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Fertilizer placement and the potential for its combination with bio-effectors to improve crop nutrient acquisition and yield

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List of abbreviations

+P, treatment with $Ca(NO_3)_2$ and $Ca(H_2PO_4)_2$ homogenously mixed in soil

ACC, 1-aminocyclopropane-1-carboxylate

ANR, Apparent Nutrient Recovery efficiency

B.atr., *Bacillus atrophaeus*

B.s.R41, *Bacillus simplex R41* (cold-adapted strain)

B.spec, *Bacillus spec*,

Bact, Bactofil (BE consortia comprising: *Azospirillum brasielnse, Azotobacter vinerólandii, B. megaterium, Bacillus polymyxa, Pseudomonas fluorescens, Sterptomyces albus*)

BE, Bio-effector or PGPM

BFDC, *Penicillium sp*. PK 112 (surfactant dispersion, "Tenside" in German);

BFOD, *Penicillium sp*. PK 112 (oil dispersion)

Broad., Fertilizer broadcasted and incorporated in soil (Field)

CAN, Calcium ammonium nitrate

CI95%, *Bias-corrected percentile bootstrap confidence intervals (95 %)* with *999 iterations* at the power $\alpha = 0.05$

ComA, CombiFectorA (consortia BE comprising: *Trichoderma. harzianum* OMG08, *Pseudomonas fluorescens, Bacillus subtilis*).

CULTAN, Controlled Long-Term Ammonium Nutrition

DAP, di-ammonium phosphate

DAS, Days after sowing

dap, Days after planting

Depot, Fertilizer placed as a subsurface localized depot

DM, dry matter

DMPP, 3, 4-Dimethylpyrazole phosphate

MAP, Mono-ammonium phosphate

Meg, MegaNit, (BE consortia comprising: *A. chroccoccum, Azospirillum ssp., B. megaterium, Bacillus subtilis*)

Mixed, Fertilizer evenly mixed in soil (Greenhouse)

NH_4^+-Depot, treatment with $(NH_4)_2SO_4$ placed as a concentrated depot

NO_3^--Mixed, treatment with $Ca(NO_3)_2$ homogenously mixed in soil

NoBE, no inoculated BE or PGPM

OmG08, *Trichoderma harzianum* OmG-08

P-Depot, treatment with placed RP-enriched manure placed as a concentrated depot

PGPMs, Plant Growth-Promoting Microorganisms

Phyl, Phylazonit (BE consortia comprising: *Azotobacter chrococcum, Bacillus megaterium*)

P-Mixed, treatment with RP-enriched manure homogenously mixed in soil

P-Mixed10cm, treatment with RP-enriched manure homogenously mixed in top 10 cm soil

Pro, *Pseudomonas* sp. DSMZ 13134, Proradix®

Rhiz, *Bacillus amyloliquefaciens* FZB42, Rhizovital42®

RLD, Root length density

RP, rock phosphate

RPE, Relative placement effect

SP11, Vitalin SP11 (consortia BE comprising: *Bacillus subtilis*, *Pseudomonas sp.*, *Streptomyces spp*., natural humic acids und extracts of the seaweed *Ascophyllum nodosum*.

SSA/**SA**, sewage sludge ash.

STD, Standard deviations

STR, struvite (magnesium ammonium phosphate, $NH_4MgPO_4 \cdot 6H_2O$)

T-22, *Trichoderma harzianum* -T22

T50, *Trichoderma harzianum* T50

TSP, Triple superphosphate

USDA, United States Department of Agriculture

WG, *Trichoderma harzianum* WG

1 Summary

Even when total nitrogen (N) and phosphorus (P) concentrations in most agricultural soils are high, the concentrations of plant-available N and P fractions are often inadequate for acceptable yield. In comparison to conventional fertilizer application by homogenous broadcast over the soil surface (with or without subsequent incorporation), fertilizer placement in defined soil areas/volumes close to seeds or crop roots is a more effective application method to enhance the plant-availability of applied fertilizers. Nevertheless, considerable root growth in subsurface nutrient patches or around concentrated fertilizer-depots (and/or improved nutrient influx rates in roots) is a prerequisite for improved uptake of placed nutrients. Furthermore, zones with intense rooting around placed fertilizer depots ("rhizosphere hotspots") with high concentrations of organic nutrients released as root exudates may be favorable for the survival and establishment of inoculated plant-growth-promoting microorganisms (PGPMs), which mobilize nutrients in soil to favor plant growth.

In the last three decades, several published field studies comparing fertilizer placement to fertilizer broadcast arrived at different and often conflicting results regarding their effects on yield and nutrient status of various crops. For this reason, the first task was to conduct a Meta-analysis on data in published peer-reviewed field studies on fertilizer placement that met a set of pre-defined criteria for inclusion. We investigated the relative effect of fertilizer placement for specific fertilizer formulations (e.g. NH_4^+ and $CO(NH_2)_2$ without or in combination with soluble P (HPO_4^{2-}; $H_2PO_4^-$); soluble K; solid or liquid manure) in a precise restricted area on surface or subsurface soil in comparison to fertilizer broadcast on yield, nutrient concentration and content in above-ground plant parts. We utilized data from a total of 40 field studies published between 1982 and 2015 (85% of studies published from 2000) that met our criteria. We used the method of "baseline contrasts" to compare different fertilizer placement treatments to fertilizer broadcast as a common control or baseline treatment. Results showed that overall, fertilizer placement led to +3.7% higher yields, +3.7% higher concentrations of nutrients in above-ground plant parts and +11.9% higher contents of nutrients also in above-ground plant parts than fertilizer broadcast application. Placement depth had a strong effect of the outcome of fertilizer placement because relative placement effects increased with increasing fertilizer placement depth. Composition of fertilizer formulations was also an important factor. High yields of fertilizer placement relative to fertilizer broadcast application were obtained for $CO(NH_2)_2$ in combination with soluble P (HPO_4^{2-}; $H_2PO_4^-$) (+27%) or NH_4^+ in combination with HPO_4^{2-}; $H_2PO_4^-$ (+15%) (Nkebiwe et al., 2016 a*: Field Crops Research* 196: 389–401).

The next aim was to investigate the effect of fertilizer placement in subsurface soil in combination with application of bio-effectors (BEs) (PGPMs and natural active substances such as humic acids and seaweed extracts) on root growth of crop plants, establishment of inoculated PGPM in the rhizosphere, grain and biomass production as well as plant nutrient status for maize (*Zea mays* L) and wheat (*Triticum aestivum* L) cultures. Through various pot and rhizobox experiments, we observed that placement of a subsurface concentrated NH_4^+-fertilizer depot stabilized with the nitrification inhibitor DMPP (3,4-di-

methylpyrazolphosphate) induced dense rooting around the depot contributing to more efficient exploitation of the depot. For this, it was crucial the N persisted in the depot mainly as poorly mobile NH_4^+, in order to induce localized depot-zone root-growth as well as favorable chemical and biological changes in the rhizosphere to improve N and P uptake by crop plants. Through *in vitro* culture experiments on solid and liquid media, we could show that via acidification of the growth media, several selected microbial BEs were capable to solubilize sparingly soluble inorganic phosphates and also that these BEs showed considerable tolerance to high concentrations of NH_4^+ und DMPP. The latter indicated a potential for the BEs to colonize plant roots in NH_4^+-rich well rooted soil zones around a subsurface NH_4^+-fertilizer depot (Nkebiwe et al., 2016 c: *Manuscript submitted*). Through further pot experiments and four others experiments as Bachelor and Master theses conduction under my supervision, we observed that certain BEs that readily solubilized tri-calcium phosphates *in vitro* were able to mobilize rock phosphate (RP) applied in soil-based substrates when N was supplied as stabilized NH_4^++DMPP, thereby contributing to enhanced P uptake and growth of maize and wheat plants. The bacterial BE *Pseudomonas sp.* DSMZ 13134 and BE consortia products containing bacteria and fungi such as CombiFectorA were good candidates. BE-induced RP-solubilzation occurred mainly in substrates with low $CaCO_3$ contents indicating low P sorption capacity for neutral and moderately alkaline soils. With CombiFectorA, maize P-acquisition from sewage sludge ash could be enhanced, thus increasing the efficiency of a sparingly soluble fertilizer based of recycled wastes. Possible explanations for the beneficial effects of best performing BEs to improve plant growth were enhanced solubility of sparingly soluble P fertilizers via acidification of the rhizosphere and release of nutrient-chelating substances as well as improvement of root growth for better spatial interception of nutrients (Nkebiwe et al., 2016 d: *Manuscript in preparation).*

Alongside, more greenhouse and two field experiments (grain maize 2014 and maize silage 2015) were designed, planned, conducted and evaluated. A peer-reviewed paper from this work has already been published (Nkebiwe et al., 2016 b: *Chemical and Biological Technologies in Agriculture* 3:15). In the greenhouse and experiments, placement of a concentrated stabilized NH_4^+-fertilizer depot led to improved root and shoot growth, and increased shoot N and P contents. Through intense root growth of maize around the NH_4^+-depot, increased root-colonization by *Pseudomonas sp.* DSMZ 13134 close to seeds could be observed. In the field, many weeks after subsurface placement of the concentrated stabilized NH_4^+-depot, it could be shown that N considerably persisted in the depot-zone as NH_4^+, which strongly induced depot-zone root growth. Placement of the NH_4^+-depot led to +7.4 % increase in grain yield of maize (2014) and +5.8% increase in maize silage yield (2015) in comparison to fertilizer broadcast. Placement of *Pseudomonas sp.* DSMZ 13134 inoculum in the sowing row let to +7.1% increase in yield of maize silage (2015) in comparison to the non-inoculated control.

In total, these results showed that precise placement of specific fertilizer formulations in combination with the application of selected PGPMs can lead to improved plant growth, improved N and P uptake with a potential to save resources.

2 Zusammenfassung

In landwirtschaftlichen Böden kommt es oft vor, dass die Konzentrationen von pflanzenverfügbarem Stickstoff (N) und Phosphor (P) für einen guten Ertrag nicht ausreichen, selbst bei hohen Konzentrationen vom Gesamt-N und -P. Im Vergleich zur üblichen breitflächigen Düngerausbringung auf der Bodenoberfläche (mit oder ohne anschließender Einarbeitung), ist die präzise Platzierung von Dünger möglichst nah an Saatgut oder Wurzel eine vielversprechende Alternative zur Erhöhung der Pflanzenverfügbarkeit von Düngemitteln. Eine gute Durchwurzelung um den Depotbereich ist allerdings eine Voraussetzung für die wirksame Platzierung von Dünger, mit dem Ziel die Nährstoffaufnahme zu verbessern und gleichzeitig günstige Bedingungen für wachstumsfördernden Bodenmikroorganismen zu schaffen.

In den letzten drei Jahrzehnten kamen zahlreiche Feldversuche zur Platzierung von Dünger im Vergleich zur breitflächige Ausbringung zu scheinbar unterschiedlichen Ergebnissen hinsichtlich Ertrag und Nährstoffaufnahme von Kulturpflanzen. Deshalb wurde zum Beginn, mit Studien die bestimmten Voraussetzungen erfüllten, eine Meta-Analyse durchgeführt,. Dabei wurde der Effekt der gezielten Platzierung von unterschiedlichen Dünger (zB. NH_4^+ oder $CO(NH_2)_2$ ohne oder mit wasserlöslichem Phosphor (HPO_4^{2-}; $H_2PO_4^-$); wasserlöslichem Kaliumr; Stallmist oder Gülle) in einen begrenzten Bereich auf der Bodenoberfläche oder im Unterboden im Vergleich zur breitflächigen Ausbringung auf Ertrag, Nährstoffgehalt und Nährstoffaufnahme bei verschiedenen Kulturpflanzen untersucht. Insgesamt wurden Ergebnissen aus vierzig Feldversuchen verwendet, die zwischen 1982 und 2015 stattfanden (85% der Versuche ab 2000). Mit Hilfe der Methode “Baseline contrasts”, haben wir unterschiedliche Behandlungen von Düngerplatzierung mit breitflächiger Ausbringung von Dünger als Kontrolle verglichen. Die Ergebnisse zeigen, dass Düngerplatzierung zu +3,7 % mehr Ertrag, +3,7% höherer Nährstoffkonzentration und +11,9% höherer Nährstoffgehalte in oberirdischer Biomasse als breitflächige Ausbringung führt. Die Tiefe der Platzierung hatte auch einen deutlichen Effekt (je tiefer, desto höherer der Platzierung-Effekt), ebenso die Kombination von Nährstoffen, mit höheren Erträgen bei der Platzierung eine Düngermischung aus Harnstoff und Phosphat (+27% Ertrag) oder Ammonium und Phosphat (+15% Ertrag) als bei der breitflächigen Ausbringung (Nkebiwe et al., 2016 a*: Field Crops Research* 196: 389–401).

Zunächst wurde der Effekt der Platzierung von Düngern in Kombination mit der Applikation von Bio-Effektoren (Pflanzenwachstums-stimulierende Mikroorganismen und natürliche, wirkaktive Stoffen wie Huminsäure und Algenextrakte) auf das Wurzelwachstum von Kulturpflanzen, die Etablierung der mikrobiellen Inokula in der Rhizosphere, Korn- und Biomassebildung, sowie Nährstoffinhalt bei Mais (*Zea mays* L) und Weizen (*Triticum aestivum* L) untersucht. Mit Hilfe von Topf- und Wurzelkastenversuchen habe ich festgestellt, dass ein konzentriertes Düngedepot aus NH_4^+ stabilisiert mit DMPP (der Nitrifikationshemmer 3,4-Dimethylpyrazolphosphat) zu einer starken Durchwurzelung und Erschließung des Düngerdepots führt. Dabei war es wichtig, dass die Stickstoffform im Depot hauptsächlich als wenig mobiles NH_4^+ besteht, sowohl um lokalisiertes Wurzelwachstum

anzuregen, als auch um günstige chemische und biologische Bedingungen für eine verbesserte Stickstoff- und Phosphataufnahme der Pflanzen zu ermöglichen. Durch *in vitro* Versuche auf feste und flüssige Kulturmedien konnte gezeigt werden, dass einige wachstumsfördernde Bakterien und Pilze in der Lage sind, schwerlösliches, unorganisches P durch Ansäuerung aufzulösen und dass sie Toleranz an hohen Konzentrationen von NH_4^+ und DMPP zeigen. Damit zeigen die wachstumsfördernde Mikroorganismen ein Potenzial den ammonium-reichen Boden um das Düngerdepot gut zu besiedeln (Nkebiwe et al., 2016 c: *Manuskript in eingereicht*). Durch eigene Topfversuche und vier betreute Bachelor- und Masterarbeit haben wir festgestellt, dass einige Trikalciumphosphat-lösende mikrobielle Bio-Effektoren das Rohphosphat- aneignungsvermögen von Mais und Sommerweizen verbessern wenn N als stabilisiertem Ammonium (NH_4^++DMPP) gedüngt ist. Dies galt besonders für bakterielle Bio-Effektoren wie *Pseudomonas sp.* DSMZ 13134 oder Kombi-Produkten aus Bakterien und Pilzen wie CombiFectorA. Dieser Effekt war deutlich in Substraten mit geringem Kalkgehalt bzw. mit geringer Sorptionsfähigkeit für Phosphat in Böden mit neutralem oder leicht erhöhtem pH-Werten. Mit CombiFectorA konnte die P-Aneignungsvermögen von Mais aus schwerlöslicher Klärschlammasche verbessert. Auf diese Weise konnte die Effizienz der schwerlöslichen Klärschlammasche als Recycling-Phosphatdünger erhöht werden. Die Verbesserung der Lösbarkeit von schwerlöslichem Phosphat durch Ansäuerung der Rhizosphäre und Ausscheidung von Chelaten wie auch die Verstärkung des Wurzelwachstums bei Mais und Sommerweizen waren mögliche Wirkmechanismen der erfolgreichsten mikrobiellen Bio-Effektoren (Nkebiwe et al., 2016 d: *Manuskript in Vorbereitung).*

Daneben wurden weitere Gewächshausversuche und zwei Feldversuche (Körnermais 2014 und Maissilage 2015) geplant, umgesetzt, ausgewertet und evaluiert. Ein peer-reviewed Paper wurde mit Ergebnissen aus diesen Versuchen publiziert (Nkebiwe et al., 2016 b: *Chemical and Biological Technologies in Agriculture* 3:15). Bei Gewächshausversuchen hat die Platzierung von stabilisiertem Ammoniumdepot zum verbesserten Wurzel- und Sproßwachstum und zu höheren Sproß-N und -P Gehalt geführt. Durch gute Erschließung des Ammoniumdepots mit Maiswurzeln wurde die Besiedlung des Inokulums *Pseudomonas sp.* DSMZ 13134 im Bereich des Düngerdepots verbessert. In den Feldversuchen konnte mehrere Wochen nach Platzierung des Düngerdepots (Ammonium+DMPP) nachgewiesen werden, dass Stickstoff hauptsächlich als NH_4^+ in dem Depotbereich verblieb, was zu verstärktem Wurzelwachstum im Depotbereich führte. Durch Platzierung von Ammoniumdepot konnte ein Ertrag von +7,4% für Körnermais (2014) und +5,8% für Maissilage (2015) in Vergleich zu breitflächiger Ausbringung erzielt werden. Auch durch die Platzierung von *Pseudomonas sp.* DSMZ 13134 unter der Saatreihe wurde +7,1% mehr Maissilage (2015) im Vergleich zur Kontrolle ohne Inokulum festgestellt.

Insgesamt zeigen die Ergebnisse, dass die präzise Platzierung von speziellen Düngemitteln und die Applikation von wachstumsfördernde Mikroorganismen zu verbessertem Pflanzenwachstum, verbesserter N und P Aufnahme und höherer Ertrag bei gleichzeitiger Ressourcensparung führen.

3 General introduction

3.1 Phosphate fertilizers for agricultural productivity

Phosphorus (P) is a major element present in different cell organelles (e.g. phospholipid plasma membranes); macromolecules (e.g. DNA and RNA); and energy transfer compounds (e.g. ATP, UTP, GTP, and the phosphate esters glucose 6-phoshate and phosphoglyceraldehyde) involved in vital processes such as nutrient transport, respiration, photosynthesis, starch and cellulose synthesis (*Hawkesford* et al. 2012). Given the importance of P to most biological processes, with increasing P supply, organisms tend to store P for later use as orthophosphates (HPO_4^{2-}/$H_2PO_4^-$) in cell vacuoles, as polyphosphates for bacteria, fungi and lower plants, and as phytate in roots, tubers, pollen, grain and seeds for higher plants (*Hawkesford* et al. 2012; *White* 2012).

In many agricultural systems, phosphate (PO_4^{3-}) (taken up by plants in the form HPO_4^{2-}/$H_2PO_4^-$) is frequently the most limiting among macronutrients for optimal crop productivity (*Grant* et al. 2001; *Hinsinger* 2001). Since the early history of agriculture, P-rich farm inputs like manure and more recently guano, bone meal, rock phosphate (RP) and phosphate-rich industrial by-products have been important P fertilizers for crop production. Today, manures and RP are still valuable P fertilizers especially in organic farming systems whereas soluble mineral PO_4^{3-} fertilizers have gained significant importance especially in intensive farming systems.

Only a small fraction of soluble PO_4^{3-} fertilizer that is applied to soil becomes available to plants over time. This is because soluble PO_4^{3-} becomes converted to less soluble and less available P-forms by fixation processes such as precipitation and adsorption reactions in soil, depending on soil pH, concentrations of reactive Ca^{2+}, Fe^{3+} and Al^{3+}, and concentrations and types of clays and organic matter present (*Hansen* et al. 2002). The fraction of applied soluble

PO_4^{3-} that is immediately plant-available to the total amount of soluble PO_4^{3-} applied as fertilizer in soil could be as high as 1:3 or less than 1:20, depending on fertilizer application rate, soil P-sorption properties and duration since fertilizer application (*Hansen* et al. 2002). In four long term studies for example, measured soluble plant-available PO_4^{3-} was <5% of total soil P for sites with no P fertilization and 6 - 87% for sites regularly fertilized with soluble PO_4^{3-} fertilizers or manure, depending on fertilization rates, soil type and texture (*Hansen* et al. 2002).

Soluble mineral PO_4^{3-} fertilizers are mostly prepared by acid-digestion of non-renewable RP mined from a few major global reserves. Known world RP reserves are disproportionately located in a few countries (72 % in Morocco and Western Sahara), among which the rate of mine production of RP is also disproportionately allocated (45% 2015 global mine production by China) (*USGS*, 2016). According to current estimates (mine production rate 218-223 Pg a^{-1} from 2014-2015 and total world reserves 69,000 Pg, 2016), global RP reserves may become depleted in about 300 years or earlier given that mine production rate is projected to increase (*USGS*, 2016). The need for agricultural products will continue to grow because of a growing global population (*Tilman* et al. 2002). Given depleting global P reserves, soluble PO_4^{3-} fertilizers required for crop production will certainly become more expensive. Resource scarcity for food production may also give way to future geo-political tensions. In this light, the challenge to adequately supply agricultural crops with PO_4^{3-} may only grow in its severity. Therefore, every promising strategy to improve PO_4^{3-} availability in crop production is worth close consideration alone and in combination with other compatible strategies.

An important approach to resolve the PO_4^{3-} insufficiency problem is using soil P reserves and P-fertilizers efficiently by adopting measures that increase the uptake-efficiency of P-fertilizers by crop plants. In the tropics and subtropics, the quantity of P apparently recovered in harvested crops during the first year of application is only about 10% the amount of soluble

PO_4^{3-} fertilizer applied (*Baligar* et al. 2001; *Raun* and *Johnson* 1999). For soluble PO_4^{3-} fertilizers, P-uptake efficiency can be considerable increased by adopting fertilizer application techniques that enhance plant P-acquisition. Through precise placement of mineral or organic phosphate fertilizers close to seeds, growth and P status of crop plants can be considerably enhanced, especially during critical early growth stages (*Grant* et al. 2001; *Bittman* et al. 2012). Nevertheless, several published field studies comparing fertilizer placement to fertilizer broadcast in the last three decades arrived at different and often conflicting results regarding their effects on yield and nutrient acquisition of field crops. This meant a knowledge gap existed regarding the effect of fertilizer placement on crop nutrition and yield in comparison to conventional fertilizer application by broadcast. Furthermore, requirements for effective fertilizer placement were still unknown.

When a complete dose of P- and N-fertilizer is placed in subsurface soil at a high rate, the point of placement is usually farther away from seeds than starter-fertilizer to avoid seed injury. In such cases, P and N uptake efficiency of the placed fertilizer depends on considerable root growth in and around the subsurface fertilizer patch or depot. It has been repeatedly shown that uptake of placed PO_4^{3-} fertilizers by crop plants can be significantly improved when a PO_4^{3-} fertilizer is placed in combination with an NH_4^+ fertilizer, likely due to a stronger localized root-growth response of NH_4^+ at the site of contact with roots than PO_4^{3-} and due to the pH-decreasing effect of NH_4^+ nutrition (*Lynch* et al. 2012; *Jing* et al. 2012; *Miller* and *Ohlrogge* 1958). Furthermore, when less expensive but less soluble P fertilizers are added to biomass during composting, their solubility and plant uptake-efficiency can be considerably enhanced (*Biswas* and *Narayanasamy* 2006; *Bustamante* et al. 2016; *Moharana* and *Biswas* 2016).

Plant P-acquisition from sparingly available P resources in soil and from P applied via fertilizer can be improved by measures that enhance P mobilization via enhancing

mineralization and solubilization of organic and inorganic P pools respectively. Plant P-acquisition from sparingly available organic and inorganic soil pools can be improved by application of bio-effectors (*Weinmann* and *Römheld* 2012). The term bio-effector describes viable plant growth-promoting microorganisms (PGPMs) such as phosphate solubilizing bacteria (PSB), mycorrhiza-helper bacteria (MHB), IAA- or ACC-deaminase-producing bacteria that improve plant growth via phytohormone signaling, and active natural substances such as humic acids and seaweed extracts that promote growth and nutrient acquisition of crops plants via several interacting mechanisms (*Altomare* et al. 1999; *Lugtenberg* and *Kamilova* 2009; *Richardson* et al. 2009; *Jiang* et al. 2012; *Zahir* et al. 2009; *Glick* 2005; *Sharma* et al. 2012). A major problem with the application foreign microorganisms in soil to promote plant growth is that their populations in soil rapidly decline as a consequence of adverse prevailing biotic and/or abiotic conditions (*van Veen et al.*, 1997). Among others, competition for nutrients is a key cause for declining populations of soil-inoculated PGPMs. If PGPMs are inoculated in densely-rooted soil, their established may be promoted by the presence of high concentrations of nutrients exuded by roots.

Furthermore, soil P turnover and plant-availability can also be strongly influenced by farm management practices such as tillage (aeration and improved soil microbial activity, incorporation of P stratified on the soil surface from broadcast fertilization), application of soil amendments like organic matter and lime (modification of soil texture and pH), cropping systems and crop rotations (*Fink* et al. 2016; *Kleinman* et al. 2015; *Redel* et al., 2007).

A complementary approach to resolve the increasingly difficult challenge of inadequate PO_4^{3-} supply to crops is to develop a more closed flow cycle of P resources by reducing losses from "farm to fork" and by increasing the value of recycling. However, P-rich materials from waste recycling or by-products from industrial processes can gain value as PO_4^{3-} fertilizers if their plant-availability and therefore efficiency can be considerable improved and if their

concentrations of impurities can be kept below the accepted threshold. Unlike manure and composts containing large pools of organically bound P that can be mineralized over the course of a few years, recycled P fertilizers like ashes from incineration of sewage sludge; P-enriched slags as by-products from the metallurgical industry (e.g. Thomas phosphate); and products from pyrolysis of biomass (e.g. biochars) are generally not soluble and only sparingly plant-available in neutral to slightly alkaline soils. On the contrary, a recycled P fertilizer like struvite ($NH_4MgPO_4 \cdot 6H_2O$) - crystalized out of solution during sewage treatment - is also sparingly soluble in water, however, more available than the ashes described above because it readily releases PO_4^{3-} into soil solution to equilibrate for P taken up by roots, thus performing as a valuable slow-release N-P-Mg fertilizer (*Ryu* et al. 2011; *Talboys* et al. 2016; *Vaneeckhaute* et al. 2015). To reduce loss of P from fertilizers to surface water bodies via runoffs (particulate P, dissolved PO_4^{3-} and suspending colloidal-dissolved organic PO_4^{3-}), subsurface placement of PO_4^{3-} fertilizers and manures is a means to overcome P stratification an the soil surface, which is associated with high risk for runoff losses (*Kleinman* et al. 2015; *Sharpley* and *Jarvie* 2012).

3.2 Placement of fertilizers and bio-effector as options to improve plant-P acquisition

A combination of improved mobilization of sparingly soluble P-forms in the rhizosphere and increased localize root growth been presented as an approach to improve plant uptake of P from labile and moderately labile pools present in soil as well as from applied fertilizers. Chemical mobilization of P (and other elements like Fe and Zn) can be improved by root exudation of protons, which acidify the rhizosphere especially by plants under ammonium nutrition (*Marschner* et al., 1986; *Marschner* et al., 1989; *Neumann* and *Römheld*, 2007). Furthermore, roots are capable of secreting metal-complexing compounds like carboxylates, which improve P solubility and regulate the concentration of phosphate-immobilizing cations like Ca^{2+}, Fe^{3+} and Al^{3+} in the rhizosphere (*Lipton* et al., 1987; *Neumann* and *Römheld* 2007).

Similarly, plant growth-promoting rhizobacteria (PGPR) (*Vassilev* et al., 2006; *Rodríguez* et al., 2006; *Lugtenberg* and *Kamilova*, 2009) and fungi (*Altomare* et al., 1999) may secrete organic acids for P mobilization. Plant growth-promoting microorganisms (PGPM) may enhance mycorrhization (*Richardson* et al., 2009) and secrete root-growth-stimulating phytohormones (*Jiang* et al., 2012) as modes of action to improve spatial P acquisition. Root growth, especially fine roots (< 2 mm ø) and root hairs, determines the capacity of plants to take up mineral nutrients and water (*Jackson* et al., 1997). However, the full potential of active mobilization of P and other less mobile nutrients by plant roots may only be exploited if those nutrients are present in the rhizosphere in considerable amounts. Placement of root-attracting fertilizers at high concentrations below the soil surface with a subsequent exploration of the fertilizer spots by plant roots may, thus, be an effective option.

In natural ecosystems, nutrients are unevenly distributed in soil and plants optimize nutrient acquisition by maximizing uptake from nutrient-rich sites whereas efforts to acquire nutrients from poor soil zones are kept low (*Hutchings* and *John*, 2004). In agro-ecosystems, fertilizer placement creates strong heterogeneity of nutrient concentrations in soil. Under such conditions, the gradients of nutrient concentrations between a nutrient depot and the surrounding soil is influenced by the method of fertilizer application, fertilization history, mobility of the nutrient in soil, and biotic and abiotic processes that transform and mobilize soil nutrients. Therefore, root and shoot growth response of plants to fertilizer placement is proposed to be stronger on soils with suboptimal fertility than on nutrient-rich ones (*Randall* and *Hoeft*, 1988; *Buah* et al., 2000; *Borges* and *Mallarino*, 2001; *Grant* et al., 2001). Plant response to placed nutrients has been shown to be strong when a considerable proportion of the total nutrient requirement of the crop plant is supplied within the subsurface nutrient depot (*Hodge*, 2004).

Below ground PO_4^{3-} patches have been shown to induce root growth towards the patch in crops such as canola, wheat (*Rose* et al., 2009), and maize (*Chassot* et al., 2001; *Qin* et al., 2005). However, plants show a stronger root-growth response within or close to nutrient patches containing soluble N fertilizers (especially NH_4^+) in comparison to soluble PO_4^{3-} fertilizers (*Lynch* et al., 2012). Furthermore, root growth towards nutrient patches is stronger for NH_4^+ than for NO_3^- or $CO(NH_2)_2$ at the area of contact with the nutrient patch (*Anghinoni* and *Barber*, 1990; *Jing* et al., 2010, 2012). Under certain abiotic stresses, uptake of NH_4^+ can be advantageous to plants compared to uptake of NO_3^-. An example is early spring in temperate regions characterized by low temperatures, reduced organic matter mineralization, slowed root growth caused by low temperature-impaired root cell wall extensibility (*Kristoffersen* et al., 2005; *Lynch* et al., 2012) as well as low light and low root carbohydrate supply. Under such suboptimal conditions, several crops preferentially take up NH_4^+ instead of NO_3^-, thus, utilizing ATP required for N assimilation more efficiently (*Covey-Crump* et al., 2002; *Tischner*, 2000; *White*, 2012).

The ability of plants to efficiently exploit placed subsurface fertilizer depots containing root-growth stimulating nutrients like NH_4^+ and PO_4^{3-} is also influenced by plant species characteristics. Plant species predisposed with high root turnover rates may be more efficient utilizers of placed nutrients that have low mobility in soil (e.g. NH_4^+ and PO_4^{3-}) because with increasing root growth the depletion zones of individual roots rapidly overlap in a nutrient patch with high root density (*Marschner* and *Rengel*, 2012). Placed NH_4^+ and/or PO_4^{3-} function as chemo-attractants of roots and as plant macronutrients taken in substantial quantities (*Hawkesford* et al., 2012; *Jing* et al., 2012; *Lynch* et al., 2012). During early plant growth stages, when P availability is crucial for plant development (*Grant* et al., 2001), placement of a PO_4^{3-} fertilizer in a small soil volume close to a growing seedling is an effective method to ensure adequate P supply to young plants. In field-grown maize (*Zea*

mays L.), for example, placement of PO_4^{3-} fertilizer (100 kg P ha^{-1}) in 3 – 6 % of the total soil volume in the row (0.28 m^3 assuming 2 rows $plot^{-1}$, 75 cm inter-row distance, 250 cm row length and 15 cm plow layer) was associated with higher shoot P concentration and shoot P content than placement in 12 – 25% of total row soil volume during early growth stages (*Lu* and *Miller*, 1993).

3.3 Fertilizer placement in potted soil compared to field soil

The enormous difference in the total volume of soil that can be rooted under field conditions in comparison to the volume in pots under controlled greenhouse/climate chamber conditions makes it inappropriate to directly compare methods to apply fertilizers in field soil to those in potted soil. In this light, homogenous mixture of a fertilizer in a potted substrate may be commensurate to placement in a similar restricted soil volume during early growth stages under field conditions when root growth is still limited. This assumption is relevant to the pot experiments described in sections 5, 6 and 7. Given that PO_4^{3-} and NH_4^+ have very low effective diffusion coefficients in soil compared to other macronutrient ions or molecules, and because they readily bind to charged surfaces in soil (*Barber* 1984; *Clarke* and *Barley* 1968; *Neumann* and *Römheld* 2012; *Pang* et al. 1973; *Schenk* and *Barber* 1979), their uptake by plants mostly depends on root interception (chemotropism) and diffusion rather than on mass flow. Therefore, in pots in which total potential rooting soil volume is very restricted, it may be expected that the higher the proportion of total soil volume that is fertilized with poorly mobile PO_4^{3-} and/or NH_4^+, the higher the quantity of fertilizer P or N that is taken up (*Anghinoni* and *Barber* 1988). Conversely, under field conditions where total potential rooting soil volume is more or less limitless, placement of a fertilizer in a restricted soil volume close to seeds or plant roots (5-10 cm) leads to higher nutrient uptake especially during early growth stages than homogenous broadcast and incorporation (*Nkebiwe* et al. 2016). Homogenous broadcast and incorporation of P-fertilizers increase contact with PO_4^{3-} -

fixing cations (Ca^{2+}, Fe^{3+}, Al^{3+}) (*Grant* et al. 2001) and enhances immobilization by soil microorganisms- even though the latter functions as an important sink and source for soil P (*Gichangi* et al. 2009; *Bünemann* et al., 2011). In contrast, placement of a PO_4^{3-}-fertilizer in a restricted soil volume reduces P fixation and immobilization. Nevertheless, for PO_4^{3-} that is extremely immobile in soil and for sparingly-soluble inorganic P-fertilizers (e.g. rock phosphates and apatite-rich incineration ashes from recycled wastes), placement in a concentrated point in soil may limit P uptake when optimum root density and maximum root influx rate is reached. Therefore, to increase the concentration of $H_2PO_4^-/HPO_4^{2-}$ in soil solution around the root system of target crop plants under field conditions, instead of broadcast and incorporation in the entire plough layer or point placement at a high concentration in a very restricted soil volume distant from seeds (to avoid seed injury), P-fertilizers may be placed by banding on the sowing row followed by incorporation as shown by (*Lu* and *Miller* 1993) where PO_4^{3-}-fertilizer was placed only in 3-6% of total soil volume in the plough layer for in maize (*Zea mays* L.) cultures. For soils with high P sorption capacity, P uptake by maize was high when the fraction of fertilized soil volume was low (*Anghinoni* and *Barber* 1980). Nevertheless, at very low fertilization rates, it is a standard farmer's practice in many regions of Europe and North America to place a soluble PO_4^{3-} and NH_4^+-fertilizer such as diammonium phosphate as a concentrated point close to seeds to improve early season P-availability without causing injury to seeds.

3.4 Aims and objectives

The main aim of this Ph.D. research was to investigate the potential for improving crop nutrient-acquisition and yield by fertilizer placement in combination with the application of bio-effectors (BEs). Maize (*Zea mays* L) and wheat (*Triticum aestivum* L.) were chosen as test crops for this research because they are important crop species in Europea as well as in

many other parts of the world. This dissertation is structured in the following order of objectives:

1. To cover the knowledge gap that exist regarding the overall effect of fertilizer placement in comparison to conventional fertilizer application by broadcast on crop nutrient acquisition and yield by conducting a comprehensive meta-analysis on field studies published in accepted peer-reviewed journals in the last three decades.
2. To explore how subsurface fertilizer placement could be used as a tool to spatially control the rhizosphere by stimulating the development of zones of intense root-growth around fertilizer depots. These "rhizosphere hotspots" are proposed as soil areas with a high potential for effective colonization by inoculated microbial BEs.
3. To investigate the ability of selected microbial BEs to solubilize different sparingly-soluble inorganic P-forms *in vitro*, as an indicator of their potential to improve plant P-acquisition from sparingly available soil P pools or from applied sparingly soluble P-fertilizers under greenhouse and field conditions.
4. To investigate the potential of microbial BEs to establish in rhizosphere hotspots by studying their survival and growth characteristics firstly *in vitro* under extreme concentrations of fertilizer ions, pH and salinity - simulating chemical conditions within and around a rich subsurface fertilizer depot - and secondly in pot-grown plants.

3.5 References

Altomare, C., Norvell, W. A., Björkman, T., Harman, G. E. (1999): Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Appl. Environ. Microbiol.* 65, 2926–2933.

Anghinoni, I., Barber, S. A. (1980): Predicting the most efficient phosphorus placement for corn. *Soil Sci. Soc. Am. J.* 44, 1016–1020.

Anghinoni, I., Barber, S. A. (1988): Corn Root Growth and Nitrogen Uptake as Affected by Ammonium Placement. *Agron. J.* 80, 799–802.

Anghinoni, I., Barber, S. A. (1990): Predicting the effect of ammonium placement on nitrogen uptake by corn. *Agron. J.* 82, 135–138.

Baligar, V. C., Fageria, N. K., He, Z. L. (2001): Nutrient use efficiency in plants. *Commun. Soil Sci. Plant Anal.* 32, 921–950.

Barber, S. A. (1984): Soil nutrient bioavailability. A mechanistic approach. Wiley, New York

Biswas, D. R., Narayanasamy, G. (2006): Rock phosphate enriched compost: An approach to improve low-grade Indian rock phosphate. *Bioresource Technol.* 97, 2243–2251.

Bittman, S., Liu, A., Hunt, D., Forge, T., Kowalenko, C., Chantigny, M., Buckley, K. (2012): Precision placement of separated dairy sludge improves early phosphorus nutrition and growth in corn (L.). *.). J. Environ. Qual.* 41, 582–591.

Borges, R., Mallarino, A. P. (2001): Deep banding phosphorus and potassium fertilizers for corn managed with ridge tillage. *Soil Sci. Soc. Am. J.* 65, 376–384.

Buah, S., Polito, T. A., Killorn, R. (2000): No-tillage corn response to placement of fertilizer nitrogen, phosphorus, and potassium. *Commun. Soil Sci. Plant Anal.* 31, 3121–3133.

Bünemann, E. K, Prusisz, B,. Ehlers, K. (2011): Characterization of phosphorous forms in soil microorganisms, in Bünemann, E. K., Oberson, A., Frossard, E.: Phosphorous in action, Biological processes in soil phosphorous cycling. Springer-Verlag Berlin Heidelberg. pp. 37–58.

Bustamante, M. A., Ceglie, F. G., Aly, A., Mihreteab, H. T., Ciaccia, C., Tittarelli, F. (2016): Phosphorus availability from rock phosphate: Combined effect of green waste composting and sulfur addition. *J. Environ. Manage.* 182, 557–563.

Chassot, A., Stamp, P., Richner, W. (2001): Root distribution and morphology of maize seedlings as affected by tillage and fertilizer placement. *Plant Soil* 231, 123–135.

Clarke, A. L., Barley, K. P. (1968): The uptake of nitrogen from soils in relation to solute diffusion. *Aust. J. Soil Res.* 6, 75–92.

Covey-Crump, E. M., Attwood, R. G., Atkin, O. K. (2002): Regulation of root respiration in two species of Plantago that differ in relative growth rate: the effect of short- and long-term changes in temperature. *Plant Cell Environ.* 25, 1501–1513.

Fink, J. R., Inda, A. V., Tiecher, T., Barrón, V. (2016): Iron oxides and organic matter on soil phosphorus availability. *Ciênc. agrotec.* 40, 369–379.

Gichangi, E. M., Mnkeni, P. N. S., Brookes, P. C. (2009): Effects of goat manure and inorganic phosphate addition on soil inorganic and microbial biomass phosphorus fractions under laboratory incubation conditions. *Soil Sci. Plant Nutr.* 55, 764–771.

Glick, B. R. (2005): Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiol. Lett.* 251, 1–7.

Grant, C. A., Flaten, D. N., Tomasiewicz, D. J., Sheppard, S. C. (2001): The importance of early season phosphorus nutrition. *Can. J. Plant Sci.* 81, 211–224.

Hansen, N. C., Daniel, T. C., Sharpley, A. N., Lemunyon, J. L. (2002): The fate and transport of phosphorus in agricultural systems. *J.Soil Water Conserv.* 57, 408–417.

Hawkesford, M., Horst, W., Kichey, T., Lambers, H., Schjoerring, J., Møller, I. S., White, P. (2012): Functions of macronutrients, in Marschner P.: Marschner's Mineral Nutrition of Higher Plants, Third edition, Academic Press, Elsevier, Amsterdam, pp. 135–178.

Hinsinger, P. (2001): Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant Soil* 237, 173-195.

Hodge, A. (2004): The plastic plant: root responses to heterogeneous supplies of nutrients. *New Phytol.* 162, 9–24.

Hutchings, M. J., John, E. A. (2004): The effects of environmental heterogeneity on root growth and root/shoot partitioning. *Anna. Bot.-London* 94, 1–8.

Jackson, R. B., Mooney, H. A., Schulze, E.-D. (1997): A global budget for fine root biomass, surface area, and nutrient contents. *Proc. Natl. Acad. Sci. (USA)* 94, 7362–7366.

Jiang, Y., Wu, Y., Xu, W., Cheng, Y., Chen, J., Xu, L., Hu, F., Li, H. (2012): IAA-producing bacteria and bacterial-feeding nematodes promote Arabidopsis thaliana root growth in natural soil. *Eur. J. Soil Biol.* 52, 20–26.

Jing, J., Rui, Y., Zhang, F., Rengel, Z., Shen, J. (2010): Localized application of phosphorus and ammonium improves growth of maize seedlings by stimulating root proliferation and rhizosphere acidification. *Field Crop. Res.* 119, 355–364.

Jing, J., Zhang, F., Rengel, Z., Shen, J. (2012): Localized fertilization with P plus N elicits an ammonium-dependent enhancement of maize root growth and nutrient uptake. *Field Crop. Res.* 133, 176–185.

Kleinman, P. J. A., Sharpley, A. N., Withers, P. J. A., Bergström, L., Johnson, L. T., Doody, D. G. (2015): Implementing agricultural phosphorus science and management to combat eutrophication. *AMBIO* 44, 297–310.

Kristoffersen, A. Ø., Riley, H., Sogn, T. A. (2005): Effects of p fertilizer placement and temperature on root hair formation, shoot growth and p content of barley grown on soils with varying p status. *Nutr. Cycl. Agroecosys.* 73, 147–159.

Lipton, D. S., Blanchar, R. W., Blevins, D. G. (1987): Citrate, malate, and succinate concentration in exudates from P-sufficient and P-stressed *Medicago sativa* L. seedlings. *Plant Physiol.* 85, 315–317.

Lu, S., Miller, M. H. (1993): Determination of the most efficient phosphorus placement for field-grown maize (Zea mays L.) in early growth stages. *Can. J. Soil. Sci.* 73, 349–358.

Lugtenberg, B., Kamilova, F. (2009): Plant-Growth-Promoting Rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556.

Lynch, J., Marschner, P., Rengel, Z. (2012): Effect of internal and external factors on root growth and development, in Marschner P.: Marschner's Mineral Nutrition of Higher Plants, Third edition, Academic Press, Elsevier, Amsterdam, pp.331–346.

Marschner, H., Römheld, V., Horst, W. J., Martin, P. (1986): Root-induced changes in the rhizosphere: Importance for the mineral nutrition of plants. *Z. Pflanzenernaehr. Bodenk.* 149, 441–456.

Marschner, H., Treeby, M., Römheld, V. (1989): Role of root-induced changes in the rhizosphere for iron acquisition in higher plants. *Z. Pflanzenernaehr. Bodenk.* 152, 197–204.

Marschner, P., Rengel, Z. (2012): Nutrient availability in soils, in Marschner P.: Marschner's Mineral Nutrition of Higher Plants, Third edition, Academic Press, Elsevier, Amsterdam, pp. 315–330.

Miller, M. H., Ohlrogge, A. J. (1958): Principles of nutrient uptake from fertilizer bands 1. Effect of placement of nitrogen fertilizer on the uptake of band-placed phosphorus at different soil phosphorus levels1. *Agron. J.* 50, 95–97.

Moharana, P. C., Biswas, D. R. (2016): Assessment of maturity indices of rock phosphate enriched composts using variable crop residues. *Bioresource Technol.* 222, 1–13.

Neumann, G., Römheld, V. (2007): The Release of Root Exudates as Affected by the Plant Physiological Status, in Pinton, R., Varanini, Z.; Nannipieri, P.: The Rhizosphere. Biochemistry and organic substances at the soil-plant interface, Ed. 2. CRC Press, Boca Raton, Florida pp. 23–72.

Neumann, G., Römheld, V. (2012): Rhizosphere Chemistry in Relation to Plant Nutrition, in Marschner, P.: Marschner's Mineral Nutrition of Higher Plants. 3rd ed. Academic Pr, United States of America, pp. 347–360.

Nkebiwe, P. M., Weinmann, M., Bar-Tal, A., Müller, T. (2016): Fertilizer placement to improve crop nutrient acquisition and yield: A review and meta-analysis. *Field Crop. Res.* 196, 389–401.

Pang, P. C., Hedlin, R. A., Cho, C. M. (1973): Transformation and movement of band-applied urea, ammonium sulfate, and ammonium hydroxide during incubation in several manitoba soils. *Can. J. Soil. Sci.* 53, 331–341.

Qin, R., Stamp, P., Richner, W. (2005): Impact of tillage and banded starter fertilizer on maize root growth in the top 25 centimeters of the soil. *Agron. J.* 97, 674–683.

Randall, G. W., Hoeft, R. G. (1988): Placement methods for improved efficiency of P and K fertilizers: A Review. *J. Prod. Agr.* 1, 70–79.

Raun, W. R., Johnson, G. V. (1999): Improving nitrogen use efficiency for cereal production. *Agron. J.* 91, 357–363.

Redel, Y. D., Rubio, R., Rouanet, J. L., Borie, F. (2007): Phosphorus bioavailability affected by tillage and crop rotation on a chilean volcanic derived ultisol. *Geoderma* 139, 388–396.

Richardson, A. E., Barea, J.-M., McNeill, A. M., Prigent-Combaret, C. (2009): Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant Soil* 321, 305–339.

Rodríguez, H., Fraga, R., Gonzalez, T., Bashan, Y. (2006): Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. *Plant Soil* 287, 15–21.

Rose, T. J., Rengel, Z., Ma, Q. F., Bowden, J. W. (2009): Crop species differ in root plasticity response to localised P supply. *J. Plant Nutrit. Soil Sci.* 172, 360–368.

Ryu, H.-D., Lim, C.-S., Kim, Y.-K., Kim, K.-Y., Lee, S.-I. (2011): Recovery of struvite obtained from semiconductor wastewater and reuse as a slow-release fertilizer. *Environ. Eng. Sci.* 29, 540–548.

Schenk, M. K., Barber, S. A. (1979): Phosphate uptake by corn as affected by soil characteristics and root morphology1. *Soil Sci. Soc. Am. J.* 43, 880–883.

Sharma, S. H. S., Lyons, G., McRoberts, C., McCall, D., Carmichael, E., Andrews, F., Swan, R., McCormack, R., Mellon, R. (2012): Biostimulant activity of brown seaweed species from Strangford Lough: compositional analyses of polysaccharides and bioassay of extracts using mung bean (Vigno mungo L.) and pak choi (Brassica rapa chinensis L.). *J. Appl. Phycol.* 24, 1081–1091.

Sharpley, A. N., Jarvie, H. P. (2012): Agricultural management, water quality & ecology: Putting practice into policy, in National Agricultural Biotechnology Council Conference Proceedings, pp. 87–116.

Talboys, P. J., Heppell, J., Roose, T., Healey, J. R., Jones, D. L., Withers, P. J. A. (2016): Struvite: a slow-release fertiliser for sustainable phosphorus management? *Plant Soil* 401, 109–123.

Tilman, D., Cassman, K. G., Matson, P. A., Naylor, R., Polasky, S. (2002): Agricultural sustainability and intensive production practices. *Nature* 418, 671–677.

Tischner, R. (2000): Nitrate uptake and reduction in higher and lower plants. *Plant Cell and Environ.* 23, 1005–1024.

USGS, (2016): Phosphate rock, Annual publication of mineral commodity summerises by The United States Geological Survey (USGS), www.usgs.gov. Downloaded from http://minerals.usgs.gov/minerals/pubs/commodity/phosphate_rock/mcs-2016-phosp.pdf on 18.10.2016

Vaneeckhaute, C., Janda, J., Meers, E., Tack, F. M. G. (2015): Efficiency of soil and fertilizer phosphorus use in time: a comparison between recovered struvite, FePO4-Sludge, digestate, animal manure, and synthetic fertilizer, in Rakshit, A., Singh, H. B., Sen, A.: Nutrient Use Efficiency: from Basics to Advances. Springer India, New Delhi, pp. 73–85.

van Veen, J. A., van Overbeek, L. S., van Elsas, J. D. (1997): Fate and activity of microorganisms introduced into soil. *Microbiol. Mol. Biol. R.* 61, 121–135.

Vassilev, N., Vassileva, M., Nikolaeva, I. (2006): Simultaneous P-solubilizing and biocontrol activity of microorganisms: potentials and future trends. *Appl. Microbiol. Biot.* 71, 137–144.

Weinmann, M., Römheld, V.: Resource Preservation by Application of BIOefFECTORs in European Crop Production (Research proposal). European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 312117 (BIOFECTOR).

White, P. J. (2012): Long-distance transport in the xylem and phloem, in Marschner, P.: Marschner's Mineral Nutrition of Higher Plants. 3rd ed. Academic Pr, United States of America, pp. 49–70.

Zahir, Z. A., Ghani, U., Naveed, M., Nadeem, S. M., Asghar, H. N. (2009): Comparative effectiveness of *Pseudomonas* and *Serratia sp*. containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.) under salt-stressed conditions. *Arch. Microbiol. (Archives of Microbiology)* 191, 415–424.

4 Fertilizer placement to improve crop nutrient acquisition and yield: A review and meta-analysis

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4.1 Abstract

In agricultural soils, plant-available nitrogen (N) and phosphorous (P) may be inadequate for crop production although total N and P concentrations are high. Therefore, N and/or P fertilizer is commonly applied to field soil by broadcast, even though broadcast does not ensure that a considerable proportion of applied fertilizer is available at the right time and place for optimal root uptake. Fertilizer placement in soil, which refers to precise application of specific fertilizer formulations close to seeds or plant roots to ensure high nutrient availability, may be a more effective alternative to broadcast application. The objectives of this paper are: (1) to summarize current techniques for fertilizer placement in soil and to identify fertilizers that are suitable for subsurface placement; and (2) to quantify the relative effects of fertilizer placement to fertilizer broadcast on crop nutrient acquisition and yield. To achieve these aims, we reviewed scientific literature on the dynamics of nutrient movement from soil into roots and studies on fertilizer placement under field conditions. Additionally, we performed three meta-analyses according to the method of *baseline contrast*s on the relative effects of fertilizer placement (Treatment) to fertilizer broadcast (Control) on yield, nutrient concentration and content in above-ground plant parts. In all, we used 1022 datasets from 40 field studies published from 1982 to 2015 (85 % of studies from 2000). Results showed that overall, fertilizer placement led to 3.7 % higher yield, 3.7 % higher nutrient concentration and 11.9 % higher nutrient content in above-ground parts than fertilizer broadcast. For $CO(NH_2)_2$ and soluble phosphate (PO_4^{3-}), NH_4^+ and PO_4^{3-}, $CO(NH_2)_2$, NH_4^+, and PO_4^{3-}, fertilizer placement led to 27.3 %, 14.7 %, 11.6 %, 3.8 % and 0.0% increase in yield in comparison to broadcast respectively. Increase in relative yield and relative nutrient uptake from subsurface placed $CO(NH_2)_2$, $CO(NH_2)_2$ and PO_4^{3-}, NH_4^+, NH_4^+ and PO_4^{3-} or K^+ tend to increase with increasing placement depth to more than 10 cm. Results show that deep subsurface placed NH_4^+ ($\pm$ PO_4^{3-}) or $CO(NH_2)_2$ ($\pm PO_4^{3-}$), K^+, solid or liquid manure is more effective to improve deep rooting, nutrient uptake and yield than broadcast. Thus, deep subsurface fertilizer placement could be one more tool to mitigate negative consequences of increasingly frequent high temperatures and drought that threaten food production globally.

Keywords: fertilizer placement; meta-analysis; yield; nutrient mobilization; nutrient uptake

4.2 Introduction

Intensive agriculture, which requires substantial amounts of soluble fertilizers and other inputs, has considerably increased global food production (*Matson* et al., 1997; *Tilman* et al., 2002). However, it has also increased the risk of negative consequences on ecosystems, climate and public health (*Delgado* and *Scalenghe*, 2008; *Matson* et al., 1997; *Tilman* et al., 2002; *Tscharntke* et al, 2012). High energy costs for ammonia synthesis (*Michalsky* and *Pfromm*, 2012) and increasing scarcity in quality and quantity of rock phosphate reserves (*Cordell* et al, 2009) suggest that synthetic mineral N and P fertilizers, and agricultural goods produced using them will become more expensive.

Overuse of soluble fertilizers in intensive production systems is often associated with low crop nutrient use efficiency (*Fan* et al., 2012). In the tropics and sub-tropics, apparent nutrient recovery efficiency (ANR) of applied mineral N, P and K within the first year of application is estimated to be less than 50%, 10% and 40% respectively (*Baligar* and *Bennett*, 1986; *Baligar* et al, 2001; *Raun* and *Johnson*, 1999). Excess fertilizer nutrients that cannot be contained in the soil matrix or in soil microbial biomass may be released to the atmosphere (e.g. NH_3, N_2O, NO_x and N_2) and to surface and/or below-ground water bodies (e.g. NO_3^-, $HPO_4^{2-}/H_2PO_4^-$) (*Fan* et al., 2012; *Tunney* et al., 1997; *Weaver* et al. 1988).

Compared to other macronutrients, plant-available P is frequently the prime limiting nutrient for plant growth in most agricultural soils (*Hinsinger*, 2001). Like any other essential nutrient, severe P deficiency, especially during early growth stages in annual plants, may lead to irreversible restrictions in plant growth and development, which may not be corrected even after optimal P supply during later growth stages, thus, limiting crop yield (*Colomb* et al. 2000; *Grant* et al., 2001).

After long-term P fertilization, plant-available P levels may become suboptimal for crop production although total P is high (*Hinsinger*, 2001). A fraction of applied P is taken up by plants and by soil microorganisms, the latter acting as an important sink and source for soil P (*Bünemann* et al., 2011; *Gichangi* et al., 2009). The other fraction may be rendered partially or fully unavailable to plants through fixation or occlusion respectively. P fixation may occur by adsorption of PO_4^{3-} and or stabilized organic phosphates (e.g. phosphate monoesters) to sorbent surfaces on soil colloids such as iron and other metal hydroxides (*Turner* et al., 2005). It may also occur through precipitation of PO_4^{3-} as sparingly soluble Ca-phosphates in calcareous soils with neutral or alkaline pH (up to about 8) (*Lu* et al., 1987; *Moody* et al., 1995) or as Fe- or Al-phosphates in acidic soils (pH below 5.5) (*Prochnow* et al., 2004; *Redel* et al., 2007).

To optimize N and P availability to crop plants especially in early growth stages, N and P fertilizer can be applied by localized placement at moderate amounts in the seeding zone or in high amounts given sufficient spacing to plants as opposed to conventional fertilizer application by homogenous broadcast over the entire soil surface, with or without subsequent incorporation (*Grant* et al., 2001; *Lu* and *Miller*, 1993; *Valluru* et al., 2010). In this paper, "fertilizer placement" refers to localized application of fertilizers to small areas on surface or subsurface soil. Early studies on fertilizer placement mainly focused on the effects on crop yields. They reported enhanced plant growth and yield for placement of N and NPK fertilizers and conflicting results for placement of P or K fertilizers (*Cooke*, 1954; *Reith*, 1954). Within the last two decades, the significance of fertilizer placement in comparison to broadcast can be appreciated through the wide range of published peer-reviewed articles on the topic. There has been much interest in the effects of fertilizer placement in comparison to fertilizer broadcast on crop performance attributes like root growth and nutrient uptake (*Hodge*, 2004; *Rose* et al., 2009; *Weligama* et al., 2008;); crop yield (*Jing* et al., 2012; *Kelley* and *Sweeney*,

2007; *Schlegel* et al., 2003) and yield quality (*Boelcke*, 2003; *Weber* et al. , 2008); and on environmental aspects like NO_3^- leaching (*Baker*, 2001; *Ruidisch* et al., 2013; *Zhou* et al., 2008); emission of N_2O (*Engel* et al., 2010; *Halvorson* and *Del Grosso*, 2012; *Pfab* et al., 2012; *Nash* et al., 2012); release of CH_4 (*Linquist* et al, 2012; *van Kessel* et al-., 2012); and volatilization of NH_3 (*Hayashi* et al., 2009; *Ma* et al., 2010; *Rochette* et al., 2009). Fertilizer placement has also gained much interest in weed management where effective fertilizer placement disproportionately favors nutrition of target crop plants and enables them to be more competitive against weeds (*Blackshaw* et al., 2002; *Légère* et al., 2013; *Melander* et al., 2005; *Petersen*, 2005). Interest in fertilizer placement can also be appreciated by continuous development of improved placement machinery (*Bautista* et al., 2001; *Nyord* et al., 2008).

In more recent decades, many studies on fertilizer placement have also reported conflicting results on its effect on crop performance in comparison to fertilizer broadcast and requirements for effective fertilizer placement remain unclear. Open questions still exist, such as: Which fertilizers and placement techniques have been shown to be consistently effective? Can placement improve the efficiency of alternative recycled N and/or P fertilizers that are usually sparingly soluble (e.g. sewage sludge ash, biogas digestates or P-rich industrial by-products, if levels of heavy metals and other impurities are acceptable)?

The objectives of this paper are: (1) to summarize current techniques for fertilizer placement in the field and to outline properties of fertilizers suitable for placement as a subsurface depot; and (2) to compare the relative effect of fertilizer placement (Treatment) to fertilizer broadcast (Control) on yield and nutrient concentration and content in above-ground biomass, for various field crops, fertilizers and placement techniques through comprehensive meta-analyses on data published from field studies within the last three decades. Fertilizer placement effect on biomass nutrient concentration is addressed in terms of post-harvest nutritional value of crops (e.g. relation of N concentration to protein content and baking

quality of bread wheat) (*Boelcke*, 2003; *Grant* et al., 2016) and not regarded as an indicator for crop nutrition or yield potential, given the general inverse allometric relation of nutrient concentration to dry biomass yield per unit area, as clearly illustrated for N concentration in different crop species (*Greenwood* et al., 1991).

4.3 Literature review

4.3.1 Methodology

In order to compile and summarize relevant information on fertilizer placement techniques and fertilizers suitable for placement, and data on yield, nutrient concentration and content in above-ground biomass from fertilizer placement in comparison to fertilizer broadcast, we used published peer-reviewed articles and reviews obtained through recognized literature databases like Scopus and EBSCO EDS-global index as well as free scientific publication servers like Google Scholar. In our comprehensive literature search, we initially employed the following keywords and their combinations: fertilizer application methods, fertilizer application techniques, fertilizer placement, nutrient placement, localized fertilizer, localized nutrient supply, soil fertilizer depot, nitrogen placement, phosphorous placement, potassium placement, manure placement, slurry placement, field soil, field crops. These searches yielded more specific keywords and technical terms that were subsequently used particularly in literature search for data used in the meta-analyses described in section 3. Further keywords and technical terms included: starter fertilizer, 2x2 fertilizer, 5x5 fertilizer, pop-up fertilizer, fertilizer band, furrow fertilizer, below-seed fertilizer, deep placement, fertilizer side-dress, fertilizer injection, CULTAN, fertilizer depot placement, N-fertilizer depot, fertilizer nests, knife fertilizer, coulter-knife fertilizer, fertilizer broadcast, broadcast-incorporated, fertilizer topdress, yield, nutrient uptake, yield quality, yield composition, maize, wheat, field experiment, field study. Using defined time ranges, priority was given to scientific papers published from recent years till 2000 before older publications were considered.

Books were obtained through the library services of the University of Hohenheim, Stuttgart, Germany.

4.3.2 Techniques for fertilizer placement

Common techniques for fertilizer placement in soil include: indirect placement by pre-treatment of seeds with fertilizers before sowing (*Peltonen-Sainio* et al., 2006; *Sekiya and Yano*, 2010); in the seed hole or furrow during seeding (*Hocking* et al., 2003), on the soil surface as a band with or without incorporation (*Kelley* and *Sweeney*, 2007); subsurface as: shallow or deep band (*Pfab* et al., 2012), in a shallow or deep trench cut in the soil ("knife" or "coulter-knife" application, *Kelley* and *Sweeney*, 2007), as shallow or deep point placement ("nest" placement, *Engel* et al., 2010) or point injection (*Sommer*, 2005; *Weber* et al., 2008) (Figure 4.1). Fertilizer bands could also be placed on or below the soil surface, on or to the side(s) of the crop row. These techniques can be applied to both inorganic and organic fertilizers (*Bittman* et al, 2012; *Dell* et al., 2011) as well as to solid, liquid and gaseous fertilizer formulations, the latter requiring special equipment to minimize gaseous losses. Effective fertilizer placement requires good timing to crop demand and environmental conditions with low risk of nutrient loss. Split fertilizer placement at key growth stages with high nutrient demand enhances nutrient uptake and crop yield (*Saleem* et al. 2009), however, it may entail higher labor and energy costs.

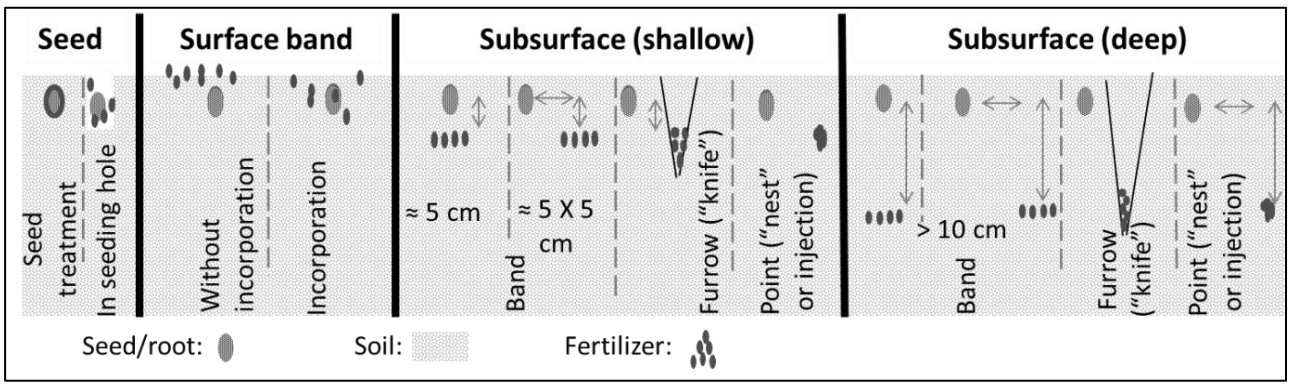

Figure 4.1. Fertilizer placement techniques

Seed placement ensures that as seed nutrient reserves become depleted, nutrients (especially macronutrients N and P) are sufficiently available during susceptible early growth stages when rooting is small. Nevertheless, high seed NH_4^+ and PO_4^{3-} rates are not advisable to avoid injury on seeds and young plants.

Surface placement without incorporation is not advisable for N fertilizers such as liquid manure, NH_4^+-fertilizer and $CO(NH_2)_2$ because it may lead to high gaseous NH_3 losses especially on alkaline or dry soils and under high air temperatures (*Adamsen* and *Sabey*, 1987; *Dell* et al., 2011; *Köhler* et al., 2003). Surface-placed fertilizers are more prone to wind and water erosion and more likely to emit undesirable odors (especially for manures) than incorporated or subsurface placed fertilizers. Although soil incorporation may reduce NH_3 volatilization from NH_4^+-fertilizer or $CO(NH_2)_2$, it increases the surface area of contact with soil microorganisms, thereby promoting biological oxidation with high risk for NO_3^- leaching and gaseous N_2O, NO_x and N_2 losses (*Malhi* et al., 2001; *Nash* et al., 2012).

Subsurface fertilizer placement may be shallow (often 5 – 10 cm) or deep (>10 cm). Similarly to seed placement, fertilizer application rates should be kept low if they are placed below ground and close to the seed row (*Zhang* and *Rengel*, 2002). If placed close to seeds, granulated fertilizers may be less harmful to seeds than fine and/or highly soluble ones due to slower nutrient release (*Olson* and *Dreier*, 1956). "Starter fertilizer" usually refers to macronutrient(s) especially NH_4^+ and PO_4^{3-} (e.g. $(NH_4)_2HPO_4$) banded only about 5 cm sideways and 5 cm below seeds, in the seeding hole or on/in the sowing row to ensure high nutrient availability during early crop development stages (*Grant* et al., 2001; *Kristoffersen* et al., 2005; *Qin* et al., 2005; *Niehues* et al., 2004). Unlike even broadcast with or without incorporation, banding reduces the surface area of contact with soil and soil microorganisms, thereby reducing PO_4^{3-} immobilization by fixation to various cations (*Grant* et al., 2001) and NH_4^+ nitrification by soil microorganisms (*Malhi* et al., 2001). NH_4^+-fertilizers containing

nitrification inhibitors may be suitable for placement (For chemical structures and inhibited reactions of nitrification and urease inhibitors tested in the field studies used in the meta-analyses described in section 3, see Table A.1, Online appendix: http://www.sciencedirect.com/science/article/pii/S0378429016302283).

Whereas fertilizers placed deep in soil with high moisture content may be more plant-available than those placed at shallow depths with less moisture (*Ma* et al., 2009; *Singh* et al., 2005), nutrients placed too deep may be less plant-available during early stages of plant growth when root density is still low at high depths.

4.3.3 Fertilizers suitable for placement as depots

To effectively place fertilizer to form a subsurface nutrient depot, we propose the following prerequisites. Fertilizer ions should:

a. *be taken up by plants in relevant quantities (macronutrients*).
b. *considerably stimulate root-growth and attract roots at the site of contact*.
c. *have limited mobility from the depot*. This is feasible for nutrients that have low effective diffusion coefficients in soil due to their adsorption properties (Table 1) although being water-soluble.
d. *be relatively stable in chemical form and plant- availability* especially at depot borders accessible to roots.
e. *be placed at an appropriate distance from the seeding zone to avoid injury to plants.*

Equation 4.1. The effective diffusion coefficient of a nutrient in soil (D_e)

$$D_e = \frac{(D_l \theta f_l dC_l)}{dC_s} \quad \text{Eqn.4.1}$$

D_l, diffusion coefficient of the nutrient in water; θ, volumetric moisture content of soil; f_l, tortuosity, the capacity of the soil to impede diffusion of non-adsorbed ions; dC_l/dC_s,; the reciprocal of the soil buffer capacity for the nutrient (*Barber*, 1984).

An effective approach is to place fertilizers at a high dose and concentration in a limited soil volume to form a nutrient depot providing high and persistent nutrient-availability during the growing season. For placement of NH_4^+ (with or without PO_4^{3-}) as a rich subsurface depot, *Sommer* (2005) proposed the term Controlled Long-Term Ammonium Nutrition (CULTAN), which describes a single application of a high phytotoxic concentration of fertilizer solution or granules at a safe distance from plant roots or seeds (*Deppe* et al., 2016). Toxic NH_4^+ concentrations inhibit NH_4^+ oxidation by soil microorganisms (*Müller* et al., 2006; *Shaviv*, 1988). Nutrient concentration in such a rich depot could be in the order of 1000 mg N or P kg^{-1} dry soil and even higher (*Lu* and *Miller*, 1993; *Pfab* et al., 2012).

Table 4.1. Effective diffusion coefficients of macronutrients in soil

Nutrient	Effective diffusion coefficient in soil (x 10^{-6} cm^2 s^{-1})	Soil texture	Volumetric moisture content (%)	Bulk density (g cm^{-3})	Subgroup (USDA soil taxonomy)	Source
$H_2PO_4^-$	0.00001 – 0.01					*Marschner* and *Rengel*, 2012; *Neumann* and *Römheld*, 2012
$H_2PO_4^-$	0.0023	Silt loam				*Barber*, 1984
$H_2PO_4^-$	0.007 – 0.025	Sandy loam				*Bhat* and *Nye*; 1973
$H_2PO_4^-$	0.0042	Loamy sand	20	1.5	Typic Udipsamment	*Schenk* and *Barber*, 1979
$H_2PO_4^-$	0.0062	Loam	24	1.2	Typic Argiaquoll	*Schenk* and *Barber*, 1979
$H_2PO_4^-$	0.0097	Silt loam	24	1.2	Ultic Hapludalf	*Schenk* and *Barber*, 1979
$H_2PO_4^-$	0.0131	Silt loam	24	1.2	Typic Halplaquoll	*Schenk* and *Barber*, 1979
$H_2PO_4^-$	0.0152	Silt loam	24	1.2	Aeric Ochraqualf	*Schenk* and *Barber*, 1979
$H_2PO_4^-$	0.0893	Silt loam	20	1.2	Aquic Argiudoll	*Schenk* and *Barber*, 1979
K^+	0.01 – 0.1					*Marschner* and *Rengel*, 2012; *Neumann* and *Römheld*, 2012
K^+	0.019	Silt loam				*Barber*, 1984
K^+	0.066	Silt loam			Typic Halplaquoll	*Barber*, 1984
K^+	0.075	Silt loam			Ultic Hapludalf	*Barber*, 1984

NH_4^+	0.157[a]	Loam	20	1.14 – 1.23		*Clarke* and *Barley*, 1968
NH_4^+	0.319 [a]	Sand	20	1.48 - 1.55		*Clarke* and *Barley*, 1968
NH_4^+	0.73	Clay loam	32			*Pang* et al., 1973
NH_4^+	0.82	Silty clay loam	42			*Pang* et al., 1973
NH_4^+	1.24	Fine sandy loam	17			*Pang* et al., 1973
$CO(NH_2)_2$	0.62	Sandy clay	52	1.22	Typic Hapludults	*Sadeghi* et al, 1989
$CO(NH_2)_2$	0.805	Silt loam	58.6	1.37	Cumulic Hapludolls	*Sadeghi* et al, 1989
NO_3^-	0.1 – 1.0					*Marschner* and *Rengel*, 2012; *Neumann* and *Römheld*, 2012
NO_3^-	1.99 [a]	Loam	20	1.14 – 1.23		*Clarke* and *Barley*, 1968
NO_3^-	2.5	Silt loam				*Barber*, 1984;
NO_3^-	4.76 [a]	Sand	20	1.48 - 1.55		*Clarke* and *Barley*, 1968

USDA, United States Department of Agriculture
[a], effective diffusion coefficient derived from equations of effective diffusion as a function of volumetric water content (*Clarke* and *Barley*, 1968)

Based on the suggested criteria, fertilizers containing the macronutrient N in the form of NH_4^+ and/or P normally in the oxidized form of PO_4^{3-} (e.g. $(NH_4)_2SO_4$, $Ca(H_2PO_4)_2$, $NH_4H_2PO_4$, $(NH_4)_2HPO_4$ and ammonium polyphosphate – $[NH_4\ PO_3]_n$) are the best candidates to be recommended for placement as a subsurface depot. NH_4^+ and PO_4^{3-} ions are both macronutrients that strongly stimulate initiation and elongation of lateral roots on the part of the root system that is within or close to their respective nutrient depots (*Anghinoni* and *Barber*, 1990; *Chassot* et al., 2001; *Drew*, 1975; *Jing* et al., 2012), also with a potential to contribute to root growth in soil zones distant from the nutrient patch (*Zhang* et al., 2000). NH_4^+ and PO_4^{3-} generally have low effective diffusion coefficients in soils (*Barber*, 1984; *Neumann* and *Römheld*, 2012). NH_4^+ readily binds to negative charges on the surface of clay minerals and becomes fixed, especially in 2:1 type clay-rich soils, when it penetrates the clay mineral interlayers and becomes trapped between its silicate sheets (*Nieder* et al, 2011). As previously indicated, PO_4^{3-} is readily fixed by adsorption to iron and other metal hydroxides or is precipitated depending on pH as Fe-, Al- and Ca-phosphates. PO_4^{3-} sorption capacity of soil can be measured from the concentration of Fe, Al and Ca cations upon extraction with Mehlich-3 solution (*Zhang* et al., 2005). According to *Sommer* (2005), high concentrations of NH_4^+ inhibit nitrification in subsurface NH_4^+-depots, thereby lowering the potential for NO_3^--related N losses. As a further step, the stability of NH_4^+ in a subsurface depot may be increased by using NH_4^+ treated with nitrification inhibitors e.g. 3, 4-Dimethylpyrazole phosphate (DMPP) (*Zerulla* et al., 2001). Through localized placement of NH_4^+ and/or PO_4^{3-} in small soil volumes, the surface area for contact to soil microorganisms (biological transformation or immobilization) or to soil minerals (for chemical transformation, adsorption, fixation or occlusion) is greatly reduced, thus, promoting nutrient stability in soil.

NO_3^-, $CO(NH_2)_2$ and K^+ do not substantially stimulate initiation and growth of lateral roots upon contact and are highly mobile in soil due to rapid diffusion and movement by mass flow. Therefore, they are less suitable candidates for placement to form a localized subsurface depot. Nevertheless, $CO(NH_2)_2$ may be placed as a depot if conditions are optimal for rapid ammonification and reduced NH_3 volatilization.

4.4 Meta-analyses of relative effects of fertilizer placement to fertilizer broadcast on crop yield and nutrient uptake

4.4.1 Prerequisites for data inclusion

Studies included in the meta-analysis fulfilled the following conditions:

- Published in an international peer-reviewed journal. Two exceptions were made specific to the fertilizer placement technique termed CULTAN (*Sommer*, 2005): a Ph.D. Thesis and a publication in a national agricultural research center journal
- Performed under field conditions
- Contained at least one fertilizer placement treatment (Treatment) and one fertilizer broadcast (or broadcast/incorporation) treatment (Control)
- Applied the same or comparable fertilizer types and application rates for Treatments and Control

To compile published peer-reviewed studies that were included in these meta-analyses, we used specific keywords to search in recognized scientific literature databases as already described in section 2.1. For yield, nutrient concentration in plant parts and nutrient uptake combined, there were 1022 datasets collected from 40 studies: six from 1982 – 1999 and 34 from 2000 – 2015. The term “dataset” refers to a pair of means ($\bar{X}$), standard deviations (S) and sample sizes (N), one for Treatment

and the other for Control derived from the same experiment. Many datasets could be retrieved because several studies were extensive e.g. *Borges* and *Mallarino* (2000) which covered 20 field tests in long-term trials and 11 field tests in short-term trials. Additionally, many studies investigated different fertilizer types, application rates, timing and techniques under different systems for cropping, rotation, irrigation and tillage.

Information about crops, soil types, fertilizer types, broadcast and placement techniques, result of fertilizer broadcast and fertilizer placement on yield, nutrient uptake and nutrient concentration in plant parts, relative effects of fertilizer placement to broadcast and source of studies are summarized in Table A.1 (Online appendix: http://www.sciencedirect.com/science/article/pii/S0378429016302283).

4.4.2 Methodology

In order to combine treatment effects across several primary independent randomized studies, a suitable method used in most meta-analyses is *baseline contrasts*, which puts the results of these studies in a common framework to enable comparison. This method expresses the effect of an experimental treatment in a study as a contrast or response ratio to the effect of a baseline or control treatment within the same study (*Akiyama*, 2010; *Piepho*, 2012). A *random effects model* with *grouping variable* was used to analyze the data (data structured in groups e.g. crop species). This model accounted for sampling error between studies and random variation in effect sizes between studies.

The whole data used in each meta-analysis could be arranged according to one of several grouping variables into different subgroups. For relative yield, five grouping variables and their subgroups included: Crop type (15: maize, winter wheat, spring

wheat, winter rye, sorghum, rice, soybean, rapeseed, turnip rape, potato, sugar beet, lettuce, cauliflower, Chinese cabbage and mixed grassland grass species); Yield component (6: grain – for cereals, oilseeds and pulses; cob – for maize only; straw – for cereals, oilseeds and pulses; above-ground biomass; tuber – for potato and sugar beet; sucrose – for sugar beet); Fertilizer type (9: ammonium, ammonium and phosphorus, N (no description), urea, urea and phosphorus, phosphorus, potassium, liquid manure and solid manure); Placement technique (11: surface band, seed, below seed, shallow band, shallow knife, shallow point placement, shallow point injection, deep band, deep knife, deep point placement and deep point injection); Placement depth (3: 0 cm, 5 –10 cm and >10 cm). For relative nutrient concentration in plant parts, six grouping variables and subgroups were: Crop type (4: maize, winter wheat, soybean and turnip rape); Plant part (3: grain – for cereals, oilseeds and pulses; leaf – youngest or ear-leaf; and above-ground biomass); Nutrient (4: N, P, K and Grain-protein); Fertilizer type (6: ammonium, ammonium and phosphorus, urea, phosphorus, potassium and liquid manure); Placement technique (6: surface band, seed, shallow band, shallow point injection, deep band and deep point injection); Placement depth (3: same as for yield). Finally, for relative nutrient uptake, five grouping variables and subgroups comprised: Crop type (10: maize, winter wheat, winter rye, sorghum, soybean, rapeseed, turnip rape, lettuce, cauliflower and mixed grassland grass species); Nutrient (4: N, P, K and S), Fertilizer type (8: ammonium, ammonium and phosphorus, urea, urea and phosphorus, phosphorus, potassium, liquid manure and solid manure); Placement technique (8: surface band, shallow band, shallow knife, shallow point injection, deep band, deep knife, deep point placement and deep point injection); Placement depth (3: same as for yield).

To perform the meta-analyses, we used the software MetaWin 2.0 (*Rosenberg* et al., 2000) to calculate effect sizes (response ratios, *ln R*) of fertilizer placement – experimental Treatment (*E*) – in relation to fertilizer broadcast –baseline or Control treatment (*C*).

Equation 4.2. Effect sizes (response ratios, *ln R*)

$$\ln R = \ln(\frac{\bar{X}^E}{\bar{X}^C}) = \ln(\bar{X}^E) - \ln(\bar{X}^C) \quad \text{Eqn.4.2}$$

The variance of effect sizes ($V_{ln\ R}$), was calculated as follows:

Equation 4.3. Variance of effect sizes (V_{lnR})

$$V_{\ln\ R} = \frac{(S^E)^2}{N^E(\bar{X}^E)^2} + \frac{(S^C)^2}{N^C(\bar{X}^C)^2} \quad \text{Eqn.4.3}$$

(Where mean, standard deviation and sample size are: $\bar{X}^E$, S^E and N^E for fertilizer placement respectively and $\bar{X}^C$, S^C and N^C for fertilizer broadcast respectively, (*Rosenberg* et al., 2000))

Standard deviations (STDs) were not reported in some studies (see details in results). Where applicable, they were calculated from reported variances, standard errors, *p*-values or *t*-values. Where not applicable, we imputed missing STDs with the average of STDs reported in other studies used in the meta-analysis. Imputation of missing STDs for the purpose of including as many data as possible in a meta-analysis has been shown to be safe and accurate (*Furukawa* et al. 2006; *Philbrook* et al., 2007). The procedure for STD imputation involved calculating the mean of reported STDs expressed as a fraction of the mean of reported means for a specific variable (e.g. 0.1969 or 19.69 % mean for maize grain yield). This number was then multiplied to the reported mean with missing STD to obtain an appropriate STD for it.

The procedure for the weighted *random effects model* analysis consisted of: 1.) Running a *fixed effects model* to produce summary statistics (mean effect size and total heterogeneity). 2.) Using the resulting summary statistics to estimate a pooled variance. 3.) Using the pooled variance to calculate *random effects*-weights for each individual study, which were then used in further calculations (*Rosenberg* et al., 2000).

For the *fixed effects model*, weighted mean effect sizes were calculated because individual studies had different sample sizes. The *fixed-effects* weight of the i^{th} study or dataset (w_i), was calculated by inverting the variance of its effect size:

Equation 4.4. Fixed-effects weight (W_i)

$$w_i = \frac{1}{V_{\ln R}} \qquad \text{Eqn.4.4}$$

The overall mean effect size ($\bar{\bar{E}}$) for all studies was given as:

Equation 4.5. Overall mean effect size ($\bar{\bar{E}}$)

$$\bar{\bar{E}} = \frac{\sum_{i=1}^{n} w_i E_i}{\sum_{i=1}^{n} w_i} \qquad \text{Eqn.4.5}$$

(n = number of studies or datasets; E_i = effect size of the i^{th} study or dataset)

The variance of the overall mean effect size ($S^2_{\bar{\bar{E}}}$) was given a function of the individual weights.

Equation 4.6. Variance of the overall mean effect size ($S^2_{\bar{\bar{E}}}$)

$$S^2_{\bar{\bar{E}}} = \frac{1}{\sum_{i=1}^{n} w_i} \qquad \text{Eqn.4.6}$$

Using $S^2_{\bar{\bar{E}}}$, the confidence interval (*CI*) around $\bar{\bar{E}}$ was calculated as follows:

Equation 4.7. Confidence interval of mean effect size

$$CI = \bar{\bar{E}} \pm t_{\alpha/2[n-1]} * S_{\bar{\bar{E}}} \qquad \text{Eqn.4.7}$$

(t = two-tailed critical value from the Student's t-distribution at the critical level α)

Total heterogeneity (Q_T) was given as:

Equation 4.8. Total heterogeneity (Q_T) (1)

$$Q_T = \sum_{i=1}^{n} w_i E_i^2 - \frac{\left(\sum_{i=1}^{n} w_i E_i\right)^2}{\sum_{i=1}^{n} w_i} = \sum_{i=1}^{n} w_i \left(E_i - \bar{\bar{E}}\right)^2 \qquad \text{Eqn.4.8}$$

Q_T was used for Q statistical test for variability in effect sizes across studies. For this, Q_T was tested against a χ^2-distribution (Chi-Square) at appropriate degrees of freedom. Presence of significant sample heterogeneity showed that variance among effect sizes was greater than expected by sampling error and therefore, the appropriate weight for each study or dataset should incorporate a *pooled study variance*.

Total heterogeneity (Q_T) is the sum of model-derived effect size heterogeneity between studies (or effect size heterogeneity between groups for data with grouping structure) (Q_M) and residual error variance (Q_E).

Equation 4.9. Total heterogeneity (Q_T) (2)

$$Q_T = Q_M + Q_E \qquad \text{Eqn.4.9}$$

For the j^{th} group of studies or datasets, the mean effect size ($\bar{E}_j$), its variance ($S^2_{\bar{E}_j}$), confidence intervals ($CI_{\bar{E}_j}$) and heterogeneity (Q_{wj}) were also calculated as shown by Eqns. 4.5, 4.6, 4.7, and 4.8-4.9 respectively.

The sum of individual group heterogeneity (Q_M) was given by:

Equation 4.10. Sum of individual group heterogeneity (Q_M)

$$Q_M = \sum_{j=1}^{m} \sum_{i=1}^{k_j} w_{ij} \left(\bar{E}_j - \bar{\bar{E}}\right)^2 \qquad \text{Eqn.4.10}$$

(m = number of groups; k_j = number of studies in the j^{th} group; w_{ij} =weight for the i^{th} study in the j^{th} group; $\bar{E}_j$= mean effect size for the j^{th} group; and $\bar{\bar{E}}$ is the overall mean effect size given in Eqn.4.5)

Residual error heterogeneity (Q_E), the sum of within-group heterogeneity, was given b

Equation 4.11. Residual error heterogeneity (Q_E)

$$Q_E = \sum_{j=1}^{m} Q_{wj} = \sum_{j=1}^{m} \sum_{i=1}^{k_j} w_{ij}\left(E_{ij} - \bar{E}_j\right)^2 \quad \text{Eqn. 4.11}$$

(Q_{Wj} = individual within-group heterogeneity; m = number of groups; k_j = number of studies in the j^{th} group; w_{ij} = weight and E_{ij} = effect size for the i^{th} study in the j^{th} group; $\bar{E}_j$= the effect size for the j^{th} group).

With Q_E known, the *pooled study variance* (between-study variance) for data with grouping structure (σ^2_{pooled}), was calculated as follows:

Equation 4.12. Pooled study variance (σ^2_{pooled})

$$\sigma^2_{pooled} = \frac{Q_E - (n-m)}{\sum_{j=1}^{m}\left(\sum_{i=1}^{k_j} w_{ij} - \frac{\sum_{i=1}^{k_j} w_{ij}^2}{\sum_{i=1}^{k_j} w_{ij}}\right)} \quad \text{Eqn. 4.12}$$

(Q_E = residual error heterogeneity from the fixed-effects model; n = total number of studies, m = the number of groups; k_j = the number of studies in the j^{th} group; w_{ij} = the fixed-effects weight for the i^{th} study in the j^{th} group)

Using σ^2_{pooled}, the *random-effects* weight of the i^{th} study or dataset ($w_{i(rand)}$) was then calculated:

Equation 4.13. Random-effects weight ($W_{i(rand)}$)

$$w_{i(rand)} = \frac{1}{V_i + \sigma^2_{pooled}} \quad \text{Eqn. 4.13}$$

These *random-effects* weights, which account for pooled variance, were then used for calculation of mean effect sizes according to Equations 4.5, 4.6 and 4.7. As a pre-requisite for meta-analysis, normality was checked for bell-shaped distribution in *Weighted Histograms* (sum of study weights per effect size class plotted against effect size classes) and for location of data points within confidence bands in *Normal Quantile Plots* (*Rosenberg* et al., 2000). Finally, the unlogged overall and group mean effect sizes, which show the relative effect of fertilizer placement to fertilizer broadcast, together with their *bias-corrected percentile bootstrap confidence intervals* with *999 iterations* at the power $\alpha = 0.05$, were reported. Any groups with less than two datasets were excluded by default settings from the meta-analysis. We used the software *SigmaPlot 12.0* (Systat Software Inc.) to create scatter plots of relative mean effects and their confidence intervals.

4.4.3 Sensitivity analyses

We conducted sensitivity analyses to investigate whether non-targeted input factors associated with individual studies affected the outcome of the meta-analysis. In addition to the grouping variables described earlier (section 3.2), the whole data used for each meta-analysis could be further arranged according to one of the following groupings: Outlier study ± 3 STDs (yes, no), Source of STD (reported or imputed), Broadcast method (surface, incorporation) and Phenological crop development stage (vegetative, reproductive, maturity). For the first and second sensitivity analyses respectively, mean effect sizes estimated using the whole dataset were compared to values estimated using datasets excluding outliers (limits, ± 3 STDs) or data for which STDs were missing. For the third and fourth, we checked whether (1.) fertilizer broadcast method (surface broadcast and broadcast/incorporation) and (2.) phenological crop development stage (vegetative, reproductive and maturity) affected

the outcome of meta-analysis. Additionally, we checked the outcome of the third and fourth sensitivity analyses by running a linear mixed model on *weighted study effect sizes* (using *random–effects* weights) as the dependent variable with broadcast method and crop development stage as independent fixed effects variables, and study as the random effects variable, using *SAS 9.4* (SAS Institute Inc., Cary, NC, USA).

To check whether there was a propensity that studies showing statistical significant results were selectively published over those that did not, a condition termed *Publication bias*, we checked for symmetry in *Funnel Plots* of effect size against sample size (widely scattered effect sizes at lowest sample size, bottom; closely placed effect sizes at highest sample size, top) (*Rosenberg* and *Goodnight*, 2005). We also looked for linearity and absence of gaps in *Normal Quantile Plots* of standardized effect sizes against normal quantiles (*Rosenberg* et al., 2000). For a numerical test that is simple to calculate and easy to interpret, we additionally used *fail-safe* numbers (N_R) according to Rosenthal's method ($\alpha = 0.05$) (*Rosenberg* et al., 2000; *Rosenberg* and *Goodnight*, 2005). N_R represents the number of additional non-significant unpublished studies (or datasets), with a mean effect size of zero, that need to be added in order to reduce combined significance of a meta-analysis to non-significant i.e. $P \geq \alpha$. $5n + 10$ (n, total number of studies) is given as a reasonable conservative critical lower limit for N_R. Nevertheless, it is recommended to check N_R results with other tests such as symmetry in *Funnel Plots*, because use of N_R only cannot adequately detect presence of publication bias (*Rosenberg* and *Goodnight*, 2005).

4.4.4 Results

4.4.4.1 Explanation

For the sake of brevity in this subsection, "placement" refers to fertilizer placement in soil using any of the techniques described in Figure 4.1 (Section 4.3.2) and "broadcast" refers to fertilizer application on the soil surface with or without incorporation. All relative mean effects of placement to broadcast are given as percentage (%) differences from broadcast. Directly after each relative mean effect, its 95% confidence interval (CI95%) is given. If CI95% was below zero, there was a negative relative placement effect (RPE) on the measured variable (i.e. Placement < Broadcast); if CI95% included zero, there was no RPE (i.e. Placement = Broadcast); and if CI95% was above zero, there a positive RPE (i.e. Placement > Broadcast). RPE was considered different between groups if their CI95% did not overlap. After the CI95%, the number of datasets in each group (n) is given. In figures, "n" is shown in brackets after the name of each group.

4.4.4.2 Yield

Overall, 772 datasets from 39 studies were used for this meta-analysis (six from 1982 to1999 and 33 from 2000 to 2015). Mean effects from all datasets showed that placement resulted in significantly higher yield than broadcast. The RPE on yield was 3.7 %, CI95% 3.1 to 4.3, n = 772 ($P < 0.00001$). Symmetrical funnel plot and high N_R (*120085*) observed suggest absence of significant publication bias. Exclusion of 17 outlier datasets did not change the outcome of the meta-analysis (3.6 %, CI95% 3.0 to 4.3, n = 755). Furthermore, there was no difference in RPE on yield between all 772 datasets and datasets with reported STDs (3.1 %, CI95% 2.3 to 3.8, n = 444) or datasets with imputed STDs (4.6 %, CI95% 3.5 to 6.0, n = 328). For broadcast methods, there was also no difference in RPE on yield between Surface broadcast (3.6

%, CI95% 2.8 to 4.2, n = 719) and Broadcast/incorporation (5.2 %, CI95% 2.2 to 9.2, n = 53). For different crop phenological growth stages, the same RPE was observed: Vegetative (5.7 %, CI95% 3.3 to 8.0, n = 133); Reproductive (3.7 %, CI95% 1.3 to 6.7, n = 13); Maturity (3.4 %, CI95% 2.7 to 4.1, n = 626). The RPE on yield for each broadcast method or phenological growth stage did not differ from the overall RPE on yield. Linear model analysis confirmed that broadcast method (*P=0.2932*) and crop development stage (*P=0.9793*) had no effect on weighted study effect sizes.

According to yield components, RPE on yield for Tubers (potato and sugar beet) (9.4 %, CI95% 4.8 to 16.0, n = 16) was higher than that for Grains (3.0 %, CI95% 2.4 to 3.6, n = 566) (Figure 4.2). Among 15 crop species analyzed, yield from placement was higher than yield from broadcast in nine species. The RPE on yield was higher in Winter wheat (9.5 %, CI95% 6.8 to 12.4, n = 112) than in Maize (4.5 %, CI95% 3.6 to 5.5, n = 408) (Figure 4.3). Other crop species for which placement had a positive effect on yield, are shown in Figure 4.3. Placement did not have an effect on yield in the following six crop species: Cauliflower, Mixed grassland grass species, Lettuce, Winter rye, Soybean and Turnip rape (Figure 4.3).

Sorted by 11 placement techniques involved, yield from placement was higher than yield from broadcast in eight placement techniques. For placement techniques with more than 100 datasets, Subsurface deep point injection showed the highest RPE on yield (6.4 %, CI95% 4.3 to 8.5, n = 114) (Figure 4.4). There was no RPE on yield for the following placement methods: Seed, Below-seed and Subsurface shallow point injection (Figure 4.4).

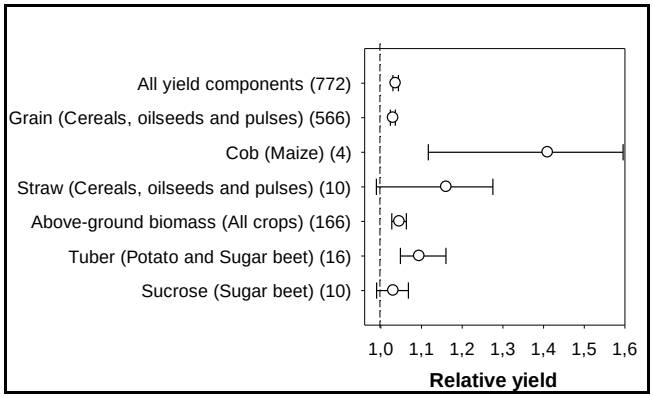

Figure 4.2. Relative yield of fertilizer placement by yield component
Y-axis, categories and number of datasets per category in brackets; **X-axis,** relative value of fertilizer **Placement** to fertilizer **Broadcast**; **Error bars**, 95 % confidence intervals; **Placement ≠ Broadcast**, if error bars do not included 1.0; Relative values of a pair of categories are different from each other if their 95% confidence intervals do not overlap.

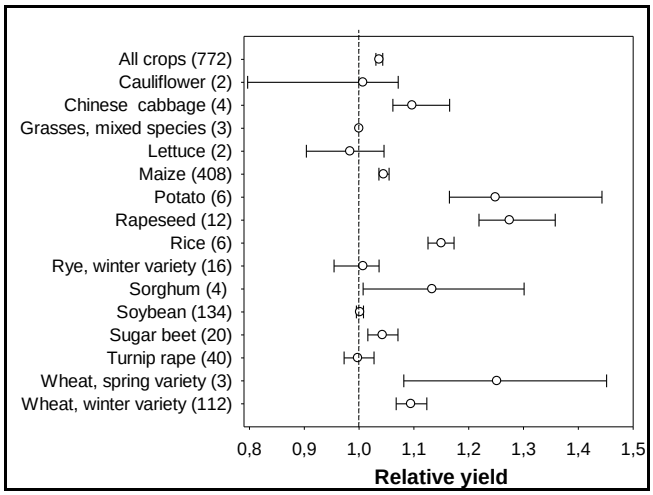

Figure 4.3. Relative yield of fertilizer placement by crop type
Y-axis, categories and number of datasets per category in brackets; **X-axis,** relative value of fertilizer **Placement** to fertilizer **Broadcast**; **Error bars**, 95 % confidence intervals; **Placement ≠ Broadcast**, if error bars do not included 1.0; Relative values of a pair of categories are different from each other if their 95% confidence intervals do not overlap.

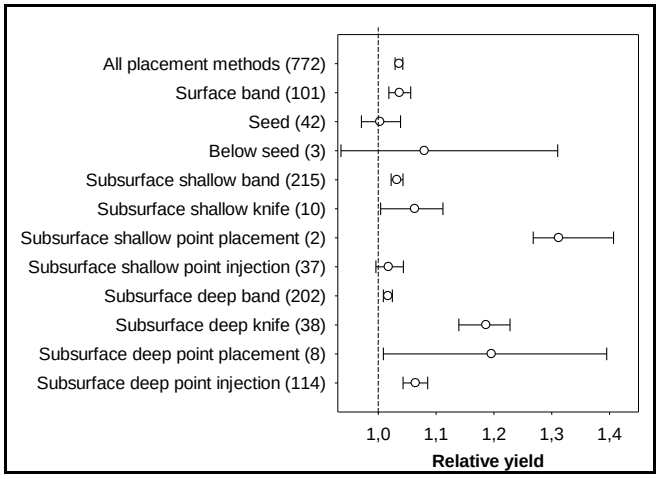

Figure 4.4. Relative yield of fertilizer placement by fertilizer placement method **Y-axis**, categories and number of datasets per category in brackets; **X-axis,** relative value of fertilizer **Placement** to fertilizer **Broadcast**; **Error bars**, 95 % confidence intervals; **Placement ≠ Broadcast**, if error bars do not included 1.0; Relative values of a pair of categories are different from each other if their 95% confidence intervals do not overlap.

Yield from placement for each placement depth was higher than that from broadcast. The RPE on yield was the same across placement depths. Nevertheless, there was a slight tendency for RPE on yield to increase with increasing placement depth: Surface placement (0 cm) (3.9 %, CI95% 1.9 to 5.6, n = 101); 0 – 5 cm (3.4 %, CI95% 2.4 to 4.6, n = 317); > 10 cm (4.1 %, CI95% 3.1 to 5.0, n = 354) (Figure 4.5).

According to fertilizer type irrespective of placement depth, there was no RPE on yield for Solid manure (7.9 %, CI95% -0.1 to 14.6, n = 6); Soluble P fertilizers (P) (0.0 %, CI95% -0.6 to 0.7, n = 136) and Undescribed soluble N fertilizers (1.4 %, CI95% -1.5 to 4.4, n = 48). Effective fertilizer types were in the following order of strongest to weakest relative RPE: Urea combined with soluble P (27.3 %, CI95% 21.7 to 34.7, n = 12); Ammonium combined with soluble P (14.7 %, CI95% 12.9 to 17.0, n =163); Liquid manure (11.6 %, CI95% 5.9 to 18.3, n = 24); Urea (11.0 %, CI95% 5.7 to 17.5, n = 64); Ammonium (3.8 %, CI95% 2.2 to 5.4, n = 134); and soluble Potassium (1.6 %, CI95% 0.8 to 2.4, n = 185). These results showed that

placement of combinations of Ammonium and soluble P or Urea and soluble P was more effective to improve yield than placement of ammonium, urea or soluble P uncombined. This occurrence can also be seen in Figure 4.5, which also shows that yield from placement of urea or ammonium (each with or without soluble P) or K tends to increase with increasing placement depth from 5 cm to more than 10cm.

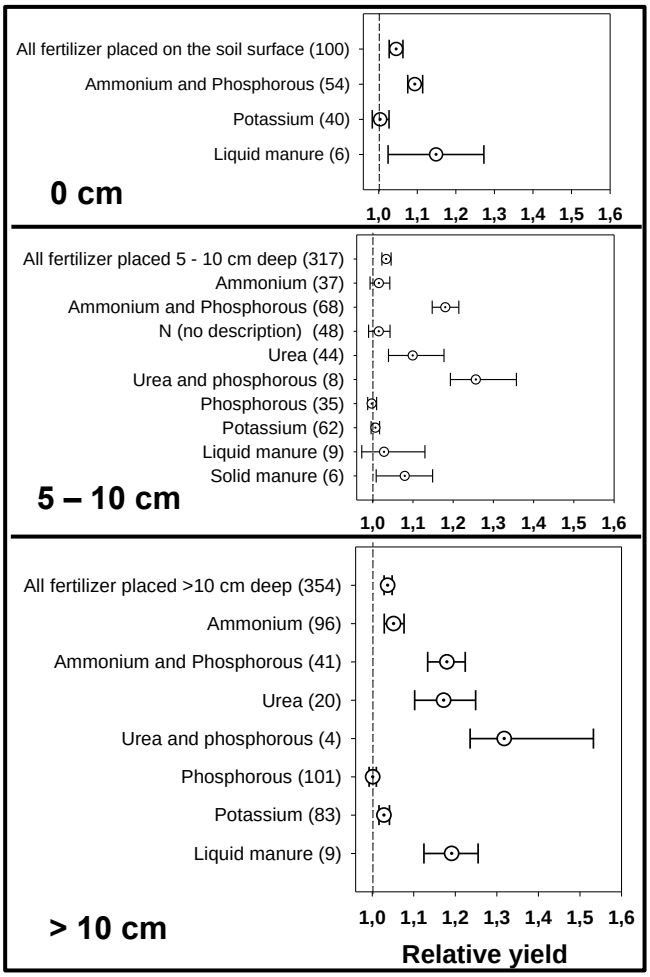

Figure 4.5. Relative yield of fertilizer placement by fertilizer type and placement depth
Y-axis, categories and number of datasets per category in brackets; **X-axis,** relative value of fertilizer **Placement** to fertilizer **Broadcast**; **Error bars**, 95 % confidence intervals; **Placement ≠ Broadcast**, if error bars do not included 1.0; Relative values of a pair of categories are different from each other if their 95% confidence intervals do not overlap.

Meaningful RPE regarding the use of nitrification inhibitors; urease inhibitors (or both); toxic concentrations of ammonium depot solutions; urea coating; or palletization of solid manure, methods used to stabilize mineral and/or organic N fertilizers with the aim to optimize N uptake and yield, could not be obtained from this meta-analysis. The reason was that the number of datasets for each of these modified or stabilized N fertilizer groups was too small in comparison to the number of datasets for unmodified N fertilizers.

4.4.4.3 Nutrient concentration in above-ground biomass

357 datasets from 11 studies (two studies published in 1982 and nine from 2000 to 2013) were used for this meta-analysis. In all, placement resulted in higher concentrations of N, P, K or grain protein in different above-ground plant parts than broadcast. For all plant parts combined, overall RPE on nutrient concentration in plant parts was 3.7 %, CI95% 2.7 to 4.9, n = 357 ($P < 0.00001$). Exclusion of six outliers or imputation of missing STDs for 190 datasets did not change the outcome of the meta-analysis. Analysis according broadcast methods (Surface broadcast 3.8%, Broadcast/Incorporation 2.1%) or crop development stages (Reproductive 2.3%, Vegetative 3.2%, Maturity 5.03%) did not change the result. Linear model analysis confirmed that broadcast method had no effect on *weighted study effect sizes* ($P=0.1093$). However, crop development stage had an effect ($P=0.0222$) with the same increasing trend from Reproductive, Vegetative to Maturity shown by the meta-analysis. There was no difference between the RPE on nutrient concentration in plant parts between each plant part (Leaf (ear or youngest developed leaf), Total above-ground biomass and Grain) and all parts combined. RPE on nutrient concentration tend to decrease slightly from Leaf (5.7 %, CI95% 3.8 to 7.7, n = 123) to Total above-

ground biomass (2.9 %, CI95% 1.4 to 4.8, n =154) to Grain (2.0 %, CI95% 0.1 to 3.9, n = 80). According to crop species, RPE on nutrient concentration in different plant parts decreased in the order: Maize (7.0 %, CI95% 5.4 to 8.7, n = 197) > Soybean (1.8 %, CI95% 0.6 to 3.0, n = 126) > Turnip rape (-4.9 %, CI95% -8.6 to -2.2, n = 24) = Winter wheat (-8.0 %, CI95% -10.7 to -4.4, n = 10). By fertilizer placement technique, the decreasing trend was: Subsurface deep point injection (7.6 %, CI95% 5.7 to 9.7, n = 149); Subsurface deep band (4.4 %, CI95% 1.7 to 7.7, n = 66); Subsurface shallow band (1.8 %, CI95% 0.0 to 3.8, n = 78); Surface band (1.2 %, CI95% 0.2 to 2.3, n = 40); Seed (-3.3 %, CI95% -7.6 to 0.3, n = 12); and Subsurface shallow point injection (-6.4 %, CI95% -9.7 to -3.2, n = 12).

There was no difference between the overall RPE on nutrient concentration in plant parts for all nutrients combined (3.7 %, CI95% 2.7 to 4.9, n = 357, also shown above) and the RPE for the following individual nutrients: Grain protein (6.3 %, CI95% 1.3 to 10.7, n = 26), K (3.4 %, CI95% 2.2 to 5.0, n = 132), N (3.8 %, CI95% 2.0 to 5.9, n = 141) and P (3.0 %, CI95% 0.7 to 5.8, n = 58). According to fertilizer type and nutrient taken up (except P), there was a tendency of RPE on nutrient concentration in plant parts to increase with increase in the fertilizer placement depth (Figure 4.6).

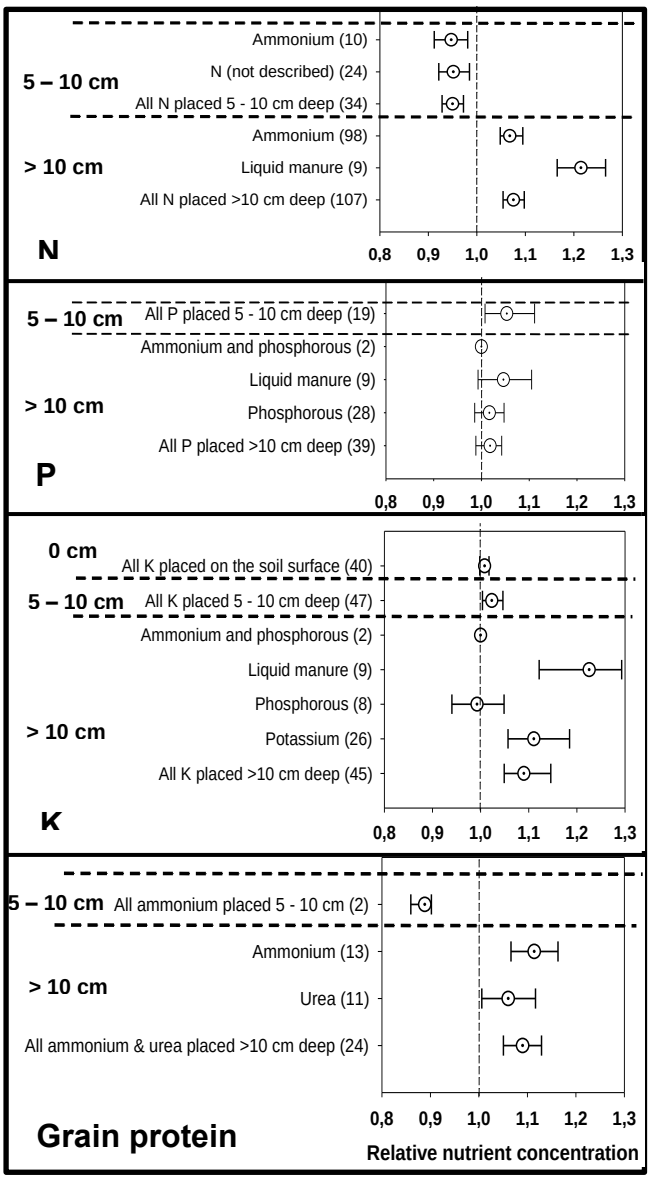

Figure 4.6. Relative nutrient concentration of plant parts by nutrient, fertilizer type and placement depth (0, 5 – 10, and > 10 cm)
Y-axis, categories and number of datasets per category in brackets; **X-axis,** relative value of fertilizer **Placement** to fertilizer **Broadcast**; **Error bars**, 95 % confidence intervals; **Placement ≠ Broadcast**, if error bars do not included 1.0; Relative values of a pair of categories are different from each other if their 95% confidence intervals do not overlap.

4.4.4.4 Nutrient content in above-ground biomass

In this study, the term "nutrient content" refers to the quantity of N, P, K and S in kilograms recovered in total or partial above-ground crop biomass per hectare of agricultural land.

The meta-analysis in this section involved 245 datasets from 22 studies (three studies published from 1993 to 1999 and 19 from 2000 to 2015). Overall, nutrient content from placement was higher than nutrient content from broadcast. The overall RPE on nutrient content was 11.9 %, CI95% 9.7 to 14.5, n =245 (*P < 0.00001*). Removal of two outlier studies did not change the outcome of the meta-analysis. For 148 datasets with reported STDs, RPE was 19.2 %, CI95% 15.5 to 23.0, n = 148. Therefore, imputation of STDs for 97 datasets resulted in an underestimation of the RPE on nutrient content. RPE on nutrient content was the same irrespective of broadcast method. According to phenological growth stage, RPE on nutrient content was higher in the vegetative growth stage than in later growth stages: Vegetative (20.3 %, CI95% 15.8 to 26.1, n =91) > Maturity (9.2 %, CI95% 6.5 to 12.0, n = 138) = Reproductive (6.5 %, CI95% 1.2 to 11.7, n = 16). Linear model analysis also confirmed that broadcast method had no effect on *weighted study effect sizes* (*P=0.1022*) and that crop development stage had an effect (*P=0.0372*), with the same decreasing trend from Vegetative, Maturity to Reproductive. RPE on nutrient content was higher in the Vegetative than in Generative growth stage (i.e. Reproductive and Maturity stages combined). By crop type only, RPE on nutrient content for different crop species were in the following order: Rapeseed (36.4 %, CI95% 30.2 to 43.3, n = 36); Turnip rape (30.3 %, CI95% 28.1 to 32.9, n = 2); Sorghum (17.7 %, CI95% 10.8 to 26.4, n = 12); Maize (12.2 %, CI95% 8.7 to 16.1, n = 112); Cauliflower (12.2 %, CI95% 9.1 to 15.4, n = 2); Winter wheat (7.2 %, CI95% 3.5 to 11.1, n = 57); Soybean (2.2 %, CI95% 0.6

to 4.1, n = 2); Mixed grass species (0.0 %, CI95% 0.0 to 0.0, n = 3); Lettuce (-2.0 %, CI95% -17.4 to 13.2, n = 2); Winter rye (-3.1 %, CI95% -9.0 to 0.8, n = 16). According to crop type and development stage the following trend of RPE on nutrient content was observed: Maize-Vegetative (19.6 %, CI95% 13.9 to 26.9, n = 60) > Maize-Generative (7.5 %, CI95% 3.4 to 12.0, n = 52); Rapeseed-Vegetative (52.6 %, CI95% 40.9 to 66.7, n = 18) > Rapeseed-Generative (29.1 %, CI95% 23.5 to 36.7, n = 18); Sorghum-Vegetative (32.7 %, CI95% 10.7 to 56.2, n = 6) > Sorghum-Generative (13.7 %, CI95% 6.4 to 20, n = 6).

In two out of eight fertilizer placement methods (Subsurface shallow point injection and Subsurface deep point injection), nutrient content from placement was the same as that from broadcast. In six placement methods, it was higher than that from broadcast. For groups with more than 50 datasets, the trend of RPE on nutrient content was: Subsurface shallow band (15.2 %, CI95% 11.8 to 19.5, n = 98) = Subsurface deep band (14.4 %, CI95% 9.8 to19.9, n = 60).

During vegetative growth, RPE on nutrient content showed the following decreasing trend according to placement depth: > 10 cm (24.9 %, CI95% 16.8 to 33.8, n = 36) = 5 – 10 cm (23.4 %, CI95% 16.2 to 32.5, n = 51) > Surface (-5.7 %, CI95% -15.4 to 6.3, n = 4). For the generative growth stage (Reproductive and Maturity combined), nutrient content for all placement depths combined (8.7 %, CI95% 6.3 to 11.2, n = 154) was higher than that for broadcast. There were no differences in relative nutrient content between placement depths.

According to fertilizer type and placement depth, there was also a tendency for the RPE on uptake of N, P and K to increase with increasing placement depth (Figure 4.7). Placement of a combination of ammonium or urea with soluble P showed a

tendency to lead to stronger RPE on N or P uptake than placement of ammonium, urea or soluble P uncombined (Figure 4.7).

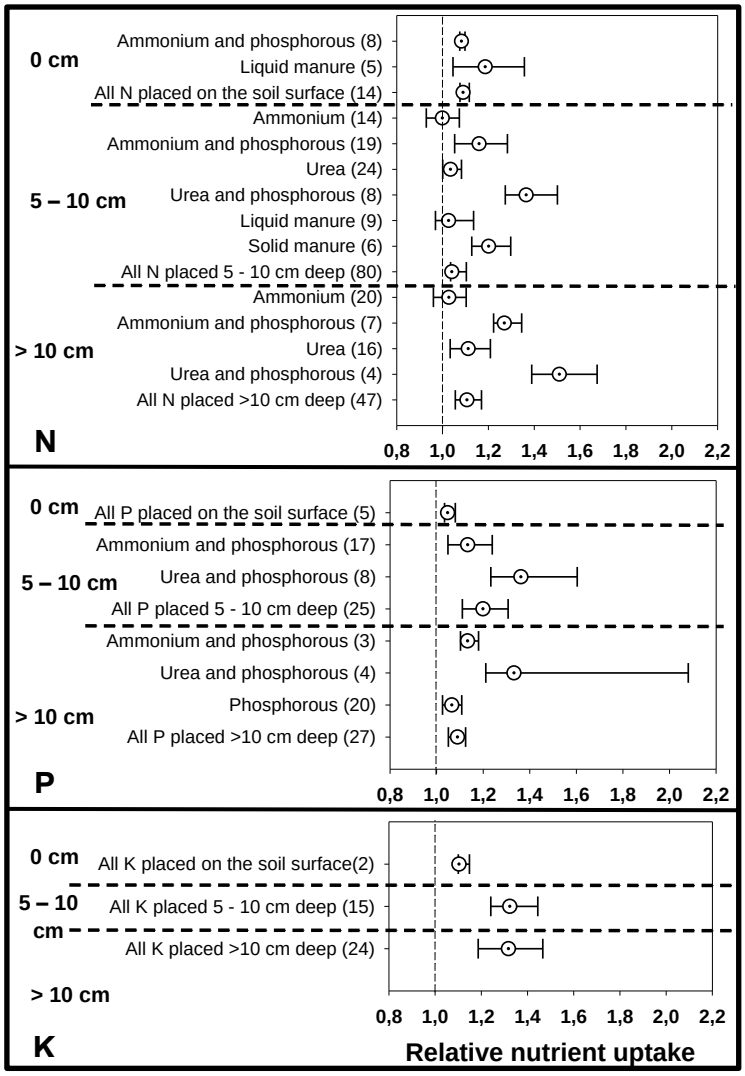

Figure 4.7. Relative contents of N, P and K in above-ground biomass by fertilizer type and placement depth (0, 5 – 10, and > 10 cm)
Y-axis, categories and number of datasets per category in brackets; **X-axis,** relative value of fertilizer **Placement** to fertilizer **Broadcast**; **Error bars**, 95 % confidence intervals; **Placement ≠ Broadcast**, if error bars do not included 1.0; Relative values of a pair of categories are different from each other if their 95% confidence intervals do not overlap.

4.5 Discussion

Subsurface placement of fertilizers close to seeds or plant roots has been shown to lead to higher nutrient uptake, higher concentration of nutrients in above-ground biomass and higher yield than homogenous broadcast of fertilizers. Likely modes of occurrence include: (1) persistence of high levels of nutrients in plant-available form close to roots; (2) stimulation of root growth close to and away from fertilizer depots based on NH_4^+, $CO(NH_2)_2$, PO_4^{3-} or their combinations for improved depot exploitation (*Arkoun* et al., 2012; *Forde* and *Lorenzo*, 2001; *Zhang* et al., 2000); (3) induction of favorable changes in chemical (*Jing* et al., 2012; *Marschner* et al., 1986; *Neumann* and *Römheld*, 2007) and biological properties of the rhizosphere (*Ghorbani* et al., 2008; *Huber* et al, 2012; *Marschner*, 2012; *Murakami* et al., 2002); and (4) reduction of nutrient loss to the environment (*Dell* et al., 2011; *Shaviv*, 1988; *Sommer*, 2003).

In accordance with prerequisite *a* formulated in section 2.3 – fertilizers suitable for placement in soil as a depot should be *taken up by plants in relevant quantities* – most published studies on fertilizer placement under field conditions that we found and utilized in our meta-analysis (shown in Table A.1, Online appendix: http://www.sciencedirect.com/science/article/pii/S0378429016302283) mainly investigated the effect of placing macronutrients N, P or K or their combinations (also organic fertilizers) on crop performance. We found little literature on placement of micronutrients in field soil (*Malhi* and *Karamanos*, 2006), given that it is not a common farming practice. Micronutrient application in soil is associated with lower nutrient recovery efficiencies than seed treatment and foliar sprays, which are more effective alternatives (*Farooq* et al., 2012).

Prerequisite *b* formulated in section 2.3, – fertilizers considered for placement as a depot should *considerably stimulate root growth and attract roots* – could also be supported. The meta-analyses showed that subsurface placement of NH_4^+ or $CO(NH_2)_2$ or both (with or without soluble phosphates, PO_4^{3-}) resulted in statistically higher (or a tendency of higher) relative yield, nutrient concentration and content in above-ground ground plant parts than subsurface placement of PO_4^{3-}. Under favorable conditions, subsurface placed $CO(NH_2)_2$ may be rapidly hydrolyzed to root growth-stimulating NH_4^+. Subsurface placement of liquid or solid manure, which also contains NH_4^+ and PO_4^{3-} led to higher yield and nutrient content in above-ground biomass than broadcast.

In disagreement to the requirement suggested at *c*, section 2.3 – suitable fertilizers for depot placement should have *limited mobility* in soil – subsurface placed $CO(NH_2)_2$ or $CO(NH_2)_2$ and PO_4^{3-} performed better than NH_4^+ or NH_4^+ and PO_4^{3-} at improving yield and biomass N or P contents. *Su* et al. (2015) observed that deep subsurface placement of $CO(NH_2)_2$ and superphosphate in winter rapeseed was associated with increased growth of lateral roots at deep soil layers as well as increased taproot diameter and length, which functioned as an important nutrient storage organ. High moisture availability in deep soil layers may promote rapid hydrolysis of $CO(NH_2)_2$ to NH_4^+ with lower mobility and stronger root growth-promotion effects. Furthermore, deep-placed $CO(NH_2)_2$ is more protected from NH_3 volatilization than one that is surface-placed or applied by broadcast and incorporated (*Ma* et al., 2010). In line with *c*, section 2.3, among all field studies reviewed, NO_3^- was placed in subsurface soil only in combination with NH_4^+, $CO(NH_2)_2$ or PO_4^{3-} or their combinations.

In divergence from the suggested prerequisites: *localized root-growth stimulation* (b, section 2.3) and *limited mobility* in soil (c, section 2.3), subsurface placement of

soluble K^+ produced statistically higher yields (>10 cm depth), K concentrations (>10 cm depth) and K content (0, 5 – 10 and > 10 cm depth) in above-ground plant parts than broadcast. This can be explained by high moisture content in deep soil layers than on the surface because K movement to roots is mainly determined mass flow and not by root interception (*Barber*, 1984). Under drought stress, it is not advisable to place any fertilizer on the soil surface or at shallow depths (*Su et al.*, 2015). Under such conditions, deep subsurface fertilizer placement has been shown to enhance resilience of crop plants to drought stress, thereby increasing yields (*Garwood* and *Williams*, 1967; *Ma* et al., 2009; *Randall* and *Hoeft*, 1988; *Singh* et al., 2005; *Su et al.*, 2015). Nevertheless, to adopt deep subsurface fertilizer placement, cost of additional mechanical power required should be considered (*Su et al.*, 2015).

The effect of placement depth on the effectiveness of fertilizer placement could be confirmed by the meta-analysis. With increasing placement depth from 0 cm to more than 10 cm, fertilizers based on NH_4^+, NH_4^+ and PO_4^{3-}, $CO(NH_2)_2$, $CO(NH_2)_2$ and PO_4^{3-}, or K^+ tend to result in an increase in yield, nutrient concentration and content in above-ground plant parts. Contrarily, placement of PO_4^{3-} without combination with NH_4^+ or $CO(NH_2)_2$, at 5-10 cm depth or >10 cm resulted in the same yield and nutrient content in above-ground biomass as broadcast. This suggests that PO_4^{3-} depots can be more efficiently exploited by plant roots if NH_4^+ or $CO(NH_2)_2$ is added to the depot to induce stronger root signaling and root-growth.

Seed treatment with fertilizer or subsurface placement of fertilizer in the seeding hole was shown to produce the same yield as broadcast. *Niehues* et al. (2004) showed that NH_4^+ placed on maize seeds at high rates (> 22 kg N ha^{-1}) led to seed or seedling damage, reduced plant density and grain yield.

Nutrient acquisition by plants from moderately or sparingly available nutrient pools in soil or from placed fertilizers may also be improved by *bio-effectors* (*Weinmann* and *Römheld,* 2012), which refer to plant growth-promoting microorganisms (PGPMs) (*Altomare* et al., 1999; *Grant* et al., 2001; *Jiang* et al., 2012; *Lugtenberg* and *Kamilova,* 2009; *Richardson* et al., 2009; *Vassilev* et al., 2006;) or active natural bio-stimulants like humic acids (*Giovannini* et al., 2013; *Muhammad* et al., 2007; *Uygur* and *Karabatak*, 2009) and seaweed extracts (*Sharma* et al., 2012). Such *bio-effectors* can be applied to seeds, aerial plant parts or soil. First field studies combining fertilizer placement and inoculation of fluorescent *Pseudomonads* as bio-effectors show promising growth-promotion effects on chickpea (*Cicer arietinum*) (*Dutta* and *Bandyopadhyay* ; 2009) and maize (*Zea mays* L.) (*Nkebiwe* et al., 2016).

4.6 Conclusion

Collectively, several field studies showed that fertilizer placement resulted in 3.7 % higher yield than broadcast and up to 27.3% for placement of urea and soluble P, and 14.7% for ammonium and soluble P. Fertilizer placement also led to higher nutrient concentrations in different plant parts by 3.7 % and nutrient content in above-ground biomass by 11.9 % than fertilizer broadcast. Deep subsurface placement of ammonium (±P) or urea (±P), potassium, solid or liquid manure (10 - 30 cm) is more effective to improve nutrient uptake and yield of field crops than broadcast with or without incorporation. This suggests that deep subsurface fertilizer placement may be an additional tool for the mitigation of negative consequences of increasingly frequent extreme weather events like high temperatures, droughts or heavy rainfall (*Parry* et al., 2004), which affect food production for an expanding global population.

4.7 References

Adamsen, F. J., Sabey, B. R. (1987): Ammonia volatilization from liquid digested sewage sludge as affected by placement in soil. *Soil Sci. Am. J.* 51, 1080–1082.

Akiyama, H., Yan, X., Yagi, K. (2010): Evaluation of effectiveness of enhanced-efficiency fertilizers as mitigation options for N_2O and NO emissions from agricultural soils: meta-analysis. *Glob. Change Biol.* 16, 1837–1846

Altomare, C., Norvell, W. A., Björkman, T., Harman, G. E. (1999): Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Appl. Environ. Microbiol.* 65(7), 2926–2933.

Anghinoni, I., Barber, S. A. (1990): Predicting the effect of ammonium placement on nitrogen uptake by corn. *Agron. J.* 82, 135–138.

Arkoun, M., Sarda, X., Jannin, L., Laîné, P., Etienne, P., Garcia-Mina, J.-M., Yvin, J.-C., Ourry, A. (2012): Hydroponics versus field lysimeter studies of urea, ammonium and nitrate uptake by oilseed rape (*Brassica napus* L.). *J. Exp. Bot.* 63, 5245–5258.

Baker, J. L. (2001): Limitations of improved nitrogen management to reduce nitrate leaching and increase use efficiency. *TheScientificWorldJOURNAL* 1, 10–16.

Baligar, V. C., Bennett, O. L. (1986): Outlook on fertilizer use efficiency in the tropics. *Fert. Res.* 10, 83–96.

Baligar, V. C., Fageria, N. K., He, Z. L. (2001): Nutrient use efficiency in plants. *Commun. Soil Sci. Plant Anal.* 32, 921–950.

Barber, S. A. (1984): Soil nutrient bioavailability. A mechanistic approach. *Wiley, New York* pp. 90–113, 218, 251.

Bautista, E., Koike, M., Suministrado, D. (2001): PM—Power and Machinery: Mechanical deep placement of nitrogen in wetland rice. *J. Agri. Eng. Res.* 78, 333–346.

Bittman, S., Liu, A., Hunt, D., Forge, T., Kowalenko, C., Chantigny, M., Buckley, K. (2012): Precision placement of separated dairy sludge improves early phosphorus nutrition and growth in corn (*Zea mays* L.). *J. Environ. Qual.* 41, 582–591.

Bhat, K. K. S., Nye, P. H. (1973): Diffusion of phosphate to plant roots in soil. *Plant Soil* 38, 161–175.

Blackshaw, R. E., Semach, G., Janzen, H. H. (2002): Fertilizer application method affects nitrogen uptake in weeds and wheat. *Weed Sci.* 50, 634–641.

Boelcke, B. (2003): Effekt der N-Injektionsdüngung auf Ertrag und Qualität von Getreide un Raps in Mecklenburg-Vorpommern, in Kücke, M.: Anbauverfahren mit N-injektion (CULTAN), Ergebnisse, Perspektiven , Erfahrungen (Beiträge des Workshops am 29. November 2001 in Braunschweig). *Bundesforschungsantalt für Landwirthschaft (FAL), Landbauforschung Völkenrode, Sonderheft 245* (*FAL Agricultural Reasearch, special issue 245*) pp. 45–53.

Borges, R., Mallarino, A. P. (2000): Grain yield, early growth, and nutrient uptake of no-till soybean as affected by phosphorus and potassium placement. *Agron. J.* 92, 380–388.

Bünemann, E. K, Prusisz, B,. Ehlers, K. (2011): Characterization of phosphorous forms in soil microorganisms, in Bünemann, E. K., Oberson, A., Frossard, E.: Phosphorous in action, Biological processes in soil phosphorous cycling. *Springer-Verlag Berlin Heidelberg, Germany*. pp. 37–58

Chassot, A., Stamp, P., Richner, W. (2001): Root distribution and morphology of maize seedlings as affected by tillage and fertilizer placement. *Plant Soil* 231, 123–135.

Clarke, A. L., Barley, K. P. (1968): The uptake of nitrogen from soils in relation to solute diffusion. *Aust. J. Soil Res.* 6, 75–92.

Colomb, B., Kiniry, J. R., Debaeke, P. (2000): Effect of soil phosphorus on leaf development and senescence dynamics of field-grown maize. *Agron. J.* 92, 428–435.

Cooke, G. W. (1954): Recent advances in fertilizer placement. II.—Fertilizer placement in england. *J. Sci. Food Agric.* 5, 429–440.

Cordell, D., Drangert, J.-O., White, S. (2009): The story of phosphorus: Global food security and food for thought. *Global Environ.Chang.* 19, 292–305.

Delgado, A., Scalenghe, R. (2008): Aspects of phosphorus transfer from soils in Europe. *J. Plant Nutr. Soil Sci.* 171, 552–575.

Dell, C. J., Meisinger, J. J., Beegle, D. B. (2011): Subsurface application of manures slurries for conservation tillage and pasture soils and their impact on the nitrogen balance. *J. Environ. Qual.* 40, 352–361.

Deppe, M., Well, R., Kücke, M., Fuß, R., Giesemann, A., Flessa, H. (2016): Impact of CULTAN fertilization with ammonium sulfate on field emissions of nitrous oxide. *Agric. Ecosyst. Environ.* 219, 138–151.

Drew, M. C. (1975): Comparison of the effects of a localised supply of phosphate, nitrate, ammonium and potassium on the growth of the seminal root system, and the shoot, in barley. *New Phytol.* 75, 479–490.

Dutta, D., Bandyopadhyay, P. (2009): Performance of chickpea (*Cicer arietinum* L.) to application of phosphorus and bio-fertilizer in laterite soil. *Arch. Agron. Soil Sci.* 55, 147–155.

Engel, R., Liang, D. L., Wallander, R., Bembenek, A. (2010): Influence of urea fertilizer placement on nitrous oxide production from a silt loam soil. *J. Environ. Qual.* 39, 115–125.

Fan, M., Shen, J., Yuan, L., Jiang, R., Chen, X., Davies, W. J., Zhang, F. (2012): Improving crop productivity and resource use efficiency to ensure food security and environmental quality in China. *J. Exp. Bot.* 63(1), 13–24.

Farooq, M., Wahid, A., Siddique, K. H. M. (2012): Micronutrient application through seed treatments. A review. *J. soil sci. plant nutr.* 12, 125–14,

Forde, B., Lorenzo, H. (2001): The nutritional control of root development. *Plant Soil* 232, 51–68.

Furukawa, T. A., Barbui, C., Cipriani, A., Brambilla, P., Watanabe, N. (2006): Imputing missing standard deviations in meta-analyses can provide accurate results. *J. Clin. Epidemiol.* 59, 7–10.

Garwood, E. A,. Williams, T. E. (1967): Growth, water use and nutrient uptake from the subsoil by grass swards. *J. Agric. Sc.* 69, 125–130.

Ghorbani, R., Wilcockson, S., Koocheki, A., Leifert, C. (2008): Soil management for sustainable crop disease control: a review. *Environ. Chem. Lett.* 6, 149–162.

Gichangi, E. M., Mnkeni, P. N. S., Brookes, P. C. (2009): Effects of goat manure and inorganic phosphate addition on soil inorganic and microbial biomass phosphorus fractions under laboratory incubation conditions. *Soil Sci. Plant Nutr.* 55, 764–771.

Giovannini, C., Garcia-Mina, J. M., Ciavatta, C., Marzadori, C. (2013): Effect of organic-complexed superphosphates on microbial biomass and microbial activity of soil. *Biol. Fertil. Soils* 49, 395–401.

Grant, C. A., Flaten, D. N., Tomasiewicz, D. J., Sheppard, S. C. (2001): The importance of early season phosphorus nutrition. *Can. J. Plant Sci.* 81, 211–224.

Grant, C. A., Moulin, A. P., Tremblay, N. (2016): Nitrogen management effects on spring wheat yield and protein concentration vary with seeding date and slope position. *Agron. J.* 108, 1246–1256.

Greenwood, D. J., Gastal, F., Lemaire, G., Draycott, A., Millard, P., Neeteson, J. J. (1991): Growth rate and % N of field grown crops: Theory and experiments. *Ann. Bot.* 67, 181–190.

Halvorson, A. D., Del Grosso, S. J. (2012): Nitrogen source and placement effects on soil nitrous oxide emissions from no-till corn. *J. Environ. Qual.* 41, 1349–1360.

Hayashi, K., Koga, N., Yanai, Y. (2009): Effects of field-applied composted cattle manure and chemical fertilizer on ammonia and particulate ammonium exchanges at an upland field. *Atmos. Environ.* 43, 5702–5707.

Hinsinger, P. (2001): Bioavailability of soil inorganic P in the rhizosphere as affected by root-induced chemical changes: a review. *Plant Soil* 237, 173–195.

Hocking, P. J., Mead, J. A., Good, A. J., Diffey, S. M. (2003): The response of canola (*Brassica napus* L.) to tillage and fertiliser placement in contrasting environments in southern NSW. *Aust. J. Exp. Agric.* 43, 1323–1335.

Hodge, A. (2004): The plastic plant: root responses to heterogeneous supplies of nutrients. *New Phytol.* 162, 9–24.

Huber, D., Römheld, V., Weinmann, M. (2012): Relationship between nutrition, plant diseases and pests, in Marschner P.: Marschner's Mineral Nutrition of Higher Plants, Third edition, *Academic Press, Elsevier, Amsterdam*, pp. 283–298.

Jiang, Y., Wu, Y., Xu, W., Cheng, Y., Chen, J., Xu, L., Hu, F., Li, H. (2012): IAA-producing bacteria and bacterial-feeding nematodes promote Arabidopsis thaliana root growth in natural soil. *Eur. J. Soil Biol.* 52, 20–26.

Jing, J., Zhang, F., Rengel, Z., Shen, J. (2012): Localized fertilization with P plus N elicits an ammonium-dependent enhancement of maize root growth and nutrient uptake. *Field Crop. Res.* 133, 176–185.

Kelley, K. W., Sweeney, D. W. (2007): Placement of preplant liquid nitrogen and phosphorus fertilizer and nitrogen rate affects no-till wheat following different summer crops. *Agron. J.* 99, 1009–1017.

Köhler, S., Bischoff, W.-A, Liebig, H.-P. (2003): Cultandüngung- ein Beitrag zum Grundwasserschutz durch Verringerung des Nitrataustrages, in Kücke, M.: Anbauverfahren mit N-injektion (CULTAN), Ergebnisse, Perspektiven , Erfahrungen (Beiträge des Workshops am 29. November 2001 in Braunschweig). *Bundesforschungsantalt für Landwirthschaft (FAL), Landbauforschung Völkenrode, Sonderheft 245 (FAL Agricultural Reasearch, special issue 245)* pp. 117–127.

Kristoffersen, A. Ø., Riley, H., Sogn, T. A. (2005): Effects of P fertilizer placement and temperature on root hair formation, shoot growth and P content of barley grown on soils with varying P status. *Nutr. Cycl. Agroecosys.* 73, 147–159.

Légère, A., Shirtliffe, S. J., Vanasse, A., Gulden, R. H. (2013): Extreme grain-based cropping systems: when herbicide-free weed management meets conservation tillage in northern climates. *Weed Technol.* 27, 204–211.

Linquist, B. A., Adviento-Borbe, M. A., Pittelkow, C. M., van Kessel, C., van Groenigen, K. J. (2012): Fertilizer management practices and greenhouse gas emissions from rice systems: A quantitative review and analysis. *Field Crop. Res.*135, 10–21.

Lu, D. Q., Chien, H. S., Henao, J., Sompongse, D. (1987): Evaluation of short-term efficiency of diammonium phosphate versus urea plus single superphosphate on a calcareous soil. *Agron. J.* 79, 896–900.

Lu, S., Miller, M. H. (1993): Determination of the most efficient phosphorus placement for field-grown maize (*Zea mays* L.) in early growth stages. *Can. J. Soil. Sci.* 73, 349–358.

Lugtenberg, B., Kamilova, F. (2009): Plant-growth-promoting rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556.

Ma, Q., Rengel, Z., Rose, T. (2009): The effectiveness of deep placement of fertilisers is determined by crop species and edaphic conditions in Mediterranean-type environments: a review. *Aust. J. Soil Res.* 47, 19–32.

Ma, B. L., Wu, T. Y., Tremblay, N., Deen, W., McLaughlin, N. B., Morrison, M. J., Stewart, G. (2010): On-farm assessment of the amount and timing of nitrogen fertilizer on ammonia volatilization. *Agron. J.* 102, 134–144.

Malhi, S. S., Grant, C. A., Johnston, A. M., Gill, K. S. (2001): Nitrogen fertilization management for no-till cereal production in the Canadian Great Plains: a review. *Soil Till. Res.* 60, 101–122.

Malhi, S. S., Karamanos, R. E. (2006): A review of copper fertilizer management for optimum yield and quality of crops in the Canadian Prairie Provinces. *Can. J. Plant Sci.* 86, 605–619.

Marschner, P., Rengel, Z. (2012): Nutrient availability in soils, in Marschner P.: Marschner's Mineral Nutrition of Higher Plants, Third edition, *Academic Press, Elsevier, Amsterdam*, pp. 315–330.

Marschner, P. (2012): Rhizosphere biology, in Marschner P.: Marschner's Mineral Nutrition of Higher Plants, Third edition, *Academic Press, Elsevier, Amsterdam*, pp. 369–388.

Marschner, H., Römheld, V., Horst, W. J., Martin, P. (1986): Root-induced changes in the rhizosphere: Importance for the mineral nutrition of plants. *Z. Pflanzenernaehr. Bodenk.* 149, 441–456.

Matson, P. A., Parton, W. J., Power, A. G., Swift, M. J. (1997): Agricultural intensification and ecosystem properties. *Science* 277, 504–509.

Melander, B., Rasmussen, I. A., Bàrberi, P. (2005): Integrating physical and cultural methods of weed control— examples from European research. *Weed Sci.* 53, 369–381.

Michalsky, R., Pfromm, P. H. (2012): Thermodynamics of metal reactants for ammonia synthesis from steam, nitrogen and biomass at atmospheric pressure. *AIChE J.* 58, 3203–3213.

Moody, P. W., Edwards, D. G., Bell, L. C. (1995): Effect of banded fertilizers on soil solution composition and short-term root growth. II. mono- and di-ammonium phosphates. *Aust. J. Soil Res.* 33, 689–707.

Muhammad, S., Müller, T., Joergensen, R. G. (2007): Compost and P amendments for stimulating microorganisms and maize growth in a saline soil from Pakistan in comparison with a nonsaline soil from Germany. *J. Plant Nutr. Soil Sci.* 170, 745–751.

Müller, T., Walter, B., Wirtz, A., Burkovski, A. (2006): Ammonium toxicity in bacteria. *Curr. Microbiol.* 52, 400–406.

Murakami, H., Tsushima, S., Shishido, Y. (2002): Factors affecting the pattern of the dose response curve of clubroot disease caused by *Plasmodiophora brassicae*. *Soil Sci. Plant Nut.* 48, 421–427.

Nash, P. R., Motavalli, P. P., Nelson, K. A. (2012): Nitrous oxide emissions from claypan soils due to nitrogen fertilizer source and tillage/fertilizer placement practices. *Soil Sci. Soc. Am. J.* 76, 983–993.

Neumann, G., Römheld, V. (2007): The release of root exudates as affected by the plant physiological status, in Pinton, R., Varanini, Z.; Nannipieri, P.: The Rhizosphere. Biochemistry and organic substances at the soil-plant interface, Ed. 2. *CRC Press, Boca Raton, Florida* pp. 23–72.

Neumann, G., Römheld, V. (2012): Rhizosphere chemistry in relation to plant nutrition, in Marschner P.: Marschner's Mineral Nutrition of Higher Plants, Third edition, *Academic Press, Elsevier, Amsterdam*, pp.347–360.

Nieder, R., Benbi, D., Scherer, H. (2011): Fixation and defixation of ammonium in soils: a review. *Biol. Fert. Soils* 47, 1–14.

Niehues, B. J., Lamond, R. E., Godsey, C. B., Olsen, C. J. (2004): Starter nitrogen fertilizer management for continuous no-till corn production contribution no. 04-099-J, K-State Research and Extension. *Agron. J.* 96, 1412–1418.

Nkebiwe, P. M., Weinmann, M., Müller, T. (2016): Improving fertilizer-depot exploitation and maize growth by inoculation with plant growth-promoting bacteria: from lab to field. *Chem. biol. technol. agric.* 3, 1–16.

Nyord, T., Søgaard, H. T., Hansen, M. N., Jensen, L. S. (2008): Injection methods to reduce ammonia emission from volatile liquid fertilisers applied to growing crops. *Biosystems Engineering* 100, 235–244.

Olson, R. A., Dreier, A. F. (1956): Fertilizer placement for small grains in relation to crop stand and nutrient efficiency in Nebraska. *Soil Sci. Soc. Am. J.* 20, 19–24.

Pang, P. C., Hedlin, R. A., Cho, C. M. (1973): Transformation and movement of band-applied urea, ammonium sulfate, and ammonium hydroxide during incubation in several Manitoba soils. *Can. J. Soil. Sci.* 53, 331–341

Parry, M. L., Rosenzweig, C., Iglesias, A., Livermore, M., Fischer, G. (2004): Effects of climate change on global food production under SRES emissions and socio-economic scenarios. *Climate Change* 14, 53–67

Peltonen-Sainio, P., Kontturi, M., Peltonen, J. (2006): Phosphorus seed coating enhancement on early growth and yield components in oat. *Agron. J.* 98, 206–211.

Petersen, J. (2005): Competition between weeds and spring wheat for 15N-labelled nitrogen applied in pig slurry. *Weed Res.* 45, 103–113.

Pfab, H., Palmer, I., Buegger, F., Fiedler, S., Müller, T., Ruser, R. (2012): Influence of a nitrification inhibitor and of placed N-fertilization on N2O fluxes from a vegetable cropped loamy soil. *Agric. Ecosyst. Environ.* 150, 91–101.

Philbrook, H. T., Barrowman, N., Garg, A. X. (2007): Imputing variance estimates do not alter the conclusions of a meta-analysis with continuous outcomes: a case study of changes in renal function after living kidney donation. *J. Clin. Epidemiol.* 60, 228–240.

Piepho, H. P., Williams, E. R., Madden, L. V. (2012): The use of two-way linear mixed models in multitreatment meta-analysis. *Biometrics* 68, 1269–1277

Prochnow, L. I., Chien, S.H., Carmona, G., Henao, J. (2004): Greenhouse evaluation of phosphorous sources produced from low-reactive Brazilian phosphate rock. *Agron. J.* 96, 761–768.

Qin, R., Stamp, P., Richner, W. (2005): Impact of tillage and banded starter fertilizer on maize root growth in the top 25 centimeters of the soil. *Agron. J.* 97, 674–683.

Randall, G. W., Hoeft, R. G. (1988): Placement methods for improved efficiency of P and K fertilizers: A Review. *J. Prod. Agr.* 1, 70–79.

Raun, W. R., Johnson, G. V. (1999): Improving nitrogen use efficiency for cereal production. *Agron. J.* 91, 357–363.

Reith, J. W. S. (1954): Recent advances in fertilizer placement. I. Fertilizer placement for swedes and turnips in Scotland. *J. Sci. Food Agric.* 5, 421–428.

Rochette, P., Angers, D. A., Chantigny, M. H., MacDonald, J. D., Gasser, M.-O., Bertrand, N. (2009): Reducing ammonia volatilization in a no-till soil by incorporating urea and pig slurry in shallow bands. *Nutr. Cycl. Agroecosys.* 84, 71–80.

Redel, Y. D., Rubio, R., Rouanet, J. L., Borie, F. (2007): Phosphorus bioavailability affected by tillage and crop rotation on a Chilean volcanic derived Ultisol. *Geoderma*, 139, 388–396.

Richardson, A. E., Barea, J.-M., McNeill, A. M., Prigent-Combaret, C. (2009): Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant Soil* 321, 305–339.

Rose, T. J., Rengel, Z., Ma, Q. F., Bowden, J. W. (2009): Crop species differ in root plasticity response to localised P supply. *J. Plant Nutrit. Soil Sci.* 172, 360–368.

Rosenberg, M. S., Adams, D. C., Gurevitch, J. (2000): MetaWin. Statistical software for meta-analysis, version 2. *Sinauer Associates, Sunderland, MA. USA.*

Rosenberg, M. S., Goodnight, C. (2005): The file-drawer problem revisited: a general weighted method for calculating fail-safe numbers in meta-analysis. *Evolution* .59, 464–468.

Ruidisch, M., Bartsch, S., Kettering, J., Huwe, B., Frei, S. (2013): The effect of fertilizer best management practices on nitrate leaching in a plastic mulched ridge cultivation system. *Agr. Ecosyst. Environ.* 169, 21–32.

Sadeghi, A. M., Kissel, D. E., Cabrera, M. L. (1989): Estimating molecular diffusion coefficients of urea in unsaturated soil. *Soil Sci. Soc. Am. J.* 53, 15–18.

Saleem, M. F., Randhawa, M. S., Hussain, S., Wahid, M. A., Anjum, S. A. (2009): Nitrogen management studies in autumn planted maize (*Zea Mays* L.) hybrids. *J. Anim. Plant Sci.* 19, 140–143.

Schenk, M. K., Barber, S. A. (1979): Phosphate uptake by corn as affected by soil characteristics and root morphology. *Soil Sci. Soc. Am. J.* 43, 880–883.

Schlegel, A. J., Dhuyvetter, K. C., Havlin, J. L. (2003): Placement of UAN for dryland winter wheat in the central high plains. *Agron. J.* 95, 1532–1541.

Sekiya, N., Yano, K. (2010): Seed P-enrichment as an effective P supply to wheat. *Plant Soil* 327, 347-354

Sharma, S. H. S., Lyons, G., McRoberts, C., McCall, D., Carmichael, E., Andrews, F., Swan, R., McCormack, R., Mellon, R. (2012): Biostimulant activity of brown seaweed species from Strangford Lough: compositional analyses of polysaccharides and bioassay of extracts using mung bean (*Vigno mungo* L.) and pak choi (*Brassica rapa* chinensis L.). *J. Appl. Phycol.* 24, 1081–1091.

Shaviv, A. (1988): Control of nitrification rate by increasing ammonium concentration. *Fert. Res*. 17, 177–188.

Singh, D. K., Sale, P. W. G., Routley, R. R. (2005): Increasing phosphorus supply in subsurface soil in northern Australia: Rationale for deep placement and the effects with various crops. *Plant Soil* 269, 35–44.

Sommer, K. (2005): CULTAN-Düngung: Physiologisch, ökologisch, ökonomisch optimiertes Düngungsverfahren für Ackerkulturen, Grünland, Gemüse, Zierpflanzen und Obstgehölze, *Verlag Th. Mann, Gelsenkirchen-Buer, Germany*.

Sommer, K. (2003): Grundlagen des " CULTAN"- Verfahrens, in Kücke, M.: Anbauverfahren mit N-injektion (CULTAN), Ergebnisse, Perspektiven , Erfahrungen (Beiträge des Workshops am 29. November 2001 in Braunschweig). *Bundesforschungsantalt für Landwirthschaft (FAL), Landbauforschung Völkenrode, Sonderheft 245 (FAL Agricultural Reasearch, special issue 245),* pp. 1–22.

Su, W., Liu, B., Liu, X., Li, X., Ren, T., Cong, R., Lu, J. (2015): Effect of depth of fertilizer banded-placement on growth, nutrient uptake and yield of oilseed rape (Brassica napus L.). *Eur. J. Agron.* 62, 38–45.

Tilman, D., Cassman ,G. K., Matson, P. A., Naylor, R. Polasky, S. (2002): Agricultural sustainability and intensive production practices. *Nature* 418, 671–677.

Tscharntke, T., Clough, Y., Wanger, T. C., Jackson, L., Motzke, I., Perfecto, I., Vandermeer, J., Whitbread, A. (2012): Global food security, biodiversity conservation and the future of agricultural intensification. *Biol. Conserv.* 151, 53–59.

Tunney, H., Carton, O.T., Brookes, P.C., Johnston, A.E. (1997): Phosphorous loss from soil to water. *CAB International, Wellingford, UK*. pp. 3, 138, 185,273 and 276.

Turner, B.L., Frossard, E., Baldwin, D.S (2005): Organic phosphorous in the environment. *CAB International, Wellingford, UK*. pp. 1–2, 75, 92–96,113, 133, 165 and 175.

Uygur, V., Karabatak, I. (2009): The effect of organic amendments on mineral phosphate fractions in calcareous soils. *J. Plant Nutr. Soil Sci.* 172, 336–345.

Valluru, R., Vadez, V., Hash, C. T., Karanam, P. (2010): A minute P application contributes to a better establishment of pearl millet (*Pennisetum glaucum* (L.) R. Br.) seedling in P deficient soils. *Soil Use Manag.* 26, 36–43.

van Kessel, C., Venterea, R., Six, J., Adviento-Borbe, M. A., Linquist, B., van Groenigen, K. J. (2012): Climate, duration, and N placement determine N_2O emissions in reduced tillage systems: a meta-analysis. *Glob. Change Biol.* 19, 33–44.

Vassilev, N., Vassileva, M., Nikolaeva, I. (2006): Simultaneous P-solubilizing and biocontrol activity of microorganisms: potentials and future trends. *Appl. Microbiol. Biot.* 71, 137–144.

Weaver, D. M., Ritchie, G. S., Anderson, G. C., Deeley, D. M. (1988): Phosphorus leaching in sandy soils .I. Short-term effects of fertilizer applications and environmental conditions. *Aust. J. Soil Res.* 26, 177–190

Weber, E. A., Koller, W.-D., Graeff, S., Hermann, W., Merkt, N., Claupein, W. (2008): Impact of different nitrogen fertilizers and an additional sulfur supply on grain yield, quality, and the potential of acrylamide formation in winter wheat. *J. Plant Nutr. Soil Sci.* 171, 643–655.

Weligama, C., Tang, C., Sale, P. W. G., Conyers, M. K., Liu, D. L. (2008): Localised nitrate and phosphate application enhances root proliferation by wheat and maximises rhizosphere alkalisation in acid subsoil. *Plant Soil* 312, 101–115.

Weinmann, M,. Römheld, V. (2012): Resource Preservation by Application of BIOefFECTORs in European Crop Production (Research proposal). European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 312117 (BIOFECTOR). *http://www.biofector.info/*

Zerulla, W., Barth, T., Dressel, J., Erhardt, K., Horchler von Locquenghien, K., Pasda, G., Rädle, M., Wissemeier, A. (2001): 3,4-Dimethylpyrazole phosphate (DMPP) - a new nitrification inhibitor for agriculture and horticulture. *Biol. Fert. Soils* 34, 79–84.

Zhang, H., Schroder, J. L., Fuhrman, J. K., Basta, N. T., Storm, D. E., Payton, M. E. (2005): Path and multiple regression analyses of phosphorus sorption capacity. *Soil Sci. Soc. Am. J.* 69, 96–106.

Zhang, M., Nyborg, M., Malhi, S. S., Solberg, E. D. (2000): Localized root growth in soil induced by controlled-release urea granule and barley nitrogen uptake. *J. Plant. Nut.* 23, 413–422.

Zhang, X. K., Rengel, Z. (2002): Temporal dynamics of gradients of phosphorus, ammonium, pH, and electrical conductivity between a di-ammonium phosphate band and wheat roots. *Aust. J. Agric. Res.* 53, 985–992.

Zhou, S.-L., Wu, Y.-C., Wang, Z.-M., Lu, L.-Q., Wang, R.-Z. (2008): The nitrate leached below maize root zone is available for deep-rooted wheat in winter wheat–summer maize rotation in the North China Plain. *Environ. Pollut.* 152, 723–730.

5 Densely rooted rhizosphere hotspots induced around subsurface NH_4^+-fertilizer depots: a home for P-solubilizing PGPMs?

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5.1 Abstract

In natural soil, populations of inoculated plant growth-promoting microorganisms (PGPMs) generally decline after application due to one or more unfavorable biotic and/or abiotic factors. Placement of a subsurface fertilizer depot that is based on concentrated stabilized NH_4^+ stimulates localized zones of dense rooting (rhizosphere hotspots). In such densely rooted soil areas, inoculated PGPMs may thrive supported by the presence of high concentrations of organic nutrients released as root exudates. Nevertheless, sometimes placement of an NH_4^+–depot does not adequately stimulate dense localized rooting due to other factors. The objectives of this study were to investigate: 1.) the effect of background soil N_{min} on localized root-growth around NH_4^+ –depots 2.) the tolerance of selected PGPMs to high concentrations of NH_4^+±DMPP (nitrification inhibitor 3, 4-dimethylpyrazole phosphate) as can be found around a rich NH_4^+ –depot; 3.) the ability of selected NH_4^+-tolerant PGPMs to solubilize inorganic P; and 4.) the establishment of the most promising NH_4^+-tolerant and P-solubilizing PGPM in soil around an NH_4^+ –depot. We conducted a rhizobox experiment with spring wheat (*Triticum aestivum L*) to investigate the effect of background N_{min} (0, 5, 20 and 60 mg N kg^{-1}) on localized root growth around a 1g NH_4^+-N depot. Through *in vitro* tests, we investigated the tolerance of selected PGPMs to 0, 2, 10, 50, 250, 1250 mM NH_4-N and 0, 0.1, 1 and 3 M NH_4-N±DMPP, of which selected candidates were tested for their ability to solubilize the tri-calcium phosphate ($Ca_3(PO_4)_2$) (Ca-P), rock phosphate (RP) or sewage sludge ash (SA). One candidate PGPM was tested in a rhizobox experiment with maize (*Zea mays* L.) for its ability to establish in soil around a ^{15}N-labelled $(NH_4)_2SO_4$+DMPP depot. Moderate background N_{min} (5 and 20 mg N kg^{-1}) improved depot-zone root growth whereas high bulk soil N_{min} (60 mg N kg^{-1}) had a negative effect. All PGPMs showed substantial tolerance to up to 1250 mM NH_4-N. Through acidification, *Pseudomonas sp.* DSMZ 13134 and *B. amyloliquefaciens* FZB42 (not *Trichoderma harzianum* T-22) solubilized Ca-P and RP whereas SA was not solubilized despite marked acidification. Placed ^{15}N-labelled $(NH_4)_2SO_4$+DMPP-depot led to increased localized rooting, rhizosphere acidification, Shoot ^{15}N signal, N and P concentrations and contents than homogenously applied $Ca(NO_3)_2$. Inoculation of *Pseudomonas sp.* DSMZ 13134 tended to increase shoot N and P concentrations, and shoot N content relative to the non-inoculated control. Higher colonization rate of *Pseudomonas sp.* DSMZ 13134 could be measured in soil 5x5 cm to seeds for the treatments with placed $(NH_4)_2SO_4$+DMPP depot than in those with homogenous supply of $Ca(NO_3)_2$. Results suggest that the survival and establishment of P-solubilizing PGPMs that are tolerant to high NH_4^+ concentrations can be enhanced if they are inoculated in soil around a concentrated subsurface NH_4^+-fertilizer depot.

Keywords: fertilizer placement; localized root-growth; PGPM; P-solubilizing bacteria

5.1.1 Background

Placement of manures or mineral fertilizers in subsurface soil has been shown to result in higher nutrient uptake and yield in different crop species than conventional fertilizer application by surface broadcast (*Federolf* et al. 2016; *Jing* et al. 2012; *Miller* and *Ohlrogge* 1958; *Nkebiwe* et al. 2016a). Among other factors, efficient exploitation of subsurface fertilizer depots depends on intense rooting around the nutrient patch and on improved nutrient uptake rates. Therefore, it is necessary that subsurface fertilizer depots contain high concentrations of nutrients like NH_4^+ or HPO_4^{2-}/ $H_2PO_4^-$ (or $CO(NH_2)_2$ if conditions for ammonification are optimal) that are poorly mobile in soil, therefore remain in the vicinity of the depot (*Barber* 1984; *Clarke* and *Barley* 1968; *Pang* et al. 1973; *Schenk* and *Barber* 1979) and strongly stimulate localized root growth at the depot upon contact with roots (*Drew* 1975; *Jing* et al. 2012). In contrasting investigations, however, it has been observed that intense rooting does not occur in a subsurface fertilizer patch based on manure or mineral N-fertilizer under both greenhouse and field conditions (*Lamb* et al. 2004; *Müller et al*. 2009, unpublished results). As an explanation of this contradiction, we hypothesized that localized root-growth around a subsurface NH_4^+-depot depends on a high nutrient concentration gradient between the nutrient patch and the background or bulk soil and that localized root-growth response at a fertilizer depot is more likely to occur in nutrient-poor soils than in nutrient-rich ones. Likewise, it is proposed to place high and even toxic concentrations of NH_4^+ in subsurface soil to form a rich N-depot certainly contrasting the N-concentration in its surrounding. In addition toxicity inhibits microbial nitrification in the depot, persistently releasing NH_4^+ to induce intense localized rooting, a method labelled Controlled Long-term Ammonium Nutrition (CULTAN) (*Sommer* 2005). An NH_4^+-Depot containing a nitrification

inhibitor (NI) such as 3, 4-dimethylpyrazole phosphate (DMPP) (*Zerulla* et al. 2001) is certainly of additional benefit to enhance the persistence of N in the form of NH_4^+ in soil.

Nevertheless, for certain plant species responding to nutrient patches by high root turnover rates within the patch (equally high root birth and death rates) e.g. *Ambrosia artemisiifolia*, no net increase in root density may be observed in the nutrient patch even weeks after subsurface fertilizer placement (*Gross* et al. 1993).

An approach to improve plant nutrient acquisition from placed subsurface NH_4^+-depots is to inoculate soil immediately surrounding the depot with plant growth promoting microorganisms (PGPMs) such as P-solubilizing bacteria.

PGPMs have been described to promote plant growth via several mechanisms and each PGPM typically improves plant growth by more than one of these mechanisms. PGPMs like *Pseudomonas sp. Bacillus sp.* or *Xanthomonas sp.* have been shown to produce the phytohormone indole acetic acid (IAA) to promote plant root-growth and /or the enzyme ACC-deaminase, which reduces ethylene levels thus delaying root senescence under stress conditions (*Jiang* et al. 2012; *Lugtenberg* and *Kamilova* 2009; *Mohite* 2013; *Shakir* et al. 2012). Additionally, these PGPMs have been shown to synthesize antifungal metabolites (e.g. various phenols and phenazines) for direct control of root pathogenic fungi in soil and low-molecular weight iron-complexing ligands (e.g. the siderophore pyoverdine) for indirect suppression of root-disease agents via competition against root pathogens for iron and other nutrients (*Mavrodi* et al. 2012; *Vassilev* et al. 2006). Other species of *Bacillus*, *Pseudomonas*, *Enterobacter* and *Trichoderma* have been shown to release protons, organic acids and chelating metabolites (e.g. Oxalic, fumaric, citric, DL-malic, DL-lactic and succinic acids) that

enhance the solubility of sparingly soluble mineral phosphates and/or secrete enzymes like phytases and phosphatases to mineralize organic phosphates in soil (*Altomare* et al. 1999; *Barea* et al. 2002; *Bashan* et al. 2013; *Panda* et al. 2016; *Pérez* et al. 2007; *Singh* and *Satyanarayana* 2012). However, for the approach to inoculate soil around subsurface NH_4^+-depots with PGPMs to be effective, PGPMs must show considerable tolerance to extreme pH, high NH_4^+ concentrations and possibly nitrification and urease inhibitors present in and around the fertilizer-depot, in order to colonize the gradually developing densely rooted rhizosphere hotspot.

The main aim of this study was to employ fertilizer placement as a tool to induce dense localized rooting in soil areas around subsurface fertilizer depots ("rhizosphere hotspot"). Rhizosphere hotspots are proposed to be soil areas with dense root-growth and a high potential for the establishment of inoculated P-solubilizing PGPMs, which may be supported by the presence of high concentrations of organic C and N nutrients released as root exudates into the rhizosphere. The objectives of this study were to investigate: 1.) the effect of background mineral N concentration on depot-zone root-growth aiming on the stimulation of dense rooting around concentrated subsurface NH_4^+-depots and; 2.) the tolerance of selected microbial bio-effectors (BEs) to high NH_4^+- concentrations (*in vitro*) comparable to those in boarder areas of concentrated subsurface NH_4^+-depots with and without the nitrification inhibitor DMPP; 3.) the potential of selected microbial BEs to solubilize different sparingly soluble inorganic P forms; and 4.) the establishment of the best performing P-solubilizing PGPM in a rhizosphere hotspot around a placed subsurface ^{15}N labelled $(NH_4)_2SO_4$+DMPP depot.

We hypothesized that: 1.) Development of rhizosphere hotspots around $(NH_4)_2SO_4$ depots depends on background substrate N_{min} and rhizosphere hotspots form when background N_{min} is low and not when it is high. 2.) In *in vitro* cultures, microbial BEs tolerate to high

concentrations of NH_4^+-N ($(NH_4)_2SO_4$ with and without the nitrification inhibitor DMPP) that are toxic even for crops species (e.g. rice, *Oryza sativa* L.) that show tolerance to NH_4^+. 3.) Microbial BEs are able to solubilize different sparingly soluble mineral phosphates in solid and liquid *in vitro* cultures. 4). Inoculation of a P-solubilizing microbial BE in the rhizosphere hotpot around a subsurface NH_4^+-depot improves BE establishment and increases P uptake by maize (*Zea mays* L.).

5.1.2 Materials and methodology

5.1.2.1 Effect of background N_{min} on root growth around subsurface NH_4^+-depots

We cultivated spring wheat (*Triticum aestivum L.* var Schirocco, KWS, 1197, 29296 Bergen, Germany) in PVC-rhizoboxes (40 x 20 x 2 cm, H x W x D). The Substrate was composed of 80 % C-horizon loess (P_{CAL}, 5 mg kg^{-1}; P_{total}, 332 mg kg^{-1}; pH ($CaCl_2$), 7.6; C_{org}, <0.3 %; N_{total} 0.02 %) and 20 % quartz sand (0.6–1.2 mm Ø) (w/w). Basic fertilization included (kg^{-1} dry soil DM): 150 mg P ($Ca(H_2PO_4)_2$), 200 mg K (K_2SO_4), 100 mg Mg ($MgSO_4$), 20 µmol Fe-EDDHA (Sequestrene138, 6 % Fe), 2.6 g Zn ($ZnSO_4$); 1 mg Cu ($CuSO_4$); 2.2 mg Mn ($MnSO_4$), 0.54 mg Mo (Na_2MoO_4) and 0.86 mg B (H_3BO_3). Moisture content was set to 26% (60-70% max. WHC). Treatments included increasing bulk soil N_{min} concentrations: 0, 5, 20, and 60 mg N kg^{-1} soil DM (NH_4NO_3). A stabilized NH_4^+-depot was placed 7 cm to the side and 16 cm below two wheat seedlings in each rhizobox. The NH_4^+ depot (1.0 g NH_4-N +0.17g NO_3-N + 0.01 g DMPP) was made by placing 3.49 g $(NH_4)_2SO_4$ in soil over which 2.5 ml solution of 1.1 M NH_4NO_3 containing DMPP(4 g L^{-1}) was pipetted. The depot was mixed in a small soil volume of 5 cm^3 (2.5 cm Ø x 2cm depth) resulting in a very high NH_4-N concentration within the depot of 78.4 mg g^{-1} soil (bulk density, 1.3 g cm^{-3}). DMPP was added at the recommended rate of 1% NH_4-N (w/w) (*Zerulla* et al. 2001). Each rhizobox contained 2.6 kg substrate.

Before transplanting, seed surfaces were disinfected by soaking in 3 % H_2O_2 for 10 min. Afterwards seeds were soaked for 10 hrs. in 2.5 mM $CaSO_4$ and pre-germinated on filter papers that were also soaked in 2.5 mM $CaSO_4$ (48 hours at 25°C in darkness). Two healthy seedlings (distinct shoot and root length ≥ 2 cm) were transplanted to each rhizobox. Greenhouse conditions were as follows: day/night length: 8h/16 h, daytime light intensity: 311 µmol $m^{-2}s^{-1}$ (ALMEMO 239-3, AHLBORN), av. daily temperature 15 °C (8-21 °C) and av. daily relative humidity 42 % (30 %-65 %) (Voltcraft, DL-141 TH). Four replicates were made per treatment and were arranged in a completely randomized design. Plants were grown for 60 days (10 February - 07 April 2013). Total root length visible through the root observation window of the rhizobox in the radial zones: 0-4, 4-8 and > 8 cm from the nutrient depot was measured at 12, 36 and 56 days after planting. Roots were drawn on a clear plastic sheet (40 X 20 cm) placed over the soil surface on the rhizobox window using a fine black water-resistant marker. Drawings were scanned (Epson Expression 10000 XL) and analyzed for total root length using *WinRhizo Pro V. 2009c* (Regent Instruments Inc., Canada). At the end of the growth period, root length density (cm cm^{-3} of substrate) could not be appropriately measured because, as shown in Fig. 2, roots grew into the nylon sheet lining the rear of the rhizobox, making it impossible to satisfactorily harvest roots. After harvesting, shoot dry weight were measured, depot and bulk soil samples were collected and frozen for later analysis of NH_4-N and NO_3-N concentrations.

5.1.2.2 PGPM tolerance to high N concentrations

The following PGPMs were cultured on various solid nutrient media with increasing N concentrations (Table 5.1):

Table 5.1. List of PGPMs

Nr.	Active microorganism	Product name	Supplier	Recommended solid nutrient media
1	*Bacillus atrophaeus*	-	ABiTEP GmbH, Berlin, Germany	Nutrient agar (NA)
2	*Bacillus simplex* R41	-	ABiTEP GmbH, Berlin, Germany	NA
3	Bacillus spec.	-	ABiTEP GmbH, Berlin, Germany	NA
4	*Penicillium sp*. PK 112	Biological Fertilizer DC	Bayer Crop Science, Germany	Malt extract peptone agar (MEP)
5	*Bacillus amyloliquefaciens* FZB42	RhizoVital ®42	ABiTEP GmbH, Berlin, Germany	Deutsche Einheitsverfahren Agar (DEV) and NA
6	*Pseudomonas sp.* DSMZ 13134	Proradix® WP	Sourcon Padena, Tübingen, Germany	King's B medium (KM)
7	*Trichoderma harzianum* OmG08	-	Hochschule Anhalt, Bernburg, Germany	MEP
8	*Trichoderma harzianum*	Trichoderma-WG	Bayer Crop Science, Germany	MEP
9	*Trichoderma harzianum* T50	Vitalin T50	Vitalin Pflansengesundheit GmbH, Ober-Ramstadt, Germany	MEP
10	*Trichoderma harzianum* T-22 (only in tests ± nitrification inhibitor DMPP)	Trianum-P	Koppert Biological Systems B.V., The Netherlands	MEP

For each culture medium, six solutions with the following NH_4^+ concentrations were prepared using deionized water: 0, 2, 10, 50, 250 and 1250 mM NH_4-N ($(NH_4)_2SO_4$). For each NH_4^+-level, the constituents per liter of medium were dissolved in the corresponding solution to a final volume of 1000 ml. The pH was then adjusted to the desired level using 1N NaOH or HCl and finally, the medium was sterilized by autoclaving at 121°C for 20 min.

The following solid media were prepared for each NH_4^+-level:

1.) **Nutrient agar** (5.0 g peptone; 3.0 g meat extract; 15.0 g agar; pH 7); 2.) **King's B medium** (From ready-made preparation (King's B medium (Basis), Carl Roth GmbH + Co. KG). (38 g medium and 10 ml glycerol in 990 ml deionized water; pH 7); 3.) **DEV medium** (Deutsche Einheitsverfahren) (10.0 g peptone; 10.0 g meat extract; 5.0 g NaCl; 18.0 g agar; pH 7.2); and 4.) **Malt extract peptone agar** (30.0 g malt extract; 3.0 g soya peptone; 15.0 g agar; pH 5.6).

At 45 °C, about 20 ml of each medium was poured into a sterile petri dish. For bacterial PGPMs, inocula were prepared as diluted suspensions in 2.5 mM $CaSO_4$. For each bacteria and NH_4^+-level, 100 µl inoculum (about 50 CFUs) was pipetted onto a petri dish and evenly spread using a Drigalski spatula. For each fungi, a small fragment of pre-cultured mycelium was placed at the center of the petri dish. There were three replicates per treatment randomly placed on the same incubator shelf (Incubator: WBT, Binder, Typ 1711509900313, Tuttlingen, Germany) and cultured at 24 ± 1°C under aerobic conditions in darkness. *Trichoderma* were grown for 48 hours and the remaining PGPMs for 1 week.

An additional incubation test on normal NH_4^+-free agar was performed with selected microbial BEs, which were each pre-incubated at 25 ± 2°C for 15 min. or 24 hrs. in

solutions containing high concentrations of NH_4^+ ($(NH_4)_2SO_4$ ± DMPP) before inoculation on normal nutrient agar. This was to investigate whether BEs could survive when applied in a concentrated NH_4^+-fertilizer solution ($(NH_4)_2SO_4$ ± DMPP) before placement in soil. *Pseudomonas sp.* DSMZ 13134, *Bacillus amyloliquefaciens* FZB42 and *Trichoderma harzianum* T-22 were tested. After pre-incubation for 15 minutes, 100 µl of each bacterial inoculum suspension in 0, 0.1, 1 or 3 M $(NH_4)_2SO_4$ ± DMPP (about 50 CFUs) was inoculated on culture media and incubated at 25 ± 2°C for 48 hrs. in darkness under aerobic conditions. For *T. harzianum* T-22, 100 µl 0, 0.1, 1 or 3 M $(NH_4)_2SO_4$ ± DMPP was pipetted on a fragment of pre-cultured mycelium and after 15 min., the mycelium fragment was placed on NH_4^+-free agar. This procedure was adopted for *T. harzianum* T-22 because the effect of $(NH_4)_2SO_4$ ± DMPP on its survival and growth could be best quantitatively analyzed by measuring the diameter of intact pieces of growing mycelium. There were four randomly placed replicates per treatment. The other duration for pre-incubation of BEs was 24 hrs. For this test, BEs were pre-incubated only in 1 or 3 M $(NH_4)_2SO_4$ ± DMPP with three replicates per treatment to reduce bulk and facilitate overall management of the incubation experiments. For 24 hrs. pre-incubation, *T. harzianum* T-22 spores were used as inoculum instead of a fragment of pre-cultured mycelium because it is more relevant to field application. However, only qualitative analysis of the survival and growth of *T. harzianum* T-22 was possible because of the absence of a distinct circular mycelium for measurement of diameter .For *T. harzianum* T-22, the diameter of the mycelium was measured. For bacteria, colony characteristics were recorded and for *Pseudomonas*, colonies were observed under UV light for typical yellow-green fluorescence.

5.1.2.3 Solubilization of inorganic phosphates by PGPMs

5.1.2.3.1 Solid media culture

To investigate the ability of selected PGPMs to solubilize insoluble inorganic phosphates, Deubel-Muromcev solid agar containing suspended precipitates of tri-calcium phosphate ($Ca_3(PO_4)_2$) (Ca-P) was prepared (*Deubel*, 1996*; Muromcev* 1958). 0.2 g K_2SO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 20 g Purified agar (Oxoid, No. LP 0028) were dissolved to a total volume of 1000 ml using deionized water. The pH of the solution was adjusted to 6.9 using and then it was autoclaved (121°C, 20 min). After cooling the solution to 60 °C, 10 g D (+) Glucose monohydrate (6887.1, Roth, Karlsruhe, Germany) dissolved in 110 ml deionized water and 1 g L-Asparagine monohydrate (11160, Fluka, Buchs, Germany) dissolved in 50 ml deionized water were added (Solution A). To precipitate non-soluble Ca-P in the medium, 2.2 g $CaCl_2 \cdot 2H_2O$ (2461.1000 Chem Solute Th.Geyer, Renningen, Germany) dissolved in 20 ml deionized water and 3.8 g $Na_3PO_4 \cdot 12H_2O$ (6578, E. Merck, Darmstadt, Germany) also dissolved in 20 ml deionized water were each autoclaved (121°C, 20 min), cooled down 60°C and simultaneously added to Solution A under continuous stirring resulting in immediate clouding of the medium through the formation of Ca-P ($Ca_3(PO_4)_2$) (Eqn. 5.1)

Equation 5.1. Preparation of ($Ca_3(PO_4)_2$)

$3CaCl_2.2H_2O + 2Na_3PO_4.12H_2O \rightarrow Ca_3(PO_4)_2 + 6NaCl + 30H_2O$ (**Eqn. 5.1**)

Between 40-45°C the medium was poured into petri dishes.

After solidification and cooling of the agar, suspensions of PGPMs in 2.5 mM CaSO4 (1×10^4 CFU or spores ml^{-1}) were inoculated and plates were incubated at 25 °C.

PGPMs included: (1) *Penicillium sp*. PK 112, (2) *Pseudomonas sp.* DSMZ 13134, (3) Vitalin SP11 (combined product: *Bacillus subtilis*, *Pseudomonas sp*., *Streptomyces spp*., natural humic acids and seaweed extract (*Ascophyllum nodosum*)) (Vitalin Pflansengesundheit GmbH, Ober-Ramstadt, Germany), (4), *Paenibacillus mucilagenosus* (ABiTEP GmbH, Berlin, Germany), (5) *Bacillus amyloliquefaciens* FZB 42, (6). *Trichoderman harzianum* T-22.

Additionally, two other media were prepared in which instead of simultaneously adding solutions of $CaCl_2 \bullet 2H_2O$ and $Na_3PO_4 \bullet 12H_2O$ at 60 °C under stirring to produce Ca-P precipitate, finely ground (< 1 mm Ø) rock phosphate (RP, 7.6 % P) or sewage sludge ash (SA, 10.3 % P) was added at the rate of 0.3 g P l^{-1}. RP was chosen because it is an important low-cost sparingly-soluble inorganic P-fertilizer (the only mineral P fertilizer allowed in organic farming) and SA was chosen as renewable low-cost sparingly-soluble P fertilizer produced from waste recycling. Only *Pseudomonas sp.* DSMZ 13134 was inoculated on RP and SA media.

After two days all petri dishes were observed for clarification of Ca-P, RP or SA along streaks and then scanned on a dark background.

5.1.2.3.2 Liquid media culture

5.1.2.3.2.1 Treatments

Based on Deubel-Muromcev solid agar, liquid media with Ca-P was prepared by simply omitting addition of agar to solidify the media. Two other liquid media were prepared in which Ca-P was replaced by either rock phosphate (RP, 7.6 %) or sewage sludge ash (SA, 10.3 %). For each of these media Ca-P, RP or SA was the only source of P present. The liquid culture conditions, sample collection and preparation, and analysis were based on methods described by (*de Freitas* et al. 1997). Treatments

included factorial combinations of three levels of sparingly soluble P fertilizers (**Ca-P**, **RP** or **SA)** and four PGPM levels as microbial bio-effector (No bio-effector (**No BE**), *Pseudomonas sp.* DSMZ 13134 (**Pro**); *Bacillus amyloliquefaciens* FZB42 (**Rhiz**); and *Trichoderma harzianum* T-22 (**T-22**). There were four replicates per treatment.

5.1.2.3.2.2 Preparation of solutions

Using sterile deionized water (autoclaved at 121° C for 20 min), 0.2 g K_2SO_4 and 0.4 g $MgSO_4.7H_2O$ were dissolved to 1000 ml and pH was adjusted 6.9 (**Basal solution**); 45.45 g D-Glucose was dissolved to 500 ml (**Glucose**); 4 g of L-Asparagine was dissolved to 200 ml (**L-Asparagine**); 5.5 g $CaCl_2.2\ H_2O$ was dissolved to 50 ml (**$CaCl_2$**); 9.5 g $Na_3PO_4.12H_2O$ was dissolved to 50 ml (**Na_3PO4**). 60 ml of Basal solution was transferred into 100 ml Erlenmeyer flasks, autoclaved and then allowed to cool down in a water bath to 80-90 °C. Glucose and L-asparagine were each heated up to 70 °C in a water bath for 30 min. $CaCl_2$ and Na_3PO4 were each autoclaved and allowed to cool down to room temperature.

5.1.2.3.2.3 Preparation liquid media

Each P form (Ca-P, RP and SA) was applied separately at the level of each experimental unit (100 ml Schott Duran flak) ensuring 0.018 g P $flask^{-1}$ because it was impossible to pour the right quantity of P from a stock suspension into each 100 ml flask due to normal rapid sedimentation of P forms after suspension. 1.2 ml $CaCl_2$ and 1.2 ml Na_3PO4 were added to 60 ml Basal solution in each flask under continuous stirring to evenly disperse the Ca-P precipitate formed. The total concentration of P was 0.3 g l^{-1} and NaCl was 1.75 g l^{-1}.

0.237 g (0.018 g P) RP (composition %: P_{Total} 7.6; $P_{Citric\ acid}$ 6.8; P_{NaHCO3} 1.7; Al 1.4; Ca 22.5; Fe 5.1; Si 7.7) or 0.174 g (0.018 g P) SA (composition %: P_{Total} 10.34; $P_{Citric\ acid}$ 4.49; Al 9.9; Ca 8.6; Fe 4.2; composition ppm: Cd 4.2; Cr 90.8; Cu 814; Ni 78.5; Pb 224; V 59.1; Zn 4004) was weighed with a semi/micro analytical balance (Mettler AT261 DeltaRange, Mettler-Toledo GmbH, Giessen, Germany) and added under continuous stirring to 60 ml Basal solution. Total P concentration was also 0.3 g P l^{-1}. 0.105 g of NaCl was added to each RP or SA flask to maintain the same NaCl concentration in Ca-P flasks (1.75 g l^{-1}.). In each Ca-P, RP or SA flask, 6.6 ml Glucose and 3 ml L-asparagine was added and swelled to mix.

5.1.2.3.2.4 Inoculation and incubation

Using sterile 2.5 mM $CaSO_4$, without PGPM (Control) or with the PGPMs: *Pseudomonas sp.* DSMZ 13134, 6.6 X 10^{10} CFUs g^{-1} (**Pro),** *Bacillus amyloliquefaciens* FZB42, 2.5 X 10^{10} Spores ml^{-1} (**Rhiz**) and *Trichoderma harzianum* T-22, 1 X 10^{9} Spores g^{-1} **(T-22**) were each sequentially diluted to produce a suspension of 1 X 10^{4} CFU or spores ml^{-1}.

For the non-inoculated control, 2 ml of sterile 2.5 mM $CaSO_4$ solution was added to each flask. For each PGPM treatment, 2 ml inoculum suspension was added. All flasks were randomly placed on two shelves in an incubator (GFL Typ 3032, Gesselschaft für Labortechnik mbH, Burgwedel, Germany) in darkness at 30 °C. To prevent sedimentation of Ca-P, RP or SA, the incubator was set to swell flasks continuously horizontally at 125 rpm.

5.1.2.3.2.5 Measurements

After 65 hrs. of incubation, 10 ml sample of medium was collected from each flask into a sterile 50 ml falcon tube after solids were allowed to settle. Tubes were

centrifuged at 5000xg for 15 min. and supernatants were carefully poured into 30 ml polyethylene bottles and stored frozen (-18 °C). For further analysis, samples were defrosted at room temperature, pH was measured (pH/Conductivity Meter, MPC227, Metler Toledo) and they were filtered through P-free blue-band filter papers (MN 640 d) for later analysis of total P concentration by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) (L.A. Chemie, University of Hohenheim).

Acid and alkaline phosphatase activity in the samples were also measured. Based on a modified p-nitrophenyl phosphate method ((*Tabatabai* and *Bremner* 1969), acid phosphatase activity was measured by dephosphorylation of 0.1 ml 150 mM p-nitrophenyl phosphate as substrate in a 1.5 ml Eppendorf tube containing 0.4 ml buffer (200 mM Na-acetate buffer, pH 5.2 for acid phosphatase or 200 mM Na-borate buffer, pH 8.2 for alkaline phosphatase), 0.4 ml deionized water and 0.1 ml liquid culture supernatant sample by incubation at 30 °C under continuous shaking. For acid phosphatase activity incubation was stopped after 30 minutes whereas for alkaline phosphatase activity, it was stopped after 45 minutes because of weak coloration observed at 30 minutes. To stop enzyme activity, 0.5 ml of 0.5 M NaOH was added immediately after incubation. For controls or blanks, a second set of tubes containing liquid media sample, buffer and was incubated as described above. After incubation, NaOH was added followed by substrate p-nitrophenyl phosphate. Both sets of samples and blanks were centrifuged (14000 rpm for 5 min.). About 800µl supernatant was pipetted into a 1 ml disposable micro cuvette and measured for absorbance of yellow coloration at 405 nm using p-nitrophenol (p-NP) standards containing volumes of p-NP stock (20 µg ml^{-1}), 500 µl 0.5 M NaOH and volumes of deionized water to top up to 1.5 ml. p-NP standards included: 0, 2, 4, 8, 12, 16 and 20 µg p-NP ml^{-1}).

5.1.2.4 Establishment of *Pseudomonas sp.* DSMZ13134 rhizosphere hotspots

The methodology for this experiment is fully described in (*Nkebiwe* et al. 2016b).Using a substrate composed of 80% loess ((P_{CAL}, 5 mg kg^{-1}; P_{total}, 332 mg kg^{-1}; pH ($CaCl_2$), 7.6; $CaCO_3$, 23.3%; C_{org}, <0.3 %; N_{total} 0.02 %))and 20% sand filled in rhizoboxes as also described in section 2.1, maize (*Zea mays* L. var Colisee) was grown to investigate the establishment of *Pseudomonas sp*. DSMZ13134 in rhizosphere hotspots around NH_4^+-depots. The substrate was optimally supplied (kg^{-1} soil DM) with 150 mg P ($Ca(H_2PO_4)_2$), 150 mg K (K_2SO_4); 50 mg Mg ($MgSO_4$); 20 µmol Fe, (Sequestrene138, 6 % Fe); 2.6 mg Zn ($ZnSO_4$) and 1 mg Cu, (CuSO4). Moisture content was 18% about 2400 g of substrate were filled into each rizobox (H x W x D: 40 x 20 x 2 cm).

Treatments included two factorial combinations of two N levels: 100 mg NO_3-N kg^{-1} soil as $CaNO_3$ homogenously mixed in the substrate (**NO_3^--Mixed**) and 100 mg NH_4-N as concentrated ^{15}N labelled stabilized $(NH_4)_2SO_4$+DMPP (64 mg N ml^{-1}) placed as a depot 5x5 cm to the maize seed (**NH_4^+-Depot**) and two BE levels: non-inoculated control (NoBE) and *Pseudomonas sp*. DSMZ13134 inoculated twice at 0 and 23 DAS each at the rate 1 x 10^9 CFUs kg^{-1} soil by placing 2.5 cm around the NH_4^+-depot or corresponding location for **NO_3^--Mixed** treatments. The ^{15}N-labelled $(NH_4)_2SO_4$ fertilizer solution was enriched using ^{15}N-$(NH_4)_2SO_4$ to10 atom %.

Concentration and contents of shoot N and P, shoot DM, rhizosphere pH, root length density and root colonization of *Pseudomonas sp*. DSMZ13134 the NH_4^+-depot or corresponding location for **NO_3^--Mixed** treatments as described in (*Nkebiwe* et al. 2016b). ^{15}N signal of dried shoot biomass was measure by Isotope ratio mass spectrometer (IRMS). During grinding of dried maize shoot samples, some

contamination of non labelled samples occurred by labelled ones. Nevertheless, large differences in ^{15}N signal between labelled and non labelled plants could be expected because of the very high ^{15}N labelling rate of the placed $(NH_4)_2SO_4$+DMPP fertilizer.

5.1.3 Results

5.1.3.1 Effect of background Nmin on root growth around subsurface NH_4^+-depots

At 12 days after planting (dap), no root-growth towards the NH_4^+-depot was observed. At 36 dap, root length (or length per unit surface area) 4 cm around the depot was low, without differences across treatments (Fig. 5.1 A). At 4-8 cm around the depot, root length was lower in soil with no background N fertilization (0 mg N kg^{-1}) than in those with 5, 20 and 60 mg N kg^{-1}. At > 8 cm around the depot, it increased with increasing background N from 0, 5 to 20 mg N kg^{-1} and then slightly decreased at 60 mg N kg^{-1}. At 56 dap in the zone > 8cm around the depot, the same trend could be observed (Fig. 5.1 B). Furthermore, there were no differences in root growth 0-4 cm and 4-8 cm around the depot across treatments at 56 dap (Fig. 5.1 B and Fig. 5.2). However, after washing away soil from the rhozoboxes, intense rooting induced by the NH_4^+-depot in distant soil volumes with less toxic NH_4^+ concentrations could be observed across all background N_{min} levels (Fig. 5.2).

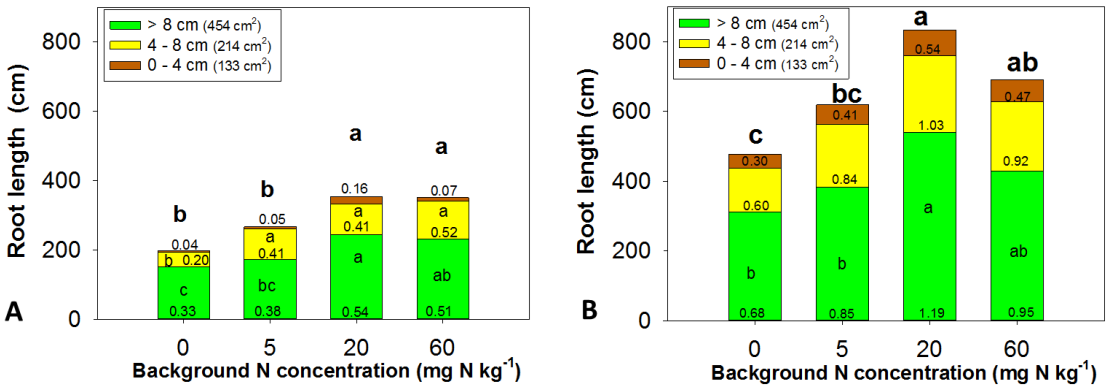

Figure 5.1. Root length around the NH4+-depot at 36 (A) and 56 (B) days after planting.
Different letters show significant difference between treatments ($P < 0.05$) (Mean ± SEM, n = 4, One Way ANOVA, Tukey test, α=0.05). Bold letters: total root length; small letters: root length within radial zones around the depot only if differences were significant. Brown, yellow and green sections of bars represent root growth at radial zones 0-4 cm, 4-8 cm and >8 cm from the depot respectively. Numbers within bars represent root length per unit surface area on the rhizobox window (cm cm^{-2}).

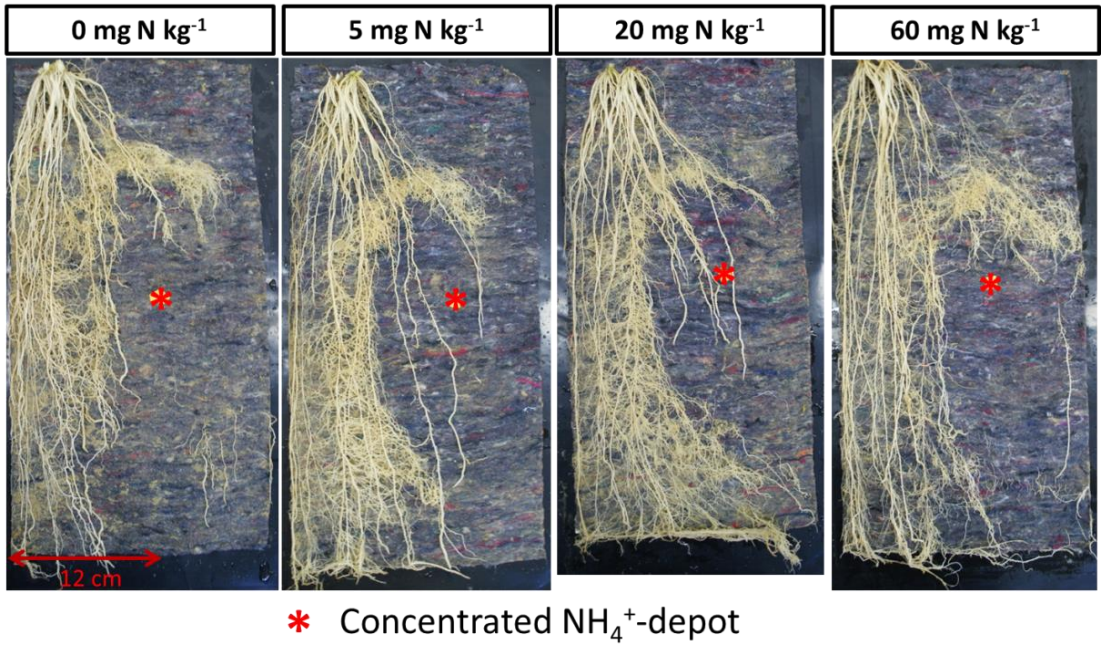

Figure 5.2. Phytotoxicity of concentrated NH_4^+ inhibited root growth in the immediate surrounding of the depot and induced intense rooting in zones with non-toxic NH_4^+ levels at 56 dap.

As expected, residual NO_3-N in the bulk soil increased with increasing background N fertilization from 0 to 60 mg N kg^{-1} whereas residual NH_4-N was low and similar across treatments (Fig. 5.3 A). Within the NH_4^+-depot, NO_3-N levels were low whereas NH_4-N levels where high, with no differences across treatments (Fig. 5.3 B).

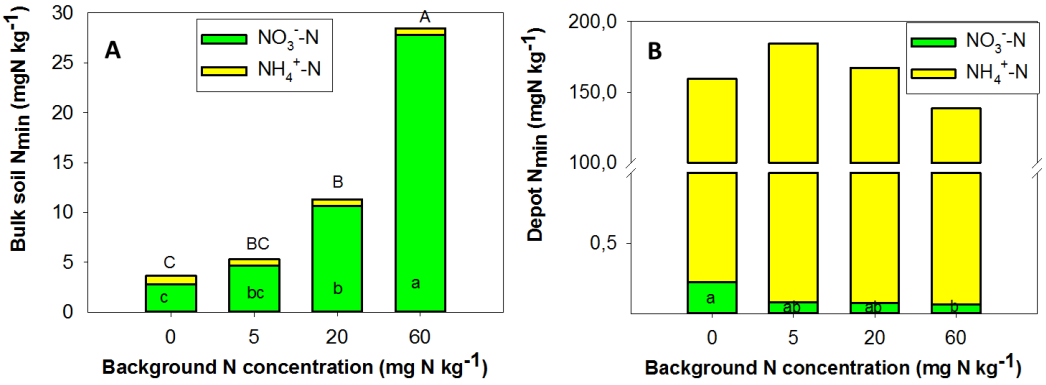

Figure 5.3. Residual Nmin in the bulk soil (A) and in the NH_4^+-depot (B) at 56 dap
Different letters show significant difference between treatments, if differences were significant (*P < 0.05*) (Mean ± SEM, n = 4, One Way ANOVA, Tukey test, α=0.05). Data for bulk soil NH_4^+-N and total N_{min} was transformed by square roots. Block letters: difference in total Nmin concentrations; small letters: difference in NH_4^+-N concentrations. Yellow and green sections of bars represent NH_4^+-N and NO_3^--N concentrations respectively.

Shoot dry matter per rhizobox at background N fertilization of 20 mg N kg^{-1} (3.7 g) was higher than those of 0 mg N kg^{-1} (2.9 g) and 5 mg N kg^{-1} (2.6 g) but not different from that of 60 mg N kg^{-1} (3.4 g) (n =4, tukey test, P < 0.05).

5.1.3.2 PGPM tolerance to high N concentrations

For all strains of *Trichoderma harzianum*, mycelia diameter showed a tendency to increase with increasing NH_4-N concentrations from 0-10 mM, followed by a decrease at 50 mM NH_4-N. Mycelium diameter at 50 mM NH_4-N was higher than the one at 250 mM, which again was higher than the one at 1250 mM (Fig. 5.4).

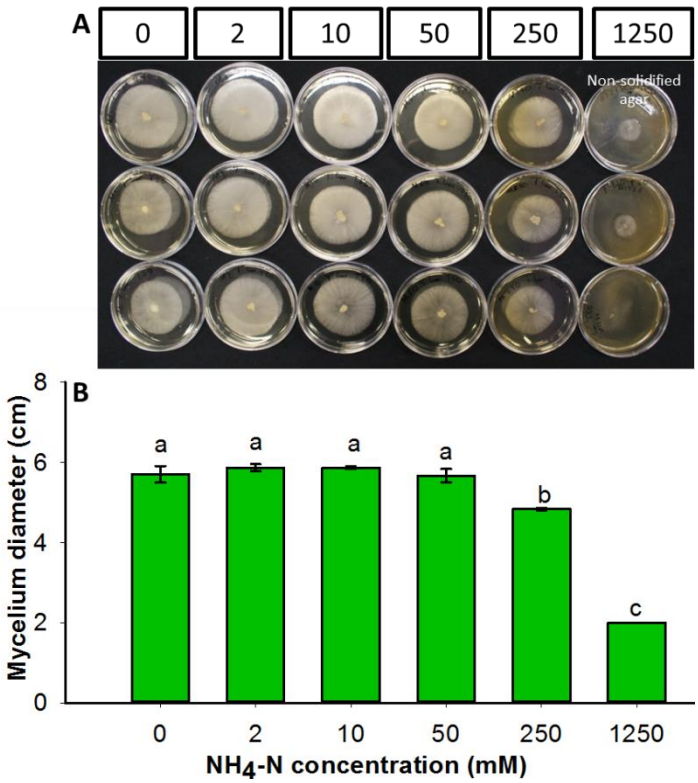

Figure 5.4. Qualitative (A) and quantitative (B) comparison of growth of *Trichoderma harzianum* T-50 on Malt extract peptone agar with increasing NH_4-N concentrations after 48 hrs.
Different letters show significant difference between treatments ($P < 0.05$) (Mean ± SE, n=3, One Way ANOVA, Tukey test, α=0.05)

After one week, there was considerable growth at 1250 mM NH_4-N for all *T. harzianum* strains meanwhile the typical green centric ring indicating conidia formation at maturity (*Mendoza* et al. 2015) could be seen only petri dishes with NH_4-N levels from 0–250 mM NH_4-N.

Penicillium sp. PK 112 grew slower than *Trichoderma.* After one week the mycelium diameter of *Penicillium sp*. PK 112 at 10 and 50 mM NH_4-N were higher than those at 0 and 2 mM NH_4-N. It increased from 2.53 cm at 0 mM NH_4-N to 2.93 cm at 50 mM NH_4-N ($P < 0.05$), after which it showed a tendency to decrease to 2.87 cm at 250 mM NH_4-N (no significant difference). Limited growth activity was observed at 1250 mM NH_4-N.

For bacterial PGPMs, considerable growth could be observed at high NH_4-N levels after one week. For *Pseudomonas sp.* DSMZ 13134 colonies were circular, yellow, smooth, glistening, entire and convex. The typical yellow pigment which was spread outwards from each colony was intense and distinct only in agar containing 0-250 mM NH_4-N. Colonies also showed typical yellow-green fluorescence under UV light. On DEV medium, *Bacillus amyloliquefaciens* FZB42 formed distinct punctiform colonies with typical spreading bio-films, which were less spread at 250 and 1250 mM NH_4-N (Fig. 5.5). There were more *B. amyloliquefaciens* FZB42 colonies at 250 mM NH_4-N (97) than at 0 mM (59) ($P = 0.017$) (Fig. 5.5). On nutrient agar, colonies of *B. amyloliquefaciens* FZB42, *B. atrophaeus*, *B. simplex* R41 and *Bacillus* spec. were less distinct and bio-films were also observed across all NH_4-N concentrations.

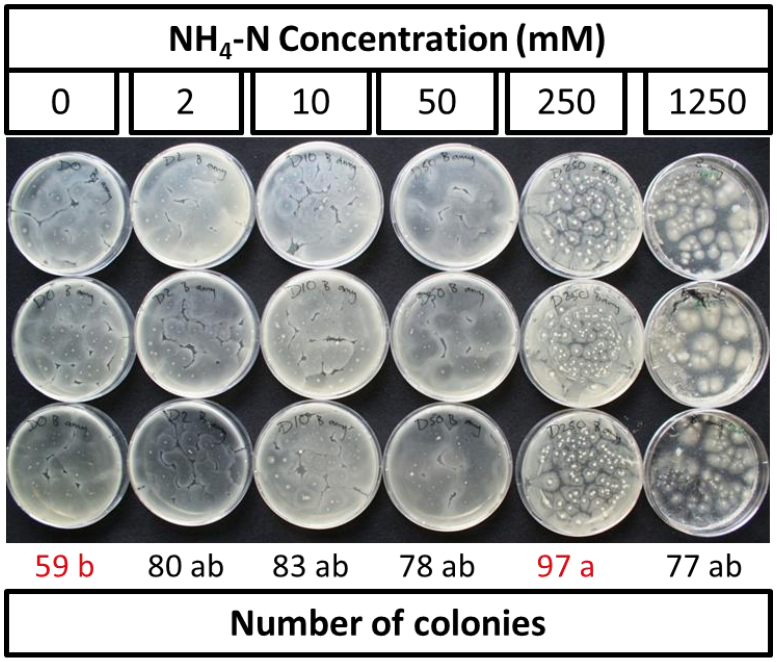

Figure 5.5. Bacillus amyloliquefaciens FZB42 grown on DEV medium
Different letters show significant difference between treatments ($P < 0.05$) (Mean ± SE, n=3, One way ANOVA, Tukey test, α=0.05)

For *T. harzianum* T-22 after 15 minutes pre-incubation of mycelium fragment in NH_4^+±DMPP, NH_4-N concentration (*P=0.007*) and presence of DMPP (*P =0.015*) affected mycelium diameter without any interaction between factors (*P=0.575*). Irrespective of the presence of DMPP, mycelium diameter at 3 M NH_4-N (4.95 cm) was less than the one at 0 M NH_4-N (5.54 cm) (*P=0.005*); and irrespective of NH_4-N level, mycelium diameter with DMPP (5.16 cm) was less than the one without (5.44 cm) (*P=0.015*) (Fig 5.6). After 15 minutes pre-incubation in 0 and 0.1 M NH_4-N, *Pseudomonas sp.* DSMZ 13134 and *B. amyloliquefaciens* FZB42 showed growth activity without any observable effect by DMPP. At 1 and 3 M NH_4-N, growth of both *Pseudomonas sp.* DSMZ 13134 and *B. amyloliquefaciens* FZB42 was suppressed (more for *Pseudomonas sp.* DSMZ 13134) and normal morphological colony characteristics where absent. Nevertheless, typical fluorescence under UV light was observed for *Pseudomonas sp.* DSMZ 13134 and typical bio-film formation was observed for *B. amyloliquefaciens* FZB42.

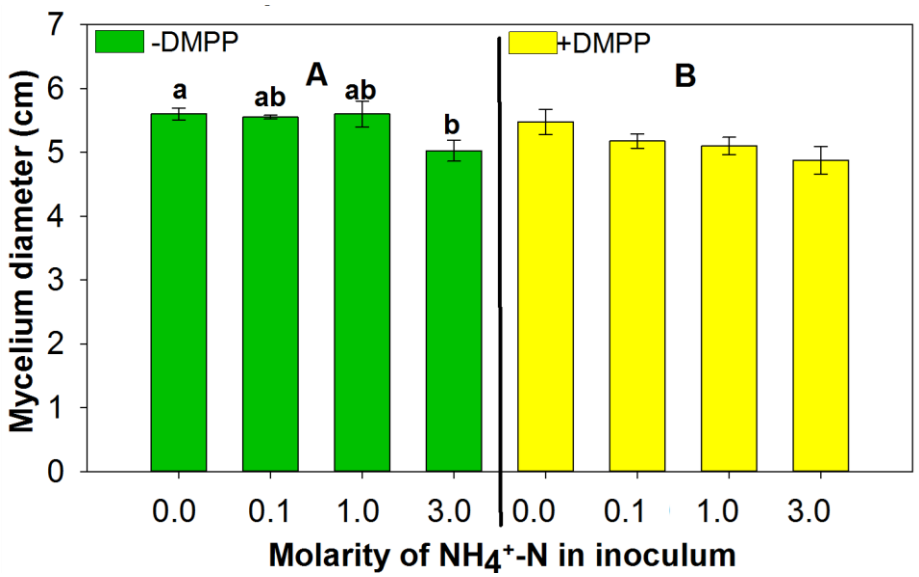

Figure 5.6. Mycelium diameter of *Trichoderma harzianum* T-22 affected by 15 minutes pre-incubation in NH_4-N solution with or without DMPP
Block letters: difference between variants with DMPP and those without (significant effect of DMPP *P=0.015*); Significant effect of NH_4^+ molarity (*P=0.007*); small letters: tendency of difference between treatments (*P=0.055*) (Mean ± SE, n=4, One and Two-way ANOVA, Tukey test, α=0.05)

After 24 hours pre-incubation of PGPM in 1 or 3M **NH_4-N** solution with or without DMPP, growth of *T. harzianum* T-22, *Pseudomonas sp.* DSMZ 13134 or *Bacillus amyloliquefaciens* FZB42 was strongly suppressed.

5.1.3.3 Solubilization of inorganic phosphates by PGPMs

All tested PGPMs except *T. harzianum* T-22 were able to solubilize Ca-P by clarifying the agar along PGPM streaks (Fig. 5.7 a-f). No visible changes could be observed along streaks of *Pseudomonas sp.* DSMZ 13134 on RP or SA agar (Fig. 5.7 i). Sedimentation of RP or SA at the bottom of the petri dishes (Fig. 5.7 g-h), suggested lack of contact between growing *Pseudomonas* on the agar surface and RP or SA at the bottom. This implied that liquid media cultures were more appropriate to test the ability of PGPMs to solubilize RP and SA.

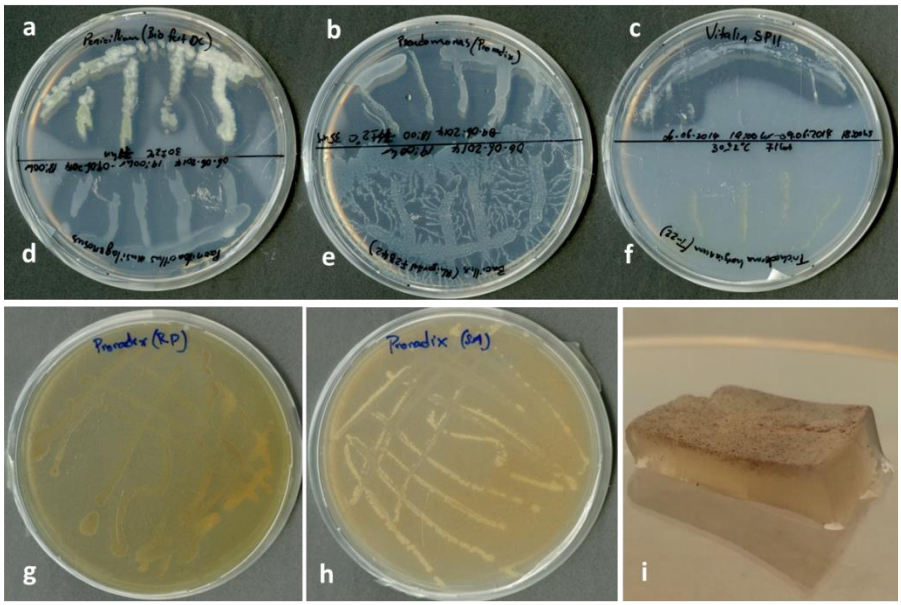

Figure 5.7. PGPMs clarified cloudy Ca-P precipitate but not rock phosphate (RP) or sewage sludge ash (SA).

Cloudy Ca-P precipitate ($Ca_3(PO_4)_2$**)** solubilized and clarified by *Penicillium sp*. PK 112 (**a**), *Pseudomonas sp.* DSMZ 13134 (**b**), Vitalin SP11 (combined product: *Bacillus subtilis*, *Pseudomonas sp*., *Streptomyces spp*., natural humic acids and seaweed extract (*Ascophyllum nodosum*)) (**c**), *Paenibacillus mucilagenosus* (**d**), *Bacillus amyloliquefaciens* FZB 42 (**e**). But it was not solubilized by *Trichoderma harzianum* T-22 (**f**). Growth of *Pseudomonas sp* did not lead to any visual changes with RP (**g**) or SA (**h**). A piece of agar inverted to show SA sediment (**i**)

The pH of the media after 65 hours varied among and within treatments. In comparison to the starting pH, there was a reduction in the pH of the nutrient medium in the following treatments: Ca-P/Pro, RP, RP/Pro, RP/Rhiz, SA, SA/Pro, SA/Rhiz and SA/T-22 (Fig. 5.8 a). The concentration of P in liquid media ($P_{conc.}$) was decreasing in the following order: Ca-P/Pro, RP/Pro, RP/Rhiz, RP/T-22 and RP. Very low $P_{conc.}$were measured in the other treatments (Fig. 5.8 b). For all treatments, pH showed a significant negative correlation with $P_{conc.}$ (*P = 0.002; r = -0.47*).

Increase in microbial biomass could be observed by the cloudy or murky appearance of the media for Pro and Rhiz variants and a thick mesh of mycelium for T-22 variants.

There was also much variability in acid and alkaline phosphatase activity (P_{ase}) among and within treatments (Figure 5.8 c and d). pH or $P_{conc.}$ did not correlate with acid or alkaline phosphatase activity.

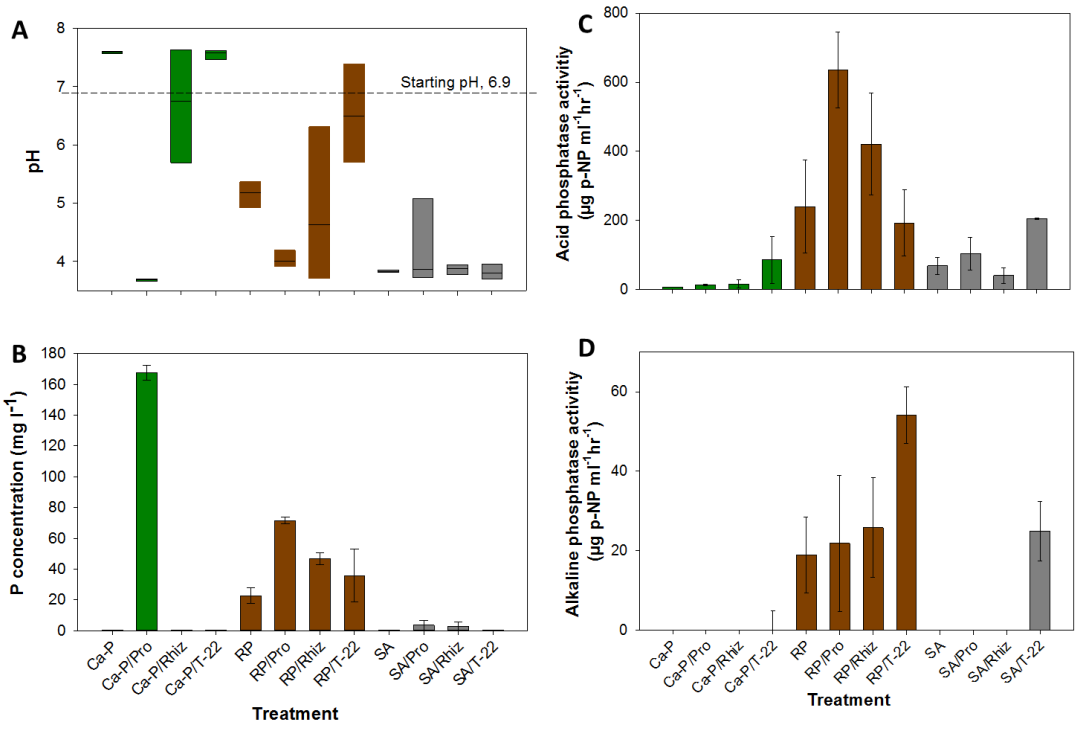

Figure 5.8. Change in pH (a), total P concentration (b)), acid- (c) and alkaline-phosphatase activity (d) in the liquid fraction of media 65 hours after onset of incubation
Ca-P, $Ca_3(PO_4)_2$; **RP**, rock phosphate; **SA**, sewage sludge ash; **Pro**, *Pseudomonas sp.* DSMZ 13134; **Rhiz**, *Bacillus amyloliquefaciens* FZB42; **T-22**, *Trichoderma harzianum* T-22. pH was inversely correlated to total P concentration (*P = 0.002*; r = -0.47)

5.1.3.4 Establishment of *Pseudomonas sp.* DSMZ13134 rhizosphere hotspots

Placement of -labelled stabilized $(NH_4)_2SO_4$ +DMPP had an effect on root length density (RLD), and rhizosphere pH 5x5 cm to seeds, shoot ^{15}N signal and shoot N and P concentrations and contents at 55DAS. Inoculation of *Pseudomonas sp.* DSMZ13134 had an effect on shoot ^{15}N signal and shoot N concentrations (Table 5.2) at 55DAS.

Table 5.2. RLD and rhizosphere pH 5x5 cm to seeds, shoot ^{15}N signal, shoots Shoot N and P concentration and content, and shoot dry matter, 55 days after sowing

	Source of variation							
	RLD 5x5 cm to seed (8 cm radius) (cm cm^{-3})	**Rhizosphere pH 5x5 cm to seed (8 cm radius)**	**δ15N (o/oo) $^{15}N/^{14}N$**	**N conc. (% DM)**	**N content (mg N plant^{-1})**	**P conc. (mg P g^{-1} DM)**	**P content (mg P plant^{-1})**	**Shoot DM (g plant^{-1})**
LS Means N*BE								
$^{14}NO_3^-$-Mixed*NoBE	6.4	5.62	170	1.83	114.0	2.18	13.7	6.35
$^{14}NO_3^-$-Mixed*Pro	6.2	5.89	148	2.22	130.1	2.32	13.6	5.91
$^{15}NH_4^+$-Depot*NoBE	10.5	4.41	25260	2.47	137.1	2.93	16.2	5.60
$^{15}NH_4^+$-Depot*Pro	9.9	4.55	21257	2.66	133.9	3.15	15.9	5.09
Standard error	0.68	0.13	831	0.13	4.69	0.146	0.62	0.420
Two-Way ANOVA								
N	***<0.001******	***<0.001******	***<0.001******	***0.002 *****	***0.014****	***<0.001******	***0.002 *****	*NS*
$^{14}NO_3^-$	6.3	5.75	159	2.03 b	122.0 b	2.52 b	13.6 b	6.13
$^{15}NH_4^+$	10.2	4.48	23259	2.57 a	135.5 a	3.04 a	16.0 a	5.35
BE	*NS*	*NS*	***0.036***	***0.051***	*NS*	*NS*	*NS*	*NS*
NoBE	8.4	5.01	12715	2.15	125.7	2.56	15.0	5.97
Pro	8.0	5.22	10702	2.44	132.0	2.73	14.7	5.50
N * BE	*NS*		*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

P values are in italics; ***NS,*** no significant difference, P ≥ 0.1; P < 0.1 is bold; * P < 0.5; ** P< 0.01; *** P< 0.001; Means not sharing the same letters are significantly different from each other, Tukey test α = 0.05; Factors and interaction is bold; ^{14}**NO_3^-**, non-isotopically labelled $Ca(NO_3)_2$ homogenously mixed in substrate; ^{15}**NH_4^+**, ^{15}N-labelled stabilized $(NH_4)_2SO_4$ +DMPP placed in substrate as a depot; **BE,** bio-effector; **NoBE**, no bio-effector; **Pro**, *Pseudomonas* sp. DSMZ 13134. ANOVA on δ15N (o/oo) $^{15}N/^{14}N$ was performed on square root transformed data. RLD, root length density

More fluorescent Pseudomonas could be recovered from the soil zone 5 x 5 cm to seed (8 cm radius) in the NH_4^+-Depot than treatment than in NO_3^--Mixed treatment. (Fig. 5.9)

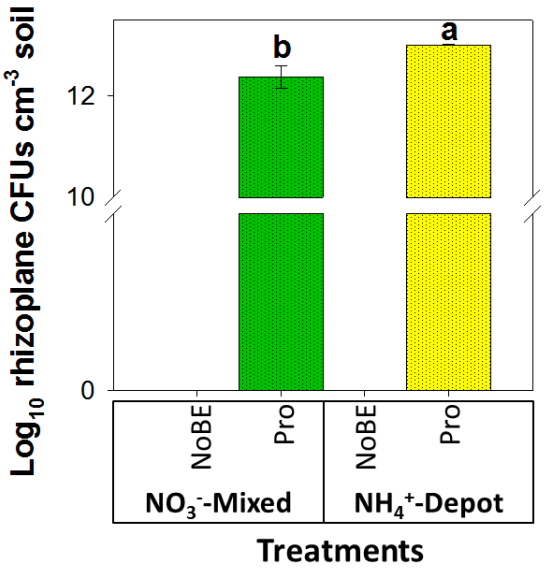

Figure 5.9. Root colonization by fluorescent Pseudomonas sp. 5 x 5 cm to seed (8 cm radius)
NO_3^--Mixed, $Ca(NO_3)_2$ homogenously mixed in substrate; **NH_4^+-Depot**, stabilized $(NH_4)_2SO_4$ placed in substrate as a depot; **BE,** bio-effector; **NoBE**, no bio-effector; **BE1**, *Pseudomonas* sp. DSMZ 13134, (t-test, P = 0.045)

5.1.4 Discussion

Root growth within the NH_4^+-depot was strongly inhibited most likely due to the presence of high phytotoxic levels of NH_4^+, as confirmed by N_{min} analysis of depot soil. Alternatively, root length per unit area on the rhizobox window was calculated. During the growth period, root length increased in all radial zones around the NH_4^+-depot. During early growth stages, root length in all zones towards the depot increased with increasing background N_{min} from 0 to 20 mg N kg^{-1} only. This was likely because at low background N_{min} levels during early growth stages, N supply to shallow root systems was inadequate for optimal root growth towards the depot. Localized root growth around the depot increased at later growth stages when the expanding N-rich boarder areas of the NH_4^+-depot became accessible as N progressively diffused outwards from the depot. This suggests that to optimally supply plants

with N especially during early growth stages, it is effective to place starter N fertilizer closer to seeds (about 5 cm below) to ensure rapid roots access(*Nkebiwe* et al. 2016a)**.** However, in case of considerable nutrient mobility from depot to seeds, it is advisable to place starter N farther from seeds to avoid harmful osmotic effects and ion toxicity (*Niehues* et al. 2004). At 56 dap, differences in root length occurred only in the zone > 8 cm, in which root growth was also most intense. Increasing root length in all radial zones around the depot with time and with increasing background N_{min} from 0 – 20 mg N kg^{-1} suggest that moderate N availability in the bulk soil favored initial root growth and subsequent root growth intensification around the NH_4^+-depot. Given very high NH_4-N levels in the depot, the high background N level of 60 mg N kg^{-1} may have led to N toxicity effects resulting in reduced overall root and shoot growth.

Although background N fertilization was with an NH_4-N share of 50%, only traces of NH_4-N could be measured in the bulk soil after 56 days. This showed that rapid nitrification of NH_4-N applied in the bulk soil occurred, resulting in residual N_{min} with an NH_4-N share of only 2.4 % (60 mg N kg^{-1}) to 11.9 % (5 mg N kg^{-1}). This can be partially explained by the absence of a nitrification inhibitor (NI) in the NH_4NO_3 used for bulk soil N fertilization. However, in soil without background N fertilization (0 mg N kg^{-1}), low N_{min} levels (2.8 mg NO_3-N kg^{-1} and 0.87 mg NH_4-N kg^{-1}) could be measured in the bulk soil with a low NH_4-N share of 23.7%, suggesting diffusion of N from the depot into the bulk soil as NH_4^+ as well as NO_3^- after nitrification. In the treatments 5, 10, 20 and 60 mg N kg^{-1}, bulk soil NH_4-N concentrations were similar to the one in the treatment without background N fertilization (0 mg N kg^{-1}) after the growth period. This confirmed that bulk soil NH_4^+ at the end of the growth period partially originated from the stabilized NH_4^+-depot directly by diffusion of NH_4^+.

Given the small substrate volume of the depot, the low clay and organic matter content of the loess-dominated substrate, the high moisture content of the substrate and high quantity of

NH_4^+ that was placed, NH_4^+ must have considerably diffused out of the initial depot boarders after sites for NH_4^+-fixation became exhausted. This explanation is supported by the wide zones around the initial depot boarders with little or no root growth likely due to presence of phytotoxic NH_4^+ levels. This is further proven by the strong decrease in the NH_4-N concentration within the depot from the estimated rate of 78.4 mg g^{-1} soil (estimated placement rate 1 g NH_4^+-N to 5 cm^3 depot soil volume (2.5 cm Ø x 2cm depth, bulk density, 1.3 g cm^{-3})) at the start of the experiment to the measured concentration of 0.18 mg NH_4-N g^{-1} after 56 days.

Despite considerable diffusion of NH_4^+ from the depot into the bulk soil during the experiment, low residual NH_4^+ concentrations measured in the bulk soil after 56 days may be explained by nitrification after diffusion of NH_4^+ out of the phytotoxic zones surrounding the depot resulting in NO_3^--formation with the outcome of even faster diffusion than NH_4^+ (*Barber* 1984; *Nkebiwe* et al. 2016a)**.** High residual NH_4^+ concentrations in the depot (0.18 mg NH_4-N g^{-1}) at the end of the growth period showed that high concentrations of $(NH_4)_2SO_4$ + DMPP in a subsoil depot inhibits biological nitrification of the placed NH_4^+ by both toxicity effects of high NH_4^+ concentrations and presence of DMPP (*Shaviv* 1988; *Sommer* 2005; *Zerulla* et al. 2001). This enabled placed N to persist in the depot zone as root growth stimulating and root attracting NH_4^+. Therefore, intense localized root-growth could be observed around a subsurface fertilizer-depot based on high concentrations of NH_4^+ stabilized with DMPP, which led to higher N and P concentrations and contents in maize shoots (*Zea mays* L.) in comparison to variants with homogenously incorporated NO_3^--fertilizer(*Nkebiwe* et al. 2016b).

This study also showed that soil zones around NH_4^+-depots with high root densities may be sites for effective root colonization by inoculated PGPMs. Growing PGPMs may be supported by the presence of organic C and N sources for growth and activity of rhizosphere

dwelling microorganisms released as root exudates (*Bonkowski* et al. 2000; *Lugtenberg* and *Kamilova* 2009; *van Overbeek* and *van Elsas* 1995; *van Veen* et al. 1997), which may be present in high concentrations in densely rooted soil.

All tested fungal and bacterial PGPMs showed normal or improved growth activity on solid nutrient agar containing as high as 50 mM NH_4-N and slightly inhibited growth at 250 mM NH_4-N except for *B. amyloliquefaciens* FZB42 and *Penicillium sp*. PK 112. In contrast to plant and animal cells, bacteria preferentially use NH_4^+ as a source of N and can be tolerant even to molar concentrations of NH_4-N, at which point growth limiting effects may be related more to osmotic or ionic imbalances than to direct toxic effects of NH_4^+ ions per se (*Müller* et al. 2006).

The threshold concentration range of 50-250 mM NH_4-N at which cultured PGPMs showed inhibited growth is above the range for NH_4^+ toxicity in sensitive crop species like barley (*Hordeum vulgare* L.) and tolerant ones like rice (*Oryza sativa* L.) grown hydroponically (*Chen* et al. 2013; *Coskun* et al. 2013; *Esteban* et al. 2016). The NH_4^+ tolerance threshold for our tested PGPMs (50-250 mM NH_4-N) range is also above the range of 2–20 mM that can be common in soil solution of most agricultural soils (*Britto* and *Kronzucker* 2002). The threshold range NH_4^+ tolerance of tested PGPMs (50-250 mM NH_4-N) is also above the NH_4-N concentrations that can be measured in soil solution extracted by centrifuging soil samples collected a few centimeters from a subsurface NH_4^+-fertilizer band (<0.001-37 mM NH_4-N and 0.4 - 91 mM NH_4-N from a distance 4-2 cm to a fertilizer band of $(NH_4)_2S0_4$) and di-ammonium phosphate respectively, after 5 days of contact of bands with soil, for tests on 5 different soil types) (*Moody* et al. 1995a; *Moody* et al. 1995b).

Furthermore, all tested PGPMs showed limited growth activity at the very high NH_4-N level of 1250 mM, which could be explained by osmotic and ionic imbalances as proposed earlier.

After 15 minutes pre-incubation of inoculum suspended in 0–3 M NH_4-N, growth of *T. harzianum* T-22 was slightly inhibited in the presence of DMPP meanwhile for *Pseudomonas sp.* DSMZ 13134 and *B. amyloliquefaciens* FZB42 after pre-incubation in 0–0.1M NH_4-N, growth activity was not affected by DMPP. After 24 hours pre-incubation in 1–3 M NH_4-N solution with or without DMPP, normal growth activity of PGPMs on solid media could no longer be achieved. This suggests that PGPMs may not be applied directly into a concentrated NH_4^+-fertilizer solution (> 50 mM NH_4-N). Alternatively, PGPMs should be inoculated in soil zones with lower NH_4-N levels in the border regions of a concentrated toxic ammonium (*Nkebiwe* et al. 2016b) or directly into the NH_4^+-fertilizer solution if it is less concentrated.

The classical test for P-solubilization on solid Ca-P media confirmed that all the tested PGPMs except *Trichoderma harzianum* T-22 were capable to solubilize inorganic P. On solid media culture, *Pseudomonas sp.* DSMZ 13134 effectively solubilized Ca-P whereas it led to no observable changes after growth on agar with rock phosphate (RP) or sewage sludge ash (SA). However, in liquid media cultures, *Pseudomonas sp.* DSMZ 13134, *Bacillus amyloliquefaciens* FZB42 and *Trichoderma harzianum* T-22 solubilized RP more than SA. Best performing PGPMs were *Pseudomonas sp.* DSMZ 13134 and *B. amyloliquefaciens* FZB42. Increase in microbial biomass, especially for *T. harzianum* T-22, suggested that the P concentrations measured in cell-free culture media filtrates represented underestimations of the P-solubilizing potential of tested PGPMs because measured P concentrations did not account for solubilized P that was subsequently immobilized in microbial biomass of *T. harzianum* T-22 (*Altomare* et al. 1999). Inoculation of T-22 did not result in considerable acidification of the Ca-P medium. Similarly to the results described by (*Altomare* et al. 1999)(2µg P ml^{-1} at pH 5, 48 hrs. incubation of medium containing 0.16 g RP-P l^{-1}), inoculation of T-22 resulted in acidification of the RP medium, however, it did not lead to

high P concentrations in media filtrates (35.7µg P ml^{-1} at pH 5.7-7.4, 65 hrs. incubation containing 0.3 g RP-P l^{-1}). This can also be explained by immobilization of solubilized PO_4^{3-} in the dense T-22 mycelium that was observed. Low pH values were measured in SA media with or without inoculated PGPM which corresponded to low P concentrations in media filtrates. In addition to immobilization of solubilized PO_4^{3-} in microbial biomass, low P concentrations in SA medium despite low medium pH may be explained by fixation of PO_4^{3-} by Al^{3+} and Fe^{3+} which may be available at low pH conditions given the high SA concentrations of Al (9.9%, 0.29 g SA-Al l^{-1}) and Fe (4.2%, 0.12 g SA-Fe l^{-1}). This explanation supports the observation that P concentrations increased with decreasing pH in the RP medium because RP had a much lower concentrations of Al (1.4%, 0.06 g RP-Al l^{-1}) and about the same concentration of Fe (5.1 %, 0.20 g RP-Fe l^{-1}) as the SA medium. We recorded pH values as low as 3.7 in cell-free filtrates of culture media inoculated with *Pseudomonas sp.* DSMZ 13134 or *B. amyloliquefaciens* FZB42. This suggested that acidification by release of protons and/or low molecular weight organic acids as reported by (*Altomare* et al. 1999; *Cunningham* and *Kuiack* 1992; *García-López* et al. 2016; *Goldstein* 1995; *Pérez* et al. 2007) may have been the likely mode of action to solubilize the inorganic P forms. This argument is supported by the significant negative correlation between pH and P concentration in the media ($P = 0.002$; $r = -0.47$). For liquid media with RP only, the correlation was even stronger ($P < 0.0001$; $r = -0.84$). Similarly to pH and total P concentration, there was high variability in acid and alkaline phosphatase activity within and between treatments. Measured acid and alkaline phosphatase activities may only be regarded as an indicator of the turnover or mineralization of organic P and not as a marker for solubilization of inorganic P. For the RP medium, there was a positive correlation between P concentration and phosphatase activity ($P = 0.015$; $r = -0.77$). Nevertheless, solubilization of inorganic P by various PGPMs in *in vitro* cultures does not guarantee improved P acquisition

and yield of plants in soil systems containing inorganic P and inoculated with a P-solubilizing PGPM (*García-López* et al. 2016). *Bashan* et al. (2013) criticized the common use of Ca-P solubilization tests as a universal test to identify potential P-solubilizing microorganisms across different soil types. To increase the chance of obtaining candidates PGPMs that can actually contribute to P nutrition of plants grown in natural soil systems, it may be more appropriate in future experiments, to test solubilization of Ca-P or rock phosphate with PGPMs isolated from alkaline soils; Fe- and Al-bound-PO_4^{3-} those from acid soils; and phytate with those from soils rich in organic matter (*Bashan* et al. 2013).

Several mechanisms for PGPMs to directly or indirectly improve acquisition of nutrients in soil by crop plants such as root-growth stimulation, nutrient solubilization and mineralization, and induction of resistance to biotic and abiotic stresses have been clearly described already (*Altomare* et al. 1999; *Barea* et al. 2002; *Bonkowski* et al. 2000; *Jiang* et al., 2012; *Jones* and *Oburger*, 2011; *Lugtenberg* and *Kamilova* 2009; *Mavrodi* et al. 2012; *Mohite* 2013; *Richardson* et al. 2009; *Singh* and *Satyanarayana* 2012; *Vassilev* et al. 2006)).

Sometimes, promised beneficial effects of several commercially available *PGPMs* are not realized even in controlled greenhouse conditions (*Schenck zu Schweinsberg-Mickan* and *Müller* 2009) or in the field (*Lugtenberg* and *Kamilova* 2009).This may be explained by rapid the decline in PGPM populations often observed after inoculation in natural soil due to competition for nutrients, predation by other soil organisms or other unfavorable biotic or abiotic factors (*van Veen* et al. 1997).

In our rhizobox experiment, placement of ^{15}N-labelled stabilized $(NH_4)_2SO_4$+DMPP as a depot led to formation of intense localized root growth around the depot (rhizosphere hotspot), which in turn led to improved N-uptake as shown by higher shoot N concentration,

^{15}N signal and N content compared to plants under homogenous supply of NO_3^-. Placement of the NH_4^+-depot also led to rhizosphere acidification, which together dense localized root growth led to higher shoot P concentration and content than in plants under homogenous supply of NO_3^-. Inoculation of *Pseudomonas sp.* DSMZ13134 in soil zones in which rhizosphere hotspots developed in response to placed root-attracting NH_4^+-depots promoted the establishment of fluorescent *Pseudomonas* (*Nkebiwe* et al. 2016b). Inoculation of *Pseudomonas sp.* DSMZ13134 showed a trend to improve shoot N concentration and content and shoot P concentration when compare to the non-inoculated control.

A possible explanation is that inoculated *Pseudomonas* may be supported by the presence of high concentrations of organic nutrients released as exudates in densely rooted soil. Improved rhizosphere-colonization of maize roots (*Zea mays* L.) around an NH_4^+-depot by rifampicin-resistant *Bacillus amyloliquefaciens* FZB42 has also been observed in a pot experiment (*Mohammad et al.*, 2016, unpublished master thesis, Nutritional Crop Physiology, University of Hohenheim).

In addition to inoculating PGPMs in NH_4^+-based rhizosphere hotspots, inoculation of PGPM strains that are adapted or compatible to specific soil properties (*Bashan* et al. 2013; *Zahir* et al. 2009), utilization of suitable nutrient additives, carrier materials and inoculation techniques may increase their survival rates of PGPMs after inoculation in soil (*van Veen* et al. 1997; *Marschner*, 2012).

5.1.5 Conclusions

Placement of a highly concentrated subsurface NH_4^+-depot induced the formation of rhizosphere hotspots with intense rooting around the depots. At high bulk soil N_{min}, localized root growth around the concentrated NH_4^+-depot may be reduced possibly because of NH_4^+-toxicity effect on root growth. PGPMs that readily solubilized sparingly soluble inorganic

PO_4^{3-} showed considerable tolerance to high NH_4^+ concentrations that are comparable to those in soil surrounding a concentrated subsurface NH_4^+-depot. Among investigated NH_4^+-tolerant PGPMs, rhizosphere hotspot establishment of *Pseudomonas sp.* DSMZ13134 was tested by inoculating it in soil around NH_4^+-depots in rhizobox-grown maize (*Zea mays* L.) resulting in improved establishment of *Pseudomonas sp* in NH_4^+-depot soil in comparison to soil with homogenous supply of NO_3^-.

5.1.6 References

Altomare, C., Norvell, W. A., Björkman, T., Harman, G. E. (1999): Solubilization of phosphates and micronutrients by the plant-growth-promoting and biocontrol fungus *Trichoderma harzianum* Rifai 1295-22. *Appl. Environ. Microbiol.* 65, 2926–2933.

Barber, S. A. (1984): Soil nutrient bioavailability. A mechanistic approach. Wiley, New York.

Barea, J. M., Toro, M., Orozco, M. O., Campos, E., Azcon, R. (2002): The application of isotopic (^{32}P and ^{15}N) dilution techniques to evaluate the interactive effect of phosphate-solubilizing rhizobacteria, mycorrhizal fungi and Rhizobium to improve the agronomic efficiency of rock phosphate for legume crops. *Nutr. Cycl. Agroecosys.* 63, 35–42,

Bashan, Y., Kamnev, A. A., de-Bashan, L. E. (2013): Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure. *Biol.Fert. Soils* 49, 465–479.

Bonkowski, M., Cheng, W., Griffiths, B. S., Alphei, J., Scheu, S. (2000): Microbial-faunal interactions in the rhizosphere and effects on plant growth. *Eur. J. Soil Biol.* 36, 135–147.

Britto, D. T., Kronzucker, H. J. (2002): NH_4^+ toxicity in higher plants: a critical review. *J. Plant Physiol.* 159, 567–584.

Chen, G., Guo, S., Kronzucker, H. J., Shi, W. (2013): Nitrogen use efficiency (NUE) in rice links to NH_4^+ toxicity and futile NH_4cycling in roots. *Plant Soil* 369, 351–363.

Clarke, A. L., Barley, K. P. (1968): The uptake of nitrogen from soils in relation to solute diffusion. *Aust. J. Soil Res.* 6, 75–92.

Coskun, D., Britto, D. T., Li, M., Becker, A., Kronzucker, H. J. (2013): Rapid ammonia gas transport accounts for futile transmembrane cycling under NH_3/NH_4^+ toxicity in plant roots. *Plant Physiol.* 163, 1859–1867.

Cunningham, J. E., Kuiack, C. (1992): Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. *Appl. Environ. Microb.* 58, 1451–1458.

de Freitas, J. R., Banerjee, M. R., Germida, J. J. (1997): Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). *Biol. Fertil. Soils* 24, 358-364.

Deubel, A. (1996)*:* Einfluß wurzelbürtiger organischer Kohlenstoffverbindungen auf Wachstum und Phosphatmobilisierungsleistung verschiedener Rhizosphärenbakterien. Dissertation., Martin-Luther-Universität, Halle-Wittenberg, Germany.

Drew, M. C. (1975): Comparison of the effects of a localised supply of phosphate, nitrate, ammonium and potassium on the growth of the seminal root system, and the shoot, in barley. *New Phytol.* 75, 479–490.

Esteban, R., Ariz, I., Cruz, C., Moran, J. F. (2016): Review: Mechanisms of ammonium toxicity and the quest for tolerance. *Plant Science* 248, 92–101,

Federolf, C.-P., Westerschulte, M., Olfs, H.-W., Broll, G., Trautz, D. (2016): Enhanced nutrient use efficiencies from liquid manure by positioned injection in maize cropping in northwest Germany. *Eur. J. Agron.* 75, 130–138,

García-López, A. M., Avilés, M., Delgado, A. (2016): Effect of various microorganisms on phosphorus uptake from insoluble Ca-phosphates by cucumber plants. *J. Plant Nutr. Soil Sci.* 179, 454–465.

Goldstein, A. H. (1995): recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by gram negative bacteria. *Biol. Agric. Hortic.* 12, 185–193,

Gross, K. L., Peters, A., Pregitzer, K. S. (1993): Fine root growth and demographic responses to nutrient patches in four old-field plant species. *Oecologia* 95, 61–64,

Jiang, Y., Wu, Y., Xu, W., Cheng, Y., Chen, J., Xu, L., Hu, F., Li, H. (2012): IAA-producing bacteria and bacterial-feeding nematodes promote Arabidopsis thaliana root growth in natural soil. *Eur. J. Soil Biol.* 52, 20–26.

Jing, J., Zhang, F., Rengel, Z., Shen, J. (2012): Localized fertilization with P plus N elicits an ammonium-dependent enhancement of maize root growth and nutrient uptake. *Field Crop. Res.* 133, 176–185.

Jones, D., L., Oburger, E. (2011): Solubilization of phosphorus by soil microorganisms, in Bünemann, E., Oberson, A., Frossard, E.: Phosphorus in Action. Biological Processes in Soil Phosphorus Cycling. Springer-Verlag, Berlin, Germany, pp. 169–198.

Lamb, E. G., Haag, J. J., Cahill, J. F. (2004): Patch–background contrast and patch density have limited effects on root proliferation and plant performance in Abutilon theophrasti. *Funct. Ecol.* 18, 836–843.

Lugtenberg, B., Kamilova, F. (2009): Plant-Growth-Promoting Rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556.

Marschner, P. (2012): Rhizosphere Biology, in Marschner P.: Marschner's Mineral Nutrition of Higher Plants, Third edition, Academic Press, Elsevier, Amsterdam, pp. 369–388.

Mavrodi, O. V., Walter, N., Elateek, S., Taylor, C. G., Okubara, P. A. (2012): Suppression of Rhizoctonia and Pythium root rot of wheat by new strains of Pseudomonas. *Biol. Control* 62, 93–102.

Mendoza, J. L. H., Pérez, M. I. S., Prieto, J. M. G., Velásquez, J. D. Q., Olivares, J. G. G., Langarica, H. R. G. (2015): Antibiosis of *Trichoderma* spp strains native to northeastern Mexico against the pathogenic fungus *Macrophomina phaseolina*. *Braz. J. Microbiol.* 46, 1093–1101.

Miller, M. H., Ohlrogge, A. J. (1958): Principles of nutrient uptake from fertilizer bands 1. Effect of placement of nitrogen fertilizer on the uptake of band-placed phosphorus at different soil phosphorus levels1. *Agron. J.* 50, 95–97.

Mohite, B. (2013): Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *J. Soil Scie. Plant Nutr.* 13, 638–649.

Moody, P. W., Aitken, R. L., Yo, S. A., Edwards, D. G., Bell, L. C. (1995a): Effect of banded fertilizers on soil solution composition and short-term root-growth .1. Ammonium-sulfate, ammonium-nitrate, potassium-nitrate and calcium nitrate. *Soil Res.* 33, 673–687.

Moody, P. W., Edwards, D. G., Bell, L. C. (1995b): Effect of banded fertilizers on soil solution composition and short-term root-growth .2. Mono-ammonium and di-ammonium phosphates. *Soil Res.* 33, 689–707.

Müller, T., Walter, B., Wirtz, A., Burkovski, A. (2006): Ammonium toxicity in bacteria. *Curr. Microbiol.* 52, 400–406.

Muromcev, G. S. (1958): Die lösende Wirkung einiger Wurzel-und Bodenmikroorganismen auf die wasserunlöslichen Kalziumphosphate (russ.). *Agrobiologija* 5, 31–36.

Niehues, B. J., Lamond, R. E., Godsey, C. B., Olsen, C. J. (2004): Starter nitrogen fertilizer management for continuous no-till corn production contribution no. 04-099-J, K-State Research and Extension. *Agron. J.* 96, 1412–1418.

Nkebiwe, P. M., Weinmann, M., Bar-Tal, A., Müller, T. (2016a): Fertilizer placement to improve crop nutrient acquisition and yield: A review and meta-analysis. *Field Crops Res.* 196, 389–401,

Nkebiwe, P. M., Weinmann, M., Müller, T. (2016b): Improving fertilizer-depot exploitation and maize growth by inoculation with plant growth-promoting bacteria: from lab to field. *Chem. Biol Technol. Agric.* 3, 1–16.

Panda, B., Rahman, H., Panda, J. (2016)*:* Phosphate solubilizing bacteria from the acidic soils of Eastern Himalayan region and their antagonistic effect on fungal pathogens. *Rhizosphere, Available online 6 August 2016, ISSN 2452-2198, http://dx.doi.org/10.1016/j.rhisph.2016.08.001.*

Pang, P. C., Hedlin, R. A., Cho, C. M. (1973): Transformation and movement of band-applied urea, ammonium sulfate, and ammonium hydroxide during incubation in several Manitoba soils. *Can. J. Soil. Sci.* 53, 331–341.

Pérez, E., Sulbarán, M., Ball, M. M., Yarzábal, L. A. (2007): Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. *Soil Biol. Biochem.* 39, 2905–2914.

Richardson, A., Barea, J.-M., McNeill, A., Prigent-Combaret, C. (2009): Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant Soil* 321, 305-339.

Schenck zu Schweinsberg-Mickan, M., Müller, T. (2009): Impact of effective microorganisms and other biofertilizers on soil microbial characteristics, organic-matter decomposition, and plant growth. *J. Plant Nutr. Soil Sci.* 172, 704–712.

Schenk, M. K., Barber, S. A. (1979): Phosphate uptake by corn as affected by soil characteristics and root morphology. *Soil Sci. Soc. Am. J.* 43, 880–883.

Shakir, M. A., Bano, A., Arshad, M. (2012): PGPR with ACC deaminase confer drought tolerance wheat. *Soil Environ.* 31, 108–112.

Shaviv, A. (1988): Control of nitrification rate by increasing ammonium concentration. *Fert. Res.* 17, 177-188.

Singh, B., Satyanarayana, T. (2012): Plant growth promotion by phytases and phytase-producing microbes due to amelioration in phosphorus availability, in Satyanarayana, T., Johri, N. B.: Microorganisms in Sustainable Agriculture and Biotechnology. Springer Netherlands, Dordrecht, pp. 3–15.

Sommer, K. (2005): CULTAN-Düngung: physiologisch, ökologisch, ökonomisch optimiertes Düngungsverfahren für Ackerkulturen, Grünland, Gemüse, Zierpflanzen und Obstgehölze. Verlag Th. Mann, Gelsenkirchen-Buer, Germany.

Tabatabai, M. A., Bremner, J. M. (1969): Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. *Soil Biol. Biochem.* 1, 301–307.

van Overbeek, L. S., van Elsas, J. D. (1995): Root exudate-induced promoter activity in *Pseudomonas fluorescens* mutants in the wheat rhizosphere. *Appl. Environ. Microb.* 61, 890–898.

van Veen, J. A., van Overbeek, L. S., van Elsas, J. D. (1997): Fate and activity of microorganisms introduced into soil. *Microbiol. Mol. Biol. R.* 61, 121–135.

Vassilev, N., Vassileva, M., Nikolaeva, I. (2006): Simultaneous P-solubilizing and biocontrol activity of microorganisms: potentials and future trends. *Appl. Microbiol. Biot.* 71, 137–144.

Zahir, Z. A., Ghani, U., Naveed, M., Nadeem, S. M., Asghar, H. N. (2009): Comparative effectiveness of *Pseudomonas* and *Serratia sp.* containing ACC-deaminase for improving growth and yield of wheat (Triticum aestivum L.) under salt-stressed conditions. *Arch. Microbiol.* 191, 415–424.

Zerulla, W., Barth, T., Dressel, J., Erhardt, K., Horchler von Locquenghien, K., Pasda, G., Rädle, M., Wissemeier, A. (2001): 3,4-Dimethylpyrazole phosphate (DMPP) - a new nitrification inhibitor for agriculture and horticulture. *Biol. Fert. Soils* 34, 79–84.

6 Improving fertilizer-depot exploitation and maize growth by inoculation with plant growth-promoting bacteria – from lab to field

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6.1 Abstract

Among other responses, plants tend to increase root growth to scavenge nutrients from more soil when soil nutrient concentrations are low. Placement of fertilizers near seeds or roots facilitates nutrient acquisition by target crop plants. Nevertheless, nutrient uptake from soil-placed fertilizer-depots depends on increased uptake rates and efficient spatial exploitation of the depot by roots. The aim of our study was to optimize exploitation of subsurface fertilizer-depots by inoculating the depot-zone with promising plant growth-promoting microorganisms (PGPMs) as bio-effectors. If included in depots, root-attracting NH_4^+ or HPO_4^{2-}/ $H_2PO_4^-$ ions may also enhance rooting within the depot, which in turn, improves survival and root-colonization by inoculated PGPMs; a consequence of high levels of microbial nutrients exuded in densely rooted soil. We tested maize (*Zea mays* L.) in two greenhouse (pot and rhizobox) and two field experiments (2014 and 2015). A core treatment was NH_4^+-fertilizer placed as a subsurface depot (**Depot**). In the field, there was also NH_4^+-fertilizer broadcasted and incorporated in soil (**Broad.**). Depot and Broad. were each with PGPM as bio-effector (**BE**) or without (**NoBE**). Bio-effectors included: *Pseudomonas* sp. DSMZ 13134 (**Pro**) and *Bacillus amyloliquefaciens* FZB42 (**Rhiz**, only in field trials). In pots, Depot with Pro led to 59% higher shoot dry matter, 50% higher shoot N content and 64% higher shoot P content than without PGPM. In rhizoboxes, higher root length density (**RLD**), lower rhizosphere pH and higher Pro-colonization rate were measured in the fertilizer depot compared to the corresponding zone for controls with homogenous NO_3^- supply. Depot led to higher shoot N and P concentrations (+26.6% N; +20.6% P) and contents (+11.1% N; +17.6% P) than control. Pro led to higher shoot N concentration (+13.5 %) than **NoBE**.

In the field, fertilizer-depot soil had higher RLD than corresponding non-depot soil. Pro led to doubled fertilizer-depot RLD in comparison to without (2014). In 2014, Depot led to 7.4% higher grain yield than Broad (not statistically significant) whereas BE broadcast had no effect. In 2015, Depot led to 5.8% higher fresh shoot biomass than Broad., below-seed placement of Pro led to higher fresh (+7.1%) and dry (+8.0%) shoot biomass than NoBE. Results show promising growth-effects of *Pseudomonas* on field-grown maize.

Keywords: Fertilizer placement, Localized root-growth, Nutrient acquisition, PGPM

6.2 Background

Of increasing importance in sustainable agriculture systems are the effective use of crop bio-stimulants (*Sharma* et al., 2012; *Halpern* et al., 2015) and/ or plant growth-promoting microorganisms (PGPMs), which have been shown to fix N (*Barea* et al., 2002), mineralize organic soil N (*Ferris* et al., 1998), stimulate plant root growth (*Mao* et al., 2006; *Jiang* et al., 2012; *Mohite* 2013) and mycorrhization (*Mauricio,* 2005; *Frey-Klett* et al., 2007; *Richardson* et al., 2009); which enhances spatial nutrient acquisition from large soil volumes; and also to induce tolerance or resistance to biotic (*Ghorbani* et al., 2008) and abiotic stresses (*Yang* et al., 2009; *Shakir* et al., 2012). Nevertheless, plant growth-promoting effects of PGPMs realized in labs and greenhouses tend to be weak or even disappear when PGPMs are tested under field conditions. PGPM ineffectiveness in the field is likely due to a suboptimal or unfavorable interaction between field-inoculated PGPM and the biotic and abiotic environment (*van Veen* et al., 1997; *Lugtenberg* and *Kamilova,* 2009).

Central to the concept of sustainable agriculture is reduction of environmental costs associated with farming. Among others, it requires responsible use of chemical fertilizers. This can be achieved through use of suitable fertilizer types and application rates timed to crop demand, seasons and weather conditions with low risk of fertilizer loss to the environment. Responsible use of chemical fertilizers also requires utilization of effective fertilizer application techniques. In contrast to conventional fertilizer application by even broadcast on the soil surface (with or without incorporation), several innovative fertilizer placement techniques have been developed, through which fertilizer can be targeted to the seed, root or canopy of young crop plants. Furthermore, fertilizer placement in soil improves fertilizer acquisition by target crop plants as opposed to weeds (*Blackshaw* et al., 2002; *Petersen,* 2005) and reduces the risk of nutrient loss to the environment. Based on fertilizer composition, application technique and timing, effective fertilizer placement can lead to

reduced leaching of nitrate to ground waters (*Ruidisch* et al., 2013), low emission of nitrous oxide (*Nash* et al., 2012), methane (*Linquist* et al., 2012) and ammonia(*Ma* et al., 2010) originating from fertilizer applied in soil. Fertilizer placement can also improve nutrient content in crop above-ground biomass as well as crop yield in comparison to conventional fertilizer broadcast (*Nkebiwe* et al., 2016).

Some of the earliest studies on fertilizer placement reported positive plant growth and yield effects (*Reith,* 1954; *Cooke,* 1954). Today, "starter" fertilizers (e.g. di-ammonium phosphate for maize), are commonly applied close to plant roots to ensure optimal N and P supply during critical early growth stages especially in cold climate regions(*Grant* et al., 2001). "Complete" fertilizer placement is also performed, in which the fertilizer need for the vegetative season is supplied as a single rich subsurface fertilizer-depot. Nevertheless, poor root-growth in the fertilizer-depot zone often limits crop nutrient acquisition from the depot. Inclusion of root-attracting nutrients like ammonium and orthophosphate ions, or inoculation of root-growth-stimulating PGPMs in the fertilizer depot zone, is a possible solution. There is evidence that fertilizer depots comprising ammonium and phosphates lead to higher N and P uptake and yield than fertilizer depots comprising either ammonium or phosphate and not both (*Nkebiwe* et al., 2016). This phenomenon is primarily due to stronger localized root growth induced within the fertilizer depot by the presence of ammonium than by phosphates (*Jing* et al., 2010). Secondly, NH_4^+-uptake from NH_4^+-rich subsurface fertilizer depots induces rhizosphere acidification around the depot-zone, which enhances plant P-acquisition in neutral to alkaline soils (*Jing* et al., 2010). Low rhizosphere pH may also modify proliferation and cell-wall mechanical properties of root cells (*Jing* et al., 2010).

(*Sommer,* 2005)proposed the term Controlled Long-Term Ammonium Nutrition (CULTAN) to describe a technique for "complete" N-fertilizer placement in which a subsurface fertilizer

depot based on toxic concentrated NH_4^+ solution is placed at a rate to cover crop N demand during the vegetation season.

Although subsurface fertilizer placement and soil-inoculated plant growth-promoting microorganisms (PGPMs) have been separately studied considerably and somewhat also separately adopted, little is known about the combination of both. We propose that root colonization by PGPMs can be enhanced if PGPMs are inoculated in rhizosphere "hotspots", developing around an NH_4^+-based fertilizer depot, due to NH_4^+-induced dense root-growth and consequently, high levels of organic nutrients for microbes released as root exudates(*Lugtenberg* and *Kamilova,* 2009; *Bonkowski* et al., 2000).

Preliminary studies on fertilizer placement in combination with inoculation of PGPMs, such as subsurface banding of inorganic P fertilizer combined with seed-inoculated PGPM(s) (*Dutta* and *Bandyopadhyay*, 2009) or subsurface banding of NPK-enriched bio-compost treated with PGPMs (*Arshad* and *Waqas,* 2011), have produced promising results.

Using maize (*Zea mays* L.) as a test crop, our objective was to investigate the effect of N fertilization by placement of an NH_4^+-depot and substrate-inoculation of the most promising PGPMs on root growth, rhizosphere modification, root-colonization by PGPM, plant growth and development, shoot nutrient concentration and content, and yield. PGPMs were selected based on initial *in vitro* laboratory tests from which promising candidates showed considerable tolerance to high levels of stabilized NH_4^+ and ability to solubilize insoluble $Ca_3(PO_4)_2$ (*Nkebiwe et al.,* 2014 submitted). We hypothesized that: (1) Placement of NH_4^+-fertilizer as a subsurface depot stimulates intense root growth around the depot, forming "rhizosphere hotspots". (2) Marked rhizosphere acidification occurs within and around the NH_4^+-depot zone. (3) Survival and colonization of inoculated PGPMs is higher in the "rhizosphere hotspot" than in the comparable soil volume with respect to plant position that is supplied homogeneously with NO_3^- fertilizer. (4) Inoculated and established PGPMs further

promote root development around the NH_4^+-depot zone. (5) Consequently, NH_4^+-depot fertilization combined with inoculated PGPMs leads to higher nutrient uptake and higher yields than NH_4^+-depot fertilization without PGPMs.

6.3 Methods

6.3.1 Greenhouse experiments

6.3.1.1 Choice of N-form for placement

A central theme in this study was N-fertilizer placement in subsurface soil to improve crop N-acquisition. Effective N-fertilizer placement required application of a suitable N-source to form a relatively stable subsurface N-depot that is sufficiently close to seeds or plant roots for optimal N-acquisition but distant enough not to impair seed germination and plant growth. Therefore, for main experimental treatments, NH_4^+ was selected over NO_3^- and $CO(NH_2)_2$ because of its low mobility in soil owing to a low effective diffusion coefficient and low mass flow (*Barber* 1984; *Anghinoni* and *Barber*, 1990; *Neumann* and *Römheld*, 2012) and also due to its ability to bind or be fixed to negatively charged sites on clay particles(*Nieder* et al., 2011). This property of NH_4^+ favorably inhibits N movement out of the depot zone to the surrounding unfertilized soil. For these reasons, it is not logical to locally place NO_3^- as a N-depot in soil because it will rapidly move out of the original spot into the surrounding soil by diffusion and mass flow (*Anghinoni* and *Barber*, 1990). NH_4^+ was also selected because it induces stronger localized root growth at the site of contact with roots than NO_3^- or $CO(NH_2)_2$ (*Anghinoni* and *Barber*, 1990; *Drew*, 1975; *Hawkesford* et al., 2012; *Jing* et al., 2012). This feature coupled with low mobility in soil makes NH_4^+ the ideal N-form to stimulate the formation densely rooted soil zones, “rhizosphere hotspots”. The NH_4^+-fertilizer chosen was further stabilized with a nitrification inhibitor (3, 4-Dimethylpyrazole phosphate (DMPP) (*Zerulla* et al., 2001) to reduce N movement out of the depot as NO_3^-. Moreover, to minimize microbial nitrification, a highly concentrated, toxic NH_4^+-depot solution was used to create a

stable persisting NH_4^+- depot in which microbial-growth, root-growth and root N uptake is initially limited to the outer boarder areas with less toxic NH_4^+ levels (*Sommer,* 2005). Therefore, in these experiments on natural-soils or soil-based substrates, localized supply of N could only be realized as a subsurface NH_4^+ -depot - with its associated effects on localized root-growth stimulation and rhizosphere acidification - and not as NO_3^-.

If the experimental treatment is localized nutrient supply, the logical control should be uniform nutrient supply (*Robinson,* 1994) given that the quantity of nutrient supplied per experiment unit (treatment or control) is the same.

For control treatments, NO_3^- (e.g. calcium ammonium nitrate, CAN) or $CO(NH_2)_2$ were suitable low-cost N-fertilizers commonly used by farmers and applied simply by broadcast and incorporation. In natural soil, NO_3^- or $CO(NH_2)_2$ is not normally and cannot be placed locally as a subsurface depot; not without the use of wax membranes (*Drew,* 1975) or other water-tight barriers(*Robinson,* 1994). This is because NO_3^- or $CO(NH_2)_2$ cannot to bind to clay particles and are very mobile soil(*Drew,* 1975). For these reasons, NO_3^- homogenously mixed in the substrate was chosen as a suitable control. It was not considered necessary to include homogenously mixed NH_4^+ and locally placed NO_3^- for the sake of completeness because with NH_4^+ nitrification and NO_3^- diffusion, as discussed, these treatments will, within a few days, become essentially the same as homogeneously mixed NO_3^-.

6.3.1.2 Pot experiment

Maize (*Zea mays* L. var Colisee, KWS, Germany) was grown in 1.6 l pots (20 cm X 10 cm Ø) under controlled root-zone temperature of 20 ± 2 °C. The substrate was based on 66 % low-P soil from a long-term unfertilized grassland (0-20 cm depth; P_{CAL}, 30 mg kg^{-1}; P_{total}, 667 mg kg^{-1}; K_{CAL}, 233 mg kg^{-1}; Mg_{CaCl2}, 66 mg kg^{-1}; pH ($CaCl_2$), 7.1; C_{org}, 2.4%; N_{total} 0.24 %) and 34 % quartz sand (0.6 -1.2 mm Ø), on weight basis. There was a control treatment without

any fertilizer (**No P**). The substrate for the treatment **NH_4^+-Depot** was fertilized as follows (kg^{-1} soil DM): 100 mg N ($(NH_4)_2SO_4$); 150 mg K (K_2SO_4); 50 mg Mg ($MgSO_4$); and 22 % H_2O (75 % max. water holding capacity). Apart from $(NH_4)_2SO_4$, which was applied in salt form as a concentrated depot (7 cm long band located 5 cm below and 5 cm to the side of the maize seed, 5 x5 cm), all other nutrients where homogenously mixed in the substrate. There were two variants of the **NH_4^+-Depot** treatment; one without PGPM and the other inoculated with *Pseudomonas sp.* DSMZ13134 as bio-effector (**Pro**). Bio-effectors (BEs) are viable plant growth-promoting microorganisms (PGPMs) and/or active natural compounds which directly or indirectly promote plant growth with a negligible direct input of nutrients and/or organic matter (*Weinmann and Römheld,* 2012). Additionally, there was a positive P control (**+P**), with its substrate fertilized similarly to that of NH_4^+-Depot described above except that N (100 mg N as $CaNO_3$) and P (150 mg as $Ca(H_2PO_4)_2$) where homogenously mixed in the substrate.

The inoculum of *Pseudomonas sp.* DSMZ13134 (Pro) was prepared from the commercially available product Proradix, which is a powder formulation of viable cells and other additives (Sourcon Padena, Tübingen, Germany). For treatment of turf, the producer recommended rate is 10 g Proradix suspended in 200 – 400 l water and applied on an area of 1000 m^2. This rate is commensurate with 4.4 x 10^6 CFUs kg^{-1} soil DM, assuming a soil bulk density of 1.5 g cm^{-3} and a treated soil depth of 10 cm. The producer also refers to higher application rates for different plant species: 1 x 10^{10} CFUs kg^{-1} soil for substrate-inoculation in pot-grown tomato or barley (*Yusran* et al., 2007; *Fröhlich* et al., 2012) and 8 x 10^{10} CFUs kg^{-1} seed for seed-inoculation in pot- and field-grown barley(*Fröhlich* et al., 2012). In an initial screening test with *c* among other bacterial and fungal PGPMs on maize, the inoculation rate of 2 x 10^8 CFUs kg^{-1} soil DM led to little or no effect on root or shoot growth in comparison to the non-inoculated controls. Therefore, in this pot experiment, we employed a high inoculation rate of

1 x 10^{11} CFUs kg^{-1} soil DM. This was to improve the chance for early root colonization in the immediate seeding-zone as well as late root colonization in the fertilizer depot zone.

Each pot was filled with 1.9 kg of substrate. For each pot of treatment Pro, half of the total quantity of inoculum was drenched over the seeding hole at sowing to ensure early root colonization and the other half was placed directly over the NH_4^+-depot to promote root colonization in the developing rhizosphere "hotspot". To prepare the inoculum for the seed-hole, Proradix (6.6 x 10^{10} CFUs g^{-1}) was suspended in 2.5 mM $CaSO_4$ to a concentration of 5 x 10^9 CFU ml^{-1} and 10 ml of the suspension (5 x 10^{10} CFUs) was applied by drenching over the seed in the seed-hole. To maintain a concentrated NH_4^+-depot, the inoculum suspension applied over the NH_4^+-depot was more concentrated than the one drenched over the seed-hole. It had a concentration of 2.5 x 10^{10} CFU ml^{-1} and 2 ml of the suspension (5 x 10^{10} CFUs) was pipetted directly over the $(NH_4)_2SO_4$ depot. The total inoculation rate for Pro pots was, therefore, 1 x 10^{11} CFUs kg^{-1} soil DM (1 kg Soil DM pot^{-1}). For other treatments, volumes of 2.5 mM $CaSO_4$ were applied accordingly. There were 4 replicates per treatment arranged in a completely randomized design. There was16 h. light and 8 h. darkness. Average daily temperature was 20 ± 2 °C (Max. 26.9 °C and min. 14.6 ° C).

The diameter of the stem base and the maximum area of the youngest fully developed leaf were measured at 55 days after sowing (DAS). At 56 DAS, SPAD values were measured on the youngest fully developed leaf (average of 6 measurements $leaf^{-1}$) using SPAD 502 Plus (Konica Minolta, Chiyoda, Japan). SPAD values represent chlorophyll concentrations, which positively correlate with leaf N-concentration.

Shoot and root biomass (65 DAS) were harvested and dried (60 °C 48 h.). Shoot N and P concentrations were measured using CN elemental analyzer and molybdate-vanadate method (*Gericke* and *Kurmies,* 1952) respectively.

6.3.1.3 Rhizobox experiment

Maize (*Zea mays* L. var Colisee) was grown in rhizoboxes (40 x 20 x 2 cm; H x W x D). The substrate was based on 80 % low-P, loess-based, C-horizon subsoil (P_{CAL}, 5 mg kg^{-1}; P_{total}, 332 mg kg^{-1}; pH ($CaCl_2$), 7.6; C_{org}, < 0.3%; N_{total} 0.02 %; $CaCO_3$, 23 %) and 20 % quartz sand (0.6 -1.2 mm Ø), on weight basis. The substrate was adequately supplied with the following nutrients (kg^{-1} soil DM): N (100 mg, $Ca(NO_3)_2$ or $(NH_4)_2(SO_4)$; P (150 mg, $Ca(H_2PO_4)_2$); K (150 mg, K_2SO_4); Mg (50 mg, $MgSO_4$); micronutrients: 20 µmol Fe, Sequestrene138, 6 % Fe; 2.6 mg Zn, $ZnSO_4$; 1 mg Cu, CuSO4); and H_2O (60 % max. water holding capacity, 18 % moisture). Each rhizobox was filled with 2.4 kg of substrate.

Treatments included two N levels: 1.) $CaNO_3$ homogenously mixed in the substrate (**NO_3^--Mixed**) and 2.) Concentrated $(NH_4)_2SO_4$, fertilizer (64 mg N ml^{-1}) stabilized with the nitrification inhibitor 3, 4-Dimethylpyrazole phosphate (DMPP) (*Zerulla* et al., 2001) (NovaTec® Solub 21, Compo Expert, Münster, Germany) placed as a depot 5 x5 cm to the maize seed (**NH_4^+-Depot**); in factorial combination with two **BE** levels: 1.) no inoculation (**NoBE**) and 2.) inoculation with (**Pro**) at the rate 1 x 10^9 CFUs kg^{-1} soil DM (x 2 applications). The inoculation rate of 1 x 10^9 CFUs or Spores kg^{-1} soil DM for bacterial bio-effectors was later recommended by project management as consistent with producer suggested rates for different soil-inoculated microbial PGPMs.

To prepare the inoculum, Proradix (6.6 x 10^{10} CFUs g^{-1}) was suspended in 2.5 mM $CaSO_4$ to a concentration of 5 x 10^8 CFU ml^{-1}. Through the rhizobox window after sowing, 3.26 ml of the inoculum suspension was pipetted on the substrate 2.5 cm around the NH_4^+-depot zone or corresponding soil zone in NO_3^--Mixed treatments. The second inoculation was performed two weeks after sowing. There were 4 replicates per treatment arranged in a completely randomized design. Greenhouse conditions were set at 16 h. light at 25°C and 8 h. darkness at 18 °C.

At 32 DAS, SPAD values on the youngest fully developed leaf, plant height and stem diameter were measured. Plants were harvested at 55 DAS. pH on the root surface 0 – 8 cm from and >8 cm away from the NH_4^+ depot or corresponding soil zone in NO_3^--Mixed treatments was assessed qualitatively for color changes with Bromocresol-purple pH-indicator agar (*Marschner* and *Römheld,* 1983; *Häussling* et al., 1985) and quantitatively by measurement of potential difference using antimony micro-electrodes (*Marschner* and *Römheld,* 1983; *Schaller* and *Fischer,* 1985).

For qualitative pH assessment with Bromocresol-purple pH-indicator agar, 1 % bromocresol-purple solution was prepared two weeks before use as recommended. For it, 1 g bromocresol-purple was suspended in 80 ml dest. water in a 100 ml Erlenmeyer flask. For dissolution to occur, 1N NaOH was added dropwise under continuous stirring ensuring with a pH meter that the pH of the solution did not exceed 9. After about 30 minutes, the pH ceased to decrease indicating complete dissolution. At that point, the pH of the solution was lowered to 6 using 1N H_2SO_4. The flask was filled up with dest. water to the 100 ml mark. Under stirring, 5g Agar was suspended and cooked in 400 ml dest. water in a 500 ml Erlenmeyer flask to completely dissolve. 5 ml of 1 % bromocresol-purple solution was added and then the flask was filled up with dest. water to 500 ml. At about 40 °C, bromocresol-purple-agar solution was then poured on a Plexiglas tray to a layer about 3 mm thick. Once solidified, the layer of agar was carefully placed over the soil surface on the rhizobox window to cover the NH_4^+-depot zone or the corresponding zone in NO_3^--Mixed treatments. After a few minutes, color change along the root surface could be observed, yellow for acidification below pH 5.2 and purple for alkalinization above pH 6.8. In order to read pH changes, color standards were prepared by mixing 50 µl pH buffer solutions (4, 5, 6, 7, 8, 9, and 10) with 450 µl bromocresol-purple-agar solution in small transparent glass-vial caps and allowed to solidify.

For quantitative potentiometric pH measurements, antimony micro-electrodes were calibrated by measuring the potential difference (200 to 500 mV) of pH buffer solutions (4, 5, 6, 7, 8, 9, and 10) and generating the following best fitting sigmoidal calibration curve with five parameters: f = y0+a/(1+exp(-(x-x0)/b))^c; R^2 = 0.99, SEM = 0.2; f = pH; x = potential difference (mV); a = 6.1419; b = 47.8570; c = 1.9364; x0 = 339.7333; and y0= 4.0089). For the regression, *SigmaPlot 12.0* was used (Systat Software Inc**.**(SSI), San Jose, California, USA).The potential difference on the root surface below the bromocresol-purple-agar was measured with a pH meter (pH 320, WTW GmbH Weilheim, Germany) connected to an antimony micro-electrode and to a reference calomel-electrode. Measured potential differences were back-transformed to pH using the sigmoidal calibration curve.

Separately, roots located within 8 cm or more than 8 cm away from the NH_4^+-Depot or corresponding zone were harvested, washed, scanned and root length and architecture analyzed using *WinRhizo Pro V. 2009c* (Regent Instruments Inc., Canada). To measure the number of rhizoplane-dwelling fluorescent Pseudomonads per unit volume of substrate in the NH_4^+ depot or corresponding zone in NO_3^--Mixed treatments, 0.5 – 1.5 g of fresh root sample were thoroughly washed with sterile deionized water (autoclaved 121° C for 20 min.), shaken with 50 ml of sterile ice-cooled 0.1 % proteose peptone and 10 sterile glass beads at 250 rpm for 15 min using autoclaved 250 ml Erlenmeyer flasks. After shaking, flasks were cooled on ice, 5 ml extracts were serially 10-fold diluted with 0.1 % proteose peptone, plated on selective Kings B medium containing 45 mg Novobiocin l^{-1} and 45 mg Penicillin l^{-1} (*Sugimoto* et al., 1990), and incubated 23 h. at 30 °C. The number of colonies were counted and the colonization rate per gram fresh root was calculated. Using colonization rate and weight of fresh roots per unit substrate volume around the fertilizer depot zone (or corresponding zone for NO_3^- treatments), we calculated the colonization rate per unit substrate volume.

Shoot and root biomass were harvested and dried (60 °C 48 h.). Shoot N and P concentrations were measured.

6.3.2 Field experiments

6.3.2.1 2014

Maize (*Zea mays* L. var Colisee) was grown on soil with moderate N_{min} and available P levels at the research station of the University of Hohenheim, Ihinger Hof, Renningen, Germany (48°44'42.3"N 8°55'26.7"E; 475 m above sea level; 688 mm av. annual rainfall; 8.8 °C mean annual daily temperature). Soil properties were Haplic luvisol, 24-28 % clay, 67-72 % silt, 4-5 % sand, pH ($CaCl_2$) 6.9, C_{org}, 1 %, N_{min}, 38 kg ha^{-1}; P_{CAL}, 120 mg kg^{-1}.There were 8 treatments (Table 6.1) arranged in a Latin rectangle design with 5 columns and 5 rows (there were 17 other treatments as part of another study). After sugar beet harvest, the soil was ploughed with a moldboard plough to 20 cm depth in autumn 2013. Plot area was 45 m² (4.5 m x 10 m) and contained 6 maize rows (75 cm inter-row distance). Data was collected only from the central four core rows (2 – 5). The first and last 1 m length of each plot and rows 1 and 6 were excluded as plot boarders.

Table 6.1. Treatments (Field experiments 2014 and 2015)

	Treatments 2014	Starter N and P	Additional P	Additional N	BE
1	Zero	-	-	-	-
2	+P	MAP	TSP	NH_4^+-Broad.	-
3	NH_4^+-Broad.	MAP	-	NH_4^+-Broad.	-
4	NH_4^+-Broad.*Pro.	MAP	-	NH_4^+-Broad.	Pro
5	NH_4^+-Broad.*Rhiz	MAP	-	NH_4^+-Broad.	Rhiz
6	NH_4^+-Depot	MAP	-	NH_4^+-Depot	-
7	NH_4^+-Depot*Pro	MAP	-	NH_4^+-Depot	Pro
8	NH_4^+-Depot*Rhiz	MAP	-	NH_4^+-Depot	Rhiz
	Treatments 2015				
1	Zero	-	-	-	-
2	+P	DAP	TSP	NH_4^+-Broad.	-
3	NH_4^+-Broad.	DAP	-	NH_4^+-Broad.	-
4	NH_4^+-Broad.*Pro	DAP	-	NH_4^+-Broad.	Pro
5	NH_4^+-Broad.*Rhiz	DAP	-	NH_4^+-Broad.	Rhiz
6	NH_4^+-Depot	DAP	-	NH_4^+-Depot	-
7	NH_4^+-Depot*Pro	DAP	-	NH_4^+-Depot	Pro
8	NH_4^+-Depot*Rhiz	DAP	-	NH_4^+-Depot	Rhiz

Starter (starter fertilizers placed 5 x 5 cm to seeds): MAP: Mono-ammonium phosphate, 17 kg N and 35 kg P ha^{-1}; DAP: di-ammonium phosphate placed 5 x 5 cm to seeds at sowing; 28.8 kg N and 32 kg P ha^{-1};TSP: Triple superphosphate broadcasted and incorporated at 10 cm

depth before sowing; 2014, 133 kg; 2015,130 kg P ha^{-1}; NH_4^+-Broad.: Stabilized $(NH_4)_2SO_4$ broadcasted over the canopy, 2014,135 kg N; 2015 100 kg N ha^{-1} ; NH_4^+-Depot: Stabilized concentrated $(NH_4)_2SO_4$ solution in water placed as a depot at 10 cm soil depth; 2014,135 kg N; 2015 100 kg N ha^{-1}; Pro: *Pseudomonas* sp. DSMZ 13134.; Rhiz: *Bacillus amyloliquefaciens* FZB42, 1 x 10^6 CFU g^{-1} soil DM.; BE application: 2014, Broadcast/incorporation 1 x 10^6 CFU g^{-1} soil DM; 2015 , Placement of a band of BE-treated pumice stones in the sowing row; 0.13 x 10^6 CFU g^{-1} soil DM

Fertilizer type, application methods and rates were as follows (Table 6.1): 1.) **MAP**: Mono-ammonium phosphate (12 % NH4-N, 22 % P) (Krista™ MAP, Yara GmbH, Germany); 17 kg N and 35 kg P ha^{-1}. MAP, was placed as "starter" fertilizer on 21 May. "Starter" fertilizer placement was performed at 5 cm to both sides of and 5 cm below the seeding zone with the assistance of GPS and additional on-site positioning tools; 2.) **TSP**: Triple superphosphate (20 % P) hand-broadcasted (20 May 2014) and incorporated at 10 cm depth the following day before sowing; 133 kg P ha^{-1}; 3.) **NH_4^+-Broad.:** Stabilized $(NH_4)_2SO_4$ (21 % NH4-N, 24 % S) broadcasted over the canopy at 5-6 leaf stage (24-25 June), (NovaTec® Solub 21, Compo Expert, Münster, Germany); 135 kg N ha^{-1}; 4.) **NH_4^+-Depot:** Concentrated stabilized $(NH_4)_2SO_4$ (21 % NH4-N, 24 % S) in water (64 g N l^{-1}) placed at 10 cm depth midway between rows 1 – 2, 3 – 4 and 5 – 6 at 5-6 leaf stage (24-25 June); 135 kg N ha^{-1}.

The bio-effectors applied included **Pro** (already described) and **Rhiz**, *Bacillus amyloliquefaciens* FZB42 (2.5 x 10^{10} spores g^{-1}), a commercially available product in liquid formulation containing spores and other additives (**Rhizovital FZB42**, ABiTEP GmbH, Berlin Germany). The producer recommended application rates are 100 – 500 ml ha^{-1} for seed-treatment and 1000 – 2000 ml ha^{-1} for soil application by drenching or spraying. These rates are commensurate with 1.7 – 8.3 x 10^6 spores kg^{-1} soil DM (for seed treatment) and 1.7 – 3.4 x 10^7 spores kg^{-1} soil DM (for soil treatment), assuming a treated soil depth of 10 cm and a bulk density of 1.5 g cm^{-3}.

Like Pro, Rhiz was also applied at a rate of 1 x 10^9 Spores kg^{-1} soil DM as recommended by project management. To apply bio-effectors, stock suspensions were freshly prepared, diluted on field site and applied on the soil surface on the same day. Bio-effectors were applied one day before sowing (20 May) and again at 2-4 leaf stage (17 June). For the first application of Pro, 1 kg of Proradix (6.6 x 10^{10} CFUs g^{-1}) was suspended in about 18 l Cl-free water to produce 20 l Pro stock suspension with a concentration of 6.75 x 10^{12} CFU l^{-1}. 2 l of stock were diluted with Cl-free water to 24 l, applied using a watering can over the soil surface and incorporated to 10 cm depth (Soil bulk density 1.5 g cm^{-3}) just before sowing on 21 May. The total quantity of Pro inoculum used in the second application (2-4 leaf stage, 17 June) was reduced as a means to reduce costs while maintaining the CFU density of 1 x 10^9 CFU kg^{-1} soil DM in the crop row. In order to achieve this, the inoculum was sprayed only over the maize row to drench the soil beneath (about 10 cm width) instead of over the entire plot area. For this, a dilute Pro stock was prepared (9.0 x 10^{11} CFU l^{-1}) from which 2 l were diluted with Cl-free water to 24 l and applied using a watering can.

For the first application of Rhiz, 5.4 kg of Rhizovital FZB42 (2.5 x 10^{10} spores g^{-1}) were suspended in about 14.6 l Cl-free water to produce 20 l of Rhiz stock suspension with a concentration of 6.75 x 10^{12} spores l^{-1}. Like Pro, Rhiz was applied at a rate of 1 x 10^9 CFU kg^{-1} soil DM. The second application of Rhiz was also performed at 2-4 leaf stage (17 June). 2 l of Rhiz stock (9.0 x 10^{11} CFU l^{-1}) were further diluted with Cl-free water to 24 l and applied over the maize row.

Using a pneumatic plot drill and positioning tools (GPS and on-site correction devices), plots were seeded at the pre-defined rows with untreated maize at the rate of 9 – 10 seeds m^{-2} on 21 May. Top dressing of $(NH_4)_2SO_4$ at 5-6 leaf stage (24-25 June) resulted in leaf injury if fertilizer was trapped on the leaf surface. Plants later fully recovered. Concentrated stabilized $(NH_4)_2SO_4$ solution placed at 10 cm depth as a depot showed no signs of injury to the plants.

Plant emergence 16 days after sowing (DAS; number of emerged plants along 2 m row length x 4), number of 2-leaf stage plants 16 DAS, BBCH 12 (measured similarly to emergence) and plant height (23 and 78 DAS; 10 successive plants row^{-1} x 4) where measured for the controls: Zero, NH_4^+-Broad. and +P. At 35 and 53 DAS (1 and 19 days after placement of NH_4^+-fertilizer depot respectively), soil samples 0 – 30 cm depth were collected from the midway point between rows 1 – 2, 3 –4 and 5 – 6 (NH_4^+-Depot zone or corresponding soil zone for non- NH_4^+-Depot treatments). N_{min} concentrations in samples were measured. SPAD (43 and 79 DAS; average of 4 measurements $leaf^{-1}$ x 5 successive plants row^{-1} x 4), ear-leaf N and P concentrations (79 DAS; 4 ear-leaf samples row^{-1} x 4) were measured. For treatments NH_4^+-Broad**.***Pro and NH_4^+-Depot*Pro at 81 DAS (47 days after placement of depot), soil core samples (30 cm L, 5 cm ∅) were collected; four samples were collected from the NH_4^+-Depot zone (or corresponding soil zone for NH_4^+-Broad**.** treatment) at midway point between rows 1 – 2, 3 –4 or 5 – 6 and four from the non-Depot zone, between rows 2 – 3 or 4 – 5. Soil samples were washed, roots were collected, scanned and analyzed (*WinRhizo Pro*). Grain was harvested on 8 Nov. (172 DAS).

6.3.2.2 2015

Maize (*Zea mays* L. var Colisee) was grown on soil at another site in Ihinger Hof research station. Like 2014, this site had moderate N_{min} and available P levels. Soil properties included: Haplic luvisol, clay loam, silty loam, pH 7.0, N_{min}, 61 kg ha^{-1}, P_{CAL}, 110 mg kg^{-1}.There were 8 treatments (Table 6.1) arranged in a completely randomized block design with 5 blocks (10 additional treatments were part of another study). Plot area was 58.5 m² (4.5 m x 13 m) with 6 maize rows (75 cm inter-row distance). Like in 2014, plot boarders were excluded during data collection.

Fertilizer types, application methods and rates included (Table 6.1): 1.) **DAP**: starter fertilizer as di-ammonium phosphate placed 5 x 5 cm to seeds at sowing (13 May); 28.8 kg N and 32 kg P ha^{-1}; 2.) **TSP:** Triple superphosphate broadcasted by hand and incorporated at 10 cm depth before sowing (11 May); 130 kg P ha^{-1}; 3.) **NH_4^+-Broad.:** Stabilized $(NH_4)_2SO_4$ broadcasted and incorporated 10 cm deep before sowing (11 May); 100 kg N ha^{-1}; 4.) **NH_4^+-Depot:** Concentrated solution of stabilized $(NH_4)_2SO_4$ in water (62.7 g N l^{-1}) placed as a depot at 10 cm depth midway between rows 1 – 2, 3 – 4 and 4 – 5 (4 – 5 leaf stage, 18 June); 100 kg N ha^{-1}.

Bio-effectors included **Pro** and **Rhiz**, each placed as a band of BE-treated pumice stones (Table 6.1). To treat pumice stones (Rotocell 0.3 – 1.5, density 320 kg m^{-3}, ROTEC GmbH & Co. KG, Mülheim-Kärlich, Germany) with BE, Cl-free water suspensions of Proradix (6.6 x 10^{10} CFUs g^{-1}) and Rhizovital FZB42 (2.5 x 10^{10}spores g^{-1}) were each prepared to a concentration of 2 x 10^{12} CFUs l^{-1} or spores l^{-1}. Each suspension was evenly applied using a pressurized hand pump sprayer at the rate of 0.23 l kg^{-1} pumice stones, which were spread on a plastic sheet (0.47 x 10^{12} CFUs or spores kg^{-1} pumice stones). Pumice stones were then turned over several times to homogenize inoculum absorption, air-dried at room temperature and applied on the field on the same day. Application was done by placement in 5-10 cm deep furrows cut in the sowing row. The application rate was 32 g pumice stones m^{-1} furrow (100 ml pumice stones m^{-1} furrow). Furrows were covered with soil and the entire plot was tilled with a rototiller to10 cm depth. The final inoculum density in soil within the sowing row was 1 x 10^9 CFU kg^{-1} soil DM (15 kg soil DM m^{-1} furrow; 10 cm row width and 10 cm row depth, soil bulk density 1.5 g cm^{-3}), which was about ten times higher the inoculum density (10 cm depth) if the inoculum was evenly applied over the entire plot area (0.13 x 10^9 CFU kg^{-1}).

On 12 May, plots were sown at the rate of 9 – 10 seeds m^{-2} as in 2014. For treatment NH_4^+-Depot only, soil samples 0 – 30 cm depth were collected from the midway point between

rows 1 – 2, 3 – 4 and 5 – 6 (NH_4^+-Depot side) and 2 – 3 and 4 – 5 (Non NH_4^+-Depot side) on 30 Jun. (48 DAS) and N_{min} concentration was measured. For treatments Zero, +P, NH_4^+-Broad., NH_4^+-Depot, NH_4^+-Depot*Pro and NH_4^+-Depot*Rhiz, plant height 48 DAS was recorded. For all treatments, plant height (71 DAS), SPAD (68 DAS), stem diameter (68 DAS, max. diameter between nodes 2 and 3, sampling was done as for plant height 2014) were collected. To measure root length density in the fertilizer depot zone for treatments NH_4^+-Broad**.** and NH_4^+-Depot at 99 DAS (63 days after placement of depot), soil core samples (30 cm L, 5.5 cm Ø) were collected, four from the NH_4^+-Depot zone (or corresponding soil zone for NH_4^+-Broad**.** treatment), midway point between rows 1 – 2, 3 –4 or 5 – 6 and four from the non-Depot zone, midway point between rows 2 – 3 or 4 – 5. Soil samples were washed, roots were collected, scanned and analyzed (*WinRhizo Pro*). On 21 Sep. (132 DAS), above-ground biomass was harvested for maize silage.

6.3.3 Statistics

For the pot and rhizobox experiments, One and Two-Way ANOVA with pair-wise comparisons (Tukey test, $\alpha = 0.05$) were performed (*SigmaPlot 12.0*, Systat Software Inc**.**(SSI), San Jose, California, USA). For field experiments, One and Two-Way ANOVA with pair-wise comparisons (Tukey test, $\alpha = 0.05$) or ANOVA on Ranks for not-normally-distributed data were performed (*SAS 9.4*, SAS Institute Inc., Cary, NC, USA).

6.4 Results

6.4.1 Pot experiment

Stem base diameter increased in the following order: NoP (8.5 mm) = NH_4^+-Depot (8.8 mm) < NH_4^+-Depot+Pro (10.8 mm) = +P (12.0 mm); and it strongly correlated with shoot P content ($r^2 = 0.83$, $P < 0.00001$). Maximum leaf area of the youngest fully developed leaf also

strongly correlated with shoot P content (r^2 = 0.71, *P < 0.00001*). SPAD increased in the following order: NoP (27.6) = +P (29.2) < NH_4^+-Depot (36.3) = NH_4^+-Depot+Pro (37.4).

After harvest, NoP plants showed the lowest shoot dry weight (4.7 g $plant^{-1}$) and shoot P content (6.1 mg P $plant^{-1}$). +P plants showed the highest shoot dry weight (11.0 g $plant^{-1}$) and shoot P content (13.9 mg P $plant^{-1}$). For NH_4^+-Depot plants, inoculation with Pro (8.9 g $plant^{-1}$) led to 59 % more shoot dry weight than without (5.6 g $plant^{-1}$) (Fig. 6.1 a). With Pro, NH_4^+-Depot plants (11.3 mg P $plant^{-1}$) had 64 % higher shoot P content than without (6.9 mg P $plant^{-1}$) (Fig. 6.1 b). Similarly, with Pro (149.9 mg N $plant^{-1}$), there was 50 % higher shoot N content in plants than without (99.8 mg N $plant^{-1}$).

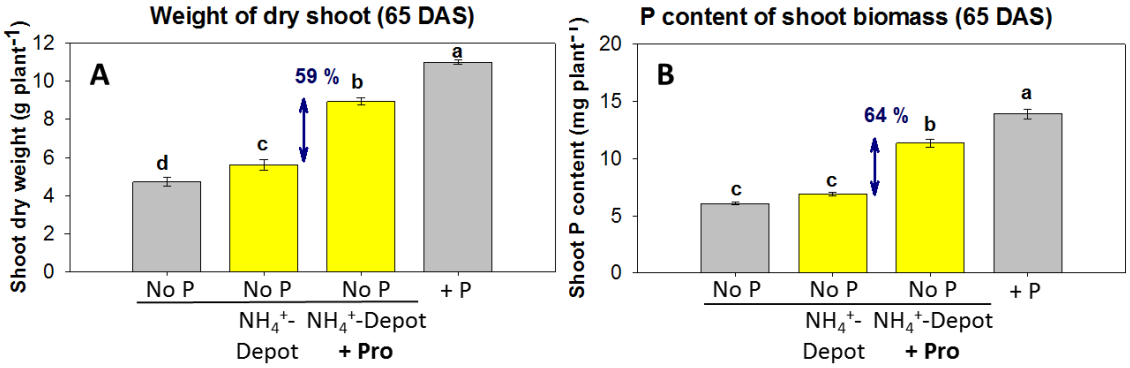

Figure 6.1. Shoot dry weight (A) and P content (B) (Pot experiment)
No P: No P fertilizer; **+P**: 100 mg NO_3-N and 150 mg soluble-P kg^{-1} soil; **NH_4^+-Depot** 100 mg NH_4-N kg^{-1} soil; **Pro**; *Pseudomonas sp.* DSMZ13134

There was no difference in the shoot P concentration between treatment pairs. Shoot N concentration increased in the following order: NoP (7.7 mg g^{-1}) < +P (10.1 mg g^{-1}) < NH_4^+-Depot+Pro(16.8 mg g^{-1}) = NH_4^+-Depot (17.9 mg g^{-1})

6.4.2 Rhizobox experiment

At 32 DAS, there was no difference in the SPAD value of the youngest fully developed leaf between pairs of treatments. BE (NoBE or Pro) had an effect on SPAD (Pro, 46.1 > NoBE, 43.6; *P = 0.040*) whereas N (NO_3^--Mixed or NH_4^+-Depot) did not. BE had an effect on plant

height (NoBE, 86.6 cm > Pro, 82.3 cm; *P = 0.012*) whereas N did not. Furthermore, N had an effect on stem diameter (NO_3^--Mixed, 11.6 mm > NH_4^+-Depot, 9.8 mm; *P = 0.008*) whereas BE did not. Stem diameter was not statistically different between treatment pairs.

At 55 DAS, there was higher root length density (RLD) in the fertilizer depot zone in treatment NH_4^+-Depot compared to the corresponding soil zone in treatment NO_3^--Mixed (Fig. 6.2 a). N had a strong effect on RLD within these zones (NH_4^+-Depot, 10.2 cm cm^{-3} > NO_3^--Mixed, 6.3 cm cm^{-3}; *P <0.001*) and BE did not. RLD in the remaining substrate volume of the rhizobox was affected by N (NO_3^--Mixed, 4.85 cm cm^{-3} > NH_4^+-Depot, 4.28 cm cm^{-3}; *P = 0.04*) (Fig. 6.2 b)and by BE (NoBE, 4.88 cm cm^{-3} > Pro, 4.26 cm cm^{-3}; *P = 0.03*), without any N * BE interaction.

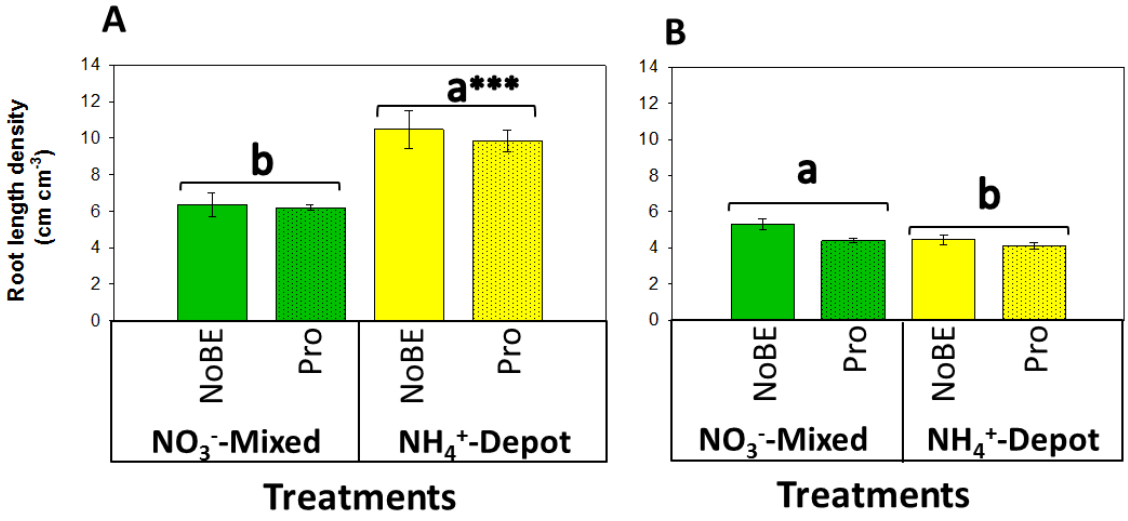

Figure 6.2. Root length density within (A) or outside (B) the NH_4^+-Depot or corresponding soil zone (Rhizobox)
NO_3^--Mixed, $Ca(NO_3)_2$ homogenously mixed in substrate; **NH_4^+-Depot**, stabilized $(NH_4)_2SO_4$ placed in substrate as a depot; **BE,** bio-effector; **NoBE**, no bio-effector; **Pro**, *Pseudomonas* sp. DSMZ 13134. Different letters between N-levels show a significant N-effect, *** P< 0.001 (Two-Way ANOVA,Tukey test, α = 0.05). There was no BE-effect within the NH_4^+-Depot zone. For RLD outside the zone, NO_3^--Mixed was higher than NH_4^+-Depot (P = 0.04) and NoBE was higher than Pro (P = 0.03)

Rhizosphere pH was lower in the NH_4^+ depot zone (NH_4^+-Depot) than in the corresponding soil zone with homogenous NO_3^- supply (NO_3^--Mixed) (Fig. 6.3 a) and only slightly lower for measurements in outer zones (Fig. 6.3 b). Rhizosphere acidification in the fertilizer depot

zone could be qualitatively confirmed by yellow coloration in Bromocresol-purple pH-indicator agar along roots growing in the NH_4^+-Depot zone (Fig. 6.3 c). Root colonization by fluorescent *Pseudomonas sp.* around the fertilizer depot zone in treatment NH_4^+-Depot was higher than that of the corresponding soil zone in treatment NO_3^--Mixed (Fig. 6.4).

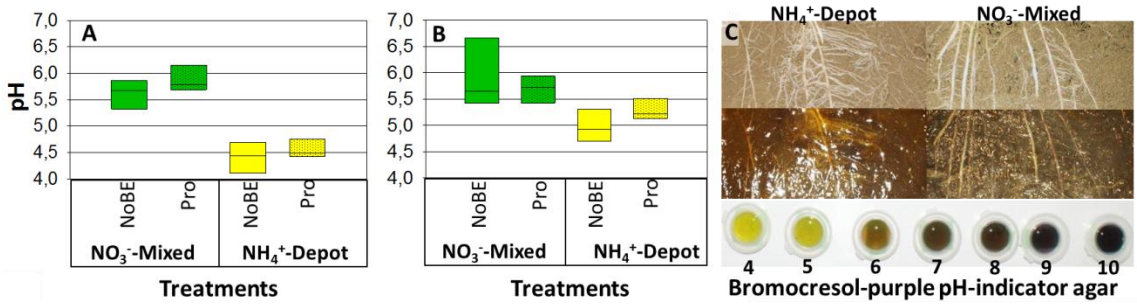

Figure 6.3. Root surface pH within (A, C) or outside (B) the NH_4^+-Depot **or corresponding soil zone (Rhizobox)**
NO_3^--Mixed, $Ca(NO_3)_2$ homogenously mixed in substrate; **NH_4^+-Depot**, stabilized $(NH_4)_2SO_4$ placed in substrate as a depot; **BE,** bio-effector; **NoBE**, no bio-effector; **Pro**, *Pseudomonas* sp. DSMZ 13134

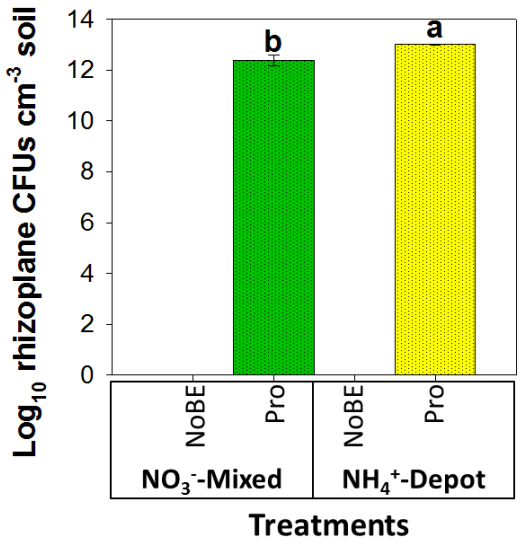

Figure 6.4. Root colonization by fluorescent Pseudomonas sp. within the NH_4^+-Depot **or corresponding soil zone (Rhizobox)**
NO_3^--Mixed, $Ca(NO_3)_2$ homogenously mixed in substrate; **NH_4^+-Depot**, stabilized $(NH_4)_2SO_4$ placed in substrate as a depot; **BE,** bio-effector; **NoBE**, no bio-effector; **Pro**, *Pseudomonas* sp. DSMZ 13134, (t-test, P = 0.045)

Maize shoots from NH_4^+-Depot treatments had higher shoot concentrations and contents of N and P than those from NO_3^--Mixed treatments (Table 6.2). There was no difference in shoot DM between pairs of treatments (Table 6.2).

Table 6.2. Shoot N and P concentration and content, and shoot dry matter, 55 days after sowing, (Rhizobox experiment)

Source of variation					
	N conc. (% DM)	**N content (mg N plant^{-1})**	**P conc. (mg P g^{-1} DM)**	**P content (mg P plant^{-1})**	**Shoot DM (g plant^{-1})**
LS Means N*BE					
NO_3^--Mixed*NoBE	1.83	114.0	2.18	13.7	6.35
NO_3^--Mixed*Pro	2.22	130.1	2.32	13.6	5.91
NH_4^+-Depot*NoBE	2.47	137.1	2.93	16.2	5.60
NH_4^+-Depot*Pro	2.66	133.9	3.15	15.9	5.09
Standard error	0.134	4.69	0.146	0.62	0.420
Two-Way ANOVA					
N	***0.002****	***0.014****	***<0.001******	***0.002 *****	*NS*
NO_3^-	2.03 b	122.0 b	2.52 b	13.6 b	6.13
NH_4^+	2.57 a	135.5 a	3.04 a	16.0 a	5.35
BE	***0.051***	*NS*	*NS*	*NS*	*NS*
NoBE	2.15	125.7	2.56	15.0	5.97
Pro	2.44	132.0	2.73	14.7	5.50
N * BE	*NS*	*NS*	*NS*	*NS*	*NS*

P values are in italics; ***NS,*** no significant difference, $P \geq 0.1$; $P < 0.1$ is bold; * $P < 0.5$; ** $P < 0.01$; *** $P < 0.001$; Means not sharing the same letters are significantly different from each other, Tukey test $\alpha = 0.05$; Factors and interaction is bold; **NO_3^-**, $Ca(NO_3)_2$ homogenously mixed in substrate; **NH_4^+**, stabilized $(NH_4)_2SO_4$ placed in substrate as a depot; **BE,** bio-effector; **NoBE**, no bio-effector; **Pro**, *Pseudomonas* sp. DSMZ 13134

6.4.3 Field experiment 2014

There was no difference in seed emergence (16 DAS), number of 2-leaf stage plants (16DAS) and plant height (23 and 78 DAS) between pairs of control treatments: Zero, NH_4^+-Broad. and +P).

One day after main N fertilization (35 DAS), soil NH_4-N concentration at 0 – 30 cm depth (fertilizer depot zone or corresponding zone) for treatment NH_4^+-Depot (304 kg NH_4-N ha^{-1}) was higher than that for NH_4^+-Broad**.** (36 kg NH_4-N ha^{-1}) and that for Zero (2.5 kg NH_4-N ha^{-1}). After 19 days (53 DAS), NH_4-N concentrations had reduced: NH_4^+-Depot (204 kg NH_4-N ha^{-1}), NH_4^+-Broad**.** (14.9 kg NH_4-N ha^{-1}) and Zero (2.15 kg NH_4-N ha^{-1}).

At 43 DAS, there was no difference in the SPAD value of the youngest fully developed leaf of plants between pairs of treatments: Zero (31.0), NH_4^+-Broad**.** (39.5), and NH_4^+-Depot (38.2). By 79 DAS, ear-leaf SPAD value for treatment Zero (50.8) was less than that for treatments NH_4^+-Depot (56.9), NH_4^+-Depot*Pro (56.3), NH_4^+-Depot*Rhiz (57.7), NH_4^+-Broad**.** (58.3) and +P (57.3) ($P<0.012$). N-fertilizer application method had an effect on ear-leaf P concentration (79 DAS) (Broad. > Depot; $P < 0.0001$, Table 6.3) whereas BE did not. Ear-leaf P concentration for treatment +P (4.90 mg P g^{-1} DM) was higher than that for other treatments. Zero had the lowest concentration (2.37 mg P g^{-1} DM), which was not different from that of NH_4^+-Depot (3.31 mg P g^{-1} DM), NH_4^+-Depot*Pro (3.40 mg P g^{-1} DM) or NH_4^+-Depot*Rhiz (3.17 mg P g^{-1} DM). Similarly, N-fertilizer application method had an effect on ear-leaf N concentration (79 DAS) (Broad > Depot; $P = 0.0029$, Table 6.3) whereas BE did not. The concentration for Zero (2.52 %) was lower than that for each of the other treatments ($P \leq 0.0485$), among which ear-leaf N concentrations were not different between pairs.

At 81 DAS, N-fertilizer application method ($P < 0.001$) and BE ($P = 0.005$) positively affected root length density (RLD) with a significant interaction between both factors ($P = 0.003$). RLD was doubled in soil on the sides of maize rows with a fertilizer depot (midway point between rows 1 – 2, 3 – 4 and 5 – 6) in comparison to those sides without (midway point between rows 2 – 3 and 4 – 5). RLD in the fertilizer-depot-zone was higher with Pro than without (Fig. 6.5 a).

N-fertilizer application method (despite NH_4^+-Depot – 7.69 Mg ha^{-1} being 7.4% higher than that of NH_4^+-Broad– 7.16 Mg ha^{-1}) and BE had no statistically significant effect on grain yield (Table 6.3). As expected, Zero produced the lowest grain yield (6.31 Mg ha^{-1}). Only NH_4^+-Depot*NoBE (8.45 Mg ha^{-1}) led to higher grain yields than Zero (*P =0.0086*).

Table 6.3. NH_4^+-application and bio-effector effects on Ear-leaf N and P, and grain yield (Field experiment 2014)

	Ear-leaf N conc. (%)	**Ear-leaf P conc. (mg g^{-1})**	**Grain (Mg ha^{-1})**
LS Means NH4*BE			
NH_4^+-Broad**.**	3.28	4.17	7.23
NH_4^+-Broad**.***Pro	3.25	4.16	7.23
NH_4^+-Broad**.***Rhiz	3.15	4.06	7.05
NH_4^+-Depot	3.02	3.31	8.35
NH_4^+-Depot*Pro	3.02	3.4	7.41
NH_4^+-Depot*Rhiz	2.91	3.17	7.36
Standard error	0.09	0.17	0.45
Two-Way ANOVA			
NH4 application tech.	***0.0029*****	***<0.0001******	*NS*
NH_4^+-Broad.	3.22 ± 0.05 a	4.13 ± 0.09 a	7.16 ± 0.28
NH_4^+-Depot	2.99 ± 0.05 b	3.29 ± 0.09 b	7.69 ± 0.29
BE	*NS*	*NS*	*NS*
NoBE	3.15 ± 0.06	3.74 ± 0.12	7.74 ± 0.34
Pro	3.14 ± 0.06	3.78 ± 0.12	7.35 ± 0.34
Rhiz	3.03 ± 0.06	3.61 ± 0.12	7.19 ± 0.34

P values are in italics; ***NS,*** no significant difference, P ≥ 0.1
; P < 0.1 is bold; * P < 0.5; ** P< 0.01; *** P< 0.001; Means ± standard errors not sharing the same letters are significantly different from each other, Tukey test α = 0.05; **NH_4^+-Broad.:** starter fertilizer as mono-ammonium phosphate (MAP) followed by broadcasting and incorporation stabilized $(NH_4)_2SO_4$ over the canopy; **NH_4^+-Depot:** Starter MAP and subsurface placement of concentrated stabilized $(NH_4)_2SO_4$ solution in water at 10 cm soil depth; **BE,** bio-effector; **NoBE,** no bio-effector; **Pro**: *Pseudomonas* sp. DSMZ 13134; **Rhiz**: *Bacillus amyloliquefaciens* FZB42; **Ear-leaf N and P** (79 DAS, BBCH 61-75) and **grain yield** (172 DAS, BBCH 89-99)

6.4.4 Field experiment 2015

There was severe soil compaction (> 15 cm depth) in many parts of the field site that led to placement of starter fertilizer and the NH_4^+- fertilizer depot often at a shallower depth than intended. Soil compaction also coincided with extreme drought and high temperatures in the summer months which caused NH_4^+- fertilizer depots to be pulled up to the soil surface forming a salt crust at several areas.

12 days after placement of fertilizer depots in treatment NH_4^+-Depot (48 DAS), soil NH_4-N concentration at 0 – 30 cm depth fertilizer depot zones (midway point between rows 1 – 2, 3 – 4 and 5 – 6; 337 kg NH_4-N ha^{-1}) was higher than that for zones without fertilizer depot (midway point between rows 2 – 3 and 4 – 5; 1.8 kg NH_4-N ha^{-1}, $P < 0.001$).

At 48 DAS, plant heights were statistically similar for NH_4^+-Depot with or without BE (NH_4^+-Depot, 93 cm; NH_4^+-Depot*Pro, 95 cm; NH_4^+-Depot*Rhiz, 93 cm). Only +P (107 cm) and NH_4^+-Broad**.** (101 cm) plants were taller than Zero plants (83 cm, $P \leq 0.0476$). At 71 DAS, N-fertilizer application method affected plant height (Broad. > Depot, $P = 0.0071$) whereas BE did not (Table 6.4). Only +P (235 cm), NH_4^+-Broad**.** (229 cm), and NH_4^+-Broad**.*** Rhiz (236 cm) plants were taller than Zero plants (210 cm, $P \leq 0.0485$).

Table 6.4. Sources of variation (Two-Way ANOVA, Field experiment 2015)

	SPAD 68 DAS	**Stem Ø 68 DAS (mm)**	**Height 71 DAS (cm)**	**Biomass F.M. (Mg ha^{-1})**	**Biomass D.M. (Mg ha^{-1})**
NH_4^+	***0.0087***	***0.0011***	***0.0071***	***0.0264***	*N.S.*
-Broad.	53.5 a	24.7 a	230.8 a	55.1 a	19.6
-Depot	52.1 b	23.4 b	222.7 b	52.1 b	18.6
BE_Band	*N.S.*	*N.S.*	*N.S.*	***0.0635***	***0.0364***
-NoBE	52.8	24.0	224.3	51.9	18.8 b
-Pro	52.4	23.9	226.4	55.6	20.3 a
-Rhiz	53.2	24.2	229.5	53.2	18.3 b
NH4 * BE_Band	*N.S.*	*N.S.*	*N.S.*	*NS*	*N.S.*

DAS, days after sowing; **Height,** plant height; **SPAD,** estimate of leaf N concentration; **Stem Ø,** stem diameter; P values are in italics; **NS,** no significant difference, $P \geq 0.1$; $P < 0.1$ is bold; Means not sharing the same letters are significantly different from each other, Tukey test $\alpha = 0.05$; **NH_4^+-Broad.:** starter fertilizer as di-ammonium phosphate (DAP) followed by broadcasting and incorporation stabilized $(NH_4)_2SO_4$ before sowing; **NH_4^+-Depot:** Starter DAP and subsurface placement of concentrated stabilized $(NH_4)_2SO_4$ solution in water at 10 cm soil depth at 4 – 5 leaf stage; **BE,** bio-effector; **Pro**: *Pseudomonas* sp. DSMZ 13134; **Rhiz**: *Bacillus amyloliquefaciens* FZB42; Above-ground biomass (BBCH 85-87)

At 68 DAS, N-fertilizer application method had an effect on SPAD for the youngest fully developed leaf (Broad. > Depot, *P = 0.0087*) whereas BE did not (Table 6.4). SPAD values for all other treatments were higher than that for Zero (49, *P ≤ 0.0336*). At 68 DAS similarly, N-fertilizer application method had an effect on stem diameter (Broad. > Depot; *P = 0.0011*) whereas BE did not (Table 6.4). Stem diameter for +P (24.4 mm), NH_4^+-Broad**.** (24.6 mm), NH_4^+-Broad**.***Pro (24.8 mm) and NH_4^+-Broad**.***Rhiz (24.7 mm) only, were higher than that of Zero (22.4 mm, *P ≤ 0.0207*).

Like in 2014 furthermore, N-fertilizer depot positively affected root length density (RLD) ($P < 0.001$). However, unlike in 2014, Pro had no effect. RLD doubled in soil in the sides of maize rows with fertilizer depot (midway point between rows 1 – 2, 3 – 4 and 5 – 6) in comparison to sides of maize rows without fertilizer depot (midway point between rows 2 – 3 and 4 – 5) (Fig. 6.5 b).

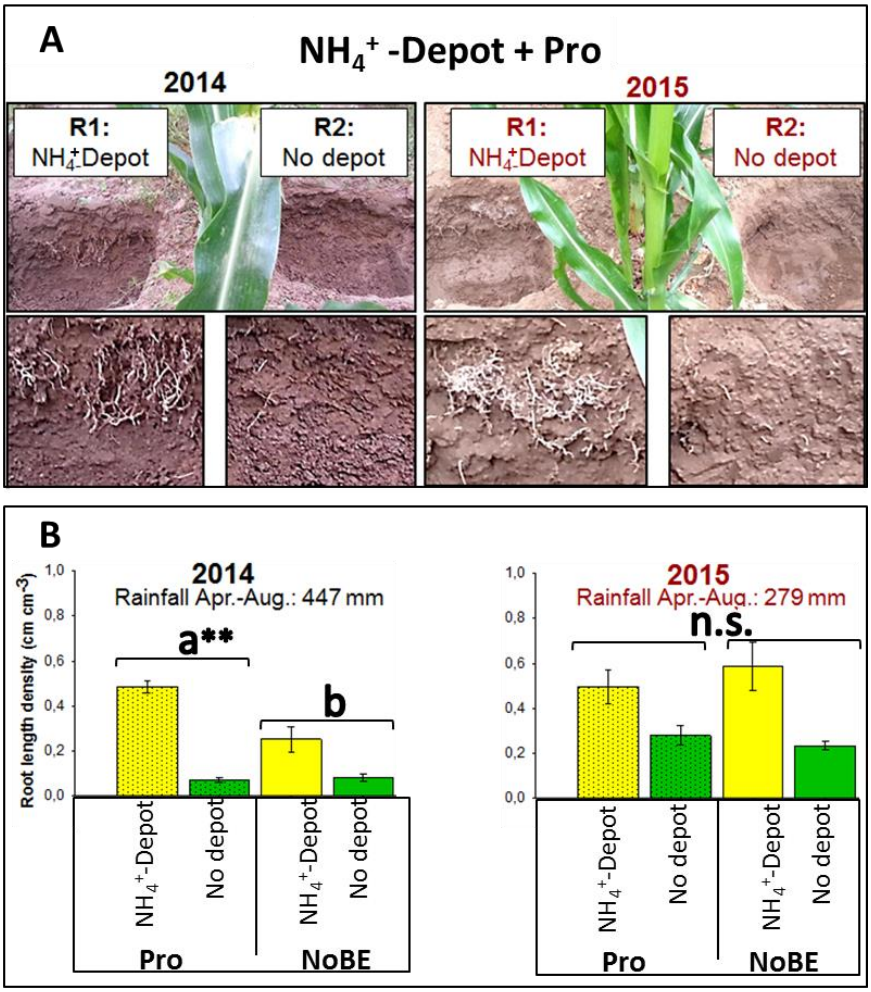

Figure 6.5. Root growth (A) and density (B) in NH_4^+-Depot **and non-Depot row-sides (Field 2014 and 2015)**
NH_4^+-Depot: Side of maize row with concentrated stabilized $(NH_4)_2SO_4$ solution placed as a depot at 10 cm depth; **No depot**: Other side of maize row without an NH_4^+ fertilizer depot; **BE,** bio-effector; **NoBE**, no bio-effector; **Pro**, *Pseudomonas* sp. DSMZ 13134. Different letters between BE-levels show a significant BE-effect, ** $P < 0.01$ (Two-Way ANOVA, Tukey test, $\alpha = 0.05$). There was no BE-effect in 2015. There was strong NH_4^+-Depot-effect on RLD in both 2014 ($P< 0.001$) and 2015 ($P< 0.001$)

N-fertilizer application method affected fresh above-ground biomass yield (Broadcast > Depot, *P=0.03*) whereas BE had only a marginal effect (Pro > NoBE, *P=0.06*) (Table 6.4). Inoculation of Pro showed a tendency to produce about 4.5 % higher fresh biomass than Rhiz (*P = 0.15*). Fresh above-ground biomass of Zero (46.0 Mg ha^{-1}) was less than that for +P (55.0 Mg ha^{-1}), NH_4^+-Broad**.***Pro (56.6 Mg ha^{-1}), NH_4^+-Broad**.***Rhiz (56.5 Mg ha^{-1}), and NH_4^+-Depot*Pro (54.6 Mg ha^{-1}) (*P < 0.0293*).

N-fertilizer application method had no effect on dry above-ground biomass whereas BE had an effect (*P=0.0364*). Banding of Pro below the seed row led to higher dry shoot biomass than banding of Rhiz or without BE inoculation (Table 6.4). Pro led 10.9 % (*P = 0.035*) higher dry biomass than Rhiz. Dry above-ground biomass for treatments +P (20.2 Mg ha^{-1}), NH_4^+-Broad**.***Pro (20.1 Mg ha^{-1}) and NH_4^+-Depot*Pro (20.5 Mg ha^{-1}) were higher than that for Zero (16.8 Mg ha^{-1}, *P < 0.0245*).

6.5 Discussion

In the pot experiment, inoculation of *Pseudomonas* strongly improved shoot P content. This was likely a result of improved plant P-acquisition from soil P pools that were previously not plant-available. Strong response of maize growth to inoculated *Pseudomonas* may have been possible due to high root colonization by *Pseudomonas* which could result from high inoculation rates and inoculation directly on the seed, seeding-hole and fertilizer depot. If N is not limiting, optimal P supply enables plants to establish large leaf areas, which increases photosynthesis and growth rate, thus, resulting in more dry-biomass production than under P limitation (*Grant* et al., 2001).

In the rhizobox experiment, higher root length density (RLD) in soil around the fertilizer depot in comparison to that in soil distant from the depot or in soil with homogenous supply of NO_3^-, was due to high concentrations of root-growth stimulating NH_4^+ present within the depot. NH_4^+ is known to strongly stimulate lateral root initiation and elongation at the site of

contact with roots (*Jing* et al., 2010; *Anghinoni* and *Barber*, 1990; *Drew,* 1975; *Jing* et al., 2012). However, the set-up of the rhizobox experiment did not make it possible to attribute the increase in RLD around the localized N-depot between localized N supply by placement and N supply as NH_4^+ differentially. Increased N-depot RLD could only be attributed to both. In our natural soil-based substrate without any water-tight barriers against mass flow and diffusion of N-sources like NO_3^- or $CO(NH_2)_2$, localized N supply could only be possible by localized placement of stabilized NH_4^+. NH_4^+ was stabilized with the nitrification inhibitor DMPP and further, by using a highly concentrated and toxic NH_4^+ solution, which also inhibits oxidation of NH_4^+ by soil microorganisms (*Shaviv,* 1988). Improved establishment of *Pseudomonas* in the fertilizer depot zone was due to increased root density in the depot-zone, which was likely associated with high levels of nutrients for rhizobacteria released as organic compounds in root exudates (*Lugtenberg* and *Kamilova,* 2009). Shoot P and N content were mainly influenced by N fertilizer form. Inoculation of *Pseudomonas* led only to a marginal increase in shoot N concentration. Rhizosphere acidification induced by NH_4^+-nutrition (*Jing* et al., 2010) is known to enhance solubility of sparingly soluble calcium phosphates in soil, which also build up after application of water-soluble phosphates on neutral to alkaline soils (*Lu* et al., 1987; *Moody* et al., 1995). Furthermore, soil acidification inhibits NH_3 volatilization from urea or NH_4^+ fertilizers placed in soil (*Ma* et al., 2013).

In the pot and rhizobox experiments, improved plant growth was associated with marked increase in shoot P content without change in shoot P concentration. Shoot P concentration stayed the same or increased marginally. An explanation could be that on the low P soils, plant P status was already in the critical range for deficiency with a threshold concentration of 0.25 – 0.4% (*Barry* and *Miller,* 1989). Under these conditions, any surplus in P supply and P uptake is immediately utilized for biomass production leading to dilution of P concentrations, which then restores the initial critical P concentrations. This suggests that positive PGPM

effects on plant growth may be more achievable on soils with moderate fertility than on very poor or highly fertile soils.

Under field conditions, placement of NH_4^+ fertilizer as a subsurface depot sustained high NH_4^+ concentrations within the depot-zone that stimulated intense depot-zone root growth. Sustained high concentrations of NH_4^+ within the depot was attributable on the one hand, to NH_4^+-stabilization effect of the nitrification-inhibitor (*Zerulla* et al., 2001) and on the other, to high, toxic NH_4^+ concentrations in the depot. In 2014, root density in the fertilizer depot-zone was higher with inoculation of *Pseudomonas* than without, indicating a potential for inoculated PGPMs to enhance root exploitation of subsurface N-fertilizer depots.

Unlike in the rhizobox experiment, N fertilization by subsurface placement of NH_4^+ as a depot did not improve shoot N and P status under field conditions. A reason could be that the fertilizer depot was closer to the maize seed in the rhizobox and pot experiments (5 x 5 cm) than in the field experiments (38 x 5 cm). Therefore, despite root growth within the fertilizer depot under field conditions at later growth stages, the distance between the depot and maize plants may have limited N acquisition from the depot. Therefore, it may be recommended that subsurface fertilizer depots should be placed as close to seeds as possible (5 x 5 cm) as long as fertilizer toxicity effects on seeds or young plants can be avoided. For this purpose, fertilizer placement in subsurface soil should be done at sowing or soon after to avoid mechanical damage to deeper and broader growing roots of plants at later growth stages.

Although field soil had moderate levels of plant-available P, additional P fertilization led to improved shoot P status (2014). However, this did not lead to improved grain yield (2014) or improved yield of above-ground biomass (2015), suggesting that P was not the most limiting nutrient in the field sites. In 2014, placement of NH_4^+ as a depot led marginally to higher grain yield than broadcast and incorporation of NH_4^+ whereas application of PGPM did not affect grain yield. A reason for the weak effect of the fertilizer depot could be the moderate

initial N_{min} level of the field soil. Furthermore, soil N_{min} likely increased later in season as soil organic matter mineralization by soil microorganisms probably increased in the warm summer months. This explanation is supported by the high yield recorded for the unfertilized control treatment.

In the field in 2015, NH_4^+-fertilizer application by broadcast and incorporation led to higher yield in fresh above-ground biomass than by placement as a subsurface depot. One reason could be that plants supplied with N by broadcast and incorporation before sowing were able to acquire more N during critical early growth stages than those supplied with N by placement of a subsurface N-depot at 5 – 6 leaf stage, more than one month after sowing. Another reason could be that severe drought that followed placement of fertilizer as a subsurface depot inhibited N acquisition. Firstly, there was insufficient moisture for optimal N uptake from the fertilizer depot, and secondly, rapid water loss from the soil caused fertilizer depot salts to be pulled up from the soil to the surface forming unavailable salt crusts.

In 2014, inoculation of *Pseudomonas* did not increase maize grain yield. It may be attributed to PGPM application technique as well as to absence of severe environmental stress factors. Application of a large quantity of inoculum (on hectare basis) as a suspension of viable cells in water by broadcast and incorporation may have been unfavorable for inoculum survival and propagation due to exposure to the biotic and abiotic environment.

In 2015, inoculation of *Pseudomonas* as a below-seed band led to higher dry above-ground biomass than inoculation with *Bacillus amyloliquefaciens* or without inoculation of PGPM. This yield increase associated with *Pseudomonas* was not linked to improved root density in the fertilizer depot or improved leaf N status. Maize growth-promotion effect of inoculated *Pseudomonas* seemed to have depended on the one hand, on high *Pseudomonas* concentrations present in the immediate surrounding of maize seeds due to placement of inoculum as a below-seed band, producing a high critical PGPM density at the root-zone

required for optimal PGPM effects on plant growth (*van Veen* et al., 1997; *Lugtenberg* and *Kamilova,* 2009). On the other hand, it may have depended on favorable protective micro-environments for inoculum survival and propagation provided by pore-spaces in pumice stones used as carrier (*van Veen* et al., 1997). Given extended drought and high temperatures on the field in 2015, plant growth-promotion by *Pseudomonas* may have occurred via induction of resistance to the prevalent abiotic stress factors. *Pseudomonas* are producers of the enzyme 1-aminocyclopropane-1-carboxylate (ACC)-deaminase, which utilizes ACC, the precursor of ethylene thereby lowering plant ethylene levels and stimulating resistance to heat and drought stress (*Glick,* 2005; *Naveed* et al., 2008; *Shaharoona* et al., 2006; *Zahir* et al., 2006).

Protective pore spaces in the pumice stone carrier employed in 2015 may have also functioned at the same time as a slow-release tool for viable cells to be progressively supplied to plant roots. Additionally, nutrients for PGPM provided in the inoculum product (skimmed milk for *Pseudomonas*) may have been protected within the pore spaces from utilization by other non-target soil microorganisms. Therefore, with pumice stones as carrier, *Pseudomonas* cells may have been able to safely multiply within the protected niche of pore spaces. It is important to note that, with respect to low inoculation rates in the field experiment, the amount skimmed milk powder present in the *Pseudomonas* inoculum formulation had no direct plant fertilization significance. Due to smaller amounts of inocula required for PGPM application as a below-seed band, high quantities of inoculum and associated high costs for application by broadcast and incorporation can be avoided.

Because PGPM effects on plant growth largely depends on viability of inoculated cells in soil (*van Veen* et al., 1997; *Lugtenberg* and *Kamilova,* 2009), it may be worthwhile to also test non-microbial bio-effectors with root growth-promoting properties in combination with placement of subsurface fertilizer depots. In this context, such non-microbial bio-effectors

may be particularly effective under conditions where PGPM activity is inhibited by unfavorable environmental conditions. Seaweed extracts with proven protective activity against abiotic stresses (*Sangha* et al., 2014) could be promising candidates.

Growth promotion effects of tested inoculated *Pseudomonas* on maize seemed also to have been determined by soil type and soil fertility level (especially for P). PGPM growth-promotion effect on maize was higher on low-P grassland soil (Pot) or low-P loess subsoil (Rhizobox) than on silty loam field soil with moderate levels of plant-available P.

Similarly to PGPM plant growth-promotion effects, growth-promotion effects of subsurface placed fertilizer depends on plant nutrient status, which in turn depends on initial soil fertility level or initial plant nutrient supply (*Grant* et al., 2001; *Buah* et al., 2000; *Borges* and *Mallarino*, 2001).

6.6 Conclusions

We hypothesized that: (1) (2) Marked rhizosphere acidification occurs within and around a "rhizosphere hotspot" formed by placement of an NH_4^+-depot in soil. (3) Survival and colonization of inoculated PGPMs is higher in the "rhizosphere hotspot" than in comparable soil zones with respect to plant position supplied homogeneously with NO_3^- fertilizer. (4) Inoculated and established PGPMs further promote root development around the NH_4^+-depot zone. (5) Consequently, NH_4^+-depot fertilization combined with inoculated PGPMs will result in higher nutrient uptake and higher yields than NH_4^+-depot fertilization without PGPMs.

Placement NH_4^+-fertilizer as a subsurface depot stimulated the formation of "rhizosphere hotspots" with intense root growth. Marked rhizosphere acidification within and around an NH_4^+-induced "rhizosphere hotspots" led to improved plant P and N uptake. Combination of fertilizer placement in subsurface soil with inoculation of the PGPM *Pseudomonas* sp. DSMZ 13134 in soil led to improved plant growth-promotion effects under greenhouse and field conditions, however, with low reproducibility. Reproducible results may be achieved through

optimization of PGPM inoculation techniques to enhance their survival in often hostile environmental conditions in field soil and through improvement of subsurface fertilizer placement to ensure optimal nutrient availability to target crop plants. PGPM application techniques involving stable dry spore formulations or viable cells in drought-resistant protective capsules or alginate may be promising options.

6.7 References

Anghinoni, I., Barber, S. A. (1990): Predicting the effect of ammonium placement on nitrogen uptake by corn. *Agron J,* 82(1):135–138.

Arshad, M., Waqas, R. (2011): Novel bacteria possessing ACC-deaminase enzyme enhances the water use efficiency in field crops. *Int Post J Sci Technol* 1 (1): 2.

Barber, S. A. (1984): Soil nutrient bioavailability. A mechanistic approach. Wiley, New York U.S.A.

Barea, J. M., Toro, M., Orozco, M. O., Campos, E., Azcon, R. (2002): The application of isotopic (32P and 15N) dilution techniques to evaluate the interactive effect of phosphate-solubilizing rhizobacteria, mycorrhizal fungi and Rhizobium to improve the agronomic efficiency of rock phosphate for legume crops. *Nutr Cycl Agroecosys* 63:35–42.

Barry, D. A. J., Miller, M. H. (1989): Phosphorus Nutritional Requirement of Maize Seedlings for Maximum Yield. *Agron J* 81(1):95–99.

Blackshaw, R. E., Semach, G., Janzen, H. H. (2002): Weed Science Society of America - Fertilizer application method affects nitrogen uptake in weeds and wheat. *Weed Sci* 50(5):634–641.

Bonkowski, M., Cheng, W., Griffiths, B. S., Alphei, J., Scheu, S. (2000): Microbial-faunal interactions in the rhizosphere and effects on plant growth. *Eur J Soil Biol* (36):135–147.

Borges, R., Mallarino, A. P. (2001): Deep Banding Phosphorus and Potassium Fertilizers for Corn Managed with Ridge Tillage. *Soil Sci Soc Am. J* 65:376–384.

Buah, S., Polito, T. A., Killorn, R. (2000): No-tillage corn response to placement of fertilizer nitrogen, phosphorus, and potassium. *Commun Soil Sci Plant* Anal 31(19-20):3121–3133.

Cooke, G. W. (1954): Recent advances in fertilizer placement. II.—Fertilizer placement in england. *J Sci Food Agric* 5(9):429–440.

Drew, M. C. (1975): Comparison of the effects of a localised supply of phosphate, nitrate, ammonium and potassium on the growth of the seminal root system, and the shoot, in barley. *New Phytol* 75(3):479–490.

Dutta, D., Bandyopadhyay, P. (2009): Performance of chickpea (*Cicer arietinum L.*) to application of phosphorus and bio-fertilizer in laterite soil. *Arch Agron Soil Sci* 55(2):147–155.

Ferris, H., Venette, R. C., van der Meulen, H., Lau, S. S. (1998): Nitrogen mineralization by bacterial-feeding nematodes: verification and measurement. *Plant Soil* 203:159–171.

*Frey-Klett P., Garbaye J., Tarkka M. (*2007): The mycorrhiza helper bacteria revisited. *New Phytol* 176(1):22–36.

Fröhlich, A., Buddrus-Schiemann, K., Durner, J., Hartmann, A., Rad, U. von (2012): Response of barley to root colonization by Pseudomonas sp. DSMZ 13134 under laboratory, greenhouse, and field conditions. *J Plant Interact* 7(1):1–9.

Gericke, S., Kurmies, B. (1952): Colorimetrische Bestimmung der Phosphorsäure mit Vanadat-Molybdat. *Z Anal Chem* 137(1):15-22.

Ghorbani, R., Wilcockson, S., Koocheki, A., Leifert, C. (2008): Soil management for sustainable crop disease control: a review. *Environ Chem Lett* 6(3):149–162.

Glick, B. R. (2005): Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiol Lett* 251(1):1–7.

Grant, C. A., Flaten, D. N., Tomasiewicz, D. J., Sheppard, S. C. (2001): The importance of early season phosphorus nutrition. *Can J Plant Sci* 81(2):211–224.

Halpern, M., Bar-Tal, A., Ofek, M., Minz, D., Muller, T., Yermiyahu, U. (2015): Chapter Two - The use of biostimulants for enhancing nutrient uptake. *Adv Agron* 130:141–174.

Häussling, M., Leisen, E., Marschner, H., Römheld, V. (1985): An improved method for non-destructive measurements of the ph at the root-soil interface (Rhizosphere). *J Plant Physiol* 117(4):371–375.

Hawkesford, M., Horst, W., Kichey, T., Lambers, H., Schjoerring, J., Møller, I. S., White, P. (2012): Functions of macronutrients. In: Marschner P., editor. *Marschner's Mineral Nutrition of Higher Plants*, 3rd ed., Academic Pr, United States of America; 135–178.

Jiang, Y., Wu, Y., Xu, W., Cheng, Y., Chen, J., Xu, L., Hu, F., Li, H. (2012): IAA-producing bacteria and bacterial-feeding nematodes promote Arabidopsis thaliana root growth in natural soil. *Eur J Soil Biol* 52:20–26.

Jing, J., Rui, Y., Zhang, F., Rengel, Z., Shen, J. (2010): Localized application of phosphorus and ammonium improves growth of maize seedlings by stimulating root proliferation and rhizosphere acidification. *Field Crop Res* 119(2-3):355–364.

Jing, J., Zhang, F., Rengel, Z., Shen, J. (2012): Localized fertilization with P plus N elicits an ammonium-dependent enhancement of maize root growth and nutrient uptake. *Field Crop Res* 133:176–185.

Linquist, B. A., Adviento-Borbe, M. A., Pittelkow, C. M., van Kessel, C., van Groenigen, K. J. (2012): Fertilizer management practices and greenhouse gas emissions from rice systems: A quantitative review and analysis. *Field Crop Res* 135:10–21.

Lu, D. Q., Chien, H. S., Henao, J., Sompongse, D. (1987): Evaluation of short-term efficiency of diammonium phosphate versus urea plus single superphosphate on a calcareous soil. *Agron J* 79(5):896–900.

Lugtenberg, B., Kamilova, F. (2009): Plant-Growth-Promoting Rhizobacteria. *Annu Rev Microbiol* 63(1):541–556.

Ma, B. L., Wu, T. Y., Tremblay, N., Deen, W., McLaughlin, N. B., Morrison, M. J., Stewart, G. (2010): On-farm assessment of the amount and timing of nitrogen fertilizer on ammonia volatilization. *Agron J* 102(1):134–144.

Mao, X., Hu, F., Griffiths, B., Li, H. (2006): Bacterial-feeding nematodes enhance root growth of tomato seedlings. *Soil Biol Biochem* 38(7):1615–1622.

Ma, Q., Zhang, F., Rengel, Z., Shen, J. (2013): Localized application of NH4 +-N plus P at the seedling and later growth stages enhances nutrient uptake and maize yield by inducing lateral root proliferation. *Plant Soil* 372(1-2):65-80.

Marschner, H., Römheld, V. (1983): In vivo measurement of root-induced ph changes at the soil-root interface: effect of plant species and nitrogen source. *Z Pflanzenphysiol* 111(3):241–251.

Mauricio, R. (2005): Mycorrhiza helper bacteria: a promising model for the genomic analysis of fungal–bacterial interactions. *New Phytol* 168:1–4.

Mohite, B. (2013): Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. *J Soil Sci Plant Nutr* 13:638–649.

Moody, P. W., Edwards, D. G., Bell, L. C. (1995): Effect of banded fertilizers on soil solution composition and short-term root-growth .2. Mono-ammonium and di-ammonium phosphates. *Soil Res* 33(4):689–707.

Nash, P. R., Motavalli, P. P., Nelson, K. A. (2012): Nitrous oxide emissions from claypan soils due to nitrogen fertilizer source and tillage/fertilizer placement practices. *Soil Sci Soc Am J* 76(3):983–993.

Naveed M., Khalid M., Jones D. L., Ahmad R., Zahir Z.A. (2008): Relative efficacy of *Pseudomonas spp.*, containing ACC-Deaminase for improving growth and yield of maize (*Zea mays L.)* in the presence of organic fertilizer. *Pak J Bot* 40:1243–1251.

Neumann, G., Römheld, V. (2012): Rhizosphere chemistry in relation to plant nutrition. In: Marschner P., editor. *Marschner's Mineral Nutrition of Higher Plants*, 3rd ed., Academic Pr, United States of America; 347–360.

Nieder, R., Benbi, D., Scherer, H. (2011): Fixation and defixation of ammonium in soils: a review. Biol Fert Soils 47(1):1–14.

Nkebiwe, P. M., Weinmann, M., Bar-Tal, A., Müller, T. (2016): Fertilizer placement to improve crop nutrient acquisition and yield: A review and meta-analysis. *Field Crops Research* 196, 389–401.

Petersen, J. (2005): Competition between weeds and spring wheat for 15N-labelled nitrogen applied in pig slurry. *Weed Res* 45:103–113.

Reith, J. W. S. (1954): Recent advances in fertilizer placement. I. Fertilizer placement for swedes and turnips in Scotland. *J Sci Food Agric* 5(9):421–428.

Richardson, A., Barea, J.-M., McNeill, A., Prigent-Combaret, C. (2009): Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. *Plant Soil* 321(1-2):305-339.

Robinson, D. (1994): The responses of plants to non-uniform supplies of nutrients. *New Phytol* 127(4):635–674.

Ruidisch, M., Bartsch, S., Kettering, J., Huwe, B., Frei, S. (2013): The effect of fertilizer best management practices on nitrate leaching in a plastic mulched ridge cultivation system. *Agr Ecosyst Environ* 169:21–32.

Sangha, J. S., Kelloway, S., Critchley, A. T., Prithiviraj, B. (2014): Seaweeds (macroalgae) and their extracts as contributors of plant productivity and quality: the current status of our understanding. *Sea Plants* 71:189–219.

Schaller, G., Fischer, W. R. (1985): pH-Änderungen in der Rhizosphäre von Mais- und Erdnußwurzeln. *Z Pflanzenernaehr Bodenk* 148(3):306–320.

Shaharoona B., Arshad M., Zahir Z. A., Khalid A. (2006): Performance of *Pseudomonas spp.* containing ACC-deaminase for improving growth and yield of maize (*Zea mays L.)* in the presence of nitrogenous fertilizer. *Soil Biol Biochem* 38(9):2971–2975.

Shakir, M. A., Bano, A., Arshad, M. (2012): PGPR with ACC deaminase confer drought tolerance wheat. *Soil Environ* 31(1):108–112.

Sharma, S. H. S., Lyons, G., McRoberts, C., McCall, D., Carmichael, E., Andrews, F., Swan, R., McCormack, R., Mellon, R. (2012): Biostimulant activity of brown seaweed species from Strangford Lough: compositional analyses of polysaccharides and bioassay of extracts using mung bean (*Vigno mungo L*.) and pak choi (*Brassica rapa chinensis L.). J Appl Phycol* 24(5):1081–1091.

Shaviv, A. (1988): Control of nitrification rate by increasing ammonium concentration. *Fert Res* 17(2):177-188.

Sommer, K. (2005): CULTAN-Düngung: physiologisch, ökologisch, ökonomisch optimiertes Düngungsverfahren für Ackerkulturen, Grünland, Gemüse, Zierpflanzen und Obstgehölze. *Verlag Th. Mann*, Gelsenkirchen-Buer, Germany; 2005.

Sugimoto, E. E., Hoitink, H. A. J., Tuovinen, O. H. (1990): Enumeration of oligotrophic rhizosphere pseudomonads with diluted and selective media formulations. *Biol Fertil Soils* 9(3):226-230.

van Veen, J. A., van Overbeek, L. S., van Elsas, J. D. (1997): Fate and Activity of Microorganisms Introduced into Soil. *Microbiol Mol Biol R* 61(2):121–135.

Weinmann M, Römheld V. (2012): Resource preservation by application of bioeffectors in european crop production (research project proposal): European Community's Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 312117 (BIOFECTOR). University of Hohenheim, 2012. www.biofector.org, 10.02.2016

Yang, J., Kloepper, J. W., Ryu, C.-M. (2009): Rhizosphere bacteria help plants tolerate abiotic stress. *Trends Plant Sci* 14(1):1–4.

Yusran, Y., Safrizal, S., Weinmann, M., Neumann, G., Müller, T., Römheld, V. (2007): Improved mycorrhisation in tomato by soil inoculation with *Pseudomonas sp.* proradix®. In: Tielkes, E., Editor. *Utilisation of Diversity in Land Use Systems: Sustainable and Organic Approaches to Meet Human Needs. Book of Abstracts*, Cuvillier Verlag, Göttingen, Germany 513–514.

Zahir Z. A., Ghani U., Naveed M., Nadeem S. M., Asghar H. N. (2009): Comparative effectiveness of *Pseudomonas* and *Serratia sp.* containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum L.)* under salt-stressed conditions. *Arch Microbiol* 191(5):415–424.

Zerulla, W., Barth, T., Dressel, J., Erhardt, K., Horchler von Locquenghien, K., Pasda, G., Rädle, M., Wissemeier, A. (2001): 3,4-Dimethylpyrazole phosphate (DMPP) - a new nitrification inhibitor for agriculture and horticulture. *Biol Fert Soils* 34(2):79–84.

7 Mobilization of sparingly –soluble soil P and alternative P-fertilizers by bio-effectors

7.1 Screening bio-effectors for ability to mobilize soil-P for improved maize growth

Dr. Brigitta Tóth, a visiting post-doc researcher, contributed by up to15% in the workload for the experiment described in this section.

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7.1.1 Background and objectives

Several microbial bio-effectors (BEs) are commercially available and many more are being isolated, characterized, tested, produced and marketed. However, the benefits of BEs promised by the producers – improvement of crop performance through mechanisms such P-mobilization, improved root growth and vitality, induction of local and systemic resistance/tolerance to biotic and abiotic stresses - are sometimes not realized even in greenhouse conditions (*Schenck zu Schweinsberg-Mickan* and *Müller* 2009) and remain frequently unachieved in field conditions (*Lugtenberg* and *Kamilova* 2009). In addition to other factors that determine and influence BE effectiveness, compatibility between BE strain and plant variety is an important factor. This has been shown for effective root colonization specifically in *Zea mays* L. var Mo17 by the BE strain *Trichoderma harzianum* T-22 (*Harman* et al. 2004; *Harman* 2006). The objectives of this experiment were: 1.) to screen various commercially available and novel microbial BEs for their ability to improve plant P acquisition from sparingly-available soil pools and therefore, promote plant growth. 2.) to investigate differences in performance for four selected strains of *Trichoderma harzianum* in pair-wise combinations with two varieties of *Zea mays* L.

7.1.2 Hypotheses

- BE inoculated in a growth substrate with low available-P levels improves P acquisition and growth of maize plants via mobilization of moderately labile soil-P and/or improved root growth for spatial P acquisition.
- Growth response of maize to *Trichoderma harzianum* inoculated in the substrate is affected by both *T. harzianum* strain and maize variety.

7.1.3 Methodology

Maize (*Zea mays* L.) was grown in 1 L pots (7 cm Ø, 25 cm height) containing a substrate composed of two parts(v/v) low-P loam soil (sieved 5 mm Ø, 713 g FM pot^{-1}; pH_{CaCl2} 6.9; P_{CAL} 35 mg P kg^{-1}: K_{CAL} 180.5 mg K kg^{-1}) that was obtained from Kleinhohhenheim, an organic farming research station of the University of Hohenheim (N 48° 44' 7.65", E 9° 12' 4.72"). 1 part quartz sand (0.6-1.2 mm Ø) was added per pot (620 g DM pot^{-1}). NO_3^- was chosen because it is commonly used by farmers (e.g. calcium ammonium nitrate) and maize was chosen as a test-crop because of its weak ability to mobilize soil P and its sensitivity to low soil plant-available P levels. Fertilization included: 100 mg NO_3-N kg^{-1} dry soil as $Ca(NO_3)_2$; and 150 mg P as soluble ($Ca(H_2PO_4)_2$ (Only for the positive P control, +P). Moisture was set to 18 % (60% max. WHC). Approximately 1400 g substrate was filled in each pot. Tested BEs included: *Trichoderma harzianum* WG (Trichoderma-WG, **WG** Prophyta GmbH, Germany, now acquired by Bayer Crops Science), *Trichoderma harzianum* OmG-08 (Tichoderma OmG-08, **OmG08**, Anhalt University of Applied Science, Germany-HS Anhalt); *Trichoderma harzianum* T50 (Vitalin T50, **T50**, Vitalin GmbH, Germany); *Trichoderma harzianum* -T22 (Trianum-P, **T-22**, Koppert, Netherlands); *Penicillium sp*. PK 112 (Biological Fertilizer DC, **BFDC**, Prophyta GmbH/Bayer Crops Science); *Pseudomonas sp.* DSMZ 13134 (Proradix®, **Pro**, Sourcon Padena, Germany); Bacillus amyloliquefaciens FZB42, Rhizovital®, **Rhiz.;** *Bacillus simplex R41* (cold-adapted strain), **B.s.R41**; *Bacillus*

atrophaeus, **B.atr.***; Bacillus spec*, **B.spec.** (the last four BEs obtained from ABiTEP GmbH, Berlin, Germany); and three BE consortia – Phylazonit, **Phyl,** (comprising: *Azotobacter chrococcum, Bacillus megaterium*); Bactofil, **Bact**, (comprising: *Azospirillum brasilense, Azotobacter vinerólandii, B. megaterium, Bacillus polymyxa, Pseudomonas fluorescens, Sterptomyces albus*) and MegaNit, **Meg**, (comprising: *A. chroccoccum, Azospirillum ssp., B. megaterium, Bacillus subtilis*) (BE consortia were obtained from Corvinus University of Budapest, Hungary).

The first set of treatments was comprised of two factorial combinations of four T. harzianum strains and two *Zea mays* L. varieties: Colisee (KWS Saat SE, Germany) and Maxxis (R.A.G.T Saaten Deutschland GmbH, Germany). The second set of treatments included the remaining nine BEs tested on *Zea mays* L. var. Maxxis. There were two non-inoculated controls: one without (**NoBE**) and one with added soluble P and wihout BE (**NoBE+P**).

Each BE was suspended in 2.5 mM $CaSO_4$ to a concentration of 1 x 10^{10} spores or CFU's L^{-1}. 20 ml of each suspension was pipetted directly on the surface of the substrate after seeding (2 x 10^8 spores or CFUs pot^{-1}). Phylazonit, Bactofil and Meganit were each diluted in 2.5 mM $CaSO_4$ and applied (also 20 ml pot^{-1}) at the rate of 1 ml kg^{-1} soil. Control pots receive 20 ml 2.5 mM $CaSO_4$.

There were five replicates per treatment arranged in a completely randomized design. Greenhouse conditions were: day/night length: 16 h/8 h; av. daytime light intensity: 428 µmol $m^{-2}s^{-1}$ (ALMEMO 239-3, AHLBORN); av. daily air temperature, 24°C (Min. 17.3 °C, Max 28.6 °C) and av. daily relative humidity, 31% (Min. 19.1%, Max. 43.9 (Voltcraft, DL-141 TH). Plants were grown for 30 days (24 May - 25 June 2013).

7.1.4 Results

There was an interaction between *T. harzianum* strain and maize variety only for shoot P concentration (Table 7.1). The best performing pairs were T50*Colisee and T.OmG08*Maxxis. With Colisee, the decreasing order of performance of *T. harzianum* strains was: T50 > T.-22 > WG>OmG08, whereas with Maxxis it was: OmG08 > T-22 > T50 >WG. Shoot P content for Maxxis was higher than that for Colisee. T50 led to the highest shoot P content whereas WG led to the lowest. Root length density for WG was less than that for other *T. harzianum* strains.

Table 7.1. Source of variation and Least square means (Two-way ANOVA, ns *P>0.1*)

	***T. harzia-num* strain:**	**WG**	**OmG08**	**T50**	**T-22**	**Maize variety**	**Colisee**	**Maxxis**	***T. harzianum* strain*Maize variety**
Shoot DM (g plant^{-1})	*0.037*	1.43	1.54	1.53	1.37	*0.046*	1.51 a	1.42 b	*ns*
Shoot P conc. (mg P g^{-1})	*<0.001*	1.53	1.63	1.72	1.74	*0.018*	1.61	1.70	*0.001*
Shoot P content (mg P plant^{-1}	*<0.001*	2.12 c	2.47 ab	2.62 a	2.38 bc	*ns*	2.41	2.40	*0.081*
Root DM (g plant^{-1})	*ns*	0.76	0.76	0.71	0.74	*ns*	0.76	0.73	*ns*
Root length density (cm cm^{-3})	*<0.001*	14.1 c	20.5 a	24.3 a	19.5 a	*<0.001*	16.4 b	22.9 a	*ns*

With the maize variety Maxxis, BEs did not lead to increased shoot biomass, shoot P content or root length density (Fig. 7.1). T. harzianum WG showed a tendency to reduce shoot P content and concentration, and root length density.

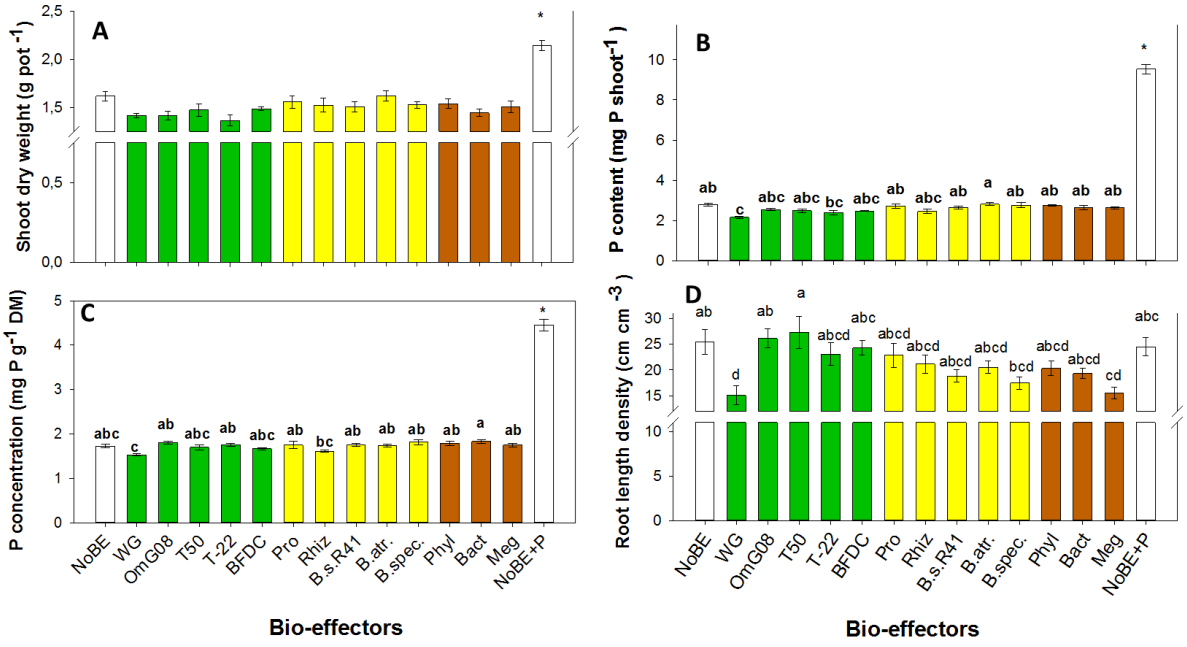

Figure 7.1. Shoot dry weight (A) and shoot P content (B) and concentration (C) and root length density (D) in response to inoculation of BEs.
Green bars, fungal BEs; yellow bars, bacterial BEs; and brown bars, BE consortia. WG, OmG08, T50 and T-22 are strains of *Trichoderma harzianum*. BFDC, *Penicillium sp*. PK 112; Pro, *Pseudomonas sp*. DSMZ 13134; Rhiz, Bacillus amyloliquefaciens FZB42; B.s.R41, *Bacillus simplex R41*; B.atr., *Bacillus* atrophaeus; B.spec., Bacillus *spec*; Phyl, Phylazonit; Bact, Bactofil; and Meg, MegaNit. Mean ± SEM, One-way ANOVA, Tukey test, α=0.05, n=5. Different letters show significant difference between treatments, ($P < 0.05$). t- test, α=0.05 *$P < 0.05$

7.1.5 Conclusion

Inoculation of BEs did not improve maize growth. Therefore, under these experimental conditions BEs were not able to contribute to mobilization of soil P or to improve root growth. Nevertheless, for *T. harzianum* only, there was strong interaction between BE strain and maize variety observed for shoot P concentration.

7.2 Growth-promoting effects of *Pseudomonas sp.* DSMZ 13134 on maize plants upon homogenous or placed application of soluble N and sparingly available P-fertilizer

7.2.1 Background and objectives

As discussed in section 3.1, for nutrients with very poor mobility in soil such as $H_2PO_4^-$ /HPO_4^{2-}, NH_4^+ or alternative P-fertilizers with poor solubility in soil (like rock phosphate, apatite-rich sewage sludge ash or struvite), placement of fertilizers in the sowing row under field conditions may be more effective to improve plant nutrient acquisition than conventional broadcast and incorporation in the entire plough layer. If placed root-attracting NH_4^+ persists in soil (stabilization of NH_4^+ by nitrification inhibitors or by use of high NH_4^+ concentrations to inhibit microbial nitrification), intense localized root growth is induced in the soil volume around the fertilizer-depot, thereby enhancing root-exploitation of nutrients in the soil volume. In comparison to field conditions, fertilizer placement in pots is less effective to create localized nutrient supply because with time, N originating from placed NH_4^+ diffuses into the entire restricted pot soil volume (with or without nitrification) and roots also naturally growth into the entire soil volume. However, for sparingly-soluble P fertilizers with even less mobility in soil than NH_4^+, placement as a concentration point in potted soil may result in more effective localized P supply even though it may not further improve P uptake when optimum root density in the concentrated P fertilizer depot is reached.

Therefore, we hypothesized that placing P-fertilizers in a concentrated point results in less P uptake and less biomass production than homogenous mixing of P in total soil volume in pots.

The objectives of this experiment were: 1.) to induce intense localized root-growth by placement of a concentrated NH_4^+-fertilizer depot. 2.) to investigate the ability of the promising BE *Pseudomonas sp.* DSMZ 13134 to improve P acquisition and shoot biomass production in maize plants under limited supply of P or supply of a mixture of sparingly soluble inorganic and organic P (rock phosphate enriched manure). 3.) to study the effect of

applying P and N-fertilizers (Placed NH_4^+ or homogenous NO_3^-) in different fractions of total substrate volume on plant growth and shoot nutrient status.

7.2.2 Hypotheses

- Placement of a fertilizer depot based on NH_4^+ or NH_4^+ combined with sparingly available P-fertilizer stimulates dense rooting around the depot (formation of rhizosphere hotspots).
- Placed NH_4^+-depot and BE enhance P acquisition and biomass production more than homogenously applied NO_3^- with or without BE.
- Application of sparingly available P-fertilizer in a large soil volume leads to higher P uptake and shoot biomass production than placement in a restricted concentrated depot.

7.2.3 Methodology:

Maize (*Zea mays L. var* Colisee) was grown in PVC pots (10 cm Ø, 20 cm H, 1.57 l) containing a substrate comprised of two parts (w/w) low-P loam soil from a long-term unfertilized grassland (pH_{CACl2} 7.1; $P_{CAL,}$ 21.6 mg P kg^{-1}; $K_{CAL,}$ 251.9 mg K kg^{-1}; C_{org}, 2.4%; N_{total}, 0.24%) and one part quartz sand (0.6-1.2 mm Ø). Moisture content was 22 % (75 % max. WHC). 1900 g of substrate was filled in each pot. Nutrients applied per kg dry soil included:150 mg K (K_2SO_4); 50 mg Mg ($MgSO_4$); 100 mg N: as $Ca(NO_3)_2$ homogenously applied in the substrate (**NO_3^- -Mixed** treatments or positive P control treatment, +**P**) or as $(NH_4)_2SO_4$ placed in a concentrated depot 5 X 5 cm to the seed (**NH_4^+-Depot**); 150 mg P: as Rock phosphate (RP)-enriched manure: 75 mg RP-P + 75 mg Manure-P) or as soluble $Ca(H_2PO_4)_2$ for the positive P control treatment, +P). RP and manure were chosen as rich inorganic and organic P fertilizers accepted in most farming systems including organic farming. *Pseudomonas sp.* DSMZ 13134 was inoculated at the rate 1 x 10^{11} CFUs kg^{-1}soil pot^{-1} (5 x 10^{10} CFUs applied to directly on the fertilizer depot and the remaining 5 x 10^{10} CFUs drenched in the seeding hole at sowing). Day/night time was 14/10 hrs. There were four

replicates arranged in completely randomized design. Root-zone temperature was controlled using a cooling system set to 20± 2 °C.

Proradix®, the commercial formulation of *Pseudomonas sp.* DSMZ 13134 (**Pro**), contains freeze-dried cells in skimmed milk powder as a nutrient additive for BE propagation after inoculation in soil. Therefore, high inoculation rates especially in pot experiments with small volumes of growth substrate may result in a meaningful input of organic nutrients and thus, a priming effect. Total N and P input by Pro inoculation at the rate 1 x 10^{11} CFUs kg^{-1}soil was:111.6 mg N and 23 mg P kg^{-1}soil. Mature sheep manure (2 yrs. old) was used. Total N input via manure at the rate of 75 mg Manure-P kg^{-1}soil was 163.5 mg N kg^{-1}soil (12 g manure kg^{-1}soil ≈ 16 tons manure ha^{-1}, 10 cm depth, 1.3 g cm^{-3} bulk density).Treatments included: **Zero,** no additional N or P fertilizer, No BE; **NH_4^+-Depot,** $(NH_4)_2SO_4$ placed as a concentrated depot (with or without **Pro)**; **NH_4^+/P-Depot,** $(NH_4)_2SO_4$ and RP-enriched manure placed as a concentrated depot (with or without **Pro); NO_3^--Mixed/P-Depot,** $Ca(NO_3)_2$ mixed homogenously in the entire soil and RP-enriched manure placed as a concentrated depot (with or without **Pro**); **NH_4^+-Depot/P-Mixed**, $(NH_4)_2SO_4$ placed as a concentrated depot and RP-enriched manure mixed homogenously in the entire soil (with or without **Pro**); **NH_4^+-Depot/ P-Mixed10cm_Pro**, $(NH_4)_2SO_4$ placed as a concentrated depot and RP-enriched manure homogenously mixed only in the top 10 cm soil layer (with **Pro)**; and **+P**, $Ca(NO_3)_2$ and $Ca(H_2PO_4)_2$ mixed homogenously in soil.

7.2.4 Results:

Placement of NH_4^+-Depot or NH_4^+/P-Depot induced dense localized rooting around the depot (Fig.7.2). For NH_4^+-Depot without P-fertilization, Pro increased shoot biomass production by 59 % shoot P content by 64% and N content by 50 % (Fig. 7.3) than with applied P-fertilizer irrespective of application method. With P-fertilizer, BE led to increases (at times marginal)

in shoot P concentration and shoot P and N content. Placement of NH_4^+ and RP-enriched manure led to higher shoot biomass and shoot P content than homogenous application of NO_3^- combined with placed RP-enriched manure. Mixing the RP-enriched manure homogenously in the entire soil volume led to higher shoot biomass and shoot P content than placing it in a concentrated depot or homogenous mixing only in the top 10 cm soil layer, irrespective of E (Fig. 7.3 A and C).

Figure 7.2. Densely rooted rhizosphere hotspot around a fertilizer depot ((NH_4^+/RP - enriched manure depot).

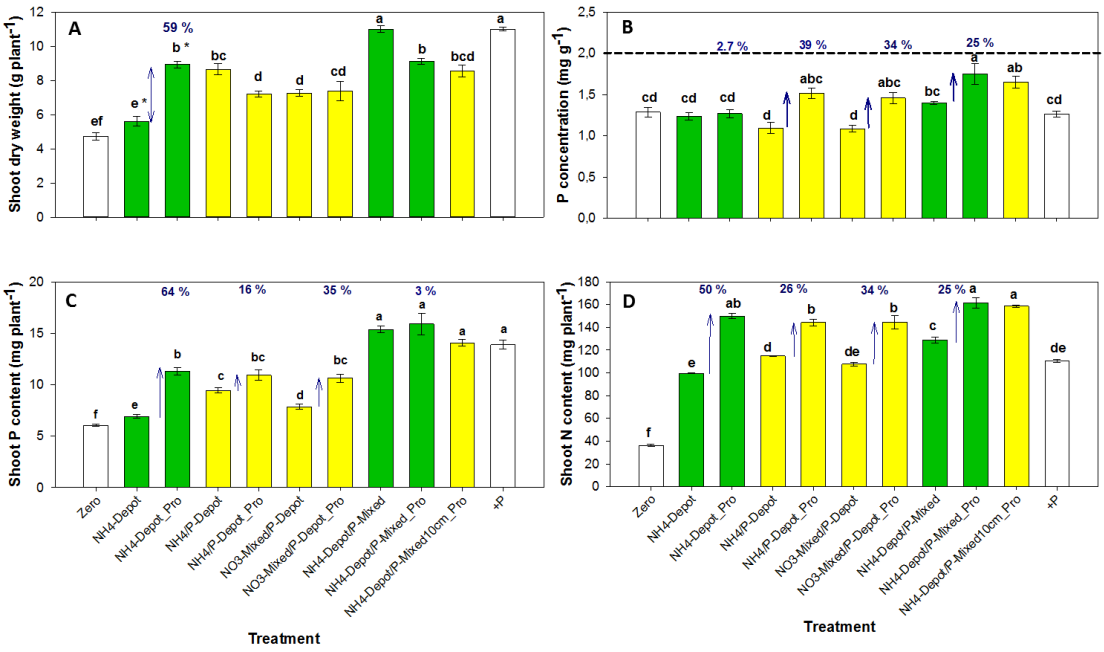

Figure 7.3. Shoot biomass (A), shoot P concentration (B), P content (C) and N content (D).

Zero, no N or P, no BE; **NH_4^+-Depot**, placed $(NH_4)_2SO_4$ depot; **NO_3^--Mixed**, $Ca(NO_3)_2$ mixed; **P-Depot**, placed RP-enriched manure depot; **P-Mixed**, RP-enriched manure homogenously applied; **P-Mixed10cm**, RP-enriched manure homogenously applied to top 10

cm; **Pro**, *Pseudomonas sp.* DSMZ 13134; **+P**, $Ca(NO_3)_2$ and $Ca(H_2PO_4)_2$ homogenously applied.

7.2.5 Conclusion:

Placement of a fertilizer depot based on NH_4^+ or NH_4^+ and RP-enriched manure induced dense localized rooting around the depot. BE improved shoot P status and shoot biomass when plant-available P was limiting. Homogenous application of RP-enriched manure in the entire substrate volume led to improved shoot growth and shoot P status in comparison to placement in a spatially restricted concentrated depot or to homogenous application only in the top 10 cm soil layer. When P-fertilizer was placed in a concentrated depot, placed NH_4^+ or homogenously applied NO_3^- had similar effects on maize growth.

7.3 Microbial bio-effectors for the mobilization of calcium phosphates in the rhizosphere

Original title: "Mikrobielle Bioeffektoren zur Mobilisierung von Calciumphosphaten in der Rhizosphäre"

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7.3.1 Background and objectives

In vitro BE-culture experiments on solid media showed that several selected BEs readily solubilize sparingly soluble $Ca_3(PO_4)$, (Ca-P) (results shown in section, 5). The aim of this experiment was investigated the ability of selected BEs to solubilize rock phosphate (RP) and/or increase root growth to contribute directly to improved P nutrition of maize plants supplied with RP. Plants were grown in a highly P buffering alkaline soil with low plant-available P. Instead of NH_4^+, NO_3^- was chosen to avoid additional improvement of RP solubility by rhizosphere acidification resulting from proton release as roots take up NH_4^+.

7.3.2 Hypothesis

BEs that readily solubilized Ca-P *in vitro* can directly contribute to improved shoot growth and shoot P status of maize plants supplied with RP via improved RP solubility and/or improved root growth.

7.3.3 Methodology:

A pot experiment was conducted on a low-P C-horizon subsoil with high P sorption capacity (P_{CAL}, 5 mg kg^{-1}; P_{total}, 332 mg kg^{-1}; pH_{CaCl2}, 7.6; C_{org}, < 0.3%; N_{total} 0.02 %; $CaCO_3$, 23 %). The substrate was made of up 80 % soil and 20% quartz sand (0.6-1.2 mm ∅) (w/w). The soil was fertilized by homogenous application of (kg^{-1} soil DM): 100 mg N, ($Ca(NO_3)_2$); 150 mg P(rock phosphate, 7.6 % P described in section 5 or $Ca(H_2PO_4)_2$) for the positive P control);

150 mg K (K_2SO_4); 50 mg Mg, ($MgSO_4$); 20 µmol Fe, (Sequestrene138, 6 % Fe); 2.6 mg Zn, ($ZnSO_4$); 1 mg Cu (CuSO4); and 18% moisture (60 % max. WHC). Each pot was filled with 2.9 kg of substrate. Tested bio-effectors included *Pseudomonas sp.* DSMZ 13134, Proradix® (**Pro**: 1x 10^9 CFU kg^{-1} soil), *Penicillium sp*. PK 112, Biological Fertilizer OD (**BFOD**, 1x 10^8 spores kg^{-1} soil), *Paenibacillus mucilaginosus* (**Paeni**, 1x 10^9 spores kg^{-1} soil) and Vitalin SP11, (**SP11**, 20 ml of 0.2% suspension kg^{-1} soil). Vitalin SP11 comprises: *Bacillus subtilis*, *Pseudomonas sp.*, *Streptomyces spp*., natural humic acids und extracts of the seaweed *Ascophyllum nodosum*. BEs were suspended in 2.5 mM $CaSO_4$, maize seeds (*Zea mays* L. var Colisee) were treated with BE suspensions, sown and then 20 ml BE suspension was inoculated in the seeding hole. There were six replicates per treatment arranged in completely randomized design. Plants were grown for 41 days (Daylight was16 hrs., 25-30°C and night 8 hrs., 18-20 °C). Pots were weighed daily and water lost was replenished with distilled water.

7.3.4 Results:

Inoculation of BEs led to lower or a tendency of lower shoot biomass (Fig. 7.4 A) , shoot P content (Fig. 7.4 B), root dry matter Fig. 7.4 C) and total root length per pot (Fig. 7.4 D) than without BEs.

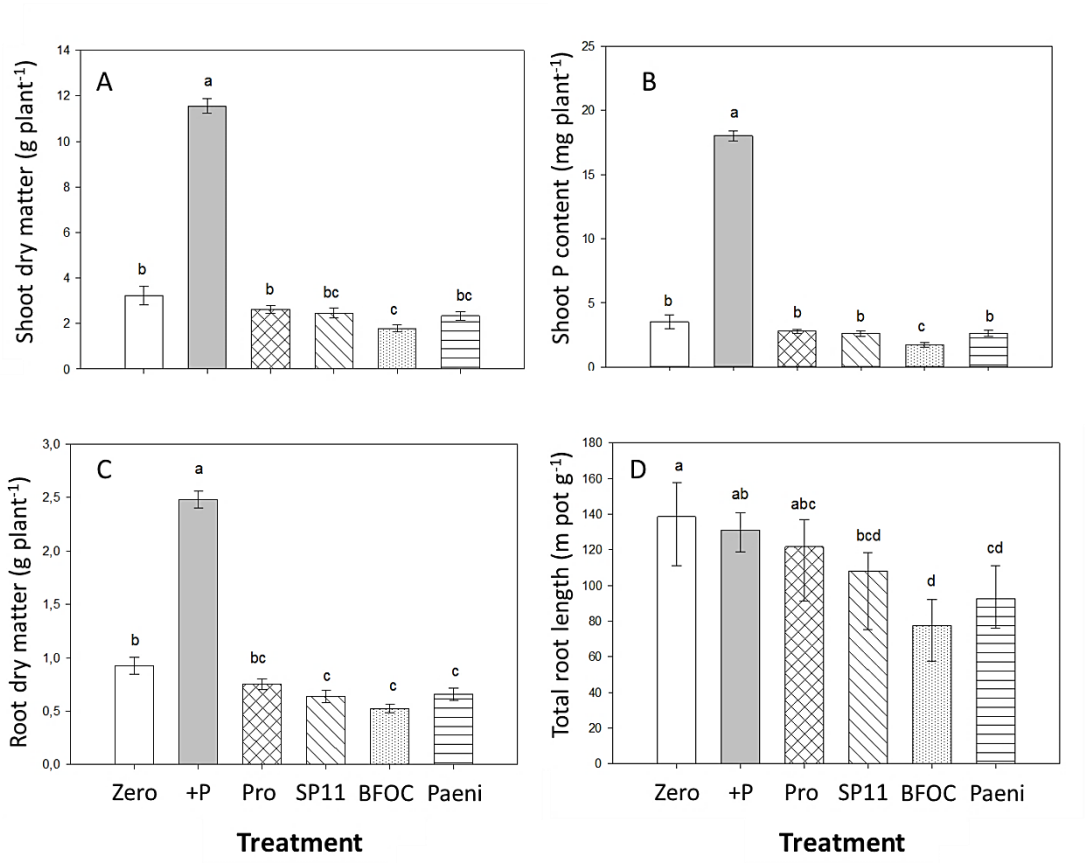

Figure 7.4. Shoot dry matter (A) and shoot P content (B), root dry matter (C) and total root length (D)
Zero, RP and no BE; **+P**, $Ca(H_2PO_4)_2$ and no BE; **Pro**, RP and *Pseudomonas sp.* DSMZ 13134; S**P11**, RP and Vitalin SP11; **BFOC**= RP and *Penicillium sp*. PK 112; **Paeni**, RP and *Paenibacillus mucilaginosus.* One-way ANOVA, Tukey test α=0.05. Different letters show significant difference *(P<0.05).*

7.3.5 Conclusion:

In disagreement with the hypothesis, BEs negatively affected plant P nutrient status and shoot growth. Possible explanations for the growth-inhibiting effect of tested BEs on maize are, competition for soil P and other nutrients between BEs and maize plants as well as utilization of organic C and N compounds released as maize root-exudate by BEs without any positive contribution in turn by BEs to promote P nutrition or growth of maize plants (parasitism).

7.4 Influence of P-solubilizing microorganisms on P acquisition from rock phosphate by maize

Original title: Einfluss P-lösender Mikroorganismen auf das Rohphosphat-aneignungsvermögen von Mais

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7.4.1 Background and objectives

Given the negative effects of tested BEs on shoot biomass production and shoot P status of maize grown on a highly P sorbing soil (reported in section 7.3 above), this experiment had the objective to investigate whether addition of organic C and N nutrients during watering could cover BE nutrient requirements thereby, reducing any competition for nutrients between BEs and plant roots while increasing BE activity for RP solubilization.

Additionally, BE effect on P acquisition from RP by maize plants was investigated on a substrate with reduced P sorption capacity. P sorption capacity of a soil is the ability of the soil to convert soluble available orthophosphates ($HPO_4^{2-}/H_2PO_4^-$) to less soluble and less available P forms by reactions such as adsorption onto charged mineral or organic surfaces and precipitation. Therefore P sorption capacity of a soil is influenced by its pH, thus contents of exchangeable Ca, Fe and Al; clay type and content; and organic matter content (*Hansen* et al. 2002). The loess subsoil used contained 23% $CaCO_3$, which at pH_{CaCl2} 7.6 implied high concentrations of exchangeable Ca were present. Low clay and organic matter contents, implied $CaCO_3$ content was the main determining factor for P sorption capacity, which could be reduced by simply diluting the loess subsoil with quartz sand.

7.4.2 Hypotheses

- Addition glucose and glycine to soil during watering alleviates negative competitive/ parasitic effects of soil-inoculated BEs on growth and P acquisition of maize plants supplied with rock phosphate in an alkaline P-sorbing substrate.
- BE-solubilization of RP with improved P nutrition of maize plants is enhanced in a substrate with low P sorption capacity.

7.4.3 Methodology:

The first pot experiment conducted was a repetition of the pot experiment in section 7.3 with daily watering of pots performed with a solution containing 5.0 mM glucose and 2.5 mM glycine instead of distilled water. The second pot experiment was different from the first in that a substrate with lower P-sorption capacity was utilized. This substrate was prepared by increasing the proportion of quartz sand from 20 to 70% and adding only 30% low-P soil (described in 7.2.3). Inoculation procedure for BE was the same as described in section 7.3.3.

Residual P concentrations in rhizosphere soil from treatments Zero, Pro and +P from the second experiment were measured by sequentially extracting 2 g air dried rhizosphere soil with 30 ml of 0.5 M NaCl/TEA (TEA, triethylamine) with the pH of the extractant adjusted from 10.8 to 7 using 2 N HCl (available P fraction) (*Tambunan* et al. 1993). After centrifugation and decanting of the supernatant, the remaining soil was extracted with 30 ml 1 M NaOH (moderately labile organic and inorganic P bound to amorphous hydroxides of Fe and Al) and finally, with 30 ml 1 M H_2SO_4 (residual non-labile P). The H_2SO_4 extract was filtered and the inorganic P concentration was analyzed using the molybdate-blue method (*Murphy* and *Riley*, 1962). pH_{CaCl2} of rhizosphere soil was also measured.

7.4.4 Results:

In the first experiment, watering with glucose and glycine did not alleviate negative BE effects on maize growth. The same negative BE effects on maize shoot growth, shoot P content, root biomass and total root length per pot observed in the experiment described in section 7.3.4 were replicated.

Although the second experiment was characterized by a general reduction in shoot biomass, shoot P content, root biomass and total root length in all treatments in comparison to the first experiment, inoculation of BE showed a significant increase (or a tendency) in all four measure variables in comparison to the non-inoculated control (Fig.7.5)

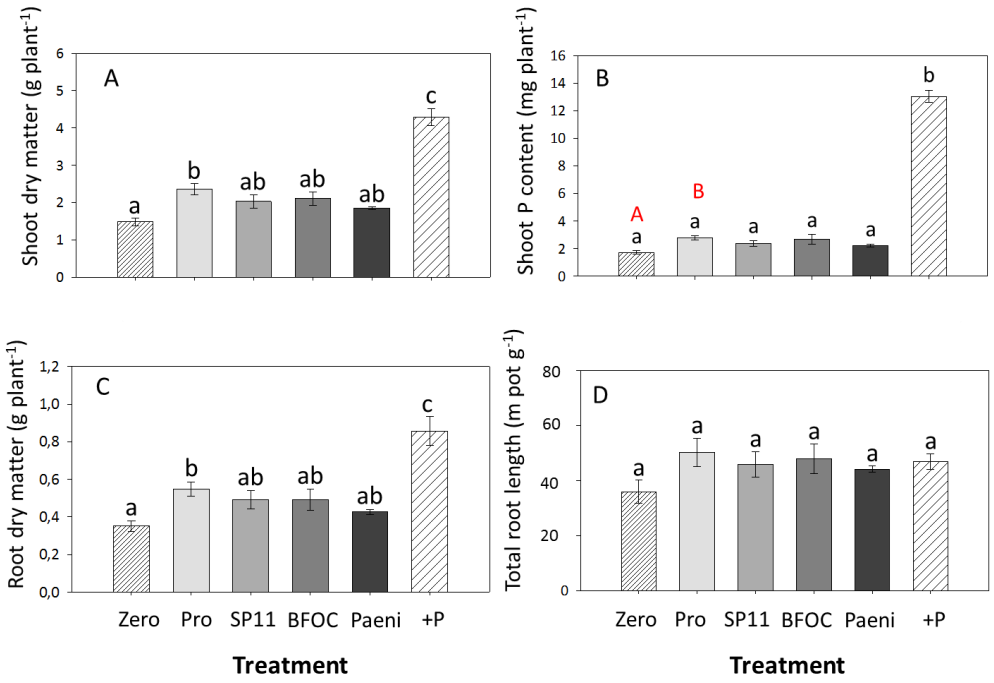

Figure 7.5. Shoot dry matter (A) and shoot P content (B), root dry matter (C) and total root length (D)
Zero, RP and no BE; **Pro**, RP and *Pseudomonas sp.* DSMZ 13134; **SP11**, RP and Vitalin SP11; **BFOC**= RP and *Penicillium sp*. PK 112; **Paeni**, RP and *Paenibacillus mucilaginosus;*. **+P**, $Ca(H_2PO_4)_2$ and no BE; One-way ANOVA, Tukey test α=0.05. Different letters show significant difference *(P<0.05).* Different bold red letters show significant difference between the non-inoculated control and Pro *(*t-test, α=0.05)

Residual P concentrations in rhizosphere soil samples from the second experiment were: Zero, 159; Pro, 156; +P, 62 mg P kg^{-1} soil. Given 70% sand content of the substrate and the resulting loose texture, it is likely that rhizosphere soil was not satisfactorily sampled with samples containing more bulk soil than tallowed. pH_{CaCl2} of rhizosphere soil from treatment +P (7.1) was slightly lower those from the other treatments (7.3).

7.4.5 Conclusion:

Addition of glucose and glycine did not result in positive BE effects on maize growth and P status of shoots. However, after reducing the P sorption capacity of the substrate by increasing the fraction of sand, all tested BEs increased (or showed a tendency to increase) total root length, root and shoot biomass, shoot P content despite overall reduction in all measured variables if compared to plant performance on the highly P sorbing substrate. The likely mechanism for improved plant growth was improved P uptake via enhanced root growth for more effective exploitation of the substrate. Nevertheless, this could not be fully confirmed because there was no reduction in the concentration of residual rhizosphere P for the best performing BE variant **(*Pseudomonas sp.* DSMZ 13134)** in comparison to the non-inoculated control partly due to sampling errors for rhizosphere soil. Despite no reduction in the pH of rhizosphere soil upon inoculation with BEs, improved solubility of RP via release of chelating low molecular weight compounds by BEs may occur.

7.5 N-form dependent solubilization of rock-phosphate in maize inoculated with microbial bio-effectors

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Date of submission: 14.12.2015

7.5.1 Background and objectives

The first part of this study was based on the second experiment described in section 7.4 above. Its aim was to investigate whether addition of $CaCO_3$ as a means to re-increase the P sorption capacity of a low-P sorbing substrate (70% sand and 30% low-P soil) supplied with rock phosphate (RP) could nullify the beneficial effects of the best performing BE *Pseudomonas sp.* DSMZ 13134 on improving maize growth and shoot P status. Furthermore, the effect of N-form (NH_4^++DMPP vs. NO_3^-) was investigated because both soil microorganisms as well as plant roots acidify their growth medium by proton exudation upon transport of NH_4^+ into cells, with the consequence of improved solubility of RP (or vice versa, reduced RP solubility due to an increase in the pH of the medium upon co-transport of NO_3^- and H^+ into cells).

A second pot experiment was conducted to screen selected promising BEs under NH_4^+ supply to a neutral clay-loam top soil on improving P acquisition of maize from RP. The clay-loam soil, which was more realistic for natural BE/Plant root interaction than the C-horizon subsoil with high P sorption capacity (used sections 7.3 and 7.4), was obtained from a cultivated arable organic farm land.

7.5.2 Hypotheses

- Addition of $CaCO_3$ to a low P sorbing substrate increases P sorption capacity, thereby abolishing positive effects of *Pseudomonas sp.* DSMZ 13134 (Pro) on P acquisition of maize plants from RP.
- Negative effects of added $CaCO_3$ on BE-assisted maize P-acquisition from RP can be neutralized by N fertilization with NH_4^+ or worsened by supply of NO_3^-.
- Growth-promotion of RP-fed maize plants by *Pseudomonas sp.* DSMZ 13134 (Pro) is stronger under N fertilization with NH_4^+ than with NO_3^-.
- With N supply as NH_4^+, selected BEs promote growth and P nutrition of maize plants fertilized with RP.

7.5.3 Methodology:

In the first pot experiment, the same basic substrate (70% sand and 30% low-P soil) and basic fertilization as described in 7.3 above was used. Treatments included a factorial combination of 2 BE levels (No Be; Pro) x 2 PO_4^{3-}-buffering levels in the substrate (0% $CaCO_3$; 25% $CaCO_3$) x 2 N-fertilizer forms (NO_3^-:100% NO_3-N as $Ca(NO_3)_2$; or NH_4^++DMPP: 80% NH_4-N as stabilized $(NH_4)_2SO_4$+DMPP and 20% NO_3-N as $Ca(NO_3)_2$). Additionally, there was a negative P control with NO_3^- and without RP (-P), a positive P control with NO_3^- and soluble $Ca(H_2PO_4)_2$ (+P), and a control to re-test BE effects under high substrate P sorption (+$CACO_3$; CC), N supply as NO_3^-, Pro and watering of pots with a solution of glucose and glycine as previously tested (section 7.3). The same inoculation procedure for *Pseudomonas sp.* DSMZ 13134 (Pro) described in section 7.2 was used.

In the second pot experiment, the substrate was composed of 70 % clay-loam soil (pH_{CaCl2} 6.8; P_{CAL} 20 mg P kg^{-1} soil) and 30 % quartz sand. $CaCO_3$ was not utilized. Basic fertilization was (kg^{-1} soil DM): 150 mg N, (as $(NH_4)_2SO_4$+DMPP or $Ca(NO_3)_2$ (for the positive P control,

+P); 100 mg P(RP or $Ca(H_2PO_4)_2$ (for +P); and 150 mg K (K_2SO_4). In addition to a non-inoculated control, seven BEs tested under P supply as RP and N supply as NH_4^+ included: *Pseudomonas sp.* DSMZ 13134 (Pro); Vitalin SP11 (SP11), *Penicillium sp*. PK 112 (BFOD), *Paenibacillus mucilaginosus* (Paeni), *Bacillus amyloliquefaciens* FZB42 Rhizovital42® (Rhiz); *Trichoderma. harzianum* T22 (T-22); and CombiFectorA: (ComA: *T. harzianum* OMG08, *Pseudomonas fluorescens*, *Bacillus subtilis*). Furthermore, the best performing BE Pro was test with RP under N supply as NO_3^- to replicate previous results on N-form effects. Two more control treatments included: one without BE and without any fertilizer (Zero); and a positive P control without BE (+P), but with supply of 100 mg N kg^{-1} ($Ca(NO_3)_2$), 100 mg P kg^{-1} P($Ca(H_2PO_4)_2$). There were five replicates in both experiments arranged in completely randomized design.

7.5.4 Results:

Once more, watering with a solution of glucose and glycine did not affect shoot and root dry matter, shoot P concentration and content in maize plants inoculated with Pro and supplied with NO_3^-, RP and $CaCO_3$ (*P>0.05*). The Factor BE affected shoot DM, $CaCO_3$ affected shoot DM and shoot P content; whereas N-fertilizer form affected all measured variables (Table 7.2 and 7.3). For the variable shoot P content, N-form and $CaCO_3$ content significantly interacted with each other. NH_4^+ improved shoot P concentration and shoot P content in the absence of added $CaCO_3$ whereas NO_3^- effect on shoot P concentration and shoot P content was not influenced by addition of $CaCO_3$.

Table 7.2. Source of variation (Three-way ANOVA) (ns *P>0.1*)

	BE	N-form	$CaCO_3$	BE*N-form	N-form*$CaCO_3$	BE*N-form*$CaCO_3$
Shoot DM	0.017	<0.001	0.045	ns	ns	ns
Shoot P concentration	ns	0.001	ns	ns	0.081	ns
Shoot P content	ns	<0.001	0.027	ns	0.040	ns
Root DM	0.069	0.001	0.052	ns	ns	ns

Table 7.3. Least square means (Three-way ANOVA)

	BE		N-form		$CaCO_3$	
	NoBE	Pro	NO_3^-	NH_4^+	25%	0%
Shoot DM (g plant^{-1})	2.8 b	3.3 a	2.5 b	3.5 a	2.8 b	3.2 a
Shoot P conc. (mg P g^{-1})	1.5	1.5	1.4 b	1.6 a	1.5	1.5
Shoot P content (mg P plant^{-1})	4.2	4.7	3.5 b	5.4 a	4.1 b	4.8 a
Root DM (g plant^{-1})	0.49	0.60	0.45 b	0.64 a	0.49	0.60

In the second pot experiment, difference in the effects NH_4^+ in comparison to NO_3^- on plant P acquisition and biomass production under inoculated Pro and supply of RP could be replicated once more. Least square means for measured variables were: Shoot DM (g plant^{-1}: NH_4^+, 9.8 a; NO_3^-, 6.8 b; *P<0.001*); Shoot P content (mg P plant^{-1}: NH_4^+, 22.2 a; NO_3^-, 17.6 b; *P=0.007*); Shoot P conc. (mg P g^{-1}: NH_4^+, 2.3 a; NO_3^-, 2.6 b; *P=0.005*); Root DM (g plant^{-1}: NH_4^+, 1.6 a; NO_3^-, 1.2 b; *P=0.007*).

Inoculation of all BEs except T-22 and BFOD resulted in higher shoot dry matter, and lower shoot P concentration (biomass dilution effect) than the non-inoculated control (Fig. 7.6).

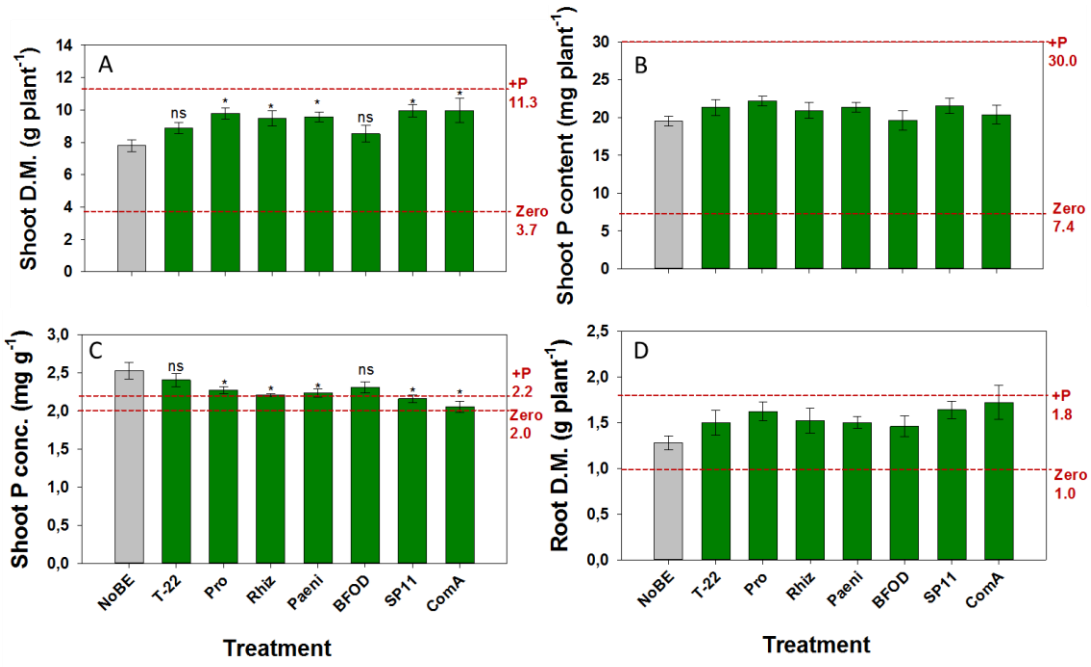

Figure 7.6. Effect of BEs on shoot dry matter (A), shoot P content (B) and P concentration (C) and root dry matter (D).
T-22, *Trichoderma. harzianum* T22; **Pro**, *Pseudomonas sp.* DSMZ 13134; **Rhiz**, *Bacillus amyloliquefaciens* FZB42; **Paeni**, *Paenibacillus mucilaginosus*; **BFOD**, *Penicillium sp*. PK 112; **SP11;** Vitalin SP11 (*Bacillus subtilis, Pseudomonas sp., Streptomyces spp*., natural humic acids und extracts of the seaweed); and **ComA**, CombiFectorA: (: *T. harzianum* OMG08, *Pseudomonas fluorescens, Bacillus subtilis*); **Zero,** non-inoculated and unfertilized control; **+P,** non-inoculated but positive P control. One way ANOVA, mean ± SEM, n = 5, Holm-Sidak test, α = 0.05, * shows significant difference between BE treatment and the non-inoculated control, *P<0.05*(NoBE)

7.5.5 Conclusion

If the P sorption capacity of a poor P-sorbing substrate is increased by adding $CaCO_3$, the positive effects of *Pseudomonas sp.* DSMZ 13134 on P acquisition and growth of maize plants supplied with RP are nullified. Supplying N as NH_4^+ contributes to mitigate the negative effects of $CaCO_3$ addition on maize growth whereas supply of N as NO_3^- does not. With N supply as NH_4^+ and P supply as RP, tested BEs containing a single bacterium strain contributed to increased maize shoot biomass whereas tested BEs with a single fungus strain did not. BE consortia containing both fungi and bacteria performed well.

7.6 Preliminary title: Effects of BE and N-form on P nutrition and growth of maize plants supplied with different sparingly soluble inorganic/recycled P fertilizers.

This work is part of an ongoing thesis for a Master of Science degree by: Ms. Shikta Kar (2016)

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7.6.1 Background and objectives

Previous experiments (sections 7.3 – 7.5) have shown that low substrate P-sorption, N supply as NH_4^+ and inoculation of bacterial BEs or BE consortia containing both bacteria and fungi promote P acquisition and growth of maize plants supplied with RP. The objective of this experiment was to investigate the ability of the most promising pair of BEs (Pro and ComA) to improved P acquisition of maize plants from native moderate- to non-labile soil P pools, RP, and two sparingly soluble recycled P-fertilizers. Once more, the effect of N-form (NH_4^+ vs. NO_3^-) was investigated for Pro under P supply as RP.

7.6.2 Hypotheses

- *Pseudomonas sp.* DSMZ 13134 (Pro) improves shoot biomass and shoot P content of maize plants supplied with RP when N is fertilized as NH_4^+ and does not when N is supplied as NO_3^-.
- With N fertilized as NH_4^+, Pro and ComA (CombiFectorA) each improve P acquisition of maize plants from recalcitrant soil pools, or applied sparingly soluble, RP, sewage sludge ash (SSA) or struvite, thereby improving shoot growth and shoot P status.

7.6.3 Methodology:

Tested P-fertilizers included: rock phosphate (RP, 7.6 % P), sewage sludge ash (SSA, 10.34 % P) and struvite (STR 19.5 % P). Like in the second experiment in section 7.5 above, the

substrate was composed of 70 % clay-loam soil (pH_{CaCl2} 6.8; P_{CAL} 20 mg P kg^{-1} soil) and 30 % quartz sand (w/w). With N supplied as stabilized NH_4^+ (100 mg NH_4-N kg^{-1} as $(NH_4)_2SO_4$+DMPP), treatments were comprised of factorial combinations of two BE levels (**Pro**, *Pseudomonas sp.* DSMZ 13134; and **ComA**, CombiFectorA: (: *T. harzianum* OMG08, *Pseudomonas fluorescens, Bacillus subtilis*)) and four P-fertilizer levels (NoP, RP, STR, SSA each at the rate100 mg P kg^{-1}). Controls included: one treatment with neither P-fertilizer nor BE (Zero); one with NO_3^- ($Ca(NO_3)_2$), RP and Pro (Pro_NO_3^-, _RP) and a positive P control (+P) with 150 mg N kg^{-1} ($Ca(NO_3)_2$) and 100 mg P kg^{-1} (soluble $Ca(H_2PO_4)_2$). There were five replicates per treatment arranged in a completely randomized design.

7.6.4 Results

With NH_4^+, Pro led to 46% higher shoot dry matter (15.2 g $plant^{-1}$) than with NO_3^- (10.4 g $plant^{-1}$) for maize plants supplied with RP. For control treatments arranged in the order: Zero, Pro_NO_3^-_RP and +P, shoot dry matter (g $plant^{-1}$) was: 8.8; 10.4 and 14.1; root length density (cm cm^{-3}) was: 3.8, 2.8 and 4.0; shoot P content (mg plant-1): 16.0, 25.5, 31.2 respectively.

Factors BE and P-fertilizer significantly affected shoot dry matter and shoot P content (with a tendency of interaction) whereas root length density was affected only by the factor P-fertilizer (Table. 7.4).

Table 7.4. Two-way ANOVA for factors BE and P-fertilizer (ns *P >0.1*)

		Shoot DM	**Shoot P content**	**Root length density**
Source of Variation	**DF**	***P***	***P***	***P***
P-fertilizer	3	*<0.001*	*<0.001*	*0.003*
BE	2	*0.025*	*<0.001*	*ns*
P-form x BE	6	*0.051*	*0.063*	*ns*

With struvite, plants attained the level of shoot DM, shoot P content and root length density as plants belonging to the positive P control treatment irrespective of inoculation with BE. Pro enhanced (or showed a tendency to enhance) shoot DM and shoot P content under no

additional P or supply of RP whereas ComA seemed to improve shoot DM for all P-forms except STR (Fig. 7.7)

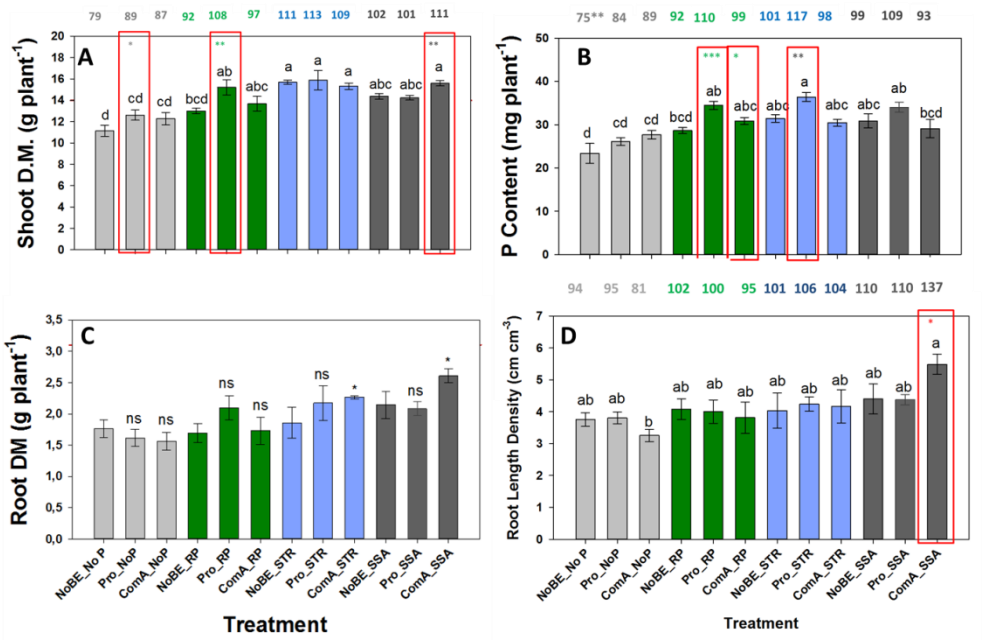

Figure 7.7. Effect of treatments with NH_4^+, BEs and P-fertilizers on shoot DM (A), Shoot P content (B), root DM (C) and root length density (D).
Different letters show significant different between treatment pairs (One way ANOVA, mean ± SEM, n = 5, Tukey test, α = 0.05), * shows significant difference between BE treatment and corresponding non-inoculated control (t test, *α = 0.05, * P<0.05, **P<0.01*). Numbers above bars show % comparison to the +P control. NoBE, no bio-effector; **Pro**, *Pseudomonas sp.* DSMZ 13134; and **ComA**, CombiFectorA (*T. harzianum* OMG08, *Pseudomonas fluorescens, Bacillus subtilis*); RP, rock phosphate; STR, struvite; SSA, sewage sludge ash.

7.6.5 Conclusions:

Once again, results showed that *Pseudomonas sp.* DSMZ 13134 **(**Pro) improves growth of maize supplied with RP when N is fertilized as NH_4^+ and not as NO_3^-. With NH_4^+, Proradix improved shoot biomass production with P supplied as RP and led to increased shoot P contents with STR, whereas ComA led to improved RLD under SSA. P acquisition by maize plants from struvite is effective without BE. Only marginal increases could be achieved by application of Pro and no growth-promotion by ComA.

7.7 BE-assisted P nutrition of spring wheat supplied with rock phosphate and placed NH_4^+

Mr. Niklas Käfer (M Sc. student at the Faculty of Agriculture, University of Hohenheim) contributed by up to 15 % to the work reported in this section. He was the only participant in a Humboldt-Reloaded project during summer semester 2015. The project was entitled "Improving phosphorous (P)-nutrition of crop plants with alternative P fertilizers and bio-effectors". It involved a pot experiment conducted at the Institute of Crop Science, department of Fertilization and Soil Matter Dynamics (340 i).

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7.7.1 Background and objectives

So far previous experiments have demonstrated positive effects of selected BEs on P acquisition from sparingly soluble P-fertilizers when N is supplied in the form of NH_4^+ only in cultures with maize (*Zea mays* L.). The response of other crop species to substrate-inoculated BEs under supply of NH_4^+ and RP is unknown. The objective of this project was to investigate BE effects under N supply as NH_4^+ on growth and P acquisition from RP in a spring wheat culture (*Triticum aestivum L*). Spring wheat is interesting as a different test crop species because it is a more effective soil P mobilizer than maize (e.g. secretions of chelating substances like siderophores in root exudates for nutrient mobilization) and less sensitive to low P availability than maize. Given knowledge from previous tests with maize, the effect of Proradix on spring wheat under different N-forms (NH_4^+/NO_3^-) was also studied.

7.7.2 Hypotheses

- Like with maize plants, *Pseudomonas sp.* DSMZ 13134 improves P acquisition and growth of spring wheat supplied with RP when N is fertilized as NH_4^+ and not when N is supplied as NO_3^-.
- Under N supply as NH_4^+, BEs improve P acquisition and growth of spring wheat plants fertilized with RP.

7.7.3 Methodology:

Spring wheat (*Triticum aestivum L.* Schirocco, KWS, Germany) was grown in 5 L pots each containing 5.7 kg substrate. The substrate was composed of 70 % low-P silt loam luvisol (Barvendorf, Lake of Constance, Germany, pH_{CaCL2} 6.4, P_{CAL} 7mg kg^{-1}; P_{NaHCO3} 8 mg kg^{-1}; P_{Total} 218 mg kg^{-1}; C_{org} 2.8 %) and 30 % quartz sand (w/w). Moisture content was 24 % (70 % max. WHC). K and Mg were sufficiently supplied (150 mg K (K_2SO_4); 50 mg Mg, ($MgSO_4$) kg^{-1} soil). BE levels tested included: NoBE, no bio-effector; **Pro**, *Pseudomonas sp.* DSMZ 13134; **Rhiz**, *Bacillus amyloliquefaciens* FZB42; **Paeni**, *Paenibacillus mucilagenosus* (each at the rate $1x10^9$ CFUs or Spores kg^{-1}); and **T-22**, *Trichoderma*. *harzianum* T22 (at the rate $1x10^8$ spores kg^{-1}). BE suspensions in 2.5 mM $CaSO_4$ were inoculated at these rates three times at 0, 24 and 34 days after sowing (DAS). P was supplied as RP (150 mg P kg^{-1} soil). There were two sets of treatments:

I. Factorial combination of 2 BE levels (NoBE; Pro) x 2 N levels (150 mg NO_3-N kg^{-1} mixed homogenously in the whole substrate as ($Ca(NO_3)_2$); 150 mg NH_4-N kg^{-1} as stabilized $(NH_4)_2SO_4$+DMPP placed by point injections at 10 cm depth);
II. Placed stabilized ($(NH_4)_2SO_4$+DMPP) at the rate 150 mg NH_4-N kg^{-1} in combination with five BE levels: NoBE, Pro, Rhiz, T-22, Paeni

N-fertilization was split to three events: 50 mg N kg^{-1} before sowing (homogenously mixed in the substrate at 0 DAS), 50 mg N kg^{-1} at 24 DAS and 50 mg N kg^{-1} at 42 DAS placed by point injection at 10 cm depth.

There were five pots per treatment arranged in a randomized block design. Plants were grown in the greenhouse and temporarily moved outdoors during good weather. The growth period lasted from 02. Jun – 04. Sep. 2015. Mean daily temperature was: 24°C (min: 8 °C, Max: 51 °C, - indoors) and daylight intensity ranged from 400-1200 µmol photons m^{-2} s^{-1} for lower intensity with indoor lamps to bright sunlight outdoors on a sunny day.

7.7.4 Results:

In most measured variables especially concentrations and contents of nutrients in biomass, NH_4^+ led to higher values than NO_3^- and Proradix also led to higher values than without BE (Table 7.5). N-form strongly interacted with BE for grain Ca concentration and content, and straw Cu content (*P<0.05*), weakly interacted for grain DM (*P=0.052*) and number of grains per pot (*P=0.061*).

Table 7.5. Relative effect (%) of NH_4^+ to NO_3^- and Pro to NoBE on shoot, straw and grain dry matter, and contents and concentrations of different nutrients at booting and senescence growth stages in shoot, straw and grain.

Effect of NH_4^+ relative to NO_3^- (%)	**Effect of Proradix relative to NoBE (%)**
Booting stage	
Shoot DM: +10% ns **Shoot concentrations:** +28% P, +5% N, +25% Zn, +18% Cu, -24% Ca, -5% Mn **Shoot contents:** +41% P, +39% Zn, +29% Cu	**Shoot DM:** +19% **Shoot concentrations:** +3% K **Shoot contents:** +23% P, +19% N, +23% K, +21% Mg, +20% Mn, +21% Cu
Senescence stage	
Root DM: +19% ns **Straw DM:** +6% ns **Grain DM:** +3% ns **Nr. grains:** -17% **TGW:** +21% **Grain concentrations:** +47% P, +11% N, +8% K, +12% Mg, -36% (Ca), +46% Zn, +15% Mn, +9% Cu **Grain contents:** +61% P, +26% N, +26% K, +22% Mg, +62% Zn, +25% Mn, +27% Cu	**Root DM:** +16% ns **Straw DM:** +12% ns **Grain DM:** +21% **Nr. grains:** +21% **TGW:** ns **Grain concentrations:** ns **Grain contents:** +20% P, +17% Zn, +14% (Ca) **Straw concentrations:** +17% Mn, -7% N

Straw concentrations: +79% P, +63Zn, +15% Cu **Straw contents:** +112% P, +14% N, +81% Zn, +27% (Cu)	**Straw contents: +26%**Mn
Two-way ANOVA (Factors: N-form and BE), tukey-test, α = 0.05. Values for nutrient concentrations and contents are shown only for significant effects (*P<0.05*), (ns, *P≥0.05*). Reductions in relative effects are shown in red. TGW, thousand grain weight. Brackets ( ) show variables with significant BE*N-form interaction.	

At booting and the senescence stages, each BE improved or showed a tendency to improve shoot biomass (Fig.7.8).

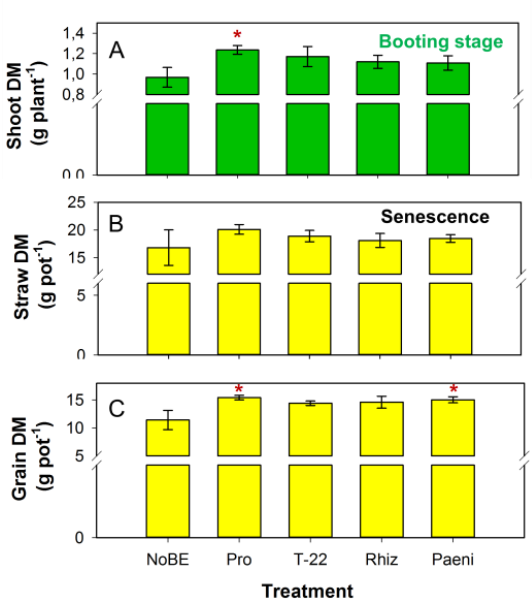

Figure 7.8. Effect of BE on shoot biomass production at the booting stage (A), straw biomass (B) and grain yield (C) at senescence stage.
t-test with NoBE, α = 0.05; ns *P≥0.05*, * *P<0.05*. NoBE, no bio-effector; **Pro**, *Pseudomonas sp.* DSMZ 13134; **Rhiz**, *Bacillus amyloliquefaciens* FZB42; **Paeni**, *Paenibacillus mucilagenosus*); and **T-22**, *Trichoderma harzianum* T22

Inoculation of BEs improved or tended to improve shoot P and N content at the booting stage, and root DM, number of grains and grain Ca content at the senescence stage (Table. 7.6).

Table 7.6. Effect of BE on shoot N and P content (Booting stage) and root biomass, grain and straw characteristics

Variable	NoBE	Pro	Rhiz	T-22	Paeni
Booting stage					
Shoot P (mg plant^{-1})	2.59	3.53*	2.90	3.24	2.92
Shoot N (mg plant^{-1})	34.0	43.3*	39.7	39.5	39.5
Senescence stage					
Root DM (g pot^{-1})	1.23	1.74	1.60	1.35	1.64
Nr. of grains	332	453**	442*	422*	460**
TGW	33.7	33.8	32.6	33.9	31.9
Straw P (mg pot^{-1})	23.6	19.9	14.5**	19.1	14.7**
Grain Ca (mg pot^{-1})	5.53	7.73**	7.4*	6.7	7.62**
t-test with NoBE, α = 0.05; ns *P≥0.05*, * *P<0.05*, ***P<0.01*					

7.7.5 Conclusion:

Like with maize plants, *Pseudomonas sp.* DSMZ 1314 improves P nutrition and growth of spring wheat when N is supplied as NH_4^+ more than when N is fertilized as NO_3^-. With NH_4^+, each inoculated BE enhanced or tended to enhance growth of spring wheat plants, with the best performing BEs being *Pseudomonas sp.* DSMZ 1314 and *Paenibacillus mucilagenosus.* In comparison to previous experiments, *Trichoderma harzianum* T22 seemed to be more growth-promoting in spring wheat cultures than in maize cultures. Improvement of nutrient status and yield of wheat plants may be explained by enhanced chemical mobilization/ solubilization of nutrients in the rhizosphere as well as by improved root growth for more effective interception of soil nutrients.

7.8 References

Hansen, N. C., Daniel, T. C., Sharpley, A. N., Lemunyon, J. L. (2002): The fate and transport of phosphorus in agricultural systems. *J. Soil Water Conserv.* 57, 408–417.

Harman, G. E. (2006): Overview of mechanisms and uses of *Trichoderma spp*. *Phytopathology* 96, 190–194.

Harman, G. E., Petzoldt, R., Comis, A., Chen, J. (2004): interactions between *Trichoderma harzianum Strain T22* and Maize Inbred Line Mo17 and effects of these interactions on diseases caused by *Pythium ultimum* and *Colletotrichum graminicola*. *Phytopathology* 94, 147–153.

Lugtenberg, B., Kamilova, F. (2009): Plant-Growth-Promoting Rhizobacteria. *Annu. Rev. Microbiol.* 63, 541–556.

Murphy, J., Riley, J. P. (1962): A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta.* 27, 31–36.

Schenck zu Schweinsberg-Mickan, M., Müller, T. (2009): Impact of effective microorganisms and other biofertilizers on soil microbial characteristics, organic-matter decomposition, and plant growth. *J. Plant Nutr. Soil Sci.* 172, 704–712.

Tambunan, D., Hedley, M. J., Bolan, N. S., Turner, M. A. (1993): A comparison of sequential extraction procedures for measuring phosphate rock residues in soils. *Fert. Res.* 35, 183–191.

8 General discussion and conclusion

8.1 Meta-analysis on fertilizer placement

Through the literature review, we identified various techniques for fertilizer placement in soil. The most effective techniques were band and "knife" placement. Subsurface fertilizer placement at a depth of more than 10 cm led to higher relative placement effects on measured variables than placement at 5-10 cm depth or to placement on surface soil. In light of these findings and those from other field studies (*Ma* et al. 2009; *Singh* et al. 2005), it should be recommended to farmers who employ fertilizer placement to consider deep subsurface placement to improve the efficiency of fertilizers. However, the cost for additional mechanical energy and possibly new machinery required for deep subsurface fertilizer placement should be considered. With increasing frequency of extreme drought events (*Parry* et al., 2004) or particularly in climate regions where topsoil rapidly dry up on hot days, deep subsurface fertilizer placement may be economically advantageous in the long-term despite initial expenses for machinery. The problem of drought could be seen in our field studies. Due to extreme drought during the crop season in 2015, yield of maize silage from fertilizer placement was -5.8% that of broadcast/incorporation. In 2014, characterized by normal rainfall, grain yield from placement was +7.4 % that of broadcast/incorporation.

The most effective fertilizer formulations for subsurface placement were $CO(NH_2)_2$ combined with PO_4^{3-}, NH_4^+ combined PO_4^{3-} or liquid manure. There was no benefit on crop performance from placing PO_4^{3-} fertilizers when not combined with a suitable N-fertilizer. A recently conducted meta-analysis on placement of PO_4^{3-} fertilizers under field conditions (247 published and unpublished studies including "grey" literature) concluded that placement led to lower yields than broadcast, especially for no-till systems (IPNI, 2016). The effectiveness of placing a combination of NH_4^+ and PO_4^{3-} in subsurface soil by banding to enhance P uptake and yield was already described in early studies on fertilizer placement with maize

(*Zea mays* L.) (*Miller* and *Ohlrogge*, 1958). Additionally, *Miller* and *Ohlrogge* (1958) observed than when NH_4^+ was placed 7-10 cm away from PO_4^{3-} fertilizer, marginal improvement in P uptake occurred only in low-P soils. This so called "improved root feeding power of banded P" when combined with NH_4^+ was not affected by soil P status. Therefore, it was attributed to favorable chemical changes caused by NH_4^+ supply or "increase in relative root sorption surfaces in the banded volume". Today both explanations of *Miller* and *Ohlrogge* (1958) have been confirmed and fully described. In our rhizobox experiment (Section 6.4.2), we could show considerable rhizosphere acidification effect of NH_4^+ nutrition of roots, which likely promoted the solubility of sparingly soluble calcium phosphates formed in the alkaline $CaCO_3$–rich substrate after addition of soluble PO_4^{3-} fertilizer. In addition, intense localized root growth at the NH_4^+-depot increased the total surface area of roots for nutrient uptake which led to improved shoot N and P concentrations and contents for maize. Furthermore, NH_4^+ may act a more potent nutrient signal than $PO_4^{3-}$for the attraction of roots towards a fertilizer band made of both NH_4^+ and PO_4^{3-}(chemotropism). This is likely because NH_4^+ is more mobile in soil than PO_4^{3-}. The consequence is improved uptake of both N and P from the band by crop plants. Consequently, we recommend farmers to place PO_4^{3-} fertilizers in subsurface soil only in combination with a suitable N-fertilizer like NH_4^+ or $CO(NH_2)_2$.

8.2 Induced rhizosphere hotspots as a "home" for inoculated microbial BEs

We were able to repeatedly show that subsurface placement of a concentrated stabilized NH_4^+-depot induced the formation of localized zones of intense root growth around fertilizer depots (rhizosphere hotspots). High NH_4^+ concentrations (64 g l^{-1}) coupled with the presence of DMPP effectively reduced biological nitrification of NH_4^+ as shown in rhizobox and field experiments. The effect of placing NH_4^+ especially at high concentrations on the induction of intense localized root growth has been previously observed and described in soil systems under greenhouse and field conditions (*Jing* et al. 2012; *Sommer* 2005). Through induced

intense localized root-growth, well-rooted soil areas can be targeted for the application of sparingly available fertilizers as well as PGPMs that improve plant-nutrient availability. Nevertheless, without good overall root growth, intense localized root growth alone may not sufficiently cover plant need for nutrients and water. Therefore, the use of fertilizer placement to spatially control the rhizosphere is regarded as a tool to accompany other measures such as ploughing that promote overall root-growth of crops.

Several beneficial effects of fertilizer placement in comparison to fertilizer application by broadcast suggest that in the future, fertilizer placement will become widely adopted. Through targeting crop plants with nutrients by subsurface fertilizer placement, crops are given the advantage to grow faster than weeds (*Blackshaw* et al., 2002; *Légère* et al., *2013*). Through fertilizer placement in subsurface soil, fixation and/or immobilization of nutrients by various ions and/or soil microorganisms could be reduced; NH_3 volatilization and runoff losses N and P could be lessened; nitrate leaching and nitrous oxide emission could be lowered; and higher fertilizer use efficiencies and crop performance is achievable (*Linquist* et al., 2012; *Ma* et al., 2010; *Nash* et al., 2012; *Ruidisch* et al., 2013).

On nutrient agar, several microbial BEs showed considerable tolerance to high concentrations of NH_4^+ irrespective of the presence of DMPP. This suggested that NH_4^+-rich rhizosphere hotspots around NH_4^+-depots in soil could be areas for effective root colonization by the PGPMs. This proposal could be confirmed after culturing root extracts on selective media for fluorescent Pseudomonads after *Pseudomonas sp***.** DSMZ 13134 (Proradix ®) was inoculated in soil around NH_4^+-depots. Another study confirmed improved root colonization of maize by a rifampicin-tolerant strain of *Bacillus amyloliquefaciens* FZB42 after it was also inoculated in soil around an NH_4^+-depot (*Mohammad et al.,* 2016, unpublished master thesis). Although these results are promising, more specific analysis of PGPM root-colonization rates by molecular techniques like qPCR with selective primers will be of additional value.

Furthermore, spatial control of the rhizosphere for establishment of inoculated PGPMs should be tested further under field conditions.

8.3 P-solubilizing BEs and potential to contribute to improved plant P-acquisition

Several microbial BEs tested positively to solubilize sparingly soluble Ca-P and RP via acidification of growth media. The fungal BE *Trichoderma harzianum* T-22 that tested negative was still capable of solubilizing Ca-P, RP and sewage sludge ash, however, solubilized P was directly utilized for the growth of the fungus. This suggests that P-solubilizing effects of some PGPMs may only contribute to plant P uptake during turnover of microbial biomass in later growth stages. Consequently, it may be important to investigate medium- and long-term effects of soil inoculated PGPMs on plant nutrient acquisition and growth. This could be done in pot experiments by growing a series of crops in the same substrate as well as through rotations in the field.

In our field experiments, tested PGPMs could not contribute to improved P nutrition of maize plants primarily because in both years, the field site had optimal concentrations of plant-available P. The positive effect of *Pseudomonas sp***.** DSMZ 13134 (Proradix ®) on maize silage yield in 2015 may be attributed to PGPM-induced tolerance to prevalent heat and drought stress in 2015 and not a direct consequence of PGPM-assisted plant P-uptake. Increase in maize silage yield in 2015 may have also been the result of different interacting PGPM effects including root growth stimulation or root disease suppression. A current challenge with the regulation of the commercialization of microbial BEs is the requirement for them to be registered in one of various categories such bio-fertilizer, bio-stimulant, bio-control or bio-pesticide. This regulation is redundant because in reality, PGPMs promote plant growth via a combination of mechanisms. For example, *Pseudomonas sp***.** DSMZ 13134 (Proradix ®), which tested as a good P-solubilizing and root-growth stimulating PGPM under

low pressure of soil pathogens, is actually registered as a bio-control agent against various soil-borne fungal pathogens in potato production.

In pot experiments, several BEs especially bacterial BEs and consortia BE products containing both bacteria and fungi effectively improved P nutrition of maize and wheat supplied with sparingly soluble P fertilizers or without added P. Improved root growth and possible enhanced solubilization of sparingly soluble P-fertilizers (via acidification and release of chelating organic acids) were main mechanisms of action. The positive BE effect on P nutrition of maize occurred only on low P-sorbing substrates especially when N was supplied as stabilized NH_4^++DMPP and not when N was supplied as NO_3^-. The use of consortia of PGPMs has the advantage that PGPMs that are best adapted to the specific biotic and abiotic condition in soil survive and colonize roots whereas those that do not gradually die and disappear. In this way, crop roots and soil determine which PGPM in the consortium survives and interacts with roots and other soil microorganisms and which do not. Furthermore, through consortia BE products, plant growth can be improved through a variety of pathways by different PGPMs at the same time.

In the medium- and long-term, more greenhouse and field experiments are required to confirm the effectiveness of combining fertilizer placement and the inoculation of PGPMs to improve crop nutrient acquisition and yield. For such field studies however, a field site with a low available P level is required. This highlights the fact that arable soils in Germany are generally well supplied with P (84 % of tested arable land samples from 2007-2013 in Baden-Württemberg had optimum plant-available P level or higher, class “C - E”) likely due to decades of adequate or more-than-required P fertilization. On the contrary, grasslands are frequently insufficiently supplied with P (only 50% of tested grassland samples from 2007-2013 in Baden-Württemberg had optimum plant-available P level or higher, class “C - E”)) (LTZ Augustenberg, 2013). This shows that grasslands in Baden-Württemberg are in more

need for improved mobilization of sparingly available soil P pools for fodder production than arable lands. This creates an opportunity to test P-solubilizing PGPMs by inoculating them in grassland soil, which usually has higher root density for the establishment of inoculated PGPMs than arable land that is regularly ploughed and often left free of plants.

8.4 Conclusion

Through this work, it could be shown for the first time that fertilizer placement leads to improved crop nutrient acquisition and higher yield than conventional fertilizer application by broadcast. This research has shown that the combination of placed NH_4^+-based fertilizers and inoculation of soil with P-solubilizing microorganisms may be a new strategy for improving nutrient acquisition by plants. Inoculation of PGPMs in an NH_4^+-fertilized substrate improves P acquisition of crop plants not only from sparingly available soil pools but also from sparingly soluble P fertilizers such as sewage sludge ash that is recycled from sewage treatment. In P-limited systems, improved P nutrition during early growth-stages ensures improved biomass production and yield.

8.5 References

Blackshaw, R. E., Semach, G., Janzen, H. H. (2002): Fertilizer application method affects nitrogen uptake in weeds and wheat. *Weed Sci.* 50, 634–641.

IPNI, (2016): Meta-analysis of phosphorus fertilizer placement and tillage interaction for corn and soybean in the U.S., 2015 Annual Interpretive Summary, International Plant Nutrition Institute, IPNI-2014-USA-4RM09. Study was funded by the 4R Research Fund of the North American 4R nutrient stewardship. Obtained from http://research.ipni.net/page/RNAP-6561 on 22.10.2016

Jing, J., Zhang, F., Rengel, Z., Shen, J. (2012): Localized fertilization with P plus N elicits an ammonium-dependent enhancement of maize root growth and nutrient uptake. *Field Crop. Res.* 133, 176–185.

Légère, A., Shirtliffe, S. J., Vanasse, A., Gulden, R. H. (2013): Extreme grain-based cropping systems: when herbicide-free weed management meets conservation tillage in northern climates. *Weed Technol.* 27, 204–211.

Linquist, B. A., Adviento-Borbe, M. A., Pittelkow, C. M., van Kessel, C., van Groenigen, K. J. (2012): Fertilizer management practices and greenhouse gas emissions from rice systems: A quantitative review and analysis. *Field Crop. Res.*135, 10–21.

LTZ Augustenberg, (2013): Grundnährstoffversorgung in Baden-Württemberg. Jahresbericht 2013. Landwirtschaftliches Technologiezentrum Augustenberg. Dowönaloaded from http://www.ltz-bw.de/pb/,Lde/1930632 on 25 October 2016.

Ma, B. L., Wu, T. Y., Tremblay, N., Deen, W., McLaughlin, N. B., Morrison, M. J., Stewart, G. (2010): On-farm assessment of the amount and timing of nitrogen fertilizer on ammonia volatilization. *Agron J* 102(1):134–144.

Ma, Q., Rengel, Z., Rose, T. (2009): The effectiveness of deep placement of fertilisers is determined by crop species and edaphic conditions in Mediterranean-type environments: a review. *Aust. J. Soil Res.* 47, 19–32.

Nash, P. R., Motavalli, P. P., Nelson, K. A. (2012): Nitrous oxide emissions from claypan soils due to nitrogen fertilizer source and tillage/fertilizer placement practices. *Soil Sci. Soc. Am. J.* 76, 983–993.

Miller, M. H., Ohlrogge, A. J. (1958): Principles of nutrient uptake from fertilizer bands 1. Effect of placement of nitrogen fertilizer on the uptake of band-placed phosphorus at different soil phosphorus levels1. *Agron. J.* 50, 95–97.

Parry, M. L., Rosenzweig, C., Iglesias, A., Livermore, M., Fischer, G. (2004): Effects of climate change on global food production under SRES emissions and socio-economic scenarios. *Climate Change* 14, 53–67.

Ruidisch, M., Bartsch, S., Kettering, J., Huwe, B., Frei, S. (2013): The effect of fertilizer best management practices on nitrate leaching in a plastic mulched ridge cultivation system. *Agr. Ecosyst. Environ.* 169, 21–32.

Singh, D. K., Sale, P. W. G., Routley, R. R. (2005): Increasing phosphorus supply in subsurface soil in northern Australia: Rationale for deep placement and the effects with various crops. *Plant Soil* 269, 35–44.

Sommer, K. (2005): CULTAN-Düngung: physiologisch, ökologisch, ökonomisch optimiertes Düngungsverfahren für Ackerkulturen, Grünland, Gemüse, Zierpflanzen und Obstgehölze. Verlag Th. Mann, Gelsenkirchen-Buer, Germany.

9 List of publications and presentations

9.1 Peer-reviewed publications

Nkebiwe, P. M., Weinmann, M., Bar-Tal, A., Müller, T. (2016): Fertilizer placement to improve crop nutrient acquisition and yield: A review and meta-analysis. *Field Crops Research* 196, 389–401 DOI: http://dx.doi.org/10.1016/j.fcr.2016.07.018

Nkebiwe, P. M., Weinmann, M., Müller, T. (2016): Improving fertilizer-depot exploitation and maize growth by inoculation with plant growth-promoting bacteria: from lab to field. *Chemical and Biological Technologies in Agriculture* 3 (1):15 DOI**:** http://dx.doi.org/10.1186/s40538-016-0065-5

Nkebiwe, P. M., Neumann G. Müller, T. (2017). Densely rooted rhizosphere hotspots induced around subsurface NH_4^+-fertilizer depots: a home for soil PGPMs? *Chemical and Biological Technologies in Agriculture* DOI: 10.1186/s40538-017-0111-y

9.2 Conference proceedings

Nkebiwe, P. M., Weinmann, M., Weber, N., Neumann, G., & Müller, T. (2015): Placement of Pseudomonas sp. DSMZ13134 around NH_4^+-based fertilizer depots in maize stimulates root exploitation of the fertilizer depot in soil. *DBGPrints Repository* (Publications of the German Soil Science Society). Proceedings from annual conference of the German Soil Science Society, "Unsere Böden - Unser Leben", 5.-10.09.2015, Münich, Germany

9.3 Oral Presentations (15-25 minutes)

Nkebiwe, P. M., Weinmann, M., Bar-Tal, A., Müller, T. (2016): **P and N placement for improved nutrient acquisition and yield: A meta-analysis.** *8th International Phosphorus Workshop (IPW8). Theme: Phosphorus 2020 – Challenges for Synthesis, Agriculture, and Ecosystems. 12 – 16 September 2016, Science Campus, Phosphorus Research, Rostock, Germany*

Nkebiwe, P. M., Weinmann, M., Bar-Tal, A., Müller, T. (2016): **P and N placement for improved nutrient acquisition and yield: A meta-analysis.** *Plant Nutrition 2016, International Conference of the German Society for Plant Nutrition – DGP (Annual Meeting. Theme: Resource efficiency: from model plants to crops and crop systems. 28 – 30 September 2016, University of Hohenheim, Stuttgart, Germany*

9.4 Poster Presentations

Nkebiwe P. M., Käfer N., Neumann G., Müller T., (2016): **BE-assisted P nutrition of wheat supplied with rock phosphate and placed NH_4^+.** *5th General Assembly Meeting, Biofector Project. (EC Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 312117). 21- 23 September 2016, Czech University of Life Sciences,Prague, Czech Republic*

Nkebiwe, P. M., Weinmann, M., Weber, N., Neumann, G., & Müller, T. (2015): **Placement of *Pseudomonas sp.* PRORADIX around NH_4^+-based fertilizer depots in maize stimulates root exploitation of the fertilizer depot in soil**. *International symposium miCROPe 2015, Theme: Microbe-assisted crop production – opportunities, challenges*

and needs. 23- 25 November 2015, Schlosschönbrunn, Vienna, Austria ***(4 min. poster talk and poster presentation)***

Nkebiwe P. M., Weinmann M., Bar-Tal A., Müller T., (2015): **Fertilizer placement to improve crop nutrient acquisition and yield: a meta-analysis.** ***4**th* *General Assembly Meeting, Biofector Project. (EC Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 312117). 30 September – 02 October 2015, Danubius-Hotel-Flamenco, Budapest, Hungary*

Nkebiwe P. M., Weber N. F., Weinmann M., Müller T., Neumann G., (2015): **NH_4^+ placement and BE application technique affect root-growth in fertilizer depots and root-colonization by BE in field-grown maize.** ***4**th* *General Assembly Meeting, Biofector Project. (EC Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 312117). 30 September – 02 October 2015, Danubius-Hotel-Flamenco, Budapest, Hungary*

Nkebiwe P. M., Weinmann M., Weber N. F., Neumann G., Müller T., (2015): **Placement of *Pseudomonas sp.* around NH_4^+-based fertilizer depots in maize culture stimulates root exploitation of the fertilizer depot.** *Plant Nutrition 2015, International Conference of the German Society for Plant Nutrition – DGP (Annual Meeting). Theme: "Boden, Nährstoffe, Wasser – Forschung für die nachhaltige und effiziente Nutzung von Ressourcen". 17 – 18 September 2015, Georg-August-Universität, Göttingen, Germany* **(3rd Poster prize)**

Nkebiwe, P. M., Weinmann, M., Weber, N., Neumann, G., & Müller, T. (2015): **Placement of *Pseudomonas sp.* PRORADIX around NH_4^+-based fertilizer depots in maize stimulates root exploitation of the fertilizer depot in soil**. *Annual conference of the German Soil Science Society, "Unsere Böden - Unser Leben", 5.-10 September 2015, Münich, Germany*

Nkebiwe P. M., Weinmann M., Weber N. F., Neumann G., Müller T., (2015): **Placement of Proradix around NH_4^+-based fertilizer depots in maize culture stimulates root exploitation of the fertilizer depot.** *Rhizosphere4 conference. Theme: Stretching the interface of life. 21-25 June 2015, Maastricht Exposition and Congress Centre Maastricht, the Netherlands*

Nkebiwe P. M., Weinmann M., Weber N. F., Neumann G., Müller T., (2015): **Placement of Proradix (BE 2) around NH_4^+-based fertilizer depots in maize culture stimulates.** *3rd General Assembly Meeting, Biofector Project. (EC Seventh Framework Programme (FP7/2007-2013) under grant agreement n° 312117). 08 - 10 October 2014, Hotel Cocumella, Sorrento, Italy*

Nkebiwe P. M., Weinmann M., Neumann G., Müller T. (2014): **Stimulation of localized root growth by enhanced establishment of placed Plant Growth-Promoting Microorganisms (PGPMs) in soil?** *Plant Nutrition 2014, International Conference of the German Society for Plant Nutrition – DGP (Annual Meeting). Theme: From Basic Understanding to Better Crops. 10 – 12 September 2014, Martin Luther University, Halle (Saale), Germany*

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11 Curriculum Vitae

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Doctoral Studies **09.2012 – present**	Fertilization and Soil Matter Dynamics (340i), University of Hohenheim Dissertation: "Fertilizer placement and the potential for its combination with bio-effectors to improve crop nutrient acquisition and yield" (Sept. 2012 – Nov. 2016)
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02.2009 – 08.2009	**Office assistant (voluntary)** • Correspondence / Registration / Receptionist Bosch Baha'i School: a Conference and Retreat Center, Santa Cruz, CA., U.S.A.
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12 Eidesstattliche Versicherung

Eidesstattliche Versicherung gemäß § 8 Absatz 2 der Promotionsordnung der

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1. Bei der eingereichten Dissertation zum Thema

Fertilizer placement and the potential for its combination with bio-effectors to improve crop nutrient acquisition and yield

handelt es sich um meine eigenständig erbrachte Leistung.

2. Ich habe nur die angegebenen Quellen und Hilfsmittel benutzt und mich keiner unzulässigen Hilfe Dritter bedient. Insbesondere habe ich wörtlich oder sinngemäß aus anderen Werken übernommene Inhalte als solche kenntlich gemacht.

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4. Die Bedeutung der eidesstattlichen Versicherung und der strafrechtlichen Folgen einer unrichtigen oder unvollständigen eidesstattlichen Versicherung sind mir bekannt.

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Stuttgart, 26 Oktober 2016 **Peteh Mehdi Nkebiwe**

Ort und Datum Unterschrift