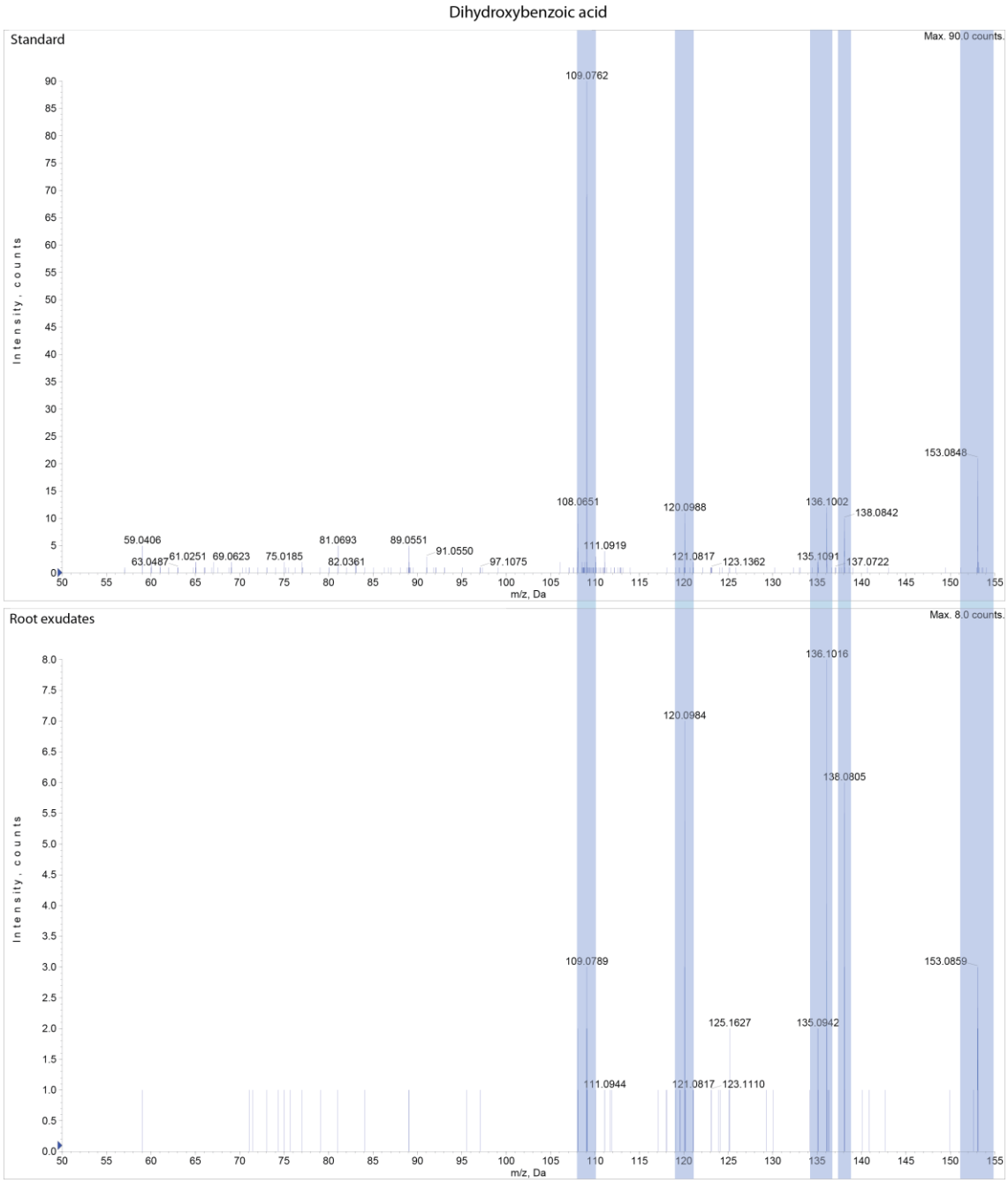

Fig. A3.5: MS-MS ion fragmentation patterns of dihydroxybenzoic acid in standard solution (top) and in collected root exudates (bottom).

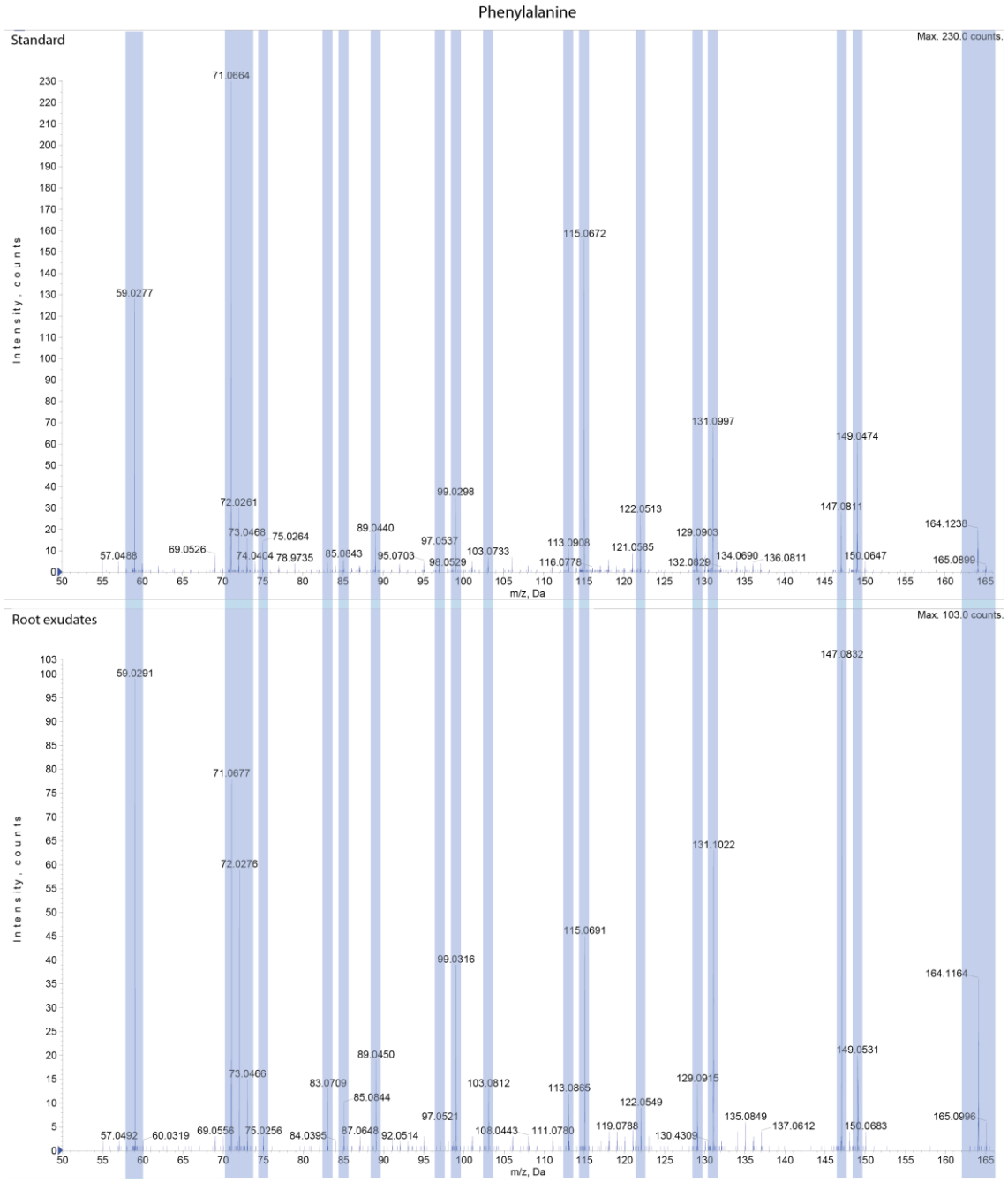

Fig. A3.6: MS-MS ion fragmentation patterns of phenyalanine in standard solution (top) and in collected root exudates (bottom).

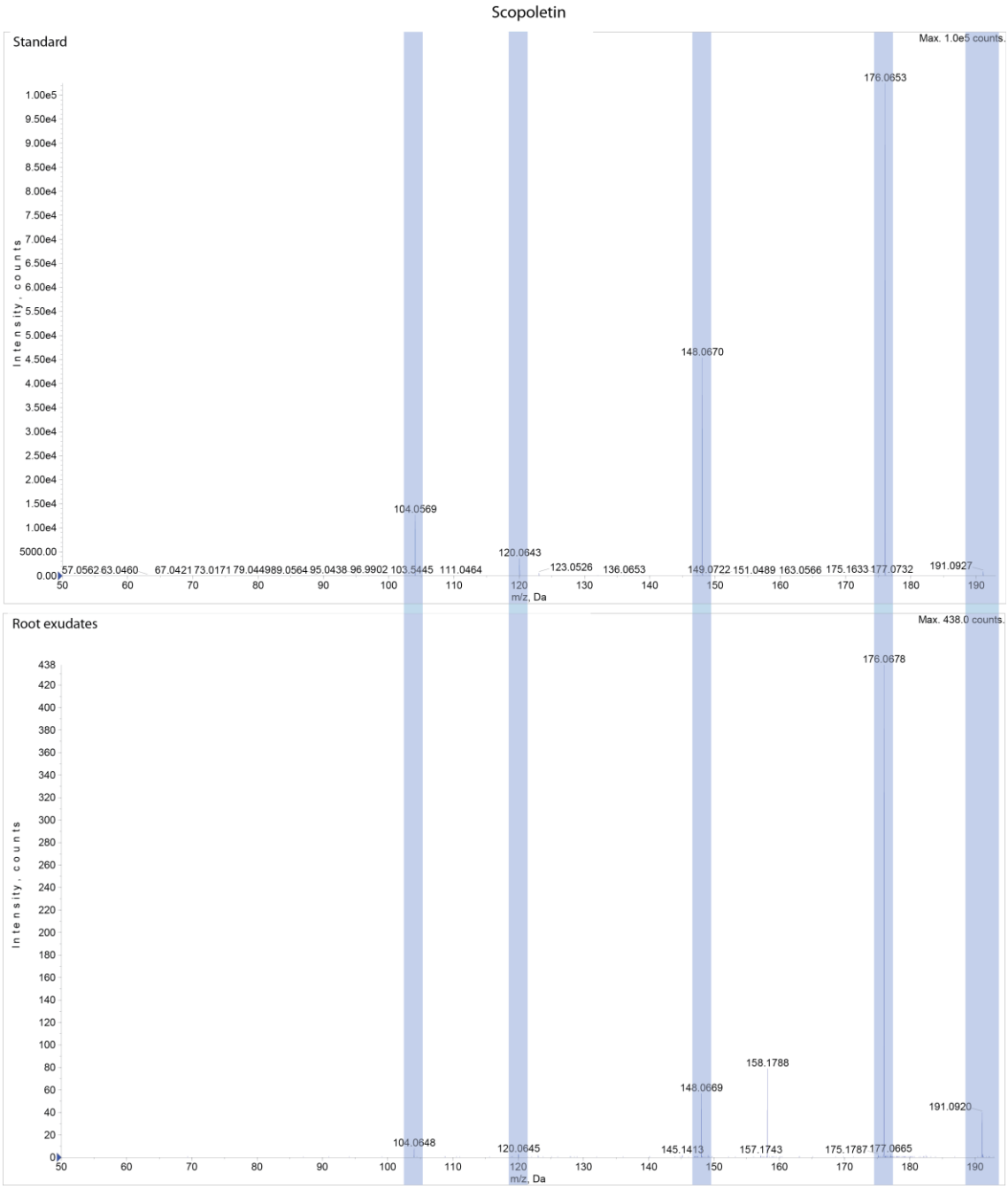

Fig. A3.7: MS-MS ion fragmentation patterns of Scopoletin in standard solution (top) and in collected root exudates (bottom).

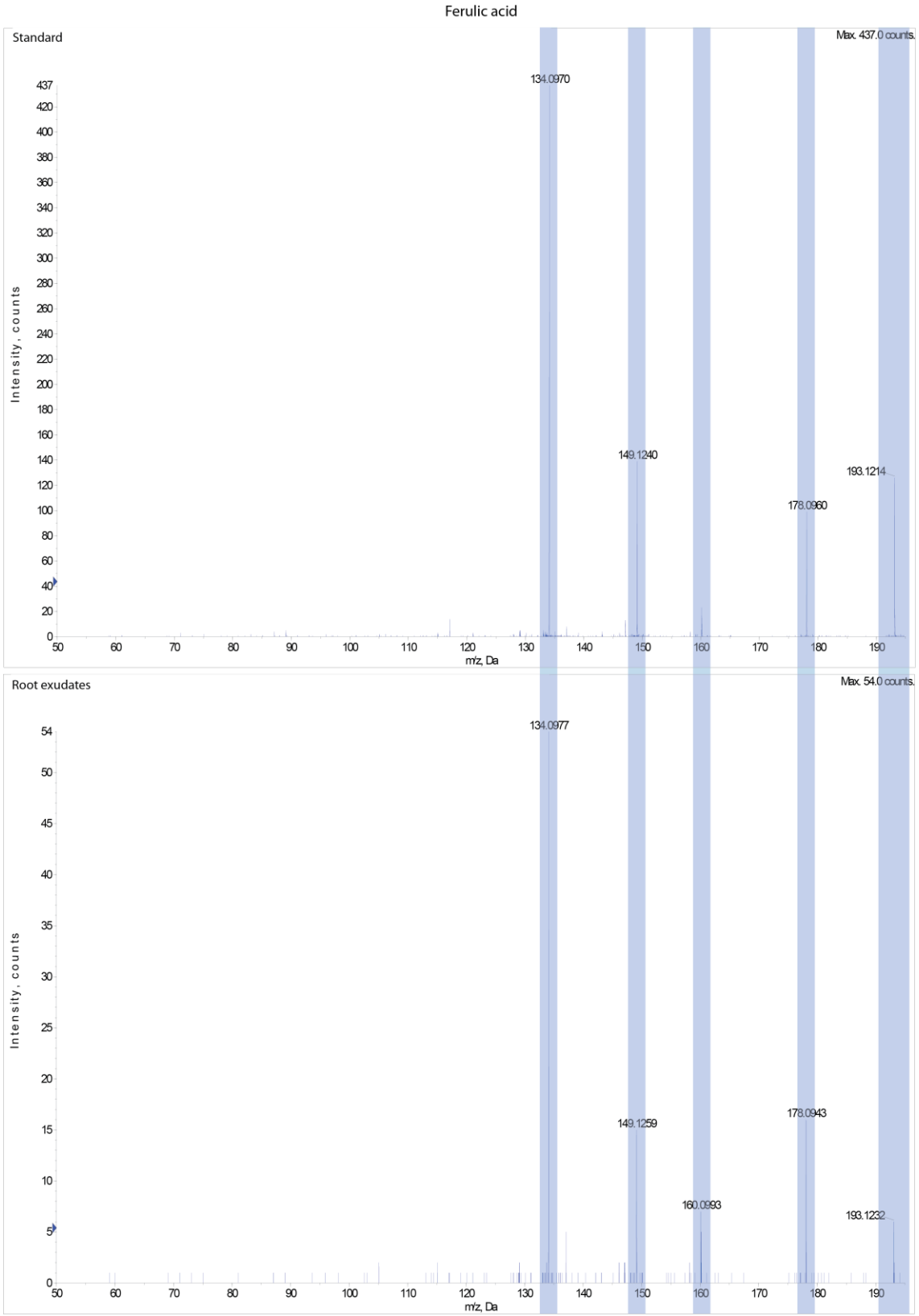

Fig. A3.8: MS-MS ion fragmentation patterns of ferulic acid in standard solution (top) and in collected root exudates (bottom).

Table A4.1: Discriminating mass bins of putatively identified carboxylic and amino acids, sugar and sugar phosphate. Compounds were either detected as hydrogen **[**M+H]+ or sodium adducts [M+NA]+. The table also includes the monoisotopic mass of each compound, the mass error between the accurate and detected mass, the affected metabolic pathways as well as the results of three-way ANOVA (n=5). ns= not significant; *= p< 0.05; **= p< 0.01; *** = p< 0.001; SP= sodium phosphate; PA= phytic acid; HA= hydroxyapatite. There was no significant effect of AMF on any mass bin; therefore it has not been listed.

Mass bin m/z		Accurate mass	Δppm	Name	Formula	Pathway	Compound group	P form	*P. putida*
[M+H]+	[M+Na]+								
117.0174973		116.011	6	Fumaric acid	C4H4O4	TCA cycle	Carboxylic acid	PA-DNA*** SP-DNA*** PA-HA** SP-HA***	ns
	144.0127758	121.0197	26	L-Cysteine	C3H7NO2S	Glycine, serine and threonine metabolism	Amino acid	PA-DNA** SP-DNA** PA-HA** SP-HA**	ns
171.0022052		169.998	18	D-Glyceraldehyde 3-phosphate	C3H7O6P	Glycolysis and pentose phosphate pathway	Sugar	PA-DNA* SP-DNA*** PA-HA* SP-HA***	ns
191.0207343		190.0114	11	Oxalosuccinic acid	C6H6O7	TCA cycle	Carboxylic acid	PA-DNA** SP-DNA*** PA-HA** SP-HA***	
227.0503707		226.0477	20	Chorismic acid	C10H10O6	Shikimic acid pathway	Carboxylic acid	PA-DNA*** SP-DNA*** PA-HA* SP-HA**	ns
		226.0477	20	Prephenic acid	C10H10O6	Shikimic acid pathway	Carboxylic acid		
	250.0702218	227.0794	6	L-Arogenate	C10H13NO5	Phenylalanine, tyrosine and tryptophan biosynthesis	Amino acid	PA-DNA** SP-DNA*** PA-HA* SP-HA***	ns

Mass bin m/z		Accurate mass	Δppm	Name	Formula	Pathway	Compound group	P form	*P. putida*
[M+H]+	[M+Na]+								
423.0853136		422.0825	10	Sucrose-6-phosphate	C12H23O14P	Starch and sucrose metabolism	Sugar phosphate	HA-DNA*** PA-DNA*** SP-DNA*** SP-HA*** SP-PA***	ns
		422.0825	10	Maltose 6'-phosphate	C12H23O14P	Starch and sucrose metabolism	Sugar phosphate		
		422.0825	10	α,α'-Trehalose 6-phosphate	C12H23O14P	Starch and sucrose metabolism	Sugar phosphate		

Table A4.2: Discriminating mass bins of putatively identified plant hormones and metabolites involved in their biosynthesis or degradation. Compounds were either detected as hydrogen **[**M+H]+ or sodium adducts [M+NA]+. The table also includes the monoisotopic mass of each compound, the mass error between the accurate and detected mass, the affected metabolic pathways as well as the results of three-way ANOVA (n=5). ns= not significant; *= p< 0.05; **= p< 0.01; *** = p< 0.001; SP= sodium phosphate; PA= phytic acid; HA= hydroxyapatite. There was no significant effect of AMF on any mass bin; therefore it has not been listed.

Mass bin m/z		Accurate mass	Δppm	Name	Formula	Pathway	Compound group	P form	*P. putida*
[M+H]+	[M+Na]+								
175.0899754		174.0793	19	Indole-3-acetaldehyde oxime	C10H10N2O	Tryptophan metabolism	Oxime	HA-DNA*** SP-DNA* PA-HA*** SP-HA*** SP-PA*	ns
		174.0793	19	Indole-3-acetamide	C10H10N2O	Tryptophan metabolism	Indole		
	182.0621684	159.0684	24	Indole-3-acetaldehyde	C10H9NO	Tryptophan metabolism	Indole	HA-DNA* PA-HA*	ns
	233.1116043	210.1256	13	Jasmonic acid	C12H18O3	alpha-Linolenic acid metabolism	Fatty acyl	HA-DNA*** PA-DNA*** PA-HA*** SP-HA*** SP-PA***	HA***
235.1267686		234.1256	25	Strigolactone ABC-rings	C14H18O3	Carotenoid and strigolactone biosynthesis	Carboxylic ester	HA-DNA*** PA-DNA*** SP-DNA*** PA-HA** SP-HA*** SP-PA***	ns
249.1438959		248.1412	18	Abscisic aldehyde	C15H20O3	Carotenoid biosynthesis	Isoprenoid	PA-DNA* SP-PA***	ns

Mass bin m/z		Accurate mass	Δppm	Name	Formula	Pathway	Compound group	P form	*P. putida*
[M+H]+	[M+Na]+								
	251.1280392	228.1361	10	Traumatic acid	C12H20O4	alpha-Linolenic acid metabolism	Carboxylic acid	HA-DNA*** PA-DNA*** PA-HA*** SP-HA*** SP-PA***	ns
	281.1442247	280.1311	20	8'-Hydroxyabscisic acid	C15H20O5	Carotenoid biosynthesis	Carboxylic acid	HA-DNA*** PA-HA*** SP-HA***	HA***
		280.1311	20	Phaseic acid	C15H20O6	Carotenoid biosynthesis	Diterpenoid		
333.2096172		332.1987	10	Gibberellin A14 aldehyde	C20H28O4	Diterpenoid biosynthesis	Diterpenoid	PA-DNA*** SP-DNA*** PA-HA** SP-HA*** SP-PA*	ns
		332.1987	10	Gibberellin A12	C20H28O4	Diterpenoid biosynthesis	Diterpenoid		
		332.1987	10	Gibberellin A53 aldehyde	C20H28O4	Diterpenoid biosynthesis	Diterpenoid		
	333.2096172	310.2144	18	13S-HpOTrE	C18H30O4	Jasmonic acid biosynthesis alpha-Linolenic acid metabolism	Fatty acyl		
347.1592883		346.1416	29	Gibberellin A29-catabolite	C19H22O6	Diterpenoid biosynthesis	Diterpenoid	HA-DNA*** PA-DNA*** SP-DNA** SP-HA*** SP-PA***	ns
		346.1416	29	Gibberellin A6	C19H22O6	Diterpenoid biosynthesis	Diterpenoid		
		346.1416	29	Gibberellin A34-catabolite	C19H22O6	Diterpenoid biosynthesis	Diterpenoid		
		346.1416	29	Gibberellin A3	C19H22O6	Diterpenoid biosynthesis	Diterpenoid		

Mass bin m/z [M+H]+	Mass bin m/z [M+Na]+	Accurate mass	Δppm	Name	Formula	Pathway	Compound group	P form	*P. putida*
405.004346		404.0022	12	Uridine diphosphate (UDP)	C9H14N2O12P2	Pyrimidine metabolism Zeatin biosynthesis	Nucleoside	HA-DNA*** PA-DNA*** PA-HA*** SP-HA*** SP-PA***	ns
509.2009122		508.1945	1	Gibberellin A3 O-beta-D- glucoside	C25H32O11	n/a	Glucoside	HA-DNA*** PA-DNA*** SP-DNA*** SP-HA*** SP-PA***	ns
527.2126633		526.205	0	Gibberellin A8 2-glucoside	C25H34O12	n/a	Glucoside	HA-DNA*** PA-DNA*** SP-DNA*** SP-HA*** SP-PA***	ns
	568.2062099	545.2333	28	Dihydrozeatin-9-N-glucoside-O-glucoside	C22H35N5O11	Cytokinin O-glucoside biosynthesis	Fatty acyl	PA-DNA* SP-DNA*** PA-HA* SP-HA***	ns

Table A4.3: Discriminating mass bins of putatively identified phenylpropanoids and flavonoids. Compounds were either detected as hydrogen **[**M+H]+ or sodium adducts [M+NA]+. The table also includes the monoisotopic mass of each compound, the mass error between the accurate and detected mass, the affected metabolic pathways as well as the results of three-way ANOVA (n=5). ns= not significant; *= p< 0.05; **= p< 0.01; *** = p< 0.001; SP= sodium phosphate; PA= phytic acid; HA= hydroxyapatite. There was no significant effect of AMF on any mass bin; therefore it has not been listed.

Mass bin m/z [M+H]+	Mass bin m/z [M+Na]+	Accurate mass	Δppm	Name	Formula	Compound group	P form	*P. putida*
133.0661114		132.0575	9	Cinnamaldehyde	C9H8O	Monolignol	SP-DNA*	SP**
195.0652841		194.0579	0	Ferulic acid	C10H10O4	Hydroxycinnamic acid	HA-DNA*** PA-HA*** SP-HA*	ns
		194.0579	0	5-Hydroxyconiferaldehyde	C10H10O4	Monolignol		
	231.0668552	208.0736	17	Sinapoyl aldehyde	C11H12O4	Monolignol	HA-DNA* PA-HA**	ns
	251.0752991	228.0786	29	Xanthyletin	C14H12O3	Coumarin	PA-DNA** SP-DNA** PA-HA** SP-HA**	ns
		228.0786	29	Seselin	C14H12O3	Coumarin		
	259.0642095	236.0685	25	5,6,7-Trimethoxycoumarin	C12H12O5	Coumarin	HA-DNA*** PA-DNA* SP-DNA*** PA-HA**	ns
287.0632973		286.0477	28	Luteolin	C15H10O6	Flavonoid	HA-DNA*** PA-DNA*** SP-DNA** PA-HA*** SP-HA***	ns
		286.0477	28	Kaempferol	C15H10O6	Flavonoid		
		286.0477	28	2'-Hydroxygenistein	C15H10O6	Flavonoid		
	293.0771225	270.0892	4	(-)-Medicarpin	C16H14O4	Flavonoid	HA-DNA** SP-HA***	ns
		270.0892	4	2'-O-Methylisoliquiritigenin	C16H14O4	Flavonoid		
		270.0892	4	Methyl-liquiritigenin	C16H14O4	Flavonoid		
		270.0892	4	Strobopinin	C16H14O4	Flavonoid		
		270.0892	4	Vignafuran	C16H14O4	Isoflavonoid		
		270.0892	4	Alloimperatorin	C16H14O4	Coumarin		
303.0913369		302.079	16	Hesperetin	C16H14O6	Flavonoid	HA-DNA***	ns
		302.079	16	Ferreirin	C16H14O6	Flavonoid		

Mass bin m/z [M+H]+	[M+Na]+	Accurate mass	Δppm	Name	Formula	Compound group	P form	*P. putida*
317.0907473		316.0767	21	2-(4-Chlorophenyl)-3-phenyl-3-(2-pyridinyl)acrylonitrile	C20H13ClN2	Stilbenoid	PA-DNA** SP-DNA*** PA-HA** SP-HA**	ns
	335.1046854	312.1209	16	4-Hydroxycinnamyl alcohol 4-D-glucoside	C15H20O7	Monolignol	PA-DNA** SP-DNA*** PA-HA** SP-HA***	ns
	343.0813547	320.0896	7	4-Coumaroylshikimate	C16H16O7	Monolignol	PA-DNA*** SP-DNA*** PA-HA*	ns
	357.0966335	334.1053	6	Byakangelicin	C17H18O7	Coumarin	HA-DNA*** PA-DNA*** SP-DNA*** PA-HA* SP-HA*** SP-PA***	ns
361.0939536		360.0845	5	Irigenin	C18H16O8	Flavonoid	PA-DNA** SP-DNA** PA-HA* SP-HA**	ns
		360.0845	5	Arcapillin	C18H16O8	Flavonoid		
		360.0845	5	Acerosin	C18H16O8	Flavonoid		
		360.0845	5	Thymonin	C18H16O8	Flavonoid		
		360.0845	5	Oxyayanin A	C18H16O8	Flavonoid		
		360.0845	5	Chrysosplenol C	C18H16O8	Flavonoid		
		360.0845	5	6-Methoxyaromadendrin 3-O-acetate	C18H16O8	Flavonoid		
	361.0939536	338.1002	12	p-Coumaroyl quinic acid	C16H18O8	Carboxylic acid	PA-DNA** SP-DNA** PA-HA* SP-HA**	ns
		338.1002	12	1-Caffeoyl-4-deoxyquinic acid	C16H18O8	Carboxylic acid		
		338.1002	12	4-p-Coumaroylquinic acid	C16H18O8	Carboxylic acid		
		338.1154	29	Wighteone	C20H18O5	Flavonoid		
		338.1154	29	(-)-Glyceollin I	C20H18O5	Flavonoid		
		338.1154	29	3,5,7-Trihydroxy-6-prenylflavone	C20H18O5	Flavonoid		

Mass bin m/z [M+H]+	[M+Na]+	Accurate mass	Δppm	Name	Formula	Compound group	P form	*P. putida*
	365.1192017	342.1315	4	Coniferin	C16H22O8	Monolignol	HA-DNA*** PA-DNA*** SP-DNA*** SP-HA*** SP-PA***	ns
373.1446638		372.142	12	Syringin	C17H24O9	Monolignol	PA-HA** SP-HA***	ns
	379.1120258	356.126	8	Kievitone	C20H20O6	Flavonoid	PA-DNA*	ns
		356.126	8	Leachianone G	C20H20O6	Flavonoid		
	401.0961417	378.1103	8	Millettone	C22H18O6	Flavonoid	HA-DNA*** PA-DNA*** SP-DNA** SP-HA*** SP-PA***	ns
411.1512316		410.1366	18	Sumatrol	C23H22O7	Flavonoid	HA-DNA*** PA-HA*** SP-HA***	ns
		410.1366	18	Toxicarol	C23H22O7	Flavonoid		
		410.1366	18	Tephrosin	C23H22O7	Flavonoid		
		410.1366	18	12a-Hydroxyrotenone	C23H22O7	Flavonoid		
	411.1512316	388.1522	23	Dihydrosamidin	C21H24O7	Coumarin		
		388.1522	23	Visnadin	C21H24O7	Coumarin		
	427.1376057	404.1471	2	Koaburanin	C21H24O8	Flavonoid	HA-DNA* PA-HA**	ns
		404.1471	2	Orientanol A	C21H24O8	Flavonoid		
441.0437397		440.0413	11	Quercetagetin 6,3',4'-trimethyl ether 3-O-sulfate	C18H16O11S	Flavonoid	PA-HA** SP-HA*	ns
445.1532402		444.142	8	Volubilin or Isovolubilin	C23H24O9	Flavonoid	PA-HA** SP-PA*	ns
		444.142	8	7-Hydroxy-5,4'-dimethoxyflavone 8-C-rhamnoside	C23H24O9	Flavonoid		
		444.142	8	Pratensin B	C23H24O9	Flavonoid		
	445.1532402	422.1729	20	Various Flavonoids	C25H26O6	Flavonoid		
	457.1692511	434.1729	15	Macaflavone II	C26H26O7	Flavonoid	PA-HA*	ns

Mass bin m/z [M+H]+	[M+Na]+	Accurate mass	Δppm	Name	Formula	Compound group	P form	*P. putida*
	509.2009122	486.2042	14	Artelastochromene	C30H30O6	Flavonoid	HA-DNA*** PA-DNA*** SP-DNA*** SP-HA*** SP-PA***	ns
527.2126633		526.1992	11	Kurzichalcolactone	C32H30O7	Flavonoid	HA-DNA*** PA-DNA*** SP-DNA*** SP-HA*** SP-PA***	ns
		526.1992	11	Kurziflavolactone A to D	C32H30O7	Flavonoid		
	527.2126633	504.2148	16	Heterophyllin	C30H32O7	Flavonoid		
		504.2148	16	Artocommunol CC	C30H32O7	Flavonoid		
		504.2148	16	Broussoflavonol D	C30H32O7	Flavonoid		
		504.2148	16	Poinsettifolin A	C30H32O7	Flavonoid		
		504.2148	16	Dorsmanin C	C30H32O7	Flavonoid		
		504.2148	16	Euchretin C	C30H32O7	Flavonoid		
	529.2067731	506.2305	24	Artelastofuran	C30H34O7	Flavonoid	HA-DNA*** PA-DNA** SP-DNA** PA-HA** SP-HA**	ns
		506.2305	24	Dorsilurin D	C30H34O7	Flavonoid		
		506.2305	24	Artelastocarpin	C30H34O7	Flavonoid		
		506.2305	24	Petalostemumol G	C30H34O7	Flavonoid		
		506.2305	24	Broussoflavonol E	C30H34O7	Flavonoid		
		506.2305	24	Lumaflavanone C	C30H34O7	Flavonoid		
		506.2305	24	Flemichin E	C30H34O7	Flavonoid		
		506.2305	24	Exiguaflavanone H	C30H34O7	Flavonoid		
	543.1990684	520.1945	28	Pinoresinol glucoside	C26H32O11	Lignan	HA-DNA* PA-DNA* SP-DNA***	ns
	650.1442837	627.1561	1	Delphinidin 3-glucosylglucoside	C27H31O17	Flavonoid	PA-DNA* SP-DNA** PA-HA** SP-HA***	ns

Mass bin m/z [M+H]+	[M+Na]+	Accurate mass	Δppm	Name	Formula	Compound group	P form	*P. putida*
877.2200062		876.2324	22	Quercetin 3-(3'''-benzoylsophoroside)-7-rhamnoside	C40H44O22	Flavonoid	PA-DNA* SP-DNA*** PA-HA*** SP-HA***	ns
		876.2324	22	Quercetin 3-rutinoside-7-(6''-benzoylglucoside)	C40H44O22	Flavonoid		
	895.2032196	872.2011	14	Kaempferol 3-(2''-feruloylglucosyl)-(1->2)-(6''-malonylglucoside)	C40H40O22	Flavonoid	HA-DNA*** PA-DNA*** SP-DNA** PA-HA*** SP-HA*** SP-PA***	ns
		872.2223	9	Kaempferol 3-(6'''-rhamnosyl-2'''-(6-malyl-glucosyl)-glucoside)	C37H44O24	Flavonoid		

Table A4.4: Discriminating mass bins of putatively identified alkaloids and terpenoids. Compounds were either detected as hydrogen **[**M+H]+ or sodium adducts [M+NA]+. The table also includes the monoisotopic mass of each compound, the mass error between the accurate and detected mass as well as the results of three-way ANOVA (n=5). ns= not significant; *= p< 0.05; **= p< 0.01; *** = p< 0.001; SP= sodium phosphate; PA= phytic acid; HA= hydroxyapatite. There was no significant effect of AMF on any mass bin; therefore it has not been listed.

Mass bin m/z		Accurate mass	Δppm	Name	Formula	Compound group	P form	*P. putida*
[M+H]+	[M+Na]+							
140.0357346		139.0269	10	6-Hydroxynicotinic acid	C6H5NO3	Alkaloid	HA-DNA** PA-DNA*** SP-DNA*** PA-HA** SP-HA*	ns
	148.0406879	125.0477	25	3,6-Dihydronicotinic acid	C6H7NO2	Alkaloid	PA-DNA** SP-DNA***	ns
203.0862282		202.0742	23	Vasicinone	C11H10N2O2	Alkaloid	SP-DNA**	ns
	205.0320827	182.044	5	7-Methyluric acid	C6H6N4O3	Alkaloid	SP-HA*	ns
		182.044	5	1-Methyluric acid	C6H6N4O3	Alkaloid		
	219.0498294	196.0596	4	3,7-Dimethyluric acid	C7H8N4O3	Alkaloid	SP-DNA** SP-HA*** SP-PA**	ns
		196.0596	4	1,7-Dimethyluric acid	C7H8N4O3	Alkaloid		
	222.1108149	199.1208	3	Tussilagine	C10H17NO3	Alkaloid	HA-DNA*** PA-DNA* PA-HA** SP-HA***	ns
236.13027		235.1208	9	Lophophorine	C13H17NO3	Alkaloid	HA-DNA*	ns
237.1045683		236.095	9	Glycosminine	C15H12N2O	Alkaloid	HA-DNA*** PA-HA*** SP-HA*** SP-PA**	ns
	237.1045683	214.1106	19	Harmaline	C13H14N2O	Alkaloid		
249.0696888		248.0586	15	Tryptanthrine	C15H8N2O2	Alkaloid	HA-DNA** PA-HA*** SP-HA***	ns
249.1438959		248.1412	18	Confertin and other terpenoids	C15H20O3	Terpenoid	PA-DNA* SP-PA***	ns
	277.1301429	254.1419	3	Elymoclavine	C16H18N2O	Alkaloid	PA-HA*	HA***

Mass bin m/z		Accurate mass	Δppm	Name	Formula	Compound group	P form	*P. putida*
[M+H]+	[M+Na]+							
	287.1580746	264.1725	12	Dehydrojuvabione	C16H24O3	Terpenoid	HA-DNA*** PA-HA*** SP-HA***	ns
	291.1069795	268.1212	11	Lysergic acid	C16H16N2O2	Alkaloid	PA-DNA** SP-DNA***	ns
	304.0928009	281.1052	5	Annolobine	C17H15NO3	Alkaloid	HA-DNA*** PA-DNA* SP-DNA* PA-HA*** SP-HA***	ns
310.1056368		309.1001	5	Lunamarine	C18H15NO4	Alkaloid	PA-DNA* SP-DNA* PA-HA* SP-HA*	ns
	310.1056368	287.1158	2	(S)-Norlaudanosoline	C16H17NO4	Alkaloid		
	325.1290423	310.1053	6	Mellitoxin	C15H18O7	Terpenoid	HA-DNA*	ns
	336.1118491	313.1215	3	Angustine	C20H15N3O	Alkaloid	PA-DNA** SP-DNA*** PA-HA* SP-HA*	ns
		313.1314	26	Acetylcaranine	C18H19NO4	Alkaloid		
	373.1446638	350.163	20	4,21-Dehydrogeissoschizine	C21H22N2O3	Alkaloid	PA-HA** SP-HA***	ns
		350.163	20	Polyneuridine aldehyde	C21H22N2O3	Alkaloid		
		350.163	20	Cathenamine	C21H22N2O3	Alkaloid		
		350.163	20	4,21-Dehydrocorynantheine aldehyde	C21H22N2O3	Alkaloid		
		350.163	20	Vomilenine	C21H22N2O3	Alkaloid		
380.1095725		379.1056	8	12-Hydroxydihydrochelirubine	C21H17NO6	Alkaloid	PA-DNA*** SP-DNA*** PA-HA*** SP-HA***	ns
402.09882		9				HA-DNA*** PA-DNA*** SP-HA*** SP-PA***	ns	

Mass bin m/z		Accurate mass	Δppm	Name	Formula	Compound group	P form	*P. putida*
[M+H]+	[M+Na]+							
	380.2253238	357.2304	15	Songorine	C22H31NO3	Alkaloid	PA-DNA** SP-DNA*** PA-HA** SP-HA***	ns
	381.1170238	358.1264	3	10-Deoxygeniposidic acid	C16H22O9	Terpenoid	PA-DNA** SP-DNA** PA-HA* SP-HA*	ns
441.3308		440.329	12	Pfaffic acid	C29H44O3	Terpenoid	HA-DNA*** PA-DNA*** SP-HA*** SP-PA***	ns
	691.3232	668.3309	4	14-Deacetylnudicauline	C36H48N2O10	Alkaloid	HA-DNA*** PA-DNA*** SP-DNA*** SP-HA*** SP-PA***	ns
		668.3309	4	Lycaconitine	C36H48N2O10	Alkaloid		

Table A4.5: Discriminating mass bins putatively identified as peptides. Peptides were either detected as hydrogen **[**M+H]+ or sodium adducts [M+NA]+. The table also includes the monoisotopic mass of each peptide, the mass error between the accurate and detected mass as well as the results of three-way ANOVA (n=5). ns= not significant; *= p< 0.05; **= p< 0.01; *** = p< 0.001; SP= sodium phosphate; PA= phytic acid; HA= hydroxyapatite. There was no significant effect of AMF on any mass bin; therefore it has not been listed.

Mass bin m/z		Accurate mass	Δppm	Formula	P form	*P. putida*
[M+H]+	[M+Na]+					
219.0966014		218.0903	4	C8H14N2O5	HA-DNA*** PA-DNA*** PA-HA*** SP-HA*** SP-PA***	ns
220.0985329		219.0855	26	C7H13N3O5	HA-DNA*** PA-DNA*** PA-HA*** SP-HA*** SP-PA***	ns
	261.0855323	238.0954	3	C11H14N2O4	HA-DNA** SP-DNA*	ns
	270.0633913	247.0804	23	C8H13N3O6	HA-DNA*** PA-DNA*** SP-DNA*** PA-HA*** SP-HA*** SP-PA***	ns
	277.1301429	254.1379	10	C11H18N4O3	PA-HA*	HA***
283.0889788		282.0852	12	C12H14N2O6	SP-DNA*	ns
294.0722106		293.0682	10	C9H15N3O6S1	PA-DNA* SP-DNA** PA-HA* SP-HA**	ns
	303.0913369	280.1059	12	C13H16N2O5	HA-DNA***	ns
310.1056368		309.0995	3	C10H19N3O6S1	PA-DNA* SP-DNA* PA-HA* SP-HA*	ns
	325.1290423	302.1379	5	C15H18N4O3	HA-DNA*	ns
335.1046854		334.0913	18	C14H14N4O6	PA-DNA** SP-DNA*** PA-HA** SP-HA***	ns
	335.1046854	312.1144	3	C14H20N2O4S		
336.1118491		335.0965	24	C11H17N3O9	PA-DNA** SP-DNA*** PA-HA* SP-HA*	ns
337.1223281		336.1104	13	C11H20N4O6S	PA-DNA** SP-DNA*** PA-HA* SP-HA**	ns
	344.0832505	321.0995	15	C11H19N3O6S1	HA-DNA*** PA-DNA** SP-DNA***	ns
349.1764268		348.1645	13	C13H24N4O7	SP-DNA***	ns
	355.1312732	332.1485	18	C16H20N4O4	SP-DNA*** SP-HA* SP-PA**	ns
		332.1332	24	C12H20N4O7		
	357.0966335	334.1125	14	C11H18N4O8	HA-DNA*** PA-DNA*** SP-DNA*** PA-HA* SP-HA*** SP-PA***	ns

Mass bin m/z		Accurate mass	Δppm	Formula	P form	*P. putida*
[M+H]+	[M+Na]+					
362.0913942		361.0878	10	C12H19N5O4S2	PA-DNA** SP-DNA*** PA-HA** SP-HA***	ns
363.1050673		362.1074	26	C12H18N4O9	PA-DNA* SP-DNA** PA-HA* SP-HA**	ns
	366.1232526	343.1314	7	C13H21N5O4S1	HA-DNA*** PA-DNA*** SP-DNA*** PA-HA*** SP-PA***	ns
371.1283441		370.1199	3	C16H22N2O6S	PA-DNA*** SP-DNA** PA-HA***	ns
	371.1283441	348.1434 348.1467 348.1281	11 20 29	C16H20N4O5 C13H24N4O5S C12H20N4O8		
371.2437092		370.2329	9	C16H30N6O4	HA-DNA** PA-HA*** SP-HA**	ns
373.1446638		372.1321	14	C19H20N2O6	PA-HA** SP-HA***	ns
	373.1446638	372.1467 350.159 350.1624	25 9 18	C15H24N4O5S C16H22N4O5 C13H26N4O5S1		
379.1120258		378.1032	4	C13H22N4O5S2	PA-DNA*	ns
	401.0961417		9		HA-DNA*** PA-DNA*** SP-DNA** SP-HA*** SP-PA***	ns
	379.2307438	356.2424	2	C17H32N4O4	PA-DNA** SP-DNA*** PA-HA** SP-HA***	ns
380.1095725		379.1049	6	C13H21N3O8S1	PA-DNA*** SP-DNA*** PA-HA*** SP-HA***	ns
	402.09882		11		HA-DNA*** PA-DNA*** SP-HA*** SP-PA***	ns
	380.1095725	357.1285	21	C13H19N5O7	PA-DNA*** SP-DNA*** PA-HA*** SP-HA***	ns
380.2253238		379.2107	19	C19H29N3O5	PA-DNA** SP-DNA*** PA-HA** SP-HA***	ns
381.1170238		380.1002	25	C12H20N4O8S	PA-DNA** SP-DNA** PA-HA* SP-HA*	ns
		380.1008 380.1188	23 23	C20H16N2O6 C13H24N4O5S2		
	383.0901917	360.0958	13	C17H16N2O7	HA-DNA** PA-DNA*** SP-DNA*** PA-HA*** SP-PA**	SP*

Mass bin m/z		Accurate mass	Δppm	Formula	P form	*P. putida*
[M+H]+	[M+Na]+					
389.1754597		388.1594	22	C15H24N4O8	HA-DNA*** PA-HA** SP-HA**	ns
		388.1706	6	C14H24N6O7		
		388.1747	16	C19H24N4O5		
	411.1512316	388.1594	6		HA-DNA***	ns
		388.1706	20		PA-HA***	
		388.1747	21		SP-HA***	
	389.1754597	366.1903	10	C17H26N4O5	HA-DNA*** PA-HA** SP-HA**	ns
411.1512316		410.1438	0	C17H22N4O8	HA-DNA*** PA-HA*** SP-HA***	ns
		410.1471	7	C14H26N4O8S		
	417.0748789	394.0947	21	C16H18N4O6S	HA-DNA*** PA-DNA*** PA-HA*** SP-HA*** SP-PA***	ns
	418.0780225	395.0933	10	C12H21N5O6S2	HA-DNA*** PA-DNA** PA-HA*** SP-HA*** SP-PA**	ns
422.1990763		421.1809	25	C15H27N5O9	HA-DNA*** PA-DNA*** PA-HA*** SP-HA*** SP-PA***	HA** PA*
		421.1961	10	C19H27N5O6		
		421.1995	18	C16H31N5O6S		
	422.1990763	399.2118	4	C17H29N5O6		
424.2156578		423.2118	8	C19H29N5O6	HA-DNA* SP-DNA* SP-HA*** SP-PA***	DNA*
	424.2156578	401.2274	2	C17H31N5O6		
427.1376057		426.1175	29	C20H18N4O7	HA-DNA* PA-HA**	ns
		426.1243	14	C14H26N4O7S2		
	427.1376057	404.1366	27	C15H24N4O7S		
		404.1518	8	C19H24N4O4S1		
		404.1543	13	C15H24N4O9		
445.1532402		444.1427	7	C16H24N6O7S	PA-HA** SP-PA*	ns
		444.158	26	C20H24N6O4S1		
	445.1532402	422.1624	3	C19H26N4O5S		
		422.1624	3	C19H26N4O5S1		
		422.1649	1	C15H26N4O10		
		422.1658	3	C16H30N4O5S2		
445.3086693		444.2948	14	C21H40N4O6	SP-HA***	ns
		444.306	10	C20H40N6O5		
450.1729452		449.158	16	C16H27N5O8S	HA-DNA* SP-DNA* PA-HA*** SP-HA***	ns
		449.1693	7	C15H27N7O7S		
		449.1733	16	C20H27N5O5S		
		449.1758	22	C16H27N5O10		
	450.1729452	427.1815	4	C16H25N7O7		
457.1692511		456.1605	3	C17H24N6O9	PA-HA*	ns
		456.1679	12	C19H28N4O7S		
	457.1692511	434.1761	8	C15H26N6O9		
		434.1801	0	C20H26N4O7		
		434.1835	7	C17H30N4O7S		

Mass bin m/z		Accurate mass	Δppm	Formula	P form	*P. putida*
[M+H]+	[M+Na]+					
	461.0537418	438.0652	1	C19H19ClN2O6S	HA-DNA*** PA-DNA*** SP-DNA*** PA-HA** SP-PA***	ns
468.2647834		467.2605	6	C19H33N9O5	HA-DNA*** PA-HA*** SP-HA***	HA***
		467.2492	17	C20H33N7O6		
	468.2647834	445.2689	14	C23H35N5O4		
		445.2649	22	C18H35N7O6		
498.2239688		497.2122	9	C21H31N5O9	SP-DNA** PA-HA** SP-HA***	ns
		497.2234	13	C20H31N7O8		
		497.2274	21	C25H31N5O6		
		497.2057	22	C20H31N7O6S		
	498.2239688	475.2278	13	C19H33N5O9		
		475.2391	8	C18H33N7O8		
		475.2431	16	C23H33N5O6		
		475.2213	26	C18H33N7O6S		
		475.2465	23	C20H37N5O6S		
	512.1481422	489.1536	10	C26H23N3O7	HA-DNA*** PA-DNA*** PA-HA* SP-HA*** SP-PA***	ns
		489.1464	24	C17H27N7O6S2		
	524.1151702	501.1352	17	C19H27N5O7S2	HA-DNA*** PA-DNA*** PA-HA*** SP-HA*** SP-PA**	ns
528.2243343		527.2128	7	C24H29N7O7	HA-DNA*** PA-DNA*** SP-DNA*** SP-HA*** SP-PA***	ns
		527.2162	1	C21H33N7O7S		
		527.2236	12	C23H37N5O5S2		
		527.205	22	C22H33N5O8S		
	528.2243343	505.2319	6	C19H35N7O7S		
		505.2325	4	C27H31N5O5		
		505.2359	1	C24H35N5O5S		
		505.2384	6	C20H35N5O10		
		505.2393	7	C21H39N5O5S2		
		505.2397	8	C21H31N9O6		
		505.2206	27	C20H35N5O8S		
529.2067731		528.2043	8	C26H32N4O6S	HA-DNA*** PA-DNA** SP-DNA** PA-HA** SP-HA**	ns
		528.2076	15	C23H36N4O6S2		
		528.189	19	C22H32N4O9S		
		528.2115	22	C20H32N8O7S		
	529.2067731	506.2046	24	C20H34N4O9S		
		506.2271	18	C18H34N8O7S		
		506.2125	9	C22H30N6O8		
		506.2159	3	C19H34N6O8S		
		506.2199	4	C24H34N4O6S		
		506.2237	11	C21H30N8O7		
543.1990684		542.1869	9	C23H34N4O7S2	HA-DNA* PA-DNA* SP-DNA***	ns

Mass bin m/z		Accurate mass	Δppm	Formula	P form	*P. putida*
[M+H]+	[M+Na]+					
543.1990684		542.1973	10	C21H30N6O11	HA-DNA* PA-DNA* SP-DNA***	ns
		542.2013	17	C26H30N4O9		
		542.2046	23	C23H34N4O9S		
		542.206	26	C24H30N8O5S		
	543.1990684	520.2064	6	C18H32N8O8S		
		520.2104	1	C23H32N6O6S		
		520.2142	8	C20H28N10O7		
		520.2169	13	C24H32N4O9		
		520.2203	19	C21H36N4O9S		
		520.2216	21	C22H32N8O5S		
548.2071342		547.2027	5	C23H29N7O9	HA-DNA*** PA-HA** SP-HA**	ns
		547.2101	18	C25H33N5O7S		
	548.2071342	525.2223	8	C26H31N5O7		
		525.2257	14	C23H35N5O7S		
		525.2046	24	C26H31N5O5S		
		525.2071	19	C22H31N5O10		
		525.2296	21	C20H31N9O8		
		525.2336	28	C25H31N7O6		
568.2062099		567.1821	29	C24H33N5O7S2	PA-DNA* SP-DNA*** PA-HA* SP-HA***	ns
		567.2152	28	C28H33N5O6S		
	568.2062099	545.2057	19	C24H31N7O6S		
		545.209	14	C21H35N7O6S2		
		545.2122	8	C25H31N5O9		
		545.2203	5	C20H35N9O5S2		
		545.2234	11	C24H31N7O8		
	689.3239742	666.3166	26	C37H42N6O6	HA-DNA*** PA-DNA*** SP-DNA* SP-HA*** SP-PA***	ns
690.3270731		689.3326	18	C39H43N7O5	HA-DNA*** PA-DNA*** SP-HA*** SP-PA***	ns
705.3039761		704.3071	14	C38H40N8O6	SP-DNA*	ns
706.308244		705.2911	13	C38H39N7O7	SP-DNA*	ns
	706.308244	683.3292	14	C34H41N11O5		