UNIVERSITY OF HOHENHEIM

Faculty of Agricultural Sciences

Institute of Crop Sciences (340h)

Prof. Dr Günter Neumann

Fertilization Strategies to Improve the Plant Growth-Promoting Potential of Microbial Bio-Effectors.

Dissertation

Submitted in fulfilment of the requirements for the degree of
"Doktor der Agrarwissenschaften"
(Dr. sc. agr./Ph.D. in Agricultural Sciences)
to the
Faculty of Agricultural Sciences presented
by

Isaac Kwadwo Mpanga

Born in Atebubu, Ghana

2019

This thesis was accepted as a doctoral dissertation in fulfilment of the requirements for the degree “Doktor der Agrarwissenschaften” (Dr. sc. agr. / PhD in Agricultural Sciences) by the faculty Agricultural Sciences of the University of Hohenheim on November 22nd 2019.

Date of oral examination: November 22nd 2019.

Examination Committee

Supervisor and Reviewer: Professor Dr Gunter Neumann

External Reviewer: Professor Dr Pavel Tlustoš

Additional Examiner: Professor Dr Torsten Mueller

Head of the Committee: Professor Dr Gunter Neumann

TABLE OF CONTENT

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

Aluminium (Al)

Ammonium (NH_4)

Bio-effectors/Biofertilizers (BE's)

Calcium (Ca)

Combifector A (CFA)

Combifector B (CFB)

European Union (EU)

Hydrogen (H)

Indole-3-acetic acid (IAA)

Integrated Plant Nutrient Management (IPNM)

Integrated Plant Nutrient Supply (IPNS)

Iron (Fe)

Nitrate (NO_3)

Nitrogen (N)

No Phosphorus (No P)

Phosphorus (P)

Phosphorus Solubilizing Microorganisms (PSMs)

Plant Growth Promoting Microorganisms (PGPM)

Plant Growth Promoting Rhizobacteria (PGPR)

PM (Poultry manure)

Positive control (P Ctrl)

Pseudomonas sp. DSMZ13134 (Px)

Rock Phosphate (RP)

Sewage sludge ash (SSA)

Triple Super Phosphate (TSP)

Ultra Micro Granule (UMG)

United States Dollar (USD)

1 SUMMARY

The use of plant growth-promoting microorganisms (PGPMs) as inoculants to support nutrient acquisition of crops is discussed as a promising strategy for improving fertilizer use efficiency, to enable crop production with less input of fertilizers, and to reduce detrimental environmental side effects related with high inputs of mineral fertilizers. However, the efficiency of PGPM-assisted cropping systems is still biased by the limited reproducibility of the expected effects under real production conditions. This can be attributed to the sensitivity of plant-PGPM interactions to environmental stress factors particularly during the phase of establishment and to limited knowledge on positive or negative interactions with the native soil microbiome and the application conditions required for successful rhizosphere colonization as a pre-requisite for beneficial plant PGPM interactions.

This study demonstrated that the combination with compatible fertilizers offers an option to promote the establishment of PGPM effects as a potential management option to improve the performance of PGPM-assisted production strategies. In a range of model experiments with maize with a limited inherent potential for root-induced P-solubilization, it was demonstrated that the acquisition of sparingly soluble Ca-phosphates could be synergistically improved by a combination of PGPM inoculants with ammonium fertilizers, stabilized with nitrification inhibitors (Chapter 4). The effect was demonstrated for PGPMs based on 15 different fungal (genus: *Trichoderma, Penicillium)* and bacterial (genus: *Bacillus*, Paenibacillus, *Pseudomonas*, *Streptomyces*) strains and strain combinations, which were largely ineffective in combination with nitrate fertilization. On average over all experiments, the PGPM-ammonium combinations with sparingly soluble Ca-P supply reached about 84% of the shoot biomass production and 80% of the shoot P accumulation as compared with positive controls fertilized with soluble P. The soil pH-buffering capacity, particularly on neutral to alkaline soils, was identified as a

limiting factor, counteracting the plant growth-promoting potential of the selected inoculants with a proven ability for Ca-P solubilization on artificial growth media. Accordingly, plants supplied with nitrate fertilization were severely P deficient and the weak host plants were unable to establish a functional association with the microbial inoculants. By contrast, stabilized ammonium fertilization triggered root extrusion of protons for charge balance of ammonium uptake, associated with rhizosphere acidification, contributing to P solubilization. This increased the P-nutritional status and vitality of the host plants, which enabled the establishment of PGPMs in the rhizosphere. Interestingly in this scenario, the contribution of the PGPM inoculants to plant P acquisition was only marginally expressed but the PGPMs stimulated root development, contributing to an improved nutrient acquisition in general (Chapter 4.1).

A closer look on the related modes of action (Chapter 4.2) revealed that ammonium fertilization stimulated the production of auxin as a key regulator for root growth, both, by the bacterial inoculants and by the roots of the host plants. While ammonium supply without PGPM inoculants had no effects on total root length, the length of the root hairs and the diameter of rhizosheaths formed by root hair-adhering soil was increased, leading to an extension of the root surface area involved in rhizosphere acidification and spatial acquisition of nutrients. Moreover, root hairs have been reported as preferential infection sites for various inoculants investigated in this study, and accordingly increased root colonization of the fungal inoculant *Trichoderma harzianum* OMG16 was recorded in combination with ammonium fertilization. By contrast, there was no evidence for increased organic acid production or a contribution of the inoculants to the acquisition of organic P sources by the release of phosphohydrolases in the investigated strains. Increased rhizosphere acidification after PGPM inoculation in combination with ammonium fertilization was observed exceptionally only in one experiment conducted on

a moderately acidic sandy soil with a low buffering capacity. However, soil pH was identified as a critical factor determining the expression of the synergistic PGPM-ammonium effects on Ca-P solubilization, which declined with increasing soil pH (Chapter 4.3). Highly-buffered calcareous soils counteracted ammonium-induced rhizosphere acidification and P mobilization as a pre-requisite for PGPM-establishment in the rhizosphere. Under these conditions, successful experiments with applications of granulated fertilizers, based on stabilized di-ammonium phosphate and PGPM inoculants, suggest that placement of starter fertilizers leading to a more concentrated ammonium effect may offer an option to overcome this problem. First field experiments suggested that beneficial effects of ammonium-assisted PGPM inoculation on P acquisition can be expected particularly on soils with low P availability and the approach was patented in 2018.

As a second approach, the combination of PGPMs with fertilizers based on products of organic waste recycling, such as municipal waste compost or composted poultry manure (PM compost), applied with the same P dose, were investigated with tomato as model plant on low P soils with contrasting pH in Ghana (Chapter 5). Interestingly, on both soils, PGPM inoculation increased the P use efficiency and early plant growth only in the combination of compost with PM but not with sole compost application. Additional supplementation with ammonium on the moderately acidic soil increased plant biomass production in PGPM inoculated plants to the same level as soluble superphosphate fertilization. Similar to the ammonium-PGPM combinations, root growth stimulation was a major PGPM effect, which improved nutrient acquisition in general. Large-scale greenhouse and open-field tomato production trials conducted in Romania and Hungary revealed reproducible effects on yield and fruit quality over three years by PGPM combinations with manure-based fertilizers (Chapter 6).

Taken together, the thesis demonstrated that the selection of compatible combinations of fertilizers and PGPM inoculants is an essential factor for the successful establishment of beneficial plant-PGPM interactions in the rhizosphere. Combinations with stabilized ammonium fertilizers or with products based on organic waste recycling, such as composted manures, have been identified as two promising examples with potential for the development of PGPM-assisted production systems.

2 ZUSAMMENFASSUNG

Der Einsatz pflanzenwachstums-stimulierender Mikroorganismen zur Unterstützung der Nährstoffaneignung von Kulturpflanzen wird als vielversprechender Ansatz diskutiert, deren Nährstoffaneignungseffizienz zu verbessern, um den Düngemitteleinsatz zu reduzieren und schädliche Umweltwirkungen in Verbindung mit intensivem Mineraldüngereinsatz zu minimieren. Allerdings ist die erfolgreiche Anwendung von PGPMs unter Praxisbedingungen nach wie vor durch mangelnde Reproduzierbarkeit der erwarteten Effekte limitiert. Dies kann zum einen auf eine hohe Sensitivität pflanzlicher PGPM-Wechselwirkungen gegenüber umweltbedingten Stressfaktoren, besonders in der sensiblen Etablierungsphase, zurückgeführt werden, aber auch auf einen noch unzureichenden Kenntnisstand im Hinblick auf förderliche oder hemmende Wechselwirkungen mit dem nativen Bodenmikrobiom, die Wurzelexsudation verschiedener Wirtspflanzenarten und die Anwendungsbedingungen, welche eine erfolgreiche Besiedelung der Rhizosphäre begünstigen, als Voraussetzung für die Etablierung pflanzenwachstums-fördernder PGPM-Wechselwirkungen.

Die vorliegende Untersuchung hat gezeigt, dass die Auswahl kompatibler PGPM-Düngerkombinationen offensichtlich Möglichkeiten bietet, die Etablierung von PGPM Effekten zu unterstützen, als mögliche Managementoption zur Optimierung PGPM-basierter Anbausysteme. Im Rahmen verschiedener Modellversuche mit Mais als Kulturpflanzenart mit limitiertem Potenzial zur wurzelinduzierten Phosphatmobilisierung, konnte gezeigt werden, dass die Aneignung schwerlöslicher Ca-Phosphate (Ca-P) in synergistischer Weise durch die kombinierte Anwendung von PGPMs mit stabilisierten Ammoniumdüngern verbessert werden konnte (Kapitel 4). Dieser Effekt konnte für PGPM Produkte auf der Basis von 15 Stämmen pilzlicher (Gattung: *Trichoderma, Penicillium*) und bakterieller (Gattung: *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Streptomyces*) Herkunft bestätigt werden, die sich dagegen in Kombination mit

Nitratdüngern als weitgehend ineffektiv erwiesen. Im Durchschnitt über alle Experimente erreichten die PGPM-Ammoniumkombinationen mit Applikation schwerlöslicher Ca-Phosphate etwa 84% der Sprossbiomasse und 80% der Spross P-Gehalte im Vergleich zu Positivkontrollen mit löslichen P Düngern. Die pH-Pufferungskapazität, besonders bei neutralen bis alkalischen Böden, wurde als limitierender Faktor identifiziert, der das pflanzenwachstums-fördernde Potenzial der ausgewählten Inokulanzien begrenzte, obwohl in allen Fällen die Lösung schwerlöslicher Ca-Phosphate auf artifiziellen Kulturmedien nachweisbar war. Wirtspflanzen mit Nitratdüngung entwickelten entsprechend deutlichen P-Mangel, und die so geschwächten Pflanzen waren offensichtlich nicht in der Lage, eine PGPM- Assoziation in der Rhizosphäre zu etablieren. Durch die stabilisierte Ammoniumdüngung wurde dagegen die wurzelinduzierte Protonenabgabe zum Ladungsausgleich der Ammoniumaufnahme stimuliert, was eine Ansäuerung der Rhizosphäre, verbunden mit der Mobilisierung schwerlöslicher Ca-Phosphate zur Folge hatte und so den P-Ernährungsstatus und damit die Vitalität der Wirtspflanzen verbesserte, die dadurch in der Lage waren, die Wurzelbesiedelung durch PGPMs zu unterstützen. Interessanterweise war dabei der direkte Beitrag der PGPMs zur P-Mobilisierung nur gering ausgeprägt. Die PGPM Inokulation führte dagegen hauptsächlich zu einer Stimulierung des Wurzelwachstums, was die Nährstoffaufnahme im Allgemeinen begünstigte (Kapitel 4.1).

Ein genauerer Blick auf die beteiligten Wirkmechanismen (Kapitel 4.2) ergab eine Ammonium-induzierte Förderung der Auxinproduktion, als hormonellen Hauptfaktor für die Regulation des Wurzelwachstums, sowohl bei den bakteriellen Inokulanzien, als auch bei der Wirtspflanze. Während die Ammoniumdüngung bei den Pflanzen ohne PGPM Inokulation jedoch keinen Effekt auf die Wurzellängenentwicklung hatte, stimulierte sie doch das Längenwachstum der Wurzelhaare und die Bildung von Wurzelscheiden aus anhaftenden Bodenpartikeln, was zu

einer Vergrößerung der Wurzeloberfläche führte, die an der Ammonium-induzierten Ansäuerung der Rhizosphäre beteiligt war und eine räumliche Erweiterung der Rhizosphäre für die Nährstoffaneignung bedingte. Darüber hinaus wurden Wurzelhaare als präferenzielle Infektionsorte für zahlreiche der untersuchten PGPMs identifiziert und entsprechend wurde z.B. bei der Inokulation mit *Trichoderma harzianum* OMG16 eine verbesserte Wurzelbesiedelung unter Ammoniumdüngung festgestellt.

Im Gegensatz dazu gab es keine Hinweise auf eine verstärkte Abgabe organischer Säuren oder einen Beitrag der untersuchten PGPM-Inokulanzien zur Aneignung organischer P Formen über die Abgabe von Phosphohydrolasen. Eine verstärkte Ansäuerung der Rhizosphäre nach PGPM-Inokulation in Kombination mit Ammoniumdüngung wurde nur in einem Ausnahmefall auf einem leicht sauren Sandboden mit geringer Pufferkapazität nachgewiesen. Jedoch wurde der Boden pH-Wert als kritischer Faktor identifiziert, der die Ausprägung der synergistischen Wirkung der PGPM-Ammonium-Kombinationen beeinflusste, deren Ausprägung mit ansteigendem Boden-pH abnahm (Kapitel 4.3). Stark gepufferte Kalkböden wirkten dabei der Ammonium-induzierten Ansäuerung der Rhizosphäre zur Verbesserung der P-Aneignung entgegen, die eine Voraussetzung für eine Etablierung der PGPM-Interaktionen darstellte. Unter diesen Bedingungen zeigten erfolgreiche Experimente mit der Applikation stabilisierter Diammoniumphosphat-Granulate mit PPGP-Inokulation einen möglichen Ansatz, diesem Problem durch platzierte Applikation als Starterdüngung zu begegnen, die einen konzentrierteren Ammoniumeffekt begünstigt. Erste Feldversuche weisen darauf hin, dass Ammonium-PGPM Kombinationen besonders auf Böden mit limitierter P Verfügbarkeit zur Aneignung schwerlöslicher Ca-Phosphate wie z.B. Rohphosphat eingesetzt werden können (Kapitel 4.1 und 6), und die Anwendung wurde im Jahr 2018 patentiert.

Als weiterer Ansatz wurde die Kombination von PGPMs mit Düngern auf Basis von Recyclingprodukten organischer Abfälle, wie Haushaltskompost und kompostiertem Geflügelmist (PM-Kompost) untersucht, die mit derselben P Dosierung appliziert wurden. Tomate wurde hier als Modellpflanze auf P armen Böden mit unterschiedlichen pH Werten in Gewächshausversuchen in Ghana verwendet (Kapitel 5). Interessanterweise verbesserte die PGPM-Inokulation die P-Nutzungseffizienz und das Pflanzenwachstum auf beiden Böden ausschließlich in der Kombination von Haushaltskompost mit PM-Kompost, aber nicht bei alleiniger Verwendung von Haushaltskompost. Auf dem moderat-sauren Sandboden (pH 5.6) führte die zusätzliche Verwendung von stabilisiertem Ammonium zu einer erhöhten Biomasseproduktion, die mit der Verwendung löslicher Superphosphatdüngung vergleichbar war. Ähnlich wie bei den Versuchen mit PGPM-Ammonium-Kombinationen wurde durch Stimulierung des Wurzelwachstums die Nährstoffaneignung im Allgemeinen gefördert. Versuche zur Gewächshauskultur und zum Feldanbau von Tomaten in Rumänien und Ungarn ergaben über drei Jahre signifikant reproduzierbare PGPM Effekte im Hinblick auf Ertragsbildung und Fruchtqualität in Kombination mit organischen Düngern auf Stallmist-, Guano-, Haar-, Feder-, und Fleischmehlbasis (Kapitel 6).

Zusammenfassend hat die vorliegende Arbeit gezeigt, dass die Auswahl kompatibler Düngemittel-PGPM-Kombinationen essentiell für die erfolgreiche Etablierung pflanzen-wachstumsfördernder PGPM-Interaktionen in der Rhizosphäre ist. PGPM-Kombinationen mit stabilisierten Ammoniumdüngern oder mit Düngern auf Basis organischer Abfallprodukte, wie z.B. Stallmistkompost wurden als zwei aussichtsreiche Beispiele mit Potenzial zur Entwicklung PGPM-unterstützter Produktionssysteme identifiziert.

3 GENERAL INTRODUCTION

Agricultural crop production plays a pivotal role in the supply of food, fibre and shelter at a global scale. It is the main source of nutrient supply for the growing population but unfortunately, it is increasingly threatened by climate variability. Consequently, arable land as a natural resource is under more intensive use than ever before, requiring high inputs of agrochemicals at levels that are not healthy for the environment anymore. This applies not only to intensive use of pesticides but also to mineral fertilizers containing essential plant nutrients, especially nitrogen and phosphorus. Limited use efficiency of crops and the challenge to adapt fertilizer inputs to the actual plant demands is still associated with unwanted nutrient losses into the environment. Eutrophication of water bodies and natural ecosystems, greenhouse gas emissions and wasting of energy and limited natural resources are among the most prominent consequences. Because of these damaging effects, many regions are introducing legislation to reduce the use of mineral fertilisers (Neumann et al., 2017).

Proposed strategies to meet these requirements comprise approaches of nutrient saving by use of fertilizers based on organic and inorganic waste-recycling products (Kirchmann et al., 2005; Stofella et al., 2014), as well as improved plant nutrient acquisition by fertilizer placement strategies close to the roots (Nkebiwe et al 2016a), exploiting the genetic potential of crops to increase nutrient use efficiency (Bonser et al. , 1996; Gahoonia and Nielsen 2004; Campos et al., 2018), and also the assistance of microbial inoculants with plant growth-promoting properties (PGPMs). Within this context, the present thesis aimed at optimising PGPM-assisted strategies to improve P acquisition of important crops (maize, tomato). It was hypothesized that compatible PGPM-fertilizer combinations are important determinants for the efficiency of plant-PGPM interactions, thereby offering management options for practical applications.

3.1 The role of Phosphorus (P) in Agricultural Production

In agricultural crop production, Phosphorus (P) is the second most important essential macronutrient after nitrogen (N). However, challenges for P fertilization, range from its limitation as a natural resource to strong fixation at the soil matrix, leading to P limitations in about 40% of world's arable lands (Vance, 2001). Apart from P limitation, another issue is the rapid immobilization of fertilizer P, leading to a situation that about 60% of applied fertilizer P is not available for plant uptake (Barrow 1980). This is due to fixation by Aluminium (Al) and Iron (Fe) in acidic soils and Calcium (Ca) in alkaline soils (Norrish and Rosser, 1983; Lindsay et al., 1989; Hinsinger, 2001; Rengel & Marschner, 2005). On average, only about 1 kg P ha^{-1} is readily available in the soil solution for plant uptake, about 600 kg P ha^{-1} is considered as labile or organic P (partly plant-available) and approximately 1800 kg P ha^{-1} is present as recalcitrant P (unavailable for plant uptake) (Hinsinger, 2001; Frossard, e., et al., 2000; Mengel. and Kirkby, 1987; Ozanne, 1980; Raghothama, 1999).

The utilization efficiency of the applied chemical P fertilizers rarely exceeds 30%, associated with high application rates to maintain yield stability. This bears the risk of over-fertilization and increases production costs. Run-off P contamination by erosion contributes to eutrophication of surface waters and finally leads to irreversible losses of P as a limited natural resource in the sediments of oceans (Sharma et al., 2013). According to Khan et al. (2009), if the accumulated P in soils due to fixation could somehow be made available to plants, this could sustain maximum world crop production for hundreds of years. Although this kind of P mining would definitely not represent a sustainable approach, strategies to counteract P fixation in soils could contribute to more efficient use of fertilizer P.

Therefore, there is an urgent need for prudent and efficient P management of already fixed P in our crop fields and the rock-phosphate reserves available to produce P fertilizers. In recent

reports, the longevity of rock phosphate reserves available for mining has been approximated to 300 years and over 1,400 years of resources (Van Kauwenbergh, 2013). The sustainable use of these identified P reserves for agriculture crop production is the way forward to meet global food demands and food security. Therefore, the call for efficient use of rock phosphate and other P sources ecologically and economically for us and the future generations, is urgently required (Sharma et al., 2013). This includes also strategies to recycle P in the ecosystem, such as: composting of organic waste materials, the use of sewage sludge, poultry and farm-yard manure. However, these P sources are frequently not consistent in their effect since various nutrients from them are not readily available for plant uptake, which is a disincentive to organic farmers regarding crop yield. Therefore, organic fertilizer application adapted to crop requirements is even more challenging as compared with conventional farming systems and the risk of unwanted nutrient losses into the environment can be even higher Moreover, similar to mineral fertilizers also fertilizers based on products of waste recycling are a potential source of problematic contaminants, such as heavy metals but also antibiotics, antibiotic resistance genes, or pathogenic microorganisms (Stofella et al., 2014; Zhou et al, 2017).

Though the organic farming practice has many benefits to the ecosystem, a yield gap of 25 – 30% as compared with the conventional farming is undisputable (Badgley & Perfecto, 2007), leading to a continually increased discrimination between the two systems without a mid-win. With the current climate variability, adopting alternative fertilization strategies such as the integrated plant nutrient management (IPNM) reported by IFA (2018) or integrated plant nutrient systems (IPNS) proposed by Debarup, et al. (2015) could be a win-win for the two systems users and the environment for food security. The IPNM or IPNS is the integrated application of organic and mineral fertilizers to help achieve the desired crop production yield by optimizing plant nutrition through soil fertility management using all possible available

resources (Debarup, et al., 2015), which could include also a more targeted integration of beneficial soil microbes.

3.2 Beneficial soil microbes and their role in agriculture.

Plant-microbial interactions are essential for plant nutrient availability and nutrient cycling in ecosystems and are also determinants for the biotic and abiotic stress tolerance of higher plants.

Accordingly, the targeted use of beneficial soil microbes, termed as plant growth-promoting microorganisms (PGPMs), has been discussed as a strategy to improve agricultural production systems (Abhilash, et al., 2016; Chauhana, et. al., 2015) and Phosphorus solubilizing Microorganisms (PSMs) (Oliveira et al., 2009; Alori, 2017).

Plant Growth-Promoting Microorganisms may positively influence plants in two ways-direct and indirect mechanisms. Directly, PGPMs may stimulate plant growth by; enhancing interactions with plant-hormonal balances, mediate atmospheric N_2 fixation, solubilizing inorganic phosphate and mineralizing organic phosphate into available forms for plant uptake (Berg, 2009; Kurepin, et al, 2014; Bhattacharyya & Jha, 2012).

3.2.1 Mobilization of organic soil P

The composition of soil organic P can comprise 4-90 % of the total P in soil (Khan et al, 2009) and is at least partially made available to plants and microorganisms through a hydrolytic cleavage by either plants or microbial exo-phosphatase enzymatic activities (Tarafdar and Claassen 1988) with the largest portion of these exo-phosphatase, derived from soil microorganisms (Tabatabai, 1994; Tarafdar et al, 2001). Though acid phosphatases are generally thought to be released from plant roots and fungi (Tarafdar et al, 2001) while bacteria released alkaline phosphatase, other studies inconsistently associate acid phosphatase activities to bacteria (Singh and Satyanarayana 2011; Kim et al, 1997). These contradictions may

be explained by possible root growth promotion by the bacteria which may result in the accumulation of acid phosphatase released by the roots. Also, phytate is one of the abundant forms of organic P in the soil that is unavailable to the plant. However, *Bacillus amyloliquefaciens FZB45* and other PGPMs are reported as a phytase producer with the ability to liberate P from phytate for plant uptake (Singh and Satyanarayana 2011; Ramírez & Kloepper, 2010; Iddris et al 2002).

3.2.2 Mobilization of inorganic soil P

Organic acids production by PGPMs has a potential role in the mobilization of inorganic P sources especially under limited P stress conditions (Jones, 1998). In an experiment with aerobic rice, the combined application of organic acids (oxalic & malic) and phosphate solubilizing bacteria (*Bacillus sp*.) increased the solubility of rock P with increased bacteria population in the treatment with organic acids without affecting soil pH (Panhwar, et al., 2013). Though the organic acids were added externally, it still gives us a clue on the potential of inorganic P solubilization by PGPMs that may be able to produce adequate organic acids in the rhizosphere. A study by Wei et al., (2018) on Tricalcium Phosphate (TCP) solubilization by bacteria communities during composting demonstrated that bacteria inoculation affected pH, total acidity and the production of organic acids with a strong advantage for TCP solubilization and P availability. Moreover, some field studies have also reported increased soil P fractions in the fields upon PGPM inoculation (Saini et al 2004; Singh, Y V Gained S 2019). In an *in vitro* study, strains of *Pseudomonas sp* were reported as organic acids producers with significantly associated effects on pH, organic matter, N, P, and K content of the soil independent on their genetic relatedness. Each strain had its own ability of producing organic acids (gluconic acid, oxalic acid, 2-keto-gluconic acid, lactic acid, succinic acid, formic acid, citric acid and malic acid)

during the solubilization of inorganic phosphates (tricalcium phosphate, Mussoorie rock phosphate, Udaipur rock phosphate and North Carolina rock phosphate) (Vyas & Gulati, 2009). Taken together, organic acids production and phosphatase activities are thought to be the major mechanisms used by soil phosphate solubilizing microbes for inorganic and organic phosphorus solubilization (Rodríguez & Fraga, 1999). However, studies on P solubilization activities under real soil conditions requires more attention as the way forward.

3.2.3 Interactions with plant hormonal balances

Soil microbial production of phytohormones such as indole-3-acetic acid (IAA) and cytokinins on growth-promoting, plant cell elongation and division effects have been well documented (Ashard and Frankenberger Jr, 1991). Several strains of soil microbes have been reported as IAA producers (Ahemad and Kibret, 2013; Gupta, et al., 2002). The plant growth promotion associated to phytohormonal production by soil microbes could be a very important factor for plant nutrition. Especially for P, which is not mobile, induced root growth can extend into hot spots for uptake. Other direct plant growth-promoting interactions of PGPMs comprise the suppression of pathogens by mechanisms of hyperparasitism and production of antibiotic compounds (Harman 2000; Harman et al 2004; Ortíz-Castro, 2009). More interestingly, volatile organic compounds have been identified in recent years with importance for signalling between PGPMs and plants for communication, growth promotion and defence mechanisms (Kai et al, 2009; Ortíz-Castro, 2009).

3.2.4 Indirect PGPM effects

Indirectly, PGPMs may increase the resistance of plants against biotic and abiotic stress factors by strengthening plant-defence mechanisms via priming effects on systemic induced resistance including oxidative stress responses, and accumulation of stress-protective compounds such as

proline, phenolics, antioxidants and bacterial volatiles. (Chatterjee et al. 2019; Ryu et al., 2004; Harman et al 2004; Ortíz-Castro, 2009).

In other instances, the PGPMs could degrade 1-aminocyclopropane carboxylic acid (ACC) as a precursor of ethylene production through the production of ACC-deaminase. This is thought to counteract excessive ethylene production induced by abiotic and biotic stress factors with inhibitory effects on plant growth (Glick, 2005; Glick, 2014).

3.3 Limitations of Plant Growth Promoting Microorganisms (PGPMs)

Although significant beneficial effects of PGPM applications are documented and conceptual models for the modes of action, mainly based on model experiments, have been developed in recent past, the major problems for practical application of PGPM inoculants as bio-fertilizers are limited efficacy, quality and consistency in producing positive results (Lesueur, et al., 2016). Their functioning seems to be highly dependent on environmental factors, such as soil type, temperature, moisture, salinity, pH and climate conditions (Rodríguez & Fraga, 1999). Furthermore, application methods that are not easily integrated into the farmer's routine practice but require extra trips to the fields and specific machinery are representing another important challenge (Malusà et al., 2016). Although P-solubilizing activity can be easily demonstrated for many bacterial and fungal PGPM strains in pure culture on artificial growth media, under natural growth conditions, high cell densities critical for significant P mobilization with benefit to the host plant can be established in the rhizosphere only when PGPMs find an adequate niche and low competition from other microorganisms. While a community of arbuscular mycorrhizal (AM) fungi has been reported to support plant PGPM interactions synergistically either by co-inoculation (Kim et al 1997) or in combination with soil-indigenous mycorrhizal communities, according to Wu et al., (2005), combined application of bacterial inoculants and AM fungi stimulated AM root infection rate but contrastingly, the AM fungi

seemingly inhibited bacterial P solubilisation. This highlights the importance of the rhizosphere competence and root colonization potential of PGPM inoculants as determinants for PGPM efficiency.

Accordingly, many reports in the literature on PGPM application failed to demonstrate consistent beneficial effects in field and greenhouse trials (Menzies et al., 2011; Lekfeldt et al., 2016; Thonar et al., 2017) with very marginal positive effects in some cases.

3.4 Research scope and concept

The present research falls under the integrated EU project BIOFECTOR, located within the 7th EU framework program, mandated to investigate perspectives for applications of so-called bio-effectors, (PGPMs and non-microbial bio-stimulants) to increase fertilizer use efficiency and stress tolerance of important agricultural (maize, wheat) and horticultural crops (tomato). The addressed fertilization systems comprised organic farming, fertilizer placement and use of fertilizers based on organic and inorganic waste recycling products in strategic combinations with suitable microbial and non-microbial bio-effectors (BEs). (http://biofector.info/about-biofector.html).

In this study, fertilization strategies were tested using an IPNM approach similarly proposed by IFA (2018). The performance of PGPM combinations with stabilized ammonium fertilizers was evaluated, based on findings that 13 different microbial strains with proven P solubilizing potential used as inoculants for maize, wheat and barley, failed to show any improvement in plant P acquisition on low P soils or after supplying sparingly soluble P sources, such as rock P, ashes and slags (Lekfeldt et al., 2016; Thonar et al., 2017; BIOFECTOR Final Report 2017). It was hypothesized that the well-documented ammonium-induced rhizosphere acidification potential (Neumann and Römheld, 2002) by triggering H^+ extrusion from plant roots and microbial cells could be a management option to induce synergistic effects between plants and

PGPM inoculants for mobilization of acid-soluble mineral P sources, such as native Ca-phosphates, rock phosphates or recycling fertilizers based on ashes and slags. Additionally, PGPM combinations were tested also with different compost and manure-based organic fertilizers.

3.5 Research questions

This PhD research project aimed at testing the IPNM efficiency of PGPM inoculants in terms of plant growth promotion, nutrient acquisition and mobilization of sparingly soluble P sources in combination with selected inorganic and organic fertilizers commonly used in agricultural and horticultural production systems, with special emphasis on the form of N supply. The research questions addressed in this context comprise

(i) The efficiency of plant PGPM interactions under the influence of different N forms (nitrate versus ammonium supply)?

(ii) Which mechanisms (auxin production, root growth promotion, acid and alkaline phosphatase activities, modifications of rhizosphere pH, organic acid production) are involved in PGPM-assisted nutrient acquisition and improved plant growth?

(iii) How applicable are PGPMs within the IPNM approach for the acquisition of different sparingly soluble inorganic and organic P sources (Rock P and organic recycling compost fertilisers)?

(iv) How applicable are PGPMs within the IPNM approach on low P soils with contrasting pH?

(v) How applicable are PGPMs within the IPNM approach in different crop species (maize and tomato)?

(vi) How effective are PGPMs under field conditions within the IPNM approach?

The research questions were addressed using standardized pot experiments under controlled laboratory and greenhouse conditions with maize or tomato as model plants and in first field experiments, as well.

3.6 References

Abhilash, P. C., Dubey, R. K., Tripathi, V., Gupta, V. K., & Singh, H. B. (2016): Plant Growth-Promoting Microorganisms for Environmental Sustainability. *Trends in Biotechnology*.

Ahemad and Kibret, (2013): Mechanisms and applications of plant growth-promoting rhizobacteria: Current perspective. *Journal of King Saud University - Science* 26, 1-20

Alori, E. T., Glick, B. R., & Babalola, O. O. (2017): Microbial phosphorus solubilization and its potential for use in sustainable agriculture. *Frontiers in Microbiology*.

Ashard M, and Frankenberger Jr WT (1991): Microbial production of Hormones. Plant and Soil, 133,1-8.

Badgley, C., & Perfecto, I. (2007): Can organic agriculture feed the world? *Renewable Agriculture and Food Systems*, *22*(2), 80–85.

Barrow N. J. (1980): Evaluation and utilization of residual phosphorus in soils. *In* Fea Khasawneh, ed, The role of Phosphorus in Soils. American Society of Agronomy, Madison, WI, pp 335-355.

Berg, G. (2009): Plant-microbe interactions promoting plant growth and health: Perspectives for controlled use of microorganisms in agriculture. *Applied Microbiology and Biotechnology*. https://doi.org/10.1007/s00253-009-2092-7

Bhattacharyya, P. N., & Jha, D. K. (2012): Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. *World Journal of Microbiology and Biotechnology*.

BIOFECTOR final report (2017): Resource Preservation by Application of BIOefFECTORs in European Crop Production (https://cordis.europa.eu/project/rcn/104445/reporting/en/ / *Download date 20/04/2019 @ 12:00*

Bonser, A. M., Lynch, J., Snapp, S. (1996): Effect of phosphorus deficiency on growth angle of basal roots in Phaseolus vulgaris. *New Phytol.* 132, 281–288. doi: 10.1111/j.1469-8137.1996.tb01847.x

Campos, P, Borie, F, Cornejo, P, López-Ráez, J. A, López-García, Á, Seguel, A. (2018): Phosphorus Acquisition Efficiency Related to Root Traits: Is Mycorrhizal Symbiosis a Key Factor to Wheat and Barley Cropping? Front. Plant Sci. 9:752. doi: 10.3389/fpls.2018.00752

Chauhana, H., D.J. Bagyaraja, G. Selvakumarb, S. P. S. (2015): Novel plant growth-promoting rhizobacteria-Prospects and potential. *Applied Soil Ecology*, *95*, 38–53.

Chatterjee, P., Kanagendran, A., Samaddar, S. et al. Planta (2019): *Methylobacterium oryzae* CBMB20 influences photosynthetic traits, volatile emission and ethylene metabolism in *Oryza sativa* genotypes grown in salt stress conditions

Debarup Das, B.S. Dwivedi, M.C. Meena, V.K. Singh, K. N. T. (2015): Integrated Nutrient Management for Improving Soil Health and Crop Productivity. *Indian J. Fert.*, *Vol. 11*(November), pp.64-83.

Frossard, E., Condron, L. M., Oberson, A., Sinaj, S., & Fardeau, J. C. (2000): Processes Governing Phosphorus Availability in Temperate Soils. *J. Environ. Qual.*, *29*, 15–29.

G*ahoonia, T. S, Nielsen N. E.* (2004): Barley genotypes with long root hairs sustain high grain yields in low-P field. *Plant Soil* 262: 55–62

Grand View Research. (2015): Biofertilizers Market Size | Industry Analysis Report, 2022. http://www.grandviewresearch.com/industry-analysis/biofertilizers-industry, ret date 02/03/2018.

Glick, B. R. (2005): Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. *FEMS Microbiology Letters*, *251*(1), 1–7. https://doi.org/10.1016/j.femsle.2005.07.030

Glick, B. R. (2014): Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiological Research*, *169*(1), 30–39.

Gupta, A., Meyer, J. M., & Goel, R. (2002): Development of heavy metal-resistant mutants of phosphate solubilizing Pseudomonas sp. NBRI 4014 and their characterization. *Current Microbiology*. https://doi.org/10.1007/s00284-002-3762-1

Harman, G. E. (2000): Myths and dogmas of biocontrol. Changes in perceptions derived from research on Trichoderma harzianum T-22. *Plant Dis.* 84:377-393.

Harman GE, Howell CR, Viterbo A, Chet I, Lorito M (2004): *Trichoderma* species: opportunistic, avirulent plant symbionts. *Nat Rev Microbiol* 2 43–56.

Hartmann A, Rothballer M, Schmid M (2008): Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. Plant Soil 312:7–14.

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

Idriss E. E, Makarewicz O, Farouk A, Rosner K, Greiner R, Bochow H, Richter T, Borriss R.(2002): Extracellular phytase activity of Bacillus amyloliquefaciens FZB45 contributes to its plant-growth-promoting effect. Microbiol.;148(Pt 7):2097–2109.

IFA. (2018): *INTEGRATED PLANT NUTRIENT MANAGEMENT : Increasing Yields And Improving SoilHealth*.https://www.fertilizer.org/images/Library_Downloads/2018_IFA_Integrated_Plant_Nutrient_Management.pdf

Jones, D. L. (1998): Organic acids in the rhizosphere - A critical review. *Plant and Soil*.

Kai M, Haustein M, Molina F, Petri A, Scholz B, Piechulla B (2009): Bacteria volatile and their action potentials. *Appl Microbiol Biotechnol. 2009 Jan; 81(6):1001-12.*

Khan, M. S., Zaidi, A., & Wani, P. A. (2009): m. In *Sustainable Agriculture* (pp. 551–570).

Kim KY, Jordan D, McDonald GA (1997): Effects of phosphate-solubilizing bacteria and vascular-arbuscular mycorrhiza on tomato growth and soil microbial activities. Biology and Fertility of Soils 26(2); 79-87.

Kirchmann, H., Nyamangara, J, Cohen, Y (2005): Recycling municipal wastes in the future: from organic to inorganic forms? Soil Use and Management 21: 152–159

Kurepin, L. V., Zaman, M., & Pharis, R. P. (2014): Phytohormonal basis for the plant growth-promoting action of naturally occurring biostimulators. *Journal of the Science of Food and Agriculture*. https://doi.org/10.1002/jsfa.6545

Lekfeldt, Stevens, J. D., Neergaard, D., Duus, J., Lekfeldt, S., Rex, M., Mercl, F., … Magid, J. (2016): Effect of bioeffectors and recycled P - fertiliser products on the growth of spring wheat. *Chemical and Biological Technologies in Agriculture*, *3*(22).

Lesueur D., Deaker R., Herrmann L., Bräu L., Jansa J. (2016): The Production and Potential of Biofertilizers to Improve Crop Yields. In: Arora N., Mehnaz S., Balestrini R. (eds) Bioformulations: for Sustainable Agriculture. Springer, New Delhi

Lindsay W. L., Vlek P. L. G. and Chien S. H. (1989): Phosphate minerals. In Minerals in soil environment, 2nd edn. Eds J B Dixon and S. B. Weed. pp. 1089–1130. Soil Science Society of America, Madison, WI, USA.

Panhwar, Q. A., Jusop, S., Naher, U. A., Othman, R., & Razi, M. I. (2013): Application of potential phosphate-solubilizing bacteria and organic acids on phosphate solubilization from phosphate rock in aerobic rice. *The Scientific World Journal*.

Malusà, E., Pinzari, F., & Canfora, L. (2016): Efficacy of biofertilizers: Challenges to improve crop production. In *Microbial Inoculants in Sustainable Agricultural Productivity: Vol. 2: Functional Applications*. https://doi.org/10.1007/978-81-322-2644-4_2

Mengel K and Kirkby E. A. (1987): Principles of Plant Nutrition. 4th edn. International Potash Institute, Bern, Switzerland, 687 p.

Menzies, N.; Harbison, D.; Dart, P (2011): Soil chemistry-facts and fiction and their influence on the fertilizer decision making process. In Proceedings of the 26th Annual Conference of the Grassland Society of NSW, Bathurst, Australia, 26–28; pp. 49–63.

Mordor Intelligence, (2017): Biofertilizers Market – Trends, Competitive Scenario and Forecasts (2017-2022) (https://www.mordorintelligence.com/industry-reports/global-biofertilizers-market-industry), retrieve data 02/03/2018.

Neumann, G.; Römheld, V (2002): Root-induced changes in the availability of nutrients in the rhizosphere. In Plant Roots the Hidden Half, 3rd ed.; Waisel, Y., Eshel, A., Kafkafi, U., Eds.; Marcel Dekker: New York, NY, USA; pp. 617–649

Neumann G., Junge H., Prebeck K. (2017): Reducing the need for artificial fertilisers. Sustainable biostimulants for Agriculture. Science Impact

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 Research 196:389-401

Norrish K. and Rosser H. (1983): Mineral phosphate. In Soils: an Australian viewpoint. pp. 335–361. Academic Press, Melbourne, CSIRO/London, UK, Australia.

Oliveira, C. a., Alves, V. M. C., Marriel, I. E., Gomes, E. a., Scotti, M. R., Carneiro, N. P., … Sá, N. M. H. (2009): Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. *Soil Biology and Biochemistry*, *41*(9), 1782–1787. https://doi.org/10.1016/j.soilbio.2008.01.012

Ortíz-Castro R., Contreras-Cornejo H A, Macías-Rodríguez L, and López-Bucio J (2009): The role of microbial signals in plant growth and development. *Plant Signal Behav.* 4(8): 701–712. doi: 10.4161/psb.4.8.9047

Ozanne P. G (1980): Phosphate nutrition of plants — general treatise. In The role of phosphorus in agriculture. Eds. F E Khasawneh, E C Sample and E J Kamprath. pp. 559-589. American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, WI, USA.

Raghothama K. G. (1999): Phosphate acquisition. Ann. Rev. Plant Physiol. Mol. Biol. 50, 665–693.

Ramírez, C. A., & Kloepper, J. W. (2010): Plant growth promotion by Bacillus amyloliquefaciens FZB45 depends on inoculum rate and P-related soil properties. *Biology and Fertility of Soils*. https://doi.org/10.1007/s00374-010-0488-2

Rengel, Z., & Marschner, P. (2005): Nutrient availability and management in the rhizosphere: Exploiting genotypic differences. *New Phytologist*, *168*(2), 305–312.

Rodríguez, H., & Fraga, R. (1999): Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnology Advances*, *17*(4–5), 319–339.

Ryu C. M, Farag M. A, Hu C. H, Reddy M. S, Kloepper J. W, Paré P. W (2004): Bacterial volatiles induce systemic resistance in Arabidopsis. Plant Physiol; 134:1017-26.

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., & Gobi, T. a. (2013): Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. *SpringerPlus*, *2*(1), 587. https://doi.org/10.1186/2193-1801-2-587

Saini V. K, Bhandan S. C and Tarafdar J. C. (2004): Comparison of crop yield, soil microbial C, N and P-fixation, nodulation and mycorrhizal infection in inoculated and non-inoculated

sorghum and chickpea crops. Field and Crops Research89: 39–47.
Singh Y. V and Gained S, (2019): Inorganic phosphorus fractions and crop productivity in response to different rates of phosphate-solubilising fungi Penicillium bilaii. *Indian Journal of Agricultural Sciences89* (2): 238–45.
Singh B. and Satyanarayana T (2011): Microbial phytases in phosphorus acquisition and plant growth promotion. Physiol Mol Biol Plants. 17(2): 93–103
Stoffella, P. J.; He, Z. I.; Wilson, S. B.; Ozores-Hampton, M.; Roe, B.E. (2014): Compost Utilization in Subtropical Horticultural Cropping Systems. In I International Symposium on Organic Matter Management and Compost in Horticulture; Biala, J., Prange, R., Raviv, M., Eds.; Acta Horticulturae: Adelaide, Australia, pp. 95–108.
Tabatabai M. A (1994): Soil Enzymes. In: Weaver RW, Angle JS, Bottomley PS, eds, (1994). Methods of Soil Analysis, Part 2, Microbiological and Biochemical Properties. Soil Science Society of America, Madison, (WI), 775-833
Tarafdar J. C and Claasen N (1988): Organic Phosphorus as a phosphorus source of higher plants through the activities of phosphatase produced by plant roots and microorganisms. Biology and Fertility of Soils. 5(4) 308-312.
Tarafdar J. C, Yadav R. S, Meena S. C (2001): Comparative efficiency of acid phosphatase originated from fungal and plant sources. Journal of Plant Nutrition and Soil Sciences. 164 (30: 279-282.
Thonar C., Lekfeldt D.L., Cozzolino, V., Kundel, D., Kulhánek, M., … Mäder, P. (2017): Potential of three microbial bio - effectors to promote maize growth and nutrient acquisition from alternative phosphorous fertilizers in contrasting soils. *Chemical and Biological Technologies in Agriculture*, 1–16. https://doi.org/10.1186/s40538-017-0088-6
Van Kauwenbergh, S. J., Stewart, M., & Mikkelsen, R. (2013): World Reserves of Phosphate Rock… a Dynamic and Unfolding Story. *Better Crops*, *97*, 18–20.
Vance, C. P. (2001): Symbiotic nitrogen fixation and phosphorus acquisition. Plant nutrition in a world of declining renewable resources. *Plant Physiol*, *1*(27), 390–397.
Vyas, P., & Gulati, A. (2009): Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent Pseudomonas. *BMC Microbiology*, *9*, 174. https://doi.org/10.1186/1471-2180-9-174
Wei Y., Zhaoa Y, Shi M, Cao Z, Lu Q, Yang T, Fan Y, Wei Z (2018): Phosphorus solubilization during composting with enriched phosphate-solubilizing bacteria inoculation. Bioresource Technology. 247, 190-199.
Wu S. C., Cao Z. H., Li Z. G., Cheung K. C., & Wong M. H. (2005): Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: A greenhouse trial. *Geoderma*. https://doi.org/10.1016/j.geoderma.2004.07.003
Zhou R., Liu X., Luo L., Zhou Y., Wei J., Chen A., Tang L., Wu H., Deng Y., Zhang F., & Wang Y. (2017): Remediation of Cu, Pb, Zn and Cd-contaminated agricultural soil using a combined red mud & compost amendment.International Biodeterioration & Biodegradation,118,73–81

4 PLANT-PGPM INTERACTIONS AS AFFECTED BY THE FORM OF N FERTILIZATION

4.1 The form of N supply determines plant growth promotion by P-solubilizing microorganisms in maize

Isaac Kwadwo Mpanga (IKM)[1*], Peteh Mehdi Nkebiwe (PMN)[1], Mira Kuhlmann (MK)[1], Vincenza Cozzolino (VC)[2], Alessandro Piccolo (AP)[2], Jörg Geistlinger (JG)[3], Nils Berger (NB)[4], Uwe Ludewig (UL)[1], Günter Neumann (GN)[1]

[1] Institute of Crop Science (340h), Universität Hohenheim, Fruwirthstraße 20, 70593 Stuttgart, Germany; UL u.ludewig@uni-hohenheim.de, PNM Mehdi.Nkebiwe@uni-hohenheim.de, GN gd.neumann@t-online.de, MK mirakulix83@gmx.de

[2]Department of Agriculture, University of Naples Federico II, Italy; VC vincenza.cozzolino@unina.it, AP alpiccol@unina.it

[3] Institute of Bioanalytical Sciences, Anhalt University of Applied Sciences, 06406 Bernburg, Germany; JG joerg.geistlinger@hs-anhalt.de

[4] Eurochem Agro GmbH, 68165 Mannheim, Germany; NB nils.berger@eurochemgroup.com

*IKM mpangaisaac75@gmail.com Tel.: +4915217489408

Published in Microorganisms. Received: 28/12/2018; Accepted: 26/01/2019; Published: 29/01/2019

Abstract: Phosphate-(P)-solubilizing microorganisms (PSM) are important drivers of P cycling in natural and agro-ecosystems. Their use as plant inoculants to improve P acquisition of crops has been investigated for decades. However, limited reproducibility of the expected effects, particularly under field conditions, remains a major challenge. This study demonstrates that the form of nitrogen fertilization has a significant impact on the performance of various fungal and bacterial PSM inoculants in maize grown on neutral to alkaline soils with limited P availability.

Under these conditions, a high soil pH-buffering capacity frequently limits the efficiency of nutrient mobilization, mediated by plant roots and microorganisms via rhizosphere acidification. In a soil pH range between 7.0 and 8.0, nitrate fertilization promoting rhizosphere alkalinization further aggravates this problem. Accordingly, in greenhouse experiments, six strains of *Pseudomonas*, *Bacillus*, *Paenibacillus*, *Streptomyces* and *Penicillium* with proven P-solubilizing potential, completely failed to promote P acquisition in maize grown on a calcareous Loess sub-soil pH 7.6 with nitrate fertilization and rock phosphate (Rock-P) as sparingly soluble P source. However, after replacement of nitrate fertilization by ammonium, stabilized with the nitrification inhibitor DMPP, five out of seven investigated PSM inoculants (comprising 12 fungal and bacterial PSM strains) exerted beneficial effects on plant growth and reached up to 88% of the shoot biomass production of a control supplied with soluble triple-superphosphate (TSP). Stabilized ammonium combined with PSM-inoculants improved P acquisition (*Trichoderma harzianum* T22, *Pseudomonas* sp. DMSZ 13134), while other strains particularly stimulated root growth (*T. harzianum* OMG16, *Bacillus amyloliquefaciens* FZB42), which promoted the acquisition also of other mineral nutrients such as N, K and Mn. A similar effect was recorded under field conditions on an alkaline clay-loam soil pH 8.6. The combination of stabilized ammonium with a range of consortium products based on *T. harzianum* OMG16, *B. amyloliquefaciens*, micronutrients and humic acids completely compensated the effect of a TSP fertilization on field establishment, nutrient acquisition and yield formation in maize, while non-stabilized urea-di-ammonium phosphate fertilization was largely ineffective. These findings suggest that the efficiency of PSM-plant interactions can be influenced by the form of N fertilization, offering promising perspectives for synergistic effects with stabilized ammonium fertilizers.

Keywords: Plant Growth-promoting Microorganisms (PGPM); P-solubilizing Microorganisms (PSM); Maize; Nitrogen; Stabilized Ammonium; N-form, DMPP; Phosphate mobilization

4.1.1. Introduction

Phosphorous (P) is the least soluble and consequently the least bio-available soil macronutrient, for higher plants. It is taken up by plant roots exclusively in the form of soluble mono-, and divalent phosphate anions (P_i) in the soil solution. Due to a high fixation potential in the form of Fe and Al oxides/hydroxides and the formation of sparingly soluble Fe-, Al-P at soil pH levels < 6, or Ca-phosphates at pH 7-8, soluble and easily plant-available soil phosphates usually comprise less than 0.1% of the total soil P [1]. Even in well-fertilized agricultural soils, the P_i concentrations in the rhizosphere soil solution hardly exceed 10 µM due to rapid fixation and root uptake [2]. Theoretical considerations on plant demands suggest that the respective equilibrium concentrations in the rhizosphere soil solution need to be replaced 20-50 times per day to meet the plant P requirements. This is not possible due to the slow diffusion-mediated desorption of sparingly soluble soil P forms [3]. Accordingly, soil-grown plants are generally facing at least latent P limitation and are largely depending on the expression of adaptive strategies to improve P acquisition. Stimulation of root growth and fine root structures, as well as mycorrhizal associations, support the spatial acquisition of soluble P_i. Root-induced changes in rhizosphere pH and the release of organic metal chelators can increase the solubility of immobilized soil P forms. Root-secretory phosphohydrolases can mediate the liberation of P_i sequestered in soil organic matter, which can comprise up to 80% of the total soil P [4]. These adaptations exhibit a large genotypic variation within plant species and cultivars. However, highly efficient P acquisition is not a widespread feature in most crops [4, 5]. Accordingly, P use efficiency in agricultural production systems hardly exceeds 30% [6]. Moreover, high fixation of fertilizer P in soils and low P acquisition efficiency of plant roots are factors provoking P over-

fertilization to maintain yield stability. This is associated with a high risk of irreversible P losses by surface run-off, eutrophication of surface waters, and wasting of P as a limited natural resource.

Soil microorganisms are important drivers of P turn over in soils, determining soil fertility and P availability for plants. Between 10 - 50 % of soil bacteria and 0.1 - 0.5 % of soil fungi are classified as P-solubilizing microorganisms (PSMs). They can mediate P mineralization but also promote the solubilization of sparingly soluble inorganic P forms and even weathering of rocks and stones [7, 8, 6]. Like plant roots, PSMs can secrete phosphohydrolases, protons, organic metal chelators and even mineral acids with proven potential to mineralize and solubilize the various P forms in soils [6]. Particularly in natural ecosystems, P acquisition of higher plants strongly depends on the activity of PSMs. Therefore, it is not surprising that recruiting of PSMs for symbiotic interactions is a widespread feature of plants in natural ecosystems and an important component of the adaptive plant strategies for P acquisition. Fungal PSMs are mainly found in ectomycorrhizal associations, while arbuscular mycorrhizae preferentially contribute to an improved spatial P acquisition of the host plants [4]. Similarly, many bacterial PSMs exhibit a high abundance in the rhizosphere of higher plants [6].

In face of the obvious importance of PSMs for P acquisition of higher plants, the concept to select highly efficient PSM strains as inoculants for improved P acquisition of crops has a long history dating back to the 1950s [9]. This is still promoted in numerous literature reviews [6, 9, 10, and 11]. However, although P-solubilizing properties of PSMs can be easily demonstrated on artificial growth media amended with sparingly soluble P sources, limited reproducibility of the expected effects under real rhizosphere conditions and particularly in field applications remains a major challenge [12]. More recent studies suggest that plant growth promotion and improved plant P acquisition cannot be regarded as a general PSM feature, and the expression

of effects seems to be highly dependent on external factors. For example, the rhizosphere competence of microbial inoculants strongly depends on their survival in the soil environment, which can be influenced by interactions with the native soil microbiome and by abiotic stress factors [13, 14]. But also, the amount and type of fertilizer supply can obviously play an important role: a recent meta-analysis by Schütz et al. [15] covering 171 publications, demonstrated plant growth-promoting effects of PSM inoculants mainly expressed in soils with moderate available P levels (25- 35 kg P ha^{-1}), while the efficiency declined at lower or higher ranges of P availability. This resembles the characteristics also of other beneficial plant-microbial interactions, such as symbiotic nitrogen fixation of Rhizobia with leguminous plants or plant interactions with arbuscular mycorrhizal fungi. Preferential performance of PSMs in combination with nitrogen-(N)-rich, manure-based fertilizers has been repeatedly reported by [16-18]. Nkebiwe et al. [19] found increased root colonisation by a PSM inoculant after ammonium depot fertilization in maize, associated with root proliferation and plant growth promotion, both, in lab and field experiments.

Based on these observations we hypothesized that the efficiency of plant-PSM interactions is influenced by the form of N fertilization. A range of pre-selected bacterial and fungal inoculants with documented P-solubilizing potential [20] was investigated in a series of pot and field experiments on soils with low P availability. Maize was selected as a host plant with a low inherent potential for mobilization of sparingly soluble soil P forms [4, 21]. Insoluble rock-phosphate was provided as a sparingly soluble P source. Nitrogen supplied in different forms frequently used in mineral fertilizers. The supplied N forms comprised nitrate, ammonium, urea or ammonium fertilizer, stabilized with the nitrification inhibitor DMPP (3, 4-dimethylpyrazole-phosphate).

4.1.2. Materials and Methods

4.1.2.1 Pot experiments on artificial sand sub-soil substrates

The first and second experiments were designed on June 10, 2014, and February 28, 2015, respectively using artificial mixtures of washed quartz sand and a calcareous Loess subsoil with high P sorption capacity, dominated by sparingly soluble Ca-P with low levels of organic matter to minimize P supply via mineralization and to ensure that plant P acquisition mainly depended on mineral P solubilization.

Substrate characteristics and fertilization

Plant-available P: P_{CAL}: 5 mg kg^{-1} [22]; pH_{CaCl2}: 7.6; C_{org}: < 0.3%; N_{total} 0.02 %; $CaCO_3$: 23 %).

The first experiment employed a mixture of 80 % soil and 20% (w/w) quartz sand (0.6-1.2 mm Ø). The substrate was fertilized by homogenous incorporation of [mg kg^{-1} substrate]: N ($Ca(NO_3)_2$) = 100; P 150 (Rock phosphate, 7.6 % P, Timac-Agro,Troisdorf Germany) or $Ca(H_2PO_4)_2$) for the positive P control); K (K_2SO_4) = 150; Mg ($MgSO_4$) = 50; Zn ($ZnSO_4$) =2.6; Cu ($CuSO_4$) = 1.0; and 20 µmol Fe kg^{-1} substrate (Sequestrene138, 6 % Fe). Each pot was filled with 2.9 kg of substrate and moisture was adjusted daily to 18% (w/w) = 60 % substrate water holding capacity (WHC).

For the second experiment, the addition of quartz sand was increased to 70% (w/w). The Rock-P fertilization was combined with two N forms at 100 mg N kg^{-1} substrate: (1) 100% NO_3-N as $Ca(NO_3)_2$, and (2) 80% NH_4-N as DMPP-(3,4-dimethylpyrazole-phosphate) - stabilized $(NH_4)_2SO_4$ (Novatec solub, Compo Expert GmbH, Münster, Germany) with 20% NO_3-N as $Ca(NO_3)_2$). A negative control without P fertilization and positive control with soluble $Ca(H_2PO_4)_2$ were included as additional treatments with nitrate fertilization. For the remaining nutrients, substrate fertilization was identical with experiment 1.

PSM inoculation and plant culture

Experiment 1: Pseudomonas sp. DSMZ 13134, Proradix®, Sourcon Padena GmbH, Tübingen, Germany (Pro: 1x 10^9 CFU kg^{-1} substrate), *Penicillium* sp. PK 112, Biological Fertilizer OD, Bayer CropScience Biologics GmbH, Wismar, Germany (BFOD, 1x 10^8 spores kg^{-1} substrate), *Paenibacillus mucilaginosus*, Abitep GmbH, Berlin, Germany (Paeni, 1x 10^9 spores kg^{-1} substrate) and Vitalin SP11, Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany (SP11, 20 ml of 0.2% suspension kg^{-1} substrate). Vitalin SP11 comprises *Bacillus subtilis*, Pseudomonas sp., *Streptomyces* spp., humic acids and extracts of the seaweed *Ascophyllum nodosum*.

Experiment 2: *Pseudomonas sp.* DSMZ 13134, Proradix® (Pro: 1x 10^9 CFU kg^{-1} substrate)

The Inoculants were suspended in 2.5 mM $CaSO_4$. Maize seeds (*Zea mays* L. var Colisee) were soaked for 10 min with the microbial suspensions, sown at 3 cm depth and thereafter 20 ml PSM suspension was inoculated into the seeding hole with two additional weekly applications close to the stem of the plants. Plants were arranged in a completely randomized design with 4 replicates per treatment for 41 days (experiment 1) and with 5 replicates for 36 days (experiment 2) under greenhouse conditions (air temperature range: 11-30°C, average 21°C) with additional light 12 h d^{-1}, average light intensity: 275 µM m^{-1} s^{-1}.

4.1.2.2 Pot experiment using field soil

The experiment was established on August 15, 2015 on an organic farming field soil with moderately low P availability, freshly collected from the A horizon at the experimental station Klein-Hohenheim, Hohenheim University, Stuttgart, Germany) to include a native top-soil microflora.

Substrate characteristics and fertilization

Soil characteristics: clay-loam, pH_{CaCl2} = 7.0; P_{CAL} = 36.7 mg P Kg^{-1}; N_{total}: 0.15%; C_{org}: 1.28%; substrate mixed with 30% (w/w) quartz sand for improvement of soil structure.

The basal fertilization comprised (mg kg^{-1} substrate): N 100 as DMPP-stabilized $(NH_4)_2SO_4$ or $Ca(NO_3)_2$, P 100 (Rock-P or $Ca(H_2PO_4)_2$ for the positive control); K 150 (as K_2SO_4) and Mg 50 (as $MgSO_4$). No micronutrient fertilisation was performed in this experiment. Each pot was filled with 3 kg of substrate and moisture was adjusted daily to 21% (w/w) = 60 % substrate water holding capacity (WHC).

PSM inoculation and plant culture

Seven PSM inoculants were tested in comparison with a non-inoculated control, with Rock-P as sparingly soluble P source in combination with N supply as DMPP-stabilized NH_4^+: *Pseudomonas* sp. DSMZ 13134 (Proradix); *Trichoderma harzianum* T22 (Trianum-P, Koppert, Biological Systems, Berkelen Rodenrijs, The Netherlands); *Penicillium* sp. PK 112 (BFOD), *Paenibacillus mucilaginosus* (Paeni), *Bacillus amyloliquefaciens* FZB42 Rhizovital42® (Abitep GmbH, Berlin, Germany); Vitalin SP11, and CombiFectorA: *Trichoderma harzianum* OMG18 + Vitabac with five *Bacillus* strains (Bactiva GmbH, Straelen, Germany) + Zn/Mn, Institute of Bioanalytical Sciences, Bernburg, Germany). Furthermore, the best performing PSM strain Proradix [20] was tested also with Rock-P and nitrate-based fertilization, as a reproduction of experiment 2, described under 2.1. Two additional non-inoculated treatments included an unfertilized control and positive control, supplied with soluble triple-superphosphate (TSP, 100 mg P kg^{-1} substrate) and $Ca(NO_3)_2$ fertilization (100 mg N kg^{-1} substrate). Inoculation was performed as described under 2.1.2. Plants were arranged in a completely randomized design with five replicates per treatment for 35 days under greenhouse conditions (air temperature range: 13-32°C, average 20°C) with additional light 12 h d^{-1}; average light intensity: 275 µM m^{-1} s^{-1}.

4.1.2.3 Field experiment

The field trial was conducted in 2016 at the "Experimental Station of the Department of Agriculture of Napoli Federico II", located at Castel Volturno, in an agricultural area 60 km north

of Naples, (CE) Campania, Italy; annual mean temperature: 15.6°C; average annual precipitation: 879 mm.

Soil characteristics and fertilization

The soil was classified as clay loam (Vertic Xerofluvent), pH_{H2O} 8.6; available $NaHCO_3$-extractable P_{Olsen} 11 mg kg^{-1} N $_{total}$: 0.13%, C_{org} 1.5%. Nitrogen and phosphate fertilization was performed (1) according to the local farmers practice (urea = 180 kg N ha^{-1} and non-stabilized di-ammonium phosphate (DAP) = 50 kg P ha^{-1}); (2) as a negative control with DMPP-stabilized ammonium sulfate (NovaTec 21 solub = 150 Kg N ha^{-1}) without additional P fertilization; (3) as a positive control with DMPP-stabilized ammonium sulfate (150 Kg N ha^{-1}) and triple superphosphate (TSP= 50 kg P ha^{-1}) and (4) combinations of DMPP-stabilized ammonium sulfate (150 Kg N ha^{-1}) with selected PSM-inoculants but without additional P fertilization.

PSM inoculation and plant culture

PSM products comprised: (1) Combifector A (a combination product of *Trichoderma harzianum* OMG16 + Vitabac = 5 *Bacillus* strains and micronutrients Zn/Mn,- Institute of Bioanalytical Sciences, Bernburg, Germany; (2) Combifector B (a combination product of *Trichoderma harzianum* OMG16, + *Bacillus amyloliquefaciens* (RhizoVital FZB42) and micronutrients Mn/Zn, Institute of Bioanalytical Sciences, Bernburg, Germany, ABiTEP GmbH, Berlin, Germany; (3) Bacillus amyloliquefaciens, Rhizovital FZB42® ABiTEP GmbH, Berlin, Germany + humic acids from composted cow manure produced on the farm in Castel Volturno and (4) a seaweed extract - *Bacillus amyloliquefaciens* seed dressing formulation provided by Group Limagrain, Saint-Beauzire, France). Combifector A and B (1+2), were applied at sowing by broadcast top-soil incorporation at a dosage of 100 g ha^{-1}, equivalent to 1 x 10^{12} fungal spores plus 1 x 10^{12} bacterial spores ha^{-1}. *Bacillus amyloliquefaciens*, Rhizovital FZB42 with humic acids at a dosage

of 5 kg ha^{-1} (3) was inoculated into the seeding row via band application, and the *B. amyloliquefaciens* - seaweed extract formulation (4) was provided in the form of pre-coated maize seeds.

The experimental area was divided into 40 m^2 plots under a randomized block design with four replications. Maize seeds (*Zea mays* L. cv 30.600, Group Limagrain, Saint-Beauzire, France) were sown at the beginning of June with a distance of about 10 cm and 75 cm inter-row distance, with a plant density of 7 plants m^{-2}. Each treatment was replicated four times. Plant establishment was monitored at the V6 stage at 42 DAS by shoot biomass determination. Final grain harvest was performed in early November.

4.1.2.4 Plant biomass and root length determination

At final harvest, the dry biomass of the shoots was determined after 3 d oven-dried at 65 °C. The roots in each pot were washed out from the soil substrate and were stored in 30% (v/v) ethanol. The roots were later separated, submerged in a water film in transparent Perspex trays, and digitalized using a flat-bed scanner (Epson Expression 1000 XL, Tokyo, Japan). Subsequently, the root length of the digitalized samples was measured using the WinRHIZO root analysis system (Reagent Instruments, Quebec, QC, Canada). Thereafter, the root samples were oven-dried for 2 d at 65 °C for the determination of the dry matter.

4.1.2.5 Shoot mineral analysis

For both experiments, plant mineral nutrient analysis was performed as follows: tomato shoot N was measured with a Vario Max CN macro-elementar analyser (Elementar Analysensysteme, Hanau, Germany). For P, K, Ca, and Mg, a microwave digestion method was employed for the wet ashing of finely ground dry plant materials (250 mg) in 1 mL of deionized water, 2.5 mL conc. HNO_3 (1:3), and 2 mL H_2O_2 (30%). Digestion was performed in a microwave digestion

system (Ethos, MLS, Leutkirch, Germany) for 1 hr and allowed to cool for 30 min. Approximately 5 g activated charcoal was added for sample decolouration, mixed well by shaking, and allowed to settle within 15 min. The samples were filtered with ashless MG 640d Blue ribbon filter paper (Macherey & Nagel, Düren, Germany). Phosphate was estimated spectrophotometrically (Hitachi LtD., Tokyo, Japan) according to [23]. Magnesium, calcium, zinc and manganese were measured by atomic absorption spectrophotometry (iCE 3000 series, Thermo Fischer, Dreieich, Germany) and potassium by flame emission spectrophotometry (Eppendorf-ELEX6361, Netheler+Hinz, Hamburg, Germany).

4.1.3. Results

4.1.3.1 Experiments on artificial growth substrates (sub-soil-sand mixtures)

To study PSM-induced mobilization of sparingly-soluble soil P, maize (cv Colisee) was used as a test crop with low adaptive potential for root-induced P solubilisation [4]. Plants were inoculated with different PSMs of fungal and bacterial origin, comprising three single-strain inoculants (*Pseudomonas* sp. DSMZ 13134 Proradix® (Pro), *Paenibacillus mucilaginosus* (Paeni), *Penicillium* sp. PK 112, Biological Fertilizer OD (BFOD) and one consortium product Vitalin SP11 with a combination of *Bacillus subtilis*, *Pseudomonas* sp., *Streptomyces* spp., humic acids and extract of the seaweed *Ascophyllum nodosum*. Pilot experiments revealed that the selected microbial PSMs were able to solubilize insoluble tri-calcium phosphates (Ca-P), rock phosphate (RP) and sewage sludge ash (SA), added to artificial growth media and Proradix was identified as the most efficient PSM strain [20]. The plants were cultivated on a calcareous Loess subsoil substrate (80% soil / 20% sand, pH 7.6) with low P availability (5 mg P_{CAL} kg^{-1}), low organic matter content < 0.3%) and sparingly soluble rock P as an exclusive P source. This experimental setup ensured that plant P acquisition was only possible after Ca-P solubilisation. However, despite the proven P-solubilizing potential, all microbial inoculants failed to stimulate P

acquisition of the test plants and even exerted inhibitory effects on plant growth in comparison with a non-inoculated control (Fig. 1A, C, and D). Accordingly, P shoot accumulation was not increased in the PSM-treated variants. By contrast, shoot biomass production increased by 300% and shoot P accumulation by 500 % in maize plants supplied with soluble $Ca(H_2PO_4)_2$ as a positive control (Fig. 1A, B).

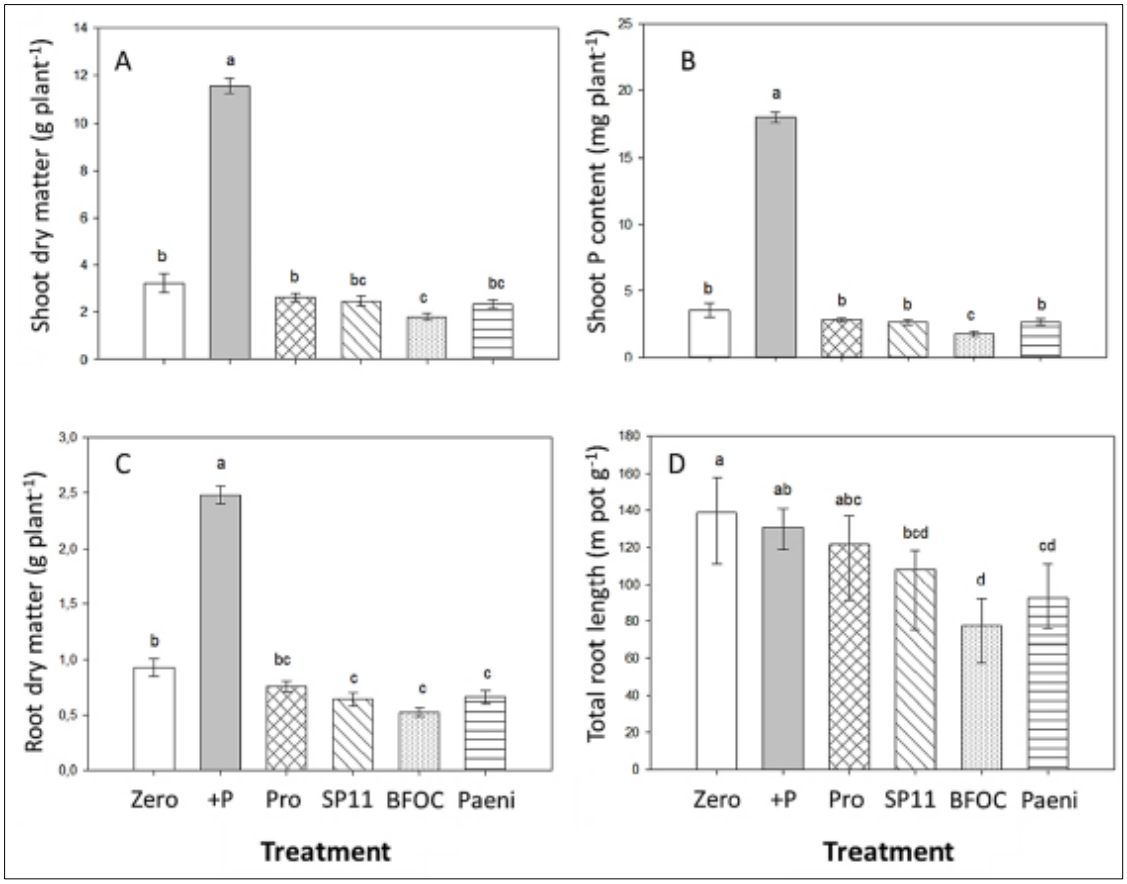


Figure 4.1: Shoot biomass (A), shoot P content (B), root dry matter (C) and total root length (D) of maize (cv Colisee) grown on a calcareous Loess subsoil (pH 7.6) - sand mixture (80/20% w/w), supplied with and without (Zero) P fertilization in the form of Rock-P (RP) or soluble $Ca(H_2PO_4)_2$ (+P) and calcium nitrate fertilization. RP variants inoculated with *Pseudomonas* sp. DSMZ 13134 Proradix (Pro); SP11, Vitalin SP11 (SP11); *Penicillium* sp. PK 112 (BFOC); and *Paenibacillus mucilaginosus* (Paeni). Means of four replicates. One-way ANOVA, Tukey test. Different letters indicate significant differences ($P < 0.05$).

Based on these results, it was hypothesized that a high pH-buffering capacity of the calcareous soil substrate with 23 % $CaCO_3$ was counteracting PSM-induced acidification of the growth medium and thereby microbial Rock-P solubilisation. To test this hypothesis, the pH buffering capacity of the growth substrate was reduced by increasing the sand content from 20 to 70% (w/w). Moreover, as an additional fertilisation treatment to N supply via calcium nitrate, a

variant with ammonium-dominated N application (80% $(NH_4)_2SO_4$, stabilized with the nitrification inhibitor DMPP + 20 % Ca $(NO_3)_2$) was included in order to promote Rock-P solubilisation by ammonium-induced rhizosphere acidification [4]. Proradix, pre-characterized as PSM with the highest P-solubilizing potential [20] was used as an inoculant.

In the variants with nitrate fertilization and Rock-P supply, PSM inoculation had no significant effect on shoot biomass production (Fig. 4.2A) and P accumulation (Fig. 4.2B) of the maize plants. Biomass production reached only 35 %, and P accumulation 24 %, as compared with the positive TSP control supplied with soluble P. Replacement of nitrate by stabilised ammonium significantly increased shoot P accumulation (Fig. 4.2B), but a significant increase in shoot biomass production by 92 % was exclusively achieved by a combination of ammonium supply with PSM inoculation (Fig. 4.2A). However, P shoot concentration and P accumulation of the ammonium variants with and without PSM inoculation were not significantly different. With the exception of the positive control supplied with soluble TSP, the P nutritional, status of the remaining variants was critical (< 0.3%, Campbell 2009) (Fig. 4.2B).

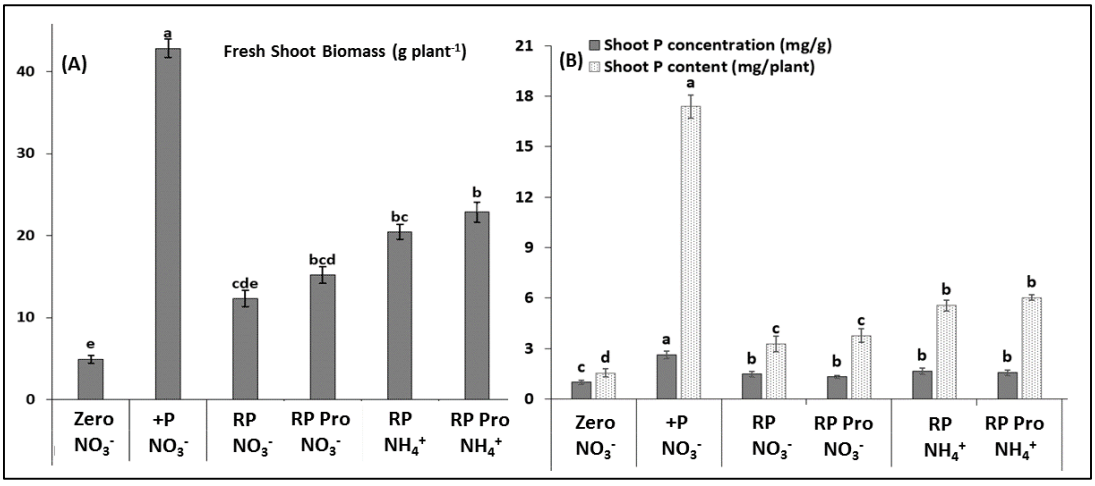


Figure 4.2: Shoot biomass (A), shoot P content and concentration (B) of maize (cv Colisee) grown on a calcareous Loess subsoil pH 7.6 - sand mixture (30/70% w/w), supplied with and without (Zero) P fertilization in the form of Rock-P (RP) or soluble $Ca(H_2PO_4)_2$ (+P). RP variants with and without *Pseudomonas* sp. DSMZ 13134 Proradix (Pro) inoculation in combination with Ca-nitrate (NO_3^-) or DMPP-stabilized ammonium (NH_4^+) fertilization. Means of five replicates. One-way ANOVA, Tukey test. Different letters indicate significant differences (P< 0.05).

4.1.3.2 Pot experiment on field soil

Since stabilized ammonium fertilization exerted beneficial effects on the plant growth-promoting potential of the PSM strain Proradix on a sand-soil substrate supplied with sparingly soluble Rock-P as major P source (Fig. 4.2), an additional experiment was conducted under more realistic conditions, using a clay-loam organic farming field soil (pH 7.0) with moderately low P availability (P_{CAL} 37 mg kg^{-1}). Phosphate was supplied as Rock-P or in the form of soluble triple-superphosphate (TSP) as a positive control. To evaluate synergistic effects of PSM inoculants with stabilized ammonium fertilization, two fungal (Trianum-P = *Trichoderma harzianum* T22, BFOD = *Penicillium* sp) and three bacterial single-strain inoculants (Proradix = *Pseudomonas* sp. DMSZ 13134; Rhizovital = *Bacillus amyloliquefaciens* FZB42 and *Paenibacillus mucilaginosus*), as well as two consortium products (SP11 and Combifector-A), pre-characterized as PSMs [20] were selected for inoculation of maize plants (cv Colisee). Proradix, characterized as the strain with the highest P-solubilizing potential [20] was investigated also in combination with nitrate fertilization.

4.1.3.3 Shoot growth and root development

Analysis of shoot biomass production revealed P as limiting nutrient, indicated by a 205 % increase after soluble TSP application as compared with the unfertilized control. Stabilized ammonium with Rock-P had a fertilizer effect of 111 %. Biomass production in the PSM-ammonium combinations was significantly increased in all variants compared with the non-inoculated control, with the exception of the two fungal strains Trianum P and BFOD. Similarly, the combination of Proradix with nitrate fertilization revealed no plant growth-promoting effects. (Fig. 4.3). Stimulation of shoot growth by PSM inoculation was associated with a clear trend for increased root length development, although the effect was significant only for the

single strain inoculant Rhizovital FZB42 (+ 32%) and the consortium product Combifector-A (+ 50%. Fig. 4.3B) after pairwise comparison with the non-inoculated control.

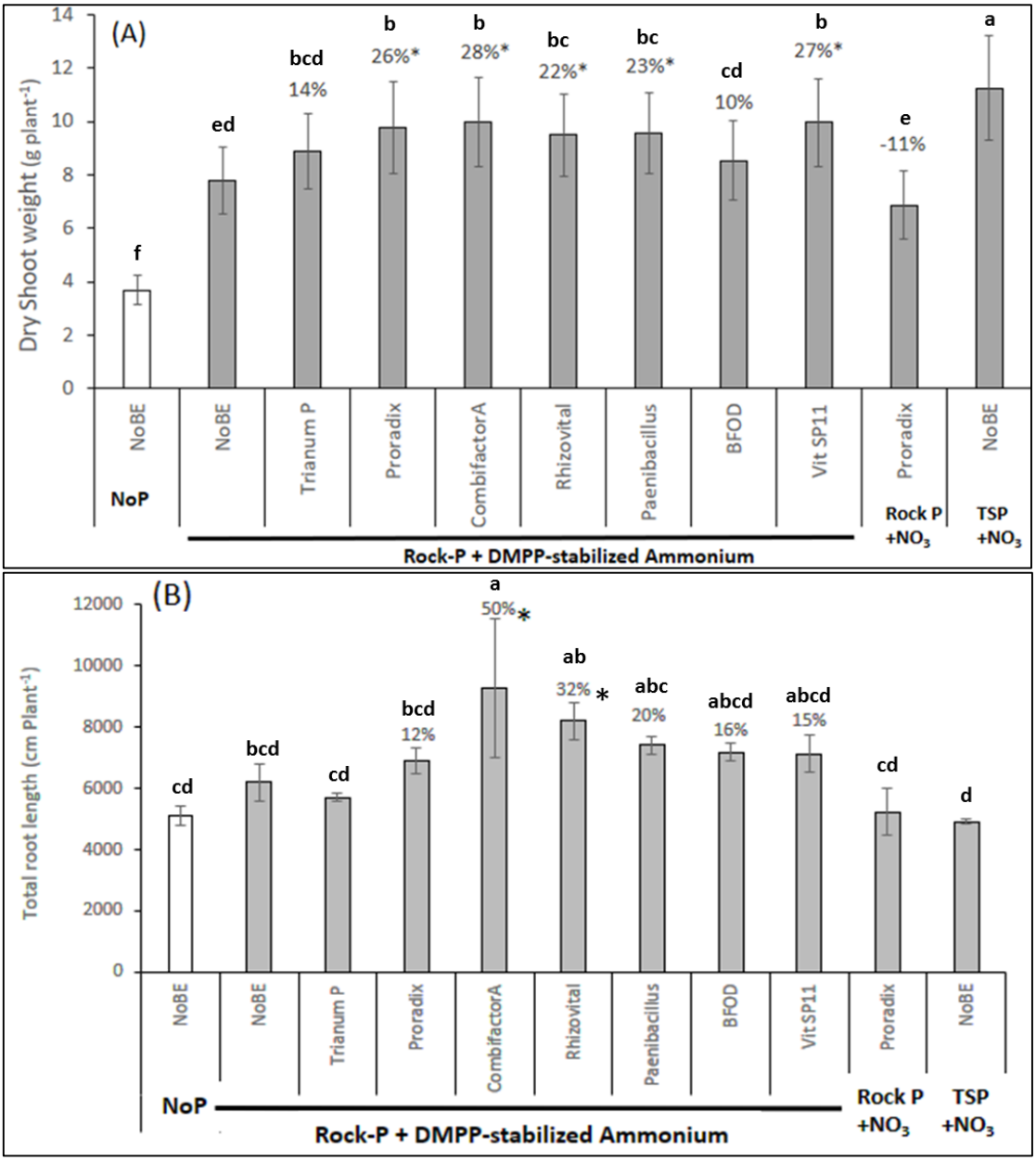


Figure 4.3: Shoot biomass (A) and total root length (B) of maize (cv Colisee) grown on a clay-loam, organic farming soil (pH 7.0), supplied with and without (No P) P fertilization in the form of Rock-P or soluble triple superphosphate (TSP). N supply in the form of Ca-nitrate (NO_3) or DMPP-stabilized ammonium. Microbial inoculants: *Trichoderma harzianum* T22 (Trianum P), *Pseudomonas* sp. DSMZ 13134 (Proradix), *Trichoderma harzianum* OMG16 + 5 Bacillus strains (Combifector-A); *Bacillus amyloliquefaciens* FZB42 (Rhizovital), *Paenibacillus mucilaginosus*, *Penicillium* sp. PK 112 (BFOD), Vitalin SP11 (VitSP11) or no inoculation (NoBE). Means of five replicates. One-way ANOVA, Tukey test. Different letters indicate significant differences ($P< 0.05$); * indicates significant differences after pairwise comparison of PSM-inoculated variants versus the non-inoculated control with ammonium fertilization (t-test, $p <0.05$).

Similar beneficial growth effects of ammonium fertilization on selected PSM strains have been recorded in additional experiments on different soils with a pH range between 5.7 and 7.9 in maize, spring wheat and tomato as target crops (summarized in supplementary data Table S1).

4.1.3.4 Mineral nutrient status

Concerning the plant nutrient status (Table 4.1), significant PSM effects were recorded for nitrogen (N), phosphate (P), potassium (K) and manganese (Mn). Magnesium and Zinc concentrations were in the sufficiency range for all treatments.

The P nutritional status of the maize plants was critical (< 0.3 %, [24]) in all investigated variants, even with soluble TSP fertilization. The combination of stabilized ammonium with Rock-P increased the P shoot concentration by 27% as compared with the unfertilized control, without a further increase by additional PSM inoculation. However, P shoot accumulation was significantly increased in the ammonium combinations with Trianum P and Proradix after pairwise comparison with the non-inoculated control (t-test, p =0.05).

The N status was critical in the ammonium-Rock-P variant (26 mg g DM^{-1}) but the N concentration reached the sufficiency range [24] for all tested PSM inoculants. Nitrogen shoot accumulation increased significantly in the Proradix-, Rhizovital-, SP11 and Combifector-A - ammonium combinations. The K status was sufficient in all treatments and shoot K accumulation was further increased by all PSM treatments except BFOD by pairwise comparison with the non-inoculated control. The manganese status of the plants supplied with Rock-P and stabilized ammonium was critical [24] but was significantly increased by 50% to the sufficiency range by PSM-inoculation.

Table 4.1: Mineral nutritional status of maize (cv Colisee) grown on a clay-loam, organic farming soil (pH 7.0), supplied with and without (No P) P fertilization in the form of Rock-P or soluble triple superphosphate (TSP) and N supply in the form of Ca-nitrate (NO_3) or DMPP-stabilized ammonium (NH_4) as affected by different PSM inoculants (see Fig, 3). Means of five replicates. One-way ANOVA, Tukey test. Different letters indicate significant differences (P< 0.05); * indicates significant differences after pairwise comparison of PSM-inoculated variants versus the non-inoculated control with ammonium fertilization (t-test, p <0.05.

Shoot mineral concentration (mg g^{-1})				
	N	P	K	Mn
No P	12.4 d	2.0 d	41.3 ab	0.02 b
NH_4_Rock-P	25.6 ab	2.5 ab	45.9 ab	0.02 b
NH_4_ Rock-P _Trianum P	35.2 ab*	2.4 abc	45.0 ab	0.03 a*
NH_4_ Rock-P _Proradix	33.9 ab*	2.3 abcd	45.9 ab	0.03 a*
NO_3_ Rock-P _Proradix	36.4 ab*	2.6 a	46.6 a	0.03 a*
NH_4_ Rock-P _Rhizovital	34.6 ab*	2.2 bcd	44.0 ab	0.03 a*
NH_4_Rock-P _Paenibacillus	31.9 b*	2.2 bcd	42.9 ab	0.03 a*
NH_4_ Rock-P _BFOD	37.4 a*	2.3 abcd	45.5 ab	0.03 a*
NH_4_ Rock-P _Vit SP11	34.2 ab*	2.2 bcd	40.8 ab	0.03 a*
NH_4_ Rock-P _CombifactorA	34.6 ab*	2.1 cd	41.2 ab	0.03 a*
NO_3_TSP	35.0 ab*	2.2 bcd	39.5 b	0.03 a*
Shoot mineral content (mg Plant^{-1})				
	N	P	K	Mn
No P	45.7 d	7.4 d	151.6 d	0.07 d
NH_4_Rock-P	271.5 bc	19.5 bc	356.6 bc	0.24 bc
NH_4_ Rock-P _Trianum P	311.7 ab	21.3 abc*	398.8 ab*	0.27 abc
NH_4_ Rock-P _Proradix	330.9 a*	22.2 ab*	448.9 a*	0.32 a*
NO_3_ Rock-P _Proradix	250.2 c	17.7 c	317.0 c	0.22 c
NH_4_ Rock-P _Rhizovital	328.1 a	20.9 abc	415.1 ab*	0.28 abc*
NH_4_ Rock-P _Paenibacillus	302.6 abc	21.4 abc	409.9 ab*	0.27 abc
NH_4_ Rock-P _BFOD	318.3 ab	19.6 bc	386.2 abc	0.27 abc
NH_4_ Rock-P _Vit SP11	339.9 a*	22.0 abc	404.7 ab*	0.29 abc*
NH_4_ Rock-P _CombifactorA	341.7 a*	20.4 bc	405.0 ab*	0.30 ab*
NO_3_TSP	289.0 abc	25.0 a	443.1 a	0.26 abc

4.1.3.5 Field experiment

To evaluate the beneficial effects of stabilized ammonium fertilization on plant-PSM interactions in maize under practice conditions, a field experiment was established at the experimental Station Castel Volturno, in an agricultural area 60 km north of Naples, (CE) Campania, Italy, on an alkaline clay loam soil (Vertic Xerofluvent) pH 8.6 with moderate P availability (11 mg kg^{-1} soil) according to P_{Olsen} extraction [25].

The investigated BEs comprised combination products of bacterial and fungal strains pre-tested in the pot experiments (Rhizovital = *Bacillus amyloliquefaciens* FZB42, Combifector A = Trichoderma harzianum OMG16 + Vitabac (5 *Bacillus* strains), Combifector B = *Trichoderma*

harzianum OMG16 and *Bacillus amyloliquefaciens* FZB42). The microbial strains were combined with Zn/Mn (Combifector A/B), humic acids (FZB42 + HA) or seaweed extract (*B. amyloliquefaciens* + SW) and applied by soil incorporation (Combi-A/B, FZB42+HA) and via seed dressing (*B. amyloliquefaciens* + SW). The treatments comprised variants without P fertilization supplied with DMPP-stabilized ammonium sulfate with or without application of microbial inoculants. A triple superphosphate combination with stabilized ammonium was included as a positive control with soluble P supply and a farmer´s practice variant with urea and non-stabilised di-ammonium phosphate fertilization was included as an additional control. An intermediate harvest during early growth of the maize plants was performed at the V6 stage (42 DAS) and final grain yield was recorded at V12.

Table 4.2: Shoot dry matter, P and N-nutritional status during early growth (42 DAS) and final grain yield of Maize (cv Limagrain 30.600) on an alkaline clay loam soil (Vertic Xerofluvent, pH 8.6) with and without (no P) P fertilization in form of triple superphosphate (TSP) or di-ammonium phosphate (DAP). Nitrogen was supplied as DMPP-stabilized ammonium sulfate (stabilized NH_4^+) or non-stabilized Urea-DAP. In the PSM variants, phosphate fertilization was replaced by selected PSM products: Combifector-A (Combi-A), Combifector-B (Combi-B), *Bacillus amyloliquefaciens* FZB42 (FZB42) + humic acids (HA), *B. amyloliquefaciens* + seaweed extract. Nutrient (P, N) data refer to shoot concentrations in % and to shoot contents per plant (data in brackets). Means of four replicates. Different letters indicate significant differences, One-way ANOVA ($p < 0.05$).

Treatment	Shoot DM 42 DAS [g]	Grain Yield [t ha-1]	Shoot-P (42 DAS) % [mg plant^{-1}]	Shoot-N (42 DAS) % [mg plant^{-1}]
Stabilized NH_4^+_no P	33.3c	15.3d	0.45a [0.15b]	3.3a [1.08c]
Stabilized NH_4^+_ TSP	41.2ab (+24%)	16.1ab (+5.2%)	0.48a [0.20ab]	3.2a [1.35ab]
Stabilized NH_4^+_Combi-A	42.4ab (+27%)	15.9ab (+3.9%)	0.47a [0.20ab]	3.3a [1.42ab]
Stabilized NH_4^+_ Combi-B	46.7a (+40%)	16.0ab (+4.0%)	0.48a [0.22a]	3.4a [1.58a]
Stabilized NH_4^+_FZB42+HA	44.4a (+33%)	16.3a (+6.5%)	0.47a [0.21a]	3.3a [1.48a]
Stabilized NH_4^+_*B. amyloliquefaciens* + seaweed extract	45.6a (+37%)	15.6bcd (+1.9%)	0.44a [0.20ab]	3.3a [1.50a]
Urea_DAP (farmers practice)	36.6c (+10%)	15.8abc (+3.2%)	0.48a [0.18ab]	3.2a [1.35ab]

During early growth, plant biomass production of the control without P fertilization supplied with stabilized ammonium was significantly increased by 24% after supplementation with soluble P (TSP). Replacing TSP application by PSM inoculation resulted in even stronger responses in shoot biomass production, with the largest effect induced by Combifector-B (+40%). Early plant growth promotion translated into a significant increase in grain yield by 0.8

t ha^{-1} (+5.2 %) in the TSP variant, while significant yield effects in the PSM treatments were recorded for CombifectorA/B and the FZB42+HA variants, with the largest effect (1.0 t ha^{-1}, + 6.5 %) in the Combifector-B-ammonium combination. The farmer´s practice fertilization with non-stabilized urea and di-ammonium phosphate had no significant effects in terms of biomass production during plant establishment and the smallest effect on final grain yield (+3.2%), as compared with the control supplied with stabilized ammonium without P fertilization. The P status was sufficient, and the N status was low to critical without significant treatment differences [24]. Shoot P accumulation was significantly increased by Combi-B and FZB42+HA application and N accumulation increased particularly in response to the PSM treatments, while this effect was less expressed in the farmer´s practice and TSP variants (Table 4.2).

4.1.4. Discussion

Understanding the contribution of PSM inoculants to plant growth promotion and the best conditions for their efficient performance at a mechanistic level is a challenge. Many studies have characterized the solubilization potential of PGPMs from sparingly soluble tri-Ca-phosphate on artificial media, followed by pot and/or field experiments with inoculated host plants on P limited soils, and successful examples of plant growth promotion are frequently interpreted as a result of microbial P solubilization [6]. There is no doubt that soil microbial activities play an important role in P mineralization and for solubilization of sparingly soluble mineral forms of soil P. However, PSM - host plant interactions involved in plant growth promotion are obviously more complex.

4.1.4.1 PGPM effects on artificial sand/sub-soil substrates

Testing a range of four PSM inoculants, based on six bacterial and fungal strains with proven potential for solubilization of sparingly soluble tri-calcium phosphate [26, 27, 20], was

performed in a culture system, based on a calcareous Loess subsoil (pH 7.6) with sparingly-soluble Ca-phosphate (Rock-P) as sole P source. Maize was used as a crop with a low inherent potential for root-induced P solubilisation [4]. Under these conditions, plant P uptake was almost exclusively dependent on Ca-P mobilization. However, all tested inoculants with P-solubilizing potential completely failed in terms of plant growth promotion and PSM-assisted P acquisition, associated with a severely P-deficient nutritional status of the host plants (1.1 mg g^{-1} shoot DM). Normal plant development required supplementation with soluble triple-superphosphate (TSP; Fig. 1). Similar results have been recently reported for experiments conducted in seven countries on soils with low-P availability and/or supply of sparingly soluble P sources, such as Rock-P, slags and ashes in four different crops (maize, barley, wheat, tomato) with 13 PSM strains [4, 17, 18, 28]. Based on these findings, it was hypothesized that on neutral to alkaline soils, a high pH buffering capacity might be a major factor, limiting the efficiency PSM-induced P solubilisation in the rhizosphere via release of protons, organic and mineral acids [6]. Similar limitations have been previously reported also for mobilization of P or Fe via root-induced rhizosphere acidification [4, 29].

To test this hypothesis, the experiment was repeated with the same calcareous sub-soil, mixed with 70% (w/w) quartz sand to reduce the pH-buffering capacity of the substrate. As an additional variant, nitrate-based N fertilization was partially replaced by stabilized ammonium, to promote rhizosphere acidification via proton release from plant roots and microorganisms for charge-balance of ammonium uptake [4]. Under the conditions of lower substrate buffering, already Rock-P supply tended to increase plant biomass production, but a significant effect compared with the unfertilized control (+207 %) was recorded exclusively in combination with the *Pseudomonas* strain DMSZ 13134 (Proradix) pre-elected as most efficient PSM strain in pilot experiments [20]. As expected, ammonium fertilization further stimulated shoot biomass

production but again a significant effect compared with the nitrate variant (+130%) was recorded only in combination with Proradix (Fig. 2A). Interestingly, in contrast to the effects on plant growth promotion, shoot P accumulation was significantly increased by Rock-P fertilization in the nitrate variant (+110 %) and by ammonium versus nitrate fertilization (+71%), without additional effects induced by PSM inoculation. This finding suggests that PSM-induced plant growth promotion was not simply a consequence of PSM-mediated P solubilisation. Taken together, the results indicated that the pH-buffering capacity of the substrate can indeed represent a limiting factor for PSM-assisted fertilization strategies to improve plant acquisition of acid-soluble Ca-P fractions in soils. The combination with stabilized ammonium fertilizers supporting rhizosphere acidification may act as a suitable strategy to promote PSM performance. However, the effects are not necessarily related to the direct promotion of the P-solubilizing potential of the PSM inoculants.

4.1.4.2 PGPM effects on field soil

Plant growth-promoting effects of microbial inoculants can be demonstrated most easily on artificial growth substrates lacking a native soil microflora with potentially competing properties in terms of root colonization. This was of course also the case in the first experiment demonstrating positive PSM effects conducted on an artificial sand-subsoil mixture. Therefore, the experiment was repeated, using a real field soil pH 6.8 with moderately low P availability (P_{CAL} 37 mg kg^{-1}) and Rock-P fertilization. Again, stabilized ammonium fertilization combined with Rock-P increased the shoot biomass compared with the unfertilized control. Shoot biomass production was further increased by combining Proradix with stabilized ammonium, which was not the case for the combination of Proradix with nitrate fertilization (Fig. 3). In this experiment, additionally, a wider range of pre-selected PSMs [20] was tested under the same conditions. The selection comprised five single-strain inoculants with two fungal (Trianum-P =

*Trichoderma harz*ianum T22, BFOD = *Penicillium* sp) and three bacterial strains (Proradix = *Pseudomonas* sp. DMSZ 13134; Rhizovital = *Bacillus amyloliquefaciens* FZB42; *Paenibacillus mucilaginosus*), as well as two consortium products (SP11 used in experiment 1 and Combifector-A (= *Trichoderma harzianum* OMG16 + Vitabac (five *Bacillus* strains) + Zn/Mn).

Except for the two fungal strains, all PSMs significantly increased shoot biomass production in combination with stabilized ammonium fertilization and reached about 88 % of the biomass of the plants supplied with soluble TSP (Fig. 3). This was associated with a clear trend for increased root length development with significant effects for FZB42 (+31%) and Combifector-A (+50%). By contrast, the two fungal inoculants and the nitrate-Proradix combination, ineffective in shoot growth promotion, also had no or only marginal effects on root growth. Like the experiment with the artificial sand-sub-soil substrate (70/30), ammonium fertilization in combination with Rock-P significantly increased the P tissue concentrations compared with the unfertilized control but no further increase was recorded after PSM inoculation (Table 4.1). Shoot accumulation of P was significantly increased after inoculation with the bacterial PSM Proradix and the fungal PSM Trianum-P, but the fungal strain had no effect on root growth or shoot biomass production (Fig. 4.3, Table 4.2). By contrast, Combifector-A with the largest impact on root length development (+50%) had no significant effect on shoot P accumulation (Fig. 4.3B, Table 4.2). The results indicated once more, that the plant growth-promoting effects of the investigated inoculants in combination with stabilized ammonium fertilization were obviously not related to direct PSM-assisted P solubilisation in the rhizosphere. However, a closer look at the mineral nutritional status of the plants revealed that P was not the only limiting nutrient, and critical levels of N (< 30 mg g^{-1} DM) and Mn concentrations (0.02 mg g^{-1} DM) were recorded in the control treatment supplied with stabilized ammonium and Rock-P fertilization (Table 4.1). The microbial inoculants increased, both, the N and Mn nutritional

status to the sufficiency range (Table 4.1) and a significant increase of N and Mn shoot accumulation was observed for the Proradix, Rhizovital, SP11 and Combifector-A - ammonium combinations by pairwise comparison with the non-inoculated control (Table 4.2), associated with increased shoot biomass production (Fig. 4.3A).

Taken together, the results suggest a scenario of synergistic interactions between fertilizer supply and plant growth-promoting properties of the selected PSM inoculants: on neutral to alkaline soils with low P availability, crops with a low inherent potential for P solubilisation are frequently facing problems of P limitation. The inoculation with PGPMs to improve plant P acquisition is not successful in this case since the weak P-deficient plants are not able to support efficient root colonization by the PSM inoculants and the establishment of functional plant-microbial interaction. The fertilization with stabilized ammonium fertilizers could partially overcome this limitation by improving the P nutritional status, probably mediated by the well-documented root-induced rhizosphere acidification [4], contributing to solubilisation of Ca-phosphates. The more vital status of these plants promoted root colonisation by the microbial inoculants, which were, in turn, able to express their plant growth-promoting potential. This is in line with previous reports on beneficial effects of P starter supply on the establishment of arbuscular mycorrhizal associations and the Rhizobium symbiosis in leguminous plants [30, 31]. It also confirms the findings of the recent meta-analysis by Schütz et al. [15], which demonstrated that plant growth-promoting effects of PSMs can be expected on soils with moderately low P availability (25-35 kg P ha^{-1}) but not on low-P soils or under sufficient P supply. Under these conditions, plant growth promotion is not necessarily caused by PSM-mediated P solubilisation. Stimulation of root growth induced by the inoculants can contribute to the acquisition of other potential growth-limiting nutrients and may also promote ammonium-induced P solubilization by the development of a larger acidifying root system.

4.1.4.3 PGPM effects under field conditions

This scenario was evaluated additionally under field conditions on an alkaline clay loam soil (Vertic Xerofluvent) pH 8.6 with a P availability (P_{Olsen}: 11 mg kg^{-1} soil) considered as moderate for maize cultivation [25, 32]. In face of the high soil pH and moderate P availability [25], no Rock-P fertilization was included into this experiment and the performance of microbial inoculants in combination with stabilized ammonium was compared without P fertilization versus TSP fertilization and fertilization according to farmers practice, which comprised di-ammonium phosphate and urea without nitrification inhibitors. Due to promising plant growth-promoting effects of PGPM combinations in the previous experiment (Fig. 4.3a), a range of consortium products were tested as microbial inoculants: Combifector-A (see 4.2) Combifector-B (*Trichoderma harzianum* OMG16 + *Bacillus amyloliquefaciens* FZB42 + Zn/Mn), *Bacillus amyloliquefaciens* FZB42 + humic acids, Bacillus amyloliquefaciens FZB42 + seaweed extract. Since many studies have demonstrated the importance of early root development as a critical trait determining yield formation of maize particularly with respect to P acquisition [33-35], special emphasis was placed on the selection of PSMs with additional root growth-promoting potential (Fig. 4.3B).

Stabilized ammonium fertilization combined with TSP significantly improved field establishment of maize indicated by a 24% increase in shoot biomass production at 42 DAS, as compared with the unfertilized control. This finding demonstrates that P availability was a growth-limiting factor. The importance of ammonium in this context is highlighted by the absence of growth-promoting effects in the farmer's fertilization practice using DAP and urea without nitrification inhibitors, leading to the rapid conversion of NH_4^+-N to nitrate in this treatment. However, even without additional P fertilization, the application of the PSM inoculants in combination with stabilized ammonium fully compensated the P fertilization

effect of TSP and reached up to 40% increased biomass production in the Combifector-B variant, which was even larger than any plant growth-promoting effect recorded in the pot experiments under controlled conditions (Table 4.2, Fig 4.3A). The effect of Combifector-A (+27%) on shoot biomass production was almost identical with the result of the pot experiment (+28%). At the time of the intermediate harvest, no treatment differences were recorded for shoot P and N concentration, but the N status was low [24]. The shoot P content and particularly shoot N accumulation significantly increased in response to TSP and PSM applications. This effect coincided with increased shoot biomass production, indicating that any surplus in nutrient uptake was immediately transformed into plant growth. Similarly, shoot accumulation of micronutrients (Zn, Mn, and Cu) significantly increased particularly in the PSM treatments (Supplementary Figure S1) without significant effects on the tissue concentrations, which reached the sufficiency range in all treatments [24]. The general stimulatory PSM effect on shoot accumulation of various macro- and micro-nutrients suggests root growth stimulation rather than P solubilization as a mode of action for the selected inoculants.

The improved field establishment during early growth finally translated into a significant increase in grain yield of 5.2% with TSP fertilization and of 6.5% in the FZB42 + humic acids variant, while farmer´s practice fertilization had smallest yield effect (+3.2%) compared with stabilized NH_4^+ variant without P supply. Large effects on early field establishment may be attributed to the limited expression of adaptive responses towards improved P acquisition during the early growth of maize [33, 4, and 21]. Localized P starter application is one of the measures to mitigate this problem [36]. Increased P availability due to ammonium-induced rhizosphere acidification in response to stabilized ammonium fertilization may induce a similar effect, followed by improved P acquisition in combination with the PSM strains with a high root growth-promoting potential, such as Combifector-A or FZB42 (Fig. 4.3B). However, nitrification

inhibitors, such as DMPP are usually active in soils only for limited time periods of several weeks due to microbial degradation [37], and also PGPM inoculants frequently exhibit only transient effects. Therefore, no direct long-lasting effects on P solubilisation can be expected. Moreover, the initial limitations in P acquisition may be at least partially compensated e.g. by more intensive rooting or the establishment of mycorrhizal associations in later stages of plant development [32] and the moderate P availability at the investigated field site. This could explain the limited translation of early growth effects into yield increases of only 5-6 %.

4.1.5. Concluding remarks

The present study demonstrates that the expression of the plant growth-promoting and P-solubilizing potential of a wide range of bacterial and fungal PSM inoculants can be selectively influenced by the form of N supply to the host plant with promising perspectives for synergistic effects with stabilized ammonium fertilization. The results clearly demonstrate that the beneficial effects are not necessarily related to a direct improvement of the P solubilizing potential of the PSM strains. It remains to be established, to which extent root-induced rhizosphere acidification in response to ammonium uptake contributes to the expression of the effects. Increased auxin production potential of the inoculants with ammonium as preferential N source [38] or a stimulatory effect on ammonium-induced proton extrusion of plant roots recently reported for selected *Bacillus* strains [39] as well as stimulation of rhizosphere acid phosphatase activities in response to a lower rhizosphere pH could provide additional explanations. It can be also expected that not only plant-PSM associations but also inoculants expressing only root growth-promoting activity would profit from the combination with stabilized ammonium fertilizers. In these cases, the formation of a larger acidifying root system may contribute to solubilisation of acid-soluble P sources (e.g. Ca-P, Rock-P, ashes, slags etc.) as well as micronutrients (Fe. Zn, Mn, Cu) at least on soils with neutral to alkaline pH. This would

not only support plant species with low inherent potential for root-induced nutrient mobilization but also the expression of adaptive mechanisms for solubilisation of sparingly soluble soil nutrients.

The finding that a wide range of different bacterial and fungal inoculants had beneficial effects on plant growth and/or nutrient mobilization in combination with stabilized ammonium fertilization raises the question whether also native populations of PGPMs could be influenced in a similar way. These interactions might at least partially contribute to the positive effects on nutrient acquisition and plant growth promotion observed in the non-inoculated controls supplied with stabilized ammonium fertilization. Apart from rhizosphere acidification, ammonium-dominated fertilization also significantly modifies the composition of root exudates compared with nitrate supply [40, 41] due to intense transcriptomic, proteomic and metabolic alterations related with the assimilation of ammonium [42, 43]. Accordingly, distinct rhizosphere microbiome effects can be expected. However, surprisingly numerous studies have addressed the impact of N fertilization intensity on soil microbial communities [44-46], while N form effects have rarely been investigated so far [47]. These aspects need to be considered for future investigations together with the impact of different soil properties, climatic conditions and genotypic differences in crop responsiveness, to evaluate the potential of stabilized ammonium fertilizers as tools to manipulate plant interactions with plant growth-promoting microorganisms.

Patents: Some result in this article has been submitted for a joint patent application in 2017 by University of Hohenheim and Eurochem Agro GmbH to the European Patent Office (application number EC70522EP SF/IRK on “Method and Composition for Improving Nutrient Acquisition of Plants”.

Supplementary Materials: The following figure is available online at www.mdpi.com/xxx/s1, Figure S1: Micronutrient shoot accumulation during early growth (42 DAS) of Maize (cv Limagrain 30.600) on an alkaline clay loam soil (Vertic Xerofluvent, pH 8.6) with and without (NoP) P fertilization in the form of triple superphosphate (TSP) or di-ammonium phosphate (DAP). Nitrogen was supplied as DMPP-stabilized ammonium sulfate or non-stabilized Urea-DAP. In the PSM variants, phosphate fertilization was replaced by selected PSM products: Combifector-A (Combi A), Combifector-B (Combi B), Bacillus amyloliquefaciens FZB42 + humic acids (FZBHA), B. amyloliquefaciens + seaweed extract (BaSE). Means of four replicates. Different letters indicate significant differences; One-way ANOVA ($p < 0.05$) title, Table S1: Effects of nitrate (NO_3) versus stabilized ammonium (Stab. NH_4) fertilization on shoot growth and yield formation of different crops with and without inoculation with microbial biostimulants (BS).

Author Contributions: Conceptualization, GN, IKM, PMN, VC, AP; Investigation, IKM, MK, VC; Resources JG, NB; Writing-Original Draft Preparation, IKM; Writing-Review & Editing, GN, AP, JG, NB, PMN, UL; Project Administration GN, UL, AP, JG; Funding acquisition UL, GN, AP, JG.

Funding: This work was funded by the European Community's FP7 Program/2007-2013 under grant agreement number 312117 (BIOFECTOR).

Acknowledgements: The authors wish to thank ABITEP GmbH, Berlin, Germany; Bayer Crop Science Biologics GmbH, Wismar, Germany; Koppert, Biological Systems, Berkelen Rodenrijs, The Netherlands; Sourcon Padena GmbH, Tübingen, Germany; Vitalin Pflanzengesundheit GmbH, Ober-Ramstadt, Germany for providing PSM strains.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

4.1.6 References

1. Zhou K, Binkley D, Doxtader KG (1992) A new method for estimating gross phosphorus mineralization and immobilization rates in soils. Plant Soil 147:243–250.

2. Schachtman DP, Reid RJ, Ayling SM. (1998). Phosphorus uptake by plants: From soil to cell. Plant Physiol 116: 447-453.
3. Marschner H. (1995) Rhizosphere pH effects on phosphorus nutrition. In: Johansen C, Lee K. K, Sharma K. K, Subbaro G. V, Kueneman E. A, eds. Genetic Manipulation of Crop Plants to Enhance Integrated Nutrient Management in Cropping Systems. 1. Phosphorus. Proceedings of an FAO/ICRISAT Expert Consultary Workshop. ICRISAT Asia Center, India. Patancheru, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics. Pp 107-115.
4. Neumann, G.; Römheld, V. Root-induced changes in the availability of nutrients in the rhizosphere. In Plant Roots the Hidden Half, 3rd ed.; Waisel, Y., Eshel, A., Kafkafi, U., Eds.; Marcel Dekker: New York, NY, USA, 2002; pp. 617–649.
5. Neumann, G., and Römheld, V. (2007): The release of root exudates as affected by the plant physiological status. In: Pinton, R., Varanini, Z., Nannipieri, Z. (eds.) The Rhizosphere: Biochemistry and organic substances at the soil-plant interface. 2nd ed. CRC Press, pp.23- 72.
6. Sharma, B.S.; Riyaz, Z.; Sayed, M.H.T.; Thivakaran, A.G (2013). Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. Springer Plus, 2, 1–14.
7. Jongmans AG, van Breemen N, Lundström U, an Hees PAW, Finlay RD, Srinivasan M, Unestam T, Giesler R, Melkerud P, Olsson M (1997) Rock-eating fungi, Nature 389: 682-683.
8. Uroz S, Calvaruso C, Turpault MP, Frey-Klett P (2009) Mineral weathering by bacteria: ecology, actors and mechanisms. Trends in Microbiology 17: 378-387.
9. Khan, A.A.; Jilani, G.; Akhtar, M.S.; Naqvi, S.M.S.; Rasheed, M. Phosphorus solubilizing bacteria: Occurrence, mechanisms and their role in crop production. J. Agric. Biol. Sci. 2009, 1, 48–58.
10. Rodríguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnology Advances 17: 319–339.
11. Alori ET, Glick BR and Babalola OO (2017) Microbial phosphorus solubilization and its potential for use in sustainable agriculture. Front. Microbiol. 8:971. doi: 10.3389/fmicb.2017.00971.
12. Menzies, N.; Harbison, D.; Dart, P (2011). Soil chemistry-facts and fiction and their influence on the fertilizer decision making process. In Proceedings of the 26th Annual Conference of the Grassland Society of NSW, Bathurst, Australia, 26–28; pp. 49–63.
13. van Veen JA, van Overbeek LS, van Elsas JD (1997) Fate and activity of microorganisms introduced into soil. Microbiol Mol Biol R. 611: 21–35.
14. Lugtenberg B, Kamilova F (2009). Plant-growth-promoting rhizobacteria. Annu Rev Microbiol. 63: 541–556.
15. Schütz, L.; Gattinger, A.; Meier, M.; Muller, A.; Boller, T.; Mäder, P.; Mathimaran, N. (2017). Improving crop yield and nutrient use efficiency via biofertilization–A global meta-analysis. Front. Plant Sci., 8.
16. Abbasi, M.K.; Musa, N.; Manzoor, M (2015). Mineralization of soluble P fertilizers and insoluble rock phosphate in response to phosphate-solubilizing bacteria and poultry manure and their effect on the growth and P utilization efficiency of chilli (Capsicum annuum L.). Biogeosciences, 12, 4607–4619.
17. Thonar, C.; Lekfeldt, J.D.S.; Cozzolino, V.; Kundel, D.; Kulhánek, M.; Mosimann, C.; Neumann, G.; Piccolo, A.; Rex, M.; Symanczik, S.; et al (2017). Potential of three microbial bio-effectors to promote maize growth and nutrient acquisition from

alternative phosphorous fertilizers in contrasting soils. Chem. Biol. Technol. Agric., 4, 7.
18. Mpanga IK, Dapaah HK, Geistlinger J, Ludewig U, Neumann G (2018): Soil type-dependent interactions of P-solubilizing microorganisms with organic and inorganic fertilizers mediate plant growth promotion in tomato. Agronomy 2018, 8, 213; doi:10.3390/agronomy8100213.
19. 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, 15.
20. Nkebiwe P.M., Neumann G., Müller T. (2017): Densely rooted rhizosphere hotspots induced around subsurface NH4+-fertilizer depots: a home for soil PGPMs? Chem. Biol. Technol. Agric. (2017) 4: 29. https://doi.org/10.1186/s40538-017-0111-y.
21. Liu H, White PJ, Li C (2016) Biomass partitioning and rhizosphere responses of maize and faba bean to phosphorus deficiency. Crop & Pasture Science 67: 847-856 http://dx.doi.org/10.1071/CP16015.
22. VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten e.V. Speyer, Germany). Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik Methodenbuch Band I Die Untersuchung von Böden, 4th ed.; VDLUFA Verlag: Darmsatdt, Germany, 1991.
23. Gericke, S.; Kurmis, B. Die kolorimetrische Phophorsäurebestimmung mit Ammonium-Vanadat-Molybdat und ihre Anwendung in der Pflanzenanalyse. Z. Pflanzenernaehr. Bodenkd 1952, 59, 235–247.
24. Campbell, R.C. Reference sufficiency ranges for plant analysis in the southern region of the United States. In Southern Cooperative Series Bulletin #394; North Carolina Department of Agriculture and Consumer Services Agronomic Division: Raleigh, NC, USA, 2009; p. 11.
25. Cottenie A. (1980) FAO Soils Bulletin 38/1, Soil and Plant Testing and Analysis, Food and Agriculture Organisation of the United Nations, Rome, Italy.
26. Gulden, R. H.; Vessey, J. K (2000). Penicillium bilaii inoculation increases root-hair production in field pea. Can. J. Plant Sci., 80, 801–804.
27. 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, 1–9, doi:10.1080/17429145.2011.597002.
28. Lekfeldt, J.D.S.; Rex, M.; Mercy, F.; Magid, K.; Tlustoš, P.; Magid, J. (2016). Effect of bioeffectors and recycled P-fertiliser products on the growth of spring wheat. Chem. Biol. Technol. Agric., 3, 22.
29. Jing J, Ruia Y, Zhanga 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 Crops Res 119: 355–364.
30. Bittman S, Kowalenko CG, Hunt DE, Forge TA, Wu X (2006) Starter phosphorus and broadcast nutrients on corn with contrasting colonization by mycorrhizae. Agronomy J. 98: 394-401.
31. Chekanaia V, Chikowoa R, Vanlauwe B (2018) Response of common bean (Phaseolus vulgaris L.) to nitrogen, phosphorus and rhizobia inoculation across variable soils in Zimbabwe. Agriculture, Ecosystems and Environment 266: 167–173.
32. Deng Y, Chen K, Teng W, Zhan A, Tong Y, Feng G, Cui Z, Zhang F, Chenet X (2014) Is the Inherent Potential of Maize Roots Efficient for Soil Phosphorus Acquisition? PLoS ONE 9(3): e90287. doi:10.1371/journal.pone.0090287.

33. Hajabbasi MA, Schumacher TE (1994) Phosphorus effects on root growth and development in two maize genotypes. Plant Soil 158: 39-46.
34. Jiang, H, Zhang J, Han Z, Yang J, Ge C, Wu Q (2017) Revealing new insights into different phosphorus-starving responses between two maize (Zea mays) inbred lines by transcriptomic and proteomic studies. Sci. Rep. 7, 44294; doi: 10.1038/srep44294.
35. Jia X, Liu P, Lynch JP (2018) Greater lateral root branching density in maize improves phosphorus acquisition from low phosphorus soil. J Exp Bot 69: 4961–4790.
36. Nkebiwe, P.M., Weinmann, M., Bar-Tal, A., Müller, T. (2016b). Fertilizer placement to improve crop nutrient acquisition and yield: a review and meta-analysis. Field Crops Research 196:389-401.
37. Benckiser G, Christ E, Herbert T, Weiske A, Blome J, Hardt M (2013) The nitrification inhibitor 3,4-dimethylpyrazole-phosphate (DMPP) - quantification and effects on soil metabolism. Plant Soil DOI 10.1007/s11104-013-1664-6.
38. Bharucha U.,Patel K.,Trivedi U.B., (2013). Optimization of indole acetic acid production by Pseudomonas putida UB1 and its effect as plant growth-promoting rhizobacteria on mustard (Brassica nigra). Agric Res. 2: 215–221.
39. Ögüt, M.; Er, F.; Neumann, G. (2011). Increased proton extrusion of wheat roots by inoculation with phosphorus solubilising microorganisms. Plant Soil, 339, 285–297.
40. Brown ME, Hornby D. (1987). Effects of nitrate and ammonium on wheat roots in gnotobiotic culture: Amino acids, cortical cell death and take-all (caused by Gaeumannomyces graminis var. Tritici). Soil Biol. Biochem., 19: 567-573.
41. Imas P, Bar-Yosef B, Kafkafi U, Ganmore-Neumann R. (1997). Release of carboxylic anions and protons by tomato roots in response to ammonium nitrate ratio and pH in nutrient solution. Plant and Soil, 191: 27-34.
42. Yang H, Menz J, Haussermann I, Benz M, Fujiwara T, Ludewig U. (2015). High and Low Affinity Urea Root Uptake: Involvement of NIP5;1. Plant Cell Physiol, 56: 1588-1597.
43. Menz J, Li Z, Schulze WX, Ludewig U. (2016). Early nitrogen-deprivation responses in Arabidopsis roots reveal distinct differences on transcriptome and (phospho-) proteome levels between nitrate and ammonium nutrition. Plant J, 88: 717-734.
44. Sommermann L, Geistlinger J, Wibberg D, Deubel A, Zwanzig J, Babin D, Schluter A, Schellenberg I. (2018). Fungal community profiles in agricultural soils of a long-term field trial under different tillage, fertilization and crop rotation conditions analyzed by high-throughput ITS-amplicon sequencing. PLoS One, 13: e0195345.
45. Babin D, Deubel A, Jacquiod S, Sørensen SJ, Geistlinger J, Grosch R, Smalla K. 2018. Impact of long-term agricultural management practices on soil prokaryotic communities. Soil Biol Biochem: (in press).
46. Yua H, Ning Ling N, Wang T, Zhub C, Wanga Y, Wanga S, Ga Q (2019). Responses of soil biological traits and bacterial communities to nitrogen fertilization mediate maize yields across three soil types Soil & Tillage Res 185: 61-69.
47. Mahmood T, Kaiser WM, Ali R, Ashraf M, Gulnaz G, Iqbal Z (2005) Ammonium versus nitrate nutrition of plants stimulates microbial activity in the rhizosphere. Plant and Soil 277:233-243.

4.2 The role of N form supply for PGPM-host plant interactions in maize

Mpanga I. K. [1], Gomez-Genao N. J.[1], Moradtalab N. [1], Wanke D. [1], Chrobaczek V. [1], Ahmed A. [1], Windisch S. [1], Hafiz F. B.[2]Geistlinger J. [2], Walker F.[3], Ludewig U. [1], Neumann G. [1]

1. Institute of Crop Sciences (340h), Universität Hohenheim, Stuttgart, 70593, Germany.
2. Institute of Bioanalytical Sciences, Anhalt University of Applied Sciences, 06406 Bernburg, Germany
3. Institute of Phytomedicine (360), Universität Hohenheim, Stuttgart, 70593, Germany.

Isaac Kwadwo Mpanga[1] mpangaisaac75@gmail.com, +4915217489408

Ninoska Gomez-Genao[1], ninoskagomez31@gmail.com

Narges Moradtalab[1], moradtalab.nersy@yahoo.com, +4917635919859

Daniel Wanke [1] daniel.wanke@uni-hohenheim.de

Victor Chrobaczek[1] victor.chrobaczek@googlemail.com

Aneesh Ahmed[1], aneesh.ahmed@uni-hohenheim.de, +4915218552590

Saskia Windisch[1], saskia.windisch@uni-hohenheim.de, +4917682141670

Joerg Geistlinger[2], joerg.geistlinger@hs-anhalt.de, +4934713551265

Fatema Binte Hafiz[2], fatema.hafiz@hs-anhalt.de, +493471 355 1267

Frank Walker[3], frank.walker@uni-hohenheim.de

Uwe Ludewig[1], u.ludewig@uni-hohenheim.de, +49 711459 22344

Gunter Neumann[1], gd.neumann@t-online.de, +49711 459 24273

Accepted in *Journal of Plant Nutrition and Soil Sciences* on 4th August 2019

Abstract: The form of nitrogen (N) supply has a significant impact on rhizosphere chemistry and root growth responses of higher plants. The respective effects are also employed as management options to improve nutrient acquisition and to minimize nutrient losses in

cropping systems. However, surprisingly little is known concerning the interactions with rhizosphere biota. In this study, we investigated the effects of selected bacterial and fungal inoculants with proven plant growth- promoting and phosphate (P) solubilizing potential (plant growth-promoting microorganisms, PGPM) in maize with nitrate or stabilized ammonium supply, on soils with limited P availability and sparingly soluble rock phosphate (Rock-P) applied as P fertilizer. The combination of the bacterial inoculants *Pseudomonas sp.* DMSZ 13134 (Proradix) and *Bacillus amyloliquefaciens* FZB42 with ammonium sulphate fertilization, stabilized with the nitrification inhibitor 3,4-dimethylpyrazole-phosphate (DMPP), resulted in a superior shoot biomass production (79 - 111%) and shoot P accumulation (109 – 235%) as compared with nitrate supply. This effect could be partially attributed to (i) ammonium-induced rhizosphere acidification via increased root extrusion of protons, (ii) promotion of root hair elongation and (iii) increased shoot concentrations of hormonal growth regulators (indole-3-acetic acid, zeatin, gibberellic acid). The effects, induced by the microbial inoculants were mainly related to increased root length development (43 - 44%), associated with a 60% increase in auxin production potential. No inoculant effects were detected on root hair elongation or on chemical modifications of the rhizosphere involved in P solubilisation, such as rhizosphere acidification, release of carboxylates or secretory phosphohydrolases. However, the ammonium-induced stimulation of root hair elongation increased preferential sites for root colonization by the selected inoculants, which may explain the increase in rhizosphere abundance of PGPMs, exemplarily recorded for the fungal inoculant *Trichoderma harzianum* OMG16 (210%). The presented data suggest a network of positive interactions between stabilized ammonium fertilization and plant growth-promoting functions of various bacterial and fungal PGPM inoculants. This offers perspectives to increase the efficiency and the reproducibility of PGPM-assisted fertilization strategies.

Keywords: Maize, nitrogen form, phosphate solubilisation, plant growth-promoting microorganisms (PGPM), rhizosphere, stabilized ammonium

4.2.1 Introduction

Plant-microbial interactions play a central role in nutrient acquisition and stress tolerance of higher plants in natural and agro-ecosystems. Negative impacts are induced by pathogens and positive effects by plant growth-promoting microorganisms (PGPMs). Accordingly, strategies to select highly efficient PGPM strains as plant inoculants, to improve nutrient acquisition and biotic and abiotic stress tolerance, has been investigated for decades. The principle effectiveness of these strategies is well-documented, particularly under controlled environmental conditions. However, limited reproducibility of the expected effects in real field applications remains a major problem (*Menzies* et al., 2011). This is not surprising since the establishment of beneficial plant-microbial interactions in the rhizosphere depends on numerous external factors. Rhizosphere competence and survival of microbial inoculants can be influenced by interactions with the native soil microbiome and by abiotic stress factors (*van Veen* et al., 1997; *Lugtenberg* and *Kamilova*, 2009). Moreover, in agricultural systems, the amount (*Dogra* and *Dudeja* 1993; *Nouri* et al., 2015; *Huang* et al., 2017; *Schütz* et al., 2017) and the type of the applied fertilizers can also play an important role. In the latter context, the preferential performance of PGPMs in combination with N-rich organic fertilizers has been repeatedly reported (*Abbasi* et al., 2015; *Thonar* et al., 2017; *Mpanga* et al., 2018; *Bradacova* et al., 2019). Recently, *Mpanga* et al. (2019) found an improved plant performance associated with a wide range of fungal and bacterial PGPMs in combination with stabilized ammonium fertilizers as compared with nitrate fertilization. Table 4.3 summarizes the effects in maize (*Mpanga* et al., 2019; *Nkebiwe* 2016), wheat (*Nkibewe* 2016) and tomato (*Mpanga* et al., 2018; *Bradacova* et al., 2019).

Table 4.3: Effects of nitrate (NO_3) versus stabilized ammonium (Stab. NH_4) fertilization on shoot growth and yield formation of different crops with and without inoculation with microbial biostimulants (BS). Proradix = *Pseudomonas* sp. DMSZ13134 (Sourcon Padena, Tübingen Germany); FZB42 = *Bacillus amyloliquefaciens* FZB42; *Paenibacillus mucilaginosus* (ABITEP, Berlin Germany), Combi-A: *Trichoderma harzianum* OMG16 + Vitabac with five Bacillus strains (Bactiva GmbH, Straelen, Germany) + Zn/Mn, Combi-B: *Trichoderma harzianum* OMG16 + FZB42 + Zn/Mn (Anhalt University of Applied Sciences, Bernburg, Germany), CRENEL = Microbial Consortia Product (Eurochem-Agro. Mannheim, Germany). HA = humic acids from composted cow manure. (Modified after *Mpanga* et al. 2019)

Effects of N-form and BS treatments (Shoot/grain biomass [g]; fruit yield [t ha^{-1}])

Microbial biostimulant (BS)	Soil / pH / P source	NO_3	NO_3+BS	Stab. NH_4	Stab. NH_4+BS	Crop, Culture system	Measured parameter	Reference
Proradix	Loess subsoil	9.3 c	14.5 b	16.9 b	20.4 a	Maize (Pot)	Shoot FW	Nkebiwe 2016
	pH 7.6 Rock-P	10.0 b	15.2 b	20.5 ab	22.9 a	Maize (Pot)	Shoot FW	Mpanga et al. 2019
Proradix	Clay loam, pH		6.9 b	7.4 b	9.8 a	Maize (Pot)	Shoot DM	Mpanga et al. 2019
Combi-B	7.0, Rock-P	2.0 b	3.2 b	4.1 ab	5.8 a	Maize (Pot)	Shoot DM	Mpanga et al. (2019)
Proradix	Clay-loam, pH 7.0, Rock-P		10.4c	13.0b	15.4a	Maize (Pot)	Shoot DM	Nkebiwe (2016)
Combi-A	Clay-loam pH	36.6*b		33.3b	42.4a	Maize (Field)	Shoot DM	Mpanga et al. 2019
Combi-B	8.6, No P	36.6*b		33.3b	46.7a	Maize (Field)	Shoot DM	(field establishment
FZB42+HA		36.6*b		33.3b	44.4a	Maize (Field)	Shoot DM	42 DAS)
Proradix	Silty loam, pH	12.5b	13.5b	11.4b	15.4a	Wheat (Pot)	Grain DM	Nkebiwe (2016)
P. mucilaginosus	6.4, Rock-P	12,5b		11.4b	15.1a	Wheat (Pot)	Grain DM	Nkebiwe (2016)
FZB42	Silty sand, pH 5.6 Rock-P			7.3b	10.0a	Tomato [Pot]	Shoot DM	Mpanga et al. (2018)
CRENEL	Silty loam pH 5.7 starter P	2.1b	2.3b	2.6b	3.4a	Maize (Pot)	Shoot DM	Mpanga et al. (2019)
CRENEL	95% Sand, pH			300b	640a	Tomato	Shoot FW	
	7.9, No P			17.2b	35.8a	(field)	Fruit yield	Bradacova et al. (2019)

*= fertilization according to farmer´s practice: urea + non-stabilized di-ammonium phosphate

Accordingly, *Nkebiwe* et al. (2016) reported increased root proliferation and plant growth promotion by PGPM inoculation combined with ammonium fertilization in maize, both, in laboratory and field experiments. Similar results were reported for P acquisition, plant growth promotion and yield formation of tomato in a drip-irrigated tomato production system with ammonium placement and inoculation of microbial consortia in the Negev desert in Israel (*Bradacova* et al., *2019*). The results of *Mpanga* et al. (2019) suggested that the beneficial effects of stabilized ammonium fertilization on PGPM performance might be at least partially attributed to an improved root-induced mobilization of P and other sparingly soluble nutrients by the well-documented rhizosphere acidification effect of ammonium fertilizers (*Neumann* and *Römheld*, 2002). Particularly on low P soils with neutral to alkaline pH or after application

of sparingly soluble Rock-P fertilizers, this resulted in a starter fertilization effect, improving the vitality status of the plants. Consequently, these plants had a superior potential to promote the establishment of beneficial plant-PGPM interactions in the rhizosphere. This is in line with previous observations on the beneficial impact of starter fertilization on the establishment of arbuscular mycorrhizal associations or the symbiosis with N_2-fixing microorganisms (*Bittman* et al., 2006; *Chekanaia* et al., 2018).

However, it remains to be elucidated whether ammonium-induced rhizosphere acidification is the only factor mediating the observed promotion of beneficial plant-PGPM interactions. Therefore, this study aims at a more detailed dissection of the obvious synergistic effects of ammonium fertilization and PGPM inoculation. Based on literature reports (*Nkebiwe* et al., 2016; *Mpanga* et al., 2019), we investigated a range of hypotheses on the potential functions of ammonium fertilization in the establishment of plant-PGPM interactions: (i) ammonium fertilization induces root-induced rhizosphere acidification, which may be intensified by PGPM inoculation) with a beneficial impact on nutrient availability. (ii) a lower rhizosphere pH stimulates rhizosphere acid phosphatase activities involved in mobilization of organic P sources ; (iii) auxin production and thereby the root growth-promoting potential of PGPMs is stimulated with ammonium as the most abundant N source; (iv) ammonium fertilization has direct beneficial effects on root growth in terms of lateral roots and root hair proliferation. This will promote spatial nutrient acquisition, nutrient mobilization via root exudates and rhizosphere acidification, as well as root colonization by PGPMs. To address these questions, a set of experiments were conducted with *maize* under controlled conditions, using a range of bacterial and fungal inoculants with high rhizosphere competence and proven potential for plant growth promotion in maize (*Nkebiwe* et al., 2016; *Thonar* et al., 2017; *Vinci* et al., 2018a.b, *Mpanga* et al., 2018; 2019).

4.2.2 Materials and Methods

4.2.2.1 Minirhizotron experiment

Plant culture

Minirhizotrons with root observation windows were prepared from PVC pipes (height 48 cm; diameter 10 cm) closed with a PVC bottom plate and longitudinally cut into two halves (Fig. 4.4A). The open parts of the half-pipes were covered with a transparent plexiglass observation window, fixed with adhesive tape (Fig. 4.4A, B). The experiment was conducted in a greenhouse with an average temperature of 26°C and 40-85 % rel. humidity throughout the culture period, using a field soil collected from the A horizon at the experimental station Kleinhohenheim (Hohenheim University, Stuttgart, Germany) and sieved to 2 mm particle size. Soil characteristics: clay-loam, pH_{CaCl2} = 7.0; P_{CAL} = 36.7 mg P Kg^{-1} soil; N_{total}: 0.15%; C_{org}: 1.28%. The soil was mixed with 30% (w/w) quartz sand for improvement of soil structure. This resulted in a moderately low P availability of the growth substrate (class B) according to the VDLUFA regulations (*VDLUFA*, 2018). Each minirhizotron was filled with 2 kg soil-sand substrate. The basal fertilization was performed with (mg kg^{-1} substrate): N 150; supplied as 3,4-dimethylpyrazole-phosphate (DMPP)-stabilized ammonium sulphate (Novatec solub, Compo Expert GmbH, Münster, Germany) or as calcium nitrate; P 100; as rock phosphate (Rock-P: TIMAC Naturphosphat 26, Timac AGRO Troisdorf Germany) or soluble P as single superphosphate (SSP) 18% P_2O_5 (Triferto, Gent, Belgium) for the positive control; K 150 (as K_2SO_4) and Mg 30 (as $MgSO_4$); *maize* L. cv Colisee (KWS Saat, Einbeck, Germany) was used as the test plant. The *Pseudomonas* sp. DMSZ13134 formulation “Proradix” (Sourcon Padena, Tübingen, Germany) was inoculated with 20 mL bacterial suspension by seed and soil drenching. Inoculation was performed by soaking the seeds in the bacterial suspension and air-drying the seeds at room temperature before sowing followed by drenching three times at

weekly intervals with $1*10^9$ cfu kg^{-1} substrate starting at the sowing date, with a total culture period of 36 days. Finally, the experiment comprised five treatments: (i)nitrate + rock-P; (ii) nitrate + rock-P + Proradix (Px); (iii) ammonium + rock-P; (iv) ammonium + rock-P ammonium + Px and (v) an additional positive control with soluble SSP fertilization and nitrate supply. Soil moisture was adjusted gravimetrically at daily intervals to 70 % of the substrate water-holding capacity. The minirhizotrons were fixed with an orientation of 45° in direction of the observation window to promote root development along the observation plane. The experiment was arranged in a randomized block design with five replicates per treatment. For statistical analysis of significant differences between treatment groups, a one-way ANOVA was employed with Tukey-test ($p<0.05$ significance level) using the SAS software 9.4 (Institute Inc., Cary, NC, USA). Pairwise comparisons were performed using the t-test.

Rhizosphere pH

Non-destructive measurements of rhizosphere pH were conducted with antimony microelectrodes of 1 mm diameter and a reference electrode using a digital pH meter (E532 Herisau, Switzerland) according to the method of Häussling et al. (1985). For each minirhizotron, measurements were taken in triplicate at 14, 20, 29 and 36 days after sowing (DAS) in the bulk soil, on root tips, along seminal roots, and lateral roots at the observation plane (Fig. 4.4C), covering the root surface and directly adhering rhizosphere soil.

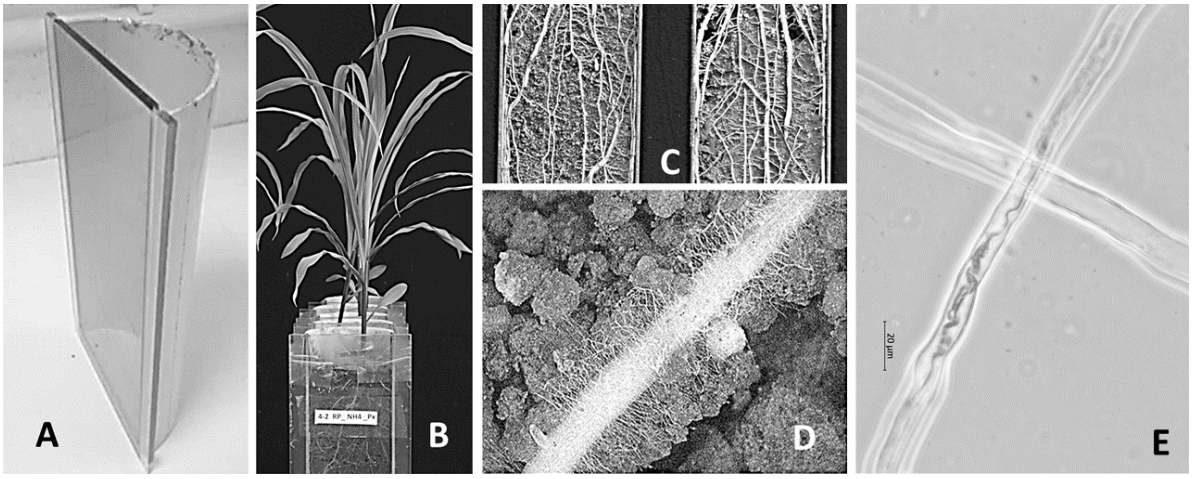


Figure 4.4: Minirhizotron with root observation window based on longitudinal cuttings of PVC tubes (A) with maize plants (B). Intense root development along the observation plane (C) and formation of rhizosheaths with root hair-adhering soil along lateral roots (D). Root hair colonization by the inoculant strain Trichoderma harzianum OMG16 (E).

Organic acids in the rhizosphere solution

Five weeks after sowing, rhizosphere soil solution was collected from 1 cm subapical root zones of seminal roots visible at the observation plane, by application of sorption filters according to the method described by Neumann et al. (2014). For each minirhizotron, sampling was conducted with five replicates and subsequently, the sorption filters were pooled. The pooled samples were re-extracted with 1 mL 80% (v/v) methanol and centrifuged at 12.000 rpm for 15 min. Aliquots of the supernatants (900 µL) were evaporated to dryness at 30°C using a SpeedVac Concentrator (Savant, Farmington, USA) and re-dissolved in HPLC elution buffer (18 mM KH2PO4, pH 2.1 adjusted with H3PO4). Organic acids were determined by RP-HPLC in the ion suppression mode, according to the method described by Haase et al. (2007). Isocratic elution with 18 mM KH2PO4, pH 2.1 was performed on a reversed-phase C-18 column (GROM-SIL 120 ODS ST, 5 µm particle size, 290 x 4.6 mm), equipped with a 20 x 4.6 mm guard column with the same stationary phase (Dr Maisch HPLC GmbH, Ammerbuch, Germany), with direct UV detection at 210 nm. Identification and quantitative determinations were conducted by comparison with known standards. Additionally, the identity of the detected compounds was

confirmed for selected samples by GC-MS profiling according to the protocol described by Neumann et al. (2014).

Rhizosphere phosphatase activities

Plants were harvested at 36 DAS and the root systems were excavated. Rhizosphere soil samples were collected by shaking off root-adhering soil, which was immediately frozen in liquid N and stored at -80°C. Acid and alkaline phosphatase activities were determined based on the p-nitrophenyl phosphate method (Tabatabai and Bremner, 1969) with modifications according to Nkebiwe, et al. (2017) with 200 mM Na-acetate buffer pH 5.2 for acid phosphatase or 200 mM Na-borate buffer pH 8.2 for alkaline phosphatase. Additional measurements were taken at the rhizosphere pH measured for plants supplied with nitrate or stabilized ammonium fertilization, using 200 mM Na-acetate buffer adjusted to the respective pH values.

Root length, root hair and rhizosheath extension

Root hair length and the diameter of rhizosheaths with root-adhering soil particles along the seminal roots and laterals (Fig. 4.4D) were recorded non-destructively along the root observation plane of the minirhizotrons by use of a video microscope (Stemi 200-c, Zeiss Oberkochen, Germany). The digitalized video photographs were analysed using the Axio Vision, software, Version 3.1.2.1 (Zeiss, Oberkochen, Germany). Root hair staining to demonstrate endophytic colonization by *Trichoderma harzianum* OMG16 (Fig. 4.4E) was performed 24 h after inoculation of *in-vitro* plantlets (14 days old rape seedlings) with a spore suspension of OMG16. Roots were stained with Fuchsin Red (0.01% acid fuchsin, 87.5% lactic acid, 6.3% glycerol in H_2O) and photographed with a Zeiss Axio Observer 3 Inverse Microscope using the ZEN 2.0 Imaging Software (Carl Zeiss, Jena, Germany).

Total root length of maize plants was determined after washing out the root systems from the soil substrate and storage in 30% (v/v) ethanol. Thereafter, roots were separated, submerged in a water film on transparent Perspex trays and subsequently digitalized using a flat-bed scanner (Epson Expression 1000 XL, Tokyo, Japan). The root length of the digitalized samples was measured by use of the WinRHIZO root analysis system (Reagent Instruments, Quebec, QC, Canada).

Shoot dry weight and P analysis

The experiment was terminated at 36 DAS, and the plant shoots were oven-dried at 60°C to constant weight for gravimetric determination of shoot dry biomass. For P analysis, 250 mg of finely ground, dried shoot material was ashed for 4 h in a muffle furnace at 500 °C. After cooling, the samples were extracted twice with 1 mL of 3.4 M HNO3 and evaporated to dryness. The ash was dissolved in 1 mL of 4 M HCl, subsequently diluted ten times with hot deionized water, and boiled for 2 min to convert meta- and pyrophosphates to orthophosphate. Spectrophotometrical determination of orthophosphate (Hitachi U-3300 spectrophotometer, Hitachi Ltd., Tokyo, Japan) was conducted according to the method of Gericke and Kurmies (1952).

4.2.2.2 Pot experiments

Plant culture

The PGPM inoculant Bacillus amyloliquefaciens FZB42 (also termed as Bacillus velezensis FZB42) Rhizovital42® liquid formulation (Abitep GmbH, Berlin, Germany) was tested in combination with Zea mays L. cv Colisee (KWS Saat, Einbeck, Germany) over a culture period of 28 d in pots with one kg of soil substrate and the fertilization regime described in section 4.2.2.1 without a positive control in this case (two seedlings per pot with thinning to one plant at 11

DAS). The PGPM inoculation was conducted by soil drenching with 20 mL bacterial spore suspension with 109 cfu kg^{-1} substrates three times at weekly intervals starting with the sowing date. The experiment was arranged in a completely randomized design (CRD) with four replicates per treatment. For statistical analysis of significant differences between treatment groups, a one-way ANOVA employed with Tukey-test ($p<0.05$ significance level) were performed using the SAS software 9.4 (Institute Inc., Cary, NC, USA).

For subsequent experiments with inoculation of the consortium product CombifectorA, based on a combination of Trichoderma harzianum OMG16 (Anhalt University of Applied Sciences, Bernburg, Germany) + Vitabac with five Bacillus strains (*Bacillus licheniformis, B. megaterium, B. polymyxa, B. pumilis* and *B. subtilis)* (Bactiva GmbH, Straelen, Germany) and for the determination of ammonium effects on the plant phytohormonal status, silty loam soils (pH 6.9 and 7.3, respectively) were used as 30 % (w/w) soil-sand mixtures (1.8 kg pot^{-1}) with the same N fertilization regime as described in section 4.2.2.1 but without additional Rock-P supply. For the determination of hormonal effects, maize cv Colisee (one plant pot-1) was cultivated for 21 d under greenhouse conditions with an average temperature of 24 °C until the final harvest for determination of shoot biomass, P shoot concentrations (4.2.2.1) and analysis of phytohormones (Moradtalab et al., 2018). The experiment was carried out in a CRD with five replicates per treatment. Pairwise comparison for significant differences was performed with the Student t-test ($p < 0.05$) using the Sigmaplot 11.0 software (SYSTAT Software Inc., Erkrath, Germany).

For the rhizosphere tracing experiment of *T. harzianum OMG16*, *Zea mays* plants cv Rolandinio (KWS Saat, Einbeck, Germany) were cultivated over 42 days (one plant pot^{-1}) under greenhouse conditions at an average temperature of 21°C with CombifectorA inoculation treatments at 7, 13 and 29 DAS, applied in 20 mL PGPM suspensions with 2.5 x 107 cfu kg-1 substrate. The

experiment was carried out in a CRD with five replicates per treatment. For statistical analysis of significant differences between treatment groups, a one-way ANOVA followed by a Tukey-test ($p<0.05$ significance level) were performed using the SAS software 9.4 (Institute Inc., Cary, NC, USA).

Bacterial indole-3-acetic acid (IAA) production

The IAA production potential of Proradix, (6.6×10^8 CFU ml^{-1}) and FZB42 (2.5×10^7 cfu ml^{-1}) was determined according to the method of (Barucha et al., 2013) with supplementation of three different N sources (KNO_3, $Ca(NO_3)_2$ and $(NH_4)_2SO_4$), at a concentration of 10 g l^{-1} in L-tryptophan-supplemented production medium.

Re-extraction of rhizosphere bacteria in the pot experiment with FZB42 inoculation was conducted 11 DAS after thinning to one seedling per pot. Five grams of root material with adhering rhizosphere soil was shaken in 45 ml of proteose-peptone medium for 1 hour. After precipitation of the rhizosphere soil, 1 ml of the supernatant was inoculated into 9 ml IAA culture broth as described by Bharucha, et al. (2013), and incubated over 48 h at 30°C on a rotary shaker at 150 rpm. Spectrophotometrical IAA determination in the culture filtrates was conducted with a modification of the Salkowski reagent according to Glickmann and Dessaux (1995), using H2SO4 instead of HCl as a solvent. Quantification was performed at a detection wavelength of 535 nm (Gordon & Weber, 1951) using an external IAA standard.

Determination of Auxin (IAA), Gibberellic acid (GA) and Cytokinin (Zeatin) in maize shoot

Analysis of IAA, GA and Zeatin in maize shoots was performed by UHPLC-MS analysis on a Velos LTQ System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) fitted with a SynergiPolar column, 4µ, 150 ∗ 3.0mm, (Phenomenex, Torrance, California, USA) according to the method described by Moradtalab et al. (2018).

4.2.2.3 Rhizosphere tracing of Trichoderma harzianum OMG16.

DNA extraction

Total root DNA was isolated as previously described by (Geistlinger et al. 2015). Briefly, maize plants (cv. Colisee) were carefully removed from the planting pots, roots were thoroughly cleaned with a soft brush and water until no residual soil remained adhered, quickly dried between paper towels and cut into small pieces. Approximately 80 mg fine roots were placed in 2 mL tubes containing 1.0 mm silica spheres including one single 0.64 cm ceramic bead (MP Biomedicals, France) and 400 µL peqGOLD lysis buffer (VWR Peqlab, Germany). Root tissue was homogenized for 3x 30 s at a speed of 6 m/s in a FastPrep 24 bead-beating system (MP Biomedicals). DNA was subsequently extracted utilizing the peqGOLD Fungal DNA Kit (VWR Peqlab), following the manufacturer's instructions. DNA was eluted in TE buffer (pH 8.0) and checked on 0.8% TAE agarose gels. DNA concentrations were determined using a Qubit® 3.0 Fluorometer and the Qubit dsDNA HS Assay Kit according to the instructions of the manufacturer (Thermo Fisher Scientific, Germany).

PCR conditions

The applicability of the isolated DNAs for appropriate PCR performance was checked by conventional PCR using the primers ITS1F and ITS4 (White et al., 1990; Gardes & Bruns, 1993) for the fungal DNA fractions of the isolated total root DNAs. PCRs were performed in 20 µL volumes containing 10 ng of extracted DNA, 0.5 µM of each primer and 2x Phusion High-Fidelity PCR Master Mix (Thermo Scientific, Germany). Amplifications were carried out in a thermal cycler (Labcycler, SensoQuest, Germany) with the following temperature profile: initial denaturation at 96°C for 3 min, followed by 33 cycles consisting of 95°C for 20 s, 56°C for 25 s and 72°C for 30 s. A final elongation step at 72°C for 5 min completed the protocol. For

quantification of the T. harzianum strain OMG16 (DSMZ accession no.: 32722) in maize root tissue, a dilution series of pure OMG16 DNA was prepared, starting from 10ng/µl followed by six 1:10 dilution steps. These calibration samples (1µl of each dilution step) were run along with the experimental root DNAs to obtain a qPCR standard curve (R^2 = 0.998). The absolute amounts in pg fungal OMG16 DNA per 10 ng of total root DNA were calculated on the basis of individual Cq- (cycles of quantification) and RFU-values (relative fluorescence units) by using the PikoReal Software 2.2 (3 planting pots as biological replicates with 3 technical PCR replicates per pot). Samples were analyzed by qPCR using 1x SYBR® Green Nucleic Acid Stain (Lonza, Switzerland). Each reaction consisted of 20 µl including 2x Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific) containing 1.5 mM MgCl2, 200 µM of each dNTP, 0.5 µM of each primer, 0.4 U of Phusion DNA Polymerase and 15 ng of total maize root DNA. Reactions were performed in a PikoReal 96 thermal cycler (Thermo Fisher Scientific) applying the following conditions: initial denaturation at 96°C for 3 min, followed by 37 cycles at 94°C for 15 sec, 60°C for 25 sec and 72°C for 20 sec. The T. harzianum OMG16-specific primer pair applied was designed from OMG16 genomic DNA sequences generated in an in-house shotgun sequencing project targeting simple sequence repeats (unpublished). The primers ThNona7for (5'-TTTCTTCGTGTTTCCCCATC-3') and ThNona7rev (5'-GACAAAGAAGCCGAGGACAG-3') flank a nona-mere repeat (GAAGTGAAG) 7 and generate with OMG16 DNA a 236 bp PCR fragment. The correct identity of the obtained fragments was checked by applying the high density melting curve function of the PikoReal device (0.1°C heating steps, melting temp. 82.7°C) and by visualizing the qPCR samples on 2% TAE agarose gels along with a 20bp ladder DNA size standard (O'RangeRuler, Thermo Scientific).

4.2.3 Results

4.2.3.1 Plant biomass and P status

Compared with nitrate fertilization, stabilized ammonium supply significantly increased shoot dry matter and the P status (P shoot accumulation and tissue concentrations) of maize plants grown on an organic farming soil pH 7.0 with moderately low P availability, supplied with Rock-P (100mg P kg^{-1} soil) as a sparingly-soluble P source. Proradix inoculation combined with stabilized ammonium fertilization significantly increased dry shoot biomass (103 %), P concentration (58.7%) and shoot P accumulation (234.9 %) as compared with the Proradix - Nitrate combination. Shoot biomass production and the nutritional P status were not significantly different from the positive control treatment supplied with soluble P (SSP, 100 mg kg^{-1} soil). The combination of Proradix with stabilized ammonium fertilization reached the highest shoot biomass and shoot P accumulation, although not significantly different from the non-inoculated control with ammonium supply (Table 4.4A).

4.2.3.2 Rhizosphere pH, organic acids and phosphatase activities

Changes in rhizosphere pH relative to the bulk soil without root contact were measured with antimony microelectrodes (1 mm diameter) at 14 and 36 days after sowing (DAS) along the seminal roots of maize plants, supplied with Rock P and nitrate or stabilized ammonium fertilization with or without PGPM (Proradix) inoculation. Ammonium supply significantly reduced the rhizosphere pH between 20 and 36 DAS by 0.7 – 0.1 pH units compared with the bulk soil, while a trend for rhizosphere alkalinisation was detectable with nitrate fertilization. A pH difference of up to one unit was recorded between plants with ammonium versus nitrate supply. However, ammonium-induced rhizosphere acidification was not intensified by Proradix inoculation (Table 4.4B).

The rhizosphere soil solution was dominated by the monocarboxylates lactate and acetate. In this case, a significant treatment effect was recorded only for the nitrate treatment with Proradix inoculation, which increased the acetic acid concentration by 61% in comparison to non-inoculated control. Typical plant di- and tricarboxylates, such as malic-, citric-, and trans-aconitic acids, tended to decline under ammonium fertilization versus nitrate supply with significant effects for t-aconitate. This trend was further intensified by Proradix inoculation. Compared with nitrate fertilization, the carboxylate concentrations in the rhizosphere soil solution with ammonium fertilization and poradix inoculation significantly declined by 80.1 % for malate, 69.5 % for trans-aconitate and by 61.1 % (n.s at α 0.05) for citrate (Table 4.4C).

Table 4.4 Effects of different N forms (Nitrate and stabilized ammonium) and PGPR inoculation (Px = Proradix, *Pseudomonas sp.* DMSZ 13134) on maize shoot biomass production and P status (A), rhizosphere pH (B), organic acid accumulation (C) and acid & alkaline phosphatase activities (D) in the rhizosphere.

A. Maize shoot dry weight, phosphate concentration and content

	Shoot dry weight [g Plant^{-1}]	Shoot P Conc. [mg g^{-1} DM]	Shoot P Content [mg Plant^{-1}]
Nitrate_Rock P	4.28 b	1.42 c	6.03 c
Nitrate_Rock P_Px	4.56 b	1.50 c	6.82 c
Ammonium_Rock P	8.10 a	2.54 a	20.63 ab
Ammonium_Rock P_Px	9.62 a	2.38 ab	22.84 a
Nitrate_Soluble P	9.46 a	2.25 ab	21.64 ab

Px= *Pseudomonas sp.,* P=Phosphate, same letter=not significant at alpha 0.05 (Tukey test in SAS)

B. Rhizosphere pH changes relative to bulk soil

	Day 14	Day 20	Day 29	Day 36
Nitrate_Rock P	+0.2	+0.0	+0.1	+0.1*
Nitrate_Rock P_Px	+0.5	+0.4	+0.1	+0.5
Ammonium_Rock P	-0.1	-0.7*	-0.6*	-0.1*
Ammonium_Rock P_Px	-0.1	-0.6*	-0.2	-0.5

Px = *Pseudomonas sp.,* P= Phosphate, "+" = pH increase in maize rhizosphere and "– "= pH decrease in maize rhizosphere. * = significant difference, t-test, p = 0.05

C. Organic acids from maize root tips [nmol h^{-1} cm^{-1} root length]

	Malic acid	Lactic acid	Acetic acid	Maleic acid	Citric acid	Succinic acid	cis-Aconitic acid	trans-Aconitic acid
Nitrate_Rock P	2.21 a	2.68 a	6.09 b	0.05 a	0.95 a	1.29 a	0.03 a	2.26 a
Nitrate_Rock P_Px	1.28 ab	2.93 a	9.81 a	0.01 b	0.62 a	0.88 a	0.01 a	0.21 b
Ammonium_Rock P	1.12 ab	2.51 a	9.41 a	0.01 b	0.83 a	1.28 a	0.02 a	0.97 b
Ammonium_Rock P_Px	0.44 b	2.98 a	8.40 a	0.02 b	0.37 a	1.47 a	0 .01 a	0.69 b

Px= *Pseudomonas sp.,* P=Phosphate, same letter=not significant at alpha 0.05 (Tukey test in SAS)

D. Rhizosphere phosphatase activities in root-adhering soil [µg p-NP g^{-1} h^{-1}]

	Acid Phosphatase	Alkaline Phosphatase	Phosphatase activity at rhizoplane pH
Nitrate_Rock P	423.6 a	111.2 bc	500.3 a
Nitrate_Rock P_Px	739.8 a	163.1 a	298.9 a
Ammonium_Rock P	558.1 a	111.8 bc	408.2 a
Ammonium_Rock P_Px	624.8 a	83.7 c	354.2 a

Px= Pseudomonas sp., P=Phosphate, same letter=not significant at alpha 0.05 (Tukey test in SAS)

No significant treatment differences were recorded for acid phosphatase activity in the rhizosphere. This applied for activity tests performed under standard conditions (pH 5.2, 30°C) and for phosphatase test conducted at the pH measured at the rhizoplane as well (pH 5.5 for nitrate fertilization and pH 4.5 under stabilized ammonium supply). However, Proradix inoculation significantly increased the rhizosphere activity of alkaline phosphatases (46.7 %) under standard conditions (pH 8,2, 30°C) in plants with nitrate fertilization, while a declining trend was recorded under stabilized ammonium supply (Table 4.4D).

4.2.3.3 Root growth and morphology

Compared with nitrate supply, stabilized ammonium fertilization had no significant effects on total root length but significantly increased the length of lateral root hairs by 46 %. This was associated with an increased diameter of rhizosheaths (56 %) (Fig. 4.5A), formed by soil particles adhering to the root hairs (Fig. 4.4D), confirmed also by a positive correlation between root hair length and rhizosheath diameter (Fig. 4.5B). Inoculation with Proradix tended to increase total root length (43%) in the treatment with stabilized ammonium supply, although the effect was not significant. The PGPM inoculation had no effect on root hair length or diameter of rhizosheaths (Fig. 4.5A).

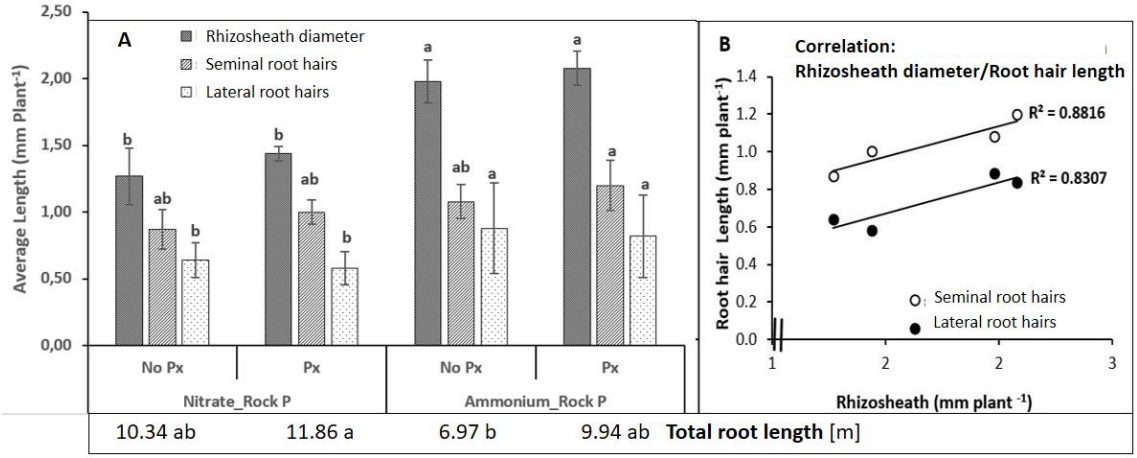


Figure 4.5: Total root length, length of root hairs along seminal and lateral roots, rhizosheath diameter (A) and the correlation between root hair length and rhizosheath diameter (B) of maize plants fertilised with stabilised ammonium sulfate or calcium nitrate and rock phosphate. (Rock-P) as sparingly soluble P source, with (Px) or without (No Px) Proradix (Pseudomonas sp. DMSZ 13134) inoculation.

Based on the findings of Bharucha et al. (2013), who demonstrated an N form-dependent production of auxins by *Pseudomonas putida* used as inoculant, with potential impact on root growth promotion of the host plant, we investigated the auxin production potential of Proradix and an additional PGPR strain FZB42 on artificial growth media supplied with different mineral N forms. For both strains, auxin production in the culture medium significantly increased in the order KNO_3 < $Ca(NO_3)_2$ < $(NH_4)_2SO_4$ (Table 4.5A).

For further investigation of N-form effects on the bacterial auxin production potential under real rhizosphere conditions, maize (2 plants pot^{-1}) supplied with nitrate or stabilized ammonium fertilization and Rock-P as sparingly soluble P source was grown on the same clay-loam soil, pH 7.0 used for the minirhizotron experiment. FZB42 was used as an inoculant. At 11 DAS, the roots of one plant were excavated and rhizosphere bacteria were extracted from root-adhering soil and the root surface (rhizoplane) into proteose-peptone medium followed by determination of the auxin production potential. The remaining plant was harvested at 28 DAS. Plant growth responses showed the same trend as observed for maize plant with Proradix inoculation grown on the same soil in the minirhizotron experiment. Compared with nitrate fertilization, stabilized ammonium supply significantly increased shoot biomass production and P shoot accumulation by 61% and 32.4%, respectively, while P shoot concentrations remained unaffected. A biomass effect of the FZB42 inoculant was detectable only in the ammonium treatment, with an increase in shoot dry matter by 41.4 % as compared with the non-inoculated control and by 105 % in comparison with the FZB42 treatment supplied with nitrate fertilization (Table 4.5 B). This was associated with a significantly higher P shoot accumulation of FZB42-inoculated plants with stabilized ammonium fertilization as compared with nitrate supply (108.9%), and a trend for increased P accumulation (57.8%) in comparison with the non-inoculated control (Table 4.5 B). However, FZB42 inoculation had no effects on P shoot

concentrations. The IAA production potential of bacterial communities, re-isolated from the maize rhizosphere, was significantly increased only in the FZB42-ammonium combination and increased by 79.2 % compared with the FZB42-nitrate treatment. This was associated with a significant increase in total root length by 89.8% (Table 4.5 B). No significant differences in IAA production or root length development were recorded in the non-inoculated controls (Table 4.5B).

Table 4.5: *N-form effects on in-vitro* indole-3-acetic acid *(IAA) production by selected PGPR strains* (Proradix, *DMSZ13134, Bacillus amyloliquefaciens* FZB42) during a 48 h incubation period **(A),** on IAA production of bacterial populations re-isolated from the rhizosphere of 11 d old maize seedlings with and without FZB42 inoculation **(B)** and on concentrations of growth hormones in the shoot tissue of non-inoculated maize plants after three weeks of growth on a clay-loam soil pH 6.9 **(C)**.

A) N-form dependent IAA production potential of PGPR strains [µg IAA ml^{-1} $48h^{-1}$]

	Proradix *Pseudomonas sp.*	*Bacillus amyloliquefaciens FZB42*
Potassium nitrate	12.1 c	12.2 c
Calcium nitrate	171.4 b	189.0 b
Ammonium sulfate	217.7 a	236. 6 a

LSM in SAS with same letters=no significant difference n=5, Tukey test alpha=0.05

B) N-form dependent IAA production potential of bacterial communities in the maize rhizosphere as related to root length development, shoot biomass production and P status of maize

	IAA production [µg ml^{-1}]	Total root length [m $plant^{-1}$]	Shoot Dry Weight [g $plant^{-1}$]	Shoot P Conc. [mg g^{-1} DM]	Shoot P Content [mg $plant^{-1}$]
Nitrate	75.4 b	3.09 b	1.8 c	1.8 a	3.1 b
Nitrate_FZB42	74.0 b	3.27 b	2.0 bc	1.7 a	3.4 b
Ammonium	82.7 b	4.31 ab	2.9 b	1.6 a	4.5 ab
Ammonium_FZB42	132.6 a	6.20 a	4.1 a	1.8 a	7.1 a

IAA=indole-3-acetic acid, FZB42=Bacillus amyloliquefaciens FZB42, P=Phosphate, same letters =no significant difference (tukey test at 0.05 in SAS)

C) N-form dependent accumulation of hormones, shoot biomass and P status of maize plants

Shoot concentration [ng g^{-1} FW]	Nitrate	DMPP-Ammonium
Auxin (IAA)	56.0 b	87.5 a
Gibberellic acid	40.7 b	76.7 a
Cytokinin (Zeatin)	2.1 b	3.2 a
Shoot FW [g $plant^{-1}$]	11.74 b	12.74 a
Shoot P [mg g^{-1} DM]	2.2 b	2.9 a

IAA=indole-3-acetic acid, in each row, different characters significant differences, t-test, p = 0.05

To address potential N form effects on the hormonal status of maize plants independent of PGPM inoculation, concentrations of auxin indole-3-acetic acid (IAA), gibberellic acid (GA) and the cytokinin zeatin (CK) were determined in the shoot tissue of three-week-old maize plants

grown on a silty loam soil pH 6.9 with calcium nitrate or DMPP-stabilized ammonium sulphate fertilization (Table 4.5 C).

The application of stabilized ammonium had a general stimulatory effect on the accumulation of growth hormones in the shoot tissue with an increase of auxin (IAA), gibberellic acid (GA), and cytokinin (zeatin) concentrations by 56.2, 88.5 and 57.1 %, respectively, as compared with the nitrate treatment. This was associated with an increased shoot P concentration (32%) close to the sufficiency threshold of 0.3 % while the plants with nitrate supply remained in the deficiency range with reduced shoot biomass (Table 4.5C).

4.2.3.4 Root colonization by PGPMs

For the microbial consortium product Combifector-A, based on a combination of *Trichoderma harzianum* OMG16 with five Bacillus strains, a specific primer was available for rhizosphere tracing of the *Trichoderma* strain. In a previous study, the strain combination has been identified as an efficient PGPM product for growth promotion of maize, both, in greenhouse and field experiments (Table 4.3). The strain-specific primer was used exemplarily to assess potential N form effects on PGPM root colonization in maize plants grown for 42 days on a clay-loam soil pH 7.3. The DNA of the endophytic *Trichoderma harzianum* strain OMG16 was traceable in the root samples of both, inoculated and non-inoculated plants close to the detection limit (Table 4.6).

Table 4.6: Root colonization with *Trichoderma harzianum OMG16* in maize plants (cv Rolandinio) grown for six weeks, with or without PGPM inoculation (Combi A) on a clay-loam field soil pH 6.9 supplied with nitrate or DMPP-stabilized ammonium fertilization (DMPP-Ammonium. Means and SD of five replicates. Different letters indicate significant differences (Tukey-Test, $p < 0.05$)

N-Form	Treatment	T. harzianum OMG16 Root colonization [pg fungal DNA 10 ng^{-1} root DNA]
Nitrate	Control	6.7 ± 3.7 b
	Combi A	6.8 ± 5.5 b
DMPP- Ammonium	Control	5.2 ± 4.1 b
	Combi A	16.1± 1.5 a

No significant differences were recorded within the treatments supplied with nitrate fertilization or non-inoculated plants with ammonium supply. However, a 210 % increase in OMG16 DNA was detected exclusively in the inoculated treatment with stabilized ammonium fertilization (Table 4.6).

4.2.3 Discussion

In this study, inoculation of maize with PGPM strains Proradix and FZB42 confirmed earlier findings on the improved performance of PGPM effects in combination with stabilized ammonium fertilizers versus nitrate fertilization, with respect to plant growth and acquisition of sparingly soluble P sources (Table 4.3). The presented results demonstrate that ammonium fertilization contributed to the synergistic interaction via direct and indirect effects on the host plant and the microbial inoculants.

4.2.3.1 Effects on rhizosphere chemistry

On the selected organic farming soil with neutral pH, sparingly soluble rock phosphate (Rock-P) supply and nitrate fertilization, the maize plants with a low inherent potential for root-induced P solubilization (Neumann and Römheld, 2002; Liu et al. 2016) suffered from severe P limitation as indicated by P shoot concentrations below 0.2 % (Campbell, 2009). The severely P-deficient plants were obviously unable to establish a functional interaction with the PGPM inoculants, indicated by the absence of inoculant effects (Tables 4.4 and 4.5). Replacement of nitrate fertilization by ammonium, stabilized with the nitrification inhibitor DMPP, resulted in the well-documented rhizosphere acidification via root-induced proton extrusion (Neumann and Römheld, 2002). The pH decline, measured at the root surface (rhizoplane) of maize plants with stabilized ammonium fertilization reached up to one pH unit recorded during a time period of 36 days with acidification maxima down to pH 4.5 (Table 4.4B). Rhizosphere acidification was obviously sufficient to mediate a significant mobilization of the sparingly soluble Rock-P

fertilizer, as indicated by an improved P status of the plants (Table 4.4A). The effect on shoot P accumulation showed a higher quantitative expression in minirhizotron culture (242%, Table 4.4A) as compared with the pot experiment conducted on the same soil (45%, Table 4.5B). This may be attributed to the extended culture period and to locally high rooting densities, induced by a 45° orientation of the minirhizotrons during cultivation, to promote root development at the observation plane (Fig. 4.4C). As demonstrated also in a previous study on local root proliferation of maize induced by placement of ammonium fertilizers (Jing et al., 2010), the resulting hotspots in rooting density can intensify ammonium-induced rhizosphere acidification, leading to more efficient solubilization of sparingly soluble Ca-phosphates.

The improved P nutritional status of the maize plants with stabilized ammonium supply was obviously sufficient to promote a successful establishment of the PGPM inoculants in the rhizosphere, with a beneficial effect on plant growth and P acquisition. The PGPM effect was most pronounced in the pot experiment with FZB42 inoculation (Table 4.5B), where root-induced rhizosphere acidification by ammonium supply and P solubilization was less expressed as compared with the minirhizotron experiment (Table 4.4A). By contrast, the PGPM effect of Proradix was less distinct in the minirhizotron experiment, associated with an intense expression of the ammonium-induced Rock-P solubilization, which increased the P status and shoot biomass production to a level slightly lower but not significantly different from the positive control supplied with soluble P. Accordingly, the additional effects by PGPM inoculation on biomass production (18.8 % n.s.) and shoot P accumulation (10.8% n.s.) were comparatively small and finally approached the biomass and P accumulation of the positive control (Table 4.4A).

Proradix has been previously characterized as PGPM strain with particularly high P-solubilizing potential, associated with intense ammonium-induced acidification of artificial growth media

(Nkebiwe et al., 2017). However, the improved performance of Proradix with respect to plant growth promotion in combination with stabilized ammonium supply instead of nitrate fertilization (Table 4.4A), could not be attributed to a contribution of the inoculant to rhizosphere acidification, which was not intensified by Proradix application (Table 4.4C). The PGPM inoculation did also not increase the concentrations of organic acids detectable in the rhizosphere soil solution (Table 4.4B), discussed as an additional mechanism for PGPM-induced P solubilization (Khan et al. 2009; Jones and Oburger, 2011). The detected di- and tri-carboxylate profiles with P solubilizing potential were dominated by carboxylates characteristic for maize root exudates, such as malate, citrate, succinate and particularly trans-aconitate (Table 4.4B, Gaume et al. 2000; Neumann and Römheld, 2002), while the monocarboxylates acetate and lactate are more typical as microbial degradation products. However, malate, citrate and succinate have been reported also as components in culture filtrates of various Pseudomonas strains during solubilization of Rock-P in liquid culture media (Vyas and Gulati, 2009). In the present study, the highest carboxylate concentrations were recorded in the rhizosphere soil solution collected from subapical root zones of maize plants with nitrate fertilization but declined significantly under stabilized ammonium fertilization, particularly in combination with the microbial inoculant (Table 4.4B). Declining organic acid exudation in response to ammonium versus nitrate supply has been similarly reported in tomato (Imas et al. 1997), due to increased intracellular consumption of carboxylates as acceptor compounds for ammonium assimilation in the root tissue (Brown and Hornby, 1987). Declining carboxylate concentrations, particularly in the rhizosphere soil solution of maize plants with Proradix inoculation and ammonium supply (Table 4.4B) may reflect the improved P status of the respective plants (Table 4.4A), since P limitation in maize is associated with stimulation of carboxylate exudation (Gaume et al., 2000; Neumann and Römheld, 2002). However, utilization

of the detected carboxylates as sole carbon sources has been reported for Pseudomonas isolates from rhizosphere soil samples (Chong et al. 2017), which may also explain the observed decline in carboxylate concentrations in the rhizosphere soil solution of the PGPM-inoculated treatment (Table 4.4B).

Secretion of phosphohydrolases by soil bacteria (alkaline phosphatases), fungi and plant roots (acid phosphatases) is an important factor mediating the hydrolysis of organic phosphate esters in soils, thereby increasing the plant-availability of phosphate anions in soils (Neumann and Römheld, 2002; Khan et al., 2009; Jones and Oburger, 2011). Comparing different fungal and bacterial PGPM strains, (Nkebiwe et al. 2017) reported particularly high secretory phosphatase activities in the growth media of the investigated PGPM strain Proradix, under conditions of P limitation. Accordingly, Proradix inoculation significantly increased the activity of alkaline phosphatase in the rhizosphere of inoculated maize plants supplied with nitrate fertilization tested under standard conditions (pH 8.2, 30°C) according to the method of Tabatabai and Bermner, (1969), with a similar trend in acid phosphatase (Table 4.4D). However, treatment differences disappeared when the enzyme test was conducted under the pH levels recorded at the root surface of maize plants supplied with nitrate (pH 5.5) and stabilized ammonium fertilization (pH 4.5), respectively (Table 4.4D). This finding suggests that at least under the real pH conditions recorded in the rhizosphere of the investigated maize plants, a contribution of PGPM-induced phosphatase production to P acquisition of the host plant seems to be unlikely.

Taken together, the results suggest that changes in rhizosphere chemistry towards improved P acquisition were mainly related to root-induced rhizosphere acidification in response to preferential ammonium uptake. However, there was no indication for a direct contribution of the PGPM inoculants.

4.2.3.2 Effects on root growth and morphology

Unlike the selective N-form effects on host plant-induced changes in rhizosphere chemistry, the application of the different N forms affected root growth and morphology via interactions both, with the host plant and with the PGPM inoculants. Stabilized ammonium fertilization significantly stimulated root hair elongation resulting in an increased diameter of rhizosheaths with root-adhering soil (Fig. 4.5A and B), increasing the extension of the rhizosphere (Nambiar, 1976, McCully, 1999). Modified root hair development in response to ammonium fertilization has been reported also in earlier studies and comprised increased length and densities (Robinson and Rorison, 1987; Kania et al. 2007) or branching of root hairs (Yang et al., 2011), and similar proliferation and elongation of root hairs are known as an adaptation to P limitation (Föhse et al., 1991). The importance of root hairs for P acquisition has been investigated by radioactive 32P tracer studies demonstrating that root hairs provided up to 68% of total P uptake in barley and were related with genotypic differences in P acquisition efficiency under field conditions (Gahoonia and Nielsen, 1998; Gahoonia et al. 1999). In combination with ammonium fertilization, stimulation of root hair development will not only increase spatial nutrient acquisition but also intensify the expression of ammonium induced rhizosphere acidification by increasing the root secretory surface area with beneficial effects on Rock-P solubilization. Stimulation of root hair growth in wheat has been previously reported after exogenous application of high auxin (IAA) concentrations (Dobbelaere et al., 1999). Accordingly, compared with nitrate fertilization, in our study, stabilized ammonium supply significantly increased the concentrations of IAA in the shoot tissue (Table 4.5C) as a source organ for IAA production, suggesting a potential relationship with the stimulatory effects on root hair development, which requires further investigation. Interestingly, stabilized ammonium fertilization also increased the shoot concentrations of hormonal shoot growth

regulators, such as root-produced cytokinin (zeatin) and gibberellic acid (GA) (Table 4.4C). This may be related to improved ammonium-induced P acquisition, resulting in positive shoot growth responses (Table 4.5C), mediated by cytokinin-, and GA-dependent signalling (Ha and Tran, 2014).

With respect to plant-microbial interactions, various reports indicate a pivotal role of root hairs for root colonization for endophytic Pseudomonas strains including Proradix (Buddrus-Schiemann et al., 2010; Buddrus-Schiemann 2008; Prieto et al., 2011). Root hair colonization has been similarly reported also for FZB42 (Fan et al., 2012) and various Trichoderma strains (Harman, 2000; Hohmann et al., 2012), including *T. Harzianum OMG16,* investigated in our study (Fig 4.4E). Therefore, ammonium-induced stimulation of root hair development observed in our study (Fig. 4.5) may also increase the root surface area attractive for colonization by the selected inoculants. Accordingly, root colonization by the *Trichoderma harzianum strain OMG16* included in the PGPM products "Combifector A and B", and successfully tested as maize PGPM inoculant in previous studies (Mpanga et al. 2018; 2019), was significantly increased by 210 % in maize plants with stabilized ammonium supply as compared with the non-inoculated control (Table 4.6). No comparable effects were detectable in plants with nitrate supply (Table 4.6).

In contrast to root hair development, stabilized ammonium fertilization had no significant effects on total root length (Fig. 4.4; Table 4.5B), but additional FZB42 inoculation significantly increased the root length of maize plants with ammonium supply over the treatment with nitrate fertilization (89.6%). For both inoculants, a similar trend for increased root length development (Fig. 4.4: 42.6%; Table 4.5B: 43.9%) was detectable also in the maize plants with ammonium fertilization in comparison with the non-inoculated controls, as similarly reported in previous studies (Mpanga et al., 2018; 2019). By contrast, PGPM (Proradix) inoculation had

no effects on root hair development (Fig. 4.5A) in accordance with other recent reports (Weber et al., 2018).

Stimulatory effects on root growth, induced by PGPM inoculants have been frequently linked with bacterial auxin production, documented also for the investigated inoculants (Budruss-Schiemann, 2010; Borris, 2015). Based on observations by Bharucha et al. (2013) on N-form dependent auxin (IAA) production of Pseudomonas putida with root growth-promoting properties, we investigated the auxin production potential of Proradix and FZB42 on peptone/yeast extract medium supplied with different mineral N forms (Barucha et al. 2013). For both PGPM strains, IAA production increased in the order KNO_3 < $Ca(NO_3)_2$ < $(NH_4)_2SO_4$ (Table 4.5A) supplied to the artificial growth media. To investigate whether this effect also applies to rhizosphere conditions, the experiment was repeated with bacterial isolates obtained from the rhizosphere of maize plants at 11 DAS, supplied with nitrate or stabilized ammonium fertilization and Rock-P as sparingly soluble P source, with and without FZB42 inoculation. The IAA production of the isolated bacteria was significantly increased exclusively in the treatment with FZB42 inoculation in combination with stabilized ammonium supply (Table 5.3B). This finding suggests that the increased auxin production potential was related with a particularly high abundance of the FZB42 inoculant in the rhizosphere samples, selectively promoted by ammonium fertilization. The increase in IAA production by 79.2%, as compared with the nitrate treatment, was associated with an 89.5% increase in total root length. The stimulation of root growth translated into an increase in P shoot accumulation and shoot biomass production by 108.8 and 105%, respectively. However, the P tissue concentration was not increased, suggesting that any surplus in P acquisition by PGPM-induced root growth promotion was immediately transformed into biomass production leading to P dilution in the shoot tissue.

4.2.3.3 Concluding remarks

The presented data suggest a network of synergistic interactions between stabilized ammonium fertilization and PGPM effects on maize growth and P acquisition. A direct effect on the mobilization of acid-soluble Ca-P in soils can be attributed to root-induced acidification of the rhizosphere soil via proton extrusion for charge-balance of ammonium uptake. However, there was no indication of an additional contribution to P solubilization, mediated by the selected bacterial inoculants, via rhizosphere acidification, the release of carboxylates or secretory phosphohydrolases. Ammonium effects on the host plant were also detected with respect to increased production of hormonal growth regulators with beneficial effects on the root (IAA) and shoot growth (zeatin, gibberellic acid) and stimulation of root hair elongation. It remains to be established, to which extent these effects are based on direct ammonium-plant interactions or on interactions with the native soil microbiome and the improved nutritional status. However, the stimulation of root hair development likely increased the root surface area available for PGPM colonization and for ammonium-induced rhizosphere acidification with beneficial effects on nutrient acquisition. The improved P nutritional status further promoted the establishment of PGPM interactions in the rhizosphere. Ammonium effects stimulated microbial auxin production, associated with beneficial effects on root length development of the host plant, further contributing to P acquisition. The data suggest that the targeted combination of PGPM inoculants with compatible fertilization strategies offers a promising strategy to manage plant-PGPM interactions. However, the expression of the effects still requires further investigation with respect to the impact of different soil types and soil pH, the role of different host plants, the most suitable inoculants and interactions with native soil microbiomes.

Patents: The combined application of PGPMs with stabilized ammonium fertilizers is part of a joint patent by the University of Hohenheim and Eurochem Agro GmbH: Neumann G.(2018): Method and Composition for Improving Nutrient Acquisition of Plants, International Publication Number WO 2018/197433 A1, Eurochem Agro GmbH, PCT/EP2018/060378.

Funding: This work was funded by the European Community's FP7 Program/2007–2013 under grant agreement number 312117 (BIOFECTOR).

Acknowledgement: The authors wish to thank Eurochem Agro GmbH for their financial support.

4.2.4 References

Abbasi, M.K., Musa, N., Manzoor, M. (2015): Mineralization of soluble P fertilizers and insoluble rock phosphate in response to phosphate-solubilizing bacteria and poultry manure and their effect on the growth and P utilization efficiency of chilli (Capsicum annuum L.). Biogeosciences 12, 4607–4619.

Bharucha, U., Patel, K., Trivedi, U.B. (2013): Optimization of indole acetic acid production by Pseudomonas putida UB1 and its effect as plant growth-promoting rhizobacteria on mustard (Brassica nigra). Agric. Res., 2, 215–221.

Bittman, S., Kowalenko, C.G., Hunt, D.E., Forge, T.A., Wu, X. (2006): Starter phosphorus and broadcast nutrients on corn with contrasting colonization by mycorrhizae. Agron. J., 98, 394–401.

Borris, R. (2015): Towards a New Generation of Commercial Microbial Disease Control and Plant Growth Promotion Products. In Principles of Plant-Microbe Interactions; Lugtenberg, B., Ed.; Springer International Publishing: Basel, Switzerland, pp. 329–337.

Bradáčová K., Florea A.S., Bar-Tal A., Minz D., Yermiyahu U., Shawahna R., Kraut-Cohen J., Zolti A., Erel R., Dietel K., Weinmann M., Zimmermann B., Berger N., Ludewig U., Neumann G., Poşta G. (2019): Microbial consortia versus single-strain inoculants: an advantage in PGPR-assisted tomato production? Agronomy, 9(2), 105. https://doi.org/10.3390/agronomy9020105

Brown ME, Hornby D. (1987): Effects of nitrate and ammonium on wheat roots in gnotobiotic culture: Amino acids, cortical cell death and take-all (caused by Gaeumannomyces graminis var. Tritici). Soil Biol. Biochem., 19: 567-573.

Buddrus-Schiemann, K. E. M. (2008): Wirkung des biologischen Pflanzenstärkungsmittels Proradix® (Pseudomonas fluorescens) auf das Wachstum von Gerste (Hordeum vulgare L. cv. Barke) und auf die bakterielle Gemeinschaft in der Rhizosphäre. Ph.D. Thesis, Ludwig-Maximilians-University, Munich, Germany.

Buddrus-Schiemann K., Schmid M., Schreiner K., Welzl G., Hartmann A. (2010): Root colonization by Pseudomonas sp. DSMZ 13134 and impact on the indigenous rhizosphere bacterial community of barley. Microbial Ecology, 60, 381–393.

Campbell, R. C. (2009): Reference Sufficiency Ranges for Plant Analysis in the Southern Region of the United States; Southern Cooperative Series Bulletin #394; North Carolina Depart. of

Agriculture and Consumer Services Agronomic Division: Raleigh, NC, USA, p. 11.
Chekanaia, V., Chikowoa, R., Vanlauwe, B. (2018): Response of common bean (Phaseolus vulgaris L.) to N, P and rhizobia inoculation across variable soils in Zimbabwe. Agric. Ecosyst. Environ., 266, 167–173.
Chong, T. M., Chen, J. W., See-Too, W. S., Yu, C. Y., Ang, G. Y., Lim, Y. L., Yin, W. F., Grandclément, C, Dessaux, Y., Chan, K. G. (2017): Phenotypic and genomic survey on organic acid utilization profile of Pseudomonas mendocina strain S5.2, a vineyard soil isolate. AMB Expr 7:138 DOI 10.1186/s13568-017-0437-7
Dobbelaere, S., Croonenborghs, A., Thys, A., Vande Broek, A., Vanderleyden, J. (1999): Phytostimulatory effect of Azospirillum brasilense wild type and mutant strains altered in IAA production on wheat. Plant and Soil 212: 153-162; dio: 10.1023/A: 1004658000815
Dogra, R. C., Dudeja, S. S. (1993): Fertilizer N and nitrogen fixation in legume-Rhizobium symbiosis Annals of biology 9:149-164
Fan, B., Borris, R., Bleiss, W., Wu, X. (2012): Gram-positive rhizobacterium Bacillus amyloliquefaciens FZB42 colonizes three types of plants in different patterns. J. Microbiol. 50:38–44.
Föhse, D., Claassen, N., Jungk, A. (1991): Phosphorus efficiency of plants. II. Significance of root hairs and cation-anion balance for phosphorus influx in seven plant species. Plant & Soil 131:261-272.
Gahoonia, T. S., Nielsen, N. E., Lyshede, O.B. (1999): Phosphorus (P) acquisition of cereal cultivars in the field at three levels of P fertilization. Plant and Soil, 211:269-281
Gahoonia T. S., Nielsen N. E. (1998): Direct evidence on participation of root hairs in phosphorus (32P) uptake from soil. Plant and Soil 198:147-152.
Gardes M., Bruns T. D. (1993): ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Molecular Ecology 2:113-118.
Gaume, A., Mächler, F., De Le´on, C., Narro, L., & Frossard, E. (2000): Low-P tolerance by maize (Zea mays L.) genotypes: Significance of root growth, and organic acids and acid phosphatase root exudation. Plant and Soil 228: 253–264
Geistlinger, J., Zwanzig, J., Heckendorff, S., Schellenberg, I. (2015): SSR markers for Trichoderma virens: their evaluation and application to identify and quantify root-endophytic strains. Diversity 7(4): 360-384.
Gericke, S., Kurmis, B. (1952): Die kolorimetrische Phophorsäurebestimmung mit Ammonium-Vanadat-Molybdat und ihre Anwendung in der Pflanzenanalyse. Z. Pflanzenernaehr. Bodenkd, 59, 235–247.
Glickmann, E., & Dessaux, Y. (1995). A Critical Examination of the Specificity of the Salkowski Reagent for Indolic Compounds Produced by Phytopathogenic Bacteria, AEM, 61(2), 793–796.
Gordon, S. A., Weber, R. P (1951): Colorimetric Estimation of Indoleacetic Acid. Plant Physiology, 261, 192-195.
Ha, S., Tran, L. S. (2014): Understanding plant responses to phosphorus starvation for improvement of plant tolerance to phosphorus deficiency by biotechnological approaches, Critical Reviews in Biotechnology, 34:1, 16-30, DOI:10.3109/07388551.2013.783549
Haase, S., Neumann, G., Kania, A., Kuzyakov, Y., Römheld, V. and Kandeler, E. (2007): Atmospheric CO_2 and the N-nutritional status modifies nodulation, nodule-carbon supply and root exudation of Phaseolus vulgaris L. Soil Biol Biochem. 39: 2208-2221.
Harman, G. E. (2000): Changes in perceptions derived from research on Trichoderma harzianum T-22 Plant Disease 84: 377-292

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 117:371–375

Hohmann, P., Jones, E. E., Hill, R. A., Stewart, A. (2012): Ecological studies of the bio-inoculant Trichoderma hamatum LU592 in the root system of Pinus radiate. FEMS Microbiol Ecol 80: 709–721. DOI: 10.1111/j.1574-6941.2012.01340.

Huang, J., Keshavarz, A. R, Tao. A., Chen, C. (2017): Efficacy of starter N fertilizer and rhizobia inoculant in dry pea (Pisum sativum Linn.) production in a semi-arid temperate environment. Soil Science and Plant Nutrition 6: 248-253

Imas, P., Bar-Yosef, B., Kafkafi, U., Ganmore-Neumann, R. (1997): Release of carboxylic anions and protons by tomato roots in response to ammonium nitrate ratio and pH in nutrient solution. Plant and Soil, 191: 27-34.

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. F. Crop. Res., 119, 355–364, doi: 10.1016/j.fcr.2010.08.005.

Jones, D., Oburger, E. (2011): Solubilization of phosphorus by soil microorganisms. In: Bünemann, E., Oberson, A., Frossard, E. (Eds.), Phosphorus in Action. Springer Berlin Heidelberg, pp. 169–198.

Kania, A., Guldner, M., Szabo, B., Kazem, s., Römheld, V., Neumann, G., Morhard, J., Evers, M., and Terlouw, T. (2007): Functional characterization of the stabilized organic turfgrass fertilizer “Marathon”. Rasen –Turf – Gazon 1/2007: 192-195.

Khan, M., Zaidi, A., Wani, P. (2009): Role of phosphate solubilizing microorganisms in sustainable agriculture - a review. In: Lichtfouse, E., Navarrete, M., Debaeke, P., Véronique, S., Alberola, C. (Eds.), Sustainable Agriculture. Springer Netherlands, pp. 551–570.

Liu, H., White, P.J., Li, C. (2016): Biomass partitioning and rhizosphere responses of maize and faba bean to phosphorus deficiency. Crop Pasture Sci. 67, 847–856.

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

McCully, M. E. (1999): Roots in soil: Unearthing the complexities of roots and their rhizospheres. Annu Rev Plant Physiol Plant Mol Biol 50:695-718.

Menzies, N., Harbison, D., Dart, P. (2011): Soil chemistry-facts and fiction and their influence on the fertilizer decision making process. In Proceedings of the 26th Annual Conference of the Grassland Society of NSW, Bathurst, Australia, 26–28, pp. 49–

Mpanga, I. K., Nkebiwe, P. M., Kuhlmann, M., Cozzolino, V., Piccolo, A., Geistlinger, J., Berger, N., Ludewig, U., Neumann, G. (2019): The Form of N Supply Determines Plant Growth Promotion by P-solubilizing Microorganisms in Maize. Microorganisms, 7, 38 *https://doi.org/10.3390/microorganisms7020038*

Mpanga, I.K., Dapaah, H.K., Geistlinger, J., Ludewig, U., Neumann, G. (2018): Soil type-dependent interactions of P-solubilizing microorganisms with organic and inorganic fertilizers mediate plant growth promotion in tomato. Agronomy, 8, 213, doi:10.3390/agronomy8100213.

Moradtalab, N., Weinmann, M., Walker, F., Höglinger, B., Ludewig, U., & Neumann, G. (2018). Silicon Improves Chilling Tolerance During Early Growth of Maize by Effects on Micronutrient Homeostasis and Hormonal Balances. Frontiers in Plant Science. https://doi.org/10.3389/fpls.2018.00420

Nambiar, E. K. S. (1976): The uptake of zinc-65 by roots in relation to soil water content and root growth. Aust. J. Soil Res 14:67-74.

Neumann, G., Römheld, V. (2002): Root-induced changes in the availability of nutrients in the rhizosphere. In Plant Roots the Hidden Half, 3rd ed.; Waisel, Y., Eshel, A., Kafkafi, U., Eds.; Marcel Dekker: New York, NY, USA; pp. 617–649.

Neumann G, Bott S, Ohler MA, Mock H-P, Lippmann R, Grosch R, Smalla K (2014): Root exudation and root development of lettuce (Lactuca sativa L. cv. Tizian) as affected by different soils. Front Microbiol. 5: 2., doi: 10.3389/fmicb.2014.00002

Nkebiwe, P.M., Neumann, G., Müller, T. (2017): Densely rooted rhizosphere hotspots induced around subsurface NH4+-fertilizer depots: a home for soil PGPMs? Chem. Biol. Technol. Agric. (2017) 4: 29. https://doi.org/10.1186/s40538-017-0111-y

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 Agriculture3:15 DOI: 10.1186/s40538-016-0065-5

Nkebiwe, P. M. (2016): Fertilizer placement and the potential for its combination with bio-effectors to improve crop nutrient acquisition and yield. Dissertation, Universität Hohenheim, Stuttgart, Germany.

Nouri, E., Breuillin-Sessoms, F., Feller, U., Reinhardt, D. (2015): Phosphorus and Nitrogen Regulate Arbuscular Mycorrhizal Symbiosis in Petunia hybrida. PLoS One 10(4): e0127472.

Ögüt, M., Er, F., Neumann, G. (2011): Increased proton extrusion of wheat roots by inoculation with phosphorus solubilising microorganisms. Plant and Soil, 339, 285–297.

Pratibha Vyas and Arvind Gulati (2009): Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate-solubilizing fluorescent Pseudomonas. BMC Microbiology 9:174 doi: 10.1186/1471-2180-9-174

Prieto, P., Schiliro, E., Maldonado-Gonzalez, M. M., Valderrama, R., Barroso-Albarracin, J. B., Mercado-Blanco, J. (2011): Root hairs play a key role in the endophytic colonization of olive roots by Pseudomonas spp. with biocontrol activity. Microbial Ecology, 62, 435–445. doi: 10.1007/s00248-011-9827-6

Robinson, D., Rorison, I. H. (1987): Root hairs and plant growth at low nitrogen availabilities. New Phytol 107:681–693

Schütz, L.; Gattinger, A.; Meier, M.; Muller, A.; Boller, T.; Mäder, P.; Mathimaran, N. (2017): Improving crop yield and nutrient use efficiency via biofertilization–A global meta-analysis. Front. Plant Sci., 8. doi:10.3389/fpls.2017.02204

Tabatabai, M. A., Bremner, J. M. (1969): Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biol Biochem.;1(4):301–7.

Thonar, C., Lekfeldt, J.D.S., Cozzolino, V., Kundel, D., Kulhánek, M., Mosimann, C., Neumann, G., Piccolo, A., Rex, M.; Symanczik, S. (2017): Potential of three microbial bio-effectors to promote maize growth and nutrient acquisition from alternative phosphorous fertilizers in contrasting soils. Chem. Biol. Technol. Agric., 4, 7.

van Veen, J.A., van Overbeek, L.S., van Elsas, J. D. (1997): Fate and activity of microorganisms introduced into soil. Microbiol. Mol. Biol. Rev., 611, 21–35. 1

VDLUFA (2018): Neue Empfehlungen des VDLUFA für die P-Düngung, VDLUFA Mitteilungen 02/2018, Verband Deutscher LandwirtschaftlicherUntersuchungs- und Forschungsanstalten e. V. (VDLUFA), Darmstadt, Germany.

Vinci, G., Cozzolino, V., Mazzei, P., Monda, H., Spaccini, R., Piccolo, A. (2018): Effects of Bacillus amyloliquefaciens and organic and inorganic phosphate amendments on Maize plants as revealed by NMR and GC-MS based metabolomics. Plant and Soil 429(10):1-14

Vinci, G., Cozzolino, V., Mazzei, P., Monda, H., Spaccini, R., Piccolo, A. (2018): An alternative to mineral phosphorus fertilizers: The combined effects of Trichoderma harzianum and

compost on Zea mays, as revealed by 1 H NMR and GC-MS metabolomics. PLoS ONE 13(12): e0209664. https://doi.org/10.1371/journal.pone.0209664

Vyas, P., Gulati, A. (2009): Organic acid production in vitro and plant growth promotion in maize under controlled environment by phosphate solubilizing fluorescent Pseudomonas. BMC Microbiology 9:174

Weber, N. F., Herrmann, I., Hochholdinger, F., Ludewig, U., & Neumann, G. (2018). PGPR-Induced Growth Stimulation and Nutrient Acquisition in Maize: Do Root Hairs Matter? Scientia Agriculturae Bohemica. https://doi.org/10.2478/sab-2018-0022

White, T. J., Bruns, T. D., Lee, S., Taylor, J. W. (1990): Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315-322. In: PCR Protocols: A Guide to Methods and Applications, eds. Innis MA, Gelfand DH, Sninsky JJ, White TJ, Academic Press Inc., New York.

Yang, N., Zhu, C., Gan, L., Ng, D., Xia, K. (2011): Ammonium-Stimulated Root Hair Branching is Enhanced by Methyl Jasmonate and Suppressed by Ethylene in Arabidopsis thaliana. J. Plant Biol. 54:92–100 DOI 10.1007/s12374-011-9147-x

4.3 Synergisms in rock-P acquisition by different forms of N fertilization and PGPMs at different soil pH

4.3.1 Acquisition of Rock Phosphate by combined application of Ammonium fertilizers and Bacillus amyloliquefaciens FZB42 in Maize as affected by Soil pH

Isaac K. Mpanga[1,2] (*mpangaisaac75@gmail.com/mpangai@arizona.edu,* +19282744826)

Uwe Ludewig[2] (u.ludewig@uni-hohenheim.de, +49 (0) 711 - 459 22344)

Harrison Kwame Dapaah[3] (harrison.dapaah@uenr.edu.gh, +233244742961)

Günter Neumann[2] (gd.neumann@t-online.de, +49 (0)711 459 24273)

1. University of Arizona Cooperative Extension, 2830 N. Commonwealth Dr, AZ 86322, Camp Verde, USA.
2. Institute of Crop Sciences (340h), Universität of Hohenheim, Fruwirthstrasse 20, Stuttgart 70593, Germany.
3. School of Agriculture and Technology, University of Energy and Natural Resources. P.O Box 214, Sunyani, Ghana.

Published: 06 April 2020 in Applied Microbiology: https://doi.org/10.1111/jam.14654

Abstract

Aims: The use of plant growth-promoting microorganisms (PGPMs) to improve plant-nutrient acquisition has a long history but reproducibility remains a challenge. Recent findings suggest an important role of suitable inoculant-fertilizer combinations for the expression of PGPM-effects, particularly with respect to nitrogen (N) supply. In face of the well-documented N form effects on rhizosphere pH, this study addressed the impact of ammonium-assisted PGPM-interactions on the acquisition of sparingly soluble calcium-phosphates as affected by soil pH.

Methods and Results: The effects of stabilized ammonium fertilization combined with the PGPM inoculant *Bacillus amyloliquefaciens* FZB42 on the acquisition of rock phosphate in maize were examined on two soils (moderately acidic-pH 5.6 and alkaline-pH 7.8). On the two contrasting soils, FZB42 improved the P status and promoted plant growth by different mechanisms. On the acidic soil, a combination of ammonium-fertilization with FZB42 increased P-acquisition by Rock P solubilization via rhizosphere acidification but P-supply in the non-inoculated control was already sufficient to meet the plant demands. By contrast, on the alkaline soil, plant growth-promotion was associated with FZB42-induced root growth stimulation.

Conclusion: The results suggest a significant impact of soil pH on performance and the mode of action of PGPM inoculants, to be considered for practical applications.

Significance and Impact of Study: The study advanced existing knowledge on PGPM-assisted P solubilization as affected by different soil properties. The results suggest perspectives for management options to be considered for efficient use of PGPMs in terms of selecting application strategies with compatible PGPM-fertilizer combinations, depending on soil pH conditions.

Keywords: Ammonium fertilizers, *Bacillus amyloliquefaciens* FZB42, maize, rock phosphate, and soil pH.

4.3.1.1 Introduction

Phosphorus (P) in an important macronutrient in crop production but P deficiencies in the world's arable lands are estimated to limit crop yield by 30-40% (Runge-Metzger, 1995; Uexküll & Mutert, 1995' Vance 2001, Vance et al. 2003), which makes application of P fertilizers obligatory to maintain yield stability. Moreover, according to Russell (1973), only 20% or less of the applied P is removed by the first years of plant cultivation while the remaining 80% are rapidly sequestered in forms with limited plant availability (Kimble et al. 2000). This bears a high-risk of P overfertilization in agricultural soils with subsequent losses by erosion and surface run-off into water bodies, contributing to eutrophication and hypoxia of the aquatic ecosystems (Bumb & Baanante, 1996; Elena et al. 2001; Runge-Metzger, 1995). Since mineral P fertilizers are produced via mining of rock phosphates (RP) as a limited natural resource, this raise concerns on sustainability aspects of mineral P fertilization. Despite variable prognoses on the longevity of RP reserves, ranging from several decades to several centuries (Van Kauwenbergh et al. 2013), improving the use efficiency of P fertilizers in agricultural production systems, remains a major challenge for the future. Proposed strategies comprise approaches of P-saving by use of P fertilizers based on organic and inorganic waste-recycling products (Kirchmann et al. 2005; Stofella et al. 2014) as well as improved plant P acquisition by fertilizer placement strategies close to the roots (Nkebiwe et al. 2016a), exploiting the genetic potential for root-induced changes in rhizosphere chemistry and root growth (Bonser et al. 1996; Campos et al. 2018; Gahoonia and Nielsen 2004), and the assistance of microbial inoculants with plant growth-promoting properties (PGPMs) (Sharma, et al. 2013). Root growth promotion by production of auxins or other signal compounds, down-regulation of excessive stress-

induced ethylene production by ACC deaminase, soil pH reduction by release of protons, organic and mineral acids as well as increased P mineralization or enhanced recruitment of other beneficial microbes are discussed as potential modes of action of PGPM-assisted P acquisition (Illmer & Schinner 1995; Kroeber et al. 2014; Mardad, et al. 2013; Thonar et al. 2017; Sharma, et al. 2013) as reported with FZB42. However, recent findings suggest that the efficiency of PGPM-assisted fertilization strategies also depends on combination with suitable fertilizers (Abbasi et al. 2015; Bradacova et al. 2019; Mpanga et al. 2018; Vinci et al. 2018a, b; Nkebiwe et al. 2016b; Thonar et al. 2017). Own preliminary work indicates preferential performance of many PGPMs in combination with ammonium instead of nitrate-based fertilizers. Positive effects on utilization of sparingly soluble Ca-P sources by combination with stabilized ammonium fertilizers have been recorded in more than 16 pot and field experiments with 24 PGPR strains or strain combinations in three crops on seven soils with pH 5.6 – 8.8 (Mpanga et al. 2019). However, so far, no studies are available with direct comparisons of soil pH effects on PGPM performance in combination with ammonium fertilization.

In this study, *Bacillus amyloliquefaciens FZB42* characterized as one of the most efficient PGPMs in the previous reports (Mpanga et al. 2018; 2019; Vinci et al. 2018a) with abilities to solubilize organic and inorganic P (Nkebiwe et al. 2017) and root growth-promoting and stress- protective properties (Borris 2015; Mpanga et al. 2018; 2019) was selected as a representative PGPM strain. Maize was used as a model plant with a low inherent capacity for root-induced P solubilization (Gaume et al. 2000; Liu et al. 2016). For comparison, the plants were grown on two low P soils with moderately acidic (pH 5.6) or moderately alkaline pH (pH 7.8) supplied with RP fertilization and stabilized ammonium. Plants with full soluble P fertilization and nitrate supply were included as positive controls.

4.3.1.2 Materials and methods

Soil Properties

Table 4.7: Physical and chemical properties of the soils from Ghana (Mpanga et al. 2018)

Soil Properties	**Soil Origin**	
	Atebubu	**Dormaa Ahenkro**
Soil pH ($CaCl_2$)	5.6	7.8
Total Nitrogen [%]	0.05	0.30
NO_3 -N [mg kg^{-1} soil]	2.4	44.2
Plant available P [mg kg^{-1} soil]	7.22 (P CAL)	2.22 (P Olsen)
Total P (ICP-OES) [mg kg^{-1} soil]	90	473
K (CAL extract) [mg kg^{-1} soil]	33.2	357
Mg ($CaCl_2$) [mg kg^{-1} soil]	110	250
Total Ca [mg kg^{-1} soil]	632	10,523
Fe (CAT extract) [mg kg^{-1} soil]	56.5	29.0
Zn (CAT extract) [mg kg^{-1} soil]	< 1	4.0
Mn (CAT extract) [mg kg^{-1} soil]	188.0	27.3
Cu (CAT extract) [mg kg^{-1} soil]	0.54	1.14
Total Carbon [%]	0.75	4.82
Humus [%]	1.23	7.89
Sand (63-2000 μm) %	66.4	44.4
Silt (2-63 μm) %	28.6	38.3
Clay (< 2 μm) %	5.0	17.3

CAL: Calcium acetate-lactate extract, CAT: Calciumchloride/-Diethylene triamine pentaacetic acid extract, ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry

Test Plant

Maize (Zea mays CV Wandataa-NBS/16/wan/wm) (Naa Bawa Seidu, Wa, Ghana) was used for the experiments. Three seeds were sown per pot and thinned to one plant of similar height and vigour at 7 days after sowing (DAS) and cultivated until 38 DAS.

Location and Culture Conditions

The experiment was carried out in a screen house (Schuch 2018) at the School of Agriculture and Technology, University of Energy and Natural Resources, Ghana. Average screen house temperature, dew point and relative humidity: 38^{o}C, 20 ^{o}C and 78% respectively. Plastic pots of 3.5L were used with 3 kg soil detailed in Table 4.7 and watered up to 70% water holding capacity after sowing the maize seeds. Watering was performed gravimetrically once a day for the first two weeks and then changed to twice a day from week three until harvested on 38 DAS.

Treatments and Fertilization

The treatments comprised; (i) no fertilization (negative control); (ii) ammonium + Rock Phosphate (RP); (iii) ammonium + RP + FZB42 and (iv) nitrate + soluble P (positive control). The P fertilizers were applied as: Rock P- (Granuphos 18% P_2O_5 (Landor, Birsfelden Switzerland) or superphosphate (single superphosphate, 18% P_2O_5, Triferto, Gent, Belgium) at 100 mg P kg^{-1} soil, Nitrogen was applied as ammonium sulfate stabilized with the nitrification inhibitor DMPP- (3,4-dimethylpyrazole-phosphate; Novatec solub, Compo Expert GmbH, Münster, Germany) at 100 mg N kg^{-1} soil). Calcium nitrate was fertilized as N form to the positive control at 100 mg N kg^{-1} soil. Potassium was applied at 100mg K kg^{-1} soil as K_2SO_4 for all treatment except the zero fertilization treatment without N, P and K. To compensate for low background nitrate levels on the acid soil N was applied as a mixture of 15% Ca $(NO_3)_2$) and 85% $(NH_4)_2SO_4$).

PGPR inoculation.

Rhizovital FZB42 fl (ABiTEP GmbH, Berlin, Germany), which is based on a liquid formulation of *Bacillus amyloliquefaciens* FZB42 also termed as *Bacillus velecensis* (2.5 * 10^{10} cfu g^{-1}) was applied three times from sowing in weekly intervals by drenching 20ml of 10^9 spores kg^{-1} soil.

Plant Biomass and Root Length

The plants were harvested at the end of the culture period and oven-dried in an oven at 60 ^{0}C until a constant weight is achieved. Rhizosphere soil was sampled by vigorous shaking root of the excavated root systems with adhering soil particles and air-dried in the screen house for later analysis of plant available rhizosphere soil P and pH. Total root length was estimated using the WinRHIZO root analysis system (Reagent instruments, Canada) after separating the roots in a water film in transparent Perspex trays and digitalized with EPSON expression 1000 XL scanner (SEIKO EPSON CORR, Japan).

Shoot N, P, K, Ca, Mg, Mn and Zn Concentration and Content

Maize shoot N was measured with a Vario Max CN macro-elementar analyser (Elementar Analysensysteme, Hanau, Germany). For P, K, Ca, and Mg, Mn, and Zn, a microwave digestion method was employed for the wet-ashing of finely ground dry plant materials (250 mg) in 1 mL of deionized water, 2.5 mL conc. 14 M HNO_3 (1:3), and 2 mL 0.185 M H_2O_2 (30%). Digestion was performed in a microwave digestion system (Ethos, MLS, Leutkirch, Germany) for 1 h and allowed to cool for 30 min. Approximately 5 g activated charcoal was added for sample to obtain a clear assay based on the method by Upreti (1984), mixed by shaking, and allowed 15 min to settle. The samples were then filtered with ashless MG 640d Blue ribbon filter paper (Macherey & Nagel, Düren, Germany). Phosphate concentration was estimated spectrophotometrically (Hitachi Ltd., Tokyo, Japan) according to Gericke and Kurmis (1952). Magnesium, Calcium, Manganese and Zinc concentrations were measured by atomic absorption spectrophotometry (iCE 3000 series, Thermo Fischer, Dreieich, Germany) and K by flame emission spectrophotometry (Eppendorf-ELEX6361, Netheler & Hinz, Hamburg, Germany). The shoot content of mineral nutrients was estimated by multiplying mineral concentrations with dry shoot biomass.

Plant-available rhizosphere soil Phosphorus and soil pH

The air-dried soils were sieved with 2mm mesh size and sub-sampled for P and pH analysis. For the slightly alkaline soil, the Olsen P method (Olsen et al. 1954) was used for soil extraction and measured with inductively coupled plasma optical emission spectrometry (ICP-OES) while on the slightly acid soil, Calcium acetate-lactate (CAL) extraction developed by (VDLUFA, 2012) was used for extraction of potentially plant-available P and measured spectrophotometrically (Hitachi Ltd., Japan) according to the method of Gericke and Kurmis, (1952).

Soil pH was measured after 1 h shaking of soil suspension in 0.01 M $CaCl_2$ in a 1:1 ratio (Digital pH-Meter E532, Metrohm Harisau, Switzerland).

Statistical analysis

The experimental set up was arranged in a completely randomized design with weekly re-arrangements of the pots. After checking the normality and residuals of the data in SAS 9.4, One-ANOVA was performed at alpha 0.05. Proc glimmix procedure was performed for significance testing while the t-test at alpha 0.05 was employed for pairwise comparisons between selected treatments and their controls. To examine the relationships between shoot P, total root length, rhizosphere pH and P, correlation analysis was carried out with the mean values obtained from the ANOVA test.

4.3.1.3 Results

Plant growth

Generally, better performance of maize plants was recorded on the pH 5.6 (Fig. 4.6 A) as compared with the pH 7.9 soil (Fig. 4.6 B). On the moderately acidic sandy soil, ammonium fertilization with Rock-P (RP) supply increased plant growth and shoot biomass production over the unfertilized control to a level not significantly different (alpha=0.05) from the positive control with full soluble P fertilization. FZB42 inoculation had no additional growth effects (Fig

4.6 A & C). Root length development was increased by the N fertilization treatments without any additional FZB42 effects (4.7).

By contrast, on the pH 7.8 soil, the ammonium fertilization increased plant growth and shoot biomass production over the unfertilized control exclusively in combination with FZB42 inoculation but reached only about 30% of the biomass compared with the positive control with soluble P supply (Fig 4.6 B & C). The same trend was recorded for root length development of the plants (Fig 4.7).

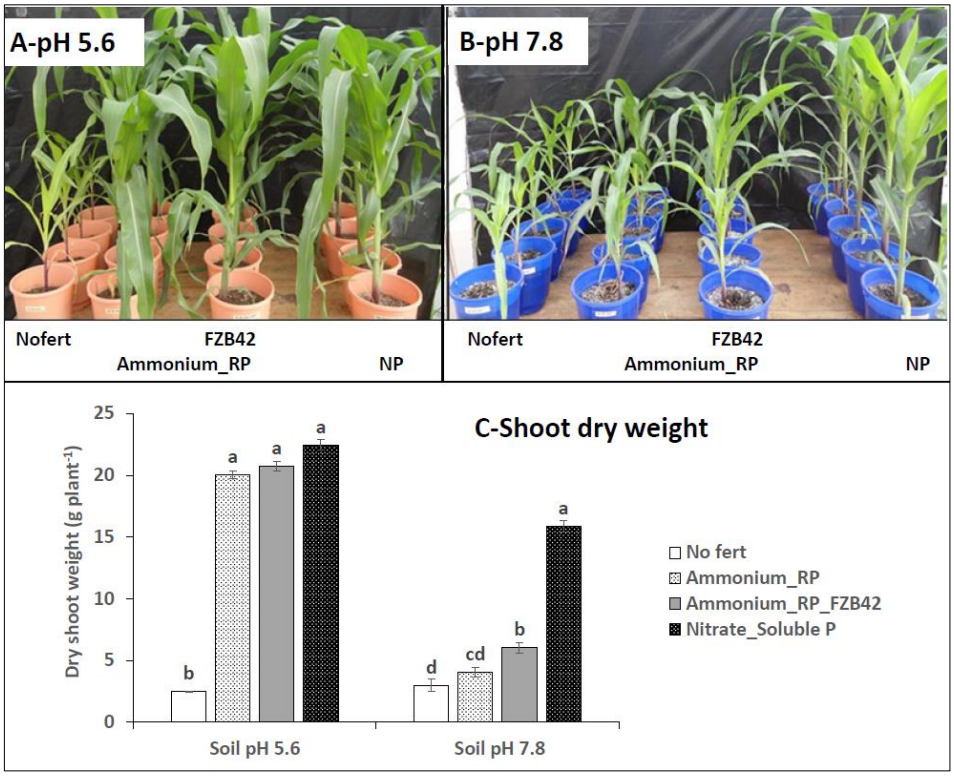


Figure 4.6: Habitus (A and B) and dry shoot weight (C) of maize supplied with DMPP-stabilized ammonium and Rock P (RP) fertilization, with and without *Bacillus amyloliquefaciens* (FZB42) inoculation, as compared with an unfertilized control (No fert) and soluble P fertilization with nitrate supply (Nitrate_Soluble P), on two soils with moderately acidic and alkaline pH. Means and SE of five replicates. For each soil, significant treatment differences (Tukey test, alpha=0.05) are indicated by different characters.

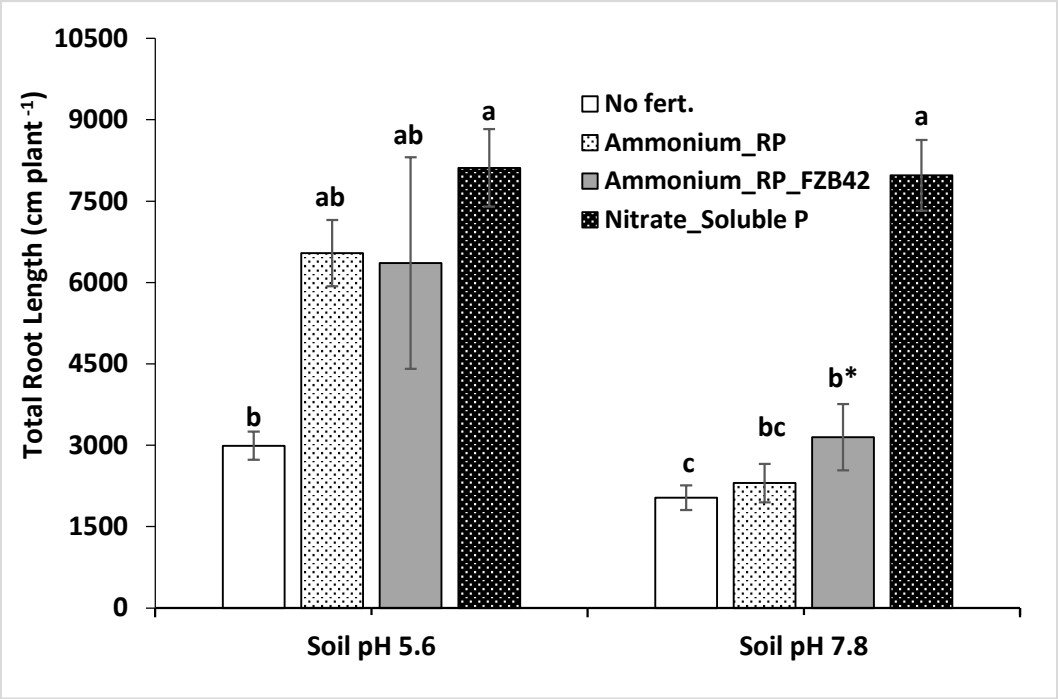


Figure 4.7: Total root length of maize supplied with DMPP-stabilized ammonium and Rock P (RP) fertilization, with and without *Bacillus amyloliquefaciens* (FZB42) inoculation, as compared with an unfertilized control (No fert) and soluble P fertilization with nitrate supply (Nitrate_Soluble P), on two soils with moderately acidic and alkaline pH. Means and SE of five replicates. For each soil, significant treatment differences are indicated by different characters (*=t-test, alpha=0.05 compared to ammonium-RP).

Rhizosphere pH, available rhizosphere P and plant nutritional status

Compared with the moderately acidic bulk soil pH (5.6), the pH of the root-adhering rhizosphere soil declined by 0.4, 0.9 and 1.4 units in the unfertilized control, ammonium-RP and ammonium-RP-FZB24 treatment, respectively with significant lower values in the ammonium-RP and ammonium-RP-FZB24 variants as compared to unfertilized control. By contrast, the plants with nitrate and soluble P fertilization significantly increased the rhizosphere pH by 0.5 units. On the pH 7.8 soil, only non-significant, marginal pH changes ≤ 0.3 were detectable (Fig 4.8).

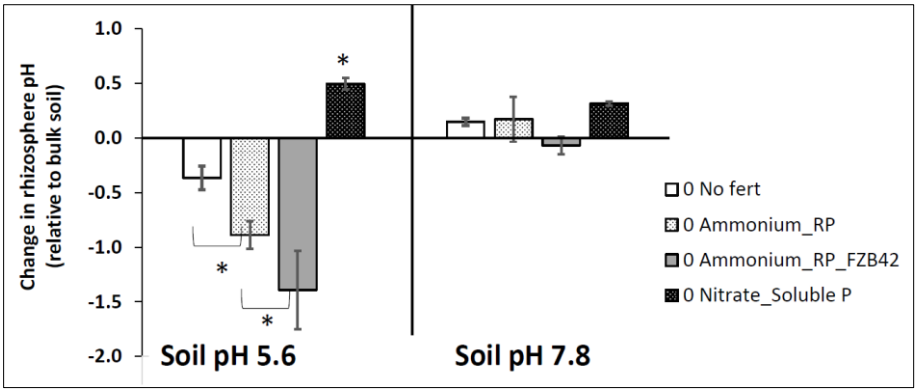


Figure 4.8: pH changes relative to the bulk soil in the rhizosphere of maize supplied with DMPP-stabilized ammonium and Rock P (RP) fertilization, with and without *Bacillus amyloliquefaciens* (FZB42) inoculation as compared with an unfertilized control (No fert) and soluble P fertilization with nitrate supply (Nitrate_Soluble P) on two soils with moderately acidic and alkaline pH. Means and SE of five replicates. * indicates significant differences (t-test, p= 0.05) compared to other treatments.

Potentially plant available CAL-P levels in the rhizosphere of the plants grown on the pH 5.6 soil showed an increasing trend in the order unfertilized control ≤ ammonium-RP < ammonium-RP-FZB42 ≤ nitrate and soluble P. No significant differences (alpha=0.05) were recorded between the ammonium-RP-FZB42 variant and the positive control with nitrate supply and soluble P fertilization (Fig 4.9 C). In the alkaline soil with nitrate and soluble P fertilization, the potentially plant-available Olsen P of the rhizosphere soil reached a level of approximately 80 mg kg^{-1} (positive control), which was significantly higher than P in the remaining variants, reaching only 18-20 mg kg^{-1} without significant treatment differences (alpha=0.05) (Fig 4.9 C).

The P-nutritional status of the plants grown on the moderately acidic soil reached the sufficiency threshold of approximately 3 mg P g^{-1} shoot DM (Campbell, 2009) in the variants with soluble P supply and in the ammonium-RP variants, both with and without FZB42 inoculation (Fig 4.9 A). However, only the ammonium-RP-FZB42 treatment reached a P shoot accumulation that was not significantly different (alpha=0.05) from the positive control with soluble P fertilization and nitrate supply (Fig 4.9 B). For the remaining nutrients K, Mg, Mn and Zn concentrations were in the sufficiency range although ammonium supply had a negative

effect on the Mg status and a positive effect on Zn and Mn shoot accumulation and tissue concentrations, without any additional effect by the microbial inoculant.

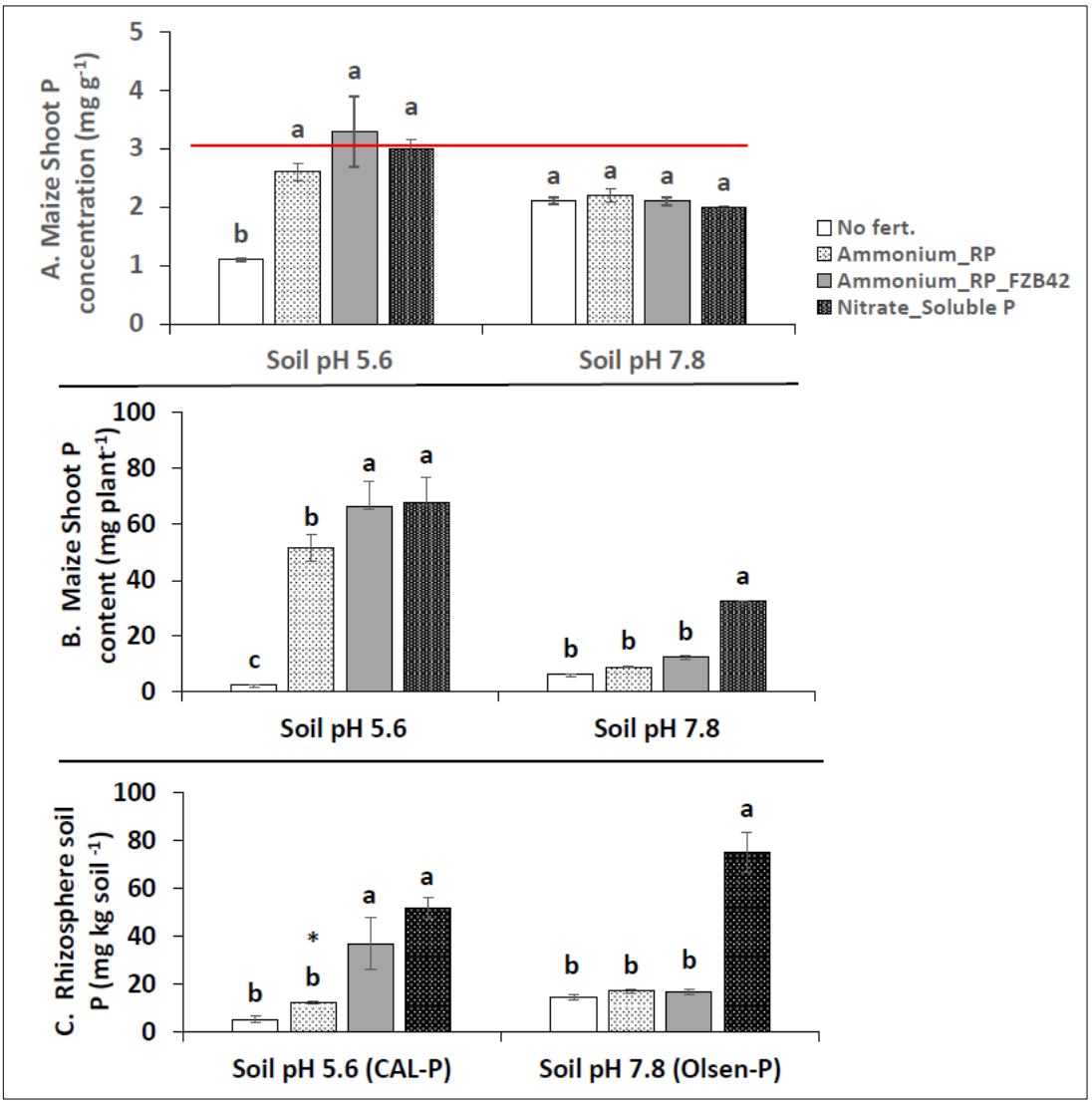


Figure 4.9: Shoot P concentration and P deficiency threshold (horizontal line) (A) shoot P content (B) and plant-available soil phosphorus in the rhizosphere (C) of maize supplied with DMPP-stabilized ammonium and Rock P (RP) fertilization, with and without *Bacillus amyloliquefaciens* (FZB42) inoculation, as compared with an unfertilized control (No fert) and soluble P fertilization with nitrate supply (Nitrate_Soluble P), on two soils with moderately acidic and alkaline pH. Means and SE of five replicates. For each soil, significant treatment differences (Tukey test, alpha=0.05) are indicated by different characters.

The N status was below the deficiency threshold of 30 mg g^{-1} shoot DM even in the variants with nitrogen supply. The highest N levels of 26 mg g^{-1} were recorded in the FZB42 inoculated variant. Ammonium fertilization induced Ca deficiency (< 25 mg g^{-1} shoot DM) and reduced Ca shoot accumulation independent of FZB42 inoculation (Table 4.8 A and B).

On the alkaline soil, the P-nutritional status remained in the deficiency range without treatment differences (Fig. 4.9 A). Only the shoot P accumulation of the positive control with soluble P fertilization was significantly increased (alpha=0.05) in comparison with the remaining variants (Fig. 4.9 B). All the remaining nutrients remained in the sufficiency range with the exception of the positive control supplied with nitrate and soluble P fertilization where the N and Mn status was close to the deficiency threshold (Table 4.8 A and B).

Table 4.8: Shoot mineral (A) concentration (conc.) and (B) content (cont.) of maize supplied with DMPP-stabilized ammonium and Rock P (RP) fertilization, with and without *Bacillus amyloliquefaciens* (FZB42) inoculation, as compared with an unfertilized control (No fert) and soluble P fertilization with nitrate supply (Nitrate_Soluble P), on two soils with moderately acidic and alkaline pH. Means and SE of five replicates. For each soil, significant treatment differences (Tukey test, alpha=0.05) are indicated by different characters.

A	N conc. (mg g^{-1})		K conc. (mg g^{-1})		Mn conc. (mg g^{-1})		Mg conc. (mg g^{-1})		Ca conc. (mg g^{-1})		Zn conc. (mg g^{-1})	
Soil pH	**5.6**	**7.8**	**5.6**	**7.8**	**5.6**	**7.8**	**5.6**	**7.8**	**5.6**	**7.8**	**5.6**	**7.8**
No fert	10.2^{b}	30.3^{a}	27.3^{ab}	47.2^{a}	0.1^{b}	0.03^{a}	4.4^{a}	3.1^{a}	19.3^{b}	34.9^{a}	0.02^{b}	0.04^{ab}
Ammonium_RP	23.0^{a}	35.7^{a}	22.7^{ab}	47.5^{a}	0.2^{a}	0.03^{a}	2.9^{b}	2.9^{a}	9.1^{c}	43.7^{a}	0.05^{a}	0.05^{a}
Ammonium_RP_FZB42	26.3^{a}	30.9^{a}	28.2^{a}	47.6^{a}	0.2^{a}	0.03^{a}	3.1^{b}	2.7^{a}	11.7^{c}	36.7^{a}	0.07^{a}	0.05^{a}
Nitrate_Soluble P	21.0^{a}	27.7^{a}	15.5^{b}	37.4^{b}	0.1^{b}	0.02^{a}	4.6^{a}	2.0^{a}	24.7^{a}	21.6^{b}	0.03^{b}	0.03^{b}

B	N cont. (mg $plant^{-1}$)		K cont. (mg $plant^{-1}$)		Mn cont. (mg $plant^{-1}$)		Mg cont. (mg $plant^{-1}$)		Ca cont. (mg $plant^{-1}$)		Zn cont. (mg $plant^{-1}$)	
Soil pH	**5.6**	**7.8**	**5.6**	**7.8**	**5.6**	**7.8**	**5.6**	**7.8**	**5.6**	**7.8**	**5.6**	**7.8**
No fert	25.0^{b}	90.4^{c}	67.8^{c}	142.5^{c}	0.2^{c}	0.1^{c}	10.9^{c}	9.0^{b}	47.7^{c}	99^{c}	0.5^{b}	0.1^{c}
Ammonium_RP	532.6^{a}	144.5^{bc}	450.7^{ab}	193.7^{bc}	3.4^{ab}	0.1^{bc}	57.9^{b}	11.7^{b}	181.9^{b}	174^{bc}	1.1^{a}	0.2^{bc}
Ammonium_RP_FZB42	427.5^{a}	183.9^{b}	561.8^{ab}	283.9^{b}	4.1^{a}	0.2^{b}	65.1^{b}	16.6^{b}	244.7^{b}	222^{b}	1.4^{a}	0.3^{ab}
Nitrate_Soluble P	471.2^{a}	439.1^{a}	350.6^{ab}	595.3^{a}	1.4^{bc}	0.3^{a}	103.3^{a}	32.0^{a}	557.0^{a}	341^{a}	0.6^{b}	0.4^{a}

4.3.1.4 Discussion

On the moderately acidic, sandy loam soil with pH 5.6, the combination of RP with stabilized ammonium fertilization was equivalent to the soluble P fertilization with nitrate supply, in terms of biomass production and the P sufficiency status (Campbell 2009). The FZB42 inoculant had no additional effects on plant growth and on the shoot P concentration. Nevertheless, the data suggest an additional contribution of FZB42 to P acquisition since the potentially, plant-available CAL-P concentrations in the rhizosphere soil and shoot P accumulation were significantly increased over the non-inoculated control (alpha=0.05). Both reached levels equivalent to the positive control with soluble P fertilization (Fig 4.9 B and C). This was associated with a declining rhizosphere pH (Fig 4.8), suggesting improved solubilization of RP due to FZB42-induced rhizosphere acidification down to pH 4.2. To our knowledge, this is the

first report suggesting a direct contribution of FZB42 to RP solubilization in the rhizosphere, which may work at least on light sandy soils with a low pH buffering capacity. Accordingly, a low pH buffering capacity can be expected for the investigated loamy sand pH 5.6 indicated by extremely low total Ca concentrations (0.06%) and low organic matter (content (0.7%). Rock P-solubilizing potential of FZB42 has been reported already on artificial growth media by Nkebiwe et al. (2016b). In face of the extremely low organic matter content of the investigated soil (0.7 %), a significant contribution of FZB42 to P mineralization via the release of secretory phosphatases seems to be less likely. However, the results need to be interpreted with some caution since also indirect effects of the inoculant cannot be excluded: i.e. Ögüt et al. (2011) reported stimulation of proton extrusion by the roots of the host plant after inoculation with certain strains of *Bacillus sp*. Also, the stimulation of root hair development induced by the inoculants (Dobbelaere et al. 1999) would increase the root surface area that is available for ammonium-induced rhizosphere acidification (Neumann and Römheld, 2002) and thereby might intensify the acidification potential of the roots. Moreover, improved recruitment of other plant beneficial microorganisms after FZB42 inoculation has been repeatedly reported in other studies (Eltlbany et al. 2019; Kröber et al. 2014, Thonar et al. 2016; Yusran et al. 2009). Under similar soil conditions, increased P solubilization from supplemented wood ash, improving the P status of maize, has been recently reported also by Mercl et al. (2018; 2019), using bacterial (*Paenibacillus mucilaginosus*) and fungal inoculants (*Penicillium sp. PK112 and Trichoderma harzianum OMG08*).

Interestingly, the significantly increased concentrations of potential plant available CAL-P in the rhizosphere and the increased P shoot accumulation in the FZB42 inoculated variant and in the positive control with soluble P supply, did not translate into any additional positive effects on shoot biomass production (Fig 4.6 A and C). Although the P status was sufficient, obviously N

limitation emerged as a growth limiting factor at the end of the culture period due to the limited pot volume, indicated by N concentrations below the deficiency threshold of 30 mg g^{-1} shoot FM in all treatments (Table 4.8 A; Campbell, 2009). Consequently, a surplus of P supply could not be transformed into biomass production due to the lack of Nitrogen. However, on this slightly acidic soil, the well-documented stimulatory effect of FZB42 on root length development in maize (Mpanga et al. 2018, 2019a, 2019b) was not detectable. One explanation could be the strong reduction of the rhizosphere pH in the ammonium-RP and particularly in the ammonium-RP-FZB42 variants down to pH 4.2-4.7, which obviously caused Ca deficiency in the respective treatments (Table 4.8 A) and may also be associated with a risk of induced Al toxicity on the weakly buffered sandy soil, both, with detrimental effects on root growth (Emanuelsson, 1984; Njoku et al. 1987; Kochian et al. 2005) . Accordingly, the highest root length was recorded in the positive control with soluble P supply and nitrate fertilization, which is possibly due to the rhizosphere alkalinization to pH 6.0 (Fig. 4.8), triggered by preferential nitrate uptake (Neumann and Römheld 2002).

Lambers et al. (2015) suggested a positive relationship between Mn shoot concentrations and carboxylate-mediated Mn solubilization in the rhizosphere. However, although the Mn concentrations increased in the variants with ammonium fertilization on the moderately acidic soil, no further increase was induced by FZB42 inoculation. This finding suggests an increased Mn status because of the well-documented Mn solubilization mediated by ammonium-induced rhizosphere acidification (Marschner 1995) whereas additional carboxylate production of the inoculated bacteria, reported for many PSMs (Sharma et al. 2013) appears unlikely.

By contrast, on the alkaline pH 7.8 soil, FZB42 inoculation had a significant (alpha=0.05) root growth promoting effect (Fig. 4.7), which translated into a moderately increased shoot biomass production (Fig. 4.6). However, the P nutritional status remained in the deficiency range, even

in the variant with soluble P supply and nitrate fertilization. Obviously, this soil had a high P fixation potential (total Ca=10,523 mg kg^{-1} soil) (Table. 4.7) that counteracted the effects of soluble P fertilization. This was reflected by a 30% reduced biomass even after supply of soluble P fertilization, as compared with the P adequate plants on the pH 5.6 soil. The high soil pH also reduced the bioavailability of micronutrients (Neumann and Römheld, 2002) and the Mn status was critical in the variant with soluble P supply (Table 4.7). Furthermore, no changes in rhizosphere pH were recorded in the variants with ammonium fertilization, probably due to a high pH buffering capacity of the respective soil, excluding chemical P solubilization via rhizosphere acidification.

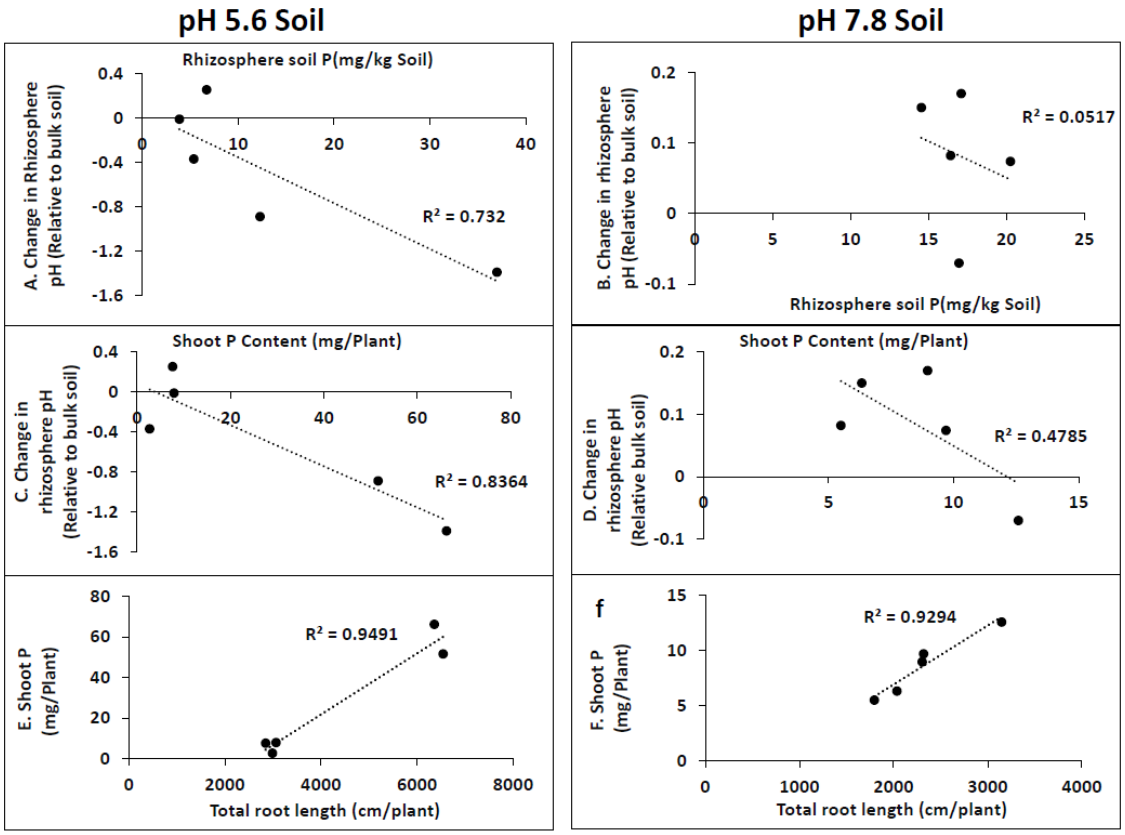


Figure 4.10: Correlations between: rhizosphere soil pH and rhizosphere available P (A and B); rhizosphere soil pH and shoot P content (C and D); shoot P content and total root length (E and F) of maize in different pH soils (left= pH 5.6 soil and right= pH 7.8 soil).Rhizosphere soil P in the slightly acid and alkaline soils were extracted by CAL and Olsen methods respectively.

Consequently, FZB42-induced root growth promotion only contributed to improved spatial acquisition of the low concentrations of soluble P but had no effects on P solubilization, resulting in a limited stimulation of plant growth. Accordingly, on both soils, P shoot accumulation was positively correlated with root length, whereas a correlation with declining rhizosphere pH was detectable exclusively on the pH 5.6 soil (Fig 4.10).

Recent studies demonstrated that limitations of ammonium-induced P solubilization by high pH buffering on alkaline soils might be overcome by ammonium-, and P-placement strategies (Nkebiwe et al. 2016a; Jing et al. 2010). Local root growth proliferation in response to the locally placed N and P supply (Drew 1975) leads to a local intensification of rhizosphere acidification, which could be enough to mediate Ca-P solubilization even at soil pH > 8 (Jing et al. 2010). Nkebiwe et al. (2016b) demonstrated that the local root proliferation can be further intensified by PGPM inoculation. Most recently Bradacova et al. (2019) found improved PGPM-mediated P acquisition in open field tomato production on a low P, pH 7.9 soil with stabilized ammonium sulfate placement without additional P supply, associated with increased biomass production and fruit yield.

Although the potential effects of other soil properties independent of soil pH cannot be excluded, the presented results suggest a strong impact of soil pH on the performance PGPM inoculants in maize. On the two investigated soils, the mode of action of a single PGPM inoculant was distinctly different, promoting P solubilization on the acidic soil and spatial nutrient acquisition under moderately alkaline soil conditions. This aspect needs to be considered for practical applications in terms of selection and application strategies of compatible PGPM-fertilizer combinations.

Acknowledgement

Funding provided by the European Community's FP7 Program/2007-2013 under grant agreement n° 312117 (BIOEFFECTOR), Landor fertilizer company (Landor, Birsfelden Switzerland) and FIAT PANIS through the Food Security Center at the University of Hohenheim in Germany. Many thanks also to the University of Energy and Natural Resources, Dormaa campus for hosting my research stay in Ghana.

Conflict of Interest

The authors have no conflict of interest regarding this manuscript.

References

Abbasi, M.K., Musa, N. and Manzoor, M. (2015) Mineralization of soluble P fertilizers and insoluble rock phosphate in response to phosphate-solubilizing bacteria and poultry manure and their effect on the growth and P utilization efficiency of chilli (*Capsicum annuum* L.). Biogeosciences12, 4607–4619.

Bonser, A.M., Lynch, J. and Snapp, S. (1996) Effect of phosphorus deficiency on growth angle of basal roots in *Phaseolus vulgaris*. New Phytol. 132, 281–288.

Borris, R. (2015) Towards a New Generation of Commercial Microbial Disease Control and Plant Growth Promotion Products. In Principles of Plant-Microbe Interactions ed. Lugtenberg, B. pp.329-337. Basel, Switzerland: Springer International Publishing.

Bradáčová, K., Florea, A. S., Bar-Tal, A., Minz, D., Yermiyahu, U., Shawahna, R., Kraut-Cohen, J., Zolti, A., Erel, R., Dietel, K., Weinmann, M., Zimmermann, B., Berger, N., Ludewig, U., Neumann, G. and Poşta, G. (2019) Microbial consortia versus single-strain inoculants: an advantage in PGPR-assisted tomato production? Agronomy, 9(2), 105

Bumb, B. and Baanante, C. (1996) The role of fertilizer in sustaining food security and protecting the environment to 2020. 2020 Vision Discussion Paper 17. Washington, DC: IFPRI.

Campbell, C. R. (2000) Reference sufficiency ranges for plant analysis in the southern region of the united states (Ed.), Southern Cooperative Series Bulletin. 394, North Carolina. Retrieved from www.ncagr.gov/agronomi/saaesd/scsb394.pdf

Campos, P., Borie, F., Cornejo, P., López-Ráez, J. A., López-García, Á. and Seguel, A. (2018) Phosphorus Acquisition Efficiency Related to Root Traits: Is Mycorrhizal Symbiosis a Key Factor to Wheat and Barley Cropping? Front. Plant Sci. 9:752. doi: 10.3389/fpls.2018.00752

Dobbelaere, S., Croonenborghs, A., Thys, A., Vande, B. A. and Vanderleyden, J. (1999) Phytostimulatory effect of Azospirillum brasilense wild type and mutant strains altered in IAA production on wheat. Plant Soil 212: 153-162; dio: 10.1023/A:1004658000815

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

Elena, M., Bennett, S. R., Carpenter, N. and Caraco, F. (2001) Human Impact on Erodable

Phosphorus and Eutrophication: A Global Perspective: Increasing accumulation of phosphorus in soil threatens rivers, lakes, and coastal oceans with eutrophication, BioSc, 51, 3, 227–234

Emanuelsson, J. (1984) Root growth and calcium uptake in relation to calcium concentration. Plant Soil, 78, 325–334.

Eltlbany, N., Baklawa, M., Guochun, D., Nassal, D., Weber, N., Kandeler, E., Neumann, G., Ludewig.,U., van Overbeek, L. and Smalla, K. (2019) Enhanced tomato plant growth in soil under reduced P supply through microbial inoculants and microbiome shifts FEMS Microbiol Ecol submitted.

Gahoonia, T. S. and Nielsen, N. E. (2004) Barley genotypes with long root hairs sustain high grain yields in low-P field. Plant Soil 262: 55–62

Gaume, A., Mächler, F., De Le´on, C., Narro, L. and Frossard, F. (2000) Low-P tolerance by maize (Zea mays L.) genotypes: Significance of root growth, and organic acids and acid phosphatase root exudation. Plant Soil 228: 253–264

Gericke, S., and Kurmis, B. (1952) Die kolorimetrische Phophorsäurebestimmung mit Ammonium-Vanadat-Molybdat und ihre Anwendung in der Pflanzenanalyse. Z. Pflanzenernaehr. Bodenkd, 59, 235–247.

Illmer, P., and Schinner, F. (1995) Solubilization of inorganic calcium phosphates - Solubilization mechanisms. Soil Biol Biochem, 27, 257–263. https://doi.org/10.1016/0038-0717(94)00190-C

Jing, J., Rui, Y., Zhang, F. and Rengel, Z., Shen, J. (2010) Localized application of phosphorus and ammonium improves growth of maize seedlings by stimulating root proliferation and rhizosphere acidification. F. Crop. Res., 119, 355–364, doi: 10.1016/j.fcr.2010.08.005.

Kimble, J. M., Ping, C. L., Sumner, M. E. and Wilding, L. P. (2000) Andisols. In: Sumner ME (ed) Handbook of soil science. CRC, Boca Raton, pp 209–224.

Kirchmann, H., Nyamangara, J. and Cohen, Y. (2005) Recycling municipal wastes in the future: from organic to inorganic forms? Soil Use Manag, 21: 152–159

Kochian, L.V., Pin, M. A. and Hoekenga, O. A. (2005) The physiology, genetics and molecular biology of plant aluminum resistance and toxicity. Plant Soil , 274, 175–195

Kröber, M., Wibberg, D., Grosch, R., Eikmeyer, F. G., Verwaaijen, B., Chowdhury, S. P., Hartmann, A., Pühler, A. and Schlüter, A. (2014) Effect of the strain *Bacillus amyloliquefaciens FZB42* on the microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing. Front. Microbiol. 5, 252.

Lambers, H., Hayes, P. E., Laliberte, E., Oliveira, R. S. and Turner, B. L. (2015) Leaf manganese accumulation and phosphorus-acquisition efficiency. Trends Plant Sci. 20, 83-90. https://doi.org/10.1016/j.tplants.2014.10.007

Liu, H., White, P. J. and Li, C. (2016) Biomass partitioning and rhizosphere responses of maize and faba bean to phosphorus deficiency. Crop Pasture Sci., 67, 847–856.

Mardad, I., Serrano, A. and Soukri, A. (2013) Solubilization of Inorganic phosphate and production of organic acids by bacteria isolated from a Moroccan mineral phosphate deposit.pdf. Afr. J. Microbiol. Res, 7(8), 626–635.

Marschner, H. (1995) Mineral nutrition of higher plants. second edition. 889pp. London: Academic Press

Mercl, F., Tejnecký. V., Ságová-Marečková, M., Dietel, K., Kopecký, J., Břendová, K., Kulhánek, M., Košnář, Z., Száková, J. and Tlustoš, P. (2018) Co-application of wood ash and Paenibacillus mucilaginosus to soil: the effect on maize nutritional status, root exudation and composition of soil solution. Plant Soil 428:105–122

Mercl, F., García-Sáncheza, M., Kulhánek, M., Košnář, Z., Száková, J. and Tlustoš, P. (2019)

Improved phosphorus fertilisation efficiency of wood ash by fungal strains Penicillium sp. PK112 and Trichoderma harzianum OMG08 on acidic soil. Appl. Soil Ecol, 147, 103360. doi.org/10.1016/j.apsoil.2019.09.010.

Mpanga, I. K., Nkebiwe, P. M., Kuhlmann, M., Cozzolino, V., Piccolo, A., Geistlinger, J., Berger, N., Ludewig, U. and Neumann, G. (2019a) The Form of N Supply Determines Plant Growth Promotion by P-solubilizing Microorganisms in Maize. Microorganisms, 7, 38

Mpanga, I. K., Dapaah, H. K., Geistlinger, J., Ludewig, U. and Neumann, G. (2018) Soil type-dependent interactions of P-solubilizing microorganisms with organic and inorganic fertilizers mediate plant growth promotion in tomato. Agronomy, 8(10), 213.

Mpanga, I. K., Gomez, N., Moradtalab, N., Ahmed, A., Geistlinger, J., Wanke, D., Walker, F., Windisch, S., Chrobaczek, V., Ludewig, U. and Neumann, G.(2019b) The Role of N form Supply on PGPM-host Plant Interaction in Maize. *J. Plant Nutr. Soil Sci. 2019, 000, 1–13.*

Njoku, B.O., Enwezor, W.O. and Onyenakwe, B.I. (1987) Calcium deficiency identified as an important factor limiting maize growth in acid ultisols of eastern Nigeria Laboratory incubation. Fertil. Res., 14, 113–123.

Neumann, G. and Römheld, V. (2002) Root-induced changes in the availability of nutrients in the rhizosphere. In Plant Roots the Hidden Half, 3rd ed.; Waisel, Y., Eshel, A., Kafkafi, U., Eds.; Marcel Dekker: New York, NY, USA; pp. 617–649.

Nkebiwe, P. M., Neumann, G. and Müller, T. (2017) Densely rooted rhizosphere hotspots induced around subsurface NH4+-fertilizer depots: a home for soil PGPMs? Chem. and Biol. Technol. Agric, 4(1), 1–16. https://doi.org/10.1186/s40538-017-0111-y

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. and 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. 3:15 DOI 10.1186/s40538-016-0065-5

Olsen, S. R., Cole, C. V., Watanabe, F. S., and Dean, L. A. (1954) Estimation of available phosphorus in soils by extraction with sodium bicarbonate. Washington DC, US Government Printing Office.

Ögüt, M., Er, F. and Neumann, G. (2011) Increased proton extrusion of wheat roots by inoculation with phosphorus solubilising microorganisms. Plant Soil. 339 (1), 285–297. https://doi.org/10.1007/s11104-010-0578-9.

Runge-Metzger, A. (1995) Closing the cycle: obstacles to efficient P management for improved global security. In H Tiessen, ed, Phosphorus in the Global Environment. John Wiley and Sons Ltd, Chichester, UK, pp 27-42.

Russell, D. W. (1973) Soil conditions and plant growth. New York: Longman Group Ltd.

Schuch, U. K. (2018) Specialty Crop Production Practices for Beginning Farmers in Arizona and the Southwest. University of Arizona Cooperative Extension publication, (no. az1774). https://extension.arizona.edu/sites/extension.arizona.edu/files/pubs/az1774-2018.pdf

Sharma, S. B., Sayyed, R. Z., Trivedi, M. H., and Gobi, T. A. (2013) Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springer Plus. 2(1), 587. https://doi.org/10.1186/2193-1801-2-587

Stoffella, P. J., He, Z. I., Wilson, S. B., Ozores-Hampton, M., Roe, B. E. (2014) Compost Utilization in Subtropical Horticultural Cropping Systems. In I International Symposium on Organic Matter Management and Compost in Horticulture; Biala, J., Prange, R., Raviv, M., ed. Acta Horticulturae: Adelaide, Australia, pp. 95–108.

Thonar, C., Lekfeldt, J. D. S., Cozzolino, V., Kundel, D., Kulhánek, M., Mosimann, C., Neumann,

G., Piccolo, A., Rex, M. and Symanczik, S. (2017) Potential of three microbial bio-effectors to promote maize growth and nutrient acquisition from alternative phosphorous fertilizers in contrasting soils. Chem. Biol. Technol. Agric, 4, 7.
Uexküll, H. R. and Mutert, E. (1995) Global Extent Development and Economic Impact of Acid Soils. Plant Soil, 171, 1-15. http://dx.doi.org/10.1007/BF00009558
Upreti, C.G. (1984) Colorimetric estimation of inorganic phosphate in colored and/or turbid biological samples: Assay of phosphohydrolases. Analyt. Biochem. 137(2), 485-492.
Van Kauwenbergh, S. J., Stewart, M., Mikkelsen, R. (2013) World Reserves of Phosphate Rock… a Dynamic and Unfolding Story. Better Crops, 97 (No. 3)
Vance, C.P. (2001) Symbiotic nitrogen fixation and phosphorus acquisition: plant nutrition in a world of declining renewable resources. Plant Physiology. 127, 390-397.
Vance, C.P., Uhde-Stone, C. and Allan, D. (2003) Phosphorus acquisition and use: critical adaptation by plants for securing non-renewable resources. New Phytologist. 15, 423-447.
VDLUFA (2012) VDLUFA Methodenbuch Band I die Untersuchung von Böden. Verband deutscher landwirtschaftlicher Untersuchungs-und Forschungsanstalten e.V. Speyer, Germany.
Vinci, G., Cozzolino, V., Mazzei, P., Monda, H., Spaccini, R. and Piccolo, A. (2018a) Effects of Bacillus amyloliquefaciens and organic and inorganic phosphate amendments on Maize plants as revealed by NMR and GC-MS based metabolomics. Plant Soil 429(10):1-14
Vinci, G., Cozzolino, V., Mazzei, P., Monda, H., Spaccini, R. and Piccolo, A. (2018b) An alternative to mineral phosphorus fertilizers: The combined effects of Trichoderma harzianum and compost on Zea mays, as revealed by 1 H NMR and GC-MS metabolomics. PLuS ONE 13(12): e0209664.
Yusran, Y., Römheld, V. and Müller, T. (2007) Effects of Pseudomonas sp." Proradix" and Bacillus amyloliquefaciens FZB42 on the establishment of AMF infection, nutrient acquisition and growth of tomato affected by Fusarium oxysporum Schlecht f.sp. radicis-lycopersici Jarvis and Shoemaker. UC Davis The Proceedings of the International Plant Nutrition Colloquium 2007. XVI. https://escholarship.org/uc/item/8g70p0zt.

4. 3.2: Soil type-dependent expression of PGPR effects in maize as affected by the form of nitrogen fertilization

Chin Tao Hsu (2018), Co-supervised Master thesis.

Date of submission: May 2018

Institute of Crop Sciences (340h), University of Hohenheim, Germany. Email: b99601040@ntu.edu.tw

4.3.2. 1 Introduction

The study of Mpanga et al. (presented under 4.3.1) suggested a significant impact of the soil pH on the synergistic performance and even on the modes of action for P acquisition mediated by the PGPM inoculant *Bacillus amyloliquefaciens* FZB42 combined with stabilized ammonium

fertilization. However, on the two investigated soils with contrasting properties, potential effects independent of soil pH cannot be excluded. To evaluate the importance of the pH effect, an additional experiment was conducted using an alternative selection of soils with contrasting pH (pH 5.6; pH 6.8 and pH 7.6) and an alternative PGPM product (CombifectorB) for a systematic comparison of N form effects (nitrate versus stabilized ammonium) on rock-P acquisition in maize.

Hypothesis: If the soil pH is a major determinant for the performance of PGPMs in combination with ammonium fertilizers, the results of Mpanga et al. (4.3.1) will be confirmed also with an alternative selection of soils with contrasting pH and with different inoculants.

6.2.2 Materials and methods

This experiment was carried out in a greenhouse located in the Institute of Crop Science at the University of Hohenheim in Germany using three soil substrates with contrasting pH. The day length was 15-16 h with average temperature, relative humidity and dew point of 29^{o}C, 41% and 14^{o}C respectively. The experiment comprised five treatments for each soil with five replicates. Each pot was filled with 2400 g of substrate, a mixture of 30% coarse sand and 70% of each soil type; (1) Sandy-loam soil mix with pH_{CaCl2}: 5.6; plant-available P: P_{CAL}: 7 mg kg^{-l} soil; C_{org}: 0.58% and N_{total}: 0.076%. (2) Clay-loam organic farming soil (Klein Hohenheim experimental station) with pH_{CaCl2}: 6.8; plant-available P: P_{CAL}: 36.7 mg P kg^{-1}; N_{total}: 0.15%; C_{org}: 1.28%. (3) Calcareous Loess subsoil with Plant-available P: P_{CAL}: 5 mg kg^{-1}; pH_{CaCl2}: 7.6; C_{org}: < 0.3%; N_{total} 0.02 %; $CaCO_3$: 23 %).

The experiment was arranged in a complete randomized design (RCD)

Fertilizer treatments:

1. **NO3_RP:** nitrate plus rock P without CFB

2. NO3_RP_CFB: nitrate plus rock P and CFB
3. NH4_RP: ammonium plus rock P without CFB
4. NH4_RP_CFB: ammonium plus rock P and CFB
5. NO3_SSP: nitrate with soluble P as single super phosphate.

Fertilization was performed with 150mg N kg^{-1} soil as Ca-nitrate (Calcinit, Yara 15.5% N; Yara, Oslo, Norway) or ammonium sulfate (Novatec 21 Solub 21% N stabilized with 0.08% DMPP (3, 4-dimethylpyrazole phosphate; Compo EXPERT GmbH, Muenster, Germany). Rock Phosphate (RP) - was applied at 150 mg P kg^{-1} soil (Granuphos 7.85 % P, Landor, Birsfelden, Switzerland). A positive control variant received soluble P (150 mg P kg^{-1} soil) as single-super phosphate SSP (Triferto, 18 % P_2O_5, Doetinchem, Netherland). In all variants, potassium was applied with 200mg K kg^{-1} soil as K_2SO_4. The microbial inoculant used in this experiment was a consortium product with proven PGPM activity documented in previous experiments (Mpanga et al. 2019). CombifectorB (CFB) is based on a combination of *Trichoderma harzianum OMG16* ($9x10^9$ spores/g) from Anhalt University of Applied Sciences, and Rhizovital FZB42 *Bacillus amyloliquefaciens* ($1x10^{11}$ cfu/g) from ABiTEP, Berlin Germany in a Kaolin formulation supplemented with Zn as $ZnSO_4.7H_2O$, Mn as $MnSO_4.H_2O$) For inoculation, 4g of CFB was dissolved in 600ml water as a stock solution and 20 ml CFB suspension was inoculated applied per pot by soil drenching in three weekly intervals starting at sowing.

6.2.3: Results

In accordance with the results of Mpanga et al. (4.3.1), on the acidic sandy loam soil pH 5.6, NH_4-induced P mobilization, associated with a significant rhizosphere acidification down to pH 4.7 (Table 4.9) was obviously sufficient to induce the same biomass production as compared with the positive control (Fig. 4.11) with full soluble P fertilization, confirmed also by a similar

P tissue concentration (Table 4.10). Accordingly, no further growth promotion was induced by PGPM inoculation. Under nitrate fertilization, the rhizosphere pH remained at 5.9 associated with a lower P availability and a reduction of biomass production and shoot P accumulation by 52 % and 74 %, respectively. Under these conditions with reduced P availability, PGPM inoculation had a moderate effect on biomass production (+28 %) and P shoot accumulation (+19%).

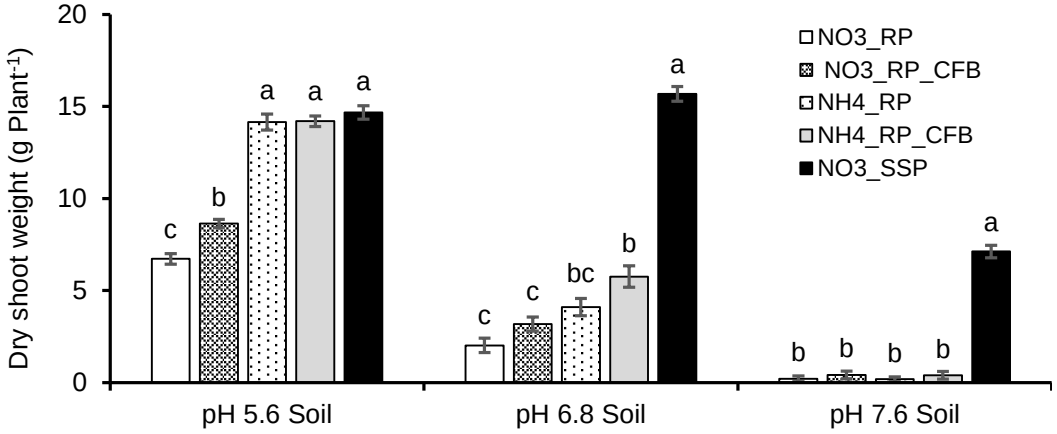


Figure 4.11: Shoot biomass production of Maize (cv Rolandinio) depending on soil pH at 42 days after sowing (DAS) with Rock-P (RP) and nitrate (NO3) vs DMPP stabilized ammonium sulfate (NH4) fertilization with and without CFB inoculation (*Bacillus amyloliquefaciens* FZB42/ *Trichoderma harzianum* OMG16 combination). Positive control with Ca-nitrate and single superphosphate (NO3_SSP)) fertilization (Means and SE of five replicates, SAS, Tukey test, for each soil, same letter indicates no significant differences at p=0.05, n=5).

Similar to the results of Mpanga et al (4.3.1) the beneficial effect of stabilized ammonium fertilization on plant biomass production declined with increasing soil pH associated with declining intensity of rhizosphere acidification and reduction of shoot P accumulation. On the pH 6.8 soil, a synergistic effect of PGPM inoculation with ammonium fertilization was detectable in terms of plant biomass production and P shoot accumulation but did not reach the positive control with soluble P fertilization. On the highly-buffered calcareous pH 7.6 soil, no ammonium-induced rhizosphere acidification was detectable. This was associated with severe growth depression and P limitation of the maize plants without the establishment of any detectable beneficial plant-PGPM interactions.

Table 4.9: Rhizosphere pH of maize (cv Rolandinio) depending on soil pH at 42 days after sowing (DAS) with Rock-P (RP) and nitrate (NO3) vs DMPP stabilized ammonium sulfate (NH4) fertilization with and without CFB inoculation. (Means and SE of five replicates, SAS, Tukey test, for each soil, same letter indicates no significant differences at p=0.05, n=5).

	Rhizosphere Soil pH		
	pH 5.6	pH 6.8	pH 7.6
NO_3_RP	5.9 a	7.2 a	7.7 a
NO_3_RP_CFB	5.9 a	7.2 a	7.7 a
NH_4_RP	4.7 b	6.9 b	7.7 a
NH_4_RP_CFB	4.7 b	6.9 b	7.6 a
NO_3_TSP	6.0 a	7.2 a	7.5 a

Table 4.10: Phosphate status of Maize (cv Rolandinio) depending on soil pH at 42 days after sowing (DAS) with Rock-P (RP) and nitrate (NO3) vs DMPP stabilized ammonium sulfate (NH4) fertilization with and without CFB inoculation. (Means and SE of five replicates, SAS, Tukey test, for each soil, same letter indicates no significant differences at p=0.05, n=5).

	P concentration (mg g^{-1})			P content (mg Plant $^{-1}$)		
	Soil pH 5.6	Soil pH 6.8	Soil pH 7.6	Soil pH 5.6	Soil pH 6.8	Soil pH 7.6
NO_3_RP	1.2 c	1.8 a	0.8 c	8.3 d	3.7 c	0.2 b
NO_3_RP_CFB	1.2 c	1.9 a	0.8 bc	9.9 c	6.1 bc	0.5 b
NH_4_RP	1.7 ab	1.9 a	0.9 abc	23.5 b	8.0 bc	0.2 b
NH_4_RP_CFB	1.5 b	1.8 a	1.0 ab	21.9 b	10.9 b	0.4 b
NO_3_SSP	1.9 a	1.5 a	1.1 a	27.1 a	22.7 a	7.7 a

6.2.4: Conclusions

This experiment confirmed the first part of the study (4.3.1), which showed that soil pH was a common factor on the five investigated soils, which affects the ability of PGPMs to induce plant growth promotion by utilization of sparingly soluble Ca-P sources in combination with ammonium-dominated fertilization. The declining efficiency with increasing soil pH reflects the intensity of ammonium-induced rhizosphere acidification depending on the buffering capacity of the soil substrate (Römheld, 1986, Fig. 4.12) as an important component of the synergistic effects of PGPM inoculation with stabilized ammonium fertilization (Mpanga et al., 2019a). On the highly-buffered calcareous subsoil without detectable rhizosphere acidification, the extremely P-deficient host plants were obviously weak to establish a functional interaction with the microbial inoculants, similar to the conditions reported for symbiotic interactions with arbuscular mycorrhizal fungi or N_2-fixing Rhizobia on extremely P-deficient soils (Bitman et al

2006; Chekanaia et al., 2018). This implicates that soil properties, such as pH and buffering capacity need to be considered for applications of PGPM-assisted strategies to improve P acquisition of crops.

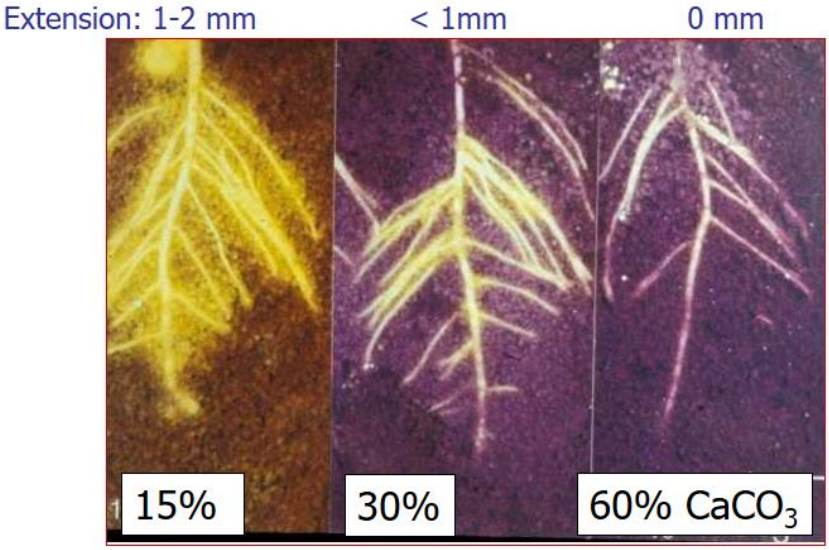


Figure 4.12: Extension of ammonium-induced rhizosphere acidification (yellow coloration of the pH indicator bromocresol purple) in chickpea as affected by increasing the substrate pH buffering capacity via liming (Römheld, 1986).

On neutral to moderately acidic soils, the combination of PGPMs with stabilized ammonium fertilizer may promote the acquisition of sparingly soluble Ca-P forms, including fertilizers based on rock-P, slags and ashes (Mpanga et al., 2018, 2019a; Nkebiwe 2016). However, on light sandy soils with low pH, buffering, the ammonium effect on P solubilization can overcompensate PGPM effects, and even negative consequences by induced Ca deficiencies or Al toxicity, as a consequence of intense rhizosphere acidification, have been observed (see 4.3.1, Sittinger, 2018). By contrast, the inefficiency of PGPM effects on highly buffered alkaline soils, due to limited expression of the ammonium-induced rhizosphere acidification (Römheld, 1986), might be overcome by ammonium placement strategies leading to localized root proliferation and consequently intensification of the acidification effect (Jing et al. 2010; Nkebiwe et al., 2016a, b; Bradacova et al., 2019)

6.2.4: References

Bittman S, Kowalenko CG, Hunt DE, Forge TA, Wu X (2006): Starter phosphorus and broadcast nutrients on corn with contrasting colonization by mycorrhizae. Agronomy J. 98: 394-401.

Bradáčová K., Florea A.S., Bar-Tal A., Minz D., Yermiyahu U., Shawahna R., Kraut-Cohen J., Zolti A., Erel R., Dietel K., Weinmann M., Zimmermann B., Berger N., Ludewig U., Neumann G., Poşta G. (2019): Microbial consortia versus single-strain inoculants: an advantage in PGPR-assisted tomato production? *Agronomy*, 9(2), 105.

Chekanaia V, Chikowoa R, Vanlauwe B (2018): Response of common bean (Phaseolus vulgaris L.) to nitrogen, phosphorus and rhizobia inoculation across variable soils in Zimbabwe. Agriculture, Ecosystems and Environment 266: 167–173.

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. F. Crop. Res., 119, 355–364, doi: 10.1016/j.fcr.2010.08.005.

Mpanga I. K., Nkebiwe P.M., Kuhlmann K, Cozzolino V., Piccolo A., Geistlinger G., Berger N., Ludewig U., Neumann G (2019): The Form of N Supply Determines Plant Growth Promotion by P-solubilizing M

Mpanga I.K,. Dapaah H.K., Geistlinger J., Ludewig U., Neumann G. (2018): Soil type-dependent interactions of P-solubilizing microorganisms with organic and inorganic fertilizers mediate plant growth promotion in tomato. Agronomy 2018, 8, 213; doi: 10.3390/agronomy8100213icroorganisms in Maize. Microorganisms 7(2): 38 https://doi.org/10.3390/microorganisms7020038 - 29

Nkebiwe P.M. (2016): Fertilizer placement and the potential for its combination with bio-effectors to improve crop nutrient acquisition and yield. Dissertation, Universität Hohenheim, Stuttgart, Germany.

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 Research 196:389-401

Nkebiwe P.M, Weinmann M., and 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. 3:15 DOI 10.1186/s40538-016-0065-5

Sittinger M. (2018): Effects of a microbial consortia product on the growth of maize (Zea mays). Master Thesis, Universität Hohenheim., Stuttgart, >Germany

Römheld V. (1986): pH changes in the rhizosphere of various crop plants in relation to the supply of plant nutrients. In: Potash Review 11: Berne, Switzerland: International Potash Institute.

4.3.3 Microgranulates as stabilized ammonium fertilizers to improve plant-PGPM interactions on alkaline soils

4.3.3.1 Introduction:

In face of the limited performance of PGPM-assisted P acquisition in combination with stabilized ammonium fertilizer on alkaline soils (4.3.1, 4.3.2, Mpanga et al. 2018) it was hypothesized that in contrast to homogenous ammonium application, the placement effect of granulated ammonium starter fertilizers will locally increase the acidification potential of the roots. This effect will promote PGPM-assisted acquisition of sparingly soluble nutrients such as

P and micronutrients and thereby plant growth even on soils with high pH-buffering capacity where broadcasted ammonium fertilizers have no effect.

4.3.3.2 Materials and Methods

Maize cv Rolandino grown on a low P soil substrate mix (Calcareous Loess subsoil pH 7.6 + sandy loam soil pH 5.6) + 20% sand (pH 6.9, P_{CAL} 3 mg/kg). Pot experiment: 2kg pots: Microgranulates provided by Eurochem Agro , Mannheim, Germany: (50 mg/kg soil) + Rock-P (50 mg P/kg soil)

- TP3136 Micro granulate; „UMG Micro Starter Max (10N, 46P, 2Zn, 1Fe)"
- TP3136 + Bacillus subtillis CH13 (TP1002)
- TP3093 Microgranulate: "UMG Micro Zeastar (7N, 32P,1K, 2.4S: 4Zn, 0.6F, Rizodyne)"
- TP 3093 + *Bacillus subtillis* CH13 (TP1002)
- NH4_RP: Homogenous Novatec solub (150 mg N/kg soil) + Rock-P (100 mg P/kg soil)
- NH4_RP + *B.subtillis* CH13 Powder 10^6 cfu/g, soil drenching 0.5% suspension)

Final harvest after 4 weeks greenhouse culture

4.3.3.3 Results

There was no indication for improved P acquisition by CH13 (TP1002) inoculation in the variant with homogenous DMPP ammonium sulfate fertilization, while CH13 significantly stimulated shoot biomass production in combination with the TP3136 microgranulates with high N and P concentrations. Phosphate limitation in the unfertilized control had significant inhibitory effects on the shoot and root growth (biomass and root length with similar effects also in the Rock-P variants with homogenous DMPP-ammonium fertilization (Table 4.11). Microgranulates significantly increased shoot and root biomass production and root length. However, *B. subtilis* CH13 (TP1002) inoculation increased shoot biomass production and root length only in the TP3136 combination (high N/P concentration) without any effects on root biomass. This finding suggests that CH13 mainly stimulated fine root production.

Table 4.11: Shoot and root dry matter and total root length of maize plants at 28 DAS fertilized with microgranules with stabilized ammonium and soluble P or separate fertilization with rock P (RP) and stabilized ammonium. LSD from SAS, Tukey test, n=5, same letters=no significant difference at p=0.05, standard errors in brackets.

	Shoot Dry Matter (g)	Root Dry Matter (g)	Total Root Length (m)
Unfert	0.69 (0.01) c	0.08 (0.01) b	8.95 (1.04) e
TP3136	1.92 (0.13) b	0.21 (0.02) a	27.51 (4.25) cbd
TP3136 _TP1002	2.41 (0.10) a	0.27 (0.02) a	39.37 (1.97) a
TP3093	1.77 (0.18) b	0.22 (0.04) a	33.06 (6.03) abc
TP3093_TP1002	1.92 (0.26) b	0.21 (0.02) a	23.40 (5.18) cd
NH4_RP	0.48 (0.03) c	0.06 (0.00) b	8.17 (0.85) e
NH4_RP_TP1002	0.66 (0.03) c	0.08 (0.01) b	15.96 (6.06) de

Analysis of root diameter classes confirmed this observation and revealed that the CH13-TP3136 combination preferentially stimulated the production of fine roots with diameters ≤ 0.2 mm). Similar trends were observed in the homogenous DMPP-ammonium variant but the effects were not significant. Interestingly the combination of CH13 with TP3093 granulates had no plant growth promoting effects and even tended to decrease fine root production (Figure 4.13).

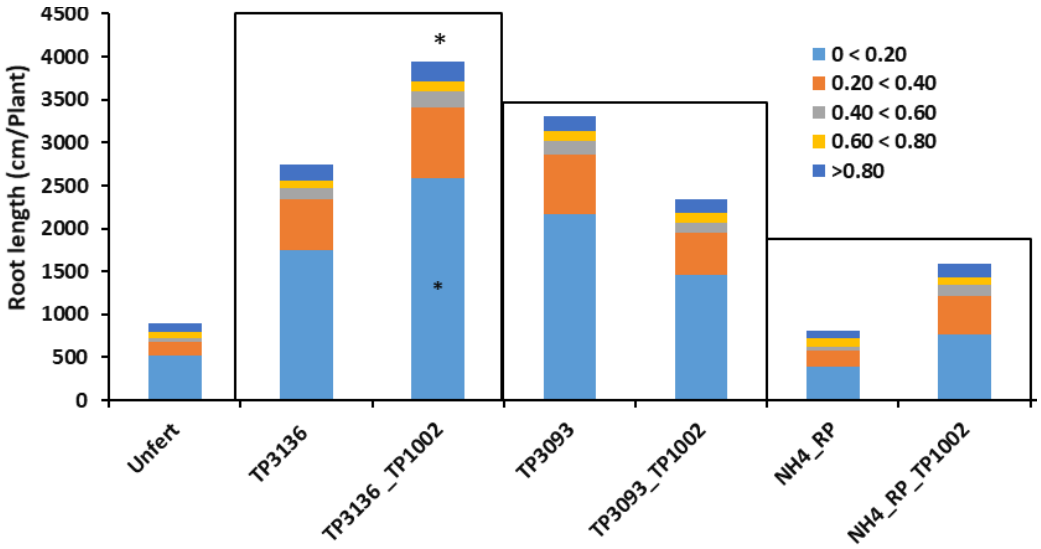


Figure 4.13: Fine root distribution of maize plants at 28 DAS fertilized with microgranules with stabilized ammonium and soluble P or separate fertilization with rock P (RP) and stabilized ammonium. LSD from SAS, Tukey test, n=5, same letters=no significant difference at p=0.05, standard errors in brackets.

Phosphate was the growth-limiting nutrient in all treatments at the end of the culture period but was significantly increased by the microgranulate treatments. In accordance with the

effects on plant growth. CH13 significantly increased P shoot accumulation particularly in combination with TP3136. A significant effect of CH13 on P accumulation was recorded in the broadcast DMPP-Ammonium / Rock-P (RP) combination but reached only 58% of the CH13-TP3136 combination and was obviously not sufficient to trigger significant plant growth responses (Table 4.12). The Zn status in the microgranulate treatments was low and Zn shoot accumulation was increased by CH13 in both microgranulate variants. Therefore, this effect was obviously not relevant for plant growth stimulation, which was observed exclusively in the CH13-TP3136 combination. CH13-TP3136 significantly increased not only accumulation of Zn and P but also of the other investigated nutrients compared with the non-inoculated control, probably a consequence of the significant stimulation (+48%) of fine root production (Fig. 4.13).

Table 4.12. Shoot concentration (A) and content (B) of maize plants at 28 DAS fertilized with microgranules with stabilized ammonium and soluble P or separate fertilization with rock P (RP) and stabilized ammonium. LSD from SAS, Tukey test, n=5, same letters indicate no significant difference at p=0.05, standard errors in brackets.

A. Shoot minerals concentration

	Macronutrients (mg $^{-1}$ g)					Micronutrients (µg^{-1} g)		
	N	P	K	Ca	Mg	Mn	Zn	Cu
Unfert	43.8 b	1.0 cd	46.4 a	9.7 a	4.2 a	85.4 a	35.7 a	9.8 b
TP3136	31.3 c	1.4 a	40.6 bc	8.1 bcd	3.8 b	50.4 d	22.4 c	6.2 d
TP3136 _TP1002	30.5 c	1.4 a	39.5 c	7.9 d	4.1 ab	53.8 d	22.2 c	6.1 d
TP3093	32.9 c	1.4 a	43.4 b	8.4 bcd	3.8 b	50.6 d	23.9 c	6.2 d
TP3093_TP1002	33.0 c	1.2 bc	43.1 b	8.5 bc	4.1 ab	54.2 d	28.3bc*	7.5 c
NH4_RP	67.2 a	0.9 d	24.7 e	8.5 b	4.0 ab	79.5 ab	35.2 a	10.9 a
NH4_RP_TP1002	65.5 a	0.9 d	27.8 d	8.1 cd	4.1 ab	69.9 c	33.3 ab	9.2 b

B. Shoot minerals content

	Macronutrients (mg $^{-1}$ Plant)					Micronutrients (µg^{-1} Plant)		
	N	P	K	Ca	Mg	Mn	Zn	Cu
Unfert	30.1 d	0.7 c	32.1 b	6.7 c	2.9 c	58.7 c	24.6 c	6.8 c
TP3136	59.7 b	2.8 ab	67.9 a	15.5 ab	7.4 b	97.2 b	42.7 b	11.9 ab
TP3136 _TP1002	73.5 a*	3.3 a*	91.7 a	19.1 a*	10.0 a*	130.3 a*	53.5 a*	14.6 a*
TP3093	57.2 b	2.5 b	79.1 a	14.7 b	6.7 b	90.7 b	42.6 b	10.8 b
TP3093_TP1002	62.6 ab	2.4 b	87.3 a	16.5 ab	7.6 b	104.7 ab	53.3 a	14.2 a
NH4_RP	32.5 d	0.4 c	15.9 b	4.1 c	1.9 c	38.1 c	16.9 c	5.3 c
NH4_RP_TP1002	42.9 c*	0.6 c	17.2 b	5.3 c	2.7 c	45.9 c	21.3 c	6.1 c

4.3.3.4 Conclusions

Taken together the experiment indicated that on the highly-buffered soil-sand substrate, homogenous application of stabilized ammonium sulfate was not able to induce significant

solubilization of Rock-P and consequently no plant growth promotion, By contrast, granulated ammonium fertilizers significantly stimulated plant growth under these conditions. To which extent this can be attributed to localized stimulation of ammonium-induced rhizosphere acidification and/or a starter fertilization effect of soluble P component of the microgranulates remains to be established.

The additional effects of Bacillus subtilis CH13 inoculation on plant growth and nutrient acquisition must be mainly attributed to stimulation of root growth and not to direct P solubilization, since CH13 inoculation was largely ineffective in the homogenous ammonium/rock-P combination. Similar to the observations reported by Mpanga et al. (2019) for various *Bacillus, Paenibacillus* and *Pseudomonas*-based inoculants, the application of microgranulated ammonium starter fertilizers increased the P status of the plants and enabled the establishment of a functional PGPR association with root growth-promoting properties. The larger acidifying root system promoted plant nutrient acquisition and plant growth. However, the reason for the exclusive expression of plant growth-promoting effects in the CH13-TP3136 combination requires further investigation and may be related to the higher ammonium content. The results confirm the hypothesis that placement of stabilized ammonium fertilizers may be an option to overcome limitations in PGPM-assisted P acquisition strategies due to high pH buffering on alkaline soils; as previously reported by Bradacova et al. (2019).

4.3.3.5 References

Bradáčová K, Florea AS, Bar-Tal A, Minz D, Yermiyahu U, Shawahna R, Kraut-Cohen J, Zolti A, Erel R, Dietel K, Weinmann M, Zimmermann B, Berger N, Ludewig U, Neumann G, Poşta G. (2019): Microbial consortia versus single-strain inoculants: an advantage in PGPR-assisted tomato production? Agronomy 9, 105; doi: 10.3390/agronomy9020105.

Mpanga IK, Dapaah HK, Geistlinger J, Ludewig U, Neumann G (2018): Soil type-dependent interactions of P-solubilizing microorganisms with organic and inorganic fertilizers mediate plant growth promotion in tomato. Agronomy 2018, 8, 213; doi: 10.3390/agronomy8100213icroorganisms in Maize. Microorganisms 7(2): 38 https://doi.org/10.3390/microorganisms7020038 - 29

5 SOIL TYPE-DEPENDENT INTERACTIONS OF P-SOLUBILIZING MICROORGANISMS WITH ORGANIC AND INORGANIC FERTILIZERS MEDIATE PLANT GROWTH PROMOTION IN TOMATO

Isaac Kwadwo Mpanga [1]*, Harrison Kwame Dapaah [2], Joerg Geistlinger [3], Uwe Ludewig [1], Günter Neumann [1]

[1] Institute of Crop Science (340h), Universität Hohenheim, Fruwirthstraße 20, 70593 Stuttgart, Germany; u.ludewig@uni-hohenheim.de (U.L.), gd.neumann@t-online.de (G.N.)

[2] School of Agriculture and Technology, The University of Energy and Natural Resources, P. O. Box 214, Sunyani, Ghana; hkdapaah@yahoo.com (H.K.D.)

[3] Institute of Bioanalytical Sciences, Anhalt University of Applied Sciences, 06406 Bernburg, Germany; joerg.geistlinger@hs-anhalt.de (J.G.)

* Correspondence: mpangaisaac75@gmail.com or isaac.mpanga@uni-hohenheim.de (I.K.M.);

Tel.: +49-15217489408

Received: 25 July 2018; Accepted: 25 September 2018; Published in Agronomy on 1 October 2018

Abstract: The use of plant growth-promoting microorganisms (PGPMs) as bio-effectors (BEs) to improve the nutrient acquisition of crops has a long history. However, limited reproducibility of the expected effects remains still, a major challenge for practical applications. Based on the hypothesis that the expression of PGPM effects depends on soil type and the properties of the applied fertilizers, in this study, the performance of selected microbial inoculants was investigated for two contrasting low-fertility soils supplied with different organic and inorganic fertilizers. Greenhouse experiments were conducted with tomato on an alkaline sandy loam of pH 7.8 and acidic loamy sand of pH 5.6 with limited phosphate (P) availability. Municipal waste compost, with and without poultry manure (PM), rock phosphate (RP), stabilized ammonium, and mineral nitrogen, phosphorus and potassium (NPK) fertilization were tested as fertilizer variants. Selected strains of *Bacillus amyloliquefaciens* (Priest et al. 1987) Borris et al. 2011

(FZB42) and *Trichoderma harzianum* Rifai (OMG16) with proven plant growth-promoting potential were used as inoculants. On both soils, P was identified as a major limiting nutrient. Microbial inoculation selectively increased the P utilization in the PM-compost variants by 116% and 56% on the alkaline and acidic soil, while RP utilization was increased by 24%. This was associated with significantly increased shoot biomass production by 37–42%. Plant growth promotion coincided with a corresponding stimulation of root growth, suggesting an improved spatial acquisition of soluble soil P fractions, associated also with improved acquisition of nitrogen (N), potassium (K), magnesium (Mg), and calcium (Ca). There was no indication for mobilization of sparingly soluble Ca phosphates via rhizosphere acidification on the alkaline soil, and only mineral NPK fertilization reached P sufficiency status and maximum biomass production. However, on the moderately acidic soil, FZB42 significantly stimulated plant growth of the variants supplied with Ca-P in the form of RP + stabilized ammonium and PM compost, which was equivalent to NPK fertilization; however, the P nutritional status was sufficiently reached only in the RP and NPK variants. The results suggest that successful application of microbial biofertilizers requires more targeted application strategies, considering the soil properties and compatible fertilizer combinations.

Keywords: Bio-effector (BE); compost; tomato; phosphate mobilization, phosphorus recovery efficiency (PRE); poultry manure (PM); biofertilizer; nitrogen; *Trichoderma harzianum; Bacillus amyloliquefaciens* FZB42

5.1 Introduction

The world's total mineral NPK fertilizer consumption is projected to reach approximately 225,000,000 metric tons by 2030, representing an average increase of 20% against levels recorded in 2010 [1], with particularly high demands in horticultural production systems. The

sole dependence on synthetic mineral fertilizers threatens the environment by high energy requirements for fertilizer production from limited natural resources, and unwanted losses by leaching, runoff, and volatilization, contributing to eutrophication and greenhouse gas emissions [2, 3]. Moreover, in developing countries, many farmers are smallholders without much income, and they frequently face problems with covering the costs for mineral fertilizers. Approaches to converting waste materials into resources, and using fertilizers based on organic and inorganic waste materials could offer more sustainable and cost-saving perspectives. Products of interest comprise composts, digestates, manures, products of waste water recycling, slags, and ashes but also less processed fertilizers, including rock phosphates. However, apart from potential contaminants (i.e., heavy metals, antibiotics, abundance of antibiotic resistance genes, or pathogenic microorganisms), the low solubility of plant nutrients, and the large proportions of nutrients sequestered in organic binding forms that are not readily available for plant uptake, are major challenges for the use of organic and inorganic waste materials as fertilizers in agricultural and horticultural practice [4, 5]. Therefore, a fertilization management plan that is adapted to the crop demand is even more complicated as compared with mineral fertilizers and is associated with a high risk of nutrient losses into the environment [4] and low fertilizer use efficiency. The use of microbial inoculants with root growth-promoting and nutrient-mobilizing properties as an option for improving plant nutrient acquisition has been discussed for decades [6–9], and may therefore also contribute to the acquisition of nutrients from fertilizers based on organic and inorganic waste materials [4]. However, a lack of reproducibility of the expected effects, particularly under field conditions, still remains a major challenge for practical applications [10]. There is increasing evidence for selective interactions between the form and the amount of fertilizers and microbial inoculants. In a meta-study covering 171 publications, Schütz et al. [11] demonstrated that P-solubilizing

microorganisms as plant inoculants were mainly effective in soils with moderate available P levels (25–35 kg P ha^{-1}) but not on low P soils or at higher P availability. In an investigation with *Bacillus*-, *Pseudomonas*-, and *Trichoderma*-based inoculants in maize on six different soils in five countries with eight different types of fertilizers based on recycling products from organic and inorganic waste materials, Thonar et al. [12] reported superior performance particularly in combination with composted animal manures. Nkebiwe et al. [13, 14] found beneficial effects by a combination of microbial inoculants with the placement of stabilized ammonium fertilizers.

Based on these findings, we hypothesised that the selection of suitable combinations of microbial inoculants with organic or inorganic fertilizers could be a key factor for the development strategies to improve the fertilizer use efficiency with the support of microbial inoculants. Therefore, in this study, we compared the performance of greenhouse tomato, supplied with different types of fertilizers (municipal waste compost, poultry manure, rock phosphate, stabilized ammonium) in combination with selected microbial inoculants with proven plant growth-promoting and phosphate-solubilizing properties, pre-selected in the studies of Thonar et al. [12] and Nkebiwe et al. [13,14]. To also consider the potential impact of different soil properties, the experiments were conducted on two contrasting soils from Ghana with low P availability, and moderately acidic and alkaline pH in face of the significance of soil pH for P fixation in soils.

5.2 Materials and Methods

5.2.1 Soil Properties

The experiments were conducted under greenhouse conditions on two contrasting soils from Ghana with pH 5.6 and 7.8 with low P availability (Table 5.1). Soil samples of the top 10 cm horizon, collected from Dormaa Ahenkro and Atebubu in the Brong-Ahafo Region in Ghana, were used for the experiments. Chemical and physical soil properties are summarized in Table

5.1. Soil characterization was performed according to the Association of German Agricultural Research and Research Institutes (VDLUFA) instructions for soil analysis [15].

Table 5.1 Physical and chemical properties of the experimental soils.

Soil Properties	**Soil Origin**	
	Atebubu	**Dormaa Ahenkro**
Soil pH ($CaCl_2$)	5.6	7.8
Total Nitrogen [%]	0.05	0.30
NO_3-N [mg kg^{-1} soil]	2.4	44.2
Plant available P [mg kg^{-1} soil]	7.22 (P CAL)	2.22 (P Olsen)
Total P (ICP-OES) [mg kg^{-1} soil]	90	473
K (CAL extract) [mg kg^{-1} soil]	33.2	357
Mg ($CaCl_2$) [mg kg^{-1} soil]	110	250
Total Ca [mg kg^{-1} soil]	632	10523
Fe (CAT extract) [mg kg^{-1} soil]	56.5	29.0
Zn (CAT extract) [mg kg^{-1} soil]	< 1	4.0
Mn (CAT extract) [mg kg^{-1} soil]	188.0	27.3
Cu (CAT extract) [mg kg^{-1} soil]	0.54	1.14
Total Carbon [%]	0.75	4.82
Humus [%]	1.23	7.89
Sand (63-2000 μm) %	66.4	44.4
Silt (2-63 μm) %	28.6	38.3
Clay (< 2 μm) %	5.0	17.3

CAL: Calcium acetate-lactate extract, CAT: Calciumchloride/-Diethylene triamine pentaacetic acid extract, ICP-OES: Inductively Coupled Plasma Optical Emission Spectrometry

5.2.2 Test Plant

The tomato (*Solanum lycopersicum* L) variety Promodoro UC 82-B (Bonanza seeds international, Yuba City, CA, USA) was used for the experiments.

5.2.3 Culture Conditions

A screen house experiment was installed at the School of Agriculture and Technology, University of Energy and Natural Resources in Sunyani, Ghana. In contrast to a greenhouse, the

walls and roofs of screen houses consist of plastic mesh to protect the plants from animals and extreme weather conditions without artificial lighting and heating devices. Solar vents were installed to provide the screen house with good ventilation. The average temperature, relative humidity, and dew-point in the screen house was 31 °C, 60%, and 18% respectively. Tomato plants were cultivated until 49 days after sowing (DAS) on the alkaline soil (experiment 1) and for 35 DAS on the acidic soil (experiment 2). Watering of the plants was performed gravimetrically once per day during the first two weeks and increased to two times in the subsequent weeks until harvest to reach a moisture content equivalent to 70% of the substrate water holding capacity.

5.2.4 Fertilization

In general, application of fertilizers was adapted according to nutrient availability indicated by the soil analysis (Table 5.1). This implicates differences in the fertilization management on the two investigated soils.

Alkaline Soil (Experiment 1)

In experiment 1 conducted on the pH 7.8 soil, P was identified as a major limiting nutrient with available Olsen P levels (Table 5.1) far below the critical range between 10 and 30 mg kg^{-1} soil reported in the literature [16]. Therefore, the P fertilization level was adjusted to 100 mg P kg^{-1} soil in all treatments. Organic fertilization was performed with a commercially available compost produced from municipal waste, or the same compost amended with poultry manure (PM compost) (Accra Compost and Recycling Plants Ltd., Accra, Ghana). The N, P, and K composition comprised 2% N, 1% P and 1% K for compost and 0.95% N, 1.35 % P and 0.94% K for compost amended with poultry manure. The fertilization rate was 10 g kg^{-1} soil for the compost fertilizer (corresponding to 200 mg N, 100 mg P and 200 mg K kg^{-1} soil), while poultry manure-amended compost was applied with 7.4 g kg^{-1} soil (corresponding to 71 mg N, 100 mg

P and 70 mg K kg^{-1} soil) and mixed thoroughly with the substrates. A negative control without fertilization and a positive control fertilized with superphosphate (single super phosphate, 18% P_2O_5, Triferto, Gent, Belgium, 100 mg P kg^{-1} soil and calcium nitrate, 100 mg N kg^{-1} soil) was also included.

Acidic soil (Experiment 2)

The acidic soil from Atebubu with pH 5.6 was also characterized by extremely low P availability (Table 5.1), far below the recommended range for maximum yield between 61 and 120 mg CAL-P kg^{-1} soil [17]. Therefore, fertilization was performed with the two compost types and with triple superphosphate as a positive control at an application rate of 100 mg P kg^{-1} soil. In the face of the lower soil pH, acid-soluble rock phosphate (Granuphos 18% P_2O_5 (Landor, Birsfelden Switzerland) was included as an additional variant. In this case, 150 mg N kg^{-1} soil of stabilized ammonium sulfate (Novatec Solub, Compo-Expert, Münster, Germany) was added as a nitrogen source to promote rock-P solubilization via ammonium-induced rhizosphere acidification. In face of the extremely low N content of the acidic soil (Table 5.1), and limited N supply recorded in experiment 1 for the organic fertilizers, additional N supplementation in form of stabilized ammonium (150 mg N kg^{-1}) soil was also performed in the case of the organic fertilizers. Calcium nitrate (150 mg N kg^{-1} soil) was added to the positive control supplied with soluble superphosphate.

5.2.5 Bioeffectors (BEs)

Alkaline Soil

In experiment 1, Combifector B (CFB), a microbial consortium product based on *Trichoderma harzianum OMG16* and *Bacillus amyloliquefaciens* FZB42 *(*also referred as *Bacillus amyloliquefaciens FZB42,* ABITEP GmbH, Berlin, Germany)*,* enriched with zinc (Zn) and manganese (Mn) (Hochschule Anhalt, Bernburg, Germany), was used as an inoculant. The

combination product with micronutrients was selected as an inoculant since the soil nutrient analysis (Table 5.1) indicated low Mn availability [18]. The CFB product was applied three times in the form of *Bacillus* spores (2×10^9 cfu kg^{-1} soil) and *Trichoderma* spores (2×10^8 cfu kg^{-1} soil), in a formulation supplemented with 2 mg Mn, and 2 mg Zn kg^{-1} soil as a suspension in water, first at the two-leaf stage of tomato seedlings during the nursery stage in small pots (50 mL), secondly, one week later during transplantation into bigger pots (2500 mL, 2 kg soil), and finally, one week after transplanting by drenching close to the stem base. In the variants without BE application, (NoBE), an equivalent amount of water was applied by weight.

Acidic Soil

In experiment 2 on the acidic soil, only the *Bacillus* FZB42-product was used as a single-strain inoculant with the same application schedule as in experiment 1.

5.2.6 Plant Biomass and Root Length

At final harvest, the dry biomass of the shoots was determined for both experiments after 3 days oven-drying at 65 °C. The roots in each pot were washed out from the soil substrate after carefully shaking off the rhizosphere soil, and they were stored in 30% (v/v) ethanol. The roots were later separated, submerged in a water film in transparent Perspex trays, and subsequently digitalized using a flat-bed scanner (Epson Expression 1000 XL, Tokyo, Japan). Subsequently, the root length of the digitalized samples was measured using the WinRHIZO root analysis system (Reagent Instruments, Quebec, QC, Canada). Thereafter, the root samples were oven-dried for 2 d at 65 °C for the determination of the dry matter.

5.2.7 Shoot N, P, K, and Mg Concentration and Content

For both experiments, plant mineral nutrient analysis was performed as follows: tomato shoot N was measured with a Vario Max CN macro-elementar analyser (Elementar Analysensysteme, Hanau, Germany). For P, K, Ca, and Mg, a microwave digestion method was

employed for the wet ashing of finely ground dry plant materials (250 mg) in 1 mL of deionized water, 2.5 mL conc. HNO_3 (1:3), and 2 mL H_2O_2 (30%). Digestion was performed in a microwave digestion system (Ethos, MLS, Leutkirch, Germany) for 1 hr and allowed to cool for 30 min. Approximately 5 g activated charcoal was added for sample decolouration, mixed well by shaking, and allowed 15 min to settle. The samples were then filtered with ashless MG 640d Blue ribbon filter paper (Macherey & Nagel, Düren, Germany). Phosphate was estimated spectrophotometrically (Hitachi LtD., Tokyo, Japan) according to Gericke and Kurmis [19]. Magnesium and calcium were measured by atomic absorption spectrophotometry (iCE 3000 series, Thermo Fischer, Dreieich, Germany) and K by flame emission spectrophotometry (Eppendorf-ELEX6361, Netheler+Hinz, Hamburg, Germany).

5.2.8 Rhizosphere Soil pH

The collected rhizosphere soil samples were air-dried and sub-sampled for pH analysis. Soil pH was measured after 1 hr shaking of a soil suspension in 0.01 M $CaCl_2$ in a 1:1 ratio (Digital pH-Meter E532, Metrohm Harisau, Switzerland).

5.2.9 Phosphorous Recovery Efficiency

Phosphorus recovery efficiency (PRE) was calculated based on the formula below:

$$= \left[\frac{\text{Shoot P content (fertilized)} - \text{Negative control (unfertilized) (mg pot}^{-1})}{\text{Fertilizer applied (mg pot}^{-1})} \right] \times 100 \quad [1]$$

5.2.10 Experimental Setup and Data Analysis

The experiments were established in a completely randomized design with five replicates per treatment in experiment 1, and four replicates per treatment in experiment 2. Statistical analysis was performed in SAS 9.4 (2016) (SAS Institute Inc., Cary, NC, USA), with the treatments as fixed variables and the measured parameters as random variables. The normality of the data

was tested using a Q-Q Plot with fit diagnostics. The proc glimmix procedure was performed to give a general overview of the comparison of specific fertilizer types to no fertilization (negative control) or soluble P (positive control) variants. The pairwise t-test was employed, to test for specific differences within fertilizer types between inoculated and non-inoculated variants at $p < 0.05$. (Supplementary Table S1).

5.3 Results

5.3.1 Experiment 1, Alkaline Soil (pH 7.8)

Plant Growth and Rhizosphere pH: At 49 DAS, both, compost and PM-compost fertilizers increased plant growth (Figure 5.1) and shoot biomass production (Figure 5.2) to a similar extent (+82%) as compared with the unfertilized control (NoFert) variant but did not reach the biomass of the plants supplied with mineral nitrate + superphosphate (NP) fertilization (+346%).

Compared with the non-inoculated control treatments (NoBE), application of the microbial inoculants (CFB) significantly increased the shoot biomass production by 123% in the unfertilized variants, and by 42% in the compost amended with poultry manure ($p < 0.05$). This was associated with significant increases, also in root biomass (Figure 5.2) and root length (Figure 5.3). The total root length of plants supplied with PM-compost and CFB inoculation was finally comparable with the plants receiving full mineral (NP) fertilization. However, in all treatments without CFB inoculation, root growth inhibition was observed in comparison with the NP variant (Figure 5.3).

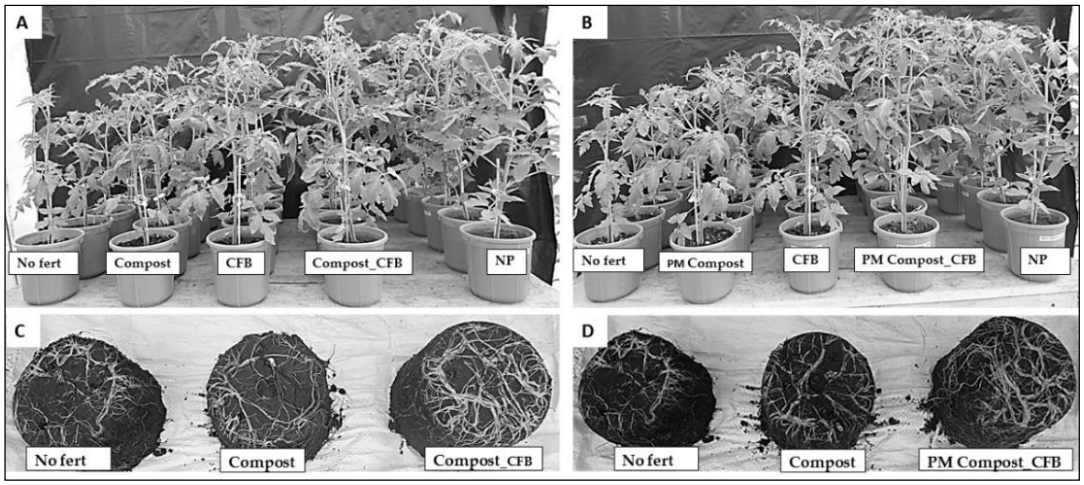


Figure 5.1. (A) Plant growth of 7-week-old tomatoes on pH 7.8 soil supplied with CFB and compost, mineral NPK fertilization (NP) or without fertilization; (B) CFB and PM compost; and root development with (C) CFB and compost; (D) CFB and PM compost. (CFB = Combifector B, PM = poultry manure, NP = calcium nitrate plus superphosphate, Nofert = unfertilized).

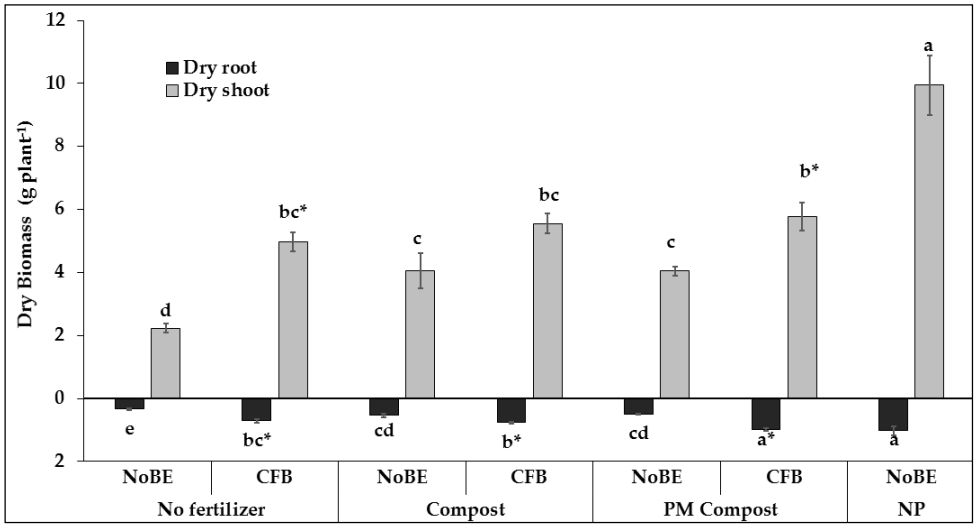


Figure 5.2. Shoot and root dry weight of 7-week-old tomatoes on pH 7.8 soil (CFB = Combifector B, PM = poultry manure, NP = calcium nitrate plus superphosphate. Means and SE of five replicates. Significant treatment differences are indicated by different letters (T-grouping (LSD) at $p = 0.05$). * = significant difference compared with the non-inoculated control (NoBE) within the same fertilizer treatment (*t*-test at $p < 0.05$).

Tomato is a plant species with a well-documented potential for rhizosphere acidification under conditions of P limitation, which is known to mediate the solubilization of Ca-phosphates in alkaline soils, while the release of carboxylates with P-solubilizing potential is negligible [20,21]. Therefore, pH measurements were conducted with root-adhering rhizosphere soil samples, collected by shaking of the root systems at final harvest. However, the maximum

decline in rhizosphere soil pH recorded on the pH 7.8 soil reached only 0.1 pH units without any significant treatment differences (Supplementary data, Figure S1).

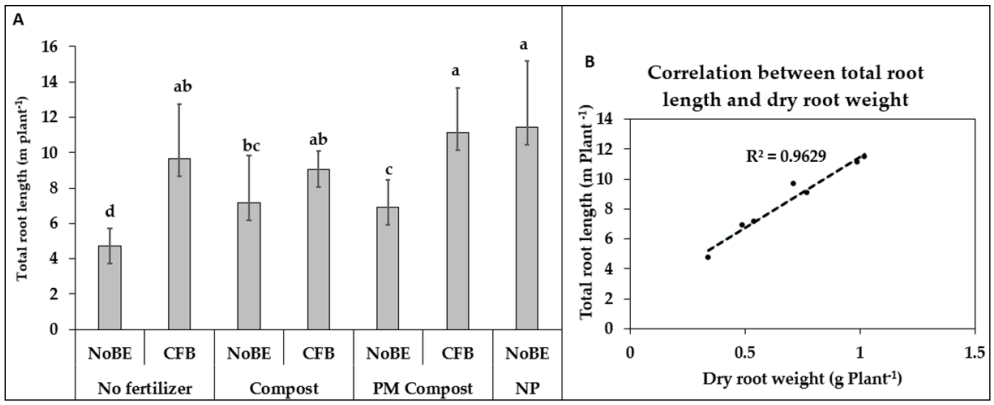


Figure 5.3. Total root length (A) and root length correlation with dry root biomass (B) of 7-week-old tomatoes on pH 7.8 soil. (CFB = Combifector B, PM = Poultry manure, NP = calcium nitrate plus superphosphate. Means and SE of five replicates. Significant treatment differences are indicated by different letters (T-grouping (LSD) at p = 0.05). * = significant difference compared with the non-inoculated control (NoBE) within the same fertilizer treatment (t-test at $p < 0.05$).

Plant Nutritional Status: Although all fertilizer treatments stimulated shoot P accumulation as compared with the NoFert variant (Table 5.2), only mineral P fertilization (NP) was able to increase the shoot P concentrations above the deficiency threshold (Figure 5.4). For nitrogen, only the PM compost variants reached the sufficiency range (Figure 5.4) and N shoot accumulation increased in the order NoFert < compost < PM-compost < NP (Table 5.2).

The microbial inoculants (CFB) further promoted P and N accumulation, with significant effects being detectable for the treatments without fertilization (NoFert) and for the PM-compost variants (Table 5.2). The nutritional status for potassium, magnesium, and calcium was sufficient in all treatments, with a trend for declining concentrations in the CFB variants (Figure 5.4). The CFB inoculation significantly increased K, Ca, and Mg shoot accumulation in the NoFert variant (Table 5.2). In the compost treatment, a significant CFB effect was recorded for Ca accumulation, and for K and Ca in the PM-compost variant.

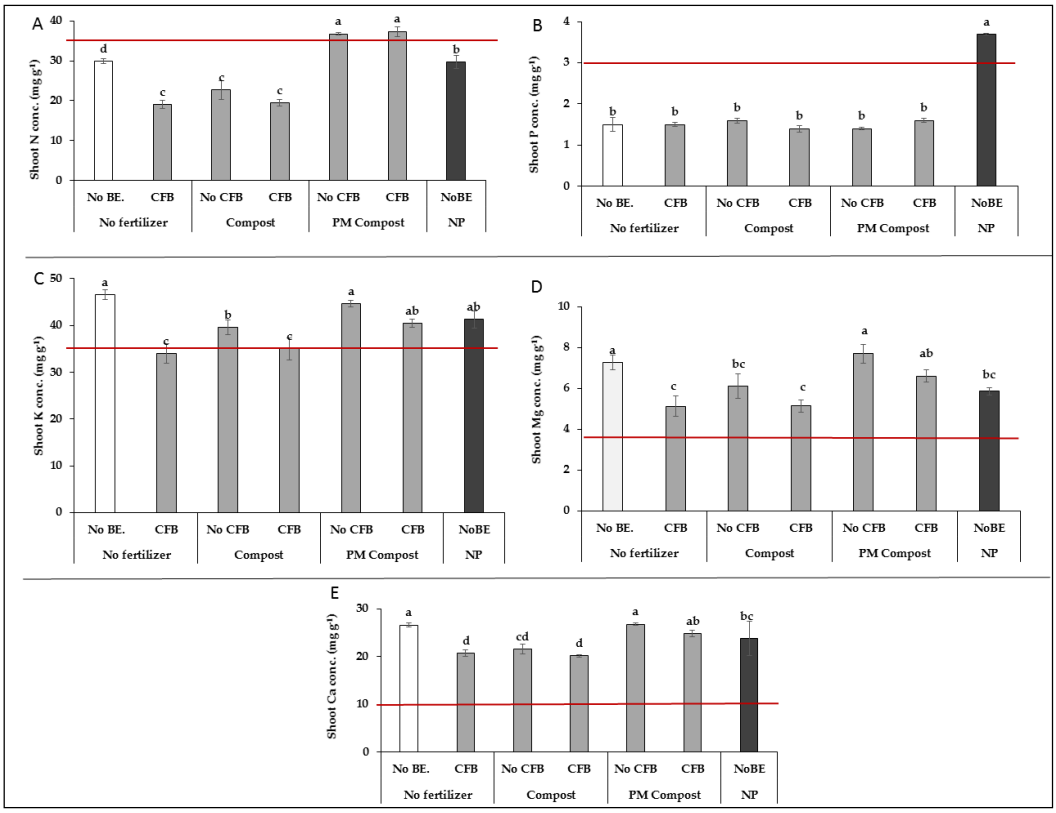


Figure 5.4. Effects of Combifector B inoculation on shoot N (A), P (B), K (C), Mg (D), and Ca (E) concentrations of 7-weeks-old tomatoes on pH 7.8 soil supplied with different fertilizers (CFB = Combifector B, PM = poultry manure, NP = calcium nitrate plus superphosphate., The red lines represent the deficiency thresholds for nutrient tissue concentrations [22]. Means and SE of five replicates. Significant treatment differences are indicated by different letters (T-grouping (LSD) at p = 0.05). * = significant difference compared with the non-inoculated control (NoBE) within the same fertilizer treatment (t-test at $p < 0.05$).

Table 5.2: Effects of Combifector B inoculation on the shoot accumulation of N, P, K, Mg, and Ca of 7-week-old tomatoes on pH 7.8 soil supplied with different fertilizers. (CFB = Combifector B, PM = Poultry Manure, NP = calcium nitrate plus superphosphate). Means and SE of five replicates. Significant treatment differences are indicated by different letters (T-grouping (LSD) at p = 0.05). * = significant difference compared with the non-inoculated control (NoBE) within the same fertilizer treatment (t-test at $p < 0.05$).

	Shoot mineral content (mg Plant^{-1})				
	N	P	K	Mg	Ca
No fertilization	66.3 e	2.7 d	102.9 d	18.0 e	62.0 e
CFB	94.2 d*	7.5 b*	169.2 c*	25.1 cd*	102.9 cd*
Compost	86.6 d	6.3 bc	157.6 c	23.5 d	85.5 d
Compost_CFB	107.3 d*	7.4 b	192.3 bc*	28.3 cd*	110.7 c*
PM Compost	148.4 c	5.8 bc	181.2 c	31.2 bc	108.6 cd
PM Compost_CFB	213.6 b*	8.7 b*	232.2 b*	37.8 b*	143.2 b*
NP_	289.1 a	37.0 a	405.1 a	57.7 a	233.7 a

5.3.2 Experiment 2, Acidic Soil (pH 5.6)

In addition to the organic fertilizers tested in experiment 1, rock phosphate (RP) fertilization was included into experiment 2, as an alternative, low-cost P source with the potential to be used on acidic soils due to improved Ca-P solubility at low soil pH. By contrast, the application of RP on neutral to alkaline soil is considered to be largely ineffective [23, 24]. Amendments of ammonium sulfate, stabilized with the nitrification inhibitor 3, 4-dimethylpyrazole phosphate (DMPP), were added to support RP solubilization by root-induced acidification in response to preferential N uptake in the ammonium form [25] and to improve the obviously sub-optimal nitrogen supply of the organic fertilizers (Figure 5.3). The *Bacillus amyloliquefaciens* strain FZB42 with the proven potential to solubilize sparingly soluble rock phosphate [14], and to promote nutrient acquisition from organic fertilizers in maize [12], was used as a microbial inoculant.

Plant Growth and Development: At 35 DAS, the fertilizer applications significantly increased plant growth (Figure 5.5) and shoot biomass production (Figure 5.6) of tomato compared with the NoFert control, in the order NoFert < compost < PM-compost < RP < superphosphate (NP).

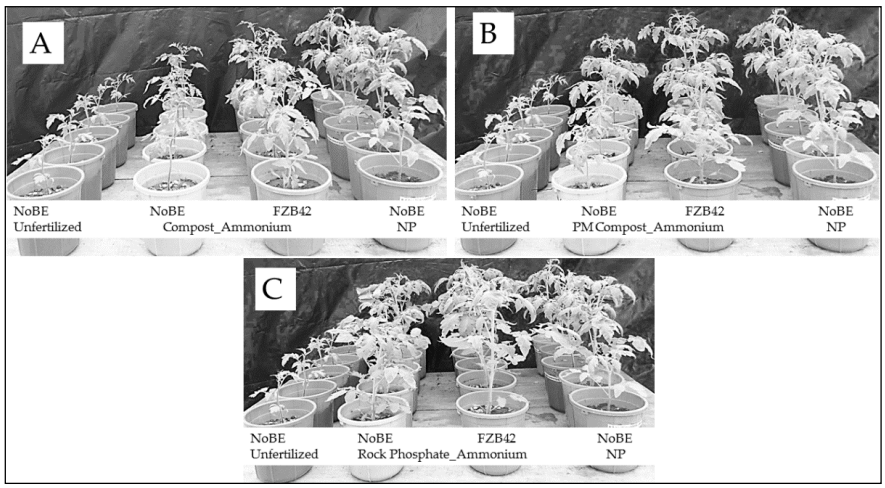


Figure 5.5. Plant growth of 4-week-old tomato on pH 5.6 soil supplied with different fertilizers and with and without *Bacillus amyloliquefaciens* FZB42 inoculation (**A**), PM compost (**B**), and rock phosphate (**C**) (NoBE = no bioeffector, PM = poultry manure, NP = calcium nitrate plus superphosphate).

Inoculation with FZB42 significantly stimulated shoot and root biomass production in the PM-Compost and RP variants (Figure 5.6) to a level that was not significantly different from the plants that were supplied with mineral superphosphate fertilization (NP). FZB-induced shoot growth promotion increased in the order Compost < PM-Compost < RP, while the stimulation of root biomass production by FZB inoculation declined in the same order (Figure 5.6).

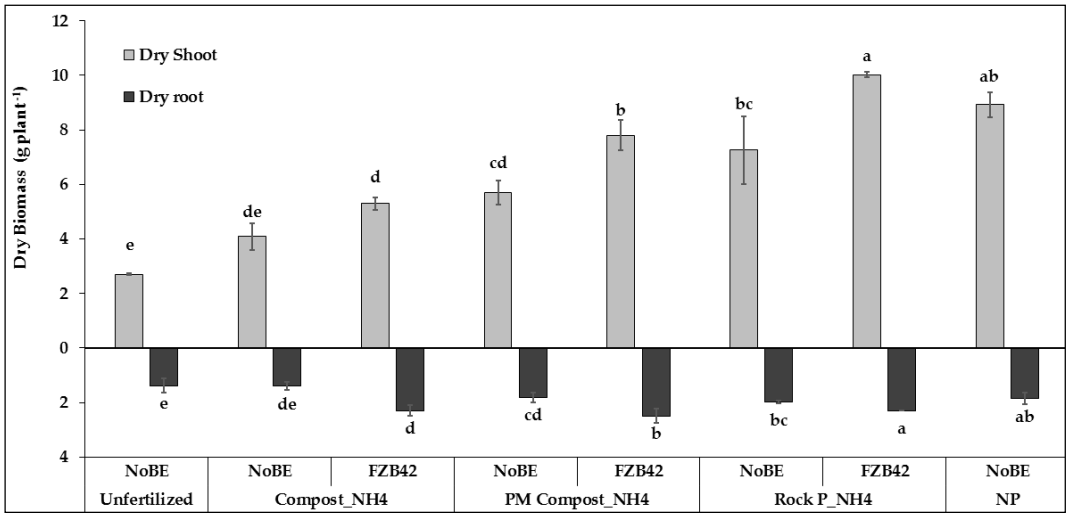


Figure 5.6. Shoot and root dry weight of 5-week-old tomatoes with FZB42 and different P sources and ammonium sulfate under acidic soil conditions (PM = poultry manure, NP = calcium nitrate plus superphosphate. Means and SE of four replicates. Significant treatment differences are indicated by different letters (T-grouping (LSD) at p = 0.05). * = significant difference compared with the non-inoculated control (NoBE) within the same fertilizer treatment (t-test at $p < 0.05$).

Plant Nutritional Status: All fertilizer treatments increased N and P accumulation in the order: NoFert < Compost < PM-compost < RP < superphosphate (NP), with significant effects for PM-compost, RP, and NP (Table 5.3). However, only RP and NP treatments exceeded the P deficiency threshold, while sufficient N supply was recorded for PM compost, RP, and NP (Figure 5.7). The nutritional status of K, Mg, and Ca was sufficient, or at least very close to the respective sufficiency thresholds in all treatments (Figure 5.7). Corresponding with plant growth stimulation (Table 5.3), the shoot accumulation of K, Mg, and Ca tended to increase in response to the fertilizer applications (Table 5.3) in the order: NoFert < compost < PM-compost < RP < NP, with significant effects for PM-Compost, RP and NP, but the tissue concentrations declined in the same order (Figure 5.7).

Inoculation with the FZB42 *Bacillus* strain significantly increased K, Mg, and Ca shoot accumulation in the PM-compost and RP variants, while significant stimulation of N and P accumulation was recorded only for the RP treatment with a similar trend under PM-compost fertilization (Table 5.3). However, N and P shoot concentrations declined after FZB inoculation. (Figure 5.7).

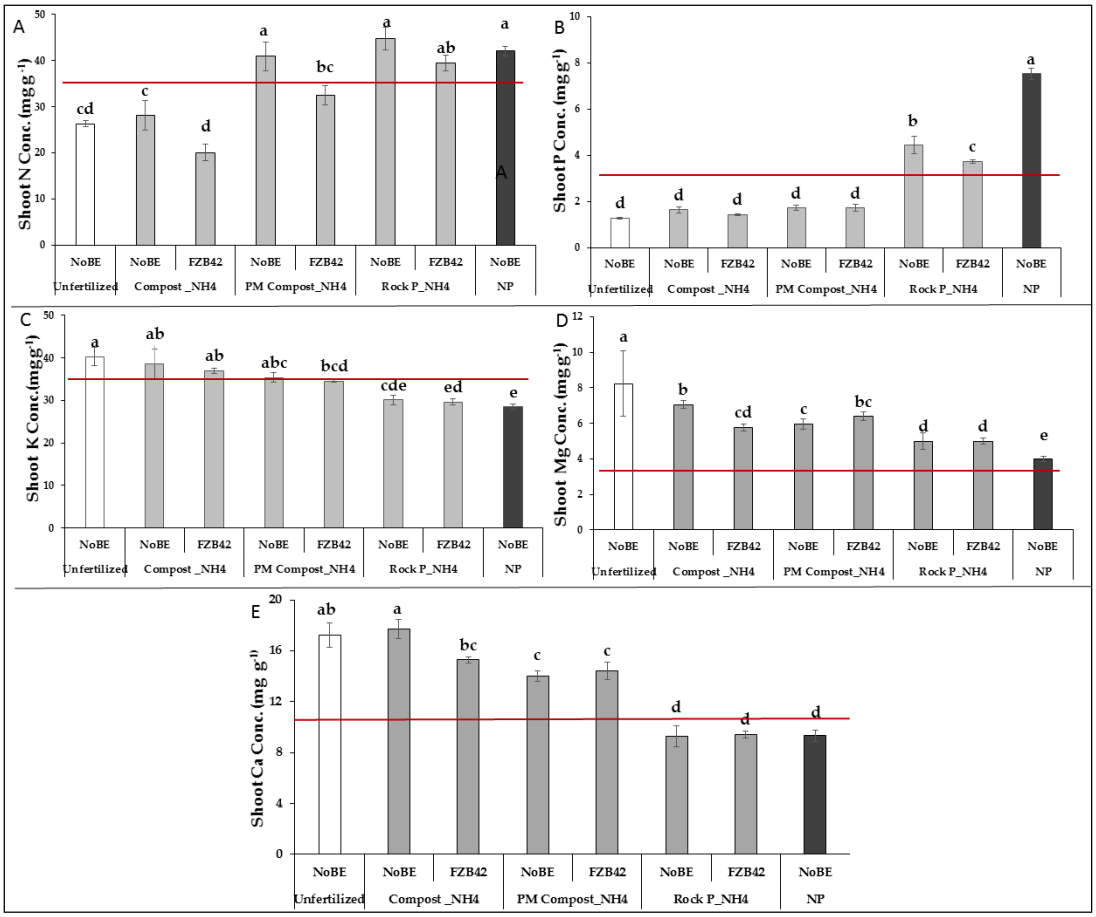


Figure 5.7. Effects of *Bacillus amyloliquefaciens* FZB42 inoculation on shoot N (A), P (B), K (C), Mg (D), and Ca (E) concentrations of 5-week-old tomatoes on pH 7.8 soil supplied with different fertilizers (Compost_NH_4 = municipal waste compost + stabilized ammonium sulfate, PM-Compost_NH_4 = municipal waste compost + poultry manure + stabilized ammonium sulfate, Rock P_NH_4 = rock phosphate +stabilized ammonium sulfate, NP = calcium nitrate plus superphosphate. The red lines represent the deficiency thresholds for nutrient tissue concentrations [22]. Means and SE of four replicates. Significant differences are indicated by different letters (T-grouping (LSD) at p = 0.05).

Table 5.3: Effects of *Bacillus amyloliquefaciens* FZB 42 inoculation on shoot accumulation of N, P, K, Mg, and Ca of 5-week-old tomatoes on pH 5.6 soil supplied with different fertilizers. (Compost_NH_4 = municipal waste compost + stabilized ammonium sulfate, PM-Compost_NH_4 = municipal waste compost + poultry manure + stabilized ammonium sulfate, Rock P_NH_4 = rock phosphate + stabilized ammonium sulfate, NP = calcium nitrate plus superphosphate). Means and SE of four replicates. Significant differences are indicated by different letters (T-grouping (LSD) at $p = 0.05$). * = significant difference compared with the non-inoculated control (NoBE) within the same fertilizer treatment (*t*-test at $p < 0.05$).

	Shoot minerals content (mg Plant^{-1})				
	N	**P**	**K**	**Mg**	**Ca**
Unfertilized	71.2 d	3.4 f	108.9 e	22.2 c	46.6 d
Compost _NH_4^+	108.9 d	6.5 ef	159.9 de	28.5 bc	71.0 c
Compost _NH_4^+_FZB42	106.2 d	7.5 ef	194.9 cd*	30.3 bc	81.0 bc
PM Compost_NH_4^+	228.3 c	9.7 de	199.9 c	33.7 b	79.6 bc
PM Compost_NH_4^+_FZB42	247.7 c	13.2 d	268.9 a*	49.3 a*	111.0 a*
Rock P_NH_4^+	314.8 b	30 c.7	214.1 bc	34.8 b	65.0 c
Rock P_NH_4^+_FZB42	394.1 a*	37.3 b	297 a	50.0 a*	94.7 b*
TSP_NO_3^- (NP)	373.8 a	66.9 a	253.5 ab	35.5 b	80.4 bc

5.4 Discussion

5.4.1 Fertilizer Effects

In this study, different types of alternative organic and inorganic fertilizers, based on compost, poultry manure, and rock phosphate, as well as conventional mineral superphosphate, were applied at the same P dosage for greenhouse tomato cultivation on two soils with limited P availability [17, 18] and contrasting pH (Table 5.1). On both soils, all tested fertilizers showed beneficial effects on plant growth (Figures 5.1, 5.2, 5.5, and 5.6). However, in contrast to conventional mineral NPK fertilization (NP), P remained the major limiting nutrient, particularly in case of the organic fertilizers (Figures 5.4 and 5.7), with shoot P concentrations in the deficiency range [22], where any surplus of P supply was immediately transformed into biomass production. This was confirmed in the case of tomato plants supplied with municipal waste compost, where a sufficient N supply was achieved by increasing the N availability of the fertilizer via additions of poultry manure and/or mineral N (NH_4^+) applications (Figures 5.4 and 5.7). In these cases, significant stimulation of plant growth was detected only when it was possible to increase additionally the shoot P accumulation, e.g., by inoculation with the plant growth-promoting microorganisms (Tables 5.2 and 5.3). The status of the remaining

macronutrients (K, Mg, and Ca) was sufficient in all treatments [21], and shoot accumulation of these nutrients increased in response to stimulation of plant growth, induced by increasing the P supply (Table 5.3), while the tissue concentrations declined at the same time (Figure 5.7), as a consequence of a dilution effect.

5.4.2 PGPM Effects

On both soils, inoculation with the selected microbial inoculants with proven PGPM potential in maize [12, 13], also improved the growth of tomato plants (Figures 5.1, 5.2, 5.5, and 5.6), and acquisition of P as a major limiting nutrient, in a soil type-, and fertilizer-specific manner.

Alkaline Soil: On the alkaline pH 7.8 soil, plants without mineral P fertilization suffered from severe P limitation, as indicated by P shoot concentrations of approximately 1.5 mg g^{-1} DM (Figure 5.4), which was far below the published deficiency threshold of 3 mg g^{-1} DM [22], and by distinct inhibition of both, shoot and root growth (Figures 5.2 and 5.3).

The inoculation with the microbial combination product Combifector B (CFB), based on strains of *Bacillus amyloliquefaciens* FZB42 and *Trichoderma harzianum* OMG16 in combination with Zn and Mn as stress-protective micronutrients [26,27], significantly increased plant growth (Figures 5.1 and 5.2) and shoot P accumulation (Table 5.2) in the unfertilized control (B), and the compost treatment amended with poultry manure (PM-compost). This effect was associated with a corresponding stimulation of root growth, reflected by increased root biomass and root length (Figures 5.2 and 5.3), suggesting an improved spatial acquisition of soluble soil P fractions. Root growth stimulation is a well-documented mechanism of plant growth promotion via microbial inoculants. This has been related to the microbial production of hormonal factors with root growth-stimulating properties, such as auxins or certain quorum-sensing molecules [9, 28], but also with the ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which counteracts excessive stress-induced ethylene

accumulation with inhibitory effects on root growth [29, 30], as a well-documented response to P deficiency in higher plants [31]. Accordingly, root growth inhibition in response to severe P limitation was recorded also in the present study (Figures 5.2 and 5.3), and both, the production of auxin and ACC deaminase have been reported for the *Bacillus* strain FZB42, which was used as an inoculant [32,33]. Similarly, auxin production and root growth stimulation is documented for *Trichoderma harzianum* [34].

Moreover, improved root development can support the establishment of arbuscular-mycorrhizal associations with important functions particularly for P acquisition, although this aspect has not been considered in the present study. In this context, it is worthwhile to mention that mycorrhizal helper functions supporting mycorrhizal root colonization have been documented for the inoculant strain *B. amyloliquefaciens FZB42* [12,35]. More recently, it has been also demonstrated that changes in root morphology can significantly modify the rhizosphere microbial diversity [36] with yet unexplored consequences for soil health and nutrient turnover in the rhizosphere.

Soils with neutral to alkaline pH are frequently characterized by limited solubility of micronutrients, such as Fe, Zn, Mn, and Cu. Zinc limitation has been identified as a major factor for limiting plant growth via increased oxidative auxin degradation due to a lack of Zn as a cofactor for the enzymatic detoxification of reactive oxygen species [26, 27]. Therefore, the supplementation of Zn via CFB application may have contributed to root growth stimulation by the inoculants on the moderately alkaline soil with pH 7.8. However, soil analysis revealed a plant-available Zn status of 4 mg kg^{-1} soil (Table 5.1), which is considered to be sufficient for plant growth [18], and accordingly, Zn limitation seems to be an unlikely scenario in this case. By contrast, the plant-available Mn concentration was suboptimal on this soil [18] but subsequent plant analysis revealed sufficient Mn status [22] of 40–50 mg kg^{-1} shoot dry matter,

independent of the CFB treatment (data not shown). These findings suggest that the plant potential for Mn acquisition was enough to cover the Mn demand, and a further contribution via CFB inoculation was not required.

The observed stimulation of root growth may also impact on P acquisition via chemical changes in the rhizosphere: in response to P limitation, plants usually increase the release of root-secretory acid phosphatases [25], which can help to hydrolyze soluble organic P forms in the rhizosphere, which are particularly abundant after application of organic fertilizers. Accordingly, root secretion of acid phosphatases is also characteristic for tomatoes exposed to P starvation [37]. Moreover, the secretion of phosphatases into growth media with limited P availability has been similarly reported for *Bacillus amyloliquefaciens* FZB42 and *Trichoderma harzianum* [14]. Consequently, a bigger root system with increased activities of both, plant and microbial rhizosphere phosphohydrolases may represent a particular advantage for acquiring P, e.g., from organic fertilizers, which are rich in organic P forms. However, in this study, a significantly improved P acquisition from organic fertilizers due to increased release of phosphatases by the microbial inoculants seems to be unlikely. This is indicated by the observation that the additional effect of BE inoculation on P accumulation (Table 5.2) and plant growth (Figure 5.2) was in the same order of magnitude with and without the application of the organic fertilizers, or even higher in the unfertilized control. This finding suggests that improved spatial soil exploitation for the uptake of soluble mineral phosphates due to the microbial promotion of root growth (Figure 5.3), and not an increase in the mineralization of organic P forms supplied with the organic fertilizers represented the major contribution of the microbial inoculants to plant P acquisition on the moderately alkaline soil.

On neutral and alkaline soils, substantial amounts of mineral P are present in the form of sparingly-soluble Ca phosphates, which can be solubilized by rhizosphere acidification and/or

organic chelators [25]. However, pH determinations of the rhizosphere soil revealed no indications for root-induced rhizosphere acidification (Figure S1), although tomato is a plant species with a known potential to acidify the rhizosphere under P limitation [20,21], and Ca–P mobilization via the acidification of artificial growth media has been similarly demonstrated both for *Bacillus amyloliquefaciens* FZB42 and *Trichoderma harzianum* [14]. Possibly, a high pH buffering capacity of the moderately alkaline soil substrate counteracted the expression of significant rhizosphere acidification effects, which is a well-known problem for plant nutrient acquisition strategies based on proton extrusion on well-buffered alkaline soils [25]. Accordingly, a limited potential of the tested microbial inoculants to contribute to plant acquisition of Ca–P under real rhizosphere conditions has been reported also in earlier studies [12, 38].

As a consequence of the general stimulation of shoot and root growth induced by the microbial inoculants; also the shoot accumulation of the non-limiting nutrients K, Mg, and Ca increased due to improved nutrient acquisition potential of a bigger root system (Table 5.2). Substantial differences were found also for the N availability of the applied fertilizers. Despite the lower total N application (see Section 2.4), the N status of plants supplied with PM-amended municipal waste compost was sufficient, while the N supply only by municipal waste compost was not adequate (Figure 5.4). This effect may be attributed to particularly high levels of readily plant-available N forms (particularly NH_4^+) reported for poultry manures [39]. Similar to K. Mg, and Ca accumulation, the microbial inoculants also increased N accumulation (Table 2), closely related to microbial effects on root growth (Figure 5.3). However, despite improved spatial nutrient acquisition by stimulation of root growth, the inoculant effect was not big enough to cover also the plant demand of P as a limiting nutrient. The P nutritional status remained in the deficiency range (Figure 5.4) and the plants could not express growth

responses comparable with the variants supplied with soluble nutrients via mineral NPK fertilization (Figure 5.2).

Acidic Soil: This scenario changed completely on the moderately acidic, sandy soil of pH 5. 6 with a lower pH buffering capacity. On this soil, plants were able to acquire sparingly soluble Ca–P applied in the form of rock phosphate. In this case, stabilized ammonium sulfate was supplied as an N source to promote root-induced rhizosphere acidification, with beneficial effects on the solubilization of Ca–P [25]. Accordingly, the P and N status reached the sufficiency range (Figure 5.7) and compared with the unfertilized control, shoot and root biomass production increased by 163% and 44% (Figure 5.5), to a level that was not significantly different from plants with full mineral fertilization (CN). This is in line with earlier reports on the efficient use of rock phosphate fertilizers with acid-soluble Ca phosphates, particularly at lower soil pH [23, 24]. On the weakly-buffered sandy soil, root extrusion of protons by the P-limited tomato plants [20, 21], additionally stimulated by ammonium fertilization [25], obviously reached a degree of rhizosphere acidification that was sufficient for rock-P solubilization. This effect was further promoted by inoculation with *Bacillus amyloliquefaciens* FZB42. Compared with the non-inoculated control, shoot P accumulation increased significantly by 25% (Table 5.3), which was associated with root growth promotion by 15% (Figure 5. 5), and which translated into an increase in shoot biomass production by 38% (Figure 5. 5). This may indicate that the development of a larger acidifying root system induced by the microbial inoculant, at least partially contributed to improved rock phosphate acquisition. However, in the face of the documented potential of the FZB42 strain for rock phosphate mobilization [14, 32], an additional contribution by microbial P mobilization cannot be excluded. Moreover, Ögüt et al. [40] demonstrated that various *Bacillus* strains are obviously able to stimulate root-induced proton extrusion of host plants supplied with ammonium fertilization, and thereby the

rhizosphere acidification potential. On the other hand, in contrast to the beneficial effects of rock-P solubilization, rhizosphere acidification may also contribute to a stronger immobilization of P adsorbed to Fe, and Al oxides and hydroxides, as a dominant sparingly soluble P form in acidic soils [25]. However, in the face of the extremely low levels of plant-available P [17] and even total P in the respective soils (Table 5.1), this potentially negative impact on P availability was obviously largely overcompensated for by the beneficial effects of ammonium fertilization and plant growth-promoting rhizobacteria (PGPR) inoculation on the solubility of rock-P.

Similar to the experiment on the alkaline soil, PGPR inoculation had a selective impact on the utilization of the organic fertilizers. Compared with the unfertilized control, the municipal waste compost and the compost amended with poultry manure (PM compost) increased P accumulation and plant biomass without significant differences between the fertilizer treatments (Tables 5. 2 and 5. 3). The additional inoculation with FZB42 significantly stimulated plant growth (Figures 5. 2 and 5. 5) and nutrient accumulation compared with the non-inoculated (NoBE) control (Tables 5.2 and 5.3), only on the soil with PM compost, with a biomass production that was not significantly different from the plants supplied with full mineral P fertilization of the NP treatments. By contrast, due to the extremely low organic C content of the acidic soil (Table 5.1), a significant contribution of native organic soil P to plant P acquisition seems to be unlikely in this case.

Obviously, on both soils with P as the major limiting nutrient, the plant growth-promoting potential of the microbial inoculants was particularly expressed in combination with manure-based organic fertilizers, as similarly reported also in earlier studies with sweet pepper [23] or maize [12]. Accordingly, with the same amount of P supply, PGPM inoculation selectively increased P recovery efficiency on the manure-amended compost (Figure5. 8). The reasons for this selective effect may be directly related to differences in P forms and the availability of the

applied organic fertilizers. However, indirect effects are also possible, due to a P-solubilizing potential that has been reported for manure components, or microbiome effects mediated by microorganisms that are involved in manure degradation [23]. The root growth-stimulating effect of the tested inoculants would consequently promote the acquisition of the increased available P fraction in the manure-amended variants.

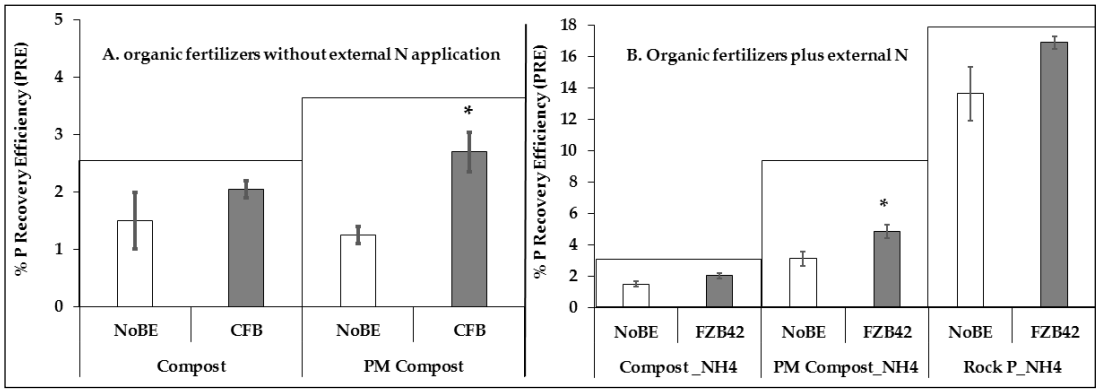


Figure 5.8. Phosphorus recovery efficiency [41] in tomatoes from different organic and inorganic fertilizers in moderately alkaline soil pH 7.8 (A), and moderately acidic soil pH 5.6 without (NoBE) and with microbial inoculation (B); CFB = Combifector B FZB42 = *Bacillus amyloliquefaciens* FZB42, Compost_NH4 = Municipal waste compost + stabilized ammonium sulfate, PM-Compost_NH4 = Municipal waste compost + poultry manure + stabilized ammonium sulfate, Rock P_NH4 = rock phosphate + stabilized ammonium sulfate. * = significant *t*-test $p < 0.05$ compared with the non-inoculated control (NoBE) in each fertilizer variant.

5.5 Conclusions

The present study suggests that inoculation with plant growth-promoting microorganisms can provide an efficient tool to increase the use efficiency of alternative fertilizers based on waste recycling products or less well-processed P fertilizers, such as rock phosphate, generating plant growth responses comparable with conventional mineral fertilization. Successful strategies may contribute to a better adaptation of fertilizer supply to the plant demands, reduce fertilizer inputs, the risk of unwanted nutrient losses with detrimental effects on the environment, thereby promoting zero waste" concepts turning waste into resources needed for crop production. However, obviously more targeted application strategies are required considering the impact of different soil properties and differences in the compatibility of the

microbial inoculants with the selected fertilizer products. These interactions seem to be much more specific than currently assumed. This is reflected e.g., by the selective impact of the PGPM inoculants on P utilization from manure-amended compost as compared with sole application of the municipal waste compost, observed on both soils, or the specific effects on Ca–P utilization on the acidic soil only. However, the results also demonstrate that even the use of less-efficient fertilizers can be further improved by enrichment strategies with compatible fertilizer components, as demonstrated for poultry manure or stabilized ammonium in the present study. The results also demonstrate that consortium products do not necessarily exhibit better performance than single-strain inoculants. A better understanding of the underlying mechanisms determining compatible interactions between fertilizers, soil properties, and PGPM inoculants may significantly contribute to the development of more reproducible application strategies, which still represents a major challenge for the use of microbial biostimulants in agricultural practice.

Supplementary Materials: The following are available online at www.mdpi.com/link. Figure S1: Tomato rhizosphere pH for alkaline soil-Experiment 1, Table S1: Type III error tables.

Author Contributions: I.K.M. responsibly planned, set up, and ran all analysis, and wrote the manuscript. H.K.D., provided supervision, and editing and reviewing of the manuscript. J.G. is a contributing author with CFB product development, and manuscript review and editing. G.N. was part of the planning, supervision, and reviewing and editing, with contributions in the write-up. U.L. was part of the supervision team and was involved in reviewing and editing of the manuscript.

Funding: Research funds were provided by The Food Security Center at the University of Hohenheim and FIAT PANIS and by the European Community's FP7 Program/2007-2013 under grant agreement number 312117 (BIOFECTOR).

Acknowledgements: The lead author thanks the Food Security Centre, the Technicians (Helene Ochott and Charlotte Haake), the PhD supervisory team, and colleagues at the Institute of Crop Sciences (340h), University of Hohenheim. Thanks also go to all the staff and colleagues at the University of Energy and Natural Resources in Ghana for hosting the lead author during a research stay, especially Nashiru.

Conflicts of Interest: "The authors declare no conflict of interest." and "The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results".

5.6 References

1. Zhang, W.J.; Zhang, X.Y. A forecast analysis on fertilizers consumption worldwide. *Environ. Monit. Assess.* 2007, *133*, 427–434. doi: 10.1007/s10661-006-9597-7.
2. Tilman, D.; Cassman, K.G.; Matson, P.A.; Naylor, R.; Polasky, S. Agricultural sustainability and intensive production practices. *Nature* 2002, *418*, 671–677.
3. Tilman, D.; Fargione, J.; Wolff, B.; D'Antonio, C.; Dobson, A. Forecasting agriculturally driven global environmental change. *Science* 2001, *292*, 281–284.
4. Stoffella, P.J.; He, Z.I.; Wilson, S.B.; Ozores-Hampton, M.; Roe, B.E. Compost Utilization in Subtropical Horticultural Cropping Systems. I International Symposium on Organic Matter Management and Compost in Horticulture; J. Biala et al. Eds.; Acta horticulturae, Adelaide, Australia: 2014; pp. 95–108.
5. Zhou, X.; Qiao, M.; Wang, F.H.; Zhu, Y.G. Use of commercial organic fertilizer increases the abundance of antibiotic resistance genes and antibiotics in soil. *Environ. Sci. Poll. Res.* 2017, *24*, 701–710.
6. Khan, A.A.; Jilani, G.; Akhtar, M.S.; Naqvi, S.M.S.; Rasheed, M. Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. *J. Agric. Biol. Sci.* 2009, *1*, 48–58.
7. Sharma, B.S.; Riyaz, Z.; Sayed, M.H.T., Thivakaran, A.G. Phosphate solubilizing microbes: Sustainable approach for managing phosphorus deficiency in agricultural soils. *Springer Plus* 2013, *2*, 1–14.
8. Sheraz M.S.; Hassan, G.I.; Samoon, S.A.; Rather, H.A.; Dar, S.A., Zehra, B. Bio-fertilizers in Organic Agriculture. *J. Phyto.* 2010, *2*, 42–54.

9. Verbon, E.; Liberman, L.M. Microbes affect endogenous mechanisms controlling root development. *Trends Plant Sci.* 2016, *21*, 218–229.
10. Menzies, N.; Harbison, D.; Dart, P. Soil chemistry-facts and fiction and their influence on the fertilizer decision making process. In Proceedings of the 26th Annual Conference of the Grassland Society of NSW, Bathurst, Australia, 26-28 July, 2011; pp. 49–63.
11. Schütz, L.; Gattinger, A.; Meier, M.; Muller, A.; Boller, T.; Mäder, P.; Mathimaran, N. Improving crop yield and nutrient use efficiency via biofertilization–A global meta-analysis. *Front. Plant Sci.* 2017, *8*. doi:10.3389/fpls.2017.02204
12. Thonar, C.; Lekfeldt, J.D.S.; Cozzolino, V.; Kundel, D.; Kulhánek, M.; Mosimann, C.; Neumann, G.; Piccolo, A.; Rex, M.; Symanczik, S.; et al. Potential of three microbial bio-effectors to promote maize growth and nutrient acquisition from alternative phosphorous fertilizers in contrasting soils. *Chem. Biol. Technol. Agric.* 2017, *4*, 7. doi: 10.1186/s40538-017-0088-6.
13. Nkebiwe, P.M.; Weinmann, M.; Müller, T. Improving fertilizer-depot exploitation and maize growth by inoculation with plant growth-promoting bacteria: From lab to field. *Chem. Biol. Technol. Agric.* 2016, *3*, 15. doi: 10.1186/s40538-016-0065-5.
14. Nkebiwe, P.M.; Neumann, G.; Müller, T. Densely rooted rhizosphere hotspots induced around subsurface NH_4^+-fertilizer depots: A home for soil PGPMs? *Chem. Biol. Technol. Agric.* 2017, *4*, 29.
15. VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten e.V. Speyer, Germany). *Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik Methodenbuch Band I Die Untersuchung von Böden*, 4th ed.; VDLUFA Verlag: Darmsatdt, Germany, 1991.
16. Bai, Z.; Li, H.; Yang, X.; Zhou, B.; Shi, X.; Wang, B.; Li, D.; Shen, J.; Chen, Q.; Qin, Q.; Oenema, O.; Zhang, F. The critical soil P levels for crop yield, soil fertility and environmental safety in different soil types. *Plant Soil* 2013, *372*, 27–37. doi:10.1007/s11104-013-1696-y.
17. VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten e.V. Speyer, Germany). *Standpunkt. Phosphordüngung nach Bodenuntersuchung und Pflanzenbedarf;* VDLUFA Verlag: Darmsatdt, Germany, 2018.
18. Landwirtschaftskammer Niedersachsen. Richtwerte für die Düngung in Niedersachsen. In *Auszug aus den Düngungsrichtlinien, Stand März 2008, Mikronährstoffe Bor, Mangan, Kupfer und Zink. Landwirtschaftskammer Niedersachsen*; Fachbereich Pflanzenbau, Hannover, Germany, 2008.
19. Gericke, S.; Kurmis, B. Die kolorimetrische Phophorsäurebestimmung mit Ammonium-Vanadat-Molybdat und ihre Anwendung in der Pflanzenanalyse. *Z. Pflanzenernaehr. Bodenkd* 1952, *59*, 235–247.
20. Pilbeam, D.J.; Cakmak, I.; Marschner, H.; Kirkby, E.A. Effect of withdrawal of phosphorus on nitrate assimilation and PEP carboxylase activity in tomato. *Plant Soil* 1993, *154*, 111–117. https://doi.org/10.1007/BF00011079
21. Neumann, G.; Römheld, V. Root excretion of carboxylic acids and protons in phosphorus-deficient plants. *Plant Soil* 1999, *211*, 121–130. doi:10.1023/A:1004380832118
22. Campbell, R.C. Reference sufficiency ranges for plant analysis in the southern region of the United States. In *Southern Cooperative Series Bulletin #394*; North Carolina Department of Agriculture and Consumer Services Agronomic Division: NC, USA, 2009; p. 79.
23. Abbasi, M.K.; Musa, N.; Manzoor, M. Mineralization of soluble P fertilizers and insoluble rock phosphate in response to phosphate-solubilizing bacteria and poultry manure and their effect on the growth and P utilization efficiency of chilli (*Capsicum annuum* L.).

Biogeosciences 2015, *12*, 4607–4619. www.biogeosciences.net/12/4607/2015/ doi:10.5194/bg-12-4607-2015
24. Kanabo, A.I.; Gilkes, R. The role of soil pH in the dissolution of phosphate rock fertilizers. *Fert. Res.* 1987, *12*, 165–173. doi: 10.1007/BF01048916.
25. Neumann, G.; Römheld, V. Root-induced changes in the availability of nutrients in the rhizosphere. In *Plant Roots The Hidden Half*, 3rd ed.; Waisel Y.; Eshel, A.; Kafkafi U; Eds.; Marcel Dekker: New York, USA, 2002; pp.617–649.
26. Cakmak, I. Possible role of Zn in protecting plant cells from damage by reactive oxygen species. *New Phytol*. 2000, *146*, 185–205.
27. Datnoff L.E., Elmer W.H., Huber D.M. *Mineral Nutrition and Plant Disease* American Phytopathological Society: St. Paul, AK, USA, 2007.
28. von Rad, U.; Klein, I.; Dobrev, P.I.; Kottova, J.; Zazimolova, E.; Fekete, A.; Hartmann, A.; Schmitt-Kopplin, P.; Durner, J. Response of Arabidopsis thaliana to N-hexanoyl-DL-homoserine-lactone, a bacterial quorum sensing molecule produce in the rhizosphere. *Planta* 2008, *229*, 73–85.
29. Soni, R.; Yadav, S.K.; Rajput, A.S. ACC-Deaminase Producing Rhizobacteria: Prospects and Application as Stress Busters for Stressed Agriculture. *microorg. Green Revolut.* 2018, *7*, 161–175.
30. Khan, A.L.; Halo, B.A.; Elyassi, A.; Ali, S.; Al-Hosni, K.; Hussain, J.; Lee, I.J. Indole acetic acid and ACC deaminase from endophytic bacteria improves the growth of Solanum lycopersicum. *Electron. J. Biotechnol.* 2016, *21*, 58–64. doi:10.1016/j.ejbt.2016.02.001.
31. Neumann, G. The role of ethylene in plant adaptations for phosphate acquisition in soils—A review. *Front. Plant Sci.* 2016, *6*, 1224. doi:10.3389/fpls.2015.01224.
32. Borriss, R. *Bacillus*, a plant beneficial bacterium. In *Principles of Plant Microbe Interactions*; Lugtenberg B. Ed.; Springer International Publishing: Cham, Switzerland, 2015; pp. 379–389.
33. Meng, Q. *Characterization of Bacillus amyloliquefaciens strain BAC03 in disease control and plant growth promotion.* ProQuest Dissertations Publishing: East Lansing, MI, USA, 2014.
34. Saber, W.I.A.; Ghoneem, K.M.; Rashad, Y.M.; Al-Askar, A.A. Trichoderma harzianum WKY1: An indole acetic acid producer for growth improvement and anthracnose disease control in sorghum. *Biocontrol Sci. Technol.* 2017, *27*, 654–676.
35. Yusran, Y.; Roemheld, V.; Mueller, T. Effects of Pseudomonas sp. "Proradix" and Bacillus amyloliquefaciens FZB42 on the establishment of AMF infection, nutrient acquisition and growth of tomato affected by Fusarium oxysporum Schlecht f.sp. radicis-lycopersici Jarvis and shoemaker. In Proceedings of the International Plant Nutrition Colloquium XVI, California, USA, 26th to 30th August, 2009; pp. 1106.
36. Saleem, M.; Law, A.D.; Sahib, M.R.; Pervaiz, Z.H.; Zhang, Q. Impact of root system architecture on rhizosphere and root microbiome. *Rhizosphere* 2018, *6*, 47–51.
37. Suen, P.K.; Zhang, S.; Sun, S.S.M. Molecular characterization of a tomato purple acid phosphatase during seed germination and seedling growth under phosphate stress. *Plant Cell Rep.* 2015, *34*, 981–992.
38. Lekfeldt, J.D.S.; Rex, M.; Mercl, F.; Magid, K.; Tlustoš, P.; Magid, J. Effect of bioeffectors and recycled P-fertiliser products on the growth of spring wheat. *Chem. Biol. Technol. Agric.* 2016, *3*, 22. doi: 10.1186/s40538-016-0074-4.
39. Rosen, C.J.; Bierman, P.M. Using manure and compost as nutrient sources for fruit and vegetable crops. Available online: https://www.plantgrower.org/uploads/6/5/5/4/65545169/manure-and-compost. Pdf (accessed on 29.05.2018date month year).

40. Ögüt, M.; Er, F.; Neumann, G. Increased proton extrusion of wheat roots by inoculation with phosphorus solubilising microorganisms. *Plant Soil* 2011, *339*, 285–297. doi: 10.1007/s11104-010-0578-9.
41. Dobermann, A.R. *Nitrogen Use Efficiency–State of the Art*; Agronomy--Faculty Publications: Nebraska, USA, 2005; pp. 1–16.

6 GENERAL DISCUSSION AND CONCLUSIONS

This PhD research project addressed perspectives to improve the efficiency of PGPM inoculants in terms of plant growth promotion, nutrient acquisition and mobilization of sparingly soluble P sources by combination with selected inorganic and organic fertilizers commonly used in agricultural and horticultural production systems. In the first part of the study, special emphasis was placed on the form of N supply (nitrate versus ammonium fertilizers stabilized with nitrification inhibitors), with the aim to promote the P-solubilizing potential of pre-selected PGPMs with P-solubilizing properties (Nkebiwe et al. 2017). The starting point was the observation that all selected fungal and bacterial PGPMs (13 strains), showing solubilization of tri-calcium phosphate and Rock-P on artificial growth media (Nkebiwe et al., 2017; Fig. 6.1), completely failed to support plant growth on low P soils with supply of sparingly soluble P sources, such as rock-P, ashes and slags (Chapter 4.1; Lekfeldt et al., 2016; Thonar et al., 2017; BIOFECTOR Final Report, 2017).

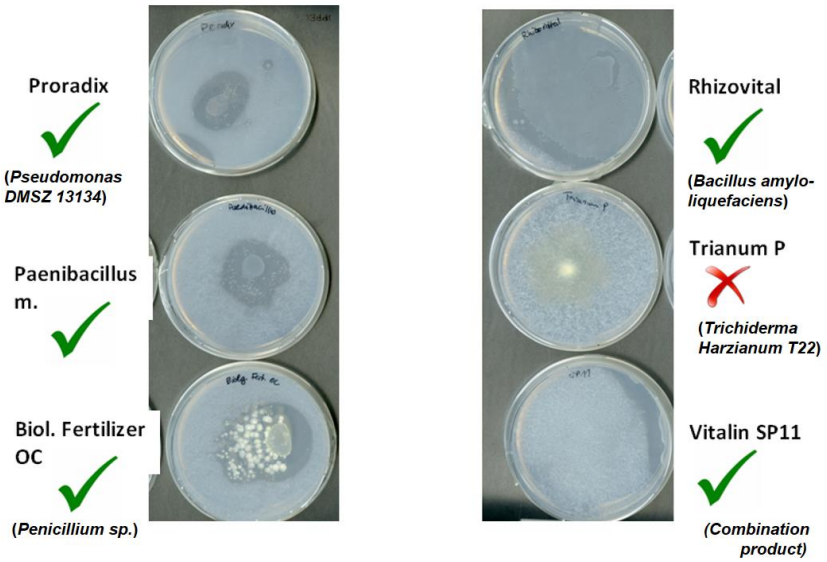


Figure 6.1. Tri-calcium phosphate solubilization of selected fungal and bacterial PGPM strains on Deubel-Muromecev medium indicated by clarification zones (Kuhlmann, 2014; Nkebiwe et al. 2017).

Based on the hypothesis that proton extrusion by roots and microorganisms, triggered by uptake of ammonium as major N source (Römheld and Marschner, 1983; Sharma et al., 2013), would further support the P solubilizing potential of the selected PGPMs, model experiments

were conducted in total with 16 fungal and bacterial strains. Maize was used as a model plant, grown on P-limited soils with DMPP (3, 4-dimethylpyrazole-phosphate)-stabilized ammonium sulfate fertilization and rock-P as sparingly soluble P source. Apart from demonstrating the principal effectiveness (Chapter 4.1), special emphasis was placed on the characterization of functional mechanisms (Chapter 4.2) and the expression under different soil conditions (Chapter 4.3), including also a series of first field experiments (Chapter 4.1; Annex, Tables 6.3 and 6.4).

The second aspect was an evaluation of PGPM interactions with different types of organic fertilizers based on compost and composted manure, stabilized ammonium and combinations thereof on different soil types using tomato as a model plant (Chapter 5).

6.1 Plant growth promotion by PGPM-ammonium interactions and related mechanisms.

In total, a beneficial effect of stabilized ammonium fertilization on performance of 16 different PGPM strains and strain combinations was demonstrated in 14 experiments with maize, wheat and tomato as host plants grown on soils with sparingly soluble Ca-P supply (Nkebiwe 2016; Mpanga et al. 2018, 2019a,b; Bradacova et al. 2019). Accordingly, also a meta-analysis based on the impact of ammonium fertilization on PGPM-induced plant growth promotion in comparison with other soluble mineral N fertilizers covering all experiments conducted within the BIOFECTOR project revealed a significant effect exclusively for stabilized ammonium fertilizers (Fig. 6.2).

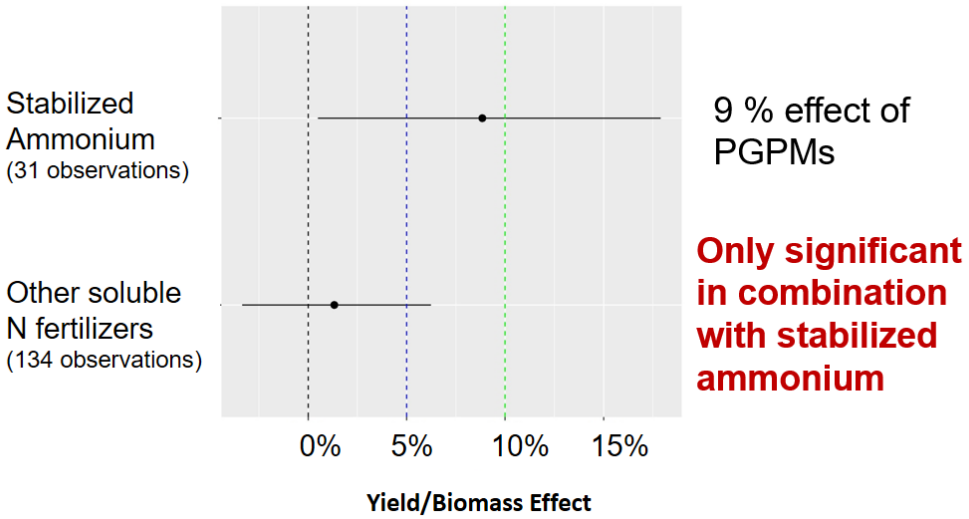


Figure 6.2. PGPM-induced plant growth promotion depending on the form of N fertilization (stabilized ammonium versus other soluble N fertilizers. Meta-analysis BIOFECTOR Project; BIOFECTOR Final Report, 2017).

Own experiments revealed that the observed plant-growth-promoting effects could be clearly related to interactions of ammonium fertilization and the PGPM inoculants. Compiling all experiments with comparisons of Rock-P acquisition under nitrate fertilization versus PGPM-ammonium combinations, revealed an increase of shoot P accumulation by 102% in the ammonium variants with PGPM inoculation, reaching about 80% of the P content obtained with soluble P fertilization (Table 6.1). However, 91% of this effect could be attributed to improved P acquisition mediated by stabilized ammonium fertilization even in non-inoculated plants and the additional PGPM effect comprised only 11% (Table 6.1). By contrast, the ammonium-PGPM combinations increased shoot biomass production on average by 69% and about half of this effect could be attributed, each to ammonium fertilization or PGPM inoculation, respectively (Table 6.1). These findings demonstrate that, although stabilized ammonium fertilization and PGPM inoculation, equally contributed to plant growth promotion on low P soils with sparingly soluble P supply, the additional PGPM effect seems to be at least not mainly caused by an improved microbial P acquisition.

Table 6.1. Additive effects of stabilized ammonium supply and PGPM inoculation (% increase in comparison with nitrate fertilization) on shoot biomass production and shoot P accumulation of the host plants. Average calculated from 13-20 experimental variants (pot and field experiments with maize, wheat, tomato and 16 PGPM inoculant strains) on low P soils with sparingly soluble Ca-P sources in comparison with the effects of soluble P fertilization (adapted from Nkebiwe, 2016; Mpanga et al., 2018; 2019a,b; Bradacova et al., 2019)

	NH_4^+ Effect (%)	PGPM Effect (%)	NH_4^+ + PGPM Effect (%)	% of Soluble P fertilization
Shoot Biomass	**35.8** ± 12.5	**31.4** ± 6.2	**68.7** ± 17.7	**84.2** ± 5.8
Shoot P Content	**90.6** ± 22.0	**11.4** ± 5.1	**102** ± 25.4	**79.1** ± 6.0

Despite a high P-solubilizing potential detectable on artificial growth media for most of the investigated strains (Fig. 6.1, Nkebiwe et al., 2017), the P mobilization effect was obviously induced by the well-documented ammonium-induced rhizosphere acidification (see also Tables 4.4; 4.9 and Fig. 4.8) mainly via root extrusion of protons (Marschner and Römheld, 1983; Neumann and Römheld 2002). This seems to be rather the cause than the consequence of plant growth stimulation induced by PGPM inoculation: on the investigated soils with low P availability, the improved P acquisition via ammonium-induced root extrusion of protons obviously provided a P starter supply, which enabled a successful rhizosphere establishment of the PGPM inoculants as pre-requisite for the expression of plant growth-promoting effects. This has been similarly demonstrated for symbiotic interactions with Rhizobia or AM fungi as plant inoculants (Bittman et al., 2006; Chekanaia et al., 2018) but was obviously not associated with a significant additional PGPM-induced P mobilization (Tab. 6.1; 6.2). Accordingly, only one out of six experiments with three P-solubilizing PGPM inoculants revealed rhizosphere acidification after PGPM inoculation in addition to the effect induced by stabilized ammonium fertilization (see Tables 4.4, 4.9 and Fig. 4.8), associated with significantly increased P accumulation by the host plant. This was the case on a moderately acidic and weakly-buffered sandy soil pH 5.8 supplied with Rock-P fertilization and *Bacillus amyloliquefaciens* FZB42 used as an inoculant. Obviously, only in this exceptional situation, the combined effects of the acidic soil pH, low soil pH buffering and increased proton extrusion by roots and inoculants were sufficient to mediate

significant additional solubilization of rock-P in comparison with the non-inoculated control, while in all other cases the inoculant effect was not detectable. There was also no indication for an increased organic acid exudation by the PGPM inoculants under rhizosphere conditions (Chapter 4.2 and 4.3). These findings suggest that the initial hypothesis, assuming synergistic effects on P solubilization by rhizosphere acidification induced by plant roots and PGPM inoculants in response to stabilized ammonium fertilization, must be rejected at least for the investigated combinations. Since in total 16 fungal and bacterial inoculant strains have been investigated with two different crops on seven different soils, it is worthwhile to assume that PGPM-assisted mobilization of sparingly soluble mineral P sources cannot be regarded as a widespread mechanism of plant growth promotion by PGPM inoculants under real rhizosphere conditions.

This view is also supported by comparing the spatial extension of ammonium-induced rhizosphere acidification and the root colonization pattern of bacterial inoculants, as shown in Figure 6.3. While ammonium-induced rhizosphere acidification intensively spreads over the whole root system (Fig. 6.3 A), colonization of soil-grown maize roots indicated by a GFP-tagged strain of *Bacillus amyloliquefacie*ns FZB42 on soil-grown roots only shows a very spotty, irregular pattern of micro-colonies on the root surface (Fig. 6.3 B). Similar results have been reported for root colonization of tomato by *B. amyloliquefaciens* FZB42 or *Pseudomonas* sp. RU47, respectively (Eltlbany et al., 2019). This implicates only a very local extension of potential rhizosphere acidification effects induced by the inoculants in the vicinity of the micro-colonies, which may explain the limited contribution to P acquisition of the whole root system (Table 6.1).

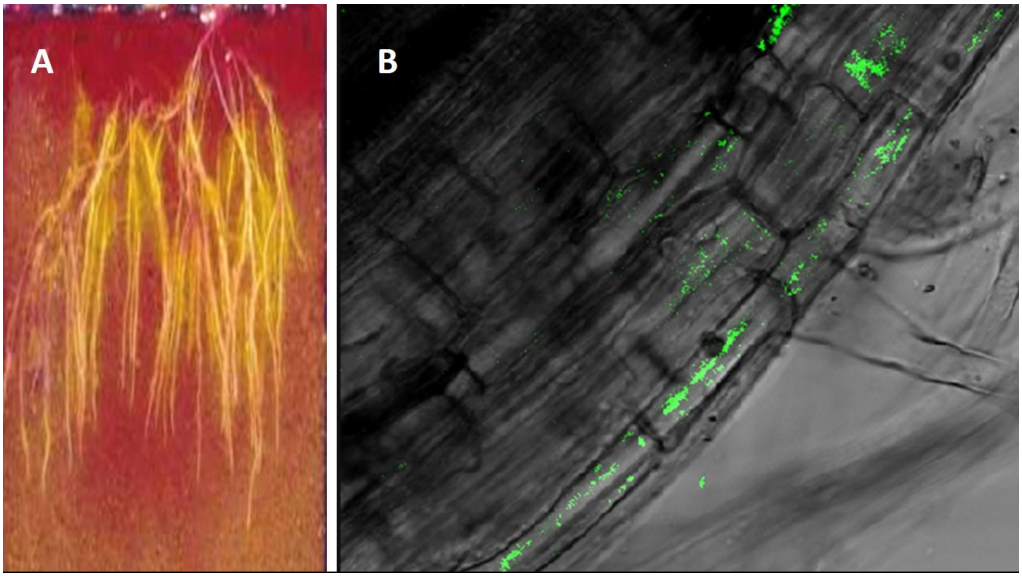


Figure 6.3. Root-induced rhizosphere acidification in response to ammonium fertilization (A) and root colonization pattern of a GFP-tagged strain of *Bacillus amyloliquefaciens* FZB42. (Source: Römheld and Marschner, 1983 and Eltlbany, 2019).

According to the findings described above, the observed effects on plant growth in combination with sparingly soluble Ca-P sources and stabilized ammonium fertilization by inoculation of the various fungal and bacterial inoculants cannot be attributed to a direct P-solubilizing effect of the inoculants. The expression of a more indirect effect is illustrated exemplarily in Table 6.2. In accordance with the summary of PGPM effects resented in Table 6.1, ammonium fertilization contributed to Rock-P acquisition in maize on a low P clay loam soil pH 7.0, while additional inoculation with various fungal and bacterial inoculants had only marginal effects. However, PGPM inoculation significantly contributed to the acquisition of other macronutrients such as N and K and this was associated with increased root length development (Table 6.2). Obviously, ammonium-induced promotion of Rock-P acquisition promoted root colonization of the PGPM with root growth promoting potential. As a result, the larger root systems supported plant growth by improved nutrient acquisition in general.

Root growth stimulation is a well-documented feature of PGPMs (Vejan et al., 2016; Berg, 2009) and is thought to be mediated by microbial production of auxins (Patten and Glick, 2002; Ahmed and Hasnain, 2010) and molecules interfering with plant-hormonal signalling, such as

certain volatile organic compounds (VOCs) (Sharifi and Ryu, 2018), quorum sensing metabolites (Hartmann et al., 2014) or enzymes involved in ethylene degradation, such as ACC deaminase (Glick, 2005; 2014).

Table 6.2 Shoot nutrient contents and total root length of maize (cv Colisee) grown on a clay-loam, organic farming soil (pH 7.0), supplied with and without (No P) P fertilization in form of Rock-P. N supply in the form of DMPP-stabilized ammonium. Microbial inoculants: *Pseudomonas sp.* DSMZ13134 (Proradix), *Trichoderma harzianum* OMG16 + 5 Bacillus strains (Combifector-A); *Bacillus amyloliquefaciens* FZB42 (Rhizovital), *Paenibacillus mucilaginosus*, Vitalin SP11 (*Bacillus subtilis*, *Pseudomonas* sp., *Streptomyces* spp., humic acids and extracts of the seaweed Ascophyllum nodosum), or no inoculation (NoBE). *indicates significant differences after pairwise comparison of PSM-inoculated variants versus the non-inoculated control with ammonium fertilization; t-test, $p < 0.05$ (adapted from Mpanga et al., 2019a).

Fertilization	Shoot P content (mg Plant^{-1})	Shoot N content (mg Plant^{-1})	Shoot K content (mg Plant^{-1})	Total root length (m Plant^{-1})
No P	7.4	45.7	151.6	5.12
NH_4^+_Rock-P	19.5	271.5	356.6	6.20
NH_4^+_Rock-P_Proradix	21.3*	330.9*	448.9*	6.90
NH_4^+_Rock-P_FZB42	20.9	328.1*	415.1*	8.21*
NH_4^+_Rock-P_Paenibac.	21.4	302.5	409.9*	7.42*
NH_4^+_Rock-P_Vitallin SP11	22.0	339.9*	404.7*	7.14
NH_4^+_Rock-P_Combi-A	20.4	341.7*	405.0*	9.30*

The present study revealed that stabilized ammonium fertilization exerted a range of direct and indirect effects favouring PGPM-induced root growth stimulation. Based on findings of Barucha et al. (2013) and Patil et al. (2011), showing increased auxin production of *Pseudomonas putida* or *Acetobacter diazotrophicus* L1 on artificial growth media with ammonium supply, this was shown also for a range of PGPM inoculants investigated in this thesis (*Pseudomonas sp.* DMSZ 13134, *Bacillus amyloliquefaciens* FZB42; Chapter 4.2). An increased auxin production potential was also detected for bacterial communities re-isolated from the rhizosphere of PGPM-inoculated plants (FZB42) associated with PGPM-induced root-growth promotion (Chapter 4.2) and this effect was strictly dependent on PGPM inoculation. Whether this was a direct inoculant effect or induced by modifications of the rhizosphere microbiome triggered by the inoculants still remains an open question. A recent study by Eltlbany et al. (2019) demonstrated that inoculation with *B. amyloliquefaciens* FZB42 increased the rhizosphere abundance also of other potentially plant-growth promoting strains of *Bacillus* and *Paenibacillus* in P-deficient tomato.

This was associated with a stimulation of adaptive P deficiency responses of the host plant, such as promotion of root development but inhibition of shoot growth, increased rhizosphere activity of acid phosphatase known to be secreted from roots of P-deficient tomato plants (Tadano & Sakai, 1991), and increased AM colonization of the larger root system in accordance with documented mycorrhizal helper functions of FZB42 (Yusran et al., 2009; Thonar et al. 2017).

Accordingly, Moradtalab et al. (2019) reported increased expression of auxin-related genes associated with increased auxin (IAA) accumulation in the root tissue of maize after inoculation with the combination product Combifector-A (*Trichoderma harzianum* OMG16 + 5 *Bacillus strains*), as one of the most efficient inoculants in terms of root growth promotion tested in this thesis (Chapter 4.1). The effects were particularly expressed in combination with stabilized ammonium fertilization (Fig. 6.4) and comprised increased expression of genes encoding the auxin transporter ZmPIN1A, the auxin-responsive transcription factor ZmArf12 and also tryptophan synthase involved in auxin biosynthesis (Fig. 6.4). Interestingly, PIN1A and ZmArf12 have not only been related with root growth stimulation but also with the regulation of responses to P deficiency, including adaptive modifications of shoot growth, induction of root secretory acid phosphatases and alterations in P homeostasis (Li et al., 2018; Wang et al., 2014). This raises the question, whether the PGPM inoculation not only induced root growth promotion by interactions with auxin biosynthesis, transport and signalling but additionally promoted the expression of P deficiency responses of the host plant as similarly observed in the study of Eltlbany et al. (2019). Accordingly, in both studies, improved P acquisition in response to PGPM inoculation was recorded. Also in face of the very localized and spotty root colonization patterns of PGPM inoculants in soil-grown plants (Eltlbany et al, 2019, Fig. 6.3), plant-PGPM interactions via hormonal signalling, usually requiring only trace amounts of signal

molecules, are more likely than direct mobilization of significant amounts of nutrients by the inoculants in the rhizosphere. However, a larger proportion of young growing roots characterized by most intense root exudation (Neumann and Römheld, 2007) may indirectly promote also beneficial rhizosphere interactions with indigenous microbial populations as demonstrated e.g. for mycorrhizal helper effects by inoculation with *Pseudomonas* sp. DMSZ 13134 (Proradix) or *Bacillus amyloliquefaciens* FZB42 by Yusran et al. (2009), Thonar et al. (2017) and Eltlbany et al., (2019).

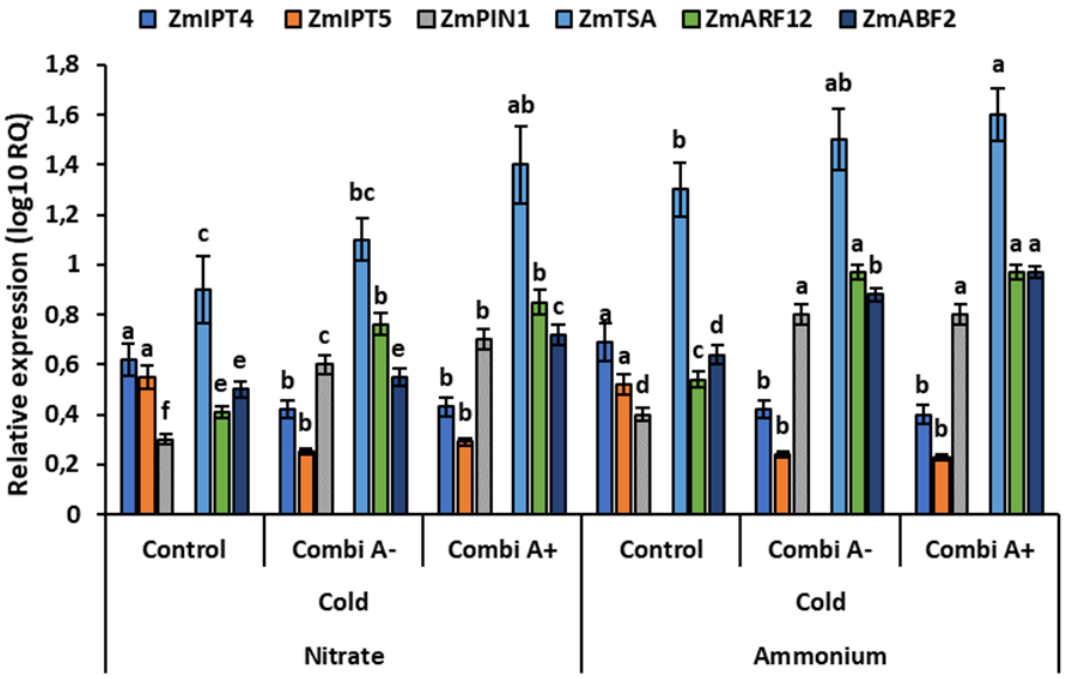


Figure 6.4. Relative expression of hormone-related genes (cytokinins, auxins, abscisic acid) in the root tissue of maize seedlings grown for six weeks on a silty loam field soil, pH 7.1 with nitrate or stabilized ammonium fertilization, after 14 d recovery from two weeks exposure to reduced root zone temperatures (12-14 ° C) with and without Combifector-A inoculation (Combi A-) and Zn/Mn supplementation (Combi A+) Combi A = Trichoderma harzianum OMG16 + 5 Bacillus strains; IPT: Isopentenyl Transferases, PIN: PIN1A, TSA: tryptophan synthase, ARF: Auxin Response Factor, ABF: Abscisic acid binding Factor. Means and SD of five replicates. Different letters indicate significant differences (Tukey-Test, $p < 0.05$) (Moradtalab et al., 2019).

However, apart from direct interactions of the PGPM inoculants with ammonium supply via stimulation of microbial or plant auxin production, also more indirect ammonium effects may contribute to the observed superior expression of PGPM effects. In Chapter 4.2 it was demonstrated that stabilized ammonium fertilization not only increased the auxin production potential of microbial inoculants but also stimulated auxin accumulation in the shoot tissue of

maize, even in absence of PGPM inoculants (Table 4.5). This may increase the responsiveness of the host plant to additional auxin supply provided by the PGPMs. However, promotion of root elongation by ammonium fertilization of non-inoculated plants was only weakly expressed as similarly reported by (Hoffmann et al., 1994) and could be mainly attributed to inoculant effects (Chapter 4.1 – 4.3). By contrast, ammonium fertilization increased root hair length and the related formation of rhizosheaths of root-adhering soil without any additional effects induced by PGPM inoculation (Fig, 4.2). Similar effects of ammonium fertilization on root hair formation have been reported also in previous studies (Kania et al., 2007; Fig. 6.5). In addition to ammonium-induced root extrusion of protons promoting P solubilization (Neumann and Römheld, 2002), this effect will increase both, the root surface area contributing to rhizosphere acidification and the extension of the rhizosphere with well-documented beneficial effects on spatial acquisition particularly of nutrients with limited solubility such as P and Fe (Neumann and Römheld, 2002; 2007).

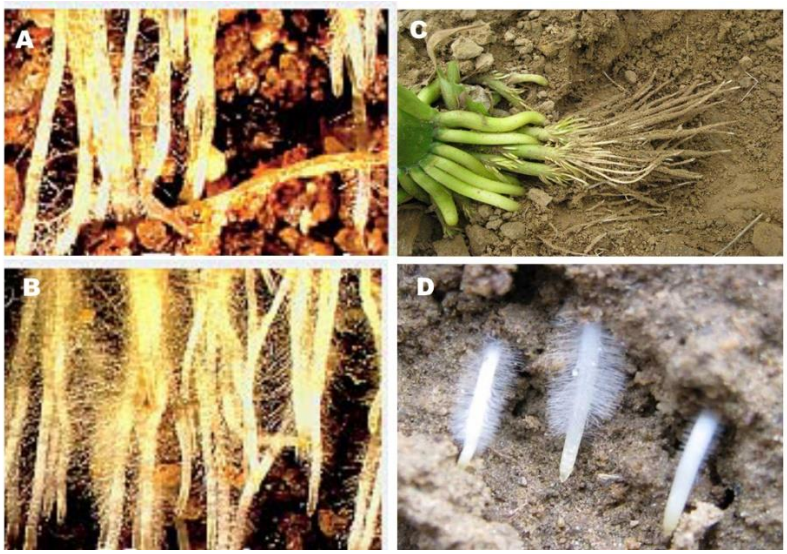


Figure 6.5. Ammonium-induced stimulation of root hair development in (A, B) *Lolium perenne* (Kania et al., 2007) and (C, D) ammonium placement under field conditions in maize (J. Shen, pers. comm.)

However, ammonium-induced promotion of root hair development may also contribute to root-PGPM interactions in the rhizosphere. For the inoculant *Trichoderma harzianum* OMG16, increased root colonization was observed in maize plants with stabilized ammonium

fertilization (Chapter 4.2). Interestingly, for this *Trichoderma* strain and also for other inoculants tested in this study, preferential colonization of root hairs has been reported in the literature (Fig. 6.6). Accordingly, stimulation of root hair development providing additional infection sites for PGPMs may at least partially explain the improved root colonization and thus superior rhizosphere establishment in response to ammonium supply.

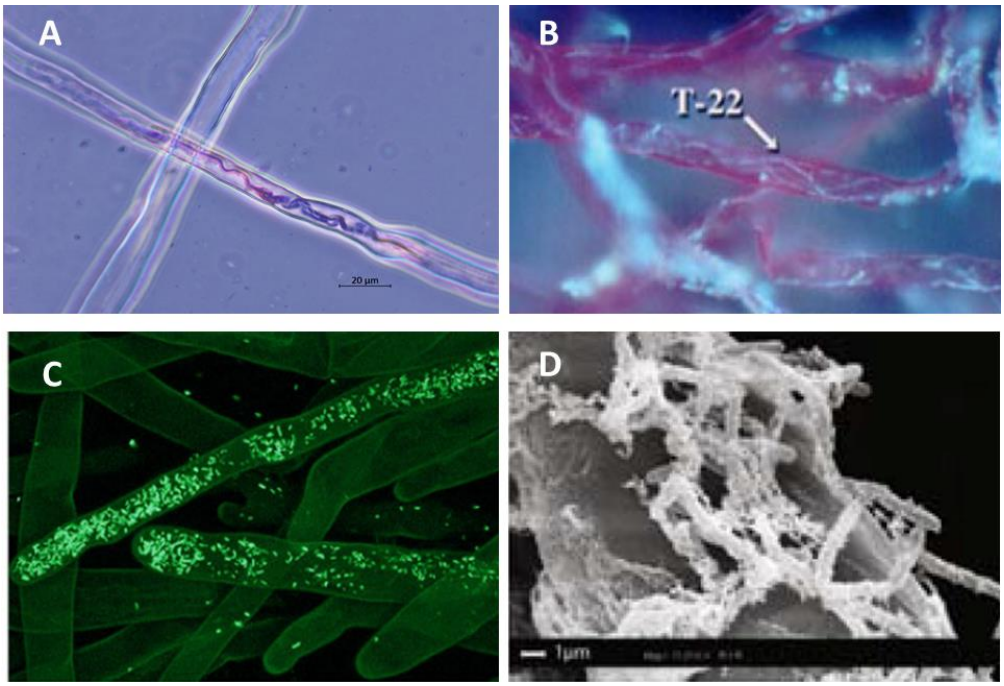


Figure 6.6. Root hair colonization by selected PGPM strains. A: *Trichoderma harzianum* OMG16 in oilseed rape (Mpanga et al., 2019b); B: *Trichoderma harzianum* T22 in maize (Harman, 2000); C: P*seudomonas fluorescens* PICF7 in olive (Prieto et al., 2011*); D: Bacillus amyloliquefaciens* FZB42 in maize (Fan et al., 2012).

Taken together, the results suggest that the combination of PGPM inoculants with stabilized ammonium fertilizers instead of nitrate can promote beneficial plant PGPM associations in a synergistic way at different levels of interaction. Root-induced rhizosphere acidification in response to ammonium supply preferentially increased the solubility of acid-soluble P forms and stress-protective micronutrients (e.g. Zn, Mn) in soils and fertilizers, providing a starter fertilization effect, which facilitated the rhizosphere establishment of PGPMs. Additionally, the microbial inoculants preferentially stimulated root development via interactions with plant-hormonal signalling. This improved spatial nutrient acquisition in general but also contributed

to the development of a larger acidifying root system. The interaction was further promoted by ammonium supply, which increased the auxin production potential of PGPM inoculants and the host plant as well and by beneficial effects on root hair development as preferential infection sites for many PGPM strains. This multi-level interaction matrix offers a platform for a wide range of PGPMs with root growth-promoting, nutrient-solubilizing or pathogen-suppressive properties, explaining the positive effects observed in combination with various fungal and bacterial inoculants. Finally, this approach has been patented under the International Publication No: WO/2018/197433 – "Method and Composition for Improving Nutrient Acquisition of Plants" as a measure to improve plant-PGPM interactions in the rhizosphere and the conditions for expression of positive PGPM effects.

6.2 Plant growth promotion by PGPM-interactions with organic fertilizers.

Investigations on perspectives to improve the utilization of fertilizers based on products of organic and inorganic waste recycling by use of PGPM inoculants revealed superior performance particularly in combination with composted animal manures in maize and tomato (Li et al, 2017; Thonar et al. 2017, Vinci et al, 2018ab; Bradacova et al. 2019) and similar results have been reported for sweet pepper (Abbasi et al., 2015) and *Majorana hortensis* (Gharib et al., 2008). Accordingly, in a meta-analysis of all experiments conducted within the BIOFECTOR project with different types of fertilizers, yield and growth effects were most intensively expressed in combinations of the investigated microbial and non-microbial biostimulants with composted manures and also with other N-rich organic fertilizers based on guano, blood-, meat- hair-, and feather-meals (Fig. 6.7).

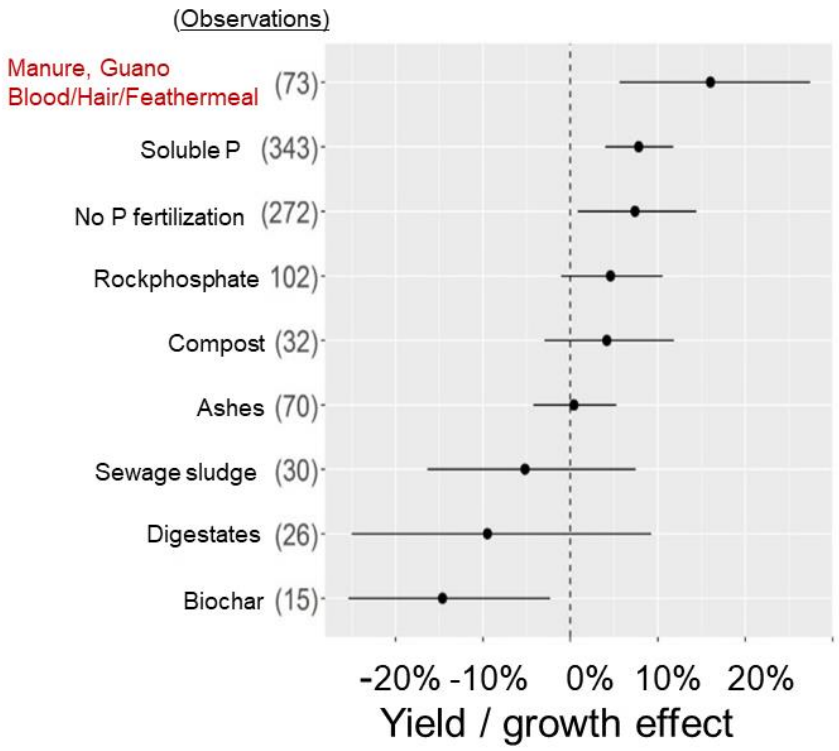


Figure 6.7. Efficiency of microbial and non-microbial biostimulants as plant inoculants in combination with organic and inorganic fertilizers. A meta-analysis of greenhouse and field experiments conducted within the framework of the BIOFECTOR project (BIOFECTOR, Final Report, 2017).

For a more systematic comparison, a greenhouse experiment was conducted with tomato on two contrasting soils (different soil texture, pH, and organic matter content) with limited P availability and organic fertilizers based on municipal waste compost or a mixture of the municipal waste compost with composted poultry manure (PM-compost), both, with and without additional application of DMPP-stabilized ammonium. The fertilizers were supplied at identical P input levels. Two PGPM products with proven plant growth-promoting potential were used as inoculants (Combifector-B = *Trichoderma harzianum* OMG16 + *Bacillus amyloliquefaciens* FZB42 or *B. amyloliquefaciens* FZB42 as single strain inoculant). While the P use efficiency (measured as P recovery efficiency in the shoot tissue) was not different for compost and PM-compost, it was significantly increased by the PGPM inoculants on both soils exclusively in combination PM-Compost, which induced plant-growth promoting effects even comparable with soluble P supply, when additionally combined with stabilized ammonium fertilization (Chapter 5). Similar to the experiments described in Chapter 4, improved P

acquisition was associated with PGPM-induced stimulation of root growth resulting also in the improved acquisition of other macronutrients such as N, K, Ca and Mg. The reasons for the preferential PGPM performance in the described organic fertilizer combinations are not entirely clear but may be related with comparatively high contents of plant-available nitrogen forms, which are easily mineralized and may thereby induce beneficial ammonium effects as described in section 6.1. Also, P availability may play a role, and particularly in case of manures, a P solubilizing potential has been described based on products of manure degradation or related changes in the soil microbiome (Abbasi et al., 2015). However, independent of the mode of action, the observed effects may provide a basis for fertilizer recommendations in organic farming systems but also for enrichment approaches of less efficient organic fertilizers as proposed for strategies of integrated plant nutrient management (IPNM) based on targeted combinations of organic and mineral fertilizers with superior performance over concepts with exclusive organic or mineral fertilization (Timsina, 2018; Wu & Ma, 2015; Chivenge et al., 2011).

6.3 Soil type-dependent expression of PGPM effects

To assess the impact of soil properties on the expression of plant-PGPM interactions, in total, three experiments have been carried out with maize and tomato on five different soils with contrasting properties, using the single-strain inoculant *Bacillus amyloliquefaciens* FZB42 and the combination product Combifector-B (*Trichoderma harzianum* OMG16 + *B. amyloliquefaciens* FZB42 + Zn/Mn supplementation). In all experiments, the soil pH has been identified as an important determinant for the expression of the investigated PGPM effects, declining with increasing soil pH (Chapter 4.3). For the approach to improve the acquisition of sparingly soluble Ca-P by combined application of stabilized ammonium fertilizers with PGPM inoculants, declining PGPM efficiency with increasing soil pH reflected the intensity of ammonium-induced rhizosphere acidification, depending on the pH buffering capacity of the

soil substrate (Fig. 4.1.2; Römheld, 1986). This was also confirmed by the improved performance of PGPM inoculants when the pH buffering capacity of a calcareous soil substrate was reduced by the addition of quartz sand (Mpanga et al., 2019a, Fig. 4.1.2). The central role of root-induced rhizosphere acidification triggered by ammonium supply, as a major factor mediating the solubilisztion of Ca-P (Table 6.1), which provided the starter fertilization for the establishment of plant-PGPM interactions, underlines the importance of the ammonium effect, which in turn depends on soil pH (Römheld, 1986). Accordingly, on the moderately acidic soils (pH 5.6) with a low pH-buffering capacity investigated in this study, ammonium-PGPM combinations were able to mediate Rock-P mobilization and plant growth to a level comparable with superphosphate fertilization (Chapter 5). In some cases, just ammonium fertilization without inoculants was already sufficient to cover the P demand of the host plants and additional PGPM effects were detectable only in terms of nutrient acquisition but not in plant growth responses (Chapter 4.3). Therefore, PGPM-ammonium combinations can be recommended as a strategy to support P acquisition from Rock-P or other sparingly soluble Ca-P fertilizers, such as ashes and slags on low P soils with moderately acidic to neutral pH. Moreover, low soil pH has been identified as one factor with inhibitory effects on nitrification (Yao, et al., 2011; Zhang et al., 2012), which may further contribute to the expression and longevity of ammonium effects on Ca-P solubilization.

However, particularly on acidic soils with a low pH buffering capacity, the strategy can induce even negative effects in cases without simultaneous application of sparingly soluble sources of Ca-P, which counteract over-acidification of the rhizosphere, induced by ammonium fertilization and can also provide Ca and Mg, as frequently limiting nutrients on acidic soils (Marschner, 1995). Over-acidification effects can even lead to root growth inhibition in response to low rhizosphere pH levels (4-5), by increasing the risk of Al and Mn toxicity and

further reduce Ca availability by cation competition with the uptake systems and increased leaching (Sittinger, 2018; Marschner, 1995).

On the other hand, on alkaline soils, the strategy is frequently limited due to a high pH buffering capacity counteracting the ammonium-induced rhizosphere acidification. However, a range of pot and field experiments indicated that this problem might be overcome by placement of suitable P and ammonium fertilizers with root attracting properties in combination with PGPMs (Chapter 4.3; Nkebiwe et al., 2016; Bradacova et al., 2019), leading to localized root proliferation and root clustering, thereby creating rhizosphere hot-spots. In these hot-spots, the higher rooting densities can increase the rhizosphere acidification potential to overcome the high pH-buffering capacity of alkaline soils (Jing et al., 2010) and also increase the root exudation potential supporting PGPM establishment in the rhizosphere (Nkebiwe et al., 2016).

Although soil pH has been identified as an important factor determining the activity and the composition of the soil microbiome (Amoo & Babalola, 2017), surprisingly little is known concerning effects of soil pH or ammonium fertilization on microbial inoculants. Soil acidity is known as a limiting factor for the establishment of rhizobia or AM symbioses due to inhibitory effects on root growth and sensitivity of non-adapted inoculant strains (Lapinskas, 2007). Accordingly, the meta-study of Schütz et al. (2017) reported improved efficiency of combined applications of microbial P solubilizers and N_2-fixers with increasing soil pH, while at high soil pH levels the efficiency of P solubilizing microorganisms was found to be inhibited by the increased pH buffering capacity of the soils. However, systematic investigations on interactions of PGPM inoculants with the soil microbiome as affected by the soil pH are still lacking. Soil organic matter (SOM) was reported as another determinant of plant PGPM interactions in the rhizosphere, with lower expression of PGPM effects with increasing SOM, which was attributed to higher competition with indigenous soil-microbial communities, more abundant in soils rich

in SOM (Schütz et al., 2017). However, this relationship was not clearly confirmed in the present study and also not in other experiments of the BIOFECTOR project (Thonar et al., 2017; BIOFECTOR Final report, 2017).

6.4 Genotypic interactions

Based already on the classical rhizosphere concept of Lorenz Hiltner (Hartmann et al., 2004; 2008), the composition of rhizosphere communities is selectively shaped by the root exudates of the host plant, serving as signal compounds, nutrient sources and pathogen protective agents with a genotype-specific composition (Berg and Smalla, 2009). However, less clear is the role of the host plant genotype as a determinant for the establishment of interactions with PGPM inoculants. Within the 150 pot and field experiments conducted in the framework of the BIOFECTOR project, there was a clear indication for superior performance of the investigated microbial and non-microbial biostimulants in combination with tomato as compared with cereal crops (Fig.6.8).

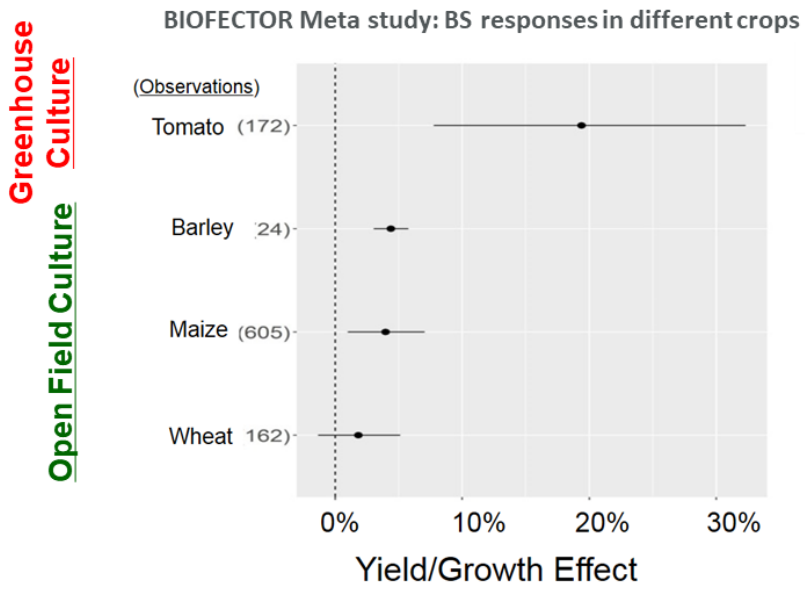


Figure 6.8. Interaction of microbial and non-microbial biostimulants as plant inoculants in combination with different crops. A meta-analysis of greenhouse and field experiments conducted within the framework of the BIOFECTOR project (BIOFECTOR, Final Report, 2017).

However, a closer look revealed that the cropping system and the related application conditions of the investigated biostimulants are a more likely cause for the observed differences. While tomato cultivation and PGPM inoculation is performed under protected greenhouse culture at least during the nursery phase (Mpanga et al., 2018; Bradacova et al., 2019), offering optimal conditions for PGPM establishment in the rhizosphere, open-field inoculation of agricultural crops (barley, wheat, maize) is more challenging, bearing a higher risk for stress factors interfering with PGPM rhizosphere establishment. This may significantly contribute to higher variability of PGPM responses in field crops. Accordingly, growing maize as a field crop under the same protected greenhouse conditions as tomato resulted in a similar expression of PGPM responses (Eltlbany et al. 2019, BIOFECTOR Final Report, 2017). Moreover, a wide host specificity has been reported in the literature for many of the investigated inoculants (Fröhlich et al. 2012; Borris, 2015; Gulden and Vessey, 2000; Harman et al., 2004).

The important role of the application conditions for PGPM performance investigated in the present study, is also reflected by the observation that different fungal and bacterial PGPMs usually showed very similar responses with respect to the presence or absence of plant growth promoting effects under a given set of culture conditions, frequently without significant differences between the different inoculants (Nkebiwe, 2016; Lekfeldt et al., 2016; Thonar et al., 2017; Eltlbany et al, 2019, Mpanga et al. 2019a; Bradacova et al. 2019). However, in contrast to the investigated PGPM functions as biofertilizers, a more strain-specific expression of effects was observed for applications of PGPMs as stress protectants, indicating superior performance of certain strains and products. Here, *Penicillium* sp. PK112 or Combifector A were particularly effective to mitigate cold stress in maize (Gómez-Muñoz et al. 2018; Ahmed, 2017), while the consortium product CRENEL showed superior performance in open field tomato production under desert conditions in Israel (Bradacova et al., 2019) and *Azotobacter chroococcum* 76A

was particularly effective to mitigate drought and salinity stress in tomato (Viscardi et al., 2016; Van Oosten et al. 2018). The differential performance of plant growth promoting effects may be explained by the observation that root growth promotion was the most widespread plant growth-promoting effect detectable for most of the investigated inoculants, which contributes to nutrient acquisition in general (Nkebiwe, 2016, Thonar et al. 2017; Eltlbany et al., 2019, Mpanga et al 2018, 2019ab) leading to beneficial PGPM effects for many different inoculants. By contrast, the promotion of stress-protective adaptations requires additional features, such as interactions with stress hormonal signalling (ABA, jasmonic and salicylic acids), induction of defence reactions against oxidative stress, accumulation of metabolites involved in osmotic adjustment or also antibiotic compounds (Ahmed, 2017; Moradtalab et al., 2019; Windisch et al., 2017; van Oosten, 2018) with an obviously higher expression variability within different PGPM strains and species.

6.5 PGPM performance under field conditions

Limited reproducibility of PGPM effects under real production conditions in the field still remains a major problem for PGPM-assisted fertilization strategies (Menzies et al., 2011). This may be at least partially attributed to the more challenging environmental conditions as compared with controlled greenhouse experiments, with a higher probability for interference by various stress factors particularly during the most sensitive phase of PGPM establishment in the rhizosphere after inoculation. Since root colonization of PGPMs depends on the supply of root exudates by the host plant, any stress factor affecting root activity is expected to affect also the establishment of plant-PGPM interactions. This may explain the lower performance in field crops as compared with greenhouse cultures shown in Fig. 6.8. However, based on the findings of the present study, also insufficient information on the most promising application conditions and PGPM-fertilizer combinations for the successful expression of PGPM effects may

play an important role. Therefore, the application strategies investigated within this thesis were tested also in a range of first field experiments conducted within the framework of the BIOFECTOR project.

6.5.1 PGPM combinations with stabilized ammonium fertilizers

Experiments on PGPM-assisted P acquisition from Rock-P and sparingly soluble Ca-P in combination with stabilized ammonium fertilizers were conducted in Germany, the Czech Republic, Italy and Israel on field sites with moderate to low P availability and a pH range between 5.2 and 8.6 in maize (4 experiments), wheat and tomato (one experiment each). PGPMs comprised strains and products successfully tested also in the pot experiments (Chapters 4 and 5). The results summarized in Table 6.3 were highly variable and PGPM yield effects over the non-inoculated variants ranged between 0 and 108 %, resulting in an average yield increase of 16.6 % ± 6.9 (SEM) over all experimental variants. The wheat effect reached 5.6 %, the average maize effect 6.1 % ± 2.5 and the average tomato effect 51 % ± 22.3, reflecting the conditions described also in Fig. 6.8 for all BIOFECTOR experiments. Significant PGPM effects on yield were recorded only on the soils with low P availability in Italy and Israel, when a significant effect of soluble P fertilization was detectable, associated also with significant effects on early growth (Tab. 6.3). This is in line with the observations of the greenhouse experiments conducted in this thesis, with positive responses of PGPM inoculation in combination with stabilized ammonium fertilizers on soils with low P availability during early growth of maize and tomato (Chapters 4 and 5). By contrast, the PGPM effects disappeared when no significant effects of soluble P fertilization were detectable (Tab. 6.3), indicating that P was not a growth-limiting factor and the P availability in the respective experiments was obviously sufficient to cover the plant demand. Since fertilization of the remaining macronutrients was performed according to the crop demands, no PGPM effects could be

expected in these cases. In this context, the responsiveness to P fertilization on a given soil seems to be a better indicator for an expected benefit by PGPM inoculation than the plant available soil P status, which does not consider a contribution of potential P mineralization effects.

Interestingly, field sites with positive PGPM responses were not only characterized by low P availability and high pH but also by Mediterranean climates. Therefore, a potential impact of additional stress factors, such as high temperatures or temporal water limitation cannot be excluded. In this context, it is also interesting to note that significant yield and early growth responses were obtained only by application of combination products based on different fungal and bacterial inoculant strains, including additions also of non-microbial bio-stimulants such as seaweed extracts and humic acids (Tab.6.3) This supports the hypothesis of a superior performance of product combinations (so-called consortia products) over single strain inoculants, due to complementary effects of the different strains particularly under more challenging environmental conditions (Nuti and Giovanetti, 2015; Sekar et al., 2016; Bradacova et al., 2019).

6.5.2 PGPM combinations with organic fertilizers

The second set of experiments was conducted in accordance with the results of Chapter 5 by combining PGPMs with selected N-rich organic fertilizers, such as manure composts, guano, meat-, hair-, and feather-meals in large scale greenhouse tomato production trials in Romania and in organic open-field tomato production in Hungary. Promising fungal and bacterial PGPM strains investigated in Chapter 4 and 5 were used as inoculants. Table 6.4 summarizes yield and early growth responses induced by the various inoculants. On both experimental sites, surprisingly intense and reproducible yield effects were recorded over three years for all investigated inoculants (Tab. 5.4), with an average increase by 56.8 % ± 5.4. The effects were

more expressed in the greenhouse experiments (72% ± 4.4) as compared with open field production (33.1 % ± 6.4). However, in both cases, PGPM inoculation was performed with two applications in a nursery phase under greenhouse conditions and directly after transplanting, respectively, similar to the tomato experiment conducted in the Negev desert in Israel (Table 6.3).

Table 6.3: Summary of crop performance in the field supplied with rock phosphate or no P, stabilized ammonium fertilization and PGPM inoculation versus soluble P supply. Proradix: *Pseudomonas* sp. DSMZ 13134; P. mucil: *Paenibacillus mucilaginosus*; FZB42: *Bacillus amyloliquefaciens* FZB42 Rhizovital42®; CombiAof crop performance in the field on soils with low or moderate P: *Trichoderma harzianum* OMG16 + Vitabac with five Bacillus strains (Bactiva GmbH, Straelen, Germany) + Zn/Mn; CombiB: *Trichoderma harzianum* OMG16 + VFZB42 + Zn/Mn; HA = humic acids; SW = seaweed extract; B. amylol: *Bacillus amyloliquefaciens* seed dressing formulation; CRENEL: microbial consortia product; Eurochem Agro GmbH, Mannheim, Germany (adopted from Bradacova et al., 2019; Mpanga et al., 2019a; BIOFECTOR Project Report, 2017)

Country	Crops	Fertilizers	Soil P levels Soil pH	PGPMs	Early growth PGPM+NH_4 response (versus NO_3)	NH_4 Yield response (versus NO_3)	PGPM Yield response (over NH_4)	Soluble P Yield response
Germany	Wheat	$NH4^+$+ RP	21 mg kg^{-1} (CAL) Low B, pH 6.6	Proradix	n.d.	6.7 % ns	5.6 % ns	0 %
Germany	Maize	$NH4^+$+ RP	52 mg kg^{-1} (CAL) Moderate C, pH 5.9	Proradix CombiB CRENEL	13 % ns 7 % ns 13 % ns	13.5 % ns	0 % (-3.5) 0 % (-3.9)	0 %
Italy	Maize	$NH4^+$ +native soil P	11 mg kg^{-1} (Olsen) Moderately low pH 8.6	Soluble P CombiA CombiB FZB42+HA B. amylol+SW	24 %* 27 %* 40 %* 39 %`* 37 %*	n.a`	 3.9 %* 4.6 %* 6.5 %* 2.0 % ns	5.2 %*
Czech Republic	Maize	$NH4^+$+ RP	58 mg kg^{-1} (CAL) Moderate C pH 5.2	Proradix P mucil. CombiB	10.3 % n.s 8.3 % n.s 12..8 % n.s	19.4 % ns	23.2 % ns 5.1 % ns 24.5 % ns	28.4 % ns
			48 mg kg^{1} (CAL) Moderate C pH 5.9	Proradix P. mucil. CombiB	0% (-1.9)ns 0% (-2.5)ns 0% (-4.0)ns	0.9 % ns	3.1 % ns 0 % ns (-1.7) 0 % ns (-7.1)	6.0% ns
Israel	Tomato	$NH4^+$native soil P	5.5 mg kg^{-1} (Olsen) Low pH 7.9	Proradix FZB42 CombiB CRENEL	13.3% ns 16.7% ns -13 % ns 113.3%*	n.a	35.5% ns 58.7% ns 1.7 % ns 108.1 %*	232. 0% *

The superior expression of PGPM effects in these trials supports the hypothesis that PGPM establishment during early growth under protected condition promotes the efficiency of plant-PGPM interactions. In contrast to the open field experiment in Israel (Tab. 6.3), the greenhouse experiment conducted in Romania (Tab. 6.4) revealed no differences in the expression of PGPM effects between single strain inoculants and consortia products (Bradacova et al., 2019),

supporting the view that benefits of microbial consortia can be mainly expected under environmental stress conditions.

Table 6.4: Summary of tomato performance in large scale greenhouse production trials and open field culture with organic fertilizers and PGPM inoculation (Adopted from Bradacova et al., 2019; BIOFECTOR Project Report, 2017)

Country	Crops / Location	Bioeffectors	Main Fertilizers	Soil P levels mg kg^{-1}	Early growth response	BE. Yield response
Hungary	Open field Hungary 2015	Trianum P T22 Proradix FZB42	Manure-compost[1] Meat/bone meal	190 – 335 (CAL) pH 7.3	n.d	2.3 % 29.1 %* 70.5 %*
	2016	Trianum P T22 Proradix FZB42			n.d	17.1 %* 37.8 %* 45.8 %*
	2017	Trianum P T22 Proradix FZB42			n.d	21.0%* 38.0%* 26.4%*
Romania	Tomato Greenhouse Romania 2015	Penicillium PK112 Proradix FZB42+R41 Vitalin AM Proradix + Vitalin	Manure-compost[1] Manure	ca 700 (CAL) pH 7.1	22.6%* 30.8%* 49.4%*	96.5%* 102.5%* 77.1%* 59.9%* 73.7%*
	2016	Penicillium PK112 Proradix FZB42+R41 CRENEL FZB42	Manure – compost[1] Guano / feather meal	ca 700 (CAL) pH 7.1	27.5%* 33.0%* 28.7%* 31.4%* 30.0%*	64.1%* 75.9 %* 62.4 %* 39.5 %* 68.6 %*
	2017	Penicillium PK112 Proradix FZB42+R41 CRENEL	Manure-compost[1] Hair / feather meal	70 (soluble P fertilization) pH 6.2	45.5 %* 70.0 %* 41.0 %* 42.7 %*	52.0 %* 83.9 %* 70.8 %* 81.0 %*

[1] nursery fertilization; * significant difference to the non-inoculated control ($p < 0.05$)

The investigated fertilizers comprised substrates with composted manures in the nursery stage, and manure and guano/meat hair and feather-meal fertilizers in the main culture. However, in contrast to earlier studies showing promising PGPM effects with composted manures on low P soils (Li et al., 2017; Thonar et al, 2017; Mpanga et al., 2018; Vinci et al. 2018a,b), P availability on the investigated field soils was moderate or even extremely high (Tab. 6.4), suggesting that in this case mitigation of P limitation was not the reason for marked yield increase induced by PGPM inoculation. A common feature of the investigated fertilizers is a high proportion of easily

plant available N forms including ammonium, which may promote the rhizosphere establishment of the PGPMs in the protected nursery phase.

During later stages of plant development these vitally established PGPM populations in the rhizosphere may further increase the use efficiency of the organic fertilizers e.g. by stimulation of root growth as similarly demonstrated in the studies of Thonar et al., 2017 and Mpanga et al., 2018). Interactions with phytohormonal balances (see e.g. Fig. 6.3) may have an impact on flowering and fruit setting in tomato (Srivastava and Handa, 2005) but may also mitigate potentially phytotoxic effects in some cases reported for manure-based nursery substrates in tomato (Nielsen and Thrup-Kristensen, 2001; Tiquia et al., 1996) and increase the biotic and abiotic stress resistance of the host plants (Bradacova et al., 2019). In all cases, PGPM inoculation strongly contributed to yield stability and enabled tomato production according to the yield potential reported for production systems based on organic fertilization (Hornischer and Koller, 2005). However, so far the potential mechanisms behind the highly reproducible yield effects in tomato PGPM-assisted production are far from being clarified and require further investigations.

6.6 PGPMs market growth trend.

The use of PGPMS as biofertilizers has a long history and started with a first patent already in 1896 on Rhizobia used as seed inoculants to increase the atmospheric nitrogen fixation potential in leguminous plants (Hartmann et al., 2008). The estimated market at USD 668.47 million in 2016 and is expected to grow up to USD 1.39 billion by 2022 (Mordor Intelligence 2017). A similar trend was reported by (Grand View Research, 2018) based on market size by product, witnessing over 15 % than in the forecast period with very high small groups.

In both reports, the high desire to reduce soil contamination and other environmental hazards from excessive agrochemical use has been identified as a key driving factor for the biofertilizers market growth. Other expected effects comprise their eco-friendliness and improved soil and plant health. However, some constraints for the market are; high-demand and easy regulatory structures for synthetic fertilizers, and less awareness among farmers about the application and benefits coupled with low product efficacy under unfavourable conditions (Mordor Intelligence, 2017; Grand View Research, 2015). The reduction in the use of mineral fertilizers by PGPM-assisted production strategies would only be successful if biofertilizers are tested and proven to be efficaciously good and consistent in quality (Lesueur, et al., 2016), which still represents a major challenge for the sector.

6.7 Concluding remarks and open questions

This thesis clearly demonstrated that the selection of compatible combinations of fertilizers and PGPM inoculants is an essential factor for the successful establishment of beneficial plant-PGPM interactions in the rhizosphere. Combinations with stabilized ammonium fertilizers or with products based on organic waste recycling, such as composted manures have been identified as two promising examples with potential for PGPM-assisted production systems. However, although principal modes of action have been identified in model experiments and first field experiments and final patenting were conducted within the framework of the BIOFECTOR project, a range of open questions still remains to be clarified for the development of practical applications:

- Based on the obtained data it was possible to develop first hypotheses concerning critical factors, determining field performance of the novel PGPM-assisted fertilization

strategies. This requires further confirmation in more extended field studies under different production conditions.

- It is also essential to conduct additional investigations on the functional mechanisms not only in controlled environments but also under field conditions. This holds true particularly for PGPM combinations with the selected organic fertilizers, where the modes of action are still largely speculative. In this context, also the potential role of interactions with the soil microbiome has so far only been exemplarily investigated in first model experiments and requires further investigation.

- Also, the economic aspects of the proposed production systems require further consideration. Cost-benefit analyses conducted within the BIOFECTOR project revealed highly profitable scenarios particularly with horticultural crops, such as tomato with high economic value where efficient and product-saving inoculation strategies are possible in nursery culture with small-size culture vessels (BIOFECTOR Project Final Report 2017). By contrast, profitability was affected by the more challenging production conditions in agricultural crops, requiring field inoculation, and bearing a higher risk of interferences with environmental stress factors, resulting in smaller effects with higher variability (Fig. 6.8). In this context, the development of efficient and cost-saving inoculation techniques, which can be integrated into the existing management practice for field crops, is essential. The combined application of inoculants with fertilizers as described in section 4.3.3 and fertilizer placement strategies may be promising steps in this direction. Seed inoculation is a product-saving approach but resulted in lower efficiency with respect to PGPM root colonisation as compared with soil drenching (BIOFECTOR, Final Report, 2017). Also, the best timing and the required number of

PGPM applications during the culture period needs to be identified more clearly. Repeated PGPM applications by soil injection close to the plant roots, as performed in the model experiments, can be easily integrated into fertigation systems frequently used in horticulture but this is hardly possible in agricultural production. Since inoculation with high inoculum densities close to the roots seems to be essential (Bradacova et al., 2019), here usually only one single inoculation at the begin of the culture period is possible e.g. by combination with underfoot placement of fertilizers (Nkebiwe, 2016) or placement into the seeding furrow. By contrast, later drenching applications directly on the soil surface will hardly reach the growing root system.

- A more restrictive practice for registration of novel PGPMs and consortia products in the future is potentially related with attempts towards a legislative harmonization of the registration practice for biostimulants in the European Union. In face of the broad host specificity, the proposed strategies for PGPM application may offer alternative options for product development and optimization, by strategic combinations of suitable fertilizers with already registered PGPM strains instead of registration of novel isolates.

6.8 References

Abbasi, M.K., Musa, N., Manzoor, M. (2015): Mineralization of soluble P fertilizers and insoluble rock phosphate in response to phosphate-solubilizing bacteria and poultry manure and their effect on the growth and P utilization efficiency of chilli (Capsicum annuum L.). *Biogeosciences* 12, 4607–4619.

Ahmed A. (2017): Effects of micronutrients, form of nitrogen supply and selected bioeffectors on early growth of maize exposed to low root zone temperature. Master Thesis, University of Hohenheim, Stuttgart, Germany.

Ahmed, A., & Hasnain, S. (2010): Auxin-producing Bacillus sp.: Auxin quantification and effect on the growth of Solanum tuberosum. *Pure and Applied Chemistry*, *82*(1).

Amoo, A. E., & Babalola, O. O. (2017): Ammonia-oxidizing microorganisms: key players in the promotion of plant growth, *17*(December), 935–947. https://doi.org/10.4067/S0718-95162017000400008

Bradáčová K., Florea A.S., Bar-Tal A., Minz D., Yermiyahu U., Shawahna R., Kraut-Cohen J., Zolti A., Erel R., Dietel K., Weinmann M., Zimmermann B., Berger N., Ludewig U., Neumann G., Poşta G. (2019): Microbial consortia versus single-strain inoculants: an advantage in PGPR-assisted tomato production? Agronomy, 9(2), 105; https://doi.org/10.3390/agronomy9020105

Berg G, Smalla K. Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol Ecol, 2009, 68(1): 1-13

Bharucha, U., Patel, K., & Trivedi, U. B. (2013): Optimization of Indole Acetic Acid Production by Pseudomonas putida UB1 and its Effect as Plant Growth-Promoting Rhizobacteria on Mustard (Brassica nigra). *Agricultural Research*, *2*(3), 215–221. https://doi.org/10.1007/s40003-013-0065

Bittman S, Kowalenko CG, Hunt DE, Forge TA, Wu X (2006): Starter phosphorus and broadcast nutrients on corn with contrasting colonization by mycorrhizae. Agronomy J. 98: 394-401.

BIOFECTOR final report (2017): Resource Preservation by Application of BIOefFECTORs in European Crop Production (https://cordis.europa.eu/project/rcn/104445/reporting/en/ 20/05/2019).

Borris, R. (2015): Towards a New Generation of Commercial Microbial Disease Control and Plant Growth Promotion Products. In Principles of Plant-Microbe Interactions; Lugtenberg, B., Ed.; Springer International Publishing: Basel, Switzerland, pp. 329–337.

Chekanaia V, Chikowoa R, Vanlauwe B (2018): Response of common bean (Phaseolus vulgaris L.) to nitrogen, phosphorus and rhizobia inoculation across variable soils in Zimbabwe. Agriculture, Ecosystems and Environment 266: 167–173.

Chivenge, P., Vanlauwe, B., & Six, J. (2011): Does the combined application of organic and mineral nutrient sources influence maize productivity? A meta-analysis. *Plant and Soil*. https://doi.org/10.1007/s11104-010-0626-5

Eltlbany N, Baklawa M, Guochun D, Nassal D, Weber N, Kandeler E, Neumann G, Ludewig U, van Overbeek L, Smalla K (2019): Enhanced tomato plant growth in soil under reduced P supply through microbial inoculants and microbiome shifts. FEMS Microbiology Ecology, submitted.

Fan B, Borriss R, Bleiss W (2012): Gram-positive rhizobacterium Bacillus amyloliquefaciens FZB42 colonizes three types of plants in different patterns. J Microbiol, 50:38–44. doi: 634 10.1007/s12275-012-1439-4

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

Glick B R (2005): Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiology Letters banner, 251(1), 1-7.

Glick B R (2014): Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiological Research, 69 (1), 30-39.

Gharib, F. A., Moussa, L. A., & Massoud, O. N. (2008): Effect of compost and bio-fertilizers on growth, yield and n essential oil of sweet marjoram (Majorana hortensis) plant. International Journal of Agriculture and Biology, 10(4), 381–387. https://doi.org/10.1.1.335.6758

Gómez-Muñoz, B., Jensen, L. S., de Neergaard, A., Richardson, A. E., & Magid, J. (2018): Effects of Penicillium bilaii on maize growth are mediated by available phosphorus. *Plant and Soil*, *431*(1–2), 159–173. https://doi.org/10.1007/s11104-018-3756-9

Gulden, R.H.; Vessey, J.K. (2000): Penicillium bilaii inoculation increases root-hair production in field pea. Can. J. Plant Sci., 80, 801–804

Hartmann A, Schmid M, Wenzel W, Hinsinger P. (2004). Rhizosphere 2004—Perspectives and Challenges—A Tribute to Lorenz Hiltner. Book of Abstracts, GSF-Bericht, GSFNational Research Center for Environment and Health, Neuherberg. 333 p.

Hartmann, A.; Rothballer, M.; Schmid, M. (2008): Lorenz Hiltner, a pioneer in rhizosphere microbial ecology and soil bacteriology research. Plant Soil, 312, 7–14.

Hartmann A, Rothballer M, Hense BA and Schröder P (2014): Bacterial quorum sensing compounds are important modulators of microbe-plant interactions. Front. Plant Sci. 5:131. doi: 10.3389/fpls.2014.00131

Harman, G.E.; Howell, C.R.; Viterbo, A.; Chet, I.; Lorito, M.; Pathology, P (2004): Trichoderma Species—Opportunistic, Avirulent Plant Symbionts. Nat. Rev. Microbiol., 2, 43–56.

Harman G. E. (2000): Myths and dogmas of biocontrol. Changes in perceptions derived from research on Trichoderma harzianum T-22. Plant Dis., 84:377–393.

Hoffmann, C., E. Ladewig, N. Claassen, A. Jungk (1994): Phosphorus uptake of maize as affected

ammonium and nitrate nitrogen - measurements and model calculations. Z. Pflanzenernähr. Bodenkunde 157:225-232.

Hornischer, U.; Koller, M.(2005): Biologischer Anbau von Tomaten. Bioland Beratung GmbH Kompetenzzentrum Ökolandbau Niedersachsen FiBl/KÖN/Bioland, Germany. Available online: https://www.bioland.de/fileadmin/dateien/HP_Dokumente/Verlag/MB_Tomaten.pdf (accessed on 20 December 2018). https://doi.org/10.1371/journal.pone.0209664

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 Crops Res., 119, 355–364.

Kania, A., Guldner, M., Szabo, B., Kazem, s., Römheld, V., Neumann, G., Morhard, J., Evers, M., and Terlouw, T. (2007): Functional characterization of the stabilized organic turf grass fertilizer “Marathon”. Rasen –Turf – Gazon 1/2007: 192-195.

Kuhlmann, (2014): Microbial bio-effectors for the mobilization of calcium phosphates in the rhizosphere. Bachelor thesis. University of Hohenheim, Stuttgart, Germany.

Lapinskas, E. B. (2007): The Effect of Acidity on the Distribution and Symbiotic Efficiency of Rhizobia in Lithuanian Soils. *Eurasian Soil Science*, 40 (4) pp. 419–425.

Lekfeldt JDS, Rex M, Mercl F, Kulhánek M, Tlustoš P, Magid J, de Neergaard A (2016): Effect of bioeffectors and recycled P-fertiliser products on the growth of spring wheat. Chem. Biol. Technol. Agric. 3:22 DOI 10.1186/s40538-016-0074-4.

Li M., Cozzolino V., Mazzei P., Monda H., Drosos M., Piccolo A (2017): Effects of microbial bioeffectors and P amendments on P forms in a maize cropped soil as evaluated by 31P-NMR spectroscopy. Plant Soil DOI 10.1007/s11104-017-3405-8

Li Z, Zhang X, Zhao Y, Li Y, Zhang G, Peng Z,Zhang J. (2018): Enhancing auxin accumulation in maize root tips improves root growth and dwarfs plant height. Plant Biotechnol J, 16(1): 86-99

Marschner H. (1995): Rhizosphere pH effects on phosphorus nutrition. In: Johansen C, Lee KK, Sharma KK, Subbaro GV, Kueneman EA, eds. Genetic Manipulation of Crop Plants to Enhance Integrated Nutrient Management in Cropping Systems. 1. Phosphorus. Proceedings of an FAO/ICRISAT Expert Consultary Workshop. ICRISAT Asia Center, India. Patancheru, Andhra Pradesh, India: International Crops Research Institute for the Semi-Arid Tropics. pp 107-115

Menzies, N., Harbison, D., Dart, P. (2011): Soil chemistry-facts and fiction and their influence on the fertilizer decision making process. In Proceedings of the 26th Annual Conference of the Grassland Society of NSW, Bathurst, Australia, 26–28, pp. 49

Mpanga I K, Nkebiwe PM, Kuhlmann M, Cozzolino V, Piccolo A, Geistlinger J, Berger N, Ludewig U, Neumann G, (2019a): The Form of N Supply Determines Plant Growth Promotion by P-Solubilizing Microorganisms in Maize. *Microorganisms 2019, 7, 38. DOI: 10.3390/microorganisms7020038*

Mpanga I K, Gomez-Genao N J, Moradtalab N, Wanke D, Chrobaczek V, Ahmed A, Windisch S, Geistlinger J, Walker F, Ludewig U, Neumann G (2019b): The role of N form supply for PGPM-host plant interactions in maize. Journal of Plant Nutrition and Soil Sciences (submitted).

Mpanga, I K, Dapaah H K, Geistlinger J, Ludewig U, and Neumann G, (2018): Soil type-dependent interactions of P-solubilizing microorganisms with organic and inorganic fertilizers mediate plant growth promotion in tomato. *Agronomy 2018, 8(10), 213*. doi.org/10.3390/agronomy8100213.

Moradtalab N, Ahmed A, Geistlinger G, Walker F, Höglinger B, Ludewig U, Neumann G (2019): Synergistic contribution of PGPM, micronutrients, and ammonium to cold stress tolerance in maize plants by regulating phytohormone homeostasis and antioxidative defense. Submitted

Nielsen, K.L.; Thorup-Kristensen, K. (2001): Growing media for organic tomato plantlet production Archived at http://orgprints.org/00001606. Acta Hortic., 664, 183–188.

Neumann, G., & Römheld, V. (2007): Root exudates and nutrient cycling, in Nutrient cycling in Ecosystems, Soil Biology, Vol. 10, eds Marschner P., Rengel Z., editors. (Berlin; Heidelberg: Springer; 123–157.

Neumann, G., & Römheld, V. (2002): Root-induced changes in the availability of nutrients in the rhizosphere. *Books in Soils, Plants, and the Environment Series*, 617–649.

Nkebiwe, P.M., Neumann, G., Müller, T. (2017): Densely rooted rhizosphere hotspots induced around subsurface NH4+-fertilizer depots: a home for soil PGPMs? *Chem. Biol. Technol. Agric*. (2017) 4: 29.

Nkebiwe, P. M. (2016): Fertilizer placement and the potential for its combination with bio-effectors to improve crop nutrient acquisition and yield. Dissertation, Universität Hohenheim, Stuttgart, Germany.

Nkebiwe P M, Weinmann M, and 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. https://doi.org/10.1186/s40538-016-0065-5

Nuti, M.; Giovannetti, G (2015): Borderline Products between Bio-fertilizers/ Bio-effectors and Plant Protectants: The Role of Microbial Consortia. J. Agric. Sci. Technol. A, 5, 305–315.

Patten, C. L., & Glick, B. R. (2002): Role of Pseudomonas putida indoleacetic acid in development of the host plant root system. *Applied and Environmental Microbiology*, *68*(8), 3795–3801.

Patil, N. B., Gajbhiye, M., Ahiwale, S. S., Gunjal, A. B., & Kapadnis, B. P. (2011): Optimization of Indole 3 - acetic acid (IAA) production by Acetobacter diazotrophicus L1 isolated from Sugarcane. *International Journal of Environmental Sciences*, *2*(1), 295–302.

Prieto P, Schilirò E, Maldonado-González MM, Valderrama R, Barroso-Albarracín JB, Mercado-Blanco J (2011): "Root Hairs Play a Key Role in the Endophytic Colonization of Olive Roots by Pseudomonas Spp. with Biocontrol Activity." Microbial Ecology 62 (2): 435–45. doi:10.1007/s00248-011-9827-6.

Römheld, V. and Marschner, H. (1983): Mechanism of iron uptake by peanut plants. I. FeIII reduction, che-late splitting, and release of phenolics. *Plant Physiol.*, 71: 949–954.

Römheld V. (1986): pH changes in the rhizosphere of various crop plants in relation to the supply of plant nutrients. In: Potash Review 11: Berne, Switzerland: International Potash Institute.

Sharifi R., Ryu C. (2018): Revisiting bacterial volatile-mediated plant growth promotion: lessons from the past and objectives for the future, *Annals of Botany*, Volume 122,(3), 349–358.

Sekar, J.; Raj, R.; Prabavathy, V.R. (2016): Microbial Consortial Products for Sustainable Agriculture: Commercialization and Regulatory Issues in India. In: Agriculturally Important Microorganisms; Singh, H.B., Sarma, B.K., Keswani, C., Eds.; Springer Science+Business Media: Singapore,; pp. 107–131.

Sharma S B, Sayyed R Z, Trivedi M H, and Gobi T A (2013): Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. Springer Plus. 2(1), 587.

Schütz, L.; Gattinger, A.; Meier, M.; Muller, A.; Boller, T.; Mäder, P.; Mathimaran, N. (2017): Improving crop yield and nutrient use efficiency via biofertilization–A global meta-analysis.

Front. Plant Sci., 8. doi:10.3389/fpls.2017.02204

Sittinger M (2018): Effects of a microbial consortia product on the growth of maize (Zea mays). Master Thesis, University of Hohenheim, Stuttgart, Germany.

Srivastava, A.; Handa, A.K. (2005): Hormonal Regulation of Tomato Fruit Development: A Molecular Perspective. J. Plant Growth Regul., 24, 67–82.

Tadano T.; Sakai H. (1991): Secretion of acid phosphatase by the roots of several crop species under phosphorus-deficient conditions, Soil Science and Plant Nutrition, 37:1, 129-140, DOI: 10.1080/00380768.1991.10415018

Tiquia, S.M.; Tam, N.F.Y.; Hodgkiss, I.J. (1996): Effects of composting on phytotoxicity of spent pig-manure sawdust litter. Environ. Pollut., 93, 249–256.

Timsina, J. (2018): Can Organic Sources of Nutrients Increase Crop Yields to Meet Global Food Demand? *Agronomy*, *8*(10), 214. https://doi.org/10.3390/agronomy8100214

Thonar C, Lekfeldt JDS, Cozzolino V, Kundel D, Kulhánek M, Mosimann C, Neumann G, Piccolo A, Rex M, Symanczik S, Walder F, Weinmann M, de Neergaard A, Mäder P (2017): Potential of three microbial bio-effectors to promote maize growth and nutrient acquisition from alternative phosphorous fertilizers in contrasting soils Chemical and Biological Technologies in Agriculture 4:7 DOI 10.1186/s40538-017-0088-6

Van Oosten M J, Emilio Di Stasio, Valerio Cirillo, Silvia Silletti, Valeria Ventorino, Olimpia Pepe, Giampaolo Raimondi and Albino Maggio (2018): Root inoculation with Azotobacter chroococcum 76A enhances tomato plants adaptation to salt stress under low N conditions. *BMC Plant Biology*, 18:205.

Vejan, P., Abdullah, R., Khadiran, T., Ismail, S., & Nasrulhaq Boyce, A. (2016): Role of plant growth promoting rhizobacteria in agricultural sustainability-A review. Molecules, 21(5).

Vinci G., Cozzolino V., Mazzei P., Monda H., Spaccini R., Piccolo A. (2018): Effects of Bacillus amyloliquefaciens and organic and inorganic phosphate amendments on Maize plants as revealed by NMR and GC-MS based metabolomics. Plant Soil 429(10):1-14

Vinci G, Cozzolino V, Mazzei P, Monda H, Spaccini R, Piccolo A (2018): An alternative to mineral phosphorus fertilizers: The combined effects of Trichoderma harzianum and compost on Zea mays, as revealed by 1 H NMR and GC-MS metabolomics. PLoS ONE 13(12): e0209664.

Viscardi S, Ventorino V, Duran P , Maggio A, De Pascale S, Mora M L , Pepe O (2016): Assessment of plant growth promoting activities and abiotic stress tolerance of Azotobacter chroococcum strains for a potential use in sustainable agriculture. Journal of

Soil Science and Plant Nutrition, 2016, 16 (3), 848-863.

Wang S, Zhang S, Sun C, Xu Y, Chen Y, Yu C, Qian Q, Jiang D A,Qi Y. (2014): Auxin response factor (OsARF12), a novel regulator for phosphate homeostasis in rice (Oryza sativa). New Phytol, , 201(1): 91-103.

Windisch S, Bott S, Ohler MA, Mock H-P, Lippmann R, Grosch R, Smalla K, Ludewig U. Neumann G. (2017): Rhizoctonia solani and bacterial inoculants stimulate root exudation of antifungal compounds in lettuce in a soil-type specific manner. Agronomy 7: 44. doi:10.3390/agronomy7020044

Wu, W., & Ma, B. (2015): Integrated nutrient management (INM) for sustaining crop productivity and reducing environmental impact: A review. *Science of the Total Environment*.

Yao, H., Campbell, C. D., & Qiao, X. (2011): Soil pH controls nitrification and carbon substrate utilization more than urea or charcoal in some highly acidic soils. *Biology and Fertility of Soils*, *47*(5), 515–522.

Yusran Y, Römheld V, Müller T. (2007): Effects of Pseudomonas sp. (Proradix) and Bacillus amyloliquefaciens FZB42 on the establishment of AMF infection, nutrient acquisition and growth of tomato affected by Fusarium oxysporum Schlecht f.sp. radicis-lycopersici Jarvis and Shoemaker. UC Davis The Proceedings of the International Plant Nutrition Colloquium. XVI. https://escholarship.org/uc/item/8g70p0zt.

Zhang, L. M., Hu, H. W., Shen, J. P., & He, J. Z. (2012): Ammonia-oxidizing archaea have more important role than ammonia-oxidizing bacteria in ammonia oxidation of strongly acidic soils. *ISME Journal*, *6*(5), 1032–1045. https://doi.org/10.1038/ismej.2011.168

7 APPENDIX

COMBINED EFFECTS OF PGPMs AND DIFFERENT N FORMS ON P ACQUISITION IN MAIZE UNDER FIELD CONDITIONS.

Introduction

Ammonium-PGPM synergisms for improved acquisition of sparingly soluble P sources have been demonstrated in various pot experiments with different crops, such as maize, wheat, tomato as described in chapter 4. This study aimed to investigate the potential of the proposed fertilization strategy in under field conditions in a silo maize production system on soil with moderate P availability and Rock-P, as well as sewage sludge ash as sparingly soluble P sources.

Materials and methods

Trial location and soil: This om-farm triall was conducted in Germany in 72160 Horb am Neckar; 48°27'19.7"N 8°42'42.8"E. The soil was a silty loam, pH ($CaCl_2$) 5.9, P_{CAL} 52.3 mg P kg^{-1} soil. Pre-crop was wheat followed by catch crops with a seed mixture of vetch, phacelia, sunflower, buckwheat, ramtilla herb and fertilized with 15-20 m^3 manure ha^{-1}.

Experimental treatments and fertilization: below are the treatments and their descriptions

1. Zero: Only DAP underfoot placement at sowing.
2. Standard (Std): Fertilizer applications according to the farmer's practice, no bio-effector treatments (60 kg N ha^{-1} as liquid cow manure and 70 kg N ha^{-1} as urea)
3. NO_3: 60 kg N ha^{-1} before sowing as calcium nitrate (Calcinit, Yara, 15.5 % N, Yara, Oslo, Norway) and 70 kg N ha^{-1} at about 5 leaves stage.
4. NH_4: Novatec solub21 (COMPO, 21 % N, COMPO Expert GmbH, Krefeld, Germany) before sowing

5. **RP + NO_3:** Rock P (Granuphos 7.85 % P, Landor, Birsfelden, Switzerland) plus nitrate
6. **RP + NO_3 + Px:** Rock P (RP) plus nitrate plus Proradix® WP (Px).
7. **RP + NO_3 + CF A:** RP plus nitrate plus CombiFector A (CFA).
8. **RP + NH_4:** Rock P (RP) plus ammonium
9. **RP + NH_4 + Px:** Rock P (RP) plus ammonium plus Proradix® WP (Px).
10. **RP + NH_4 + CF A:** RP plus ammonium plus CFA.
11. **Positive Ctrl. + NO_3^-:** single super phosphate (Triferto, 18 % P_2O_5, Doetinchem, Netherland) plus nitrate, no bio-effector.
12. **Positive Ctrl. + NH_4:** single supper phosphate plus nitrate, no bio-effector.
13. **SSA + NH_4:** Sewage sludge ash (SSA) with 7.63 % P plus ammonium, no bio-effector.
14. **SSA + NH_4 + Px:** Sewage sludge ash (SSA) with 7.63 % P plus ammonium plus Px.

All fertilizers were broadcasted and incorporated into the soil with underfoot placement of N (21.6 kg N ha^{-1}) and P (ca. 24 kg P ha^{-1}) as di-ammonium phosphate (18 % N, 46 % P2O5 ≙ 20 % P) at sowing, 5 cm x 5 cm beside the seeding row. Total N and P fertilization for all treatments were 150 N kg ha-1 and 130 kg P ha^{-1}.

Maize variety and sowing date: Jessy, S 230 (Silo- und Energiemais, ADVANTA, Edemissen, Germany) was planted on April 28th, 2016 at a density of Kernels m^{-2} with row distance of 75 cm, 6 m sowing machine and depth of 6 cm.

Tested bio-effector products: 1) Proradix® WP, (SOURCON PADENA, Tübingen, Germany), active ingredient: *Pseudomonas* sp. DSMZ 13134 (6.6 * 10^{10} colony forming units g^{-1}). 2) Combifector A is a consortium product of *Trichoderma harzianum* OMG15 with 5 bacillus strains (Bactiva GmbH, Strelen Germany) and supplementation of micronutrients (Zn, Mn) (Institute of Bioanalytical Sciences (IBAS), Bernburg, Germany.

Soil and plant sampling and mineral analysis: Soil samples were collected from five random spots at 15 cm depth in each plot, mixed together and subsampled for N min, P CAL analysis. For the Shoot mineral analysis, maize leaves adjacent the uppermost ear were sampled at tasselling stage randomly from each plot according to the recommendation by (Campbell, 2000; Mills and Jones 1996). Soil N_{min} and P $_{CAL}$ analysis were done according to the Association of German Agricultural Research and Research Institutes (VDLUFA) instructions for soil and plant shoot analysis protocols (VDLUFA, 1991). Shoot minerals (N, P, K, Mg, Ca) were done with the same procedure and instrumentation as reported by (Mpanga et al., 2018).

Experimental design: Randomized block design with 5 repetitions, plot size of 6 m width by 7 m length with 8 rows by 75 cm row distance (Fig. 7.1). The data analysis was done in SAS with Tukey test at p=0.05.

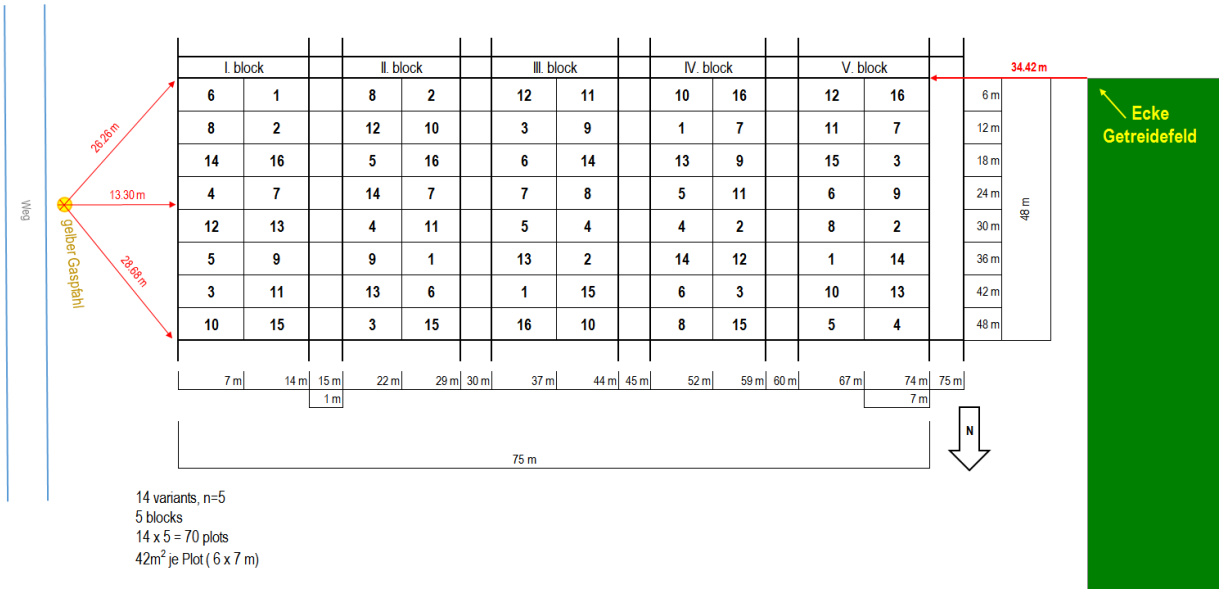


Figure 7.1 Experimental plan

Results and Discussion

There were no significant differences among all the treatments in terms of final above ground dry biomass and P, K, Mg and Ca tissue concentrations recorded in the tasselling stage, without any effects of the PGPMs. No differences in the P status of the plants existed even between the

zero control without fertilization and the positive controls with soluble P standard fertilization (Table 7.2).

Table 7.2: Above ground dry biomass at final harvest and mineral macronutrient status (tasselling stage mid of July) of field silage maize inoculated with PGPMs and different N-forms and Rock P, sewage sludge ash (SSP) (SSA) or single super phosphate as P sources.

	Visual Evaluation	Dry biomass (tons ha^{-1})	N (mg g^{-1})	P (mg g^{-1})	K (mg g^{-1})	Ca (mg g^{-1})	Mg (mg g^{-1})
No fertilization (Zero)	6.9 ab	22.6 a	29.2 c	2.9 a	31 a	35.2 a	74.9
Cattle slurry + Urea (Std)	7.2 ab	25.9 a	31.5 bc	3.6 a	32 a	34.9 a	68.0
No P_NO3	6.5 b	22.5 a	32.5 abc	2.6 a	30 a	34.7 a	72.7
RP_NO3	7.1 ab	23.0 a	31.7 bc	2.6 a	33 a	33.2 a	72.9
RP_NO3_Px	7 ab	22.9 a	31.8 bc	2.5 a	30 a	32.5 a	74.0
RP_NO3_CFA	6.9 ab	24.0 a	32.2 abc	2.6 a	32 a	32.4 a	74.2
SSP_NO3 (Positive control)	7.3 ab	23.3 a	33.7 ab	2.8 a	31 a	34.7 a	73.2
No P_NH4	7.5 ab	25.0 a	32.0 abc	3.0 a	31 a	34.7 a	73.2
RP_NH4	7.3 ab	26.1 a	33.9 ab	2.6 a	31 a	30.0 ab	64.1
RP_NH4_Px	8 ab	25.3 a	35.0 a	2.8 a	30 a	34.7 a	75.8
RP_NH4_CFA	7.6 ab	25.2 a	33.2 ab	2.7 a	29 a	33.4 a	65.2
SSA_NH4	7.4 ab	24.5 a	33.7 ab	2.8 a	30 a	33.8 a	73.0
SSA_NH4_Px	7.4 ab	24.0 a	33.6 ab	2.7 a	30 a	32.8 a	76.5
SSP_NH4 (Positive control)	8.1 a	24.5 a	34.4 ab	2.8 a	30 a	32.3 a	70. 0

Standard (Std) is farmer's practice, No Phosphorus (No P), Rock Phosphate (RP), Pseudomonas strain of bacteria (Px), CombiFectorA (CFA), Positive control with single super phosphate and nitrate (SSP), Nitrate (NO3), ammonium (NH4), Sewage sludge ash (SSA). (Same letters, no difference, SAS, Tukey test, n=5 at 0.05)

On the selected field site with moderate P availability (P_{CAL} 52 mg kg^{-1}; Class C: sufficient), obviously P was not a growth-limiting nutrient and accordingly, even soluble P fertilization (SP) resulted in a non-significant increase of biomass yield only by 3% as compared with the N fertilized controls without P supply. Therefore, larger BE effects could not be expected in this case.

Interestingly, in the Rock-P variants with stabilized ammonium fertilization, BE treatments increased the soil N_{min} concentrations (Fig. 7.3B) recorded by the mid of July 2018. This effect could not be attributed to reduced N leaching losses due to ammonium adsorption since it was not observed in the non-inoculated control. The results rather suggest an effect of the BE inoculants on N mineralization by interactions with the indigenous microflora. It also shows

that the DMPP-stabilized ammonium fertilization was still effective under field conditions at 12 weeks after sowing, demonstrating high stability of the nitrification inhibitor.

Stabilized ammonium fertilization with SSA showed a very clear increase of PCAL in the rhizosphere similar to the positive control with soluble P fertilization but different from the rock P treatments, suggesting a high potential for ammonium-induced P solubilisation with SSA as a P source, reaching the effect of soluble P application.

However, although SSA under ammonium fertilization and the positive controls under both N-forms revealed significant differences over the rock P and no P variants, this did not translate into increased shoot growth and P concentration and may be explained to non-limiting P in this field (Figure 7. 3A).

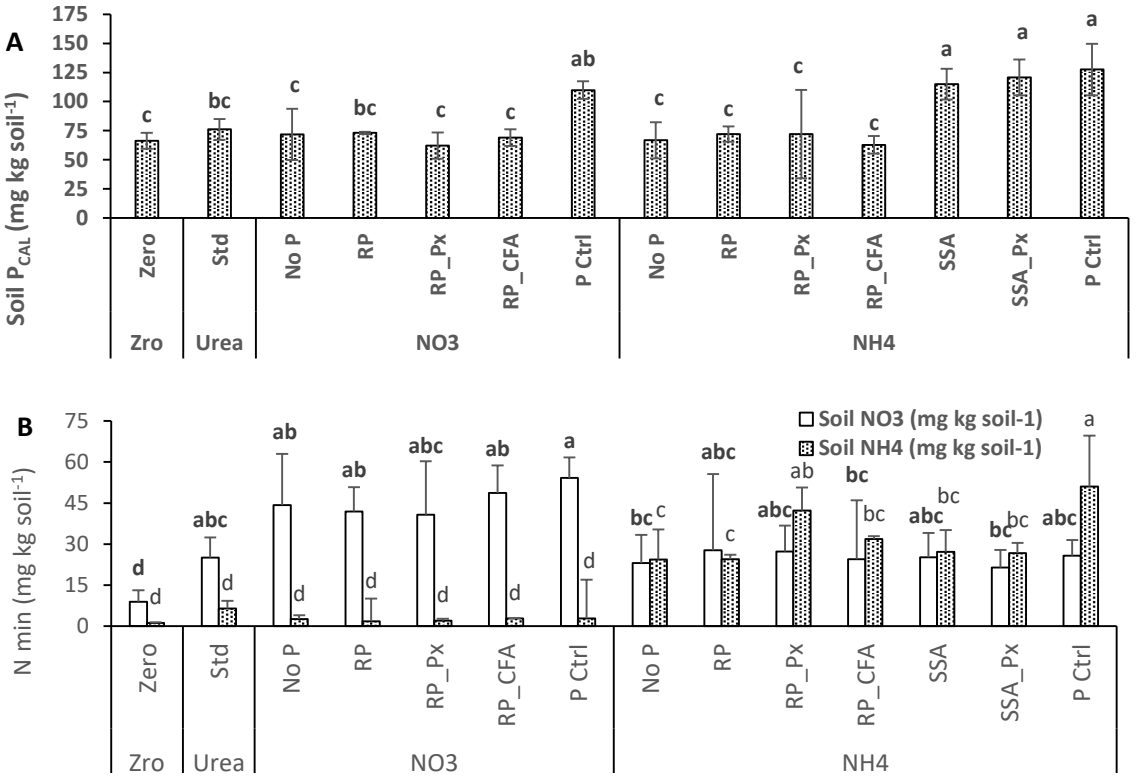


Figure 7.3: Effects of N-form combinations with PGPM on soil P_{CAL} (A) and N_{min} (B) under field conditions with different P sources. Standard (Std), No Phosphorus (No P), Rock Phosphate (RP), Pseudomonas strain of bacteria (Px), CombifectorA (CFA), Positive control with single super phosphate and nitrate (P Ctrl), Sewage sludge ash (SSA). Sampling conducted at 12 weeks after sowing. (Same letters, no difference, SAS, Tukey test, n=5 at 0.05).

References

Campbell, R.C. (2009). Reference sufficiency ranges for plant analysis in the southern region of

the United States. In Southern Cooperative Series Bulletin #394; North Carolina Department of Agriculture and Consumer Services Agronomic Division: NC, USA; p. 79.

Mills HA, Jones JB Jr. 1996. Plant analysis handbook II: a practical sampling, preparation, analysis, and interpretation guide. Athens (GA): Micro-Macro Publishing.

Mpanga IK, Nkebiwe PM, Kuhlmann M, Cozzolino V, Piccolo A, Geistlinger J, Berger N, Ludewig U and Neumann G, (2019). N-form dependent solubilization of rock-phosphate in maize inoculated with microbial bioeffectors. Master thesis, University of Hohenheim, Germany.

Mpanga, I., Dapaah, H., Geistlinger, J., Ludewig, U., Neumann, G., Mpanga, I. K., … Neumann, G. (2018). Soil Type-Dependent Interactions of P-solubilizing Microorganisms with Organic and Inorganic Fertilizers Mediate Plant Growth Promotion in Tomato. *Agronomy*, *8*(10), 213. https://doi.org/10.3390/agronomy8100213

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.

Kar S. (2016). Effects of BE and N-form on P nutrition and growth of maize plants supplied with different sparingly soluble inorganic/recycled P fertilizers. Master thesis, University of Hohenheim, Germany.

VDLUFA (Verband Deutscher Landwirtschaftlicher Untersuchungs-und Forschungsanstalten e.V. Speyer, Germany) (1991). Handbuch der Landwirtschaftlichen Versuchs- und Untersuchungsmethodik Methodenbuch Band I Die Untersuchung von Böden, 4th ed.; VDLUFA Verlag: Darmsatdt, Germany.

8 CURRICULUM VITAE

Isaac Kwadwo Mpanga

Summary

Soil and Plant Sciences specialist (Agronomist) with excellent teaching pedagogy experiences and data management, analysis and interpretations with SAS, manuscripts writing and presentations.

Office address

University of Hohenheim
Institute of crop sciences (340h)
Fruwirthstrasse 20, 70599 Stuttgart, Germany

Personal data

Date of Birth: 21-09-1981 Place of Birth: Atebubu Marital Status: Married
Tel: +4915217489408 Email: mpangaisaac75@gmail.com

Education

04 - 2016 to 11-2019	**Crop Sciences (PhD)** Institute of Crop Sciences (340h) University of Hohenheim, Germany **Dissertation: Fertilization Strategies to Improve the Plant Growth-Promoting Potential of Microbial Bio-Effectors.**
10 - 2013 to 12 - 2015	**Crop sciences (MSc) (GPA 3.85 out of 4.0)** Institute of Crop Sciences (340h) University of Hohenheim, Germany
08 - 2008 to 07 - 2012	**Agriculture EDU. (BSc) (GPA 3.45 out of 4.0)** College of Agriculture The University of Edu. Winneba, Ghana
10 - 2000 to 06 - 2003	**Education and Teaching (3-Year Post Secondary)** Dambai Teacher Training College University of Cape Coast, Ghana
08-1996 to 06 - 1999	**Kete-Krachi Secondary Technical School, Ghana** General Agriculture with Mathematics, English & Integrated Science (Physics, Chemistry, Biology and Computer Science).
08 - 1993 to 06 - 1996	**Borae No2. Junior Secondary School, Ghana** General Education with Mathematics, English and Sciences

Work Experience	
12-2019 to Present	Faculty (Area Associate Agent) at University of Arizona, USA Research and Extension programing for Commercial Horticulture and Small Acreage in Yavapai and Coconino.
04 -2016 to 03 -2019	Research Fellow at University of Hohenheim, Germany • Field, greenhouse, lab trials • Data analysis, results interpretation and reporting • Manuscripts writing and publication in journals • Research funds Proposals writing and supervision • Collaboration with Farmers and industry
01 - 2016 to 03 - 2016 11 - 2017 to 02 - 2017	Teaching Internship and Research Stay at Uni. of Energy and Natural Resources, Ghana • Taught, supervised and assessed BSc students in Fundamentals of Horticulture • Conducted experiments in greenhouse and lab • Report writing and accounting
09 - 2003 to 08 - 2013	Teacher at Ghana Education Service, Ghana • Taught and managed students at all levels • Examined and assessed student performance • Planned and Supervised all extra curriculum activities
06 - 2011 to 12 - 2011	Extension intern at Ghana Cocoa board, Ghana • Compiled regional cocoa data for grading activities • Regional Storekeeper

Awards: Field research fund. Awarded by FIAT PANIS through Food Security Centre, University of Hohenheim, Germany

Skills: Teaching Pedagogy, Research Methods, writing and speaking, and Computer Skills such as Microsoft suite, SAS, Sigma plot, Social media handles, Mendley referencing tool.

9 AFFIDAVIT

Affidavit pursuant to Sec. 8(2) of the University of Hohenheim's doctoral degree regulations for Dr.sc.agr.

1. For the dissertation submitted on the topic "**Fertilization Strategies to Improve the Plant Growth-Promoting Potential of Microbial bio-Effectors"** I hereby declare that I independently completed the work.

2. I only used the sources and aids documented and only made use of permissible assistance by third parties. In particular, I properly documented any contents which I used - either by directly quoting or paraphrasing - from other works.

3. I did not accept any assistance from a commercial doctoral agency or consulting firm.

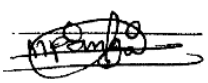
4. I am aware of the meaning of this affidavit and the criminal penalties of an incorrect or incomplete affidavit.

I hereby confirm the correctness of the above declaration. I hereby affirm in lieu of oath that I have, to the best of my knowledge, declared nothing but the truth and have not omitted any information.

12/22/2019, Cottonwood, Az, USA

…….

Date and Place



……

Signature

10 ACKNOWLEDGEMENTS

To God be the glory and praise for the victories in all my endeavor of life with the gift of wisdom.

To my supervisor (Prof. Dr Gunter Neumann), I pour out my deepest appreciation for your mentorship, guidance and training. Your support will forever be remembered in special ways. I also thank all other supervisors and colleagues at the Institute of Crop Sciences (340h) and the University of Hohenheim for any form of contribution that has brought me to this level.

Prof. Dr Harrison Kwame Dapaah (Vice Chancellor, University of Energy and Natural Resources, Ghana), your act of kindness initiated to support my master's degree in Germany has yielded a PhD thesis today and you will always be celebrated for this great achievement. You have been a great mentor and inspiration and all I wish for you and your family is excellence in all areas of your life.

I thank my dear wife (Sabrina Ava Campbell who is currently at the United States Air Force Basic Military Training Academy) and my family (the Mpanga family at Borae No2, Ghana) for their love, care, support, understanding and encouragement in all cases especially my mother (Grace Ndombe), father (Mpanga Bignabil), my siblings (Pastor Daniel, Veronica, Agnes, Abena and Mansa), my nieces and nephews. I know I did not have time for you for many years now because of this journey but you still standing right by my side in all and I love you all for your unconditional love. To the Pentecost International Worship Center (PIWC) in Stuttgart, Germany and in Sunyani, Ghana, thank you for your prayer support and spiritual empowerment in the Kingdom of God.

Finally, to my friends that I still have, because you tolerated my absence on this journey, I thank you for your encouragement and all forms of support. Hopefully, I can catch up with the loss times for us to build together.