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The functional role of phosphorus-mobilizing bacteria in the rhizosphere of tomato and maize

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1 Summary

Phosphorus (P) is an essential plant nutrient. Its sufficient availability in soil is a prerequisite for successful plant production. However, global P reserves are being increasingly exploited and surplus P applied by P fertilization is steadily accumulating in the form of plant-unavailable P compounds in arable soils. Future plant production will therefore require a more effective and sustainable P fertilization regime. One promising approach is the use of phosphorus-mobilizing bacteria (PMB), which are able to mobilize P in soil through mineralization or solubilization so effectively that plant P supply is improved. Increases in plant growth and P uptake by the addition of PMB have been reported, but the heterogeneity of PMB's effectiveness when tested under a wide range of environmental conditions has revealed a lack of understanding of PMB's functional mechanisms in soils and plants. In addition, potential additional plant growth-promoting attributes of PMB, such as secretion of phytohormones or interactions with indigenous soil microbes, make it difficult to distinguish P mobilization-dependent and -independent mechanisms. However, an understanding of PMB's functional mechanisms is necessary to evaluate both the potential and limitations of their use as well as to develop practical application recommendations. This thesis aimed to provide a better understanding of PMB's functional mechanisms in soil; the foci here were mechanisms and interactions of P mineralization with indigenous soil microorganisms. We aimed to identify P mineralization-dependent and -independent as well as direct and indirect mechanisms of PMB on soil and plants. To this end, three rhizobox experiments were performed in the greenhouse using tomato and maize as the test plants and *Pseudomonas* sp. RU47 (RU47) as the PMB. To identify effective P mineralization beyond the level of endogenous microbial activity, a treatment using unselectively cultivated soil bacteria for inoculation was included. Furthermore, the addition of devitalized RU47 cells provided the opportunity to identify indirect mechanisms. In all three rhizobox experiments the activities of acid and alkaline phosphomonoesterases in rhizosphere and bulk soil were determined, as the latter could be clearly identified as being of microbial origin. Effects on microbial community structure in soil were estimated by denaturing gradient gel electrophoresis (DGGE) and/or phospholipid fatty acid analysis. For deeper investigations of potential effects on microbial population composition and possible dependencies on soil conditions, a fourth experiment was performed using maize, three different *Pseudomonas* strains possessing PMB abilities, and three different soils varying in parameters which included organic C, pH, and P content. Microbiome shifts in soil were quantitatively determined via quantitative PCR using domain- (bacteria, archaea, fungi) and six bacterial phylum-specific primers.

Our experiments showed that tomato plants grown under low P availability soil conditions improved in both growth and P uptake when viable RU47 cells were added. This effect was accompanied by increased alkaline phosphatase activity (PA) in the rhizosphere. We also observed plant growth-promotion effects and a trend of increased PA by the addition of dead RU47 cells. Based on DGGE results, which indicated the promotion of indigenous rhizobacteria, we assume a priming effect induced by the addition of C sources in the form of bacterial residues (dead RU47), which resulted in increased indigenous microbial activity in the rhizosphere. In each rhizobox experiment viable RU47 cells were able to colonize the rhizosphere at high abundances, persisting up to 50 days after sowing. We found indications of phytohormonal influences with the addition of both viable and dead RU47 cells, but this was more pronounced in dead than in viable RU47 treatments. Increasing P availability in soil by mineral P fertilization seemed to improve RU47's ability to colonize and persist, which was shown by an increased RU47 abundance in both rhizosphere and bulk soils. However, despite an observable slight tendency, strengthened plant growth-promotion that positively correlated with improved RU47 abundance in the rhizosphere could not be detected. In general, colonization by viable RU47 cells did not significantly affect microbial community structure, either in the rhizosphere or in bulk soil. Using three different PMB strains, including RU47, in three contrasting soils, inoculation effects on the microbial community occurred heterogeneously, differing between the strains, soils, and time. Changes at the domain level were due primarily to nutrient availability in the soil, which differed between the soils and over time. Individual shifts in microbial community structure occurred more frequently in the rhizosphere than in bulk soil, but colonizing PMB neither increased bacterial abundance in rhizosphere bacteria, nor displaced copiotrophic rhizobacteria (indicative of C competition).

In conclusion, this thesis demonstrated that various PMB mechanisms involved in plant growth and P uptake enhancement run in parallel. P mineralization-dependent and -independent as well as direct and indirect mechanisms are overlapping; the dominant mechanism seems to depend on existing environmental conditions. PMB are able to mineralize P effectively from soil, and this offers a promising approach for their use in exploiting organic soil P for plant nutrition. Despite highly abundant colonization, however, effects of viable PMB on indigenous soil microbial community structure are low and temporary, implying low ecological risks to their use with respect to microbial diversity in arable soils.

2 Zusammenfassung

Phosphor (P) ist ein essentieller Pflanzennährstoff, dessen ausreichende Verfügbarkeit im Boden mitentscheidend für eine ertragreiche Pflanzenproduktion ist. Die zunehmende Verknappung der globalen P-Ressourcen sowie die steigende P-Anreicherung, in Form von für die Pflanze nicht nutzbaren P-Verbindungen, in agrarwirtschaftlich genutzten Böden zeigen die Dringlichkeit nach einer effizienteren und nachhaltigeren P-Düngung in der zukünftigen Pflanzenproduktion. Einen vielversprechenden Ansatz bieten hierbei Phosphor-mobilisierende Bakterien (PMB). PMB sind in der Lage mittels Mineralisation und Demineralisation P im Boden effektiv zu mobilisieren und für die pflanzliche Aufnahme verfügbar zu machen. Positive Pflanzeneffekte durch den Einsatz von PMB konnten bereits mehrfach nachgewiesen werden. Dennoch, das noch unzureichende Wissen über die Wirkmechanismen von PMB auf Boden und Pflanze führt zu unterschiedlichen Ergebnissen bei abweichenden Versuchs- und Anbaubedingungen. Potentielle, das Pflanzenwachstum beeinflussende Eigenschaften (z.B. Phytohormonsekretion oder mikrobielle Interaktionen) erschweren zusätzlich eine Differenzierung von P-mobilisationsabhängigen und -unabhängigen Mechanismen. Das Verständnis über die Funktionsmechanismen von PMB ist jedoch grundlegend, um die Möglichkeiten und Grenzen ihres praktischen Einsatzes abschätzen und standortangepasste Anwendungsempfehlungen entwickeln zu können. Ziel dieser Arbeit war es, zu einem besseren Verständnis der Funktionsmechanismen von PMB im Boden beizutragen. Die Schwerpunkte lagen hierbei sowohl auf den Mechanismen der P-Mineralisation als auch auf bodenmikrobielle Interaktionen. Angestrebt wurde die Identifizierung von P-mineralisationsabhängigen und -unabhängigen sowie direkten und indirekten PMB-Mechanismen. Es wurden drei Wurzelkastenversuche unter Verwendung von Tomate und Mais als Testpflanzen und *Pseudomonas* sp. RU47 (RU47) als PMB-Stamm durchgeführt. Zur Identifizierung einer über die endogene mikrobielle Bodenaktivität hinausgehenden P-Mineralisation wurde jeweils eine Behandlungsgruppe mitgeführt, in der undifferenzierte Bodenbakterien für die Inokulation verwendet wurden. Die Unterscheidung von direkten und indirekten Mechanismen erfolgte durch die Applikation von abgetöteten RU47-Zellen in einer weiteren Behandlungsgruppe. Es wurde jeweils die saure und alkalische Phosphomonoesteraseaktivität (PA) in Rhizosphäre und Umgebungsboden gemessen, wobei die alkalische PA einem mikrobiellen Ursprung zugewiesen werden kann. Die Untersuchung der Auswirkungen auf die mikrobielle Bodengemeinschaft wurde mittels der denaturierenden Gradienten-Gelelektrophorese (DGGE) und/oder der Phospholipidfettsäuren-Analyse durchgeführt. Für genauere Untersuchungen hinsichtlich möglicher Effekte auf die mikrobielle Bodengemeinschaft sowie deren Beeinflussung durch

unterschiedliche Bodeneigenschaften wurde ein viertes Experiment unter Verwendung von Mais als Versuchspflanze durchgeführt. Dieser Versuch enthielt drei *Pseudomonas* Stämme mit PMB-Eigenschaften sowie drei in ihren Bodeneigenschaften (z.B. organischer Kohlenstoff (C)-Gehalt, P-Gehalt, pH-Wert) unterschiedliche Böden. Effekte auf die mikrobielle Bodengemeinschaft wurden quantitativ mittels qPCR unter Verwendung von Domain- (Bakterien, Archaeen, Pilze) und sechs Bakterienphylum-spezifischer Primerpaaren bestimmt.

Mit lebenden RU47-Zellen behandelte Tomatenpflanzen zeigten unter P-limitierten Bodenbedingungen eine Verbesserung im Pflanzenwachstum und der P-Aufnahme. Gleichzeitig wurde eine Erhöhung der alkalischen PA in der Rhizosphäre nachgewiesen. Ein pflanzenwachstumsverbessernder Effekt sowie eine erhöhte PA konnte auch bei der Verwendung abgetöteter RU47-Zellen beobachtet werden. An Hand der DGGE-Ergebnisse konnte hier auf eine Förderung endogener Rhizobakterien, vermutlich durch von Zugabe von leicht verfügbarem C in Form von bakteriellen Zellrückständen, geschlossen werden, welche die mikrobielle Aktivität in der Rhizosphäre erhöhte („Priming-Effekt“). Lebende RU47-Zellen zeigten in allen Wurzelkastenversuchen eine hohe Rhizosphären-Kompetenz; hoch abundant nachweisbar bis zu 50 Tage nach Aussaat. Es wurden Hinweise auf phytohormonelle Effekte in beiden RU47-Behandlungsgruppen gefunden, diese waren jedoch stärker ausgeprägt in der Gruppe, in der tote RU47-Zellen verwendet wurden. Bei erhöhter P-Verfügbarkeit im Boden (P-Düngung) konnte sowohl in der Rhizosphäre als auch im Umgebungsboden eine erhöhte RU47-Abundanz beobachtet werden. Trotz beobachteter Tendenzen, ein positiv mit der RU47 Rhizosphären-Abundanz korrelierender, pflanzenwachstumsverbessernder Effekt wurde nicht nachgewiesen. Die Besiedlung lebender RU47-Zellen führte weder in der Rhizosphäre noch im Umgebungsboden zu einer Veränderung der mikrobiellen Bodengemeinschaft. Die Verwendung drei verschiedener PMB-Stämme, darunter auch RU47, in drei verschiedenen Böden zeigte einzelne Inokulationseffekte auf die mikrobielle Gemeinschaft, welche jedoch stark zwischen den Stämmen, Böden und der Zeit variierten. Änderungen auf Domainniveau folgten vorwiegend der Nährstoffverfügbarkeit im Boden und variierten somit zwischen den Böden und Probezeitpunkten. Einzelne Inokulationseffekte auf die mikrobielle Bodengemeinschaft wurden häufiger in der Rhizosphäre als im Umgebungsboden beobachtet. Trotz hoher Rhizosphären-Kompetenz wurde weder ein Anstieg der Rhizobakterien-Abundanz noch eine Verdrängung copiotropher Rhizobakterien (C-Konkurrenz) dokumentiert.

Zusammenfassend demonstriert diese Arbeit, dass verschiedene, das Pflanzenwachstum und die P-Versorgung beeinflussende PMB-Mechanismen parallel ablaufen. P-

mineralisationsabhängige und -unabhängige sowie direkte und indirekte Mechanismen verlaufen überlappend; die Ausprägung als dominanter Mechanismus scheint hierbei abhängig von den vorliegenden Umweltbedingungen zu sein. PMB sind in der Lage P effektiv im Boden zu mineralisieren und bieten damit einen vielversprechenden Ansatz organische P-Quellen im Boden für die Pflanzenernährung nutzbar zu machen. Trotz hoher Rhizosphären-Kompetenz beeinflussen PMB die mikrobielle Bodengemeinschaft nur gering und temporär, was geringe ökologische Risiken hinsichtlich der mikrobiellen Diversität im Boden bei ihrer praktischen Nutzung birgt.

3 General introduction

3.1 The looming phosphorus crisis

Phosphorus (P) is worldwide the second most commonly used plant nutrient in agriculture after nitrogen (N). P plays a key role in nearly all plant metabolic processes, including photosynthesis, respiration, and energy transfer (Khan *et al.* 2010, Sharma *et al.* 2013). Thus, an adequate supply of P in soil is required for high plant productivity. Due to the rapidly growing world population and increasing demand for food and forage crops, the consumption of P fertilizers has more than quadrupled during the last century (FAOSTAT 2014). This trend continues to rise, as the global population is expected to reach 9.7 billion people by the year 2050 (Newbold 2017). However, P is a non-renewable element that is sourced primarily from the phosphatic mineral apatite, found in sedimentary and igneous ores (Fixen and Johnston 2012, Pufahl and Groat 2017). Nearly all commercial phosphate fertilizers are based on phosphate rock, deposits of which are located mainly in the USA, China, Russia, and North Africa, and are increasingly exploited (Van Kauwenbergh 2010). Although phosphate rock is also used for various products in manufacturing industries (e.g. flame retardants, plasticisers, and batteries) its use as fertilizer accounts for more than 80 % of the total (Cordell and White 2011, Withers *et al.* 2015 a). Current global phosphate rock reserves are estimated by the U.S. Geological Survey (2017) at ca. 68,000,000 kt. Based on current consumption, these reserves will run out in 200-300 years at the latest (Pufahl and Groat 2017). However, this time period shortens considerably under the scenario of a continuing rise in P consumption, already posing a challenge to future agriculture. This situation is exacerbated by the unbalanced P cycle in soil. Although in most agricultural soils a large quantity of P has accumulated as a consequence of intensive P fertilization, only 0.1 % of the total P is present in a soluble form available for root uptake (Zhou *et al.* 1992, Richardson 2001). Orthophosphates originating from chemical P fertilizers are rapidly adsorbed to soil mineral surfaces, precipitated by free trivalent aluminium (Al) and iron (Fe) in soil solution (Havlin *et al.* 1999, Sharma *et al.* 2013), or immobilized in soil organic matter (Richardson 2001, Richardson and Simpson 2011). In addition, a decreasing response in crop yield with increasing soil P status has been observed (SCOPE 1995, SCOPE 2014), making the application of chemical P fertilizers, especially in well-fertilized European soils, increasingly inefficient and uneconomical (Schoumans *et al.* 2015). Continued accessibility of P at the lowest possible cost is a major concern for countries having no P reserves of their own, such as Europe (Cooper *et al.* 2011, Withers *et al.* 2015 b). Ironically, a growing environmental problem is the leakage of P into water bodies through its discharge into groundwater where it affects aquatic biodiversity and human health by increasing eutrophication (Smith and

Schindler 2009, Withers *et al.* 2015 a). This description of the current global P situation clearly demonstrates the urgent need for a more effective and sustainable P fertilization regime, especially in European agriculture.

Most P applied to agricultural soils as inorganic P fertilizer and manure is stored as surplus P (Owen *et al.* 2015). For the period from 1965 to 2007, cumulative P inputs to European soils were estimated as total ca. 1115 kg ha^{-1}, while the plant-available portion was determined to be only 360 kg ha^{-1} (Sattari *et al.* 2012, Owen *et al.* 2015). Sattari *et al.* (2012) estimated that if the surplus P fixed in soil and which is not plant-available was accounted for in nutrient planning, the requirement for inorganic P fertilizers could be reduced by 50 %. A promising approach to exploit this stored soil P is the use of bio-fertilizers and bio-inoculants. Bio-fertilizers are defined as products that may contain plant nutrients or specific organic components that stimulate microbial activity and thereby the recovery of plant-unavailable nutrients fixed in soil (Erro *et al.* 2007, Owens *et al.* 2015). Bio-inoculants are specific strains or consortia of microorganisms that are able to improve a plant's growth and health; those which specifically increase P mobilization in soil are termed phosphorus-mobilizing microorganisms (PMM; Ahmad *et al.* 2013, Owen *et al.* 2015). Given the current situation of increasing demand and scarcity of P reserves, the global market of products promising to improve P fertilization's efficiency is growing. This is especially true of the market for bio-inoculants, which is estimated to grow at a rate of 10 % per year (Berg 2009). However, results in the literature on the effects of bio-inoculants in general, and PMM in particular, on plant growth and nutrient supply, have been inconsistent. Variability in PMM effects have been reported not only with respect to which strains/consortia were used, but also to growing and soil conditions, as well as crops and even crop varieties which were tested (e.g. Chanway and Nelson 1988, De Freitas *et al.* 1997, Bais *et al.* 2006, Delfin *et al.* 2015).

Contradictory results as well as heterogeneity of experimental conditions and procedures are due to the fact that the underlying functional mechanisms of PMM are still poorly understood. Therefore, understanding PMM's mechanisms can provide insights into both limits and opportunities for their use. This is a key prerequisite for determining PMM's usability in agriculture - and for taking the first step toward addressing the looming P crisis.

3.2 Soil microorganisms

Soil microorganisms play a crucial role in P cycling by mediating the availability of P to plants (Richardson 2001). Plant P uptake from soil solution, in the form of inorganic P, occurs actively; orthophosphates (mainly $H_2PO_4^-$ and HPO_4^{2-}) are taken up by high-affinity transporters which are located at the root epidermis and are expressed in response to P

deficiency (Bucher 2007, Richardson and Simpson 2011). However, phosphate exists in soil predominately in fractions that are unavailable for plant uptake. They are adsorbed to mineral surfaces, precipitated with calcium (Ca), Al, and Fe, associated/adsorbed to soil organic matter, or incorporated into organic biomass (Richardson and Simpson 2011). Soil microorganisms in general, and PMM in particular, are able to mobilize orthophosphate from these P forms primarily for their own use but in doing so provide P for plant uptake. Thus, the targeted application of soil microorganisms offers a promising approach for the recover of plant-available orthophosphate from stored P in soil, enabling its exploitation for plant nutrition. Indeed, this concept is not new. As early as 1948, Gerretsen improved the P supply to canola plants by adding pure cultures of soil bacteria which solubilized precipitated forms of Ca phosphate. During the 1950s a bio-fertilizer named Phosphobacterin (Menkina 1956, Cooper 1959, Menkina 1963), which contains spores of *Bacillus megaterium* was commonly used by farmers in the USSR and several eastern countries. Its use resulted in increased organic P mineralization in soil and improved crop yields of up to 70 % (Yung 1954, Smith *et al.* 1961, Mishustin and Naumova 1962). Though still an emerging technology, the use of arbuscular mycorrhizal fungi (AMF) also has a history of more than 30 years showing auspicious results in improving plant P supply (Gianinazzi and Vosátka 2004, Faye *et al.* 2013). However, AMF effects are based on a plant-fungi symbiotic interaction. Depending on the AMF family, a successful infection/colonization of the plant roots may take up to two months (Hart and Reader 2002). The use of more rapidly growing/colonizing, and thus potentially more effective, phosphorus-mobilizing bacteria offers more promise under modern agricultural conditions, which are characterized by changing crops and short crop seasons and where P deficiency is problematic in the early stages of plant growth.

3.3 Phosphorus-mobilizing bacteria (PMB)

PMB can be defined as soil- or rhizobacteria that effectively mobilize the limited soluble P in soil through mineralization of organic P and/or solubilization of inorganic P (Jones and Oburger 2011, Owens *et al.* 2015), yielding benefits for plant growth and P nutrition. PMB are specific bacterial strains/consortia isolated from natural rhizosphere communities and belonging mainly to the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Enterobacter* (Rodríguez and Fraga 1999, Fankem 2006). Plant growth-promoting effects by the addition of PMB have been reported several times (for review see Rodríguez and Fraga 1999, Gyaneshwar *et al.* 2002, Khan *et al.* 2007, 2009 a, Harvey *et al.* 2009, Owens *et al.* 2015). However, effects on plants described in those studies have varied due to differences in experimental conditions and procedures. These differences have made it difficult to derive recommendations on PMB's practical use in plant production. Aggravating this situation, it is

possible that the absence of plant growth-promoting effects of PMB have been less frequently reported. This information, if available, could help indicate which conditions are required for successful colonization and plant effects. However, more important than testing various PMB under a wide range of plant and soil conditions, is to better understand PMB's underlying functional mechanisms, making it possible to draw conclusions more rapidly about PMB strains' effectiveness in a given location. There remains a lack of knowledge about PMB's mechanisms of P mobilization in soil and their role in plant growth-promotion. In general, two direct and two indirect mechanisms are under discussion. Solubilization of inorganic P and mineralization of organic P in soil by the added PMB belong to the direct mechanisms. Potential interactions with indigenous soil microbes (which alone improve P availability in soil by solubilization and mineralization), and bacterial release of phytohormones which improve plants' P acquisition in soil by increased root growth and activity, are considered indirect PMB mechanisms and are explained in more detail in the following chapters.

3.3.1 Mineralization of organic P

Organic P may constitute 4 - 90 % of the total P in soil (Khan *et al.* 2009 a). Utilization of organic P by plants and microorganisms requires hydrolytic cleavage by exo-phosphatase enzymes that may be either plant or microbial in origin (Tarafdar and Claassen 1988). Soil microbes produce various phosphatases having the capacity to mineralize P from phytate, myo-inositol hexakisphosphate, and other isomers that are the dominant forms of organic P in many soils (Lim *et al.* 2007, Turner 2007, Richardson and Simpson 2011). Up to 60 % of the total organic P in soil may be hydrolysed by phosphatases, mainly by phytase (Bünemann 2008). The main portion of exo-phosphatases in soil is derived from microorganisms (Tabatabai 1994, Tarafdar *et al.* 2001, Dodor and Tabatabai 2003). While the ability of PMB to mobilize P by solubilization of inorganic P is comparatively well studied, less is known about their capacity to mineralize organic P in soil. However, Kim *et al.* (1997), in a field experiment with tomato plants, reported increased activity of acid phosphatase in soil after the addition of *Enterobacter agglomerans*. Kaur and Reddy (2014) observed a plant growth-promoting effect on wheat and maize in the field accompanied by increased activities of phosphomonoesterase, phytase and dehydrogenase after inoculations with *Pantoea cypripedii* and *Pseudomonas plecoglossicida* into soil.

Increased mineralization of organic P in the rhizosphere may also be a result of a microbial priming effect. Rhizosphere-colonizing PMB utilize root exudates as an easily available C source. Due to the coupled incorporation of C, N and P into microbial biomass (Cleveland and Liptzin 2007), an increase in C incorporation increases mobilization of N and P from soil

organic matter (Cheng 2009, Richardson and Simpson 2011). However, interactions between PMB and plants are still poorly understood; for example, it is still not known whether increased P mineralization in the rhizosphere by the addition of PMB is independent of endogenous microbial turnover of organic matter, since effects of PMB on plant growth and P mobilization have to date been compared only with non-inoculated controls.

3.3.2 Solubilization of inorganic P

The ability of PMB to solubilize P sorbed to inorganic P compounds is most often characterized as the "organic acid theory" (Owens *et al.* 2015). This is the release of organic acids/organic acid anions which includes two follow-up mechanisms: first, lowering of pH, which directly dissolves P by proton extrusion; and second, ligand exchange resulting in solubilization of adsorbed P (Ryan *et al.* 2001, Oburger *et al.* 2011). The designation of bacterial strains/consortia as phosphorus-solubilizing bacteria is based mainly on laboratory experiments which have demonstrated their ability to acidify a culture medium containing added Ca, Fe or Al phosphate, and to release organic anions, such as citrate, gluconate, oxalate, and succinate (for review see Khan *et al.* 2007). Improved plant growth and P nutrition by the addition of phosphorus-solubilizing bacteria has been reported in several studies (for review see Rodríguez and Fraga 1999, Sharma *et al.* 2013). For instance, Vyas and Gulati (2009) identified five strains belonging to *Pseudomonas trivialis*, *P.* sp., and *P. poae* as highly effective in the release of organic acids (especially gluconic and succinic acid) during solubilization of tricalcium and rock phosphate. In a pot experiment using sandy-loam soil amended with a single super-phosphate, these strains increased plant growth and nutrient uptake in maize. However, laboratory assays demonstrating individual mechanisms involved in bacterial P solubilization cannot simply be transferred to natural conditions in soil. Direct *in situ* evidence of soil/plant linkages with plant growth and nutrient promotion to a specific PMB's P solubilization capacity are rare. Also, comparison with non-inoculated controls makes it difficult to differentiate endogenous from PMB-derived P mobilization processes in soil. P can be solubilized by each redox activity of microorganisms; for instance, bacterial siderophores (Fe^{3+} reduction) also release P bound to Fe (Thiele-Brun 2006). Bacterial NH_4^+ assimilation, in which excreted H^+ decreases the pH (Illmer and Schinner 1992), can also provide an important contribution to plant-available P in soil. It is likely that a number of different P mobilization (including mineralization) processes run in parallel; the association of plant growth with particular mechanisms is difficult to identify and may differ between the strains/consortia and soil conditions.

3.4 Plant growth-promoting attributes of PMB

Besides enhancing P availability for plants in soil by P mobilization, PMB are able to produce metabolites such as phytohormones and antifungal agents that improve plant growth and health. For instance, the bacterial strain *Pseudomonas* sp. NBRI 4014 was identified not only as a potent phosphorus-solubilizer, but also as a synthesizer of high levels of the phytohormone indole acetic acid (IAA; Gupta *et al.* 2002). As is the case with all auxins, IAA affects plant cell elongation and division. Ramírez and Kloepper (2010) were able to demonstrate increased plant growth and P uptake in Chinese cabbage by inoculation with the phytase- and IAA-producing strain *Bacillus amyloliquefaciens* FZB45. A hormone-derived plant growth-promoting effect may also indirectly improve plant P supply; auxins in particular can alter root branching and root hair development and thus improve plant P acquisition in soil (Holguin *et al.* 1999, Richardson 2001). Furthermore, phytohormones are involved in plant responses to stress (Yang *et al.* 2009). Under stress conditions, the phytohormone ethylene endogenously regulates plant homeostasis and results in decreased root and shoot growth (Glick *et al.* 2007). The bacterial release of 1-aminocyclopropane-1-carboxylate (ACC) deaminase degrades the ethylene precursor ACC, which in turn reduces plant stress and uninhibited growth (Glick *et al.* 2007, Saleem *et al.* 2007, Yang *et al.* 2009, Glick 2014). Thus, phytohormonally-derived capacities of PMB may enhance plant tolerance to stresses such as drought or salinity and reduce their negative effects on plant growth. For instance, Barnawal *et al.* (2013) demonstrated in a pot experiment with fenugreek under drought conditions that inoculation with the PMB strain *Bacillus subtilis* LDR2, which produces high levels of ACC deaminase, significantly reduced ACC levels in plants, alleviating ethylene-induced damage and increasing nutrient uptake and plant growth.

Besides pathogen suppression by colonization, many PMB strains exercise bio-control activities (for review see Vassilev *et al.* 2006). For instance, Jha *et al.* (2009) identified three *Pseudomonas* strains (*P. aeruginosa* BFPB9, *P. plecoglossicida* FP12, and *P. mosselii* FP13) which were capable of solubilizing tricalcium phosphate with organic acids and producing, as well, hydrogen cyanide (HCN), highly effective against fungal plant pathogens. In addition, these strains were shown to synthesize IAA (Jha *et al.* 2009), which also plays a role in pathogen defence in plants (Brown and Hamilton 1992, Hamill 1993, Hahn and Strittmatter 1994, Droog 1997). Thus, plant growth-promoting effects of PMB involve mechanisms that can occur independently of P mobilization; however, clearly distinguishing these mechanisms is difficult to do in plant experiments. It is therefore still unclear whether and to what degree plant growth-promotion by PMB contributes to the plant growth and P supply improvements that have been observed in previous studies.

Similar to the bio-control activities of PMB that reduce potential negative effects on plant growth, there are also capacities that influence heavy metal pollution in soil. To circumvent metal stress, soil microorganisms, including PMB, have evolved mechanisms that enable them to tolerate the uptake of metal ions (Khan *et al.* 2009 b). These mechanisms include the accumulation and sequestration of metal ions inside the bacterial cell as well as their transformation into less toxic forms (Wani *et al.* 2008, Khan *et al.* 2009 b). These mechanisms reduce metal toxicity in contaminated soils and thus improve plant growth and health. In a laboratory experiment, Wani *et al.* (2007 a) identified *Bacillus* species PSB 1, PSB 7, and PSB 10 as capable of tolerating chromium (Cr) concentrations up to 550 µg mL^{-1} and of reducing Cr concentrations of up to 87 % in medium. These strains also tested positive for phosphorus-solubilization as well as production of IAA, siderophores, HCN, and ammonia in both the absence and presence of Cr (Wani *et al.* 2007 a). In addition, Rajkumar and Freitas (2008), in a pot experiment with castor oil plants grown in nickel, copper and zinc contaminated soils, observed increases in shoot and root biomass by inoculation with the IAA-producing PMB strain *Pseudomonas jessenii* M15.

3.5 Interactions of PMB with indigenous soil microbes

Besides pathogen suppression by colonization and defence due to bio-control activities, PMB can also stimulate the relationship between the plant and beneficial rhizospheric bacteria and fungi such as mycorrhizal fungi. Several studies have reported increased plant growth and P nutrition effects when PMB were co-inoculated with mycorrhizal fungi (e.g. Kim *et al.* 1997, Suri *et al.* 2011, Najjar *et al.* 2012, Vafadar *et al.* 2014). Saxena and Jha (2014) were able to demonstrate synergistic interactions between the PMB strain *Burkholderia cepacia* BAM-6 and the AMF *Glomus etunicatum* in a pot experiment with wheat under limited soil P availability. Co-inoculations of these two organisms resulted in higher plant yields and nutrient uptake (P and N) than when either PMB or AMF was used alone for inoculation (Saxena and Jha 2014). Furthermore, PMB are also able to promote indigenous mycorrhizal fungi in soil. For instance, *Pseudomonas* sp. DSMZ 13134 (Proradix®), which possesses both phosphorus-solubilizing and bio-control capabilities (Miller *et al.* 2010, Buddrus-Schiemann *et al.* 2010, Fröhlich *et al.* 2012) was shown to significantly increase mycorrhizal root colonization of Paraserianthes seedlings (Yusran *et al.* 2009).

Beneficial effects on plants have also been reported when PMB were co-inoculated with N_2-fixers such as *Azospirillum* (Belimov *et al.* 1995) or *Phyllobacterium* (Rojas *et al.* 2001). Wani *et al.* (2007 b) demonstrated, in a field experiment with chickpeas, highest seed yield and grain protein (P uptake) when plants were inoculated with a combination of two N_2-fixing

bacteria; *Mesorhizobium ciceri* RC4, and *Azotobacter chroococcum* A10, as well as the phosphorus-solubilizing strain *Bacillus spp.* PSB9. In addition, Yu *et al.* (2012) reported highest plant biomass, P, and N uptake as well as maximum concentrations of plant-available P and N in the soil of walnut seedlings when *Pseudomonas chlororaphis* (PMB) was co-inoculated with *Arthrobacter pascens* (N_2-fixer). However, co-inoculation with *Bacillus megaterium* (PMB) and *A. pascens* did not increase these parameters, indicating specific interactions between PMB and N_2-fixers (Yu *et al.* 2012). Similar results were reported by Şahin *et al.* (2004), who observed that beneficial effects of PMB-N_2-fixer combinations on plant growth varied significantly depending on environmental, plant, and soil conditions as well as the bacterial strains used.

More generally, PMB can change the composition of microbial biomass in soil. Increased abundance of soil bacteria after PMB addition was reported by Shishido and Chanway (1998), and an increase in fungal populations was observed by Vivas *et al.* (2003). Sundara *et al.* (2002) reported an increased population of indigenous PMB in soil of field-grown sugarcane when the phosphorus-solubilizing bacterium *Bacillus megaterium* var. phosphaticum (Bardiya and Gaur 1972) was applied; this effect was increased when PMB inoculation was combined with rock phosphate fertilization. In addition, Canbolat *et al.* (2006) demonstrated in a pot experiment with barley that inoculation of the N_2-fixing and phosphorus-solubilizing *Bacillus* strains M-13 and RC01 not only increased plant growth and P supply but also the total abundance of bacteria, fungi and indigenous PMB in soil. Thus, PMB may promote indigenous PMB in soil. However, underlying mechanisms, such as the release of specific attractants as well as interactions, are still unknown.

In summary, considering the current combination of scarcity and simultaneous increase in demand for mineral P fertilizers while surplus P continues to accumulate in soil, the use of PMB offers a promising approach for improving the future efficiency of P fertilization in plant production. However, the effectiveness of PMB appears subject to various conditions including soil properties, indigenous microbial community structure, environmental conditions, and plant species. Since the underlying functional mechanisms of PMB, which are diverse and can be differently expressed in individual strains, remain poorly understood, their effectiveness in practical farming is extremely difficult to implement. Therefore, the aim of this study was to verify the role of plant growth-promotion by PMB to mobilize P in soil as well as to distinguish PMB-specific mechanisms from endogenous microbial processes in soil. To ensure broad applicability of our results, maize (*Zea mays* L.), one of the most important globally cultivated crops, and tomato (*Solanum lycopersicum* L.), important in European vegetable cultivation, were used as the test plants. We selected PMB strains

belonging to the genera *Pseudomonas*, which have been proven to improve plant growth of these cultivars. Ours is the first study which, to our knowledge, used both viable and devitalized PMB cells for inoculation. This made it possible to distinguish between effects of viable PMB cells (active growth/cell metabolism) from those due to indirect plant growth-promoting effects of dead cells (such as increased C availability). Our experiments also included a treatment using unselectively cultivated soil bacteria for inoculation, which enabled us to differentiate between endogenous and PMB-specific microbial processes in soil. Since organic P is the dominant fraction of P found in soils (Ron Vaz *et al.* 1993, Shand *et al.* 1994), this study focused on PMB's abilities to mineralize organic P compounds under limited P availability soil conditions. In addition, P mineralization was also demonstrated under soil conditions that had been improved in P availability. The capacity of PMB to colonize and persist in the rhizosphere in sufficiently high numbers is likely a prerequisite for provision of beneficial effects to plants (Bellis and Ercolani 2001, Barret *et al.* 2011). Several studies have observed an increased plant growth-promoting effect when PMB were co-applied with mineral P fertilizers such as rock phosphate (Bardiya and Gaur 1972*,* Yu *et al.* 2011, Kaur and Reddy 2014, 2015). This was assumed to be associated with an increase in potentially soluble inorganic P in soil. However, we suggest that improving the initial conditions for PMB to colonize the rhizosphere successfully may also strengthen its plant beneficial effects, and this can be positively correlated with increased PMB abundance. In general, the colonization and persistence of introduced rhizobacteria may affect the microbial community structure in soil, especially in the rhizosphere. We assumed that indigenous rhizobacteria are displaced when competing with PMB for C sources. Furthermore, due to bio-control or mycorrhiza-helper capacities, fungal growth in soil is significantly affected by the introduced PMB.

Taken together, the objectives of this study were to test the following overarching hypotheses. (1) PMB's ability to improve plant growth and P uptake includes P mobilization-dependent and -independent as well as direct and indirect mechanisms, which run in parallel. Under limited plant-available P soil conditions, PMB's capacity to effectively mineralize P from soil constitute the main mechanism. (2) Increased P mineralization is PMB-specific, greater than the level of endogenous P mineralization in soil. (3) Improving nutrient availability in soil increases PMB abundance in soil, leading to improved plant growth-promoting effects. (4) The addition of PMB results in microbiome shifts. The displacement of copiotrophic rhizobacteria as well as PMB-specific antifungal or mycorrhiza-helper capacities occur independent of present soil conditions, but manifest differently in the different soils.

4 Outline of the thesis

Increases in plant growth and P uptake by the addition of PMB have been reported in several studies (Chabot *et al.* 1996, Kim *et al.* 1997, Sundara *et al.* 2002, Hussain *et al.* 2013, Surapat *et al.* 2013). Using the bacterial strain *Pseudomonas* sp. RU47 (RU47), which had previously been shown to enhance plant growth of tomato (Eltlbany *et al. in preparation*), the aim of our first experiment was to verify the role of P mineralization in plant growth-promotion by RU47. To distinguish P mineralization-dependent and -independent as well as direct and indirect mechanisms of RU47 effects, treatments using unselectively cultivated soil bacteria and devitalized RU47 cells for inoculation were included as treatments. We performed a greenhouse experiment over a period of 39 days with tomato plants grown under low P availability soil conditions. To determine P mineralization in soil, phosphatase activity (PA) in the rhizosphere was estimated during plant growth by the method of soil *in situ* zymography. Additionally, enzyme analyses were performed with bulk soil samples after final harvest. To estimate effects on microbial community structure, denaturing gradient gel electrophoresis technique was performed on rhizosphere samples, while microbiome shifts in bulk soil were tested by phospholipid fatty acid (PLFA) analysis. We assumed that the addition of viable RU47 cells would result in a plant growth-promoting effect based primarily on increased P mineralization, improving tomatoes' P uptake. The addition of dead RU47 cells or an unselective bacterial mix would also increase microbial activity in soil, but plant growth-promotion would be less pronounced compared with the viable PMB treatment. We also assumed that changes in microbial community structure would be most pronounced in treatments to which living bacteria were added. Results of the first experiment revealed a promotion effect on plant growth and P uptake when viable RU47 cells were added; RU47 abundance in soil was positively correlated with increased alkaline PA in the rhizosphere. Plant growth-promotion which was observed in the dead RU47 treatment indicated phytohormonal effects. Groups of indigenous rhizosphere bacteria were significantly promoted by bacterial residues in the dead RU47 treatment, while microbial community structure was less affected when viable RU47 or unselective soil bacteria were added.

Optimal P nutrition in the seedling stage is crucial to high yields in maize production (Barry and Miller 1989). Thus, our second experiment, conducted over the short-term (14 days) with maize in the greenhouse under limited P availability soil conditions, addressed the question of whether the addition of viable RU47 cells is sufficient to improve maize's P uptake in the early growth stages. Once again, RU47-specific active P mineralization processes were identified by phosphatase analyses and compared with enzyme activities of indirect (dead RU47) as well as endogenous (bacterial mix) processes. Potential changes in microbial

community composition were identified by PLFA analysis. We hypothesized that the addition of viable RU47 increases maize seedling growth and P uptake as a result of an RU47-originated increase in soil PA. In contrast, we expected that dead PMB cells or non-specific added soil bacteria would not improve maize P nutrition. We demonstrated that RU47 is able to colonize the rhizosphere of maize successfully within 14 days. Plant growth-promoting effects were not observed, but trends of increased PA as well as indications of phytohormonal effects in both dead and viable RU47 treatments were detected.

While increased plant growth-promoting effects by co-application of PMB and rock or tricalcium phosphate (Bardiya and Gaur 1972, Yu *et al.* 2011, Kaur and Reddy 2014, 2015) are generally assumed to be associated with an increase in potentially soluble inorganic P in soil, we also proposed that the use of easily available P fertilizers improves PMB's initial colonization conditions, resulting in increased abundance and thus a stronger plant growth-promotion effect. To verify this assumption, we performed a greenhouse experiment with tomato plants, which were grown under both limited and improved P availability soil conditions. The experiment was conducted over a period of 50 days and included as well a bacterial mix and a dead RU47 inoculation treatment. We hypothesized that initially high phosphate fertilization increases the plant growth-promoting effect of viable RU47 cells compared with those under low P soil conditions, while no P fertilization effect would occur with the use of dead RU47 cells. Measurements of PA in both rhizosphere and bulk soil would clarify whether a stronger plant effect of viable RU47 is based on increased PA (since P applied by fertilization is rapidly incorporated into bacterial biomass and becomes limiting), or P mineralization, independent of RU47 abundance-associated plant growth-promoting attributes such as the excretion of phytohormones. Although not significant, RU47 abundance was enhanced by improved P availability in soil. Plant growth-promotion was not strengthened by increased P fertilization. Under low P availability soil conditions, the addition of viable RU47 improved plant growth and P uptake, accompanied by enhanced alkaline PA in the rhizosphere. Trends of increased P mineralization were also found in the dead RU47 treatment, as well as indications of phytohormonal effects.

Since various conditions, such as soil properties, influence PMB's effect on plants, we assumed that effects on microbial community structure may differ between different soils, providing insight into potential microbial interactions associated with plant growth-promotion. To prove this hypothesis, in our fourth experiment we performed a greenhouse experiment with maize over a period of 56 days using three different *Pseudomonas* strains possessing PMB abilities and three soils differing in parameters such organic C, pH, and P content. By the use of domain- (bacteria, archaea, and fungi) as well as bacterial phyla-specific primers

in quantitative PCR, microbial community composition was determined in extracted rhizosphere and bulk soil DNA. We hypothesised that the active colonization of PMB affects rhizosphere community composition due to displacement of indigenous copiotrophic rhizobacteria. In particular, individual inoculation effects on soil microbial structure, such as known antifungal or mycorrhiza-helper capacities, will manifest differently in the varying soils. Our fourth set of experiments showed that individual inoculation effects varied heterogeneously between the PMB strains added, the contrasting soils used, and time. Changes in microbial biomass and composition were due primarily to nutrient availability in the soil substrates, which differed between the soils and over time.

5 Effects of phosphorus-mobilizing bacteria on tomato growth and soil microbial activity

Parts of this chapter are published in Nassal D, Spohn M, Eltlbany N, Jacquiod S, Smalla K, Marhan S, Kandeler E (2017) Effects of phosphorus-mobilizing bacteria on tomato growth and soil microbial activity. Plant and Soil 1-21. The published version of this article is included in the appendix (12).

5.1 Introduction

While it is well known that rhizosphere processes are important for plant P acquisition (Jones and Darrah 1994, Hinsinger 2001), the processes underlying growth promotion by beneficial microorganisms are not yet well understood. Since organic P is often the dominant form of P found in soils (Ron Vaz *et al.* 1993, Shand *et al.* 1994) and may constitute up to 90 % of the total P in soil (Khan *et al.* 2009 a), P mineralisation is a pre-requisite to convert organic P into a plant available form. P mineralisation is catalysed by extracellular phosphatases produced by microorganisms and plants. While microorganisms produce both acid and alkaline phosphatases, plants produce only acid phosphatases (Dick *et al.* 1983, Juma and Tabatabai 1988, Nannipieri *et al.* 2011). Microbial and plant P acquisition occur in different zones of the rhizosphere. Plant uptake of P occurs mostly at the root tip and in the proximal elongation zone, whereas microbial P uptake is highest in the root hair zone (Marschner *et al.* 2011). Using zymography in a rhizobox experiment, Spohn and Kuzyakov (2013 a) demonstrated the spatial separation of acid and alkaline phosphatase activity (PA) in the rhizosphere of lupines. While acid PA was associated with the root, alkaline PA was more widely distributed in the bulk soil (Spohn and Kuzyakov 2013 a, Spohn *et al.* 2013, Spohn *et al.* 2015, Hofmann *et al.* 2016). Microbial phosphatases comprise the major share of phosphatases in soil (Tabatabai 1994, Tarafdar *et al.* 2001), contributing significantly to the P supply of plants (Frossard *et al.* 2000, Oehl *et al.* 2004). However, with respect to P foraging, the plant-microbial relationship can be competitive as well as mutualistic (Harte and Kinzig 1993, Richardson *et al.* 2009, Richardson and Simpson 2011). Hence, without phosphate fertilizers, P supply is generally not sufficient for effective crop production in most agricultural soils.

Phosphorus-mobilizing bacteria (PMB) are beneficial bacteria that effectively mobilize P through solubilization of sorbed P pools and mineralization of organic P compounds which are otherwise not readily available. Application of PMB to soils can therefore be a promising approach for improving P fertilization efficiency in agriculture. Plant growth-promoting effects

resulting from targeted application of high-concentrations of PMB strains such as *Bacillus*, *Pseudomonas*, and *Rhizobium* in soils limited in P availability for plants have been documented in several studies (Chabot *et al.* 1996, Sundara *et al.* 2002, Kaur and Reddy 2014). Whether future use of PMB can improve P nutrition of arable crops and vegetables remains to be tested. Three different microbially driven functional mechanisms are currently being explored. First, added PMB may catalyze the hydrolysis of organic P compounds by the release of phosphatases (Tarafdar and Claassen 1988, Tabatabai 1994). Second, PMB may solubilize bound inorganic P into easily available phosphates by secreting organic acids which would reduce rhizosphere pH. Organic acids as well as bicarbonates, carboxylates, and other anions biotically released may function as exchange ligands (Kpomblekou-a and Tabatabai 1994, Deubel *et al.* 2000, Jones and Oburger 2011). Third, added PMB may interact with other beneficial indigenous microbes, optimizing P mobilization in soil (Belimov *et al.* 1995, Zaidi *et al.* 2003). Although the role of PMB during P solubilization has been investigated (Kim *et al.* 1997, Khan *et al.* 2007, Fankem *et al.* 2008), the importance of enzymatic cleavage of organic P resources by PMB, especially under limited P availability soil conditions, has been less well studied. Kaur and Reddy (2014) demonstrated that enhanced wheat and maize growth after inoculation of an agricultural field with *Pantoea cypripedii* and *Pseudomonas plecoglossicida* was accompanied by an increase in phosphomonoesterase, phytase and dehydrogenase activities in soil. In addition, Eltlbany *et al.* (*in preparation*), conducted a pot experiment with tomato plants under limited soil P conditions and found considerably enhanced plant growth following inoculation with *Bacillus amyloliquefaciens* FZB42 spores (RhizoVital®) as well as *Pseudomonas* sp. DSMZ 13134 (Proradix®) or *Pseudomonas* sp. RU47. PA tended to increase in the rhizosphere; alkaline phosphomonoesterase with the addition of each of the two commercial products, and acid phosphomonoesterase with the addition of *Pseudomonas* sp. DSMZ 13134 and *P.* sp. RU47. It is possible that the plant growth-promoting function of these three different bacterial strains is based mainly on their enhanced phosphatase production in the rhizosphere of plants. Since the formulation of the commercial products (i.e. the carrier matrix; culture media, skimmed milk powder, or gum arabic) may also affect microbial P mineralization, we selected *P.* sp. RU47 (RU47) as the model organism, omitting any formulation. To distinguish dependent and independent P-mobilizing mechanisms, we used both viable and devitalized PMB strains in the first study. To exclude apparent plant growth-promoting effects of the PMB due to increased microbial activity by addition of living soil bacteria, an inoculation treatment using a mix of soil bacterial isolates (bacterial mix) was also evaluated. The following hypotheses were tested. (1) Added viable RU47 successfully colonizes the soil and leads to a plant growth-promoting effect. (2) The plant growth-promoting effect of viable

RU47 under low P availability soil conditions is based on enhanced PA leading to enhanced P availability in soil and increased uptake by plants. (3) Added viable RU47 dominates colonization of the rhizosphere, leading to spatially distinct zones of enriched alkaline/acid PA and to a shift in microbial community composition.

5.2 Materials and methods

5.2.1 Rhizobox experiment

The experiment was performed under low P availability soil conditions using RU47 as the PMB, and tomato (*Solanum lycopersicum* L. var. Mobil) as the test plant. To exclude apparent plant growth-promoting effects of the PMB due to increased microbial activity from having added living soil bacteria and/or cell compounds which could affect the P efficiency of plants and indigenous soil microbes, two additional inoculation treatments were conducted, composed of either an inoculation of native soil bacteria (bacterial mix) or dead RU47 cells (dead RU47). Details of microorganism cultivation and inoculation are described in 5.5.2. An optimally P-fertilized (200 mg kg^{-1}), non-inoculation treatment was added as control. Hence, the experiment consisted of four treatments, with four replicates per treatment. Tomato plants were grown in rhizoboxes with inner dimensions of 28.0 cm x 4.5 cm x 16.5 cm, and filled with a soil substrate composed of Luvisol topsoil and quartz sand (0.2 - 1.4 mm) in a ratio of 1:1 (w/w). The Luvisol was considered as a heavy loam soil and had the following characteristics: pH 7.1 ($CaCl_2$), 26.2 % sand, 52.2 % silt, 21.6 % clay, 2.3 % total C, 2.0 % organic C, 1.8 mg NH_4^+ kg^{-1}, 53.0 mg NO_3^- kg^{-1} and 24.1 mg P (Olsen) kg^{-1}. The soil, selected on the basis of its low concentration of plant-available P (calcium acetate lactate [CAL] extraction of 20 mg kg^{-1}), was taken from an unfertilized grassland located on the campus of the University of Hohenheim (Stuttgart, Germany). Each rhizobox was filled with 1918.0 g dry matter (DM) of sieved (< 5 mm) soil substrate. Before sowing, the soil substrate was optimally fertilized with respect to N (100 mg kg^{-1}; $Ca(NO_3)_2$), K (150 mg kg^{-1}; K_2SO_4) and Mg (50 mg kg^{-1}; $MgSO_4$) and adjusted to a water holding capacity of 50 %. Although the study aimed to determine the effects of PMB under low P soil conditions, a slight P fertilization of 50 mg kg^{-1}($Ca(H_2PO_4)_2$) was applied to all treatments, excluding the optimally P-fertilized treatment, in order to achieve successful germination. Three tomato seeds were sown at a depth of 1-2 cm directly into each rhizobox and thinned to one plant per rhizobox after germination. In order to promote root growth along the hinged wall, rhizoboxes were placed at a 50 ° inclination. To avoid light-derived influences on root growth and behaviour, all boxes were wrapped in aluminium foil. The experiment was conducted for 39 days under greenhouse conditions. Rhizoboxes were distributed randomly and placed on wooden planks

to exclude contamination by leaking irrigation water. Plants were watered to maintain a water holding capacity of 50 % until 25 days after sowing, with water content checked gravimetrically on a daily basis. Watering was performed using deionized water (H_2O_{deion}), applied in 5 mL steps to avoid leakage along rhizobox edges.

5.2.2 Bacterial cultivation and inoculation

RU47 (Adesina *et al.* 2007) was cultured in King's B liquid medium (King *et al.* 1954) with 50 mg L^{-1} added rifampicin (resistance by spontaneous mutation) at 28.5 °C in an incubator shaker (SM 30 Control; Edmund Bühler, Hechingen, Germany) for 24 h; cultivation vessels were wrapped in aluminium foil to protect the antibiotic from light. Although different from the normally recommended growing conditions of *Pseudomonas* strains (e. g. Xue *et al.* 2013), best results in maintaining the exponential growing phase (to ensure inoculation by viable cells) and greater time flexibility in inoculation preparation were achieved under the growing conditions selected here. Bacterial mixes were grown in glucose-enriched (2 g L^{-1}) LB-Lennox liquid medium (Bertani 1951, Lennox 1955) at 28.5 °C for 24 h (incubator shaker) using a sample of the untreated soil as the inoculum. Glucose enrichment was chosen in order to avoid C limitation of bacterial growth. After incubation, all cultures were centrifuged (4700 g min^{-1}) for 10 min. Pellets were washed twice and re-suspended in sterile 0.3 % NaCl solution. In the treatments using the bacterial mix, remaining soil components were removed by trapping on folded filter paper (grade 4) before cell washing. Cell suspensions were photometrically measured (BioPhotometer, Eppendorf, Germany) and adjusted to an OD_{600} = 1.0 corresponding to a cell density of approximately 10^9 cell mL^{-1}, as described in Xue *et al.* (2013). However, overestimates of cell density resulting from soil-derived turbid material remaining in cell suspensions containing bacterial isolates cannot be fully excluded. The killing of RU47 cells, which were used in one of the treatments, was performed as follows: bacterial suspension (OD_{600} = 1.0) was placed in a sterile Erlenmeyer flask and boiled for 1 min on a heating plate. To minimize volume loss, the flask was covered and cooled to room temperature to exclude volume error before being used for inoculation. Pretests confirmed that this procedure was sufficient to kill RU47 cells, as plating exhibited no growth of RU47.

Plants were inoculated three times, each with a cell density of 10^9 cells mL^{-1} (OD_{600} = 1.0). The first inoculation was conducted by seed coating. Under gentle and continuous vortexing, 5 µL of cell suspension was successively added to five tomato seeds. The volume required for entire seed coating had been tested with ink (Pelikan, Pottendorf, Austria) before starting the experiment. Seed coating was controlled by using three of the inoculated seeds followed by washing with 1 mL sterile 0.3 % NaCl solution and plating 100 µL of the suspension on King's B-Agar medium (50 mg rifampicin L^{-1}) in three dilution stages. Plates were incubated

at 28.5 °C until growing colonies were unequivocally countable on the agar (after approximately 36 h). The second inoculation was applied directly after seed germination, and the last inoculation was applied one week later. Both inoculations were performed with 6 mL kg^{-1} soil substrate DM, directly applied to the soil surface to simulate farm practice. To avoid a watering effect, the P-fertilized control was inoculated with 0.3 % NaCl solution with corresponding volumes per inoculation. Viability and unviability of the RU47 cells used as well as sterility of the 0.3 % NaCl solution were checked by plating and subsequent incubation at 28.5 °C for 48 h after every inoculation.

5.2.3 Plant properties

Plant analyses during the growth period

Stem diameter, leaf number and area, shoot height and P deficiency symptoms were recorded at a temporal interval of minimum two and maximum four days, starting 20 days after sowing. While stem diameter and leaf area (length × width) were measured using a precision pocket vernier caliper (150 mm, Format, Wuppertal, Germany), shoot height, defined as the vertical length from stem base to youngest leaf's tip, was measured by a ruler. P deficiency symptoms were defined as the expression of violet discoloration on the undersides of leaves and determined as a percentage of total leaf area.

Plant analyses after harvest

Shoots of every replicate was separately and carefully cut from soil surface using a sterilized (70 % ethanol) scalpel. Shoots were briefly rinsed with H_2O_{deion} to remove adhering dust followed by drying at 60 °C in separate aluminium trays for 3 days to estimate the dry weight.

The determination the plant-bound phosphorus in tomato shoots was performed by a sequential microwave digestion based on Kalra *et al.* (1989) followed by a photometric measurement of molybdenum blue. Grinded samples with a range of 0.1 - 0.3 g dry matter were filled into Teflon containers adding 1 mL H_2O_{deion}, 2.5 mL HNO_3 and 2 mL H_2O_2, respectively. After soaking for 1 h, samples were incinerated at 70 °C (3 min) and 210 °C (62 min) at 1400 W, respectively, using an ETHOS.lab microwave (MLS, Leutkirch, Germany). The diluted suspensions (1:1 H_2O_{deion}) were filtered (blue ribbon filter), diluted again (1:3 H_2O_{deion}) and subsequently photometrically measured using Murphy and Riley colour reagent (Murphy and Riley 1962) at 710 nm using a microplate absorption reader (ELx808; BioTek Instruments Inc., Winooski, VT, USA). The calibration was performed using the following final concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg P L^{-1} prepared by a K_2HPO_4 solution.

5.2.4 Soil sampling

Rhizosphere and bulk soil samples were immediately put on ice for short-term storage. While DNA was directly extracted from the rhizosphere soils (see 5.2.5), bulk soil samples were sieved (< 2 mm), after which aliquots of each replicate were frozen at -20 °C until analyses.

5.2.5 Tracing RU47 and analyses of microbial community composition

DNA extraction

DNA extraction of the rhizosphere was performed at the Institute of Epidemiology and Pathogendiagnostic (Julius Kühn-Institut, Braunschweig, Germany). Rhizosphere DNA was extracted according to Schreiter *et al.* (2014 c) with some modifications. Briefly, after removing loosely adhering soil by vigorously shaking the roots, the complete root systems of one replicate per treatment were combined, then cut into pieces of approximately 1 cm length and carefully mixed. Five g of cut roots were transferred to a Stomacher bag, homogenized in a Stomacher 400 Circulator (SewardLtd, Worthing, UK) for 1 min at high speed after adding 15 mL sterile 0.3 % NaCl; supernatant was then collected in a Falcon tube. This step was repeated twice, the combined supernatants (45 mL) of three Stomacher homogenizations were centrifuged at 10,000 g for 15 min, after which pellets were frozen and stored at -20 °C. Total community DNA (TC-DNA) was extracted from 0.5 g of rhizosphere pellets using the Fast DNA SPIN Kit for Soil® (MP Biomedicals, Heidelberg, Germany) after a harsh lysis step as described by the manufacturer. The TC-DNA was purified with GENE CLEAN SPIN Kit® (MP Biomedicals, Heidelberg, Germany) according to the manufacturer's instructions and diluted 1:10 with 10 mM Tris HCl, pH 8.0, before use.

TC-DNA extraction of bulk soil was performed using 250 - 350 mg fresh soil using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions. DNA concentration was measured, and purity of the extract was determined spectrophotometrically (NanoDrop 2000, Thermo Scientific, Waltham, MA, USA).

Tracing RU47

Tracing the inoculated RU47 cells was done using a TaqMan® assay with a 5'-labelled 6-FAM double-quenched (BMN-Q530) probe (biomers.net, Ulm, Germany) in a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Used probe and primer sequences (Eltlbany *et al. in preparation*) were developed and provided by the Institute of Epidemiology and Pathogendiagnostic (Julius Kühn-Institut, Braunschweig, Germany). Reaction recipe and thermal-cycling conditions were adapted to a commercial master mix (TaqMan® Fast Advanced Master Mix, Thermo Fisher Scientific, Waltham, MA,

USA) following manufacturer's instructions with some modifications. The reaction composition was as follows; final concentrations are given in parentheses: 10 µL TaqMan® Fast Advanced Master Mix (1 ×), 2 µL DNA (10 ng), 5 µL nuclease-free water, 1 µL each primer (1.8 µM) and 1 µL (0.5 µM) probe. The thermal profile of the TaqMan® assay was as follows: 95 °C for 10 min (initial denaturation), 95 °C for 30 sec followed by 54 °C for 30 sec for 40 cycles. Based on the standard curve, the absolute quantity of RU47 copies was calculated (copies ng^{-1} DNA).

Denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene and ITS fragments amplified from TC-DNA

16S rRNA gene fragments were PCR-amplified from TC-DNA of rhizosphere samples using the bacterial primers F984-GC and R1378, as described by Heuer *et al.* (1997). Amplification of ITS fragments was done according to Weinert *et al.* (2009). PCR products were analysed by DGGE. The gradient of the DGGE gel was performed as described in Weinert *et al.* (2009), and electrophoresis conditions as well as the silver staining procedure were done according to Heuer *et al.* (2001).

Analysis of the DGGE fingerprints

Bacterial and fungal DGGE fingerprints were evaluated with GELCOMPAR II version 6.5 (Applied Maths, Sint-Martens-Latem, Belgium). Normalization and background subtraction were based on each DGGE gel image (Schreiter *et al.* 2014 b). The Pearson correlation index (*r*) for each pair of lanes within a gel was calculated as a measure of similarity between the fingerprints, and the clustering of patterns was calculated using the unweighted pair group method using average linkages (UPGMA). Pearson indices were also used to test for significant differences between the fingerprints of the bacterial or fungal communities by the PERMTEST software (10,000 simulations) according to Kropf *et al.* (2004).

Phospholipid fatty acid (PLFA) analysis

For determination of microbial community structure PLFA profiles were used. Four g of fresh soil were taken for lipid extraction and fractionation according to the alkaline methylation method by Frostegård *et al*. (1991). The resulting PLFA methyl ethers were dissolved with isooctane and measured by gas chromatograph using an Auto System XL (PerkinElmer, Waltham, MA, USA), where a HP-5 capillary column, a flame ionization detector and helium as the carrier gas was used. Fatty acid methyl esters (FAME) were identified using their retention time based on fatty- and bacterial-acid methylester-mix (Sigma–Aldrich, St. Louis, MO, USA). Quantification was calculated using an internal FAME standard, which had been

added before methanolysis. PLFA's division into bacteria and fungi are based on Frostegård and Bååth (1996), Zelles (1999) and Kandeler *et al.* (2008). Within bacteria PLFAs were grouped into gram$^+$, represented by i15:0, a15:0, i16:0, and gram$^-$, specified by cy17:0 and cy19:0. The amount of total bacterial PLFAs was calculated by the sum of gram$^+$ and gram$^-$ and 16:1ω7. Fungal PLFA was represented only by 18:2ω6,9.

5.2.6 Enzyme assays

Soil in situ zymography

Soil *in situ* zymography uses membranes coated with methylumbelliferyl (MUF)-substrates which become fluorescent during enzyme cleavage, yielding information about the distribution of exoenzymes in soil. Distributions of alkaline and acid phosphomonoesterase (EC 3.1.3) were analysed by soil *in situ* zymography using an approach similar to that described in Spohn and Kuzyakov (2014). All replicates were analysed by zymography at intervals of seven days, starting 18 days after sowing. MUF-phosphate (4-MUF, Sigma–Aldrich, St. Louis, MO, USA) was used as substrate; a 12 mM solution was prepared and used to coat polyamide membranes, with diameter 14.2 cm, and pore size 0.45 µm (Sartorius, Göttingen, Germany). Substrate solution was prepared using modified universal buffer (MUB) adjusted to pH 11 for alkaline PA, and pH 6.5 for acid PA. Coated membranes were laid flat onto opened rhizoboxes which were separated from soil particles by an underlying layer of fresh 1 % agarose gel (1 mm thick). Soil zymography was performed for each enzyme separately on the same rhizobox; first, acid PA was evaluated due to its affinity with the soil's pH of 7.4; second, alkaline PA was assayed. This order was maintained throughout the experiment. The possible loss of alkaline phosphatases by diffusion into the agarose gel or membrane during measurement of the acid PA cannot be excluded. In contrast to Spohn and Kuzyakov (2014), an incubation time of 35 min, adjusted to achieve the best practical contrast obtained by imaging, was used. Incubations were performed at a constant temperature of 20 °C; membranes were covered by aluminium foil to minimize liquid loss during incubation time. After incubation, membranes were placed on an epi-UV-desk (Desaga, Sarstedt, Nümbrecht, Germany) in the dark, and viewed at 360 nm wavelength. After being photographed with a digital camera (D60, Nikon, Tokyo, Japan) image processing and analysis of the zymograms were done using the open source software ImageJ. Digital images were transformed to 8-bit and multiplied by a factor of 1.25 to enhance the contrast. Images were transformed into false colors to create a color representation of enzyme activity, as given in Fig. 5.S1. Calculation of enzyme activity was based on a linear function using a calibration curve fitted to different concentrations of 4-methylumbelliferone (0, 35, 70, 130, 200, 240 µM). Image processing of calibration

zymograms was adapted to the modifications made with the soil zymograms. Calculation of enzyme activity was based on mean gray values obtained for each concentration in the calibration curve. As there was no distinct separation observed in enzyme activity between root and surrounding soil, the mean activity of the total incubated area was calculated.

Analyses of enzyme activities in bulk soil after final harvest

In addition to weekly conduced soil *in situ* zymography during vegetation period, samples of the harvested bulk soil were used for analyses of potential alkaline as well as acid phosphatase (EC 3.1.3) activity using 4-MUF (Sigma-Aldrich, St. Louis, MO, USA) according to Marx *et al.* (2001). The assay performed was very close to the method described in Poll et al. (2006) with the alteration of using MUB instead of 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer to ensure the comparability with the results obtained by the zymographies. Contrary to Niemi and Vepsalainen (2005), pretests of this study could demonstrated that the stability of 4-MUF phosphate in alkaline pH ranges (pH 8 - 12) is constantly maintained over time (2 h) when MUB instead of MES buffer is used. Acid phosphatase was measured at pH 6.5, alkaline phosphatase at pH 11. Soil suspension was prepared by adding 1 g fresh soil into 50 mL of sterile H_2O_{deion} and dispersed by ultrasonication for 2 min with 50 J s^{-1} sonication energy. The suspensions were continuously stirred using a magnetic stir plate while 50 µL aliquots were dispensed into 96-well microplate (PP F black 96 well; Greiner Bio-one, Kremsmünster, Austria), followed by the addition of 50 µL of MUB buffer (pH 6.5 or pH 11) and 100 µL of 1 mM substrate solution. Standards were mixed with 50 µL of soil suspension and an appropriate volume of buffer to give final concentrations of 0, 100, 200, 500, 800 and 1200 pmol $well^{-1}$. Microplates were incubated at 30 °C. Fluorescence was measured after 0, 30, 60, 120 and 180 min at 360/460 nm wavelength using a microplate fluorescence reader (FLx800, BioTek Instruments Inc., Winooski, VT, USA).

The activity of three enzymes involved in the C and N cycle were also measured based on the use of MUF substrates (4-MUF; Sigma–Aldrich, St. Louis, MO, USA): β-D-glucosidase (EC 3.2.1.21), β-xylosidase (EC 3.2.1.37) and β-N-acetylglucosaminidase (EC 3.2.1.52) according to Marx *et al.* (2001). Enzyme activity was measured in autoclaved MES buffer (pH 6.1).

5.2.7 Microbially bound C and P

The microbial biomass C (C_{mic}) in soil was estimated with the chloroform fumigation extraction method based on Vance *et al.* (1987) according to Mackie *et al.* (2014). Briefly, each sample was split into two subsamples, each comprising 10 g of fresh soil, one fumigated by the use of ethanol-free chloroform under vacuum in a desiccator for 24 h, the

other non-fumigated. The released C was extracted with 40 ml 0.025 M K_2SO_4 on a horizontal shaker (250 U min^{-1}) followed by centrifugation (4400 × g), each for 30 minutes. C concentration was analysed in a 1:4 dilution of the supernatant using a TOC-TNb Analyzer Multi-N/C 2100S (Analytik Jena, Jena, Germany). Inorganic C was removed by adding 1 M HCl to the extracted samples before measurement (Pausch and Kuzyakov 2012). C_{mic} concentration was calculated by the difference between the C concentrations of the fumigated and non-fumigated subsamples using the *kEC* factor of 0.45 (Joergensen 1996) to account for incomplete cell lysis by chloroform. The estimation of microbial biomass P (P_{mic}) was done by liquid fumigation extraction with anion-exchange resin membranes (Kouno *et al.* 2002) using hexanol instead of liquid chloroform (Bünemann *et al.* 2004). A fresh weight of soil corresponding to 2 g dry matter was used for fumigated and non-fumigated subsamples of each sample. Pretests for this study had indicated that the observed variability in P adsorption behaviour of soil depended on total P concentration in the soil solution to be analysed. Thus, the use of identical soil weights in all subsamples is required in order to obtain an accurate correction factor for P retained by soil after fumigation. Fumigation and extraction were performed according to Bünemann *et al.* (2004). Extracted P was mixed with Murphy and Riley color reagent (Murphy and Riley 1962) and H_2O_{deion} in a ratio of 1:1:4 (v/v), respectively. P concentration was photometrically measured at 710 nm using a microplate absorption reader (ELx808; BioTek Instruments Inc., Winooski, VT, USA). Calibration was performed using K_2HPO_4 in the following final concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg P L^{-1}. To determine the amount of P retained by soil particles and complexation after fumigation incubation, a defined P concentration (K_2HPO_4), which was equal to the measured P concentrations in fumigated subsamples (µg P g^{-1}), was added to additional non-fumigated but otherwise identically treated subsamples. The ratio of recovered P to added P was used to calculate the P_{mic} concentration as follows:

$$P_{mic}[\mu g\, g^{-1}] = \frac{(P_{fumigated}\,[\mu g\, g^{-1}] - P_{non-fumigated}[\mu g\, g^{-1}])}{(P_{recovered}\,[\mu g\, g^{-1}] / P_{added}[\mu g\, g^{-1}])}$$

Given values of water-extractable in soil P (P_{H2O}) correspond to the P concentration (µg P g^{-1}) determined in the non-fumigated subsamples. However, as the anion-exchange resin membranes used compete for P adsorption by soil particles, it cannot be assumed that given P_{H2O} values completely represent the plant-available P fraction.

5.2.8 Mineral N

To determine the concentrations of ammonium (NH_4^+) and nitrate (NO_3^-) in soil, undiluted (0.5 M K_2SO_4) soil extracts from non-fumigated samples used for the C_{mic} determination were colorimetrically measured on an Autoanalyzer III (Bran + Luebbe, Norderstedt, Germany).

5.2.9 Statistical analyses

Homogeneity of variance was tested by the Levene-test. Significance of differences was tested by ANOVA followed by the Tukey HSD-test, where $p < 0.05$ was considered as the threshold value for significance. In case of variance heterogeneity, the Games-Howell-test was used for pairwise comparison, where $p < 0.05$ was also considered as significantly different. Statistical analyses were performed using SPSS Statistics 22 (IBM 2013).

5.3 Results

5.3.1 Tracing RU47

The highest copy numbers of the RU47-specific sequence detected by qPCR were found in TC-DNA from soil inoculated with RU47 (Table 5.1). The RU47 copy number in this treatment was significantly higher than the average number of copies found in all other treatments; by a factor of four in bulk soil, and 18 in rhizosphere soil (Table 5.1). The RU47-specific DNA sequence could be detected in all treatments; however, the distribution of sequence frequency between the treatments differed between rhizosphere and bulk soils. Bulk soil inoculated with dead RU47 cells resulted in the second highest RU47 abundance, while the lowest value was recorded in the control (Table 5.1). In the rhizosphere, quantities of the RU47-specific sequence copies detected were ranked in the following order high to low: RU47 > control > bacterial mix > dead RU47 (Table 5.1). On average, a 1300-fold higher quantity of RU47 was detected in rhizosphere than in bulk soil DNA.

Table 5.1 Abundance of *P.* sp. RU47-specific DNA sequence in rhizosphere and bulk soil DNA of tomato plants 39 days after sowing under the following treatments: one non-inoculation (control) and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Values are presented as mean ± standard error (SE) of four replicates. Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the treatments are marked by lowercase letters. Cp stands for copies.

		Control		**Bacterial mix**		**Dead RU47**		**RU47**	
RU47 abundance	Unit	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Rhizosphere	[cp ng^{-1} DNA]	2747.5 ^{b}	± 2108.6	1984.9 ^{b}	± 659.1	279.6 ^{b}	± 49.0	30199.4 ^{a}	± 2925.9
Bulk soil	[cp ng^{-1} DNA]	0.8 ^{b}	± 0.3	2.1 ^{b}	± 0.7	3.1 ^{b}	± 0.7	21.1 ^{a}	± 1.9

5.3.2 Plant growth and soil nutrients

In comparison to the bacterial mix treatment, inoculations with RU47 or dead RU47 cells resulted in significantly enhanced plant growth, as shown by higher stem diameter, leaf number (Table 5.2), and shoot biomass (Fig. 5.1). Furthermore, we observed trends of increased shoot height and leaf area (Table 5.2). Symptoms of P deficiency (violet discoloration on the leaves) were less obvious in plants receiving both RU47 treatments than in the bacterial mix treatment but were not significantly different from the bacterial mix (Table 5.2). A trend of increased P uptake was observed in plants inoculated with RU47 and dead RU47 cells (Fig. 5.1). Plants of the optimally P-fertilized control and bacterial mix treatments had concentrations of about 4 g P kg^{-1}, which represented an adequate P supply for tomato plants before flowering. Plants inoculated with dead RU47 cells had an optimal concentration of 6 g P kg^{-1}, whereas plants inoculated with RU47 exhibited a remarkably low P tissue concentration of 2 g P kg^{-1}, but this was an improvement in absolute uptake compared to the bacterial mix (data not shown). In measurements of water-extractable P in soil, bulk soil samples of both RU47 treatments had a 2.3-fold higher P concentration than samples inoculated with the bacterial mix, and about one fourth of the P concentration measured in the optimally P-fertilized non-inoculation control (Table 5.2). No significant treatment effects on NH_4^+ and NO_3^- concentrations in bulk soil were observed (Table 5.2). However, NO_3^- concentrations were negatively correlated with shoot biomass (Pearson's $r = -0.7$; $p < 0.05$).

Table 5.2 Summarized plant and soil properties of tomato plants 36 and 39 days after sowing (DAS) under the following treatments: one non-inoculation (control) and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Values are presented as mean ± standard error (SE) of four replicates. Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the treatments are marked by lowercase letters. Please note the different time points of plant observation and soil sampling. Percentage of violet discolouration on the undersides of the leaves is based on total leaf area, coded as follows: 0 % = 0, > 0 - 25 % = 1, > 25 - 50 % = 2, > 50 - 75 % = 3, > 75 - 100 % = 4

		Control		**Bacterial mix**		**Dead RU47**		**RU47**	
	Unit	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Plant properties (36 DAS)									
Shoot height	[cm]	33.8 ^{a}	± 0.6	17.2 ^{b}	± 2.8	27.1 ^{a}	± 0.7	26.4 ^{ab}	± 1.8
Stem diameter	[cm]	0.5 ^{a}	± 0.0	0.3 ^{b}	± 0.0	0.5 ^{a}	± 0.0	0.5 ^{a}	± 0.0
Leaf number	-	5.5 ^{a}	± 0.3	3.3 ^{b}	± 0.3	5.0 ^{a}	± 0.0	4.8 ^{a}	± 0.3
Leaf area	[cm^2]	775.6 ^{a}	± 11.3	205.4 ^{b}	± 84.2	570.6 ^{ab}	± 34.8	567.8 ^{ab}	± 101.7
Violet discolouration	-	0.0 ^{b}	± 0.0	2.5 ^{a}	± 0.5	0.8 ^{ab}	± 0.5	1.0 ^{a}	± 0.0
Bulk soil properties (39 DAS)									
pH ($CaCl_2$)	-	7.2 ^{b}	± 0.0	7.5 ^{a}	± 0.0	7.5 ^{a}	± 0.0	7.5 ^{a}	± 0.0
P_{H2O}	[mg kg^{-1}]	100.5 ^{a}	± 5.5	9.6 ^{b}	± 5.1	22.4 ^{b}	± 2.0	22.6 ^{b}	± 1.0

Molar microbial C:P ratio	-	305.1	±	92.1	55.1	±	16.1	80.4	±	8.5	73.7	± 7.2
NH_4^+	[mg kg^{-1}]	1.9	±	0.6	2.1	±	0.4	1.5	±	0.2	1.1	± 0.1
NO_3^-	[mg kg^{-1}]	141.7	±	4.0	159.5	±	3.5	142.5	±	5.4	150.6	± 4.5
$Gram^+$ PLFAs	[nmol FAME g^{-1}]	8.0	±	0.3	8.0	±	0.2	8.9	±	0.8	8.6	± 0.3
$Gram^-$ PLFAs	[nmol FAME g^{-1}]	1.2	±	0.0	1.2	±	0.0	1.3	±	0.1	1.3	± 0.1
Bacterial PLFAs	[nmol FAME g^{-1}]	14.2	±	0.5	14.2	±	0.3	15.7	±	1.4	15.2	± 0.6
Fungal PLFA	[nmol FAME g^{-1}]	0.4	±	0.0	0.4	±	0.0	0.4	±	0.1	0.4	± 0.0
Acid phosphomonoesterase	[nmol g^{-1} h^{-1}]	73.8	±	20.1	73.7	±	28.7	105.5	±	10.3	114.6	± 13.9
Alkaline phosphomonoesterase	[nmol g^{-1} h^{-1}]	475.9	±	33.5	503.8	±	38.2	518.5	±	31.1	584.1	± 19.0
ß-glucosidase	[nmol g^{-1} h^{-1}]	188.7	±	10.5	181.9	±	4.0	187.5	±	12.6	196.0	± 9.4
N-acetyl-ß-glucosaminidase	[nmol g^{-1} h^{-1}]	63.3	±	9.3	45.1	±	3.9	41.4	±	3.1	43.4	± 1.6
ß-xylosidase	[nmol g^{-1} h^{-1}]	13.2	±	3.2	34.1	±	17.6	17.9	±	1.3	17.5	± 1.4

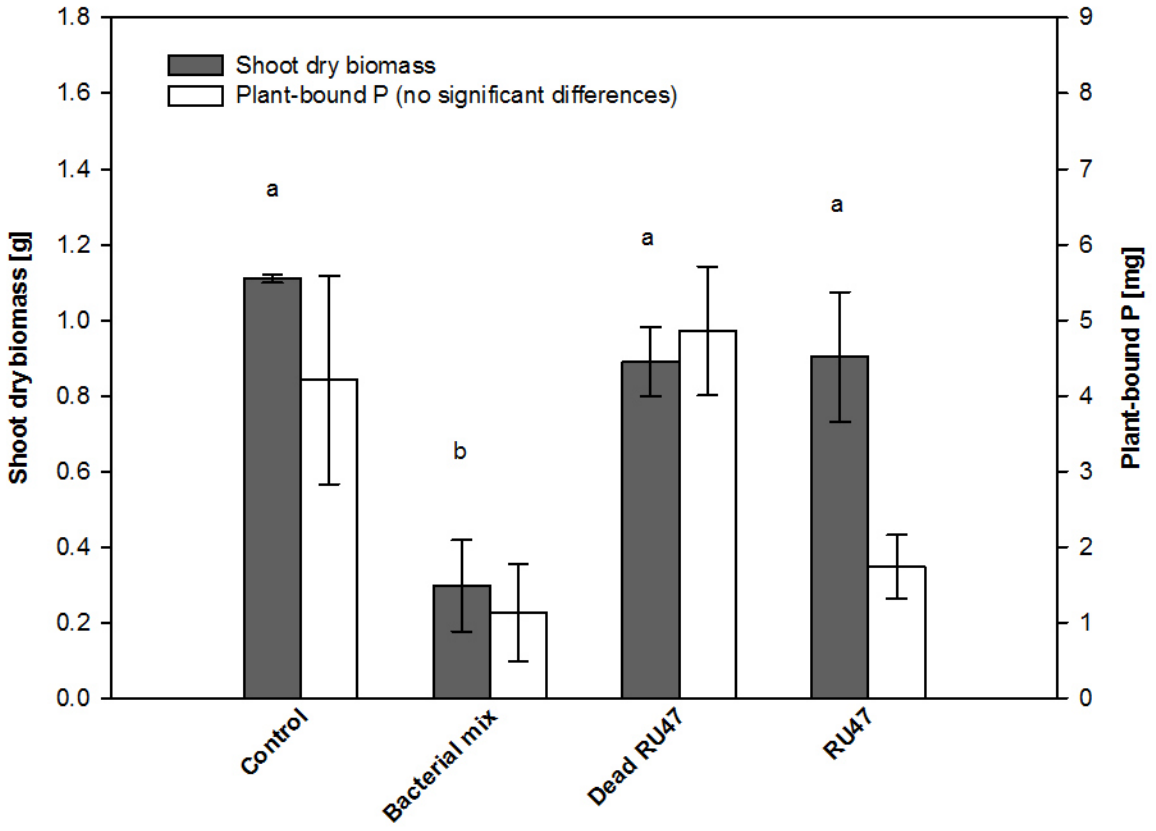

Figure 5.1 Shoot biomass (dry weight) and plant-bound P of tomato plants 39 days after sowing under the following treatments: one non-inoculation (control) and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4); significant differences (Tukey-HSD, $p < 0.05$) between the treatments are designated by lowercase letters.

5.3.3 Enzyme activities involved in P, C, and N cycle

The addition of living RU47 significantly increased alkaline PA in the rhizosphere of tomato on days 25-26 and 31-32 after sowing (Fig. 5.2 a). Alkaline PA in the rhizosphere of plants inoculated with living RU47 significantly increased over time, showing highest activity at 25-26 days after sowing, whereas the temporal pattern was stable in the dead RU47 treatment (Fig. 5.2 a). The activity of acid phosphatase was marginally less than that detected for alkaline PA (Fig. 5.2 a, Fig. 5.2 b). Acid PA increased slightly over time; significant increases of 21 % (bacterial mix) and 15 % (RU47) could be observed in the treatments to which living bacteria were added (Fig. 5.2 b). As expected, acid PA in the rhizosphere was positively correlated with plant properties (e.g. shoot height, Pearson's $r = 0.6$; $p < 0.00$). Potential alkaline and acid PA in homogeneous bulk soil samples after final harvest indicated highest activities in both RU47 treatments (Table 5.2). Alkaline PA in rhizosphere soil was positively correlated with the abundance of RU47 DNA sequence in rhizosphere and bulk soils (Pearson's $r = 0.6$; $p < 0.05$). RU47 inoculation did not influence enzyme activities in bulk soil

involved in C and N cycling (Table 5.2). Nevertheless, the bacterial mix treatment stimulated activities of the mainly fungus-derived β-xylosidase by more than 100 % (Table 5.2).

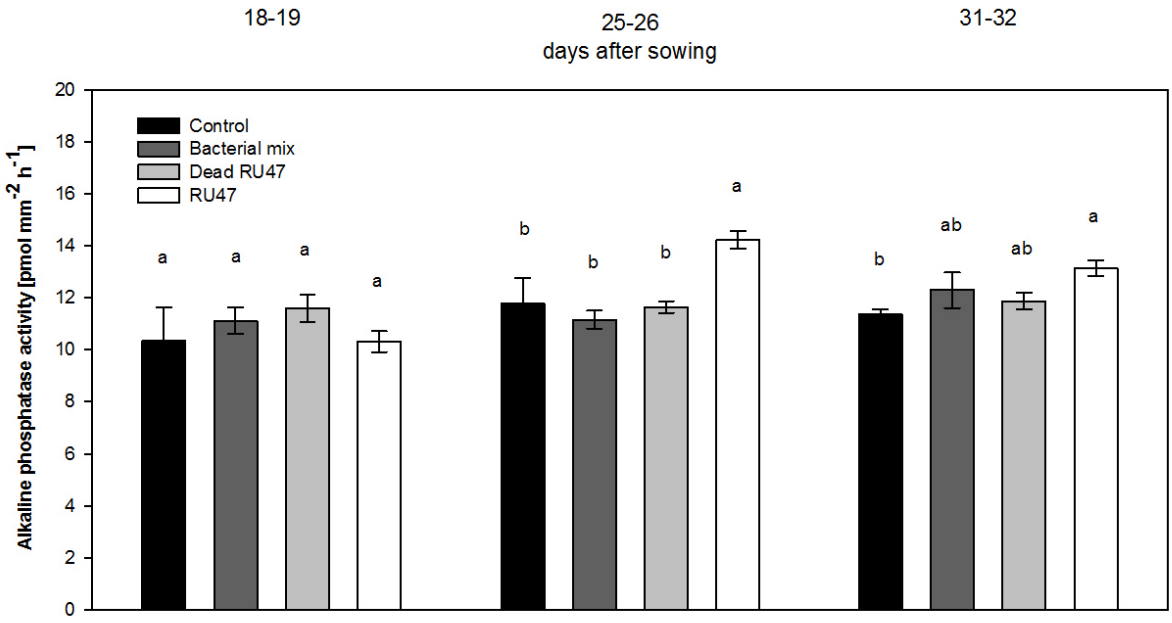

Figure 5.2 a Alkaline phosphomonoesterase activity during different growth stages of tomato plants: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead, or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4). Letters indicate significant differences (Tukey-HSD, $p < 0.05$) between the treatments, tested individually for each growth stage.

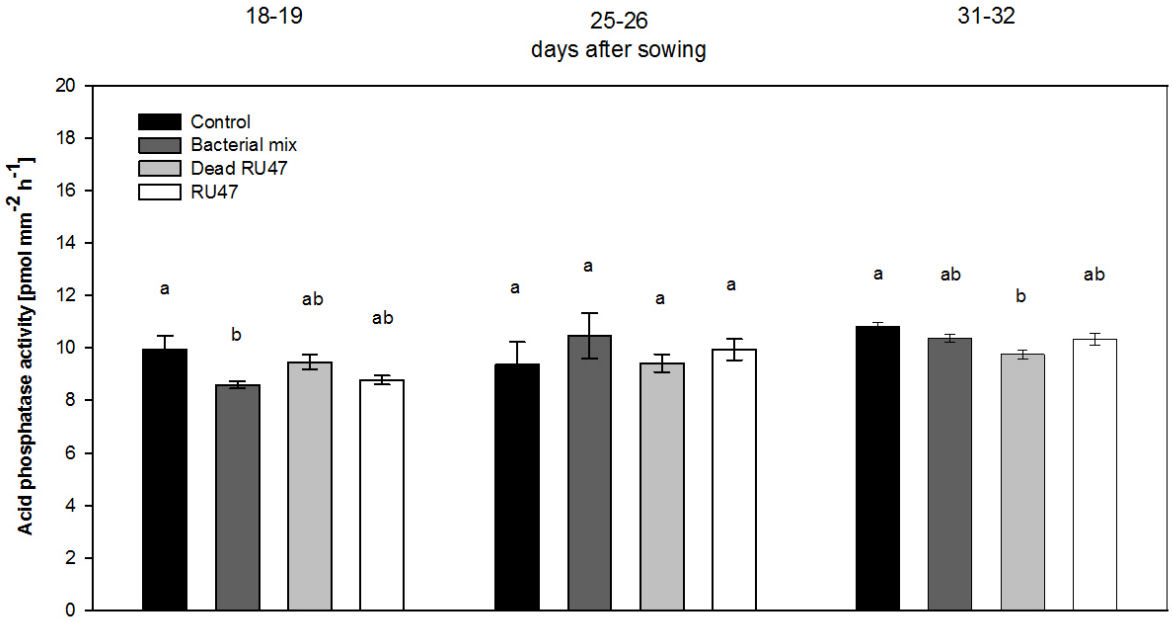

Figure 5.2 b Acid phosphomonoesterase activity during different growth stages of tomato plants: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead, or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4). Letters indicate significant differences (Tukey-HSD, $p < 0.05$) between the treatments tested individually for each growth stage.

5.3.4 Microbial biomass

RU47 inoculation did not influence microbial C content of the bulk soil (Fig. 5.3). Microbial biomass P was almost equal in all inoculation treatments and significantly higher than values detected in the control (Fig. 5.3). Thus, the calculated atomic C:P ratio of 305 in microbial biomass in the P-fertilized control was much higher (4.4 times) than the average C:P ratios of all other treatments (Table 5.2).

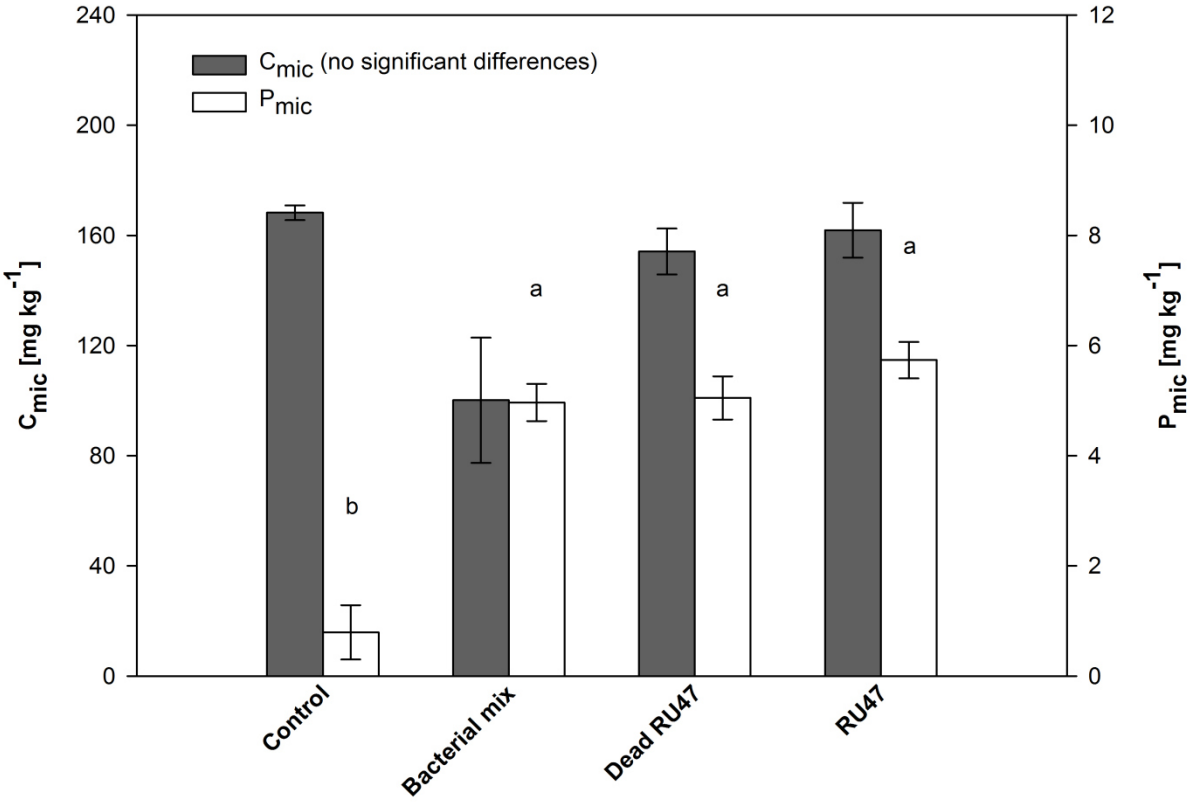

Figure 5.3 Microbially bound carbon (C_{mic}) and phosphorus (P_{mic}) in bulk soil of 39 day old tomato plants: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead, or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4); significant differences (Tukey-HSD, $p < 0.05$) between the treatments are designated by lowercase letters.

5.3.5 DGGE

In DGGE profiles, the treatment with RU47 revealed a strong band with an electrophoretic mobility like that of RU47, and a low bacterial diversity; this was in contrast to the treatment using dead RU47 cells (Fig. 5.4 a). UPGMA analysis showed that the rhizosphere samples of tomato plants grown in soil inoculated with RU47 clustered together; fingerprints of the plants grown in soil inoculated with dead RU47 formed a distinct cluster as well, but this cluster also contained the fingerprint of one control sample (Fig. 5.4 b). Highest bacterial diversity as well as an RU47-specific band was observed in the control and in the bacterial mix treatment

(Fig. 5.4 a). The permutation test revealed significant differences between the control and RU47 treatments with d-value 30.3 (Table 5.3). Moreover, high d-values indicating large differences were observed between the fingerprints of the dead RU47 and viable RU47 (43.2), and between the bacterial mix and dead RU47 (28.4) (Table 5.3). Fungal DGGE fingerprints exhibited diverse patterns with high variation both within and between treatments; only the fungal fingerprints of the control soils formed a distinct cluster (Fig. 5.5 a, Fig. 5.5 b). Significant but small differences were observed only between the fungal fingerprints of the control treatments and the bacterial mix, dead RU47, and RU47 with values of 8.9, 12.1 and 7.3, respectively (Fig. 5.5 a, Table 5.3).

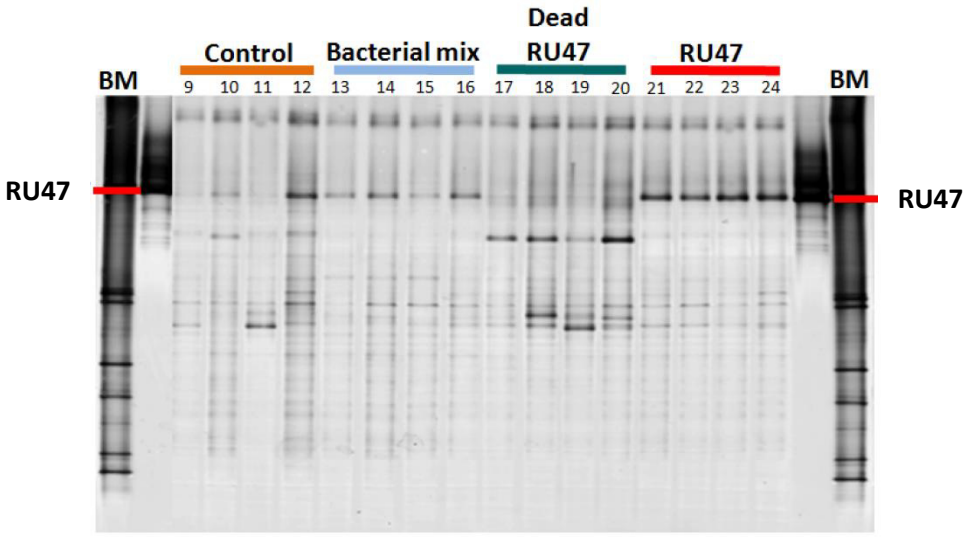

Figure 5.4 a DGGE fingerprints of bacterial 16S rRNA gene fragments from community DNA obtained from rhizosphere of tomato plants 39 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. BM stands for bacterial marker.

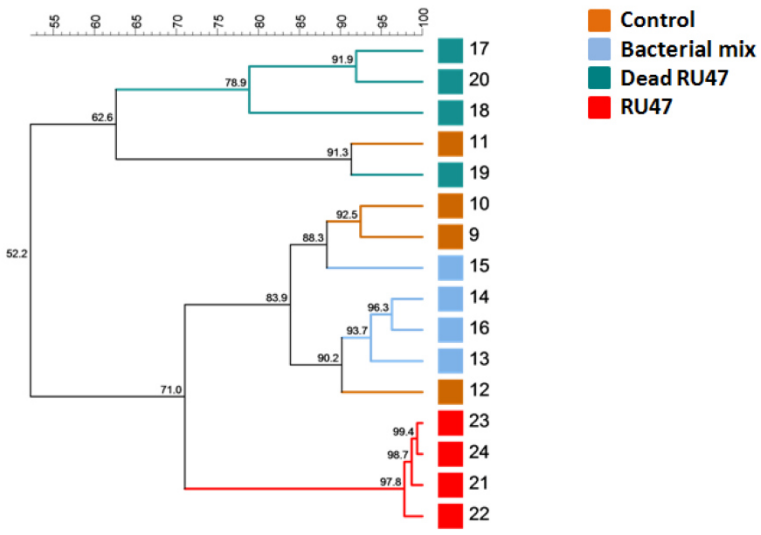

Figure 5.4 b Bacterial DGGE fingerprints (Fig. 5.4 a) corresponding to UPGMA dendrogram based on Pearson similarity matrix (Heuer *et al.* 2001). Bacterial fingerprints were from community DNA obtained from rhizosphere of tomato plants 39 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells.

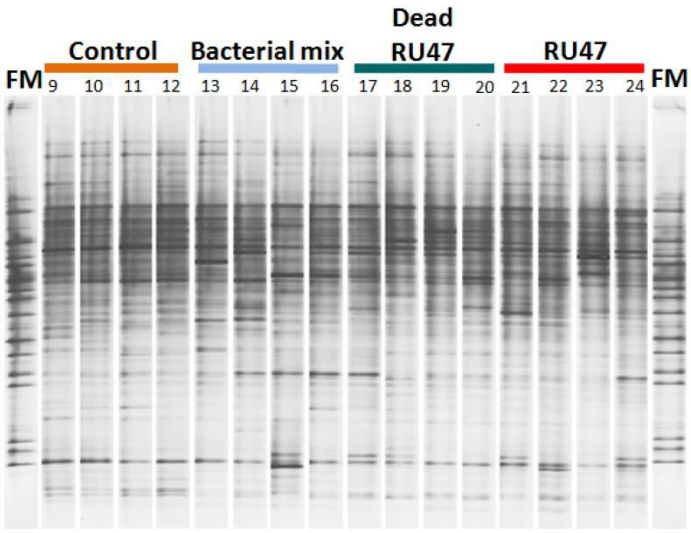

Figure 5.5 a DGGE fingerprints of fungal ITS fragments from community DNA obtained from rhizosphere of tomato plants 39 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. FM stands for ITS marker.

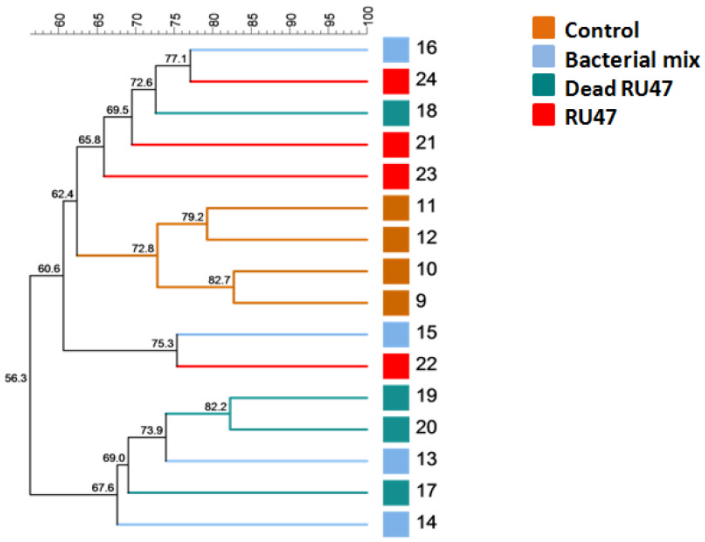

Figure 5.5 b Fungal DGGE fingerprints (Fig. 5.5 a) corresponding to UPGMA dendrogram based on Pearson similarity matrix (Heuer *et al.* 2001). Fungal fingerprints were from community DNA obtained from rhizosphere of tomato plants 39 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells.

Table 5.3 Percent dissimilarity (d-value) between rhizosphere DGGE fingerprints from community DNA obtained from rhizosphere of tomato plants 39 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells.

	Differences in the rhizosphere between the treatments	
Pairwise comparison	**16S rRNA gene**	**ITS**
Control *vs* bacterial mix	3.2	8.9*
Control *vs* dead RU47	8.5	12.1*
Control *vs* RU47	30.3*	7.3*
Bacterial mix *vs* dead RU47	28.4*	-2.1
Bacterial mix *vs* RU47	19.0*	2.1
Dead RU47 *vs* RU47	43.2*	7.0

* Significant difference ($p \leq 0.05$) according to Kropf *et al.* (2004)

5.3.6 PLFA

The addition of RU47 did not result in significant shifts in microbial community composition based on PLFA patterns (Table 5.2). However, while bulk soils of the control and bacterial mix treatments exhibited identical PLFA patterns, abundances of bacterial PLFAs were higher in bulk soil inoculated with dead RU47 or RU47 cells by 11 and 7 %, respectively (Table 5.2). Abundances of PLFAs representing gram$^+$ bacteria were higher by around 9 % in treatments using dead RU47 and living RU47 compared to the control and bacterial mix treatments (Table 5.2).

5.4 Discussion

Plant growth

In tomato plants inoculated with RU47, not only stem diameter and leaf number but also 3-fold higher shoot biomass was observed in comparison to plants grown in soils containing the bacterial mix (Table 5.2, Fig. 5.1). Therefore, the present study confirms the results of Kim *et al.* (1997), who documented a 2-fold higher plant biomass in 35 day-old tomato plants inoculated with *Enterobacter agglomerans* cells compared to the non- inoculated control. However, in our study, a growth-promoting effect was also observed in tomato plants inoculated with dead RU47 cells (Fig. 5.1, Table 5.2). The difference between the treatments RU47 and dead RU47 makes it possible to estimate whether potential plant growth-promotion was a result of direct or indirect mechanisms. Direct mechanisms can include, for example, the production of phosphatases by RU47 resulting in an improved supply of P by plants. Indirect mechanisms include the release of cell-derived phytohormones or other compounds which may stimulate indigenous microbes and their activity in soil. These indirect mechanisms are discussed in more detail below.

Tracing RU47

The strain RU47 was originally isolated from a soil which had previously been reported as suppressive to phytopathogenic fungi (Adesina *et al.* 2007). The qPCR primer system used for detection and quantification of RU47 was developed based on the draft genome sequence of RU47 (Ding *et al. unpublished*). Based on a comparative analysis of *Pseudomonas* genomes published at the time, this sequence was assumed to be RU47-specific. However, RU47 sequences were also detected in soil inoculated with the bacterial mix, with dead RU47 cells, and in the control soil. It is likely that taxonomically closely related *Pseudomonas* belonged to the indigenous bacterial community of the soil used in the experiment. In our study, the inoculated RU47 could successfully be detected by qPCR, as RU47 was significantly more abundant in the treatments with viable RU47 cells both in the rhizosphere and in bulk soil than in all other treatments, including the control soil. The significantly higher copy numbers of the RU47-specific sequence in rhizosphere as compared to bulk soils of all treatments may have indicated higher competency of RU47-like populations in the rhizosphere, but observed differences may also have been due to differences in processing rhizosphere and bulk soil samples. High abundances of RU47 in the rhizosphere soils of this study are in accordance with Adesina *et al.* (2009) and Schreiter *et al.* (2014 b, c). Both studies investigated the ability of RU47 to colonize the rhizosphere of lettuce either in growth chambers or under field conditions. However, in these studies,

cultivation-dependent methods were used. The RU47 is a spontaneous rifampicin-resistant mutant; this makes sensitive and specific detection of RU47 in rhizosphere and bulk soil possible using selective plating. Unfortunately, selective plating was not used in the present experiment. Commercial as well as non-commercial PMB strains, including RU47, were isolated from indigenous microbial communities associated with soils and plants. In contrast to genetically modified strains, specific and sensitive monitoring of naturally occurring strains is more difficult. The literature, though sparse, suggests that survival of inoculants such as PMB is difficult to track and that the inoculants exhibit great temporal and spatial dependency. A temporal decrease in abundance has frequently been reported (Kim *et al.* 1997, Dey *et al.* 2004, Hameeda *et al.* 2008, Meyer *et al.* 2017). For instance, Meyer *et al.* (2017) documented a loss of more than 99 % of the inoculated *Pseudomonas protegens* CHA0 cells within 40 days. Kim *et al.* (1997) determined that *Enterobacter agglomerans* found in the rhizosphere of non-inoculated tomato plants (35 days after sowing) corresponded to almost 50 % of the abundance in the inoculation treatment. These findings are somewhat consistent with our results, in that the relative abundance of RU47 in the treatments not inoculated with RU47 corresponded to 8 % (rhizosphere) and 7 % (bulk soil) of the abundance detected in the treatment to which RU47 was added (Table 5.1).

Improved P supply

Data on effects of PMB addition on plant growth and P uptake are rare and somewhat inconsistent. However, Egamberdiyeva (2007) and Kumar *et al.* (2013) demonstrated improved P uptake in maize and mustard respectively due to addition of single PMB strains. These findings are consistent with the results of our study, which showed a trend of increased P uptake in tomato plants inoculated with dead RU47 and viable RU47 (Fig. 5.1). In evaluating plant P uptake, P tissue concentration is the meaningful value because differences resulting from variations in plant growth are excluded. Variations in plant growth may therefore explain the adequate P tissue concentration of 0.4 % (calculated from data of P uptake and shoot biomass given in Fig. 5.1) that was observed not only in the optimally P-fertilized control but also in the plants inoculated with a bacterial mix. In the bacterial mix treatment, the lowest amounts of available P in the soils (from small starter P fertilization at the beginning of the experiment) were taken up by the plants in comparison to the other treatments, and this P was not enough to maintain growth (Fig. 5.1). The previously incorporated P was concentrated in the small biomass, resulting in apparent adequate initial P tissue concentration of 0.4 %, but this was a concentration effect of low tissue biomass. As plants grew, the initially adsorbed P was no longer available, and these plants then exhibited P deprivation, as indicated by violet discoloration of leaves (Table 5.2). Plants inoculated with

RU47 had a P tissue concentration of 0.2 %, which is in close agreement with the data reported by Kim *et al.* (1997) but may also indicate competition for available P between added bacteria and plant. This assumption is supported by the optimal P tissue concentration of 0.6 % found in plants which were inoculated with dead RU47 cells. In these plants competition was reduced, while highest P_{mic} values were determined in soil of the viable RU47 treatment, a condition in which competition between plants and bacteria is expected to be highest (Fig. 5.3). An improved P supply by the addition of RU47 or dead RU47 was also observed by a 2-fold higher P_{H2O} concentration compared to the bacterial mix treatment (Table 5.2). However, a fertilizing effect due to addition of dead RU47 cells can be excluded since N and P concentrations in cell suspension (OD_{600} = 1) were determined as 41.5 and 0.9 µg mL^{-1}, respectively, corresponding to a total N and P addition of less than 0.6 mg kg^{-1} (data not shown). These values are negligible in comparison to the initial slight P fertilization (50 mg kg^{-1}) and the optimal fertilized control (200 mg kg^{-1}).

Improved P mobilization in soil

An improved P supply by PMB, including some *Pseudomonas* strains, has been reported in several studies (for overviews see Rodríguez and Fraga 1999, Khan et al. 2007, 2009 a, Harvey *et al.* 2009). For instance, Malboobi *et al.* (2009) documented effective mobilization of inorganic and organic phosphate compounds by *Pseudomonas putida* P13 in culture media. Similar findings were reported by Pastor *et al.* (2012). They observed growth stimulation of tomato seedlings by the addition of *P. putida* PCI2 and were able to identify this strain as positive for PA and highly effective for solubilizing Al- and Ca-bound phosphates. In our study, inoculations with RU47 resulted in increased alkaline phosphomonoesterase activity in the rhizosphere of tomato plants (Fig. 5.2 a). Moreover, enzyme activity increased from 18-19 to 25-26 days after sowing, likely due to increasing bacterial colonization and P depletion. In contrast, alkaline PA in the rhizosphere inoculated with dead RU47 remained stable (Fig. 5.2 a). These findings suggest increased P mineralization by microbial phosphatases produced by viable RU47. Supporting this, alkaline PA was positively correlated with the abundance of RU47 (Pearson's r = 0.6; $p < 0.05$) in the rhizosphere, whereas acid PA was not influenced by RU47 inoculation (Fig. 5.2 b). Zymography revealed generally similar activity levels for alkaline and acid PA, which is in accordance with Spohn *et al.* (2015). They determined the PA in the rhizosphere of barley grown under low and adequate P soil conditions and observed a similarity of approximately 90 % between alkaline and acid PA. In contrast to the experiment of Spohn *et al.* (2015), we observed no distinct separation between roots and surrounding soil (Fig. 5.S1) and also generally lower PA (Fig. 5.2 a, Fig. 5.2 b). This may be attributable to the comparatively fine roots of tomato plants as compared

to barley. Lower enzyme activity levels in comparison to the values of Spohn *et al.* (2015) may have been due to the addition of quartz sand in the present experiment.

PA-data determined by soil *in situ* zymography indicated the spatial and temporal distributions of enzyme activity in the rhizosphere (soil area) during different growth stages of the tomato plants. Enzyme analyses performed after final harvest enabled us to gain additional information about the potential PA in bulk soil (soil body) at a single time point. Measured highest alkaline and acid PA values in soil inoculated with RU47 after final harvest agreed with our soil zymography results (Table 5.2, Fig. 5.2 a, Fig. 5.2 b). In comparison to the bacterial mix treatment, alkaline PA increased by 16 % (Table 5.2). These results are in agreement with those obtained by Kaur and Reddy (2014), who documented increases in alkaline PA of 31 % due to the addition of *Pseudomonas plecoglossicida* in soil of wheat plants. These findings reinforce the evidence for improved P mineralization by the addition of RU47. Nevertheless, bulk soil inoculated with dead RU47 cells also revealed increased PA (Table 5.2), despite the efficiency of our RU47 cell death method and the denaturation of phosphatases. The increased PA observed in the dead RU47 treatment may have been due to enhanced growth and activity of previously dormant microbial populations through the addition of bacterial residues (dead RU47 cells) to soil and thus a supply of fresh organic matter (FOM), also known as the priming effect (Bingeman *et al.* 1953, Fontaine *et al.* 2003).

Interactions with indigenous soil microbes and hormone-derived effects

Bacterial DGGE profiles (Fig. 5.4 a) confirmed the competence of living RU47 in the rhizosphere, and its occurrence in the natural rhizosphere microbial community was additionally supported by the quantitative PCR data (Table 5.1). However, no RU47-specific band was observed in the bacterial DGGE fingerprint of rhizosphere DNA inoculated with dead RU47 (Fig. 5.4 a). This was confirmed by results measured by the more sensitive TaqMan® assay, indicating the lowest RU47 abundance in the dead RU47 treatment (Table 5.1). This observation clearly indicates destruction of DNA due both to boiling and, more likely, to microbial degradation in soil. The latter was confirmed by the fact that in the bacterial DGGE fingerprint of the dead RU47 treatment, numerous dominant bands were observed which were absent or much less distinctly expressed in all other treatments (Fig. 5.4 a). We suggest, therefore, that inoculation with dead RU47 cells, and thus the addition of FOM to soil, resulted in a priming effect that enhanced bacterial populations specializing in the decomposition of FOM (Griffiths *et al.* 1998, Fontaine *et al.* 2003). This, in turn, could have resulted in the growth of bacterial populations responding to the nutrient spike accompanied by increased microbial PA, resulting in the observed improved P supply for the tomato plants in this treatment (Table 5.2).

Although we found no significant effects on PLFA patterns, bulk soil inoculated with RU47 cells showed a minor increase in bacterial abundance, especially of gram$^+$ bacteria (Table 5.2). This may indicate a microbiome shift as a result of RU47 inoculation, a finding also reported by Schreiter *et al.* (2014 a). Although plants treated with RU47 showed comparably low bacterial diversity in DGGE fingerprints (Fig. 5.4 a), the PLFA analysis was done with bulk soil; its microbial community structure could have differed materially from that of the rhizosphere (Maloney *et al.* 1997, Smalla *et al.* 2001). It is possible that an increased population of indigenous PMB in bulk soil may have been due to the addition of RU47. An increase in abundance of indigenous PMB after the application of specific PMB strains has also been reported by Sundara *et al.* (2002) and Canbolat *et al.* (2006). With respect to the increased microbial PA and improved P supply determined in bulk soils of both RU47 treatments (Table 5.2), there may be a complementary PMB-attracting effect in plant growth-promotion of RU47. To solve this open question, functional gene analyses of phosphomonoesterases could clarify identities of the main producers of different phosphomonoesterases, but these analyses were not possible in the present study.

Plant growth-promotion can be strongly influenced by modulation of the phytohormone level of the plant. Several studies have shown that many soil bacteria, including *Pseudomonas*, are able to synthesize phytohormones or the enzyme ACC deaminase, affecting the plant's hormonal balance and thus its growth and response to stress, ensuring the bacteria's supply of C resources from root exudation (for review see Tsavkelova *et al.* 2006, Glick 2012, 2014). Hence, the plant growth-promotion observed in both RU47 treatments was likely due to a hormonally mediated effect. Rajkumar and Freitas (2008) reported a strong effect of ACC deaminase production by *P. jessenii* M6; this was also detected for RU47 (Smalla 2016, *personal communication*). But, due to enzyme inactivation in the dead RU47 treatment, an ACC deaminase effect was negligible here. However, it is known that phytohormones such as auxins and cytokinins can remain stable after heating to 121 °C (Murashige and Skoog 1962, Kumar and Singh 2009). Therefore, it is entirely possible that co-extracted thermally stable phytohormones produced by RU47 before they were killed were added through inoculation of dead RU47 cells. Furthermore, the addition of dead RU47 cells and thus a supply of FOM in the form of bacterial residues may have promoted indigenous soil microorganisms synthesizing phytohormones. Taken together, the improved P supply in plants inoculated with dead RU47 or viable RU47 may have been due to phytohormones, stimulating root growth and activity, and improving P acquisition in soil.

5.5 Conclusion

This study demonstrated that addition of RU47 improves the P supply and subsequent growth of tomato plants under P-limited growing conditions. Furthermore, it indicated enhanced production of alkaline phosphatase by RU47. This is the first study to compare the effects of adding vital and devitalized RU47 cells to plants and soil. Both treatments resulted in improved P supply and plant growth promotion. The plant growth-promoting effect was likely caused by increased PA in the rhizosphere of tomato amended with viable RU47. In the dead RU47 treatment, the bacterial populations which proliferated in response to the added resource may have contributed to improved P supply and growth promotion via other mechanisms. Thus, the use of RU47 offers a promising approach for more efficient P fertilization in agriculture. In contrast to our hypothesis that the colonization of RU47 leads to spatially distinct zones of increased PA in the rhizosphere, no clear differences in rhizosphere and bulk soil were found. This was likely due to the fine roots of tomato plants and homogeneously distributed enzyme activity of the topsoil used in the treatments. We found no significant effects of RU47 on soil microbial community structure as determined by PLFAs, but we detected significant shifts using DGGE. Our study shows that RU47 increases microbial PA in soils with low P availability and leads to growth promotion of tomato plants.

Supplemental Material

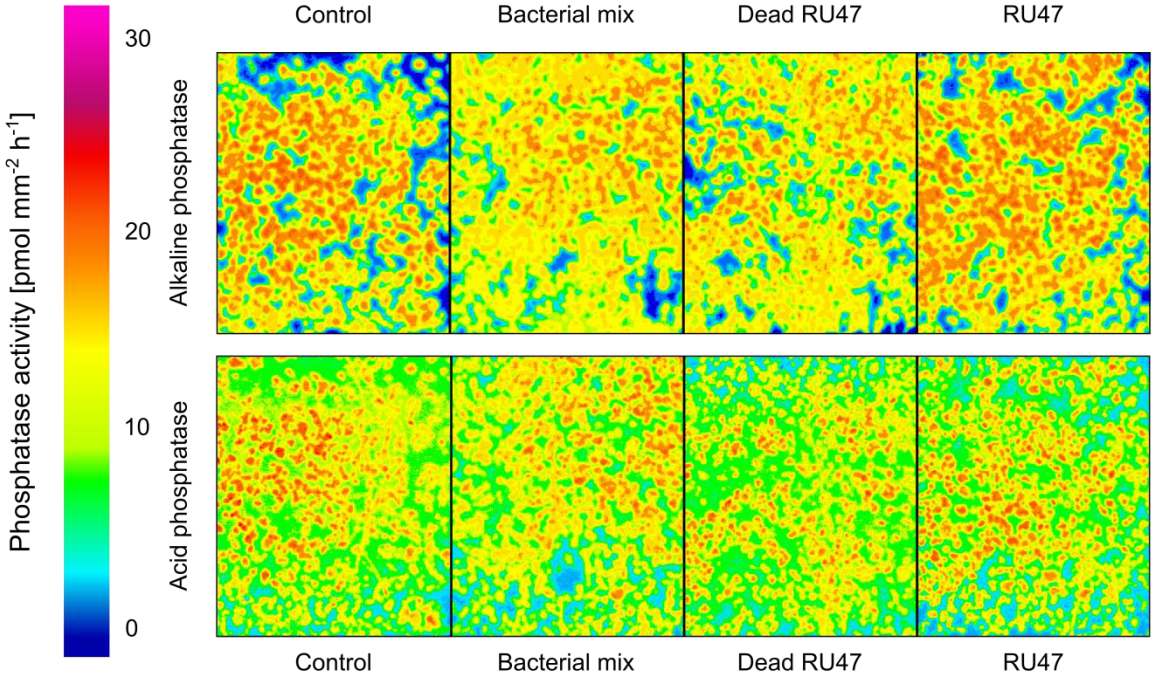

Figure 5.S1 Soil zymograms of alkaline (pH 11) and of acid phosphomonoesterase (pH 6.5) in the rhizosphere of tomato plants 31-32 days after sowing (together with the calibration line) under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Each treatment is represented by one replicate.

6 Short-term effects of phosphorus-mobilizing bacteria on maize growth and soil microbial activity

6.1 Introduction

Maize (*Zea mays* L.) is one of the most cultivated arable crops in the world; the top three maize-producing countries, the United States, China, and Brazil, alone produce around 563-717 million metric tons per year (Ranum *et al.* 2014). Due to its increasing use for bioethanol world wide corn production has significantly increased in the last ten years (Ranum *et al.* 2014). However, maize places high demands on fertilization, especially in its early growth stage. Using ^{32}P, a study by Nadeem *et al.* (2011) demonstrated that 96 % of the endosperm phytate representing the main P source in a maize seed was hydrolysed within the first seven days after sowing; P uptake by the roots starts only five days after sowing. An adequate P supply in the early growth stages of maize plants is a crucial factor for a high productivity; the kernel number especially is most sensitive to P nutrition during the period of sowing to 6-leaf-stage (Barry and Miller 1989). While in conventional farming the under-root fertilisation with di-ammonium phosphate has proven to effectively suppress P deficiency in maize seedlings, this method is not possible in organic farming due to legal restrictions. Furthermore, the increasing scarcity of rock phosphate resources has made the development of an organic and more efficient P fertilization in agriculture even more urgent. In this respect, the use of PMB, meaning beneficial bacteria, to effectively mobilize insoluble P compounds in soil, offers a promising approach. Positive effects by the targeted application of PMB strains, usually strains from the genera *Bacillus*, *Pseudomonas, Azospirillum* and *Rhizobium*, on plant growth in P limited soils have been reported several times (Chabot *et al.* 1996, Sundara *et al.* 2002, Kaur and Reddy 2014). However, the underlying functional mechanisms are not clearly understood.

In principal, three different microbially driven mechanisms of P mobilization can be defined: First, the enzymatic hydrolysis of organic P compounds (Tarafdar and Claassen 1988, Juma and Tabatabai 1988, Nannipieri *et al.* 2011); second, the ability to solubilize bound P by the release of chelating substance, organic acids and protons (Kpomblekou-a and Tabatabai 1994, Jones and Oburger 2011); and third, interactions with beneficial indigenous microbes optimizing P mobilization in soil (Belimov *et al.* 1995, Zaidi *et al.* 2003). Thus, identifying the main mechanisms of PMB is prerequisite for their successful and efficient use in agriculture in the future. In a previous study, using RU47 as the PMB and tomato as the test plant, we conducted a novel experiment inoculating vital as well as dead PMB cells to distinguish

between P mobilizing dependent and independent mechanisms (5). Results of this 39-day experiment under low P soil conditions revealed a plant growth-promoting effect and improved P supply not only by the addition of RU47 but also of dead RU47 (5.3). The effect of RU47 was clearly associated with an increased microbial PA, but we also found indications of a simultaneous hormone-derived effect increasing plant P acquisition by the roots; this was likely the main mechanism when dead RU47 was added (5.3). However, so far, the ability of microbial inoculants to colonize the rhizosphere at sufficient abundance has been described as the crucial factor for their beneficial effects on plants (De Bellis and Ercolani 2001, Barret *et al.* 2011). Based on the tomato experiment, and with regard to the existing challenge in corn production, we wanted to verify whether RU47 is able to improve maize P supply in the short and sensitive period of earliest growth stage by a colonization-dependent or -independent mechanism. Therefore, we performed a short-term rhizobox experiment with maize repeating all conditions performed in the tomato experiment (5.2.1) and hypothesised: (1) Inoculated RU47 as well as dead RU47 improves plant growth. (2) The plant growth-promoting effect by the addition of RU47 is based on an improved P supply. (3) RU47 dominantly colonizes the rhizosphere leading to spatially distinct zones of enriched microbial PA.

6.2 Materials and methods

6.2.1 Rhizobox experiment

The experiment was performed under low P availability soil conditions using *Pseudomonas* sp. RU47 as the PMB and maize (*Zea mays* L. var. Colisee) as the test plant. To exclude apparent plant growth-promoting effects of the PMB as a result of increasing microbial activity by adding living soil bacteria and/or cell compounds affecting the P efficiency of plant and indigenous soil microbes, two additional inoculation treatments were conducted, each comprising an inoculation of unselectively cultivated indigenous soil bacteria (bacterial mix) or dead RU47 cells. Details of bacteria cultivation and inoculation are briefly described in 6.2.2. Furthermore, two non-inoculation treatments were added serving as the controls. First, an optimally P-fertilized (200 mg kg^{-1}; $Ca(H_2PO_4)_2$) control and second, a no-plant variant (00-control) allowing an insight into microbial activity in soil when root and inoculation associated influences are absent. Hence, the experiment consisted of five treatments, whereby each treatment comprised four replicates. Maize plants grown in rhizoboxes with an inner size of 28.0 cm x 4.5 cm x 16.5 cm, filled with a soil substrate composed of Luvisol topsoil and quartz sand (0.2 - 1.4 mm) in a ratio of 1:1 (w/w). The Luvisol was considered as a heavy loam soil and was selected on the basis of its low concentration of plant available

phosphorus (CAL extraction of 20 mg kg^{-1}). The soil was taken from unfertilized grassland located on the campus of the University of Hohenheim; its soil characteristics are given in detail in 5.2.1. Each rhizobox was filled with 1918.0 g DM of sieved (< 5 mm) soil substrate; before sowing soil substrate was optimally fertilized regarding N, K and Mg. Application of fertilization was exactly done as performed in the tomato experiment (5) and is given in detail in 5.2.1. Excluding the optimally P-fertilized treatment, an application of 50 mg P kg^{-1} ($Ca(H_2PO_4)_2$) was add to each rhizobox enabling a successful germination. Three maize seeds were sown with a depth of 4 cm directly in each rhizobox and thinned out after germination (one plant per rhizobox). In order to make roots grow along the hinged wall, rhizoboxes were placed with a 50 ° inclination. In order to avoid light-derived influences on root's growth and behaviour, all boxes were wrapped in aluminium foil. The experiment was conducted for 14 days under greenhouse conditions. Rhizoboxes were placed randomly on wooden planks to exclude contaminations by leaking irrigation water. Plants were constantly watered maintaining a water holding capacity of 50 %; the water content was checked gravimetrically on a daily basis. Watering was performed using deionized water (H_2O_{deion}), successively applied in 5 mL steps to avoid direct leakage along rhizobox edges.

6.2.2 Bacterial cultivation and inoculation

RU47 was cultured in King's B liquid medium (King *et al.* 1954) added with rifampicin (50 mg L^{-1}), bacterial mix in a glucose-enriched (2 g L^{-1}) LB-Lennox liquid medium (Bertani 1951, Lennox 1955) at 28.5 °C for 24 h, respectively. Bacteria cells were washed twice using sterile 0.3 % NaCl solution and subsequently adjust to a cell density of 10^9 cell mL^{-1} (OD_{600} = 1.0, Xue *et al.* 2013) by the use of a photometer (BioPhotometer, Eppendorf, Germany). The devitalisation of RU47, which were used in one of the treatments, was performed by boiling for 1 min on a heating plate. Pretests have proven that this procedure was sufficient to kill RU47 completely (plate tests). Further details of cell cultivation and preparation are given in 5.2.2.

Plants were twofold inoculated with a cell density of 10^9 cell mL^{-1} (OD_{600} = 1.0). A first inoculation was conducted by seed coating. For this purpose, 25 µL of a cell suspension were slowly added to five maize seeds and gently vortexed. Seed coating was controlled by using three of the inoculated seeds followed by washing with 1 mL sterile 0.3 % NaCl solution and plating 100 µl of the suspension on King's B-Agar medium (50 mg rifampicin L^{-1}) in three dilution stages, respectively. Plates were incubated at 28.5 °C until growing colonies were unequivocally countable on the agar (approximately after 36 h). A second inoculation was performed directly after seed germination using 6 mL cell suspension per kg DM soil

substrate, directly applied on the soil surface to simulate farmer's practice. To avoid watering effects, control treatments were inoculated with 0.3 % NaCl solution with corresponding volumes per inoculation, respectively. Viability and unviability of used RU47 as well as sterility of 0.3 % NaCl solution were controlled by plating and subsequent incubation at 28.5 °C for 48 h after every inoculation.

6.2.3 Plant sampling and analyses

In order to determine the root area, visible roots grown along the hinged wall were photographed using a digital camera (D60, Nikon, Tokyo, Japan) 14 days after sowing. Photographs of each rhizobox were taken in identical distance and angle including a benchmark. Image processing and analysis were done using the open source software GIMP. The digital images were transferred to 8-bit; roots were selected by colour (threshold 16.0) followed by calculating the root area based on the number of pixels. At the end of the experiment, shoots of every replicate were cut using a sterilized (70 % ethanol) scalpel. Shoots were briefly rinsed with H_2O_{deion} water to remove adhering dust followed by drying at 60 °C in separate aluminium trays for 3 days to estimate the dry weight.

Determination the P concentration in maize shoots was performed by a sequential microwave digestion according to Kalra *et al.* (1989) followed by a photometric measurement according to the detailed description given in 5.2.3. Briefly, H_2O_{deion}, HNO_3 and H_2O_2 were added to the ground samples with a range of 0.01 - 0.04 g dry matter, filled into Teflon containers and were subsequently sequentially incinerated by the use of a ETHOS.lab microwave (MLS, Leutkirch, Germany). The filtered and diluted samples were photometrically measured using Murphy and Riley colour reagent (Murphy and Riley 1962). Measurement was performed at 710 nm using a microplate absorption reader (ELx808; BioTek Instruments Inc., Winooski, VT, USA). The calculation of P concentration is based on a linear function using the following final concentrations of 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg P L^{-1} prepared with K_2HPO_4.

6.2.4 Soil sampling

Rhizosphere and bulk soil samples were directly put on ice for short-term storage. While the rhizosphere soil was directly used for DNA extraction (see 6.2.5), bulk soil samples were sieved (< 2 mm), where each replicate was aliquoted and freeze at -20 °C until analysing.

6.2.5 Tracing RU47

DNA extraction

DNA extraction of the rhizosphere was performed at the Institute of Epidemiology and Pathogendiagnostic (Julius Kühn-Institut, Braunschweig, Germany). The complete root system as well as adhering rhizosphere soil of each maize plant as was used for DNA extraction based on a method of Schreiter *et al.* (2014 c); a detailed description of extraction preparation is given in 5.2.5. The rhizosphere DNA was extracted using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) followed by a purification (GENE CLEAN SPIN Kit®, MP Biomedicals, Solon, OH, USA) and dilution with 10 mM Tris HCl (pH 8.0) at a ratio of 1:10 before use. The DNA extraction of bulk soil was performed based on a weight of 250 - 350 g fresh soil using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following manufacturer's instructions. A spectrophotometer (NanoDrop 2000; Thermo Scientific, Waltham, MA, USA) was used to measure the DNA concentration and control the extract's purity.

Tracing RU47

Inoculated RU47 cells were traced by a TaqMan® assay using a 5'-labelled 6-FAM double-quenched (BMN-Q530) probe (biomers.net, Ulm, Germany) in a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Used sequences of primers and probe (Eltlbany *et al. in preparation*) were developed and provided by the Institute of Epidemiology and Pathogendiagnostic (Julius Kühn-Institut, Braunschweig, Germany). Details of reaction composition and thermal profile are given in 5.2.5. Based on the standard curve the absolute quantity of RU47 sequences (copies ng^{-1} DNA) was calculated.

6.2.6 Enzyme assays

Soil in situ zymography

Soil *in situ* zymography enables information about the distribution of exoenzymes in soil using membranes coated with MUF substrates which become florescent while enzyme cleavage. The distribution of alkaline as well as acid phosphomonoesterase (EC 3.1.3) was analysed by soil *in situ* zymography based on the method developed by Spohn and Kuzyakov (2014). A more detailed description of the assay performed is given in 5.2.6. Briefly, MUF-phosphate (4-MUF; Sigma–Aldrich, St. Louis, MO, USA) was used as substrate; preparing a 12 mM solution for coating the polyamide membranes with a diameter of 14.2 cm and a pore size of 0.45 µm (Sartorius, Göttingen, Germany). Substrate solution was prepared using MUB buffer adjusted to pH 11 (alkaline PA) and pH 6.5 (acid PA). Soil zymography was performed 14 days after sowing by using each replicate of all planted rhizoboxes. Each enzyme was measured separately on the same rhizobox beginning with

the acid PA, and followed by the alkaline PA. The coated membranes were incubated for 35 min at a constant temperature of 20 °C. After incubation the removed membranes were lightened at 360 nm wavelength in the dark (epi-UV-desk, Desaga, Sarstedt, Nümbrecht, Germany) and subsequently photographed with a digital camera (D60, Nikon, Tokyo, Japan). Image processing and analysis of the zymograms were done using the open source software ImageJ. The digital images were transformed to 8-bit, enhancing the contrast multiplied by the factor of 1.2 and transformed in false colours. To determine a spatially distinct average PA of root zone and surrounding soil enzyme image of each rhizobox was subdivided using the colour selection tool (threshold 16.0) of the open source software GIMP. As there was no distinct separation in alkaline PA between root zone and surrounding soil observed, the mean activity of the total incubated area was calculated, respectively. The calculation of enzyme activity is based on a linear function using a calibration line comprising different concentrations of 4-methylumbelliferone (0, 35, 70, 130, 200, 240 µM). Image processing of calibration zymograms was adapted to the modifications made with the soil zymograms. Calculation of enzyme activity bases on mean gray values given for each concentration in the calibration line.

Analyses of enzyme activities in bulk after final harvest

In addition to the soil *in situ* zymography, samples of the harvested bulk soil were used for analyses of potential alkaline as well as acid phosphatase (EC 3.1.3) activity using MUF-P (4-MUF; Sigma–Aldrich, St. Louis, MO, USA) according to Marx *et al.* (2001). The assay performed corresponds precisely to the description given in 5.2.6. Briefly, 1 g fresh soil was dissolved in 50 ml of sterile water and dispersed by ultrasonication (50 J s^{-1} sonication energy for 2 min). An aliquot of 50 µL was pipetted into a 96-well microplate (PP F black 96 well; Greiner Bio-one, Kremsmünster, Austria) and mixed with MUB buffer (pH 6.5 and 11) and 1 mM substrate solution at a ratio of 1:1:2. Microplates were incubated at 30 °C. Fluorescence was measured after 0, 30, 60, 120 and 180 min at 360/460 nm wavelength using a microplate fluorescence reader (FLx800, BioTek Instruments Inc., Winooski, VT, USA). PA was calculated based on a linear function using a calibration line comprising different concentrations of 4-methylumbelliferone (0, 100, 200, 500, 800 and 1200 pmol $well^{-1}$).

The activity of three enzymes involved in the C and N cycle were also measured based on the use of fluorescent 4-methylumbelliferone substrates (4-MUF; Sigma–Aldrich, St. Louis, MO, USA): β-D-glucosidase (EC 3.2.1.21), β-xylosidase (EC 3.2.1.37) and β-N-

acetylglucosaminidase (EC 3.2.1.52) according to Marx *et al.* (2001). Enzyme activity was measured in autoclaved MES buffer (pH 6.1).

6.2.7 Microbial biomass

Determination of microbial biomass C (C_{mic}) in bulk soil was performed by chloroform fumigation extraction method (Vance *et al.* 1987) according to Mackie *et al.* (2014) using *kEC* factor of 0.45 (Joergensen 1996). A detailed description of this method is given in 5.2.7.

6.2.8 Phospholipid fatty acid (PLFA) analysis

Microbial community structure was determined using PLFA profiles based on the alkaline methylation method of Frostegård *et al.* (1991). Lipid extraction and determination of fatty acid methyl esters (FAMEs) were performed according to Mackie *et al.* (2015). A detailed description of PLFA analysis is given in 5.2.5. The divisions of PLFAs into bacteria and fungi were based on Frostegård and Bååth (1996), Zelles (1999) and Kandeler *et al.* (2008). Within bacteria, PLFAs were grouped into Gram-positive ($gram^+$), represented by i15:0, a15:0, i16:0, and Gram-negative ($gram^-$), specified by cy17:0 and cy19:0. Total bacterial PLFAs were calculated by the sum of $gram^+$ and $gram^-$ plus 16:1ω7. Fungal PLFA was represented by 18:2ω6,9.

6.2.9 Statistical analyses

Homogeneity of variance was tested by the Levene-test. Significance of differences was tested by ANOVA followed by the Tukey HSD-test, where $p < 0.05$ was considered as the threshold value for significance. In case of variance heterogeneity, the Games-Howell-test was used for pairwise comparison, where also $p < 0.05$ was considered as significantly different. Statistical analyses were performed using SPSS Statistics 22 (IBM 2013).

6.3 Results

6.3.1 Tracing RU47

The abundance of RU47 was 17 times higher in the rhizosphere than in bulk soil (Table 6.1). We detected a significantly higher quantity of RU47-specific DNA sequence in rhizosphere and bulk soil of maize plants inoculated with RU47 in comparison to all other treatments (Table 6.1). The second highest abundance was determined in the treatment, in which dead RU47 cells were added (Table 6.1). The RU47-specific DNA sequence was detected in the rhizosphere of all treatments; in bulk soil the detection of RU47 was negative in the 00-

control and less than 1 copy ng^{-1} DNA in control as well as bacterial mix treatment (Table 6.1).

6.3.2 Plant response

Maize plants of the optimally P-fertilized, non-inoculation control showed a significantly increased shoot biomass compared to the shoot biomasses of all other treatments (Table 6.2). Plants inoculated with dead or viable RU47 cells revealed identical shoot biomasses corresponding to 2 and 7 % of the shoot biomass determined in the P-fertilized control and bacterial mix treatment, respectively (Table 6.2). The highest root area was observed in optimally P-fertilized control followed by the treatment where the bacterial mix was added (Table 6.2). In comparison to these treatments, plants inoculated with dead or viable RU47 cells showed root areas decreasing by about 40 to 50 %, respectively (Table 6.2). Maize plants, which were grown under optimally P-fertilized soil conditions (control), revealed a 53-fold higher P uptake compared to all other treatments (Table 6.2). Highest P concentrations in tissue were determined in plants which were inoculated with viable RU47 cells (Table 6.2). The lowest P tissue concentration was found in the treatment using dead RU47 cells which was corresponded to a decrease by 62 % compared to the relative P uptake determined in shoots of the RU47 treatment (Table 6.2).

6.3.3 Enzyme activities

The spatial distribution of PA in the rhizosphere was estimated by soil *in situ* zymography. In total, we observed no significant differences in alkaline or acid PA between the treatments (Fig. 6.1). Maize plants at the age of ten days after sowing showed two times higher acid PA in the root zone than in surrounding bulk soil (Fig. 6.1). In the root zone of control plants a slight decrease by around 16 % compared to the average root acid PA of all other treatments was found (Fig. 6.1). Alkaline PA did not differ significantly between root zone and surrounding soil (Fig. 6.2). Therefore, the mean activity of the total incubated area was calculated, respectively. Determining of potential alkaline and acid PA in homogenous bulk soil samples after final harvest, we found highest alkaline PA in both RU47treatments (Fig. 6.3). In total, the acid PA was four to five times lower than the alkaline PA; highest value was determined in the 00-control (Fig. 6.3). Varying inoculations did not influence the activity of ß-glucosidase and N-acetyl-ß-glucosaminidase in bulk soil (Table 6.2). However, the addition of both dead and viable RU47 cells enhanced the activity of ß-xylosidase (Table 6.2).

Table 6.1 Abundance of *P.* sp. RU47-specific DNA sequence in rhizosphere and bulk soil DNA of maize plants 14 days after sowing under the following treatments: two non-inoculation (00-control and control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Values are presented as mean ± standard error (SE) of four replicates. Significant differences (Games-Howell, $p < 0.05$) between the treatments are marked by lowercase letters. Cp stands for copies.

		00-Control		**Control**		**Bacterial mix**		**Dead RU47**		**RU47**	
RU47 abundance		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Rhizosphere	[cp ng^{-1} DNA]	-	-	118.6 ^{b}	± 70.26	22.8 ^{b}	± 10.5	192.5 ^{b}	± 72.5	3612.0 ^{a}	± 515.7
Bulk soil	[cp ng^{-1} DNA]	0.0 ^{b}	± 0.0	0.1 ^{b}	± 0.2	0.2 ^{b}	± 0.1	18.7 ^{b}	± 4.5	212.1 ^{a}	± 17.1

Table 6.2 Summarized plant and bulk soil properties of maize plants 14 days after sowing under the following treatments: two non-inoculation (00-control and control) and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Values are presented as mean ± standard error (SE) of four replicates. Significant differences (Tukey-HSD, $p < 0.05$) between the treatments are marked by lowercase letters.

	00-Control		**Control**		**Bacterial mix**		**Dead RU47**		**RU47**	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Plant properties	-	-								
Shoot dry biomass [g]	-	-	1.1 [a]	± 0.0	0.3 [b]	± 0.0	0.02 [b]	± 0.0	0.02 [b]	± 0.0
Root area [mm]	-	-	434.7	± 75.1	332.2	± 44.7	256.1	± 74.9	273.0	± 81.8
Absolute P uptake [mg shoot^{-1}]	-	-	12.9	± 1.5	0.3	± 0.1	0.2	± 0.1	0.3	± 0.1
Relative P uptake [mg g^{-1}]	-	-	12.4	± 1.5	14.0	± 0.1	9.2	± 0.1	14.9	± 0.1
Bulk soil properties										
pH ($CaCl_2$)	7.4	± 0.0	7.1	± 0.0	7.4	± 0.0	7.4	± 0.0	7.4	± 0.0
Gram$^+$ PLFAs [nmol FAME g^{-1}]	3.0 [a]	± 0.6	2.6 [b]	± 0.6	2.7 [b]	± 0.6	2.6 [b]	± 0.6	2.6 [b]	± 0.6

Gram$^-$ PLFAs [nmol FAME g^{-1}]	1.0^{a} ± 0.3	1.0^{ab} ± 0.3	0.9^{b} ± 0.2	0.9^{ab} ± 0.3	0.9^{ab} ± 0.3
Bacterial PLFAs [nmol FAME g^{-1}]	4.3^{a} ± 0.5	3.9^{b} ± 0.5	3.9^{ab} ± 0.5	3.9^{b} ± 0.4	3.8^{b} ± 0.4
Fungal PLFA [nmol FAME g^{-1}]	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
ß-glucosidase [nmol g^{-1} h^{-1}]	204.6 ± 6.7	198.6 ± 10.8	180.7 ± 3.4	188.4 ± 2.9	195.6 ± 9.3
N-acetyl-ß-glucosaminidase [nmol g^{-1} h^{-1}]	53.4 ± 2.8	52.5 ± 2.7	46.1 ± 1.1	51.1 ± 1.8	54.1 ± 4.5
ß-xylosidase [nmol g^{-1} h^{-1}]	21.1^{ab} ± 0.9	17.7^{b} ± 0.9	19.4^{ab} ± 1.0	21.6^{a} ± 0.6	22.6^{a} ± 0.5

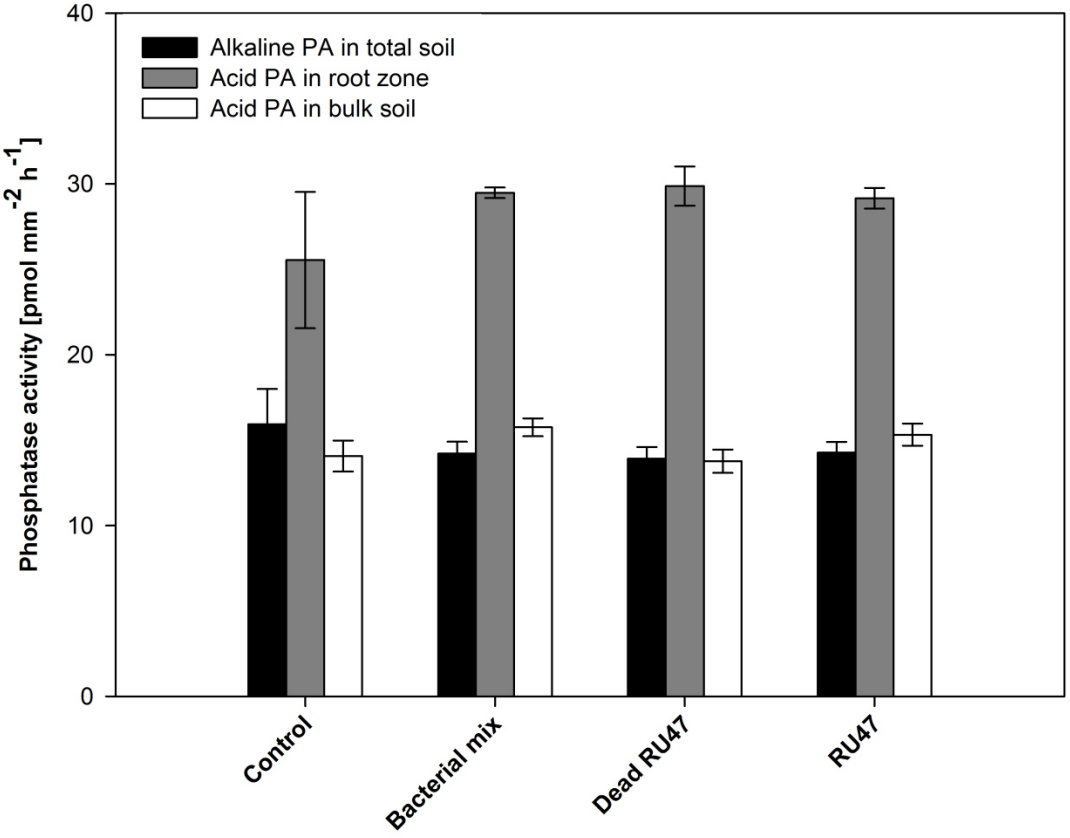

Figure 6.1 Phosphomonoesterase activity (PA) in different soil areas of maize plants ten days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4). Differences between the treatments were not significant (Tukey-HSD/Games Howell, $p < 0.05$).

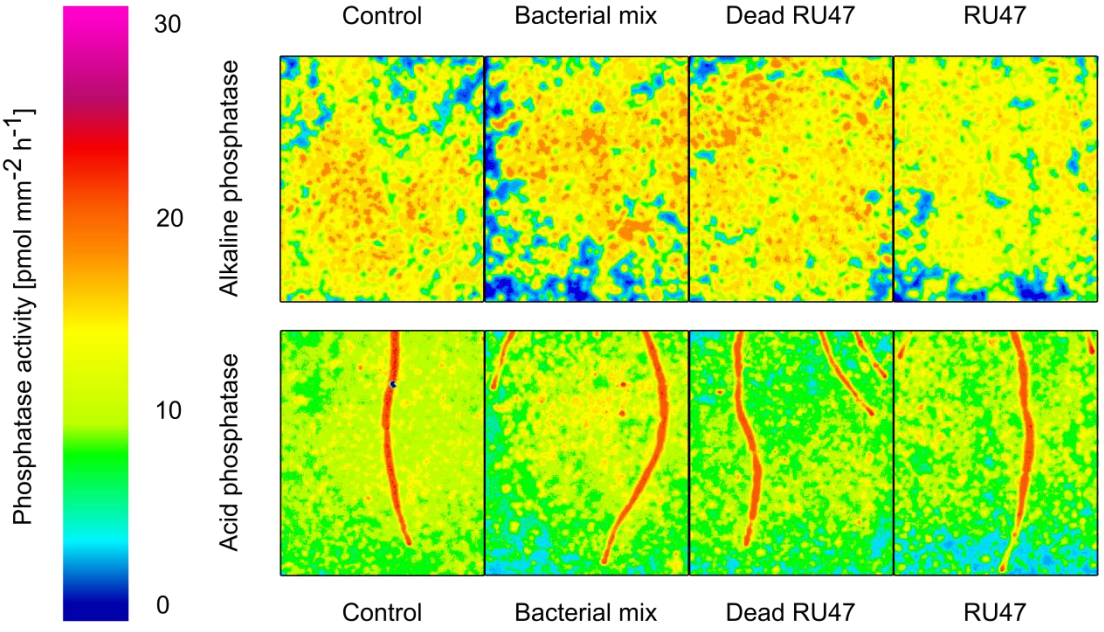

Figure 6.2 Soil zymograms of alkaline (pH 11) and of acid phosphomonoesterase (pH 6.5) in the rhizosphere of maize plants ten days after sowing (together with the calibration line) under the following treatments: one non-

inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Each treatment is represented by one replicate.

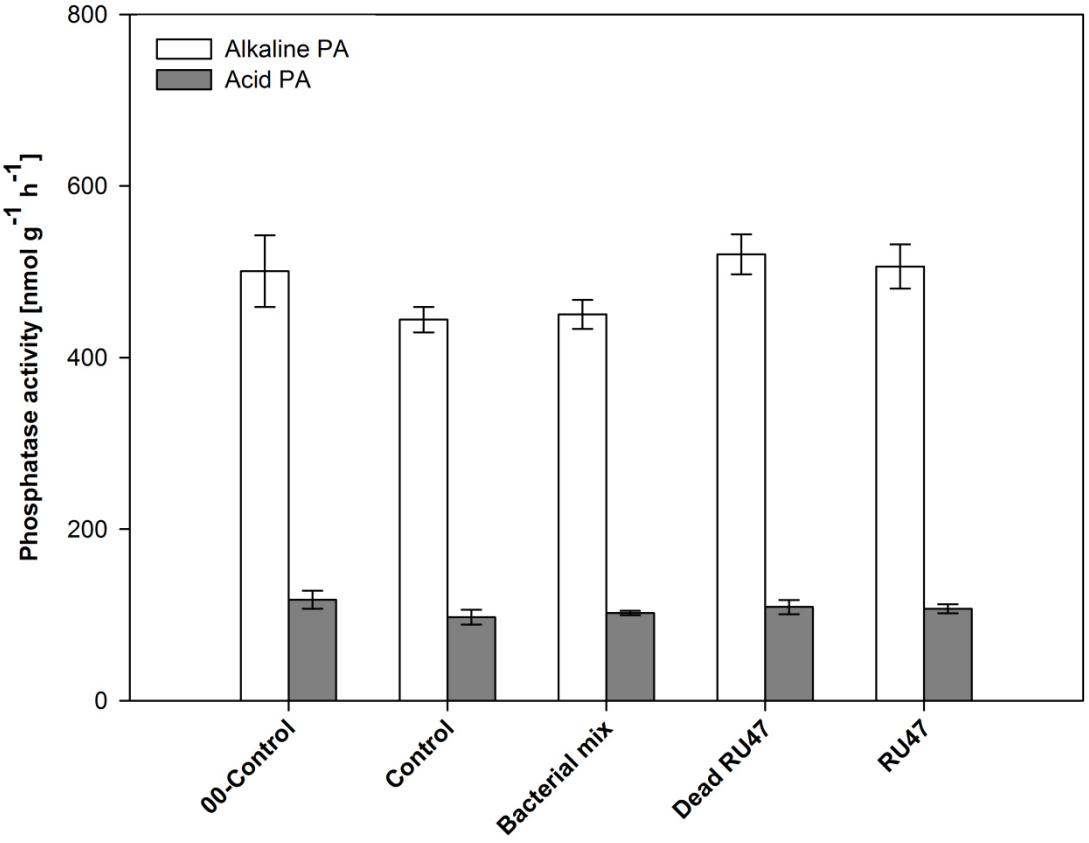

Figure 6.3 Phosphomonoesterase activity (PA) in bulk soil of maize plants 14 days after sowing under the following treatments: two non-inoculation (00-control and control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4). Differences between the treatments were not significant (Tukey-HSD/Games Howell, $p < 0.05$).

6.3.4 Microbial biomass and community structure in soil

We found the highest microbial biomass C in soil inoculated with a bacterial mix (Fig. 6.4). In comparison to this, microbial biomass in bulk soil of the RU47 treatment was by about 36 % lower and represented the lowest C_{mic} value of all treatments (Fig. 6.4). However, differences in microbial biomass between the treatments were not significant (Fig. 6.4). The PLFA analysis revealed a significantly increased bacterial abundance by around 10 % in bulk soil of the 00-control compared with the average bacterial abundance determined in all other treatments with the exception of the bacterial mix treatment (Table 6.2). This shift accompanied increases in abundances of gram$^+$ and gram$^-$ bacteria by 12 and 9 %, respectively (Table 6.2). Fungal abundance as analysed by PLFA did not differ significantly between the treatments (Table 6.2).

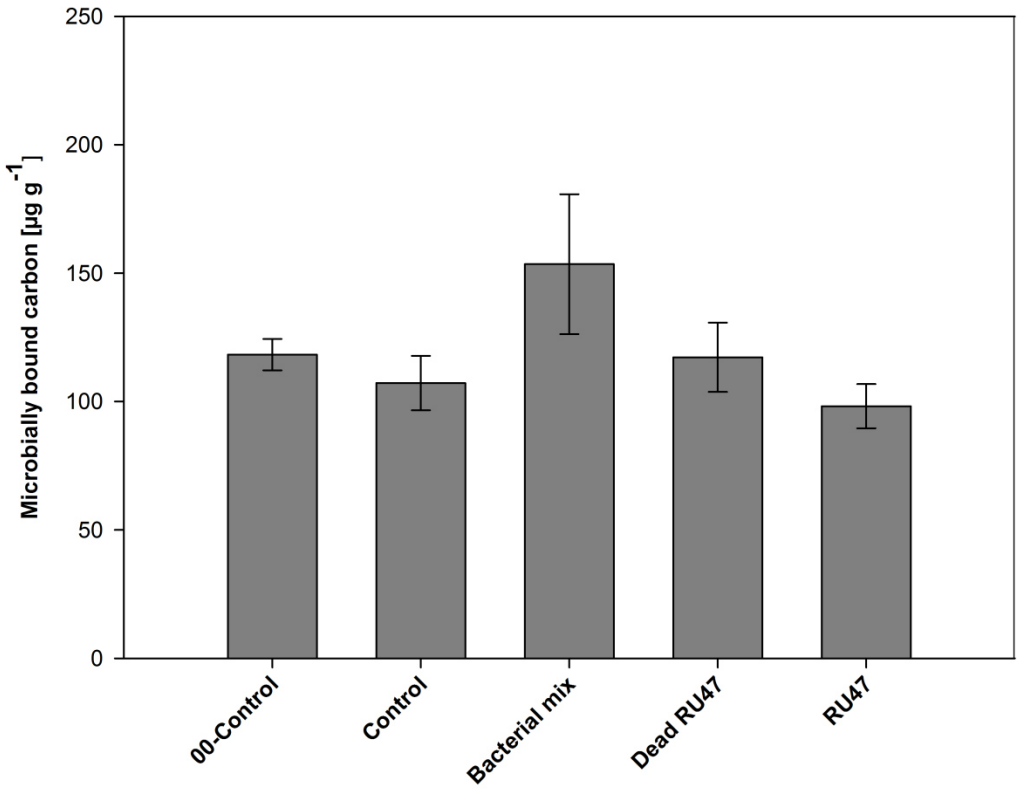

Figure 6.4 Microbially bound carbon (C_{mic}) in bulk soil of maize plants 14 days after sowing under the following treatments: two non-inoculation (00-control and control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4). Differences between the treatments were not significant (Tukey-HSD, $p < 0.05$).

6.4 Discussion

Tracing RU47

Several studies have reported little persistence of added PMB in soil (Kim *et al.* 1997, Hameeda *et al.* 2008, Meyer *et al.* 2017). However, in our study, RU47 was detectable, with highest abundances in the RU47 treatment (Table 6.1). Furthermore, we observed a significantly higher abundance in rhizosphere than in bulk soil (Table 6.1). This finding is not only in accordance with the tracing results of our tomato experiment (Table 5.1) but also with studies of Adesina *et al.* (2009) and Schreiter *et al.* (2014 c), who demonstrated a high rhizosphere competence of RU47 in lettuce soil. Since PMB strains, including RU47, are isolated from native microbial rhizosphere communities, a natural occurrence in non-inoculation treatments cannot be excluded. Kim *et al.* (1997) reported an abundance of *Enterobacter agglomerans* in the rhizosphere of non-inoculated tomato plants that corresponded to almost 50 % of the abundance in the inoculation treatment. These findings are in accordance with our observation detecting RU47 in the rhizosphere of all treatments corresponding to as much as 3 % of the abundance, which was determined in the treatment

using RU47 (Table 6.1). In this context, it has to be considered that the DNA of heat-devitalised dead RU47 is still detectable. Also a potential co-cultivation of RU47 in the unselective LB medium, which was used for the bacterial mix treatment, cannot be excluded.

Plant growth

Although improved plant biomass by the addition of PMB has been documented several times (Chabot *et al.* 1996, Kim *et al.* 1997, Sundara *et al.* 2002), shoot biomass data of the present study could not confirm these findings (Table 6.2). On the contrary, maize plants inoculated with RU47 revealed the lowest shoot biomass of all treatments (Table 6.2). As anticipated, shoot biomass of the optimally P-fertilized control plants were significantly increased (Table 6.2). This observation conforms to Mollier and Pellerin (1999), who demonstrated that the shoot growth of maize is highly limited by P supply even at an early growth stage. We found a reduced root area in plants of both RU47 treatments (Table 6.2). However, when the root:shoot ratio (mm^2:mg) was calculated, a 13 times higher root:shoot ratio was observed in plants, which were inoculated with dead RU47 or RU47. This finding may indicate an RU47-derived root growth stimulation. Hajabbasi and Schumacher (1994) as well as Mollier and Pellerin (1999) reported a short-term root growth stimulation effect in maize as a result of an early reduction of shoot growth after P deprivation that provided more carbohydrates for root growth. Considering the low root:shoot ratio of 1.2 determined in plants inoculated with a bacterial mix but also grown under P-limited soil conditions, the high root:shoot ratio of 13.2 documented in both RU47 treatments was more likely due to PMB released phytohormones such as auxins and gibberellins, which stimulated the root growth. This explanation is consistent with several studies demonstrating the ability of many soil bacteria to synthesize phytohormones or ACC deaminase; both affect a plant's hormonal balance and thus its growth, development, and response to stress (for review see Tsavkelova *et al.* 2006, Glick 2012, 2014). However, due to the enzyme inactivation in the treatment where dead RU47 cells were added, an ACC deaminase effect can be excluded here.

P uptake and enzyme activity

Results of plant growth and P uptake responses to addition of PMB are rare and somewhat inconsistent. However, Egamberdiyeva (2007) demonstrated improved P uptake in maize due to the addition of *Pseudomonas alcaligenes* PsA15. These findings agree in part the results of our study, which showed highest P tissue concentration in plants inoculated with viable RU47 cells (Table 6.2). However, shoot biomass of plants in the bacterial mix

treatment exhibited similar P concentrations (Table 6.2). This observation indicates that plant P supply is driven by microbial active processes. In a previous rhizobox experiment, the addition of RU47 resulted in an increased alkaline PA (Fig. 5.2 a); these findings could not be confirmed by the results of the soil *in situ* zymography in this study (Fig. 6.1). However, zymography was performed three days after the second inoculation indicating a still relatively low RU47 abundance and thus microbial activity. It is equally likely that the optimally supplied bacteria cells (King's B medium) were not P-limited, suggesting that increased microbial PA was not present while performing zymography. Zymograms revealed a strongly root-associated acid PA (Fig. 6.1), which is fully in agreement with previous studies (Kandeler *et al.* 2002, Spohn and Kuzyakov 2013 a, Spohn *et al.* 2015). Furthermore, we observed hotspots of acid PA especially at the root tips (Fig. 6.2), which is consistent with observations of Spohn *et al.* (2015), who evaluated this finding as a confirmation of a more general finding that the release of organic compounds by the plant is highest at the root zone (Watt *et al.* 2006, Hinsinger *et al.* 2009). Our observation that acid PA decreased due to P fertilization (Fig. 6.1) also is in line with data from the literature (Olander and Vitousek 2000, Sinsabaugh *et al.* 2008, Spohn *et al.* 2015). Compared to studies in which PA was determined by soil zymography (Spohn and Kuzyakov 2013 a, Spohn and Kuzyakov 2014, Spohn *et al.* 2015) we found no distinct separation in alkaline PA between root and surrounding soil (Fig. 6.2). This is likely due to the homogeneously distributed enzyme activity of the topsoil used for soil substrate. Moreover, low RU47 root colonization and no microbial P limitation when the zymography was performed may also explain the finding of no distinct separation in alkaline PA in the rhizosphere. Enzyme analysis performed four days after zymography revealed highest alkaline PA in bulk soil in both RU47 treatments (Fig. 6.3). These findings indicate that the addition of RU47 may enhance P mineralization in soil improving plant P supply. Despite the observed trend of increased alkaline PA in bulk soil, plant P uptake in the dead RU47 treatment was reduced (Fig. 6.3, Table 6.2). This finding cannot be explained conclusively; it should be borne in mind that in short-term experiments changes recorded in soil microbial activity do not inevitably lead to measurable effects on plant properties. The addition of dead RU47 or RU47 cells resulted in increased ß-xylosidase activity (Table 6.2). This enzyme, decomposing xylan and oligosaccharide, though produced mainly by fungi is more effectively utilized by bacteria (Romaní *et al.* 2006). P mineralization can be driven by microbial need for C (Spohn and Kuzyakov 2013 b), which is a possible explanation for observed increases in PA patterns in previous studies (Turner and Wright 2014, Zhang *et al.* 2014, Spohn *et al.* 2015). However, due to the unaffected ß-glucosidase activity an increased C requirement may be excluded (Table 6.2). Rather, this observation invites the

speculation as to whether soil microorganisms in general, and PMB in particular (viable RU47), also use ß-xylosidase to acquire P from organic compounds such as glycoproteins and proteoglycans, both of which contain xylose as well as P, the P subsequently hydrolysed by phosphatases. This is in accordance with the unaffected fungal PLFA abundance as well as the simultaneous increase in microbial PA determined in both RU47 treatments (Table 6.2, Fig. 6.3); improved P mineralization also by the addition of dead RU47 is likely attributable to indigenous PMB, which is discussed in more detail in the following chapter.

Microbial biomass and community structure in soil

In contrast to previously recorded microbial biomass C data in arable soils (Anderson and Domsch 1989, Sparling 1992) as well as C_{mic} values determined in the 39-day tomato experiment (Fig. 5.3), microbial biomass C observed in this study was relatively low (Fig. 6.4). This can be attributed mainly to the addition of quartz sand, which decreased both microbial biomass and organic C concentration. Bulk soil inoculated with a bacterial mix had the highest C_{mic} value (Fig. 6.4); however, increases in bacterial or fungal abundances could not be determined by PLFA (Table 6.2). Highest bacterial abundance was determined in the 00-control, which can be attributed to the absence of competition for nutrients between soil bacteria and plants (Table 6.2). Despite the demonstrated persistence of RU47, which is categorized as gram$^-$, no shifts in PLFA pattern were observed (Table 6.2). These findings are consistent with the PLFA data of our previously performed tomato experiment (Table 5.2). However, previous studies reported an increased population of indigenous PMB after the targeted application of specific PMB strains (Sundara *et al.* 2002, Canbolat *et al.* 2006). With regard to the improved enzyme activity observed in both RU47 treatments, a PMB-promoting effect by RU47-derived attractants or phytohormones modulating root exudation cannot be excluded on the basis of PLFA analysis. Regardless of bacterial-bacterial interactions, PMB are assumed to interact synergistically with mycorrhizal fungi (Kim *et al.* 1997, Zaidi *et al.* 2003). Furthermore, strains of *P. fluorescens* and *P. putida* have been previously identified as mycorrhiza-helper bacteria (Gamalero *et al.* 2008, Labbé *et al.* 2014) supporting the assumption that PMB simultaneously use multiple mechanisms to improve P acquisition in soil. Since linoleic acid (18:2ω6,9) is only incorporated in cell membranes of *Ascomycetes*, *Basidiomycetes* and *Zycomycetes* (Joergensen and Wichern 2008) data on fungal abundance from our PLFA analysis provided no information about (vesicular-) arbuscular mycorrhization (VAM). In order to get an idea of potential changes in VAM by the addition of RU47, we used DNA extracted from rhizosphere and bulk soil after final harvest in a nested PCR amplifying *Glomeromycota*-specific DNA sequence based on the procedure

described by Lee *et al.* (2008). Subsequent evaluation of the electrophoretically separated VAM-amplicons revealed no treatment differences in rhizosphere DNA, but a less pronounced mycorrhization in bulk soil that was added with viable RU47 cells (data not shown). This observation could be attributed to a fungal suppression effect such as that documented by Adesina *et al.* (2009) using RU47 as a bio-control agent against *Rhizoctonia solani* in lettuce roots. However, soil microbes are subject to constantly changing environmental conditions, hence any changes in microbial population must be regarded as temporary (Bashan 1999). Furthermore, with respect to the short experimental duration, allowing insufficient time for mycorrhization (Beyene 1996), a VAM-promoting effect by RU47 cannot be excluded based on the present data. Indeed, the role of mycorrhizal fungi in PMB's ability to improve P availability to plants is an upcoming topic; thus investigations on the mycosphere should be considered in further studies.

6.5 Conclusion

Our study demonstrates that RU47 is able to colonize the rhizosphere and bulk soil of maize seedlings within 14 days. The addition of RU47 did not result in plant-growth promoting effects. Although not significant, we observed a trend of an improved P supply in plants. Plants inoculated with dead or viable RU47 cells showed increased root:shoot ratios, indicating a hormonal impact on plants by phytohormones released from RU47 cells or by indigenous soil bacteria. Contrary to our hypothesis, RU47 colonization did not lead to spatially distinct zones of increased PA in the rhizosphere. This can likely be attributed to the short temporal distance between the time points of inoculation and soil *in situ* zymography and should be taken into consideration in further studies. However, we found a tendency toward increased alkaline PA in bulk soil inoculated with dead or viable RU47 cells. We found no significant effects of RU47 on soil microbial biomass and community structure at the PLFA level, implying that within 14 days the addition of RU47 did not quantitatively affect the microbial colonization in bulk soil. Shifts in microbial community structure may appear on a lower taxonomic level than class. Taking together, RU47 may have the potential to improve P supply at the sensitive early growth stage of maize; whether the early inoculation by dead or viable RU47 cells are sufficient to promote plant growth in later growth stages should be tested in longer-term experiments.

7 Effects of phosphorus-mobilizing bacteria on growth of tomato and soil microbial activity under varying P availability in soil

7.1 Introduction

Microorganisms play an essential role in mediating nutrient availability to plants due to their ability to solubilize and mineralize nutrients bound in the soil complex. Especially in view of the increasing scarcity of phosphorus (P) resources, the microbial contribution to the soil P cycle has become a focus of interest in developing new approaches for more efficient and resource-saving P fertilization in future crop production. Phosphorus is, after nitrogen (N), the second most frequently limiting macronutrient for plant growth (Richardson 1994, Marschner 1995). However, plant P uptake is almost exclusively limited by the availability of orthophosphates (predominantly HPO_4^{-2} and $H_2PO_4^{-1}$) in soil solution (Richardson 2001), representing the main challenge to practical P fertilization. Unlike other nutrients, P applied as phosphate fertilizer is rapidly adsorbed, precipitated or converted into inorganic or organic P compounds, thereby rendered unavailable or barely accessible for plant uptake (Holford 1997, Richardson 2001). Furthermore, phosphate concentrations in soil solution (~1 to 10 µM) are in equilibrium with the solid phase and are maintained by physical-chemical reactions (Barber 1980, Richardson 2001), with the consequence that additional applications of P shift P equilibrium status toward the P immobilization phase rather than providing more plant available P. Thus, in most soils, only 0.1 % of the total P is plant available (Illmer and Schinner 1995, Holford 1997), which clearly demonstrates an urgent need for new approaches to exploit the immobilized P in soil for crop production.

Fortunately, soil microorganisms in general, and PMB in particular, are able to efficiently mobilize phosphate anions from organic and inorganic P compounds bringing plant available orthophosphates into the soil solution (Richardson 1994, Richardson and Simpson 2011). Plant growth-promoting effects by the targeted application of highly concentrated PMB strains (mainly from genera *Pseudomonas*, *Bacillus* and *Azospirillum*) under P limited soil conditions have been reported in several studies (Kim *et al.* 1997, Chabot *et al.* 1996, Sundara *et al.* 2002). However, soil bacteria effectively compete with plants for available orthophosphates in soil solution (Richardson and Simpson 2011). Thus, it can be assumed that PMB are also – especially at their early colonizing stage during which P-consuming active biochemical processes such as cell expansion and the release of exo-enzymes or organic acids mobilizing P – in strong competition with plants for orthophosphates. Investigating the effects of phosphorus-solubilizing bacteria, Yu *et al.* (2011) and Kaur and

Reddy (2014) observed a reinforced plant-growth promoting effect by soil inoculated with PMB strains when that soil was P-fertilized using rock phosphate or tricalciumphosphate. The amount of P that can potentially be mobilized depends on the amount of immobilized P in soil, but also may create a reduced competitive situation for P at the early colonization stage, which in turn may enhance PMB's viability and P-mobilizing potential. Here, two possible relationships are under discussion. First, initial P fertilization increases PMB abundance, which is subsequently positively correlated with PMB-derived active P-mobilizing processes when P becomes increasingly limited. Second, the initially high incorporation of P into bacterial biomass, which effectively protects available P from soil reactions (Olander and Vitousek 2004), decreases with increasing P limitation from the dying PMB cells as plant-available orthophosphates (Macklon *et al.* 1997).

In a previous study, using RU47 as the PMB and tomato as the test plant grown under low P availability soil conditions, we implemented a novel approach, inoculating soil with viable as well as dead RU47 cells to gain a clearer picture of direct and indirect P mineralization (5). This experiment revealed a plant growth-promoting effect and improved P supply not only by the addition of RU47 but also of dead RU47, while the beneficial effect of RU47 cells was clearly associated with increased microbial PA in soil (Fig. 5.1, Table 5.2, Fig. 5.2 a). The plant growth-promoting effect observed in the addition of dead RU47 cells indicates either a phytohormone-derived effect or interactions with indigenous microorganisms. Based on these results, and given the observed strengthening effect by P fertilization reported in other studies (Yu *et al.* 2011, Kaur and Reddy 2014, 2015), we wanted to verify whether RU47's previously documented beneficial effects could be increased by enhanced P fertilization. Therefore, we performed a rhizobox experiment over a period of 50 days to test the influence of modified P fertilization on the effects of RU47 on growth and P uptake of tomato plants. We hypothesized that (1) increased P availability in soil improves the survival and colonization success of RU47, resulting in its increased abundance. Because an increase in RU47 abundance could also lead to greater bacterial demand for C resources, and bacterial PA is regulated not only by P but also by C availability in soil (Zang *et al.* 2014, Spohn *et al.* 2015), we further hypothesized that: (2) The addition of viable RU47 cells in a highly P-fertilized soil would result in increased release of RU47-derived phosphatase. We therefore additionally hypothesized that: (3) The increase in RU47 abundance under high P soil conditions is accompanied by an increased release of phytohormones, resulting in a strengthened plant growth-promoting effect, while a plant growth-stimulating effect of dead RU47 treatment would not be affected by P fertilization since it would not result in an increase in release of phytohormones.

7.2 Materials and methods

7.2.1 Rhizobox experiment

The experiment was performed using RU47 as the PMB and tomato (*Solanum lycopersicum* L. var. Mobil) as the test plant. To distinguish among RU47-derived and endogenous P mineralization as well as direct and indirect mechanisms, two additional inoculation treatments were included, comprising either an inoculation of unselectively cultivated indigenous soil bacteria (bacterial mix) or dead RU47 cells (dead RU47). Details of bacteria cultivation and inoculation are described in 2.2. A non-inoculation treatment served as the control. The experiment was performed applying two different P fertilizer doses; 50 mg P kg^{-1} (low P), and 200 mg P kg^{-1} (high P), before sowing. Hence, the experiment contained eight treatments in total, with each treatment comprising five replicates. Tomato plants were grown in rhizoboxes with inner dimensions of 28.0 cm x 4.5 cm x 16.5 cm which were filled with a soil substrate composed of Luvisol topsoil and quartz sand (0.2 - 1.4 mm) in a ratio of 1:1 (w/w). Rhizoboxes were inclined by 50°, forcing the roots to grow along the inclined wall, which additionally enhanced P limitation, since P acquisition at the rooting depth was reduced. The Luvisol, classified as a heavy loam soil, had the following characteristics: pH 7.1 ($CaCl_2$), 26.2 % sand, 52.2 % silt, 21.6 % clay, 2.3 % total C, 2.0 % organic C, 1.8 mg NH_4^+ kg^{-1}, 53.0 mg NO_3^- kg^{-1} and 24.1 mg P (Olsen) kg^{-1}. The soil, selected on the basis of its low concentration of plant available phosphorus (CAL extraction of 20 mg kg^{-1}), was taken from an unfertilized grassland located on the campus of the University of Hohenheim (Stuttgart, Germany). Each rhizobox was filled with exactly 1918.0 g DM of sieved (< 5 mm) soil substrate. Before sowing, the soil substrate was optimally fertilized with respect to N (100 mg kg^{-1}), K (150 mg kg^{-1}) and Mg (50 mg kg^{-1}) and adjusted to a water holding capacity of 50 %. Three tomato seeds were sown to a depth of 4 cm directly into each rhizobox and thinned after germination to one plant per rhizobox. To avoid light-derived influences on root growth and thus plant behaviour, all boxes were wrapped with aluminium foil. The experiment was conducted for 50 days under greenhouse conditions, with an average temperature of 20 °C and humidity of 53 %. Rhizoboxes were randomly distributed and placed on wooden planks to exclude contamination by leaking irrigation water. Plants were constantly watered to maintain a water holding capacity of 50 %; the water content was checked gravimetrically on a daily basis. Watering was performed using deionized water (H_2O_{deion}), successively applied in 5 mL steps to avoid direct leakage along rhizobox edges.

7.2.2 Bacterial cultivation and inoculation

RU47 was cultured in King's B liquid medium (King *et al.* 1954) added with rifampicin (50 mg L^{-1}); bacterial mix was prepared in a glucose-enriched (2 g L^{-1}) LB-Lennox liquid medium (Bertani 1951, Lennox 1955) and both were held at 28.5 °C for 24 h. Bacterial cells were washed twice using sterile 0.3 % NaCl solution and subsequently adjusted to a cell density of 10^9 cell mL^{-1} (OD_{600} = 1.0, Xue *et al.* 2013) by the use of a photometer (BioPhotometer, Eppendorf, Germany). The devitalisation of RU47 cells, which were used in one of the treatments, was performed by boiling for 1 min on a heating plate. Pretests have proven that this procedure was sufficient to kill RU47 cells completely (plate tests). Further details of cell cultivation and preparation are given in 5.2.2.

Plants were inoculated three times, each with a cell density of 10^9 cell mL^{-1} (OD_{600} = 1.0). The first inoculation was conducted by seed coating. Under gentle and continuous vortexing, 5 µL of cell suspension was successively added to five tomato seeds. The volume required for entire seed coating had previously been tested with ink (Pelikan, Pottendorf, Austria) before starting the experiment. Seed coating was controlled by using three of the inoculated seeds followed by washing with 1 mL sterile 0.3 % NaCl solution and plating 100 µl of the suspension on King's B-Agar medium (50 mg rifampicin L^{-1}) in three dilution stages. Plates were incubated at 28.5 °C until growing colonies were unequivocally countable on the agar (after approximately 36 h). The second inoculation was performed directly after seed germination using 6 mL cell suspension kg^{-1} soil substrate DM, directly applied onto the soil surface to simulate farm practice. To avoid watering effects, control treatments were inoculated with 0.3 % NaCl solution with corresponding volumes per inoculation. Viability and unviability of used RU47 cells as well as sterility of 0.3 % NaCl solution were controlled by plating and subsequent incubation at 28.5 °C for 48 h after every inoculation.

7.2.3 Plant sampling and properties

Before harvesting, the shoot height, defined as the vertical length from stem base to youngest leaf tip, was measured with a ruler. Subsequently, each plant was separately and carefully cut into blossom, leaf, shoot and root biomass using a sterilized (70 % ethanol) scalpel. The surface material was briefly rinsed with H_2O_{deion} water to remove adhering dust, and then dried at 40 °C in separate aluminium trays for 14 days to estimate dry weight. Roots were carefully washed (H_2O_{deion}), then dried at 60 °C for 5 days. Stem diameter (fresh) was measured using a precision pocket vernier caliper (150 mm, Format, Wuppertal, Germany). For determination of leaf area the separated leaves of each replicate were photographed

using a digital camera (D60, Nikon, Tokyo, Japan). All pictures were taken from identical distances and angles, and included a benchmark. Image processing and analysis were done using the open source software GIMP. Digital images were transferred to 8-bit. Leaves were selected by colour (threshold 16.0) followed by calculation of leaf area based on the number of pixels.

P incorporated into shoot biomass was extracted by incineration and subsequent acidulation as described by Gericke and Kurmies (1952 a, 1952 b). Briefly, a weight of 250 mg dry matter was incinerated at 500 °C for 4 h followed by threefold digestion with HNO_3 (21.7 %). After the addition of HCl (12.3 %), extracts were diluted to a ratio of 1:10 with H_2O_{deion}, filtered and subsequently photometrically measured using Murphy and Riley colour reagent (Murphy and Riley 1962). Measurement was performed at 710 nm using a microplate absorption reader (ELx808; BioTek Instruments Inc., Winooski, VT, USA). The calculation of P concentration was based on a linear function using the following final concentrations: 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10 mg P L^{-1} prepared with K_2HPO_4.

7.2.4 Soil sampling and properties

After sampling, rhizosphere and bulk soil samples were immediately put on ice, stored short-term (20 h) at 8 °C, and sieved (< 2 mm) the following day. Each sample was aliquoted and frozen at -20 °C until analysis. Plant available P (P-CAL) in soil was determined in bulk soil samples. This analysis was conducted by the Analytical Chemistry Unit (Core Facility Hohenheim) of the University of Hohenheim (Stuttgart, Germany).

Total C, organic C (C_{org}), N, and NO_3^- in bulk soil were measured by the Analytical Chemistry Unit (Core Facility Hohenheim) of the University of Hohenheim (Stuttgart, Germany). To provide a rough assessment of soil substrate properties, the respective averages values (across the treatments) were calculated and shown on Table 7.S1.

7.2.5 Tracing of RU47

DNA extraction

DNA was extracted from rhizosphere and bulk soil based on a weight of 250 - 350 g fresh soil using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following manufacturer's instructions. A spectrophotometer (NanoDrop 2000; Thermo Scientific, Waltham, MA, USA) was used to measure DNA concentration and to determine purity of the resulting extract.

Tracing of RU47

Inoculated RU47 cells were traced with a TaqMan® assay using a 5'-labelled 6-FAM double-quenched (BMN-Q530) probe (biomers.net, Ulm, Germany) in a 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). Sequences used (Eltlbany *et al. in preparation*) were developed and provided by the Institute of Epidemiology and Pathogendiagnostic (Julius Kühn-Institut, Braunschweig, Germany). Reaction recipe and thermal-cycling conditions were adapted to the TaqMan® Fast Advanced Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions with some modification. As already in given 5.2.5, the reaction composition was as follows; final concentrations are given in brackets: 10 µL TaqMan® Fast Advanced Master Mix (1 ×), 2 µL DNA (10 ng), 5 µL nuclease-free water, 1 µL each primer (1.8 µM) and 1 µL (0.5 µM) probe. The thermal profile of the TaqMan® assay was: 95 °C for 10 min (initial denaturation), 95 °C for 30 sec followed by 54 °C for 30 sec for 40 cycles. Based on the standard curve, the absolute quantity of RU47 copies was calculated. Quantity of RU47 is given in copies ng^{-1} DNA.

7.2.6 Phosphatase activity (PA)

Determination of the potential activities of acid and alkaline phosphatase (EC 3.1.3) in rhizosphere and bulk soil samples was conducted using 4-methylumbelliferone substrates (4-MUF; Sigma-Aldrich, St. Louis, MO, USA) according to Marx *et al.* (2001). The assay performed corresponds precisely to the description given in 5.2.6. Briefly, 1 g fresh soil was dissolved in 50 mL of sterile water and dispersed by ultrasonication (50 J s^{-1} sonication energy for 2 min). An aliquot of 50 µL was pipetted into a 96-well microplate (PP F black 96 well; Greiner Bio-one, Kremsmünster, Austria) and mixed with MUB buffer (pH 6.5 and 11) and 1 mM substrate solution at a ratio of 1:1:2. Microplates were incubated at 30 °C. Fluorescence was measured after 0, 30, 60, 120 and 180 min at 360/460 nm wavelength using a microplate fluorescence reader (FLx800, BioTek Instruments Inc., Winooski, VT, USA). PA was calculated based on a linear function using a calibration line comprising different concentrations of 4-methylumbelliferone (0, 100, 200, 500, 800 and 1200 pmol $well^{-1}$).

7.2.7 Statistical analyses

Homogeneity of variance was tested by the Levene-test. A two-factor ANOVA was used to estimate the effects of the factors 'inoculation' and 'P fertilization' separately as well as to determine interactions between them. Results of this test are summarized in Table 7.S2.

Significance of differences between the inoculation treatments was tested separately for each P fertilization group using the Tukey-test. The Games-Howell-test was used in case of variance heterogeneity. The effect on P fertilization within an inoculation treatment was tested by ANOVA or Welch-Test, whereby the latter was used in case of variance heterogeneity. The threshold value for significance was generally considered as $p < 0.05$. Statistical analyses were performed using SPSS Statistics 22 (IBM 2013).

7.3 Results

7.3.1 Tracing RU47

No RU47-specific DNA sequences were detectable in soils of either the control or the bacterial mix at either P treatment level (Table 7.1). Highest RU47 abundances were observed in the treatments in which RU47 was used for inoculation (Table 7.1). Sequence quantity determined in soil with dead RU47 treatments was detectable, but about 69 % lower than in RU47 treatments. In general, the quantity of RU47-specific DNA sequence was higher in rhizosphere than in bulk soil (Table 7.1). Rhizosphere and bulk soil of plants, which were inoculated with RU47 and grown under high P soil conditions, revealed three and 12 times higher RU47 abundance than abundances determined in the low P trial (Table 7.1).

7.3.2 Plant properties

We observed no plant growth-promoting effect in shoot or root biomass of RU47, in either the low or high P trial (Fig. 7.1, Fig. 7.2). However, each inoculation treatment including the bacterial mix showed a trend of increased shoot biomass in both P trials (Fig. 7.1). Under low P soil conditions, highest shoot and root biomass were found in the treatment using a bacterial mix for inoculation (Fig. 7.1, Fig. 7.2). Compared with the control, an increase in shoot biomass by about 16 and 59 % and a decrease in root biomass by about 17 % was observed in plants inoculated with dead or viable RU47 cells, respectively (Fig. 7.1, Fig. 7.2). Under high plant available P soil conditions, the two highest shoot and root biomass values were determined in the treatments using dead or viable RU47. Although not significant, both RU47 treatments exhibited increases in shoot and root biomass by about 28 and 71 %, in comparison with the control (Fig. 7.1, Fig. 7.2). P fertilization significantly affected plant growth in the dead RU47 treatment, with increased shoot and root biomass under improved P fertilization (Fig. 7.1, Fig. 7.2). The addition of viable RU47 cells increased tomato shoot height, leaf area and blossom biomass under low plant available P soil conditions (Table 7.2). In the high P trial, no plant growth-promoting effects were observed (Table 7.2). Although not significant, plants treated with dead or viable RU47 cells had higher values in

shoot height, leaf number, and leaf area than all other treatments (Table 7.2). Determination of P concentrations in shoot biomass indicated an improved P supply in plants grown under P-limited soil conditions and inoculated with dead RU47 or viable RU47 cells, but was statistically significant only for dead RU47 (Fig. 7.3 a). This effect was not observed in the high P trial (Fig. 7.3 a). Improved P fertilization increased the P concentration in shoot biomass except in the treatment to which dead RU47 cells were applied (Fig. 7.3 a). In both P trials, the mass of absolute P bound in the shoot biomass tended to increase with each bacterial inoculation. The addition of viable RU47 cells led to an increase in absolute P uptake by about 65 % only under low P soil conditions compared with the control (Fig. 7.3 b). Absolute P uptake was increased by improved P fertilization in both the bacterial mix and dead RU47 treatments (Fig. 7.3 b).

Table 7.1 Abundance of *P.* sp. RU47-specific DNA sequence in rhizosphere and bulk soil DNA of tomato plants 50 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. The experiment was performed under low (low P) and high (high P) P fertilization soil conditions. Values are presented as mean ± standard error (SE) of five replicates. Significant differences (Games-Howell, $p < 0.05$) between the inoculation treatments of each P fertilization group are marked by lowercase letters. Differences between the P fertilization groups within an inoculation treatment were not significant (ANOVA/Welch-Test, $p < 0.05$). Cp stands for copies.

			Control		**Bacterial mix**		**Dead RU47**		**RU47**	
RU47 abundance		Unit	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Rhizosphere										
	Low P	[cp ng^{-1} DNA]	0 ^{b}	± 0	0 ^{b}	± 0	77.6 ^{a}	± 12.4	128.7 ^{ab}	± 51.2
	High P	[cp ng^{-1} DNA]	0 ^{b}	± 0	0 ^{b}	± 0	49.7 ^{a}	± 9.1	394.5 ^{ab}	± 200.3
Bulk soil										
	Low P	[cp ng^{-1} DNA]	0 ^{b}	± 0	0 ^{b}	± 0	9.6 ^{a}	± 1.9	20.9 ^{ab}	± 8.7
	High P	[cp ng^{-1} DNA]	0 ^{b}	± 0	0 ^{b}	± 0	15.4 ^{a}	± 3.2	245.9 ^{ab}	± 113.9

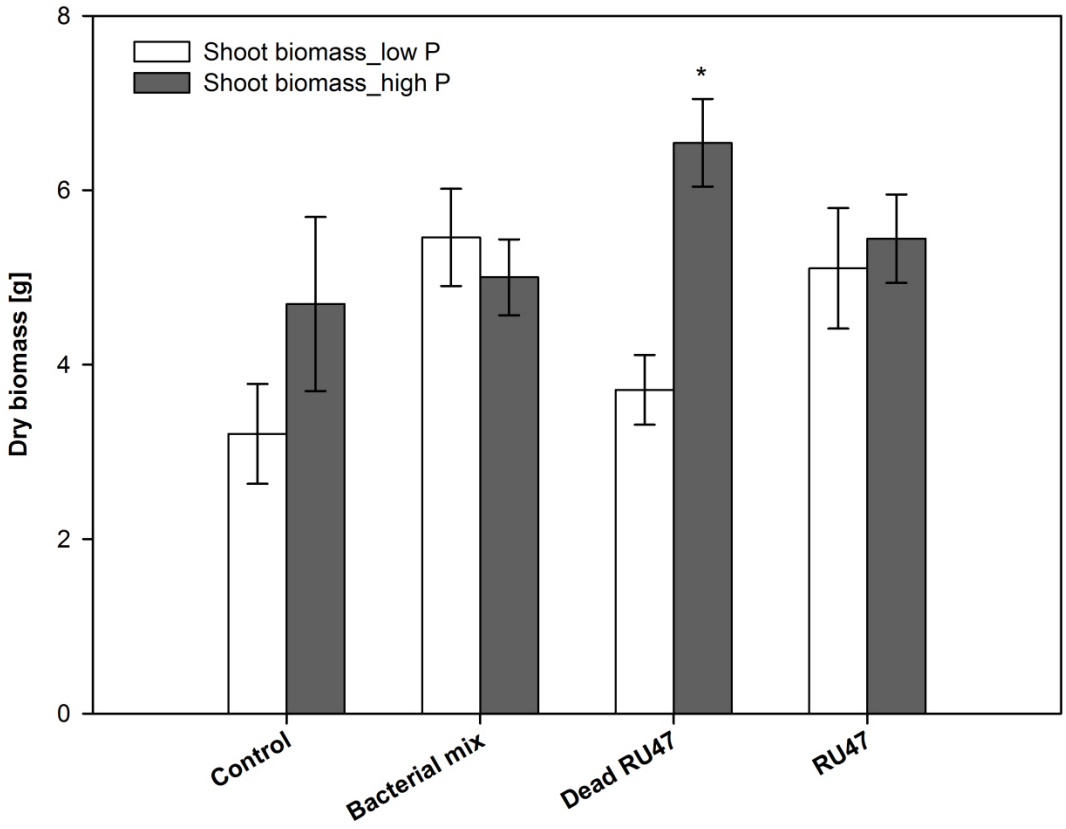

Figure 7.1 Shoot biomass (dry weight) of tomato plants 50 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. The experiment was performed under low (low P) and high (high P) P fertilization soil conditions. Error bars indicate standard error (n = 5). Differences between the inoculation treatments of each P fertilization group were not significant (Tukey-HSD, $p < 0.05$). Significant differences (ANOVA, $p < 0.05$) between the P fertilization groups within an inoculation treatment are marked with asterisks.

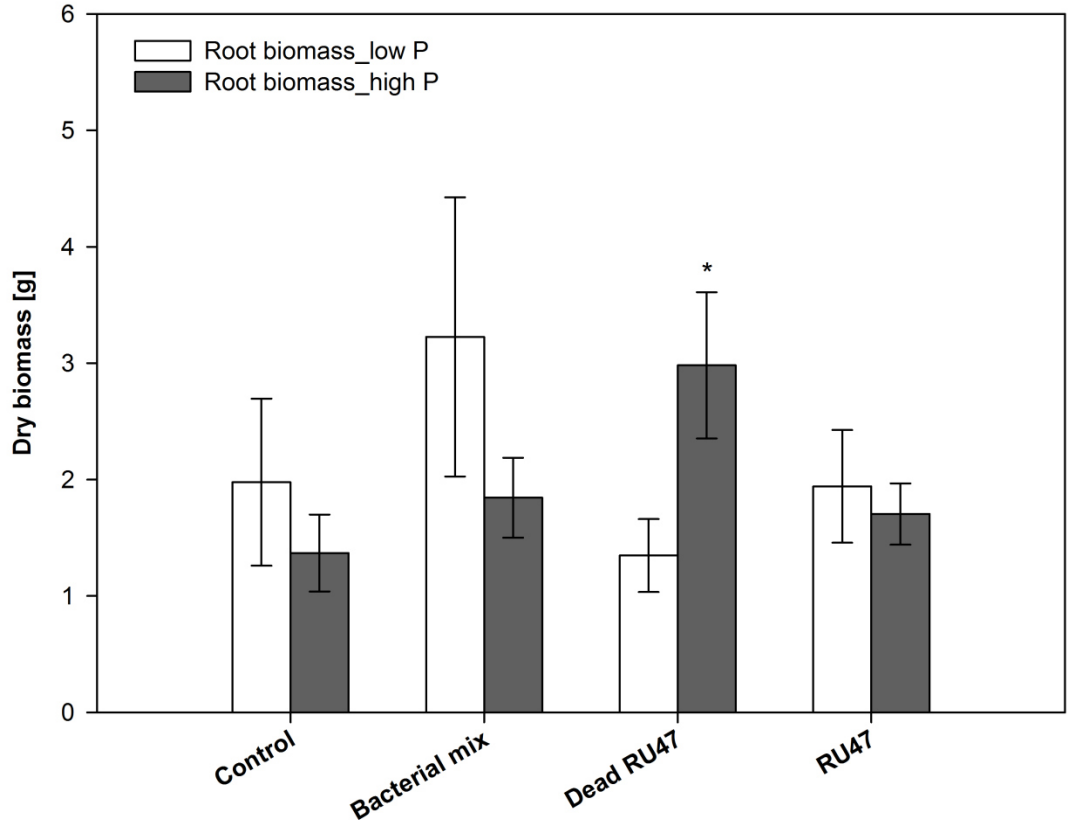

Figure 7.2 Root biomass (dry weight) of tomato plants 50 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. The experiment was performed under low (low P) and high (high P) P fertilization soil conditions. Error bars indicate standard error (n = 5). Differences between the inoculation treatments of each P fertilization group were not significant (Tukey-HSD, $p < 0.05$). Significant differences (ANOVA, $p < 0.05$) between the P fertilization groups within an inoculation treatment are marked with asterisks.

Table 7.2 Summarized plant and bulk soil properties of of tomato plants 50 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. The experiment was performed under low (low P) and high (high P) P fertilization soil conditions. Values are presented as mean ± standard error (SE) of five replicates. Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the inoculation treatments of each P fertilization group are marked by lowercase letters. Significant differences (ANOVA/Welch-Test, $p < 0.05$) between the P fertilization groups of an inoculation treatment are marked with asterisks.

		Control		**Bacterial mix**		**Dead RU47**		**RU47**	
	Unit	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Plant properties									
Shoot height									
Low P	[cm]	49.9 ^{c}	± 1.7	55.5 ^{a*}	± 1.1	48.6 ^{bc}	± 1.0	52.7 ^{ab}	± 1.8
High P	[cm]	51.2 ^{a}	± 2.0	50.5 ^{a}	± 1.0	52.7 ^{a*}	± 1.1	52.5 ^{a}	± 1.3
Stem diameter									
Low P	[cm]	0.4 ^{a}	± 0.0	0.5 ^{a}	± 0.0	0.5 ^{a}	± 0.0	0.5 ^{a}	± 0.0
High P	[cm]	0.5 ^{a}	± 0.0	0.5 ^{a}	± 0.0	0.5 ^{a}	± 0.0	0.5 ^{a}	± 0.0
Leaf number									
Low P	-	7.6 ^{a}	± 0.7	8.4 ^{a}	± 0.5	8.0 ^{a}	± 0.3	9.0 ^{a}	± 0.3
High P	-	9.0 ^{a}	± 0.7	8.4 ^{a}	± 0.4	10.4 ^{a*}	± 0.7	9.4 ^{a}	± 0.7

Leaf area						
	Low P	[dm²]	7.5 ^{b} ± 0.9	10.9 ^{a} ± 0.5	9.0 ^{ab} ± 0.6	10.6 ^{a} ± 0.9
	High P	[dm²]	10.3 ^{a} ± 1.2	10.8 ^{a} ± 0.6	12.5 ^{a*} ± 1.2	12.2 ^{a} ± 0.6
Blossom biomass						
	Low P	[mg]	15.7 ^{b} ± 4.5	30.6 ^{b} ± 8.7	44.8 ^{ab} ± 16.8	85.8 ^{a*} ± 8.5
	High P	[mg]	33.6 ^{a} ± 10.7	61.0 ^{a*} ± 8.1	61.8 ^{a} ± 14.4	34.1 ^{a} ± 10.1
Bulk soil properties						
pH ($CaCl_2$)						
	Low P	-	7.4 ^{a*} ± 0.0	7.4 ^{a*} ± 0.0	7.4 ^{a*} ± 0.0	7.4 ^{a*} ± 0.0
	High P	-	7.2 ^{a} ± 0.0	7.2 ^{a} ± 0.0	7.2 ^{a} ± 0.0	7.2 ^{a} ± 0.0
P-CAL						
	Low P	[mg kg^{-1}]	33.0 ^{a} ± 1.7	27.9 ^{a} ± 2.5	37.8 ^{a} ± 1.5	34.5 ^{a} ± 2.2
	High P	[mg kg^{-1}]	109.9 ^{a*} ± 10.2	98.5 ^{a*} ± 5.1	94.2 ^{a*} ± 8.0	95.9 ^{a} ± 8.4
P-CAL in total P						
	Low P	[%]	17.0 ^{a} ± 1.0	12.9 ^{a} ± 1.3	15.6 ^{a} ± 0.6	15.2 ^{a} ± 0.8
	High P	[%]	33.0 ^{a*} ± 3.2	31.3 ^{a*} ± 1.9	28.7 ^{a*} ± 1.8	29.8 ^{a} ± 3.0

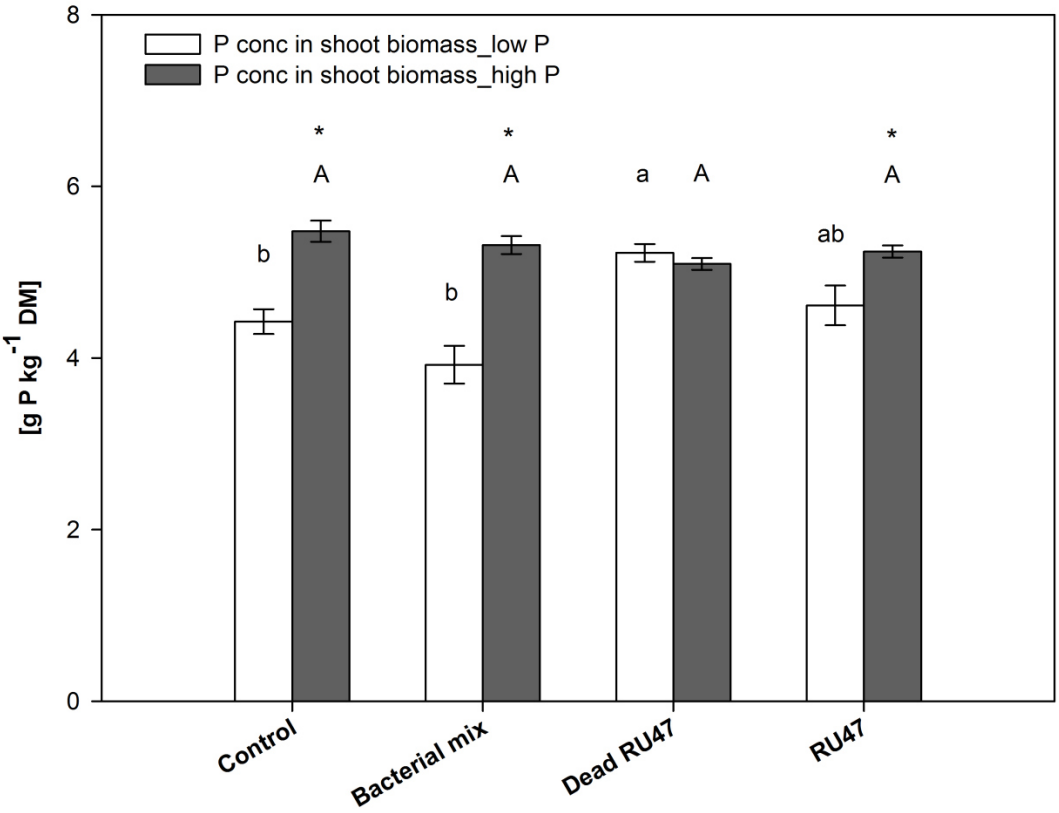

Figure 7.3 a P tissue concentration in shoots of tomato plants 50 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. The experiment was performed under low (low P) and high (high P) P fertilization soil conditions. Error bars indicate standard error (n = 5). Significant differences (Tukey-HSD, $p < 0.05$) between the inoculation treatments of each P fertilization group are marked by lower case (low P) and uppercase letters (high P). Significant differences (Welch-Test, $p < 0.05$) between the P fertilization groups within an inoculation treatment are marked with asterisks.

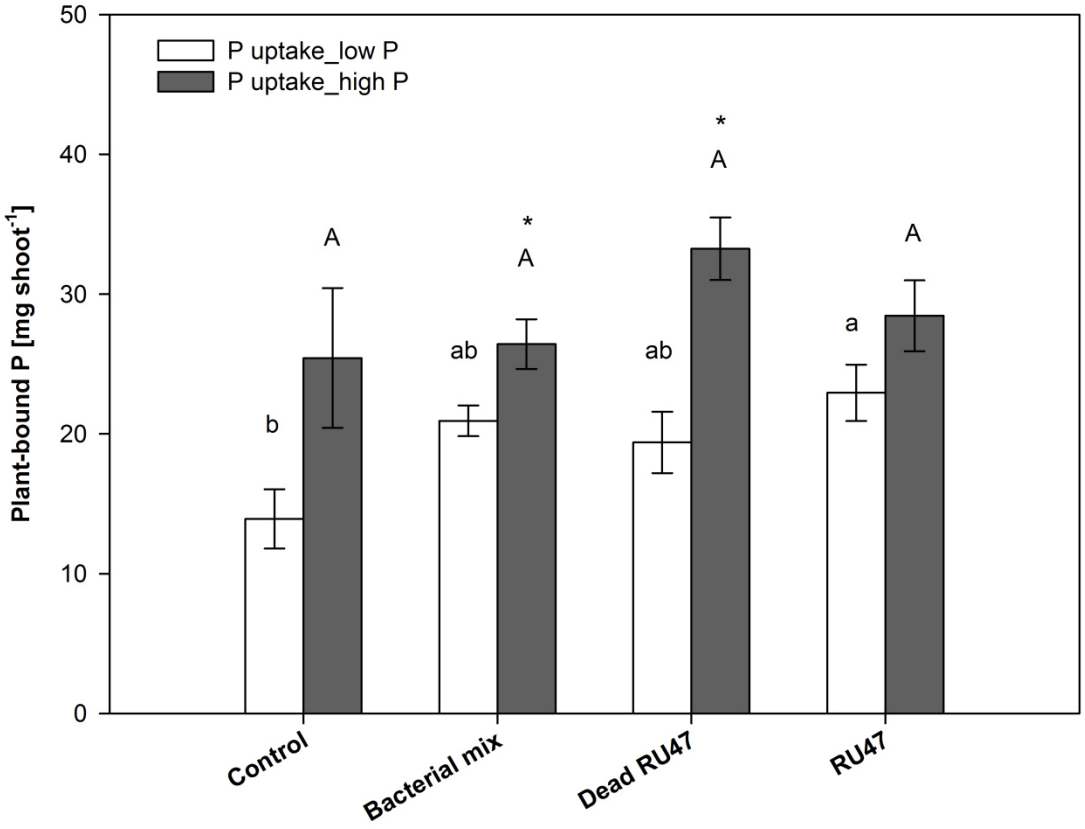

Figure 7.3 b Absolute P uptake in shoots of tomato plants 50 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. The experiment was performed under low (low P) and high (high P) P fertilization soil conditions. Error bars indicate standard error (n = 5). Significant differences (Games-Howell, $p < 0.05$) between the inoculation treatments of each P fertilization group are marked by lower case (low P) and uppercase letters (high P). Significant differences (Welch-Test, $p < 0.05$) between the P fertilization groups within an inoculation treatment are marked with asterisks.

7.3.3 Phosphatase activity (PA)

Acid and alkaline PA in the rhizosphere were higher than in the bulk soil of all treatments (Fig. 7.4 a, Fig. 7.4 b). Significant differences in PA between the different inoculation treatments were observed only in the rhizosphere (Fig. 7.4 a, Fig. 7.4 b). Under low plant-available P soil conditions, the bacterial mix increased acid PA, while alkaline PA was enhanced by the addition of dead RU47 or RU47 (Fig. 7.4 a, Fig. 7.4 b). In the high P trial, highest acid PA was found in the bacterial mix treatment, whereas the addition of dead RU47 cells significantly increased the alkaline PA (Fig. 7.4 a, Fig. 7.4 b). Varying P fertilization did not affect PA in the rhizosphere (Fig. 7.4 a, Fig. 7.4 b). However, in the bulk soil of the bacterial mix treatment we found a significantly enhanced acid PA in the low P trial (Fig. 7.4 a). Activities of alkaline phosphatase determined in rhizosphere and bulk soils of the RU47 treatment (low P) were positively correlated with RU47 abundance in the rhizosphere (Pearson's $r = 0.5$; $p < 0.05$). Under high P soil conditions, only bulk soil's alkaline PA was positively correlated with rhizosphere RU47 abundance (Pearson's $r = 0.5$; $p < 0.05$).

7.3.4 Soil properties

Increased P fertilization lowered the pH in bulk soil (Table 7.2) but we did not observe any inoculation effects on pH or P availability in soil, either under low or under high P soil conditions (Table 7.2). However, under low P soil conditions, treatments using dead or viable RU47 cells showed trends of increased P-CAL values. This trend was not confirmed, however, when we calculated the concentration of P-CAL as a percentage of total soil P (Table 7.2). In the high P trial, bulk soils of plants treated with dead or viable RU47 cells had the lowest concentrations of plant-available P (Table 7.2). In the high P group, P-CAL values were negatively correlated with alkaline PA in bulk soil (Pearson's $r = -0.5$; $p < 0.05$). Except in the treatment to which viable RU47 cells were applied, improved P fertilization increased the P availability for plants in soil (Table 7.2).

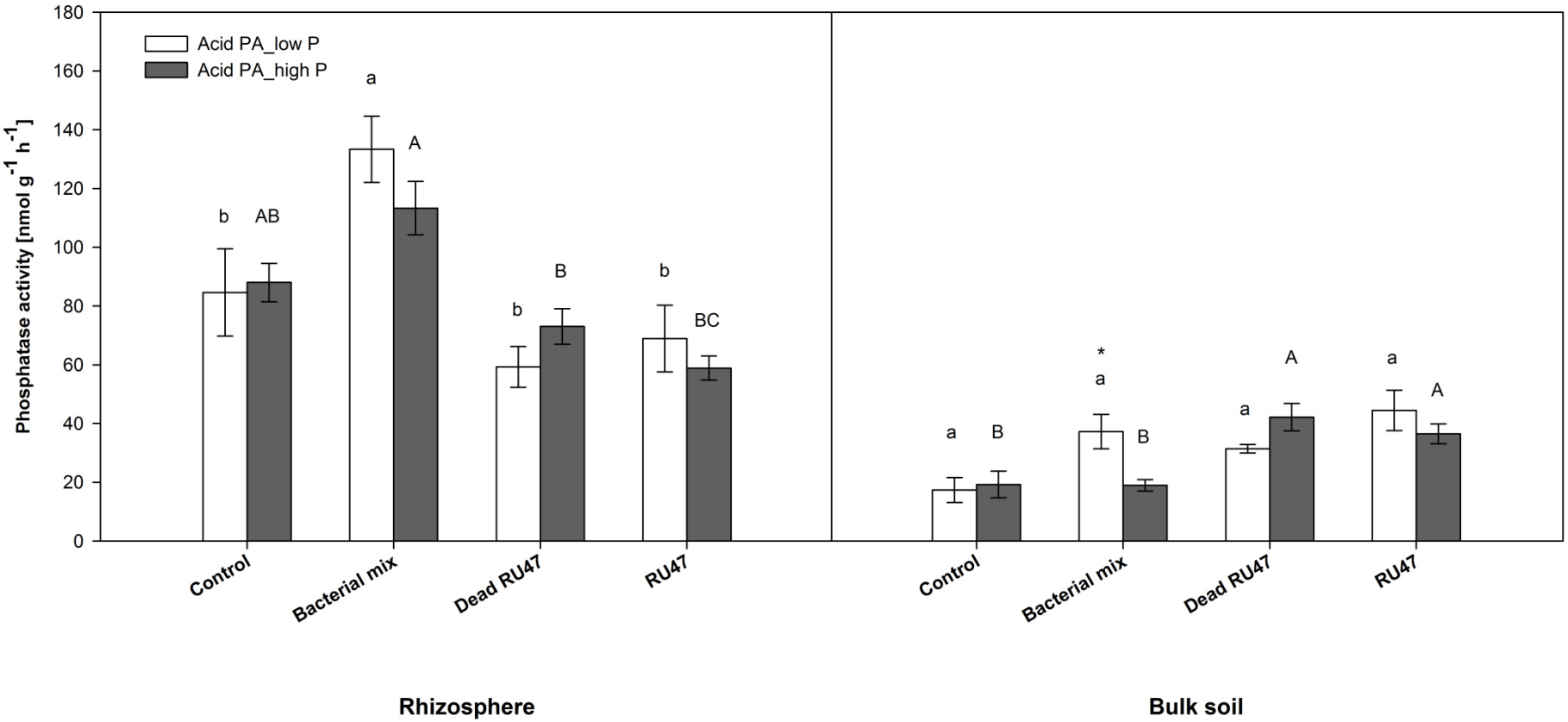

Figure 7.4 a Acid phosphomonoesterase activity (PA) in rhizosphere and bulk soil of tomato plants 50 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. The experiment was performed under low (low P) and high (high P) P fertilization soil conditions. Error bars indicate standard error (n = 5). Significant differences (Tukey-HSD, $p < 0.05$) between the inoculation treatments of each P fertilization group are marked by lower case (low P) and uppercase letters (high P). Significant differences (ANOVA, $p < 0.05$) between the P fertilization groups within an inoculation treatment are marked with asterisks.

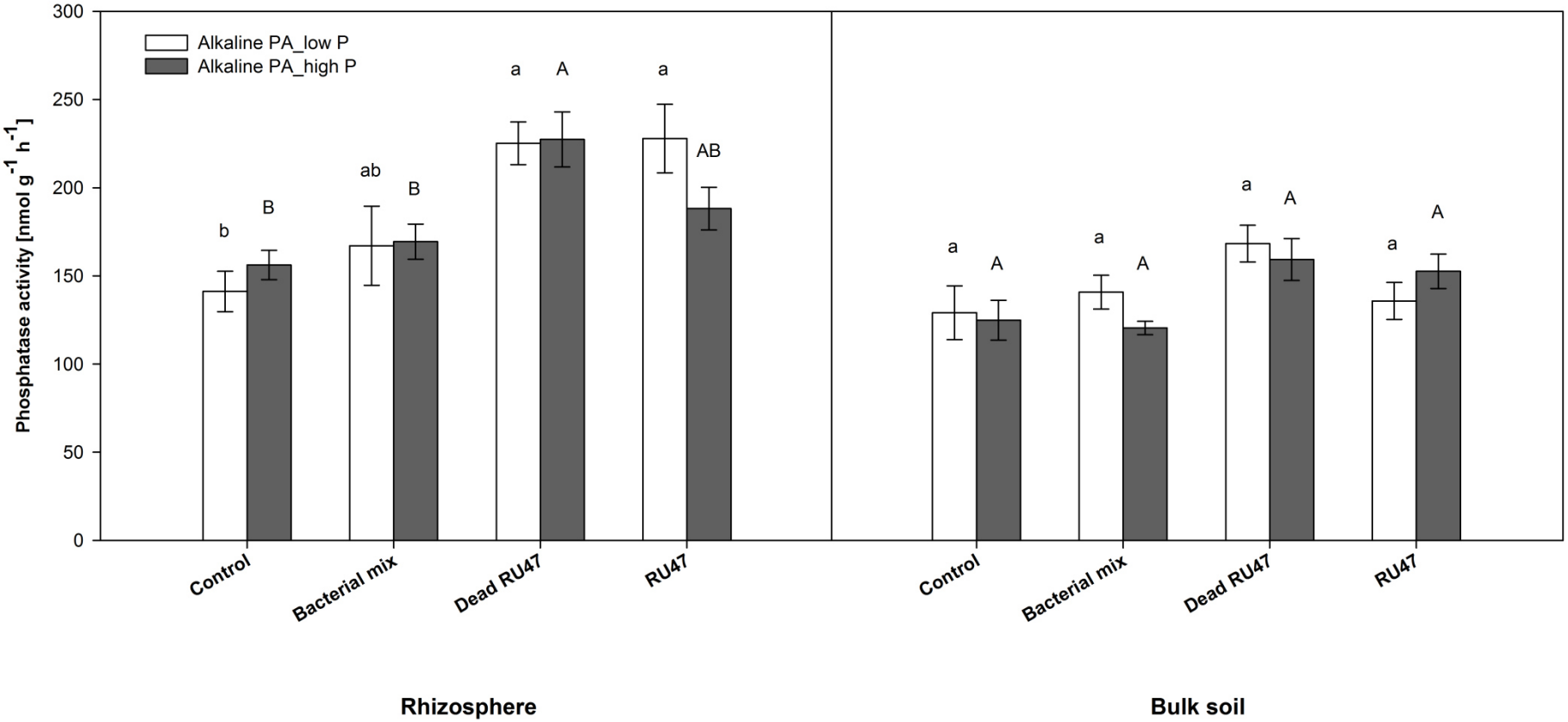

Figure 7.4 b Alkaline phosphomonoesterase activity (PA) in rhizosphere and bulk soil of tomato plants 50 days after sowing under the following treatments: one non-inoculation (control), and three inoculation treatments; unselectively cultivated soil bacteria (bacterial mix), dead RU47, or viable RU47 (RU47) cells. The experiment was performed under low (low P) and high (high P) P fertilization soil conditions. Error bars indicate standard error (n = 5). Significant differences (Tukey-HSD, $p < 0.05$) between the inoculation treatments of each P fertilization group are marked by lower case (low P) and uppercase letters (high P). Significant differences (ANOVA, $p < 0.05$) between the P fertilization groups within an inoculation treatment are marked with asterisks.

7.4 Discussion

Tracing RU47

Since tracing methods, mainly performed by molecular biological techniques, are still relatively new, little information is available about PMB's survivability in soil. However, in most studies, a temporal decrease in the recovery rate of the added PMB strain in soil has been reported (Kim *et al.* 1997, Dey *et al.* 2004, Hameeda *et al.* 2008, Meyer *et al.* 2017). For instance, Meyer *et al.* (2017) observed a loss of more than 99 % of the inoculated *Pseudomonas protegens* CHA0 abundance within 40 days. In our study, the quantity of RU47-specific DNA sequence was detectable even 50 days after sowing and highest abundances were detected in treatments to which viable RU47 cells were added (Table 7.1). Furthermore, we found up to six times higher RU47 abundance in rhizosphere than in bulk soil (Table 7.1), which is not only in agreement with tracing results of our previous experiment (Table 5.1, Table 6.1) but also with studies by Adesina *et al.* (2009) and Schreiter *et al.* (2014 c), who demonstrated a high rhizosphere competence of RU47 in lettuce. PMB strains are isolates from indigenous microbial soil communities of plants. Thus, their natural occurrence in soil and therefore also in non-inoculation treatments is entirely likely and was also observed by Kim *et al.* (1997), Adesina *et al.* (2009) and also in our previous 39-day tomato experiment using RU47 (Table 5.1). Surprisingly, despite using the same soil and identical inoculation procedure, an RU47-specific DNA sequence was not detectable in non-inoculation treatments of this study (Table 7.1). This observation is most likely due to the temporary increase in microbial nutrient and C source limitation in pots and, or as a result, intensified dominance of native soil microbes (50 *vs.* 39 days experiment duration) leading to competitive displacement of the indigenous RU47 population in soil. RU47 abundance determined in treatments using viable RU47 cells in this study corresponded to not more than 1 % of the quantity found in the previous 39-day tomato experiment. Thus, a displacement of RU47 is highly probable. Although not significant, the finding of higher RU47 abundance in the high P trial (Table 7.1) confirms our hypothesis that improved P availability in soil improves the survival and colonization success of RU47. In both P trials, an RU47-specific DNA sequence could be detected in rhizosphere and bulk soils of plants inoculated with dead RU47 cells (Table 7.1). Extracellular DNA as well as nucleases can be adsorbed by surface-reactive soil particles, resulting in a significant reduction/inhibition of DNA degradation in soil (Khanna and Stotzky 1992, Demanèche *et al.* 2001, Pietramellara *et al.* 2009). Lorenz and Wackernagel (1987) reported that the amount of DNA absorbed in quartz sand increased when salt concentration and pH value (5 to 9) increased. Due to the high

percentage of quartz sand (50 %), the mineral fertilization which resulted in increased pH (7.2-7.4), and the salt concentration in our soil substrate, adsorption of free DNA molecules was very likely and this may explain their detectable persistence in the dead RU47 treatment. Furthermore, DNA contained in dead RU47 cells which were applied to the soil was present with other cellular components (wall debris, lipids, proteins etc.) prolonging DNA degradation in soil.

Improved plant growth

Growth-promoting effects of PMB on plants grown under P limited soil conditions have been reported previously (Chabot *et al.* 1996, Kim *et al.* 1997, Sundara *et al.* 2002). In agreement with these results, we found significant increases in shoot height, leaf area and blossom biomass in tomato plants grown under low P availability soil conditions and inoculated with viable RU47 cells (Table 2). Although not significant, a tendency toward improved plant growth by the addition of RU47 was also observed in the high P trial (Fig. 7.1, Fig. 7.2, Table 7.2). This finding corresponds to the higher RU47 abundance determined in this treatment (Table 7.1). Taking into account that only trends were observed, these results support our hypothesis that improved nutrient status in soil increases RU47's ability to persist and expand, leading to an increased ability to promote plant growth. Since P-mobilizing mechanisms that improve plant P supply can be excluded under high P soil conditions, a tendency toward enhanced plant growth is likely caused by phytohormones released by the colonizing RU47. Soil bacteria, including bacteria belonging to the genus *Pseudomonas*, are able to synthesize phytohormones and ACC deaminase, affecting plant growth, development, and response to stress, and ensuring the bacteria's supply of C resources from root exudation (for review see Tsavkelova *et al.* 2006, Hayat *et al.* 2010, Glick 2012, 2014). For instance, Rajkumar and Freitas (2008), who observed a growth-promoting effect on castor oil plants after the addition of *P. jessenii* M6, were able to identify this strain as highly effective in ACC deaminase production. High ACC deaminase activity was also detected for RU47 (Smalla 2016, *personal communication*). Supporting the assumption of an RU47-derived hormonal influence, we observed a decreased root:shoot ratio of 0.4 in plants grown under limited P availability soil conditions and inoculated with viable or dead RU47 cells compared with the ratio of 0.6 found in all other treatments (calculated from data of root and shoot biomass given in Fig. 7.1 and 7.2). This finding is in accordance with Arkhipova *et al.* (2007), who reported that by stimulating the shoot growth of lettuce after the addition of cytokinin-producing bacteria, a lowered root:shoot ratio resulted. Auxin and cytokinin are known to be heat stable (Murashige and Skoog 1962, Kumar and Singh 2009), thus

phytohormones produced by RU47 before killing might be co-extracted and added through inoculation of dead RU47 cells. Cytokinin plays an important role in the regulation of plant cell division and expansion (Ivanova and Rost 1998, Francis and Sorrell 2001) and is antagonistic to abscisic acid (ABA), which inhibits plant growth and flower formation (Levy and Dean 1998, for review see Ha *et al.* 2012). Based on these facts and particularly with regard to the increased blossom biomass documented in both RU47 treatments of the low P trial (Table 7.2), a cytokinin effect by RU47 is very likely.

In both P trials, we also observed a trend of improved plant growth by the addition of dead RU47 cells, especially under high P soil conditions (Fig. 7.1, Fig. 7.2, Table 7.2). Plant growth-promotion after the addition of dead RU47 cells was also observed in our previous tomato experiment (Fig. 7.1). In general, these findings indicate that plant growth was stimulated by phytohormones produced by RU47 and/or indigenous soil microbes which may have been promoted by the addition of C resources in the form of bacterial residues to the soil. These additional C resources may account for our finding that under high P soil conditions, trends of plant growth-promotion were more pronounced in the dead RU47 treatment than in the treatment using viable RU47 cells (Fig. 7.1, Fig. 7.2, Table 7.2). Here, a potential competition effect for substrates and habitats in the rhizosphere between indigenous microbes and the colonizing RU47 bacteria may have played an additional role in reducing plant promotion in the viable RU47 treatment. Supporting the likelihood of an interaction with indigenous microorganisms in the dead RU47 treatment, quantities of RU47-specific DNA sequence determined at both P levels in the dead RU47 treatment were almost equal (Table 7.1). Our observation of increased plant growth by the inoculation of an unselective bacterial mix (Table 7.1) is in contrast to plant growth data recorded in our previous study (Fig. 7.1, Fig. 5.1). Apart from the different growing conditions between these experiments, bacteria were also freshly cultivated using an aliquot of soil before each inoculation. Differences in microbial community composition between each inoculation are likely, and furthermore, a co-cultivation of indigenous PMB in the bacterial mix cannot fully be excluded.

Increased P availability

At both P levels, we observed increased P uptake in the treatments using dead RU47 or viable RU47 cells (Fig. 7.3 b). Under low plant-available P soil conditions, the addition of RU47 resulted in enhanced absolute P uptake, but P concentration in shoot biomass was only significantly increased by the use of dead RU47 (Fig. 7.3 a). This can be explained by a concentration effect. Since plants inoculated with dead RU47 cells had lower shoot biomass

than those treated with viable RU47 cells (Fig. 7.1), the incorporated P was less diluted in shoot biomass leading to an increased P concentration in plants of the dead RU47 treatment (Fig. 7.3 a). Determination of the P tissue concentration (shoot biomass) showed that the plants grown under P limited soil conditions and inoculated with dead RU47 or RU47 had values of 0.52 and 0.46 %, respectively, which corresponds to an adequate P supply for tomato plants at the beginning of flowering, according to Gauch (1972) and Ermochin (1972). However, in the shoot biomass of plants inoculated with a bacterial mix, a P tissue concentration of 0.39 % was determined; this concentration is associated with a low P supply (Gauch 1972, Ermochin 1972). These findings indicate that the addition of dead RU47 or viable RU47 cells improved plant P supply; the more improved P supply by the addition of dead RU47 confirms data determined in our previous tomato experiment (Fig. 5.1), and may have been due to absence of competition for available P in soil between RU47 and plant. Plants grown in the high P trial had P tissue concentration values of around 0.53 %, indicating an improved P supply by higher P fertilization; however, major differences between the inoculation treatments were not observed.

Under low P soil conditions, we found increased alkaline PA in the rhizosphere of plants inoculated with dead RU47 or viable RU47 cells; in the high P trial this effect was only significant in the dead RU47 treatment (Fig. 7.4 b). Increased alkaline PA was also observed in our previous tomato experiment conducted under low P availability soil conditions, also using dead RU47 and viable RU47 for inoculation (Fig. 5.2 a, Table 5.2). In contrast to acid phosphatase, which is synthesized by plant roots as well as by soil microorganisms, alkaline phosphatase is produced exclusively by microorganisms (Dick *et al.* 1983, Juma and Tabatabai 1988, Nannipieri *et al.* 2011). Thus, our findings clearly demonstrate improved microbial PA by the addition of viable RU47 cells. However, despite successful devitalisation of cells and denaturation of phosphatases, the effect of dead RU47 on alkaline PA may have been a result of RU47-derived promotion of indigenous microbes leading to enhanced microbial activity in soil. Increased abundance of indigenous PMB by the addition of specific PMB strains was previously reported by Sundara *et al.* (2002) and Canbolat *et al.* (2006). Considering the increased microbial PA as well as the improved P supply and plant growth determined in both RU47 treatments (Fig. 7.4 b, Fig. 7.3 b, Fig. 7.1), a PMB-attracting effect, observed in both P fertilization trials, could play a complementary role in plant growth-promotion by RU47. Nevertheless, in contrast to our hypothesis, alkaline PA determined in soil inoculated with RU47 did not increase with improved P fertilization (Fig. 7.4 b). Although we observed enhanced RU47 abundance (Table 7.1), PA data did not confirm a higher release of phosphatase. Consistent with alkaline PA data, we observed a tendency of

increased P-CAL concentrations in the bulk soil of plants grown under P limited soil conditions and inoculated with dead RU47 or viable RU47 (Table 7.2). However, the opposite was observed in the high P trial, likely due to the increased P removal by these plants, which had highest shoot biomass compared with all other inoculation treatments in this trial (Table 7.2, Fig. 7.1). The various inocula had no effect on pH values measured in bulk soil (Table 7.2), either in low or in high P trials. Thus, a P solubilizing mechanism decreasing the pH in soil by the microbial secretion of organic acids (Kpomblekou-a and Tabatabai 1994, Jones and Oburger 2011) cannot be assumed - at least not in bulk soil. As expected, pH value in the highly P-fertilized bulk soil decreased significantly due to the dissociation of phosphoric acid (Table 7.1).

7.5 Conclusion

Our study demonstrated that under P limited growing conditions the addition of RU47 increased microbial PA in the rhizosphere and was accompanied by improved P nutrition and growth of tomato plants. Functional gene analyses of phosphomonoesterases could clarify whether this effect is based on increased phosphatase excretion by RU47 or indigenous microbes (interaction) and should be considered in further studies.

Although not significant, improved P nutrition and plant growth were also observed with the addition of dead RU47, suggesting a simultaneously acting hormonal effect caused by phytohormones released from lysed RU47 cells or indigenous soil microbes promoted by dead RU47 (attractants or nutrient spike). With respect to the results of a previous tomato experiment under low P soil conditions that also found plant growth-enhancing tendencies with the addition of dead RU47, further research should focus more on hormonal effects of PMB on plant growth and P supply.

Increased P fertilization improved the abundance of RU47 in both rhizosphere and bulk soil. However, RU47's promoting effects on soil and plant were not increased by the higher abundance. Since an initially high incorporation of P into the RU47 bacterial biomass effectively protects available P from soil reactions, a plant growth-promoting effect in long-term when P becomes limited remains a possible benefit. Thus, simultaneous determinations of microbially bound P and P-CAL in soil could clarify the role of P immobilization by PMB in growth-promoting of plants.

Effects of phosphorus-mobilizing bacteria on growth of tomato and soil microbial activity under varying P availability in soil

Supplemental material

Table 7.S1 Soil substrate (Luvisol topsoil mixed with quartz sand in a ratio of 1:1 [w/w]) characteristics analysed at the end of the experiment (50 days). Values are presented in mean ± standard error (SE) of 40 replicates.

Element	Unit	Mean		SE
C	[%]	0.8	±	0.0
C_{org}	[%]	0.6	±	0.0
N	[%]	0.1	±	0.0
NO_3^-	[mg kg^{-1}]	29.0	±	9.2

Table 7.S2 Results (*p*-values) of two-factor analysis of variance testing significant effects of inoculation (ino), P fertilization (P-fert), and interaction of inoculation and P fertilization (ino * P-fert).

Variable	Ino	P-fert	Ino * P-fert
Rhizosphere RU47 abundance	0.01	0.38	0.06
Bulk soil RU47 abundance	0.01	0.05	0.02
Shoot biomass	0.11	0.02	0.06
Root biomass	0.51	0.73	0.11
Shoot height	0.03	0.42	0.01
Stem diameter	0.30	0.03	0.66
Leaf number	0.22	0.01	0.16
Leaf area	0.03	0.00	0.18
Blossom biomass	0.04	0.68	0.00
P conc in shoot biomass	0.01	0.00	0.00
P uptake in shoot biomass	0.07	0.00	0.28
Rhizosphere acid PA	0.00	0.63	0.31
Bulk soil acid PA	0.00	0.29	0.02
Rhizosphere alkaline PA	0.00	0.63	0.28
Bulk soil alkaline PA	0.01	0.59	0.39
pH in bulk soil	0.61	0.00	0.96
P-CAL conc in bulk soil	0.72	0.00	0.74
P-CAL conc in total bulk soil P [%]	0.64	0.00	0.68

8 Effects of plant growth-promoting rhizobacteria on the indigenous soil microbial community structure of maize in three contrasting Swiss soils

8.1 Introduction

Plant growth promoting-rhizobacteria (PGPR) are able to improve plant nutrient acquisition and act as bio-control agents. Thus, their targeted application offers a promising approach for the future to effectively decrease the application of chemical fertilizers and pesticides in agriculture (Vessey 2003, Lucy *et al.* 2004, Bashan and de-Bashan 2010). This approach is becoming increasingly significant, as the world population, and thus the need for food, continues to grow while mineral phosphorus (P) resources, required to produce phosphate fertilizers, are becoming increasingly limited (Cordell *et al.* 2009). Based on a classification by Martinez-Viveros *et al.* (2010) PGPR are divided into endophytic living bacteria (Rhizobiaceae), which invade the root system to form nodules (Wang and Martinez-Romero 2000) and PGPR that live in the rhizosphere, on the rhizoplane or in the spaces between cells of the root cortex (Bhattacharyya and Jha 2012), such as strains from the genera *Pseudomonas*, *Azospirillum* and *Bacillus* (Gray and Smith 2005). Positive effects on plant growth and health from the application of PGPR have reported several times (for review see Dutta and Podile 2010, Beneduzi *et al.* 2012, Bhattacharyya and Jha 2012, Santoyo *et al.* 2012). The underlying functional mechanisms of PGPR on plants, especially with respect to an improved nutrient supply, are many and not yet fully clarified. However, PGPR, including PMB, can directly affect plant nutrient acquisition by mobilizing soil-bound nutrients (esp. orthophosphate), indirectly by the release of phytohormones that increase root growth and activity (Shaharoona *et al.* 2006, El Zemrany *et al.* 2007), or by promotion of indigenous soil microbes such as mycorrhizal fungi that themselves are beneficial to plant nutrient status (Frey-Klett *et al.* 2007).

Maize (*Zea mays* L.) is globally one of the most cultivated crops (Ranum *et al.* 2014) placing high demands on soil nutrients, especially P, in its early growth stages (Barry and Miller 1989, Postma and Lynch 2011). Although maize has shown a positive response to PGPR applications (Gholami *et al.* 2009, Shaharoona *et al.* 2006, Walker *et al.* 2011), inconsistent effects, depending on the strains added, plant varieties, and soil properties, have prevented a large-scale use of PGPR in current corn production (Fuchs *et al.* 2000, Shaharoona *et al.* 2006, Egamberdiyeva 2007, Mosimann et al. 2017). In a study examining its effect on wheat,

however, Mäder *et al.* (2011) reported an enhanced effect of PGPR in a soil characterized by relatively low microbial biomass and activity. The ability of an inoculant to colonize the rhizosphere at a sufficiently high abundance is a prerequisite for its beneficial effects on plants (Bellis and Ercolani 2001, Barret *et al.* 2011); the lower the competition with indigenous microbes for nutrients and habitats the higher the probability of PGPR to dominate colonization. Depending on their rhizosphere competence, PGPR are able to change the microbial community structure in soil significantly, either by suppressing or producing toxins (esp. biocontrol-capable PGPR), or by the promotion of beneficial plant-microbe symbioses (Bhattacharyya and Jha 2012). For instance, Toro *et al.* (1997) demonstrated that establishment of the indigenous arbuscular mycorrhizal fungus *Glomus intraradices* was promoted by the addition of both *Enterobacter* sp. and *Bacillus subtilis* in soil. Furthermore, Shishido and Chanway (1998) and Vivas *et al.* (2003) observed an increased population of soil bacteria and fungi after the addition of phosphorus-mobilizing PGPR strains. However, the impact of PGPR on soil microbial community structure has had inconsistent reports in the literature, indicating that the use of different PGPR strains and varying soil properties lead to different results (Lottmann *et al.* 2000, Herschkovitz *et al.* 2005, Johansen and Olsson 2005, Mosimann et al. 2017).

The aim of this study was to verify whether PGPR, including PMB, affect microbial community structure in the rhizosphere and bulk soil of maize plants, and furthermore, whether shifts in the microbial community do vary between different PGPR strains and soil properties. Due to its high proportion of phosphorus-mobilizers, three strains of the genus *Pseudomonas* (*P. fluorescens* Pf153, *P.* sp. RU47, and *P.* sp. DSMZ 13134) were chosen for a pot experiment with maize. The experiment was performed using three different Swiss soils varying in nutrient availability, pH value, and soil organic matter content. Besides the determination of bacterial, archaeal and fungal abundances in soil, the population of six bacterial phyla (*Alphaproteobacteria*, *Betaproteobacteria*, *Acidobacteria*, *Bacteroidetes*, *Gemmatimonadetes* and *Firmicutes*) which dominate in European soils (Janssen 2006, Buddrus-Schiemann *et al.* 2010) was measured via quantitative PCR. The following hypotheses were tested. (1) Due to root colonization, the addition of PGPR leads to increased bacterial abundance in the rhizosphere, while the bacterial population in bulk soil is less affected. (2) Effects of PGPR on microbial community structure in the rhizosphere are less pronounced in soils characterized by a high indigenous microbial biomass. (3) Based on the increasing competition for C during PGPR colonization, the abundance of copiotrophic bacterial phyla decreases, while oligotrophic bacterial populations remain unaffected.

8.2 Materials and Methods

The experiment conducted was performed by the Department of Soil Sciences of the Research Institute of Organic Agriculture (FiBL) in Frick (Switzerland). The experimental setup and procedure (without *P.* sp. RU47) is described in Mosimann *et al.* (2017). Microbial cultivation and inoculation, determination of plant properties, and determination of PGPR's persistence in the rhizosphere/rhizoplane were performed by FiBL, while soil sampling and analyses were conducted by the Institute of Soil Science and Land Evaluation of the University of Hohenheim in Stuttgart (Germany).

8.2.1 Pot experiment

Maize plants (*Zea mays* L. var. Colisee) were inoculated with *Pseudomonas fluorescens* Pf153 (Fuchs 1993), *P.* sp. RU47 (Adesina *et al.* 2007) and *P.* sp. DSMZ 13134 (Buddrus-Schiemann *et al.* 2010) and grown in separate soil substrates using the soils Buus, DOK-M and Le Caron (Table 8.1). Details of bacterial cultivation and inoculation are described in 8.2.2. A non-inoculated treatment served as the control. Each treatment was composed of eight replicates, of which half were harvested after four, and the second half after eight weeks. Maize plants were grown in plastic pots (3 L, Rosentopf Soparco, Hortima AG, Switzerland) filled with the equivalent of 2.5 kg soil substrate as dry matter (DM) composed of the topsoil of the respective soil (Table 8.1) and quartz sand (0.6 - 1.2 mm) in a ratio of 1:2 (w/w). To stabilize soil conditions, prepared soil substrates were incubated at 15 °C (± 2 °C) four weeks before potting. Subsequently, each pot was fertilized as follows; N (33.3 mg kg^{-1}; $Ca(NO_3)_2$), K (55.3 mg kg^{-1}; Kalimagnesia containing 30 % K_2O) and P (16.7 mg kg^{-1}; rock phosphate). Three maize seeds were directly sown to a depth of 2 cm and thinned out after germination to one plant per pot. Pots were randomly distributed and placed on saucers to exclude contamination from leaking irrigation water. Maize plants were grown in a climate chamber with 14 h light (Hg/Na lamps; 30,000 lux) at 22 °C and 10 h dark at 19 °C; daily watering used tap water to maintain a water holding capacity of 40 % (Mosimann 2013, *personal communication*).

The soils Buus, DOK-M and Le Caron were chosen due to their contrasting parameters, such as pH, texture, organic C and P content (Table 8.1). Buus soil was taken from a field that has been organically managed (IFOAM 2014, Demeter International e.V. 2016) for the past 30 years. The DOK-M soil belongs to the 36-year-old "DOK" system comparison trial (Mäder *et al.* 2000, 2002) conventionally managed with only the addition of mineral fertilizers. Le Caron soil originates from a 5-year-long grass/clover ley that was converted to a conventionally

managed, no-tillage field in 2011. Classification and characteristics of the three soils are listed in Table 8.1.

8.2.2 Bacterial cultivation and inoculation

Pseudomonas strains used for inoculation were obtained from one commercial product, Proradix® (Sourcon Padena, Tübingen, Germany) that contains *P.* sp. DSMZ 13134 (Buddrus-Schiemann *et al.* 2010), and the two non-commercial strains *P. fluorescens* Pf153 (Fuchs *et al.* 2000) and *P.* sp. RU47 (Adesina *et al.* 2007).

Plants were inoculated immediately after sowing. Five mL of bacterial suspension was added to each planting hole; in the case of the non-inoculation control, 5 mL tap water was used. To prepare the inocula, strains were separately grown in King's B liquid medium (King *et al.* 1954) at 25 °C for 24 h with shaking (150 rpm). Afterwards, 50 µL of bacterial culture was transferred to 50 mL M1 media (Fuchs *et al.* 2000) and re-grown while shaking (150 rpm) at room temperature overnight. Subsequently, bacterial culture's optical density (OD) was measured at 600 nm (UV/Vis) while an OD of 0.1 was estimated as 1×10^8 CFU mL^{-1}. Based on producer's specifications, the following cell densities per mL bacterial suspension were used for inoculation: 3.9×10^8 CFU mL^{-1} (Pf153), 8.5×10^8 CFU mL^{-1} (RU47), and 7.3×10^8 CFU mL^{-1} (Proradix).

8.2.3 Plant properties

Before harvesting, shoot height, number of leaves and SPAD values were recorded for each replicate. SPAD value, an indirect measure of chlorophyll content and thus plant health with respect to nutrient supply (especially N), was measured for each leaf using a SPAD meter (SPAD-502, Konika, Minolta, Osaka, Japan). The mean value per plant was used for statistical analysis. Maize plants were harvested four and eight weeks after sowing (WAS). Shoots of every replicate were carefully cut from the soil surface. To preventing contamination, plants were harvested within their treatment group starting with the non-inoculation control. Dry weight of shoot biomass was determined by drying in separate paper bags at 65 °C for 24 h.

Table 8.1 Characteristics of soils before being used in the present pot experiment. Soil analyses were performed by the Analytical Chemistry Unit of the Core Facility Hohenheim (University of Hohenheim, Stuttgart, Germany). Determination of total C and N as well as organic C was done by the use of an elemental analyser (EA). Total P was determined by inductively coupled plasma optical emission spectrometry (ICP-OES); amount of P_2O_5 that enables the estimation of plant-available P in soil was extracted by calcium acetate lactate (CAL).

Soil	**Geographic origin**	**Texture**			**pH**	**C_{total}**	**C_{org}**	**N_{total}**	**N_{min}**		**P_{total}**	**P_2O_5**
		Clay	**Sand**	**Silt**	($CaCl_2$)	(EA)	(EA)	(EA)	**NH_4^+**	**NO_3^-**	(ICP-OES)	(CAL)
		(%)	(%)	(%)		(%)	(%)	(%)	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})	(mg kg^{-1})
Buus	Buus (Switzerland)	29.9	3.9	66.2	6.6	2.8	2.6	0.3	0.9	27.0	999.2	15.0
DOK-M	Therwil (Switzerland)	16.7	2.7	80.6	5.7	1.3	1.3	0.2	1.0	36.0	730.5	53.0
Le Caron	Epiquerez (Switzerland)	29.9	3.5	66.6	4.8	2.5	2.4	0.3	1.4	73.0	1035.3	37.0

Based on Mosimann *et al.* (2017)

8.2.4 Soil sampling and analyses

Rhizosphere and bulk soil were sampled separately for each replicate. Soil samples were immediately put on ice and cooled at 8 °C for short-term storage. After sieving (< 2 mm) aliquots with a fresh weight of 5 g were used to determine the water content (105 °C for 24 h). Samples were stored at -20 °C until analysis.

Soil microbial C, N and P

To determine microbial biomass C (C_{mic}) and microbially bound N (N_{mic}), the chloroform fumigation extraction method (Vance *et al.* 1987) according to Mackie *et al.* (2015) was used. C_{mic} and N_{mic} were calculated using *keC* 0.45 and 0.54 as extraction factors (Joergensen 1996), respectively. The determination of microbial biomass P (P_{mic}) was performed by liquid fumigation extraction with anion-exchange resin membranes (Kouno *et al.* 2002) using hexanol instead of liquid chloroform (Bünemann *et al.* 2004). A more detailed description of the chloroform fumigation extraction method based on Vance *et al.* (1987) as well as P_{mic} estimation is given in 5.2.7. Microbial C, N and P were determined in bulk soil only.

8.2.5 Quantitative determination of microbial community structure

DNA extraction

DNA extraction of rhizosphere and bulk soil were done using 250 - 350 mg fresh soil with the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) following producer's instructions. A spectrophotometer (NanoDrop 2000; Thermo Scientific, Waltham, MA, USA) was used to measure the DNA concentration and extract purity.

Quantitative PCR (qPCR)

Microbial community structure in soil was determined by quantifying the DNA abundance of bacteria, archaea and fungi as well as six bacteria phyla via qPCR (7500 Fast Real-Time PCR System, Thermo Fisher Scientific, Waltham, MA, USA) in extracted rhizosphere and bulk soil DNA. SYBR® Green intercalating double-stranded DNA was used to quantify the amplicons; the domain- and phylum-specific primers as well as thermal cycling conditions are listed in Table 8.2. To achieve a final volume of 15 µL, the following components were used;: 7.5 µL SYBR® Green PCR Master Mix (2 ×; Thermo Fisher Scientific, Waltham, MA, USA), 4.125 µL ultra-pure water, 1.5 µL DNA (5 ng μL^{-1}), 0.75 µL each of forward and reverse primers (10 µM), and 0.375 µL T4gp32. To measure the abundance of bacterial and archaeal 16S rDNA, 1 µL of template DNA was used; the difference in final volume was balanced with

ultra-pure water. Standard curves were performed with serial dilutions of a known amount of plasmid DNA containing the sequence of the respective gene. Based on the standard curve, the absolute quantity of copies was calculated (copies g^{-1} soil).

8.2.6 Statistical analyses

Homogeneity of variance was tested by the Levene-test. Significance of differences was tested by ANOVA followed by the Tukey HSD-test, where $p < 0.05$ was defined as the threshold value for significance. In case of variance heterogeneity, determination of significance of differences used the Welch-Test, and the Games-Howell-test was used for pairwise comparisons. Here, $p < 0.05$ was also considered as significantly different. These statistical analyses were performed using SPSS Statistics 22 (IBM 2013). Variance components analysis (VCA) was used to identify which factors (inoculation, soil, time) explained most of the variance in each of the variables. This analysis was carried out with R statistics version 3.2.1 (R Core Team 2015) using “lme” and “VarCorr” functions of the “nlme” package (Pinheiro *et al.* 2013).

Table 8.2 qPCR primers and thermal profiles. NC stands for number of cycles.

	Primer	Thermal profile	NC	Reference
Bacteria (16S rDNA)	341F	95°C – 10 min	1	Muyzer *et al.* 1993, López-Gutiérrez *et al.* 2004
	534R	95 °C – 15 s, 60 °C – 30 s, 72 °C – 30 s, 75 °C – 30 s	35	
Archaea (16S rDNA)	Ar109F	95 °C – 10 min	1	Lueders and Friedrich 2000, Rasche *et al.* 2011
	Ar912R	95 °C – 15 s, 52 °C – 1 min, 72 °C – 1 min, 75 °C – 30 s	40	
Fungi (ITS)	314F	95 °C – 10 min	1	White *et al.* 1990, Ditterich *et al.* 2013
	534R	95 °C – 15 s, 52 °C – 30 s, 72 °C – 30 s, 76 °C – 30 s	35	
Alphaproteobacteria	Eub338	95 °C – 10 min	1	Fierer *et al.* 2005
	Alpha685	95 °C – 15 s, 60 °C – 30 s, 72 °C – 30 s, 79 °C – 30 s	35	
Betaproteobacteria	Eub338	95 °C – 10 min	1	Fierer *et al.* 2005
	Bet680	95 °C – 15 s, 55 °C – 30 s, 72 °C – 30 s, 76 °C – 30 s	35	
Bacteroidetes	798cfbF	95 °C – 10 min	1	De Gregoris *et al.* 2011, *this study*
	cfb967R	95 °C – 15 s, 61.5 °C – 30 s, 72 °C – 30 s, 75 °C – 30 s	35	

Firmicutes	Lgc353	95°C – 10 min	1	Fierer *et al.* 2005
	Eub518	95 °C – 15 s, 60 °C – 30 s, 72 °C – 30 s, 79 °C – 30 s	35	
Acidobacteria	Acid31	95 °C – 10 min	1	Fierer *et al.* 2005
	Eub518	95 °C – 15 s, 55 °C – 30 s, 72 °C – 30 s, 81 °C – 30 s	35	
Gemmatimonadetes	Gem440	95 °C – 10 min	1	Philippot *et al.* 2009
	Eub518	95 °C – 15 s, 58 °C – 30 s, 72 °C – 30 s, 78 °C – 30 s	35	

8.3 Results

8.3.1 Plant response

The addition of different *Pseudomonas* strains did not generally lead to improved plant growth, either four or eight weeks after sowing (Fig. 8.1, Table 8.3). However, in plants grown in soil Le Caron and harvested eight weeks after sowing the addition of Proradix and RU47 resulted in increased shoot biomass values, and each inoculation increased the shoot height (Fig. 8.1, Table 8.3). Four weeks after sowing, variation in plant growth was strongly affected by the factor soil; shoot biomass and height observed were significantly different between each soil treatment (across the inoculation treatments; $p < 0.05$) showing highest plant growth in the soil DOK-M. This effect was not observed in plants harvested eight weeks after sowing (Fig. 8.1, Table 8.3).

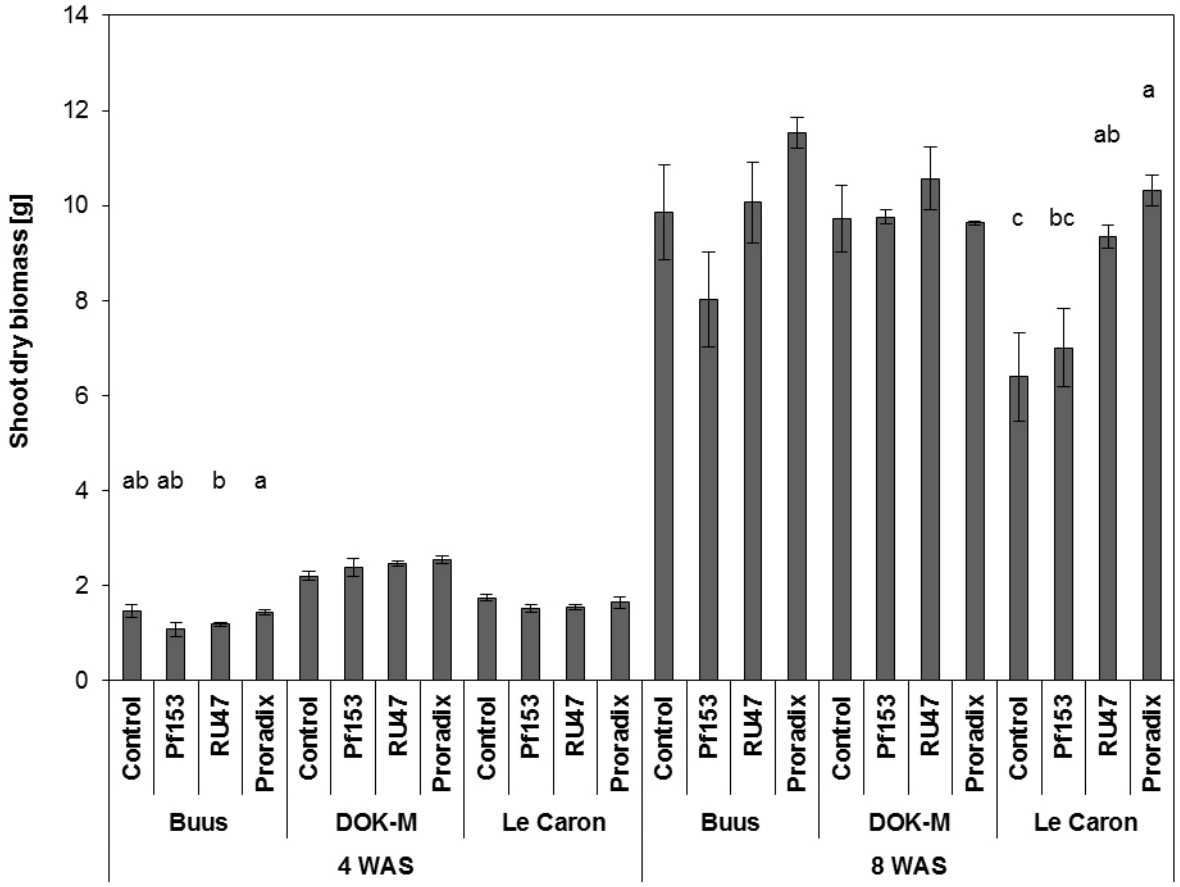

Figure 8.1 Shoot dry biomass from maize plants of one non-inoculation (control), and three inoculation treatments: *Pseudomonas fluorescens* Pf153 (Pf153), *P.* sp. RU47 (RU47) and *P.* sp. DSMZ 13134 (Proradix). Plants grown in three different soils (Buus, DOK-M, Le Caron) and were harvested four and eight weeks after sowing (WAS). Error bars indicate standard error (n = 4). Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the inoculation treatments within one soil are marked by lowercase letters.

8.3.2 Responses of microbial C, N and P in bulk soil

Various inocula rarely affect the amount of microbially bound C, N and P in bulk soil of maize plants, at four or eight weeks after sowing (Table 8.3). However, in soil Le Caron, sampled eight weeks after sowing, the addition of Pf153 led to increased microbial biomass (Table 8.3). At both four and eight weeks after sowing, highest C_{mic}, N_{mic} and P_{mic} values were found in soil Buus, while lowest values were for the most part detected in soil DOK-M (Table 8.3). Furthermore, we observed decreased C_{mic} and N_{mic} values from the first to the second harvest by 29 and 41 %, respectively, across the three soils (Table 8.3). In contrast, microbial biomass P remained unaffected by time (Table 8.3).

To identify which factors, i.e. inoculation, soil, and time (4 and 8 WAS) explained most of the variance in microbially bound C, N and P, a variance components analysis (VCA) was conducted. Based on this analysis, no effect of inoculation (0 %) on C_{mic} and N_{mic} values was found; 5 % of the variance in P_{mic} was explainable by inoculation (data not shown). Moreover, the quantities of microbially bound C, N and P determined in the bulk soil of maize plants were primarily affected by the factor soil, with explained variances of 72, 65 and 42 %, respectively (Table 8.S1). With respect to time, no explainable variance in P_{mic} was determined, while time explained 19 and 23 % of the variance in microbially bound C and N, respectively (Table 8.S1).

Table 8.3 Plant and soil properties of maize plants using one non-inoculation (control), and three inoculation treatments: *P. fluorescens* Pf153 (Pf153), *P.* sp. RU47 (RU47), and *P.* sp. DSMZ 13134 (Proradix). Plants grown in three different soils (Buus, DOK-M, Le Caron) and were harvested four and eight weeks after sowing (WAS). Values are presented as mean ± standard error (SE) of four replicates. Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the inoculation treatments within one soil are marked by lowercase letters.

		Shoot height [cm]		SPAD		Number of leaves		C_{mic} [mg kg^{-1}]		N_{mic} [mg kg^{-1}]		P_{mic} [mg kg^{-1}]	
		Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
4 WAS													
Buus	Control	63.4	±1.8	27.0	±0.8	5.5^{ab}	±0.3	206.9	±4.1	34.5	±2.9	9.8	±1.1
	Pf153	55.5	±3.3	27.9	±0.7	4.8^{ab}	±0.3	222.5	±5.4	34.5	±0.6	9.0	±0.6
	RU47	58.8	±1.8	27.9	±0.6	5.0^{b}	±0.0	218.7	±6.2	36.2	±2.0	9.8	±0.4
	Proradix	62.4	±1.2	28.6	±0.6	6.0^{a}	±0.0	220.1	±7.6	34.5	±2.2	7.5	±0.5
DOK-M	Control	67.5	±0.8	29.2^{ab}	±1.1	6.0	±0.0	77.6	±14.0	13.9	±1.4	3.9	±0.6
	Pf153	68.6	±1.2	28.8^{b}	±0.8	6.0	±0.0	97.8	±3.4	14.2	±0.7	10.3	±2.3
	RU47	70.6	±1.4	29.4^{b}	±0.6	6.0	±0.0	98.2	±5.4	12.7	±0.3	3.4	±0.5
	Proradix	72.0	±1.8	34.0^{a}	±0.6	6.0	±0.0	96.7	±1.5	13.6	±0.5	5.4	±0.8
Le Caron	Control	66.6	±1.7	27.9^{a}	±0.4	6.0	±0.0	140.3	±15.2	15.4	±4.0	4.7^{ab}	±0.5
	Pf153	63.6	±1.8	25.4^{b}	±0.4	5.8	±0.3	96.2	±6.4	11.1	±2.1	3.8^{ab}	±0.4
	RU47	64.6	±0.8	26.0^{ab}	±0.5	5.8	±0.3	121.7	±17.0	7.8	±1.5	3.0^{b}	±0.4
	Proradix	66.1	±2.3	28.1^{ab}	±1.0	6.0	±0.0	131.5	±12.0	15.7	±2.1	5.6^{a}	±0.3
8 WAS													
Buus	Control	95.8^{ab}	±1.8	20.7^{ab}	±2.2	9.8	±0.3	163.1	±9.3	20.8	±1.1	8.9	±1.4
	Pf153	93.8^{b}	±2.3	22.2^{b}	±2.3	9.5	±0.3	160.2	±3.4	19.9	±0.4	9.1	±1.6
	RU47	98.0^{ab}	±1.7	19.0^{ab}	±2.3	10.0	±0.4	156.2	±8.9	19.9	±1.5	7.4	±0.8
	Proradix	103.4^{a}	±1.9	13.0^{a}	±0.3	10.8	±0.3	157.4	±8.9	20.8	±1.2	6.9	±0.3
DOK-M	Control	97.6	±3.2	11.0	±0.3	10.8^{ab}	±0.3	65.8	±7.0	6.4	±0.5	3.2^{ab}	±0.4
	Pf153	96.3	±2.1	10.6	±0.5	10.0^{b}	±0.0	69.7	±6.3	7.8	±0.7	6.1^{a}	±0.6
	RU47	99.0	±1.1	10.9	±0.6	10.5^{ab}	±0.3	62.2	±8.0	6.2	±0.9	5.2^{ab}	±0.8
	Proradix	95.4	±1.6	10.5	±0.3	11.5^{a}	±0.3	74.2	±13.3	9.8	±3.3	2.6^{b}	±0.9

Le Caron	Control	84.3 ^{c} ±1.7	23.3 ^{a} ±0.4	10.0 ^{ab} ±0.4	61.6 ^{b} ±9.1	5.9 ±2.1	3.6 ±0.4
	Pf153	92.4 ^{b} ±2.2	23.8 ^{a} ±0.6	9.5 ^{ab} ±0.5	99.3 ^{a} ±10.0	10.3 ±1.3	6.0 ±0.8
	RU47	98.9 ^{ab} ±2.4	21.7 ^{ab} ±0.7	10.0 ^{b} ±0.0	68.0 ^{ab} ±5.1	6.5 ±0.4	7.3 ±1.4
	Proradix	101.9 ^{a} ±0.6	20.0 ^{b} ±0.5	11.0 ^{a} ±0.0	91.7 ^{ab} ±9.5	10.0 ±1.4	5.1 ±1.2

8.3.3 Effects on microbial community structure

The addition of three different *Pseudomonas* strains did not generally affect the abundances of bacteria, archaea, or fungi in soil. Individual effects of inoculation with the different strains were observed, however, in both rhizosphere and bulk soil, in different soils, and in harvest dates (Fig. 8.2 a, Fig. 8.2 b). In the rhizosphere of plants grown in Buus or Le Caron and harvested eight weeks after sowing, significant increases in archaeal and fungal abundances were found under the addition of Pf153 (Fig. 8.2 a). In the rhizosphere of plants grown in DOK-M and harvested eight weeks after sowing, inoculation with RU47 led to increases in bacterial and archeal abundances (Fig. 8.2 a). Proradix increased archeal abundance in the rhizosphere of Buus sampled four weeks after sowing (Fig. 8.2 a). In bulk soil sampled eight weeks after sowing, the addition of RU47 increased bacterial and archeal abundances in Buus, while archeal abundance was also enhanced by Proradix in DOK-M sampled four weeks after sowing (Fig. 8.2 b). In both rhizosphere and bulk soils as well as at both harvest dates, highest abundances of bacteria and archaea were found in soil Buus, while lowest values were determined in Le Caron (Fig. 8.2 a, Fig. 8.2 b). Fungal abundances shifted over time; in plants harvested four weeks after sowing, highest fungal abundances in both rhizosphere and bulk soil were found in soil DOK-M. In plants harvested eight weeks after sowing, highest fungal abundances were detected in soil Buus (Fig. 8.2 a, Fig. 8.2 b). In general, microbial abundance decreased over time; from the first to second harvest, abundances of bacteria, archaea and fungi in the rhizosphere decreased by approximately 54 % (Fig. 8.2 a). In bulk soil, declines in bacterial and fungal abundances of approximately 64 % were observed, while archaeal abundance was reduced by only 7 % (Fig. 8.2 b).

VCA indicated no effect of inoculation (0 %) on bacterial or fungal growth, in either rhizosphere or bulk soil (Fig. 8.3). However, inoculation explained 3 and 1 % of the variance in archaeal abundance in rhizosphere and bulk soil, respectively (Fig. 8.3). While archaeal growth was primarily affected by soil, which explained up to 90 % of the variance, the abundances of bacteria and fungi were primarily influenced by time (Fig. 8.3). The respective dominant influencing factors on bacterial, fungal and archaeal abundances were stronger in bulk soil than in the rhizosphere (Fig. 8.3).

Inoculation by the different *Pseudomonas* strains weakly influenced the abundances of the six bacterial phyla analysed in this study; in general, individual effects of the strains on bacterial communities were more pronounced in rhizosphere than in bulk soil, and in the second harvest compared with the first (Table 8.4 a, Table 8.4 b). Each *Pseudomonas* strain

added led to an increased abundance of *Gemmatimonadetes* in the rhizosphere of maize plants grown in Le Caron and harvested eight weeks after sowing (Table 8.4 a). In treatment Pf153 increased abundances of *Betaproteobacteria* and *Acidobacteria* were observed as well (Table 8.4 a). The addition of RU47 resulted in decreased *Alphaproteobacteria* abundance in the rhizosphere of Buus sampled four weeks after sowing, while abundances of *Betaproteobacteria* and *Acidobacteria* increased significantly in both soil DOK-M and Le Caron sampled eight weeks after sowing (Table 8.4 a). In Le Caron the addition of RU47 led to increased growth of *Firmicutes* as well (Table 8.4 a). In bulk soil, only one inoculation affected the bacterial community; the addition of Pf153 to soil DOK-M resulted in a significant decrease in *Bacteroidetes* abundance four weeks after sowing. (Table 8.4 b). In general, highest bacterial abundance was found in soil Buus; here the phyla *Bacteroidetes* and *Acidobacteria* dominated (Table 8.4 a, Table 8.4 b). Abundances of *Alphaproteobacteria*, *Betaproteobacteria* and *Bacteroidetes* were primarily affected by time; in general, their abundances decreased by approximately 80 % from the first to second harvest (Table 8.4 a, Table 8.4 b).

VCA revealed that abundances of *Betaproteobacteria*, *Bacteroidetes* and *Firmicutes* were not influenced (0 %) by inoculation, either in rhizosphere or in bulk soil (Fig. 8.4). However, inoculation explained up to 3 % of the variance in abundances of *Alphaproteobacteria*, *Acidobacteria* and *Gemmatimonadetes* in soil (Fig. 8.4). Growth of *Firmicutes*, *Acidobacteria* and *Gemmatimonadetes* was primarily affected by the factor soil, which explained their abundances of about 54 and 65% in rhizosphere and bulk soil respectively (Fig. 8.4). By contrast, the factor time (4 and 8 WAS) explained up to 90 % of the variance in *Alpha-* and *Betaproteobacterial* abundances (Fig. 8.4). The abundance of *Bacteroidetes* was influenced by time and soil; VCA explained the observed variance in *Bacteroidetes* growth of about 32 % by the factor time, and 28 % by the factor soil in both rhizosphere and bulk soil (Fig. 8.4).

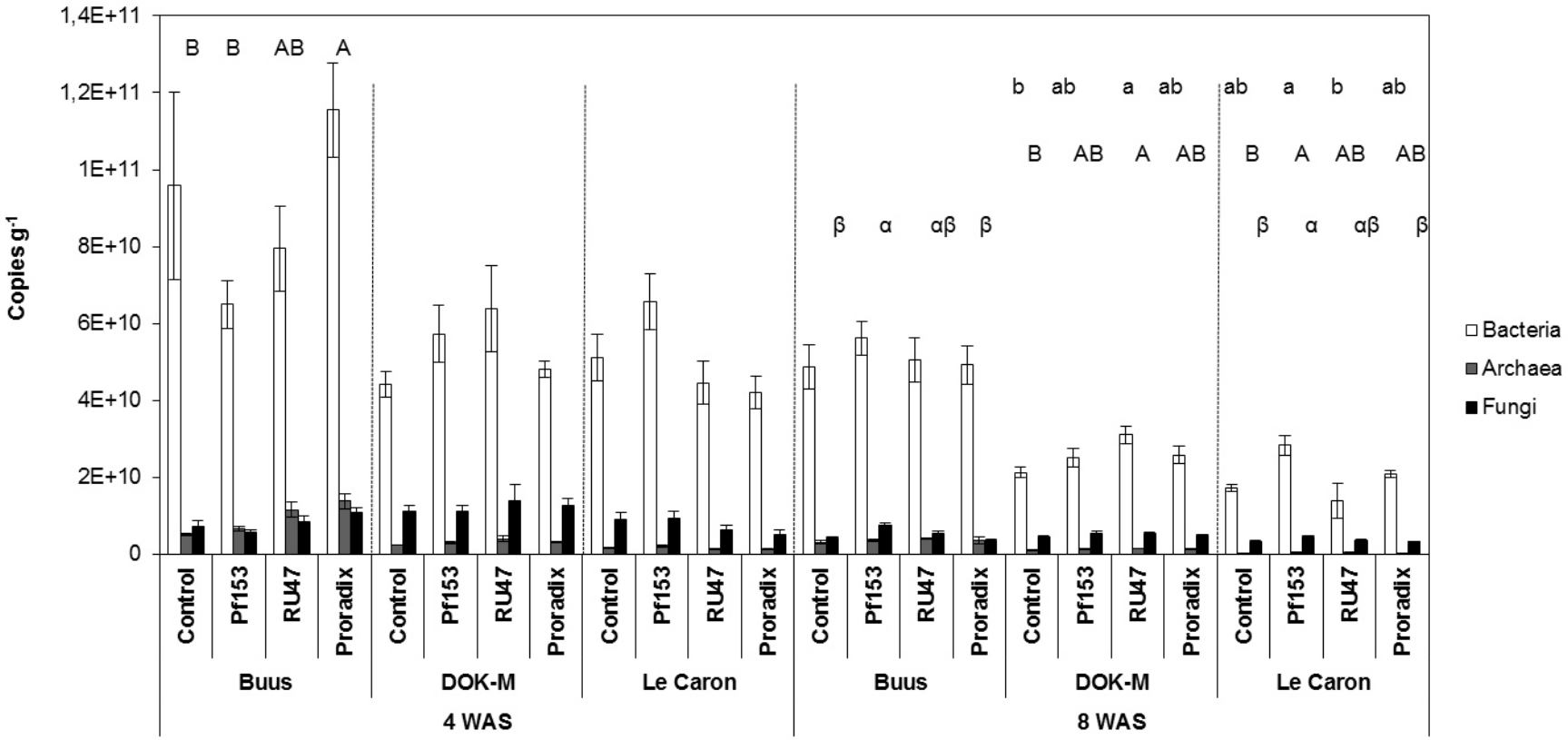

Figure 8.2 a Abundance of domain-specific DNA sequences in the rhizosphere of maize plants using one non-inoculation (control), and three inoculation treatments: *Pseudomonas fluorescens* Pf153 (Pf153), *P.* sp. RU47 (RU47), and *P.* sp. DSMZ 13134 (Proradix). Plants grown in three different soils (Buus, DOK-M, Le Caron) and were harvested four and eight weeks after sowing (WAS). Values are presented as mean (n = 4); error bars indicate standard error. Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the inoculation treatments within one soil and harvest date are marked by lowercase letters for bacteria, uppercase letters for archaea and Greek letters for fungi.

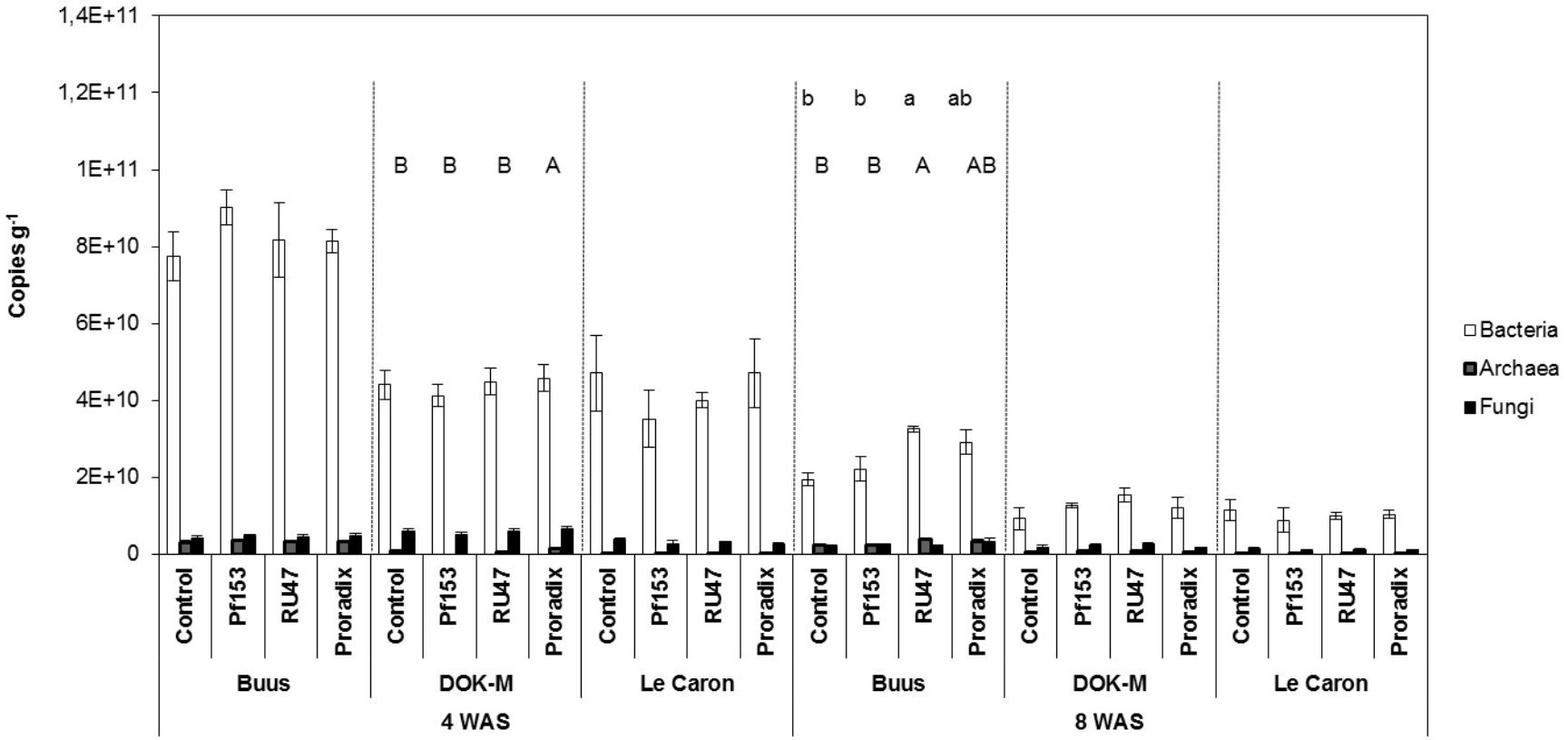

Figure 8.2 b Abundance of domain-specific DNA sequences in bulk soil of of maize plants using one non-inoculation (control), and three inoculation treatments: *Pseudomonas fluorescens* Pf153 (Pf153), *P.* sp. RU47 (RU47), and *P.* sp. DSMZ 13134 (Proradix). Plants grown in three different soils (Buus, DOK-M, Le Caron) and were harvested four and eight weeks after sowing (WAS). Values are presented as mean (n = 4); error bars indicate standard error. Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the inoculation treatments within one soil and harvest date are marked by lowercase letters for bacteria and uppercase letters for archaea. Differences in fungal abundance were not significant.

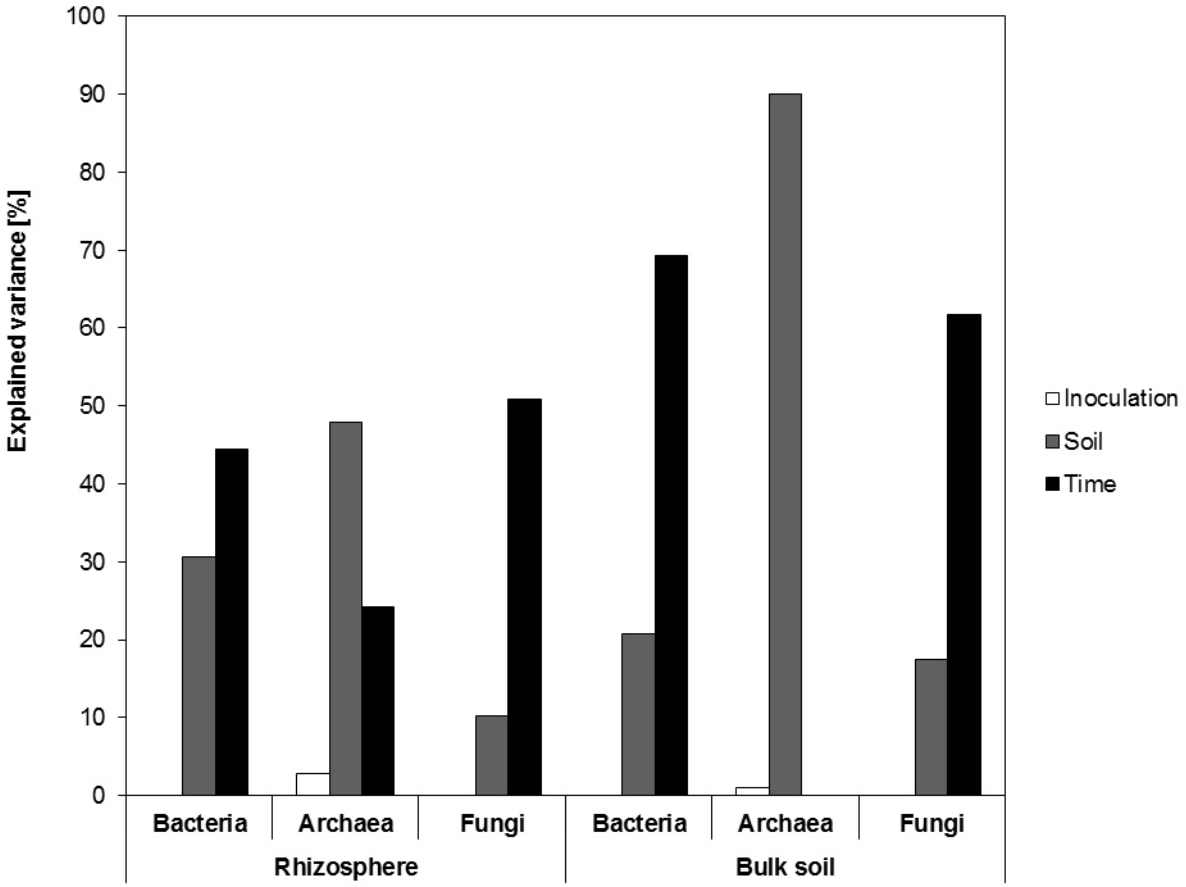

Figure 8.3 Variance components analysis showing the percent explained variance for inoculation (non-inoculation [control], *P. fluorescens* Pf153 [Pf153], *P.* sp. RU47 [RU47], *P.* sp. DSMZ 13134 [Proradix]), soil (Buus, DOK-M, Le Caron) and time (four and eight weeks after sowing) for abundances of bacteria, archaea and fungi in rhizosphere and bulk soil.

Table 8.4 a Abundance of phylum specific DNA sequences in the rhizosphere of maize using one non-inoculation (control), and three inoculation treatments: *P. fluorescens* Pf153 (Pf153), *P.* sp. RU47 (RU47) and *P.* sp. DSMZ 13134 (Proradix). Plants grown in three different soils (Buus, DOK-M, Le Caron) and were harvested four and eight weeks after sowing (WAS). Values are presented as mean ± standard error (SE) of four replicates. Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the inoculation treatments are marked by lowercase letters. Cp stands for copies.

		Alphaproteobacteria		***Betaproteobacteria***		***Bacteroidetes***		***Firmicutes***		***Acidobacteria***		***Gemmatimonadetes***	
		Mean	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**
4 WAS		**[cp g^{-1}]**		**[cp g^{-1}]**		**[cp g^{-1}]**		**[cp g^{-1}]**		**[cp g^{-1}]**		**[cp g^{-1}]**	
Buus	Control	3.E+09 ^{a}	± 4.E+08	3.E+10	± 4.E+09	5.E+10	±1.E+10	2.E+09 ^{ab}	±3.E+08	2.E+10 ^{ab}	±3.E+09	9.E+08 ^{ab}	± 1.E+08
	Pf153	3.E+09 ^{a}	± 6.E+08	2.E+10	± 3.E+09	5.E+10	±2.E+10	1.E+09 ^{a}	±1.E+08	2.E+10 ^{a}	±1.E+09	8.E+08 ^{b}	± 8.E+07
	RU47	6.E+08 ^{b}	± 4.E+08	2.E+10	± 3.E+09	4.E+10	±1.E+10	1.E+09 ^{ab}	±1.E+08	2.E+10 ^{ab}	±3.E+09	1.E+09 ^{ab}	± 1.E+08
	Psp13134	2.E+09 ^{ab}	± 3.E+08	3.E+10	± 4.E+09	5.E+10	±1.E+10	2.E+09 ^{b}	±1.E+08	3.E+10 ^{b}	±3.E+09	1.E+09 ^{a}	± 1.E+08
DOK-M	Control	1.E+09	± 2.E+08	2.E+10	± 1.E+09	2.E+10	±6.E+09	1.E+09	±1.E+08	1.E+10	±5.E+08	5.E+08	± 4.E+07
	Pf153	2.E+09	± 3.E+08	2.E+10	± 2.E+09	2.E+10	±5.E+09	1.E+09	±2.E+08	1.E+10	±1.E+09	6.E+08	± 1.E+08
	RU47	2.E+09	± 3.E+08	3.E+10	± 8.E+09	2.E+10	±6.E+09	1.E+09	±3.E+08	1.E+10	±2.E+09	8.E+08	± 2.E+08
	Proradix	2.E+09	± 3.E+08	2.E+10	± 2.E+09	2.E+10	±5.E+09	1.E+09	±7.E+07	1.E+10	±7.E+08	6.E+08	± 5.E+07
Le Caron	Control	3.E+09	± 6.E+08	2.E+10 ^{ab}	± 3.E+09	7.E+09	±3.E+09	2.E+09	±3.E+08	1.E+10	±8.E+08	9.E+08	± 8.E+07
	Pf153	4.E+09	± 6.E+08	2.E+10 ^{b}	± 2.E+09	1.E+10	±4.E+09	2.E+09	±1.E+08	1.E+10	±9.E+08	1.E+09	± 6.E+07
	RU47	3.E+09	± 6.E+08	1.E+10 ^{ab}	± 2.E+09	8.E+09	±3.E+09	1.E+09	±2.E+08	8.E+09	±1.E+09	8.E+08	± 1.E+08
	Proradix	3.E+09	± 9.E+07	1.E+10 ^{a}	± 2.E+09	8.E+09	±1.E+09	2.E+09	±2.E+08	7.E+09	±1.E+09	7.E+08	± 1.E+08
8 WAS													
Buus	Control	6.E+08	± 1.E+08	7.E+09	± 8.E+08	1.E+10	±3.E+09	1.E+09	±9.E+07	2.E+10	±2.E+09	1.E+09	± 9.E+07
	Pf153	8.E+08	± 1.E+08	8.E+09	± 7.E+08	1.E+10	±3.E+09	2.E+09	±1.E+08	2.E+10	±2.E+09	1.E+09	± 2.E+08
	RU47	6.E+08	± 1.E+08	7.E+09	± 4.E+08	1.E+10	±3.E+09	2.E+09	±2.E+08	2.E+10	±1.E+09	1.E+09	± 5.E+07
	Proradix	7.E+08	± 1.E+08	7.E+09	± 8.E+08	1.E+10	±4.E+09	2.E+09	±2.E+08	2.E+10	±2.E+09	1.E+09	± 2.E+08
DOK-M	Control	2.E+08	± 3.E+07	5.E+09 ^{b}	± 2.E+08	3.E+09	±1.E+09	7.E+08 ^{ab}	±5.E+07	7.E+09 ^{b}	±5.E+08	6.E+08 ^{b}	± 3.E+07
	Pf153	1.E+08	± 6.E+07	6.E+09 ^{ab}	± 5.E+08	2.E+09	±5.E+08	8.E+08 ^{a}	±6.E+07	8.E+09 ^{b}	±6.E+08	5.E+08 ^{b}	± 9.E+07
	RU47	2.E+08	± 2.E+07	6.E+09 ^{a}	± 4.E+08	5.E+09	±1.E+09	1.E+09 ^{a}	±6.E+07	1.E+10 ^{a}	±7.E+08	8.E+08 ^{a}	± 7.E+07
	Proradix	2.E+08	± 2.E+07	5.E+09 ^{ab}	± 3.E+08	2.E+09	±5.E+08	6.E+08 ^{b}	±3.E+07	8.E+09 ^{b}	±5.E+08	6.E+08 ^{ab}	± 4.E+07

Le Caron	Control	2.E+08	± 3.E+07	3.E+09 [b]	± 9.E+07	1.E+09	±3.E+08	1.E+09 [b]	±9.E+07	4.E+09 [b]	±3.E+08	6.E+08 [b]	± 4.E+07
	Pf153	4.E+08	± 7.E+07	4.E+09 [a]	± 3.E+08	3.E+09	±6.E+08	1.E+09 [ab]	±1.E+08	7.E+09 [a]	±4.E+08	1.E+09 [a]	± 6.E+07
	RU47	4.E+08	± 1.E+08	4.E+09 [ac]	± 2.E+08	4.E+09	±1.E+09	2.E+09 [a]	±1.E+08	7.E+09 [a]	±4.E+08	9.E+08 [a]	± 2.E+07
	Proradix	4.E+08	± 7.E+07	3.E+09 [bc]	± 1.E+08	3.E+09	±9.E+08	1.E+09 [ab]	±5.E+07	5.E+09 [ab]	±2.E+08	8.E+08 [a]	± 2.E+07

Table 8.4 b Abundance of phylum specific DNA sequences in bulk soil of maize using one non-inoculation (control), and three inoculation treatments: *P. fluorescens* Pf153 (Pf153), *P.* sp. RU47 (RU47) and *P.* sp. DSMZ 13134 (Proradix). Plants grown in three different soils (Buus, DOK-M, Le Caron) and were harvested four and eight weeks after sowing (WAS). Values are presented as mean ± standard error (SE) of four replicates. Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the inoculation treatments are marked by lowercase letters. Cp stands for copies.

		Alphaproteobacteria		***Betaproteobacteria***		***Bacteroidetes***		***Firmicutes***		***Acidobacteria***		***Gemmatimonadetes***	
4 WAS		**Mean [cp g^{-1}]**	**SE**	**Mean [cp g^{-1}]**	**SE**	**Mean [cp g^{-1}]**	**SE**	**Mean [cp g^{-1}]**	**SE**	**Mean [cp g^{-1}]**	**SE**	**Mean [cp g^{-1}]**	**SE**
Buus	Control	2.E+09	± 3.E+08	2.E+10	± 1.E+09	3.E+10	±1.E+10	8.E+08	±7.E+07	2.E+10	±1.E+09	5.E+08	± 4.E+07
	Pf153	2.E+09	± 5.E+08	3.E+10	± 4.E+09	3.E+10	±1.E+10	9.E+08	±6.E+07	2.E+10	±2.E+09	6.E+08	± 5.E+07
	RU47	2.E+09	± 6.E+08	2.E+10	± 3.E+09	3.E+10	±1.E+10	8.E+08	±1.E+08	2.E+10	±3.E+09	6.E+08	± 4.E+07
	Proradix	2.E+09	± 5.E+08	2.E+10	± 2.E+09	2.E+10	±1.E+10	8.E+08	±2.E+07	2.E+10	±2.E+09	5.E+08	± 3.E+07
DOK-M	Control	9.E+08	± 1.E+08	2.E+10	± 1.E+09	8.E+09 [a]	±3.E+09	5.E+08	±6.E+07	6.E+09	±6.E+08	3.E+08	± 2.E+07
	Pf153	7.E+08	± 1.E+08	1.E+10	± 8.E+08	0.E+00 [b]	±0.E+00	5.E+08	±3.E+07	5.E+09	±5.E+08	3.E+08	± 2.E+07
	RU47	8.E+08	± 7.E+07	2.E+10	± 1.E+09	7.E+09 [ab]	±2.E+09	5.E+08	±4.E+07	7.E+09	±6.E+08	3.E+08	± 1.E+07
	Proradix	8.E+08	± 1.E+08	1.E+10	± 6.E+08	7.E+09 [ab]	±1.E+09	5.E+08	±4.E+07	6.E+09	±8.E+08	3.E+08	± 1.E+07
Le Caron	Control	2.E+09	± 5.E+08	1.E+10	± 2.E+09	3.E+09	±2.E+09	9.E+08	±2.E+08	9.E+09	±1.E+09	5.E+08	± 1.E+08
	Pf153	1.E+09	± 3.E+08	1.E+10	± 3.E+09	3.E+09	±8.E+08	7.E+08	±2.E+08	7.E+09	±2.E+09	5.E+08	± 1.E+08
	RU47	2.E+09	± 2.E+08	1.E+10	± 1.E+09	3.E+09	±1.E+09	7.E+08	±7.E+07	7.E+09	±3.E+08	4.E+08	± 6.E+06
	Proradix	2.E+09	± 6.E+08	1.E+10	± 2.E+09	5.E+09	±2.E+09	8.E+08	±1.E+08	9.E+09	±2.E+09	5.E+08	± 8.E+07

8 WAS

Buus	Control	1.E+09	± 2.E+08	2.E+06	± 2.E+05	2.E+09	±4.E+08	1.E+09	±9.E+07	1.E+10	±1.E+09	7.E+08	± 9.E+07
	Pf153	1.E+09	± 3.E+08	2.E+06	± 1.E+05	3.E+09	±1.E+09	1.E+09	±1.E+08	2.E+10	±1.E+09	7.E+08	± 5.E+07
	RU47	1.E+09	± 2.E+08	1.E+06	± 4.E+05	3.E+09	±9.E+08	1.E+09	±7.E+07	2.E+10	±2.E+09	9.E+08	± 7.E+07
	Proradix	1.E+09	± 3.E+08	2.E+06	± 2.E+05	3.E+09	±1.E+09	1.E+09	±2.E+08	1.E+10	±2.E+09	7.E+08	± 1.E+08
DOK-M	Control	2.E+08	± 5.E+07	9.E+05	± 3.E+05	6.E+08	±3.E+08	4.E+08	±1.E+08	3.E+09	±1.E+09	2.E+08	± 8.E+07
	Pf153	4.E+08	± 4.E+07	1.E+06	± 1.E+05	1.E+09	±3.E+08	6.E+08	±1.E+07	5.E+09	±4.E+08	2.E+08	± 8.E+07
	RU47	4.E+08	± 8.E+07	1.E+06	± 1.E+05	1.E+09	±4.E+08	6.E+08	±8.E+07	6.E+09	±9.E+08	4.E+08	± 5.E+07
	Proradix	2.E+08	± 4.E+07	9.E+05	± 3.E+04	5.E+08	±2.E+08	4.E+08	±1.E+08	4.E+09	±3.E+08	2.E+08	± 2.E+07
Le Caron	Control	3.E+08	± 8.E+07	9.E+05	± 2.E+05	4.E+08	±1.E+08	9.E+08	±2.E+08	4.E+09	±1.E+09	3.E+08	± 3.E+07
	Pf153	4.E+08	± 2.E+08	7.E+05	± 3.E+05	3.E+08	±2.E+08	6.E+08	±3.E+08	3.E+09	±1.E+09	3.E+08	± 1.E+08
	RU47	3.E+08	± 6.E+07	8.E+05	± 6.E+04	6.E+08	±3.E+08	8.E+08	±8.E+07	5.E+09	±3.E+08	5.E+08	± 3.E+07
	Proradix	4.E+08	± 8.E+07	7.E+05	± 8.E+04	6.E+08	±3.E+08	7.E+08	±7.E+07	3.E+09	±4.E+08	4.E+08	± 5.E+07

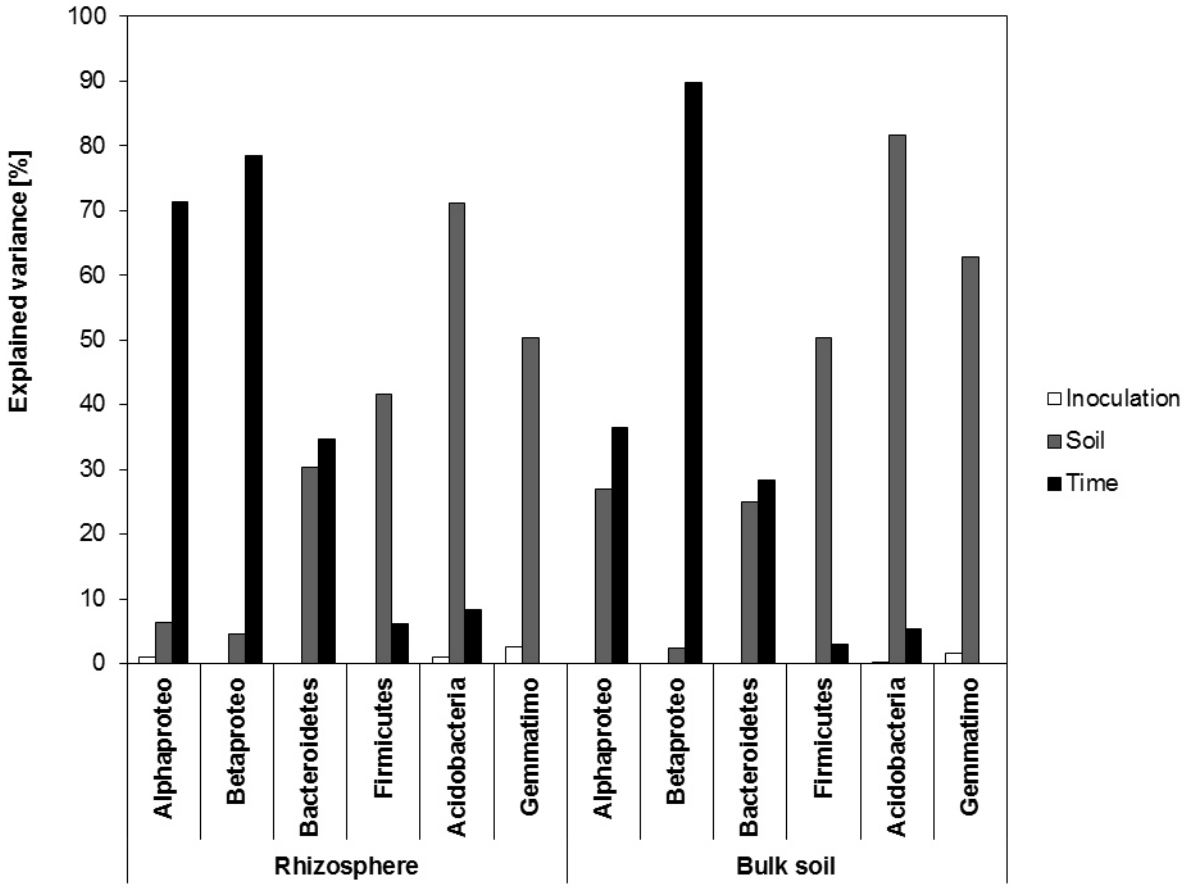

Figure 8.4 Variance components analysis showing the percent explained variance for for inoculation (non-inoculation [control], *P. fluorescens* Pf153 [Pf153], *P.* sp. RU47 [RU47], *P.* sp. DSMZ 13134 [Proradix]), soil (Buus, DOK-M, Le Caron) and time (four and eight weeks after sowing) for abundances of *Alphaproteobacteria*, *Betaproteobacteria*, *Bacteroidetes*, *Firmicutes*, *Acidobacteria* and *Gemmatimonadetes* in rhizosphere and bulk soil.

8.4 Discussion

Effects on plant growth

Inoculation by the three *Pseudomonas* strains did not improve growth of maize overall. However, a plant growth-promoting effect of each inoculation was observed in soil Le Caron (8 WAS), shown by significantly increased shoot heights (Table 8.3); furthermore, the addition of Proradix and RU47 also increased shoot biomass (Fig. 8.1). PGPR's ability to improve plant growth is due primarily to their competence in colonizing the rhizosphere at sufficiently high abundance for an extended period (Barret *et al.* 2011, Bellis and Ercolani 2001). Mosimann *et al.* (2017), who have traced two of the three *Pseudomonas* strains added 4 weeks after sowing, determined Pf153 and Proradix abundances of 1×10^4 and 1.6×10^4 CFU g^{-1} root fresh matter, respectively. Despite low variability in abundances between the soils, Mosimann *et al.* (2017) found improved persistence of both in the soil Buus, and

which was significant for Pf153. A plant growth-promoting effect of Proradix observed in plants grown in Le Caron could not, therefore, be associated with an increased abundance of this strain. As previously noted by Mosimann *et al.* (2017), although Proradix is known as an effective solubilizer of tricalcium phosphate (Miller *et al.* 2010, Buddrus-Schiemann *et al.* 2010, Fröhlich *et al.* 2012), it is highly unlikely that this characteristic contributed significantly to enhanced plant growth, since in acidic soils like Le Caron (Table 8.1) phosphate is bound mainly to Al and Fe compounds (Jones 1998, Gyaneshwar *et al.* 2002). Proradix is commonly used as a bio-control agent, but pathogen-independent plant growth-promoting effects have been observed in other studies (Yusran *et al.* 2009, Fröhlich *et al.* 2012, Eltlbany *et al. in preparation*). However, the underlying mechanisms of plant growth promotion by Proradix have not yet been fully elucidated and cannot be explained by the present study. Despite its demonstrated survival success in all soils, we found reduced plant growth in Pf153 treatments for the most part (Fig. 8.1, Table 8.3). Mosimann *et al.* (2017) assumed that this was most likely due to Pf153's ability to produce cyanide (Fuchs 1993, Compant *et al.* 2010), which provides it with a competitive advantage against native microorganisms but which may negatively affect plant growth.

Effects in microbial biomass and community structure

The addition of various inocula did not affect the quantities of microbially bound C, N and P in bulk soil; these values were primarily affected by soil (Table 8.3, Fig. 8.S1). As anticipated, highest amounts of C_{mic}, N_{mic} as well as P_{mic} were found in the organically managed soil Buus, which is characterized by a high percentage of organic C (C_{org}), suggesting high microbial abundance and activity, while lowest values were determined in the comparably C_{org}-poor soil DOK-M (Table 8.1). Our observation that the quantities of C_{mic} and N_{mic}, and therefore microbial biomass, decreased from the first to second harvest was most likely due to a decrease in nutrients in the soil substrates (Table 8.3). However, P_{mic} values remained unaffected by time (Table 8.3, Fig. 8.S1) resulting in shifts in atomic C:P and N:P ratios. The calculation of atomic C:P and N:P ratios were based on the microbial biomass data, given in Table 8.3, enabling us to gain a first insight into potential shifts in microbial community structure. We observed a decrease in C:P and N:P values, from 59 to 44 and 7 to 5, respectively, from the first to the second harvest. Values of the first harvest corresponded almost exactly to the average nutrient ratio in microbial biomass in terrestrial ecosystems (Cleveland and Liptzin 2007). Microbial biomass atomic C:P and N:P ratios of the second harvest indicated a dominance of bacterial cells (Reiners 1986); however, we observed deceased bacterial abundances (Fig. 8.2 b). Nevertheless, microbial biomass stoichiometry

can vary significantly between different groups of microorganisms (Reiners 1986, Paul and Clark 1996, Cleveland and Liptzin 2007). Thus, our finding may indicate a time-dependent shift in microbial community structure and could be associated with our observation of a relatively stable bulk soil archaeal population with simultaneous decreases in bacterial and fungal abundances by approximately 64 % from the first to second harvest (Fig. 8.2 b).

In line with our findings on the microbial biomass and microbial nutrient status in bulk soil, here, only minor effects of inoculation on soil bacterial, archaeal, and fungal abundances were observed (Fig. 8.3). Although inoculation effects differed between the strains added, soils, and harvest dates, effects were more pronounced in the rhizosphere than in bulk soil (Fig. 8.2 a, Fig. 8.2 b), which corresponds to our hypothesis that PGPR primarily affect rhizosphere microbial composition due to microbial colonization of roots. Persistence of the PGPR strain added was examined and proven for Pf153 and Proradix (Mosimann *et al.* 2017). The strain DSMZ 13134 (Proradix) is known as a mycorrhiza-helper bacterium (Yusran *et al.* 2009), while the cyanide-producing strain Pf153 (Fuchs 1993, Fuchs *et al.* 2000) as well as RU47 have been reported to be effective fungicidal biocontrol agents (Adesina *et al.* 2009, Schreiter *et al.* 2014 c). However, despite its demonstrated persistence, Proradix did not affect the abundance of fungi in soil. RU47 also did not affect fungal abundance but surprisingly, Pf153 improved fungal growth in two cases (Fig. 8.2 a, Fig. 8.2 b). These findings are in accordance with Mosimann *et al.* (2017), who estimated colonization by native arbuscular mycorrhizal fungi and observed no effect of inoculation as well. Tracing Pf153 and Proradix, Mosimann *et al.* (2017) found their improved persistence in the soil Buus, and this was statistically significant for the strain Pf153. The soil Buus was taken from an organically managed field site comprising a crop rotation of 50 % grass-clover; accordingly, this soil is rich in nutrients (primary organically-bound) and characterized by high microbial biomass (Table 8.1, Table 8.3) suggesting intensified competitive conditions for colonizing PGPR. However, in agreement with the assumption of Mosimann *et al.* (2017), the nutritional richness of this soil may have improved the persistence of Pf153 and Proradix in Buus. Notwithstanding the above, contrary to our hypothesis, which expected a less pronounced PGPR effect on soil microbial communities in soils characterized by high indigenous microbial populations, we did not even find an intensified inoculation effect in the soil where higher Pf153 and Proradix abundances were determined.

The addition of the three PGPR strains rarely influenced bacterial community structure in the soil (Table 8.4 a, Table 8.4 b, Fig. 8.4). However, individual inoculation effects occurred differently depending on the strain added, the soil, and which harvest date was examined

(Table 8.4 a, Table 8.4 b). In line with results of the determination of microbial composition at the domain level, we observed individual inoculation effects on bacterial communities more frequently in rhizosphere than in bulk soil (Table 8.4 a, Table 8.4 b). This observation was expected since each strain used in this study is described as highly rhizosphere competent (Von Felten *et al.* 2010, Buddrus-Schiemann *et al*. 2010, Adesina *et al.* 2009). Also, other studies have found marginal to no effects of PGPR on bacterial community composition in the rhizosphere of plants, with plant age appearing to be the major factor controlling microbial community structure (Scherwinski *et al.* 2008, Piromyou *et al.* 2011, Chowdhury *et al.* 2013, Kröber *et al.* 2014). Similar findings have also been reported by Schreiter *et al.* (2014 b), who investigated the effects of RU47 on bacterial community composition in the rhizosphere of lettuce in a long-term field experiment (3 years) composed of three different soil types (diluvial sand, alluvial loam and loess loam) at the same field site. Despite slight but statistically significant effects of RU47 on bacterial community structure, these effects were much less pronounced than the influence of the different soil types. This is also in accordance with our results, which showed that abundances of *Firmicutes*, *Acidobacteria* and *Gemmatimonadetes* were primarily affected by the factor soil (Fig. 8.4). Bacteria belonging to the *Acidobacteria* and *Gemmatimonadetes* phyla are considered oligotrophic organisms, not dependent on easily available substrates (Fierer *et al.* 2007, Nemergut *et al.* 2010, Ditterich *et al.* 2016). This fully confirms our observation that highest abundances of *Acidobacteria* and *Gemmatimonadetes* were found in soil Buus, which is characterized by high C_{org} and lowest N and P availability in comparison to all other soils (Table 8.1, Table 8.3, Table 8.4). Water content did not differ much between the various soil substrates in this our study (data not shown). DeBruyn *et al.* (2011) reported a negative correlation between soil moisture and *Gemmatimonadetes* abundance, indicating a competitive advantage under dry soil conditions. However, bacterial abundances of all phyla determined were positively correlated to water content, with the highest correlation coefficient among *Gemmatimonadetes* (Pearson's $r = 0.4$; $p < 0.00$), in contradiction to their findings. We observed a copiotrophic life-strategy in *Alpha-* and *Betaproteobacterial* abundances, with reduced abundances in bulk soil compared with the rhizosphere, as well as a decreased abundances by time as nutrient availability became limited in the soil substrates (Table 8.4 a, Table 8.4 b, Fig. 8.4). A copiotrophic nutritional strategy was also reported for *Alpha-* and *Betaproteobacteria* as well as for *Bacteroidetes* by Fierer *et al.* (2007). However, in our study, the abundance of *Bacteroidetes* was affected similarly by time and by soil (Fig. 8.4). Nevertheless, not all members of a phylum follow the same life-strategies; copiotrophic or oligotrophic categories may not necessarily apply to certain taxa (Fierer *et al.* 2007). Based

on the assumption that bacterial groups employing a copiotrophic life-strategy in this study mainly belonged to the *Alpha*- and *Betaproteobacteria* phyla, minor to no effects of added PGPR strains to their abundances were observed (Fig. 8.4). This finding refutes our hypothesis that due to increasing C limitation during PGPR colonization abundances of copiotrophic bacteria will be decreased. However, it must be considered that bacterial community composition is represented by six phyla in this study. Effects of PGPR on other, yet-to-be-determined taxa (e.g. *Delta*- and *Gammaproteobacteria*) or copiotrophic bacterial groups belonging to different phyla cannot be excluded and should be considered in further studies.

8.5 Conclusion

Our study demonstrated that inoculations with various PMB-capable PGPR strains did not affect microbial populations or community structure in either rhizosphere or bulk soil under maize. Individual inoculation effects occurred more frequently in rhizosphere than in bulk soil, but varied between the strains added, the contrasting soils, and the two harvest dates. Instead, differences in microbial biomass and composition at the domain level were due primarily to nutrient availability in the soil substrate, which differed between the soils and harvest dates. PGPR did not increase bacterial abundance in rhizosphere bacteria; furthermore, there was no evidence that due to increasing C limitation during PGPR colonization inoculations of PGPR led to decreased abundances of copiotrophic bacteria in the rhizosphere. However, bacterial community composition was represented by six phyla in this study. Shifts in bacterial community structure among other bacterial phyla as well as at levels of resolution lower than that of taxa cannot be excluded and should by taken into consideration in further studies.

Supplemental material

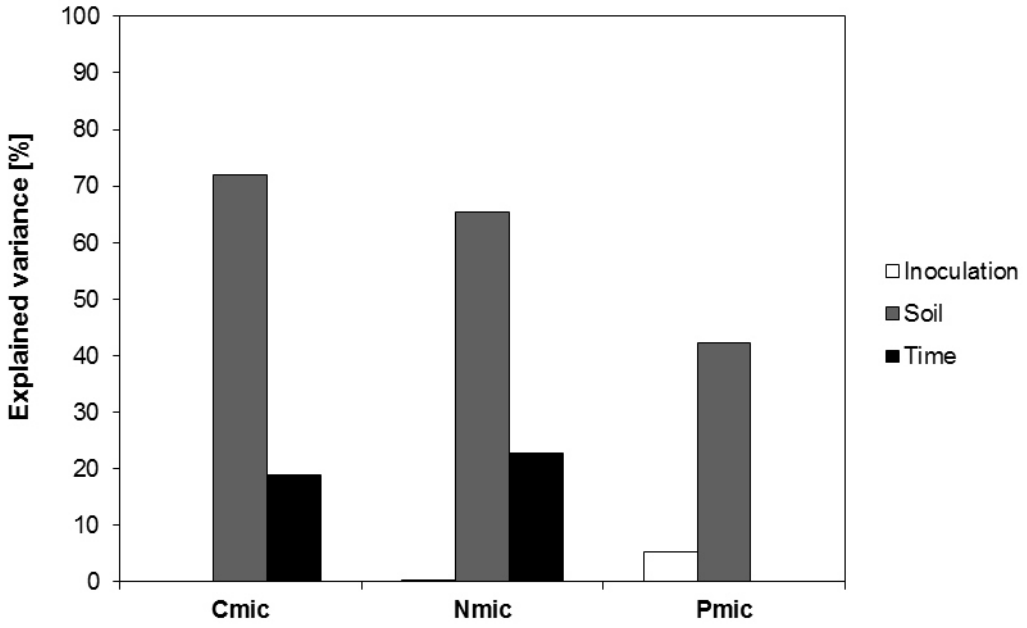

Figure 8.S1 Variance components analysis showing the percent explained variance for inoculation (non-inoculation [control], *P. fluorescens* Pf153 [Pf153], *P.* sp. RU47 [RU47], *P.* sp. DSMZ 13134 [Proradix]), soil (Buus, DOK-M, Le Caron) and time (four and eight weeks after sowing) for concentrations of microbially bound C (C_{mic}), N (N_{mic}), and P (P_{mic}) in bulk soil.

9 General Discussion

9.1 PMB persistence

In most studies investigating effects of PMB on growth and nutrient uptake of plants, the persistence of PMB introduced into soil has not been examined. However, of those studies which have been able to track the added PMB, most have reported a temporal decrease in soil (Kim *et al.* 1997, Dey *et al.* 2004, Hameeda *et al.* 2008, Meyer *et al.* 2017). For instance, Hameeda *et al.* (2008), who used *Serratia marcescens* EB 67 for inoculation in a greenhouse experiment with maize, observed a population decrease of 36 % within 84 days in rhizosphere soil. Meyer et al. (2017), using *Pseudomonas protegens* CHA0 in a pot experiment with ryegrass, reported a recovered abundance in soil of less than 1 % within 40 days. These reports indicate that neither successful rhizosphere colonization nor persistence for an extended time period can be expected when PMB are applied to plant or soil. Rather, PMB's survivability in soil is likely to depend on host plant, environmental, and soil conditions, as well as the indigenous microbial community competing with PMB for habitat, C sources and nutrients. In all our rhizobox experiments we not only recovered the PMB RU47 in soil up to 50 days after sowing, but also recorded highest abundances, higher in the rhizosphere than in bulk soil, in treatments where viable RU47 cells were added (Table 5.1, Table 6.1, Table 7.1). Our experiments clearly demonstrated the high rhizosphere competence of RU47; it was able to colonize and persist in soil of tomato and maize plants under greenhouse conditions. These findings provide useful information regarding RU47's applicability in plant production, especially as the three leading European tomato producers are Italy, Spain, and Greece (FAOSTAT 2014) where tomato cultivation takes place mainly under greenhouse conditions. Adesina *et al.* (2009) and Schreiter *et al.* (2014 b, c) had previously demonstrated high rhizosphere competence of RU47 in lettuce, and also identified its effective antifungal capacity against *Rhizoctonia solani*. The ability of a bio-control inoculant such as PMB to colonize the rhizosphere at sufficiently high abundance is crucial for pathogen suppression and defence (Bellis and Ercolani 2001, Barret *et al.* 2011). Thus, the proven rhizosphere competence of RU47 on tomato offers a promising approach for bio-control against soil-borne fungal plant pathogens in general, and *Rhizoctonia solani* in particular, a fungus which also infects tomato roots. Taken together, our results are of great practical relevance, but limited by the current use of soilless substrates (e.g. rock wool or coconut fibre), which is increasing in commercial vegetable production, including tomato, under glass (Jankauskienė *et al.* 2015).

Under high plant-available P as compared to low plant-available P soil conditions, we observed an increase in RU47 abundance in both rhizosphere and bulk soil (Table 7.1). This was in agreement with our hypothesis that improved initial soil conditions (nutrient availability) improve PMB's ability to successfully colonize the soil and thus increase their beneficial effects on plants. Similar observations were previously reported by Kaur and Reddy (2015), who co-applied *Pantoea cypripedii* or *Pseudomonas plecoglossicida* together with rock phosphate. They observed, apart from the increased PMB abundance and microbial activity in soil, plant growth-promoting effects on wheat and maize. Increased plant growth-promoting effects by co-application of PMB and P fertilizers (mainly rock phosphate) were also reported by Yu *et al.* (2011) and Kaur and Reddy (2014), and are assumed to be associated with an increase in inorganic P compounds, which improved PMB's P-solubilizing activity and competitiveness with indigenous soil microbes. By introducing viable RU47 cells into highly $Ca(H_2PO_4)_2$ fertilized soil, we were able to demonstrate that increased nutrient availability in soil is sufficient to increase PMB abundance in soil – even without improved competitiveness regarding microbial P solubilization mechanisms in soil. Based on these results, and including data from previous studies using rock/tricalcium phosphate, we can recommend co-applications of PMB with mineral P fertilizers both for biocontrol and to improve nutrient availability more generally.

In two of three rhizobox experiments, we detected RU47-specific DNA sequences in non-target treatments, meaning positive detections in treatments to which no RU47 cells were added (Table 5.1, Table 6.1). Since the majority of PMB, including RU47, have been isolated from natural rhizosphere bacterial communities associated with different plant species (Rodríguez and Fraga 1999, Fankem 2006), the presence of RU47 in soil which was also used to cultivate the unselective bacterial mix may indicate that it is part of the natural bacterial rhizosphere communities of tomato and maize. The RU47-specific DNA sequence also remained detectable in the dead RU47 treatments of each rhizobox experiment (Table 5.1, Table 6.1, Table 7.1). During decomposition of lysed cells in soil, factors such as high salt concentration (fertilization), high pH values (> 5), and percentage of quartz sand increase the probability that enzymes (proteases, nucleases) as well as free DNA are adsorbed to surface-reactive soil particles. This in turn increases the persistence of both lysed cells and their DNA in soil (Lorenz and Wackernagel 1987, Khanna and Stotzky 1992, Demanèche *et al.* 2001, Pietramellara *et al.* 2009). Accordingly, we recommend that, in addition to high-sensitivity molecular biological tracing techniques, re-cultivations of the PMB added from soil using selective media should be performed. Use of both tracing methods would clarify not only whether positive detections of PMB-specific DNA sequences in non-target treatments originated from viable PMB cells, but also whether potential positive plant

growth-promoting effects are associated with viable PMB cell processes or with indirect mechanisms, such as interactions with cell-decomposing microbes or phytohormones co-applied with the dead PMB cells.

9.2 Effects on plant growth and P nutrition

We were able to demonstrate that applications of viable RU47 cells increase the growth and P uptake of tomato plants grown under reduced plant-available P soil conditions (Fig. 5.1, Table 5.2, Fig. 7.1, Table 7.2, Fig. 7.3 a, Fig. 7.3 b). These findings are in accordance with previous studies in which growth-promoting effects of PMB on different plant species were reported (Chabot *et al.* 1996, Kim *et al.* 1997, Sundara *et al.* 2002, Hussain *et al.* 2013, Surapat *et al.* 2013). For instance, Hussain *et al.* (2013), testing five PMB strains belong to the genera *Burkholderia*, *Bacillus*, *Pseudomonas*, and *Flavobacterium* in a pot experiment with maize, observed increases in plant biomass and kernel yield of up to 42 and 33 %, respectively. In addition, Surapat *et al.* (2013) reported increases in growth and P uptake of chili plants inoculated with *Burkholderia tropica* KS04. Since RU47 had been used as an antifungal bio-control agent in previous studies (Adesina *et al.* 2007, 2009, Schreiter *et al.* 2014 b, c), the proven ability to promote growth and P uptake in tomato plants offers an additional application in practical farming. However, although RU47 was identified as highly effective against the fungal plant pathogen *Rhizoctonia solani* on lettuce roots, increases in plant growth were not observed (Adesina *et al.* 2009). Eltlbany *et al.* (*in preparation)* using the same soil and tomato variety as was used in our rhizobox experiments, also observed a plant growth-promoting effect with the addition of RU47. This suggests that RU47's beneficial effects on plant growth may depend on the host-plant and/or soil conditions, and should be considered in further studies. However, the use of RU47 offers a promising practical application. Nevertheless, its practical use is not without risk. RU47 is resistant to rifampicin, tetracycline, ampicillin, and chloramphenicol (Eltlbany *et al. in preparation*). Therefore, its practical application also may negatively influence microbial diversity in soil, and its antibiotic resistance genes might be transferred (via horizontal gene transfer) to other bacteria, including human pathogens (Forsberg *et al.* 2012).

In accordance with our hypothesis that improved nutrient availability in soil increases PMB's survivability and thus its beneficial effects on plants, we observed increases in plant growth in tomato plants which were inoculated with viable RU47 cells and grown under high plant-available P soil conditions (Fig. 7.1, Fig. 7.2, Table 7.2). Similar findings were also reported by Yu *et al.* (2011), who inoculated 1-year-old walnut seedlings with *Pseudomonas chlororaphis* W24, *Bacillus cereus* W9, and *Pseudomonas fluorescens* W12 and observed an

increase in plant growth when PMB was co-applied with tricalcium phosphate. Our observation that RU47 abundance increased when soil was not reduced in P availability (Table 7.1) indicates active RU47-mechanisms promoting plant growth. Since P-mobilizing mechanisms improving plant P supply can be excluded under high plant-available P soil conditions, the tendency toward enhanced plant growth can likely be attributed to phytohormones secreted by RU47. This is discussed in more detail in the following chapter. A derivative recommendation to increase P fertilization when PMB are added to soil does not seem appropriate given the goal of reducing of mineral P fertilizers in agriculture. However, improved PMB colonization includes rapid incorporation of P into the bacterial biomass, effectively protecting available P from soil reactions and serving as a temporary P sink for plants (Olander and Vitousek 2004). Furthermore, the greater the PMB population, the greater the amount of P that can be mobilized when P becomes limited in soil. Thus, subsequent improved P mobilization by PMB resulting in growth and nutrition-promoting effects on plants over the long-term are possible. Our maize experiment found no growth-promotion effects by the addition of RU47 on maize seedlings 14 days after sowing (Table 6. 2). An optimal P supply in the early stages of plant development is crucial for ensuring high yields in maize production (Mollier and Pellerin 1999). Yields in kernel number and weight are especially sensitive to P in the early growth stages, which extend from sowing to the 6-leaf-stage (Berry and Miller 1989). In general, however, to evaluate PMB's beneficial effects on plants in a practical context, data on yield properties would be required and should be considered in future studies, since beneficial effects of RU47 on later growth and P nutrition of maize cannot be excluded. Although we demonstrated an increase in biomass and P nutrition on tomato plants by the addition of RU47, increases in fruit yield could not be conclusively determined.

As the first study to use dead PMB strains, we also observed improved plant growth and P nutrition on tomato plants by the addition of dead RU47 cells, especially under reduced available P soil conditions (Fig. 5.1, Table 5.2, Table 7.2, Fig. 7.3 a, Fig. 7.3 b). These findings indicate that plant growth was stimulated by phytohormones produced by RU47 and/or indigenous soil microbes which may have been promoted by the addition of C sources in the form of bacterial residues to the soil. The latter may explain why, under high P soil conditions, plant growth-promotion trends were more pronounced in the dead RU47 treatment than in the treatment using viable RU47 cells. Potential competition for substrates and habitats in the rhizosphere between indigenous microbes and the colonizing viable RU47 bacteria may be an additional factor reducing plant promotion in the viable RU47 treatment. The possibility of using dead RU47 cells in practical farming offers a major production advantage since the formulation of viable RU47 is still not possible (Voigt 2014,

personal communication). PMB, which do not form spores as, e.g. *Bacillus* does, are difficult to produce commercially since their viability must be ensured. Future studies should test whether other PMB strains also demonstrate plant growth-promoting capabilities in their devitalized forms. We found indications of the underlying mechanisms, including plant impacts by RU47-derived phytohormones and interactions with indigenous microbes, increasing microbial activity in soil and/or secretion phytohormones themselves. These mechanisms are described in more detail in the following sections.

9.3 Effects on P mineralization in soil

We demonstrated that the addition of viable RU47 cells increased alkaline PA in the rhizosphere of tomato plants, especially under reduced P soil conditions (Fig. 5.2 a, Fig. 7.4 b). The ability to mineralize organic P compounds effectively had previously been shown in laboratory experiments using *Pseudomonas putida* P13 and *P. putida* PCI2 by Malboobi *et al.* (2009) and Pastor *et al.* (2012), respectively. Data on effects of PMB on PA in soil are rare; however, Kaur and Reddy (2015), for instance, reported significant increases in both acid and alkaline PA in the rhizosphere of wheat and maize plants by inoculation of *P. plecoglossicida* PSB-5. While acid phosphomonoesterase is produced by plant roots and microbes, alkaline phosphomonoesterase is synthesized exclusively by microorganisms (Dick *et al.* 1983, Juma and Tabatabai 1988, Nannipieri *et al.* 2011). Thus, considering the demonstrated rhizosphere competence of RU47 (Table 5.1, Table 6.1, Table 7.1), our findings clearly show that increased P mineralization in the rhizosphere is due to microbial processes and is, very probably, RU47-derived, providing important insights into functional mechanisms of PMB. However, we also found increased alkaline PA in treatments using dead RU47 cells (Fig. 6.3, Fig. 7.4 b). Since the devitalisation of cells was ensured by plating tests, RU47-derived PA can be excluded in the dead RU47 treatments. The observed effect may have been due to enhanced growth and activity of previously dormant microbial populations through the addition of bacterial residues (dead RU47 cells) to soil and thus a supply of FOM, known as the priming effect (Bingeman *et al.* 1953, Fontaine *et al.* 2003). A priming effect driven by another mechanism can also occur in the rhizosphere when viable PMB are added. Rhizosphere-colonizing PMB utilize root exudates as an easily available C source. Since microbial incorporation of C, N, and P are coupled (Cleveland and Liptzin 2007), an increase in C incorporation increases mobilization of N and P from soil organic matter (Cheng 2009, Richardson and Simpson 2011). Until recently, it was unclear whether increased P mineralization in soil by the addition of PMB was independent of endogenous microbial turnover of organic matter, since effects of PMB on plant growth and P mobilization have, to date, been compared only with non-inoculated controls. By including a treatment in

which a mix of unselectively cultivated soil bacteria were used for inoculation, we are able to state that microbial PA determined in both viable and dead RU47 treatments are above the level of endogenous microbial P mineralization (Fig. 7.4 b). The proportion of organic P in soil ranges between 4 and 90 % (Khan *et al.* 2009 a). The use of RU47 can therefore be recommended, especially in soils possessing an appropriate proportion of organic P. Soil microorganisms mineralize P in soil more frequently by phytase than by phosphomonoesterase (Bünemann 2008, Aseri *et al.* 2009). An increase in phytase activity in soil after the addition of PMB (*Pantoea cypripedii*, *Pseudomonas plecoglossicida*) was previously reported by Kaur and Reddy (2014, 2015). Since more P is bound in the form of phytic acid and its salts (6 mol P mol^{-1} phytate) than in other organic P compounds in soil, and since phytase is more effective than other phosphatases (Richardson 1994, Bünemann 2008), enhanced secretion of phytase by PMB provides considerable potential for exploitation of organic P in plant production. Thus, future studies should focus more on phytase activity of PMB, especially of RU47, which has already shown to be effective in increasing phosphomonoesterase activity in soil. However, since, at least in case of the dead RU47 treatments, it is very likely that indigenous microbes are responsible for the increased P mineralization in soil, molecular-based techniques making use of homologies at the sequence level of bacterial phosphatase-encoding genes (including phytase; Lim *et al.* 2007) could identify the main phosphatase producers in a microbial community. Therefore, the extension of functional gene analyses in future studies would contribute to both the elucidation of PMB's mechanisms and quantification of their contribution to increased P availability in soils.

9.4 Phytohormonal effects

As the first study to use dead PMB to distinguish active mechanisms of PMB from indirect effects, we were able to demonstrate a plant growth-promoting effect by the addition of dead RU47 cells. Indications of a hormonally-derived effect were found not only in treatments to which dead RU47 cells had been added, but also in treatments using viable RU47 cells. The change in root:shoot ratios observed in both RU47 treatments, and in both maize and tomato experiments, should be emphasized here. Arkhipova *et al.* (2007) were able to demonstrate a stimulation of shoot growth in lettuce by the addition of cytokinin-producing bacteria. Cytokinins as well as auxins are the major phytohormones regulating plant growth and development (Ivanova and Rost 1998, Berleth and Sachs 2001, Francis and Sorrell 2001). Cytokinin plays a crucial role in cell division and expansion, and in organogenesis. Furthermore, cytokinin inhibits the effects of ABA, which in turn inhibits plant growth and flower formation (Levy and Dean 1998; for review see Ha *et al.* 2012). The assumption of a

phytohormonal effect, especially by cytokinin, is supported by results of our 50-day tomato experiment under limited P availability soil conditions, which revealed an increase in blossom biomass in both RU47 treatments, and which were significant for the viable RU47 treatment (Table 7.2). It is well documented that soil bacteria, including PMB, are able to synthesize phytohormones which enable them to ensure or increase their C sources from root exudation by influencing a plant's growth or its response to stress (for review see Tsavkelova *et al.* 2006, Hayat *et al.* 2010, Glick 2012, 2014). Evidences of synthesizing phytohormones or phytohormone-related compounds of PMB are mostly known for IAA (auxin) and ACC deaminase (e.g. Gupta *et al.* 2002, Rajkumar and Freitas 2008, Ramírez and Kloepper 2010). PMB's ability to synthesise plant-effective levels of cytokinin have been less well studied. However, García de Salamone *et al.* (2001) were able to identify *Pseudomonas fluorescens* G2018 as highly productive in synthesizing the three cytokinins isopentenyl adenosine, trans-zeatin ribose, and dihydrozeatin riboside. Apart from recommending the study of RU47's ability and degree of phytohormone production (especially auxin and cytokinin), we cannot currently say whether a potential hormonal effect is due to RU47 or to indigenous microbes. In general, three options are under discussion. First, phytohormones produced by RU47 affected plant growth. While in viable RU47 treatments the colonizing RU47 cells actively synthesised phytohormones promoting plant development, the plant growth effect observed in the dead RU47 treatments was due to phytohormones produced by RU47 before killing (culture medium), which were co-extracted and applied together with the dead cells to soil. RU47 cells were devitalized by boiling (5.2.2). Since auxin and cytokinin are known to be heat-stable (Murashige and Skoog 1962, Kumar and Singh 2009), a co-application of phytohormones produced by RU47 is theoretically possible. The second option is that indigenous soil microbes promoted by RU47 attractants or an enhanced C source in the form of the dead cells synthesized effective levels of phytohormones in the dead RU47 treatments, while phytohormones in the viable RU47 treatment were RU47-derived. The third option, also in the viable RU47 treatments, is the possibility that a phytohormonal effect was caused predominantly by indigenous soil microorganisms interacting with RU47. Although this could not be clarified in this study, our results provide an important contribution to the untangling of diverse functional mechanisms of PMB in soil. The ability of RU47 to affect a plant's hormonal balance may extend its potential for application in practical farming. It has been shown that RU47 is able to produce high levels of ACC deaminase (Smalla 2016, *personal communication*). Degrading the ethylene precursor ACC, this enzyme decreases plant stress level resulting reduced growth (Glick *et al.* 2007, Saleem *et al.* 2007, Yang *et al.* 2009, Glick 2014). Apart from its established PMB and bio-control

potential, RU47 may also offer a promising approach for application in areas characterized by stress conditions, such as drought or salinity.

Apart from phytohormones and phytohormone-related compounds, volatile organic compounds (VOCs) also play key roles in plant growth promotion. Many bacterial species, including those belonging to the genera *Pseudomonas,* produce VOCs affecting plant growth (Kai and Piechulla 2009, 2010, Bailly and Weißkopf 2012) and reducing plant response to stress (for overview see Yang *et al.* 2009). For instance, Gutiérrez-Luna *et al.* (2010) demonstrated that plant growth and root architecture of *Arabidopsis thaliana* were modulated by differential VOC emissions of three rhizobacterial strains belong to the genera *Bacillus*. In addition, Park *et al.* (2015) identified in *Pseudomonas fluorescens* SS101, which was isolated from the rhizosphere of wheat, 11 different VOCs; three of these, 13-Tetradecadien-1-ol, 2-Methyl-n-1-tridecene, and 2-Butanone, significantly increased growth of tobacco plants. Thus, VOCs emitted by RU47 or by interacting indigenous soil bacteria may also have played a role in the plant growth-promotion observed in this study. VOCs may even make a crucial contribution to PMB's plant growth-promotion attributes and should be further investigated, especially considering that they could be tailored to the needs of particular areas, such as those characterized by abiotic stress conditions.

9.5 Interactions with indigenous microorganisms

A successful rhizosphere colonization and persistence of PMB in soil inevitably implies situations of competition with indigenous microorganisms for C sources and nutrients. Thus, it can be expected that the PMB introduced to a soil may, due to biological displacement, affect microbial community structure in the soil, especially in the rhizosphere. Furthermore, the bio-control potential of PMB, including RU47, mostly affects fungal plant pathogens (Adesina *et al.* 2007, 2009, Schreiter *et al.* 2014 b, c), and may therefore decrease the fungal population in soil. Interestingly, we found few or no trends in shifts in microbial community structure by the addition of viable RU47 cells. In our 39-day tomato experiment, a slight increase in bacterial abundance, especially of gram$^+$ bacteria, was observed in the bulk soil of both dead and viable RU47 treatments (Table 5.2). However, this finding may indicate that shifts in microbial community structure due to introduction of RU47 occur on a lower taxonomic level than PLFA patterns can discern and as already reported by Schreiter *et al.* (2014 a). Supporting our assumption that the addition of dead RU47 promoted indigenous soil bacteria, bacterial DGGE fingerprinting revealed numerous dominant bands in this treatment (Fig. 5.4 a). The extent to which these bacteria are responsible for the observed plant growth-promotion cannot be clarified at present. The absence or lower abundance of

these dominant bands in the treatment using viable RU47 cells indicate clearly that indigenous bacteria in the rhizosphere of tomato plants were not promoted by RU47-specific attractants, which would be the case if dominant bands had been detected in the viable RU47 treatment. It was more likely due to a spike of C source and nutrients in the form of dead bacterial cells. Linking all information obtained from our rhizobox experiments, it can be assumed that the plant growth-promotion effect of dead RU47 cells is due to grazing by indigenous soil bacteria, which increases P mineralization in soil (priming effect), secretes phytohormones and/or VOCs, and results in enhanced plant growth and P uptake. However, this implies that effective use of dead PMB is highly dependent on the bacterial community structure present in the soil. If the use of dead PMB is to be considered for practical farming, the main mechanisms of this plant growth-promoting effect must be clarified in advance. In this case, it would be crucial to know which roles RU47-specific attributes play (e.g. phytohormones) in plant growth-promotion or whether the addition of bacterial residues alone is sufficient to trigger a priming effect in soil.

Our results demonstrated that the addition of viable RU47 cells promoted growth and P uptake of tomato plants, accompanied by increased PA (Fig. 5.1, Fig. 5.2 a, Table 5.2, Fig. 7.3 b, Fig. 7.4 b, Table 7.2). Although no interactions with indigenous microorganisms were found, potential interactions on a lower phylogenetic level than was explored in this study cannot be excluded. Molecular-based techniques using conserved regions in bacterial functional genes which are associated with P mobilization in soil (including P solubilization), combined with next-generation sequencing, could identify not only the main mechanisms of PMB, but also the primary and secondary players in P mobilization, revealing potential interactions with indigenous soil microorganisms. As previously mentioned, interactions with indigenous microorganisms imply a high degree of dependency on existing soil microbial community structure. Using three different PMB strains belonging to the genera *Pseudomonas*, including RU47, in three contrasting soils, we demonstrated that individual inoculation effects differed between the strains, soils, and time (Fig. 8.2 a, Fig. 8.2 b, Fig. 8.3, Table 8.4 a, Table 8.4 b, Fig. 8.4). However, individually occurring shifts in the microbial communities were more pronounced in rhizosphere than in bulk soil (Fig. 8.2 a, Fig. 8.2 b, Table 8.4 a, Table 8.4 b), which is in accord with the known high rhizosphere competence of each strain used (Von Felten *et al.* 2010, Buddrus-Schiemann *et al.* 2010, Adesina *et al.* 2009). However, community structure in the soil was primarily influenced by soil conditions and influenced by PMB over the long term. It should be emphasized that despite its proven antifungal bio-control capability (Fuchs 1993, Fuchs *et al.* 2000, Adesina *et al.* 2009) no effect on fungal abundance was found in the RU47 treatments, whereas Pf153 even promoted fungal growth in two cases (Fig. 8.2 a). Similar results were found in the case of

Proradix, which contains the strain *P.* sp. DSMZ 13134, and which has been identified as an effective mycorrhiza-helper bacterium in a plant experiment with Paraserianthes (Yusran *et al.* 2009), but it did not affect fungal abundance in the present study (Fig. 8.2 a, Fig. 8.2 b). These findings support the assumption that effects of PMB on soil microbial community structure differ strongly between the single strains, host-plant, soil, and environmental conditions. For instance, Zhang *et al.* (2016) demonstrated that not only AMF can stimulate indigenous PMB, but also that the form of interaction between them can depend on P availability conditions. They showed that under adequate P availability, the AMF *Rhizophagus irregularis* release C into the environment, promoting PMB (*Rahnella aquatilis* HX2) growth and activity. This was followed by increased P mobilization benefiting AMF P supply. Under limited P availability conditions, AMF and PMB competed for P, accompanied by absence of a stimulation effect (Zhang *et al.* 2016).

Apart from potential interactions with indigenous microorganisms, co-inoculations of PMB with AMF or N_2-fixing bacteria are often reported to be more effective on plant growth and P uptake than applied as single inoculations (e.g. Kim *et al.* 1997, Rojas *et al.* 2001, Wani *et al.* 2007 b, Saxena and Jha 2014). Our study clearly demonstrated that RU47 possess PMB-properties. Furthermore, their antifungal capacity does not negatively affect indigenous soil fungi, including AMF (Mosimann *et al.* 2017). Thus, testing RU47 in co-inoculation with AMF or N_2-fixing bacteria would provide not only important insights into interaction mechanisms of PMB, but also make possible the evaluation of RU47's potentials and limits in practical usage.

10 General Conclusions

As the first study to use devitalized PMB cells for inoculation, we observed plant growth and P uptake-promotion effects on tomatoes grown under limited available P soil conditions by the addition not only of viable, but also of dead RU47 cells. Estimating enzyme activity in rhizosphere and bulk soil, we were able to link enhanced plant growth and P supply to increases in microbial PA in the rhizosphere. By the use of unselectively cultivated soil bacteria for inoculation, we demonstrated that the increased PA observed in both RU47 treatments occurred independently of endogenous microbial P mineralization in soil. Thus, our findings go beyond previous work by providing approaches that not only identified the PMB-specific effects on soil microbial activity, but also differentiated between direct and indirect PMB effects on soil and plant. We found evidence of a hormonal influence on plants by the addition of viable and dead RU47 cells. We assume that improvement in plant growth and P supply by the addition of viable RU47 cells was primarily due to their increased secretion of phosphatases, while bacterial phytohormones also had an influence but played only a secondary role here. Observed shifts in a rhizosphere's bacterial community structure when dead RU47 cells were added made it possible to conclude that indigenous soil bacteria were stimulated by the addition of a C source and nutrients in the form of bacterial residues, and that this resulted in increased P mineralization in soil. However, we assume that plant growth-promotion was caused primarily by hormonally-derived effects here. We can only speculate about the origin of these phytohormones. It is conceivable that either heat-stable phytohormones, which were produced by RU47 before cell devitalisation (boiling) were co-extracted and thus co-applied to the soil, or that phytohormones were secreted by the stimulated bacterial population in the rhizosphere. Nevertheless, this study was able to identify *in vivo* PMB capacities of RU47, which was formerly known as a bio-control inoculant. Viable RU47 cells were shown to be highly rhizosphere-competent on maize and tomato roots, persisting up to 50 days after sowing. Thus, our findings offer a promising approach for the use of RU47 in practical plant production, especially in tomato cultivation, which, in this study, yielded the greatest plant effects. Organically bound P constitutes a large part of fixed P in soil. Against the backdrop of the impending P crisis, RU47's ability to mineralize P effectively from soil provides encouraging approaches for its use in exploiting immobilized soil P for plant nutrition. Accordingly, this could lead to reduced demand for mineral P fertilizers, making P fertilization in agriculture more efficient and preserving a limited resource.

While previous studies have reported a strengthened plant growth-promoting effect when PMB were co-applied with mineral P fertilizers (rock or tricalcium phosphate), we can

suggest that amendments of easily available phosphate may also increase RU47's abundance in both rhizosphere and bulk soil. We could not quantify an increase in plant growth-promoting effect, however, but merely a trend which was likely phytohormonally-derived, within the 50 days of experimental duration. However, initially high incorporation of P into the RU47 bacterial biomass ensures that P will be available through soil reactions and thus it can act as a temporary P sink for plants. A P mobilization-derived plant growth-promoting effect in the long-term, as plant-available P in soil becomes limited, is conceivable and may provide an additional, although secondary use for rhizosphere competent PMB through their capacity to decrease the continuing accumulation of fixed P in arable soils. Simultaneous determinations of bacterially bound P and plant-available P in future long-term experiments could clarify the role of P immobilization by PMB in growth-promotion of plants.

Investigating the effects of PGPR, including PMB, on microbial community structure as well as its dependence on soil conditions, we showed that individual inoculation effects varied among the PMB strains added, the contrasting soils used, and time. Changes in microbial biomass and composition on the domain level (bacteria, archaea, and fungi) were due primarily to nutrient availability in the soil substrate, which differed between the soils and over time. Although individual inoculation effects on microbial community structure occurred more frequently in the rhizosphere than in bulk soil, colonizing PGPR neither increased bacterial abundance in rhizosphere, nor provided evidence of displacement of C-competing copiotrophic rhizobacteria. Thus, our findings demonstrate that the ecological impacts of specifically introduced rhizobacteria are much lower than assumed – at least at the domain and phylum levels. Based on these results, we conclude that plant-beneficial interactions with indigenous soil microbes, such as suppression and defence of plant pathogens, as well as the promotion of AMF, may be strongly dependent on existing soil conditions or on the soil microbial community.

Taken together, we conclude that various PMB mechanisms increasing plant growth and P uptake run in parallel. P mobilization-dependent and -independent as well as direct and indirect mechanisms are overlapping. Which mechanism is dominant at any given time seems to depend on the existing soil and environmental conditions. PMB-specific plant growth-promotion effects can be induced without guaranteeing PMB's survivability and highly abundant persistence in soil. Stimulating the growth and activity of indigenous soil bacteria by additional C sources (bacterial residues) may result in at least as much of an increase in P mineralization as that induced by the addition of viable, highly rhizosphere competent PMB cells. PMB's ability to effectively mineralize P from soil plays an important role in their beneficial effects on plants. However, plant growth is also affected by bacterial

phytohormones and this should be more strongly focused on in future studies investigating the effects of PMB on plants. Effects of PGPR, including PMB, on microbial community structure in soil are small and temporary; permanent microbiome shifts may actually occur at a lower phylogenetic level than that of phylum.

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12 Appendix: Nassal *et al.* (2017)

The manuscript 'Effects of phosphorus-mobilizing bacteria on tomato growth and soil microbial activity' by Dinah Nassal, Marie Spohn, Namis Eltlbany, Samuel Jacquiod, Kornelia Smalla, Sven Marhan and Ellen Kandeler which was published in Plant and Soil in 2017 is reprinted on the following 21 pages.

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REGULAR ARTICLE

Effects of phosphorus-mobilizing bacteria on tomato growth and soil microbial activity

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Abstract
Aims The aim of our study was to clarify whether inoculating a soil with *Pseudomonas* sp. RU47 (RU47) bacteria would stimulate the enzymatic cleavage of organic P compounds in the rhizosphere and bulk soil, promoting plant growth. Adding either viable or heat treated RU47 cells made it possible to separate direct from indirect effects of the inoculum on P cycling in soil and plants.
Methods We performed a rhizobox experiment in the greenhouse with tomato plants (*Solanum lycopersicum*) under low P soil conditions. Three inoculation treatments were conducted, using unselectively grown soil bacteria (bacterial mix), heat treated (HT-RU47) and viable RU47 (RU47) cells, and one not inoculated, optimally P-fertilized treatment. We verified plant growth, nutrient availability, enzyme activities and microbial community structure in soil.
Results A plant growth promotion effect with improved P uptake was observed in both RU47 treatments. Inoculations of RU47 cells increased microbial phosphatase activity (PA) in the rhizosphere.
Conclusions Plant growth promotion by RU47 cells is primarily associated with increased microbial PA in soil, while promotion of indigenous Pseudomonads as well as phytohormonal effects appear to be the dominant mechanisms when adding HT-RU47 cells. Thus, using RU47 offers a promising approach for more efficient P fertilization in agriculture.

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Introduction

While it is well known that rhizosphere processes are important for plant P acquisition (Jones and Darrah 1994; Hinsinger 2001), the processes underlying growth promotion by beneficial microorganisms are not yet well understood. Since organic P is often the dominant form of P found in soils (Ron Vaz et al. 1993; Shand et al. 1994) and may constitute up to 90% of the total P in soil (Khan et al. 2009), P mineralisation is a prerequisite to convert organic P into a plant available form. P mineralisation is catalysed by extracellular phosphatases produced by microorganisms and plants. While microorganisms produce both acid and alkaline phosphatases, plants produce only acid phosphatases (Dick et al. 1983; Juma and Tabatabai 1988; Nannipieri et al. 2011). Microbial and plant P acquisition occur in different zones of the rhizosphere. Plant uptake of P occurs mostly at the root tip and in the proximal elongation zone, whereas microbial P uptake is highest in the root hair zone (Marschner et al. 2011). Using zymography in a rhizobox experiment, Spohn and Kuzyakov (2013) demonstrated the spatial separation of acid and alkaline phosphatase activity (PA) in the rhizosphere of lupines. While acid PA was associated with the root, alkaline PA was more widely distributed in the bulk soil (Spohn and Kuzyakov 2013; Spohn et al. 2013; Spohn et al. 2015; Hofmann et al. 2016). Microbial phosphatases comprise the major share of phosphatases in soil (Tabatabai 1994; Tarafdar et al. 2001), contributing significantly to the P supply of plants (Frossard et al. 2000; Oehl et al. 2004). However, with respect to P foraging, the plant-microbial relationship can be competitive as well as mutualistic (Richardson et al. 2009). Hence, without phosphate fertilizers, P supply is generally not sufficient for effective crop production in most agricultural soils.

Phosphorus-mobilizing bacteria (PMB) are beneficial bacteria that effectively mobilize P through solubilization of sorbed P pools and mineralization of organic P compounds which are otherwise not readily available to the plant. Application of PMB to soils can therefore be a promising approach for improving P fertilization efficiency in agriculture. Plant growth-promoting effects resulting from targeted application of high-concentrations of PMB strains such as representatives from *Bacillus*, *Pseudomonas,* and *Rhizobium* in soils limited in P availability for plants have been documented in several studies (Chabot et al. 1996; Sundara et al. 2002; Kaur and Reddy 2014). Whether future use of PMB can improve P nutrition of arable crops and vegetables remains to be tested. Three different microbial-driven functional mechanisms are currently being explored. First, added PMB may catalyze the hydrolysis of organic P compounds by the release of phosphatases (Tarafdar and Claassen 1988). Second, PMB may solubilize bound inorganic P into easily available phosphates by secreting organic acids which would reduce rhizosphere pH. Organic acids as well as bicarbonates, carboxylates, and other anions biotically released may function as exchange ligands (Kpomblekou-a and Tabatabai 1994; Deubel et al. 2000; Jones 2011). Third, added PMB may interact synergistically with other beneficial indigenous microbes, like mycorrhizal fungi or N_2-fixing bacteria optimizing P mobilization in soil (Belimov et al. 1995; Zaidi et al. 2003; Zhang et al. 2016). Although the role of PMB during P solubilization has been investigated (Kim et al. 1997; Khan et al. 2007; Fankem et al. 2008), the importance of enzymatic cleavage of organic P resources by PMB, especially under P-limited conditions, has been less well studied. Kaur and Reddy (2014) demonstrated that enhanced wheat and maize growth after inoculation of an agricultural field with *Pantoea cypripedii* and *Pseudomonas plecoglossicida* was accompanied by an increase in phosphomonoesterase, phytase and dehydrogenase activities in soil. In addition, Eltlbany et al. (under review), conducted a pot experiment with tomato plants grown in soil with reduced P fertilization and found considerably enhanced plant growth following inoculation with *Bacillus amyloliquefaciens* FZB42 spores (RhizoVital®) as well as *Pseudomonas* sp. DSMZ 13134 (Proradix®) or *Pseudomonas* sp. RU47. PA tended to increase in the rhizosphere; alkaline phosphomonoesterase with the addition of each of the two commercial products, and acid phosphomonoesterase with the addition of *Pseudomonas* sp. DSMZ 13134 and *Pseudomonas* sp. RU47. It is possible that the plant growth-promoting function of these three different bacterial strains is based mainly on their enhanced phosphatase production in the rhizosphere of plants. Since the formulation of the commercial products (i.e. the carrier matrix; culture media, skimmed milk powder, or gum arabic) may also affect microbial P mineralization, we selected *Pseudomonas* sp. RU47 (RU47) as the model organism, omitting any formulation.

Whereas the addition of viable cells of RU47 should clarify direct mechanisms (e.g. enzyme production by the PMB), the addition of heat treated PMB strains should allow testing indirect mechanisms (e.g. via endogenous microorganisms).To exclude apparent plant growth-promoting effects of the PMB due to increased microbial activity by addition of living soil bacteria, an inoculation treatment using a mix of soil bacterial isolates was also evaluated. The following hypotheses were tested. (1) Added viable RU47 cells successfully colonize the soil and lead to a plant growth-promoting effect. (2) The plant growth-promoting effect of viable RU47 under P-deficient soil conditions is based on enhanced PA leading to enhanced P availability in soil and increased uptake by plants. (3) Added viable RU47 dominates colonization of the rhizosphere, leading to spatially distinct zones of enriched alkaline/acid PA and to a shift in microbial community composition which were analyzed by illumina sequencing of 16S rRNA gene fragments amplified from total community DNA.

Materials and methods

Rhizobox experiment

The experiment was performed under low P availability soil conditions using *Pseudomonas* sp. RU47 (RU47) as the PMB, and tomato (*Solanum lycopersicum* L. var. Mobil) as the test plant. We established four treatments to account for the response of plants to heat treated (HT) or viable PMB as well as to account for the possible plant growth stimulation by P fertilization. To exclude apparent plant growth-promoting effects of the PMB due to increased microbial activity from having added living soil bacteria, which could affect the P efficiency of plants, a treatment was performed using unselectively cultivated soil bacteria for inoculation (bacterial mix). To verify the effects of HT and viable PMB on plant growth and nutrition, heat treated RU47 cells (HT-RU47), and viable RU47 (RU47) were used for inoculation in two different treatments. In order to evaluate effects of P fertilization on plant growth, we conducted an optimally P-fertilized non-inoculation treatment (P-fertilized). Details of microorganism cultivation and inoculation are described in 2.2. Although the study aimed to determine the effects of PMB under low plant available P soil conditions, in order to achieve successful germination, a slight P fertilization of 50 mg kg^{-1} was applied to all treatments, excluding the optimally P-fertilized (200 mg kg^{-1}) non-inoculation treatment. Hence, the experiment consisted of four treatments, with four replicates per treatment. Tomato plants were grown in rhizoboxes with inner dimensions of 28.0 cm × 4.5 cm × 16.5 cm, and filled with a soil substrate composed of Luvisol topsoil and quartz sand (0.2–1.4 mm) in a ratio of 1:1 (*w*/w). The Luvisol was considered as a heavy loam soil and had the following characteristics: pH 7.1 ($CaCl_2$), 26.2% sand, 52.2% silt, 21.6% clay, 2.3% total C, 2.0% organic C, 1.8 mg NH_4^+ kg^{-1}, 53.0 mg NO_3^- kg^{-1} and 24.1 mg P (Olsen) kg^{-1}. The soil, selected on the basis of its low concentration of plant available P (calcium lactate extraction of 20 mg kg^{-1}), was taken from an unfertilized grassland located on the campus of the University of Hohenheim (Stuttgart, Germany). Each rhizobox was filled with 1918.0 g dry matter (DM) of sieved (< 5 mm) soil substrate. Before sowing, the soil substrate was optimally fertilized with respect to N (100 mg kg^{-1}), K (150 mg kg^{-1}) and Mg (50 mg kg^{-1}) and adjusted to a water holding capacity of 50%. Three tomato seeds were sown at a depth of 1–2 cm directly into each rhizobox and thinned to one plant per rhizobox after germination. In order to promote root growth along the hinged wall, rhizoboxes were placed at a 50 ° inclination. To avoid light-derived influences on root growth and behaviour, all boxes were wrapped in aluminium foil. The experiment was conducted for 39 days under greenhouse conditions. Rhizoboxes were distributed randomly and placed on wooden planks to exclude contamination by leaking irrigation water. Plants were watered to maintain a water holding capacity of 50% until 25 days after sowing, with water content checked gravimetrically on a daily basis. Due to small loss of soil while conducting the soil in situ zymography (2.6), from 25 days after sowing each rhizobox was watered with the same volume of 20 mL. This volume corresponded to the average volumes of water used for watering at 23 days after sowing; volumes were increased up to 35 mL when radiation and temperature have risen in the greenhouse. Watering was performed using deionized water (H_2O_{deion}), applied in 5 mL steps to avoid leakage along rhizobox edges.

Microbial cultivation and inoculation

RU47 (Adesina et al. 2007) was cultured in King's B liquid medium (King et al. 1954) with 50 mg L^{-1} added

rifampicin (resistance by spontaneous mutation) at 28.5 °C in an incubator shaker (SM 30 Control; Edmund Bühler, Hechingen, Germany) for 24 h; cultivation vessels were wrapped in aluminium foil to protect the antibiotic from light. We modified the growth conditions of *Pseudomonas* described by Xue et al. (2013) to maintain the exponential growing phase (to ensure inoculation by viable cells) and to have greater time flexibility during inoculation preparation. Briefly, we followed the following protocol: Bacterial mixes were grown in glucose-enriched (2 g L^{-1}) LB-Lennox liquid medium (Bertani 1951; Lennox 1955) at 28.5 °C for 24 h (incubator shaker) using a sample of the untreated soil as the inoculum. Glucose enrichment was chosen in order to avoid C limitation of bacterial growth. After incubation, all cultures were centrifuged (4700 *g* min^{-1}) for 10 min. Pellets were washed twice in sterile 0.3% NaCl solution and resuspended in sterile 0.3% NaCl solution. In the treatments using the bacterial mix, remaining soil components were removed by trapping on folded filter paper (grade 4) before cell washing. Cell suspensions were photometrically measured (BioPhotometer, Eppendorf, Germany) and adjusted to an $OD_{600} = 1.0$ corresponding to a cell density of approximately 10^9 cells mL^{-1}, as described in Xue et al. (2013). However, overestimates of cell density resulting from soil-derived turbid material remaining in cell suspensions containing bacterial isolates cannot be fully excluded. The killing of RU47 cells, which were used in one of the treatments, was performed as follows: bacterial suspension ($OD_{600} = 1.0$) was placed in a sterile Erlenmeyer flask and boiled for 1 min on a heating plate. To minimize volume loss, the flask was covered and cooled to room temperature to exclude volume error before being used for inoculation. Pre-tests confirmed that this procedure was sufficient to kill RU47 cells, as plating exhibited no growth of RU47.

Plants were inoculated three times, each with a cell density of 10^9 cells mL^{-1} ($OD_{600} = 1.0$). The first inoculation was conducted by seed coating. Under gentle and continuous vortexing, 5 μL of cell suspension was successively added to five tomato seeds. The volume required for entire seed coating had been tested with ink (Pelikan, Pottendorf, Austria) before starting the experiment. To prevent drying of the inoculant, coated seeds initially remained in the closed Falcon tubes, which were used performing the seed coating, and were immediately sown (i.e. within less than 5 min). Success of seed coating (i.e. viability and cell concentration of RU47) was controlled by using three of the inoculated seeds followed by washing with 1 mL sterile 0.3% NaCl solution and plating 100 μL of the suspension on King's B-Agar medium (50 mg rifampicin L^{-1}) in three dilution stages. Plates were incubated at 28.5 °C until growing colonies were unequivocally countable on the agar (after approximately 36 h). The second inoculation was applied directly after seed germination, and the last inoculation was applied one week later. Both inoculations were performed with 6 mL kg^{-1} soil substrate DM, directly applied to the soil surface to simulate farm practice. To avoid a watering effect, the P-fertilized non-inoculation treatment was inoculated with 0.3% NaCl solution with corresponding volumes per inoculation. Viability and unviability of the RU47 cells used as well as sterility of the 0.3% NaCl solution were checked by plating and subsequent incubation at 28.5 °C for 48 h after every inoculation.

Plant properties

Plant analyses during the growth period

Stem diameter, leaf number and area, shoot height and P deficiency symptoms were recorded at temporal intervals of minimum 2 and maximum 4 days, starting 20 days after sowing. While stem diameter and leaf area (length x width) were measured using a precision pocket vernier caliper (150 mm, Format, Wuppertal, Germany), shoot height, defined as the vertical length from stem base to youngest leaf's tip, was measured by a ruler. P deficiency symptoms were defined as the expression of violet discoloration on the undersides of leaves and determined as a percentage of total leaf area.

Plant analyses after harvest

Shoots of every replicate were separately and carefully cut from the soil surface using a sterilized (70% ethanol) scalpel. Shoots were briefly rinsed with H_2O_{deion} to remove adhering dust, then dried at 60 °C in separate aluminium trays for 3 days to estimate dry weight.

Determination of plant biomass P in tomato shoots was performed by sequential microwave digestion based on Kalra et al. (1989) followed by photometric measurement of molybdenum blue. Ground samples were transferred into Teflon containers to which 1 mL H_2O_{deion}, 2.5 mL HNO_3 and 2 mL H_2O_2 were added. After soaking for 1 h, samples were incinerated at 70 °C

(3 min) and 210 °C (62 min) at 1400 W, using an ETHOSlab microwave (MLS, Leutkirch, Germany). The diluted (1:1 H_2O_{deion}) and filtered (blue ribbon filter) suspensions were photometrically measured after a dilution of 1:3 H_2O_{deion} using Murphy and Riley color reagent (Murphy and Riley 1962) at 710 nm in a microplate absorption reader (ELx808; BioTek Instruments Inc., Winooski, VT, USA).

Soil sampling

Rhizosphere and bulk soil samples were immediately put on ice for short-term storage. While DNA was directly extracted from the rhizosphere soils (see 2.5), bulk soil samples were sieved (< 2 mm), after which aliquots of each replicate were frozen at −20 °C until analyses.

Tracing RU47 and analyses of microbial community composition

DNA extraction

DNA was extracted according to Schreiter et al. (2014b) with some modifications. Briefly, after removing loosely adhering soil by vigorously shaking the roots, the complete root systems of one replicate per treatment were combined, then cut into pieces of approximately 1 cm length and carefully mixed. Five g of cut roots with tightly adhering soil were transferred to a Stomacher bag, homogenized in a Stomacher 400 Circulator (Seward Ltd., Worthing, UK) for 1 min at high speed after adding 15 mL sterile 0.3% NaCl; supernatant was then collected in a Falcon tube. This step (same 5 g of root material) was repeated twice, the combined supernatants (45 mL) of three Stomacher homogenizations were centrifuged at 10,000 *g* for 15 min, after which pellets were frozen and stored at −20 °C. The use of Stomacher method to detach microbial cells adhering to root and rhizosphere does not fully exclude a co-extraction of plant cells; however, pre-tests of this method revealed only minor contaminations with plant DNA. Total community DNA (TC-DNA) was extracted from 0.5 g of rhizosphere pellets using the Fast DNA SPIN Kit for Soil® (MP Biomedicals, Heidelberg, Germany) after a harsh lysis step as described by the manufacturer. The TC-DNA was purified with GENE CLEAN SPIN Kit® (MP Biomedicals, Heidelberg, Germany) according to the manufacturer's instructions and diluted 1:10 with 10 mM Tris HCl, pH 8.0, before use.

Amplicon sequencing of 16S rRNA gene amplicon from TC-DNA

Detailed procedure describing amplification of 16S rRNA genes, sequencing, quality trimming and annotation was described previously (Nunes et al. 2016), respecting best practices guide lines (Schöler et al. 2017). Briefly, the ~460-bp fragment covering hypervariable regions V3–V4 of the small ribosomal subunit gene was amplified, tagged and sequenced using 2 × 250 bp paired-end high-throughput sequencing using illumina miseq Reagent Kits version 2 and Illumina® MiSeq® platform (Illumina, San Diego, CA, USA). Four biological replicates were sequenced for each of the four conditions tested, namely P-fertilized, bacterial mix, HT-RU47, RU47 (Table 1). Since no alpha-diversity estimation is performed in this study, the raw count data was used with appropriated biostatistic procedures accounting for uneven sequencing depth (Fig. S1) to avoid loss of information problems arising from rarefaction (McMurdie and Holmes 2014). A redundancy analysis (RDA) was performed on the profiles after relative abundance and log10 transformation to account for uneven sequencing depth and disparities between abundant and rare species using previously described methodology (Nunes et al. 2016). Major phylogenetic changes were detected at the phylum and class levels by means of ANOVA with a false discovery rate correction test (FDR, $p < 0.05$). Operational taxonomic units (OTUs) responding significantly across experimental design were extracted using previously described methodology (Jacquiod et al. 2017) using an analysis of deviance (AOD) after generalized linear modelling (GLM) of the raw counts using negative binomial distribution (nb) with 1000 resampling iterations with residual variance, using the package mvabund (nbGLM, likelihood ratio test, $p < 0.05$, Wang et al. 2012). This method was recently suggested as one of the most accurate way to extract significantly responding OTUs by minimizing the risk of error (Thorsen et al. 2016). A generalized heatmap of dominant (relative abundance >0.1%) and significantly responding OTUs was generated using previously described methodology (Jacquiod et al. 2016). A supporting table with the relative abundance of dominant *Pseudomonas* OTUs found in this study is provided in (Table S1). Sequencing fastq files were deposited in the Sequence read Archive (SRA) under the accession number SRP125744 (BioProject: PRJNA420007).

Table 1 List and description of 16S rRNA gene amplicon sequencing samples generated in this study

Name	Code	Description	Replicate	Sequences
P-fertilized_a	PC1	P-fertilized, non-inoculation	R1	27,143
P-fertilized_b	PC2	P-fertilized, non-inoculation	R2	23,947
P-fertilized_c	PC3	P-fertilized, non-inoculation	R3	36,680
P-fertilized_d	PC4	P-fertilized, non-inoculation	R4	30,721
Bacterial mix_a	BM1	Bacterial mix	R1	10,090
Bacterial mix_b	BM2	Bacterial mix	R2	16,727
Bacterial mix_c	BM3	Bacterial mix	R3	20,614
Bacterial mix_d	BM4	Bacterial mix	R4	21,026
HT-RU47_a	HT1	Heat treated *Pseudomonas* sp. RU47	R1	20,787
HT-RU47_b	HT2	Heat treated *Pseudomonas* sp. RU47	R2	28,839
HT-RU47_c	HT3	Heat treated *Pseudomonas* sp. RU47	R3	26,321
HT-RU47_d	HT4	Heat treated *Pseudomonas* sp. RU47	R4	26,280
RU47_a	RU1	*Pseudomonas* sp. RU47	R1	10,493
RU47_b	RU2	*Pseudomonas* sp. RU47	R2	22,608
RU47_c	RU3	*Pseudomonas* sp. RU47	R3	23,818
RU47_d	RU4	*Pseudomonas* sp. RU47	R4	33,953

Phospholipid fatty acid (PLFA) analysis

Microbial community structure was determined using PLFA profiles based on the alkaline methylation method of Frostegård et al. (1991). Lipid extraction and determination of fatty acid methyl esters (FAMEs) were performed according to Mackie et al. (2015). The divisions of PLFAs into bacteria and fungi were based on Frostegård and Bååth (1996), Zelles (1999) and Kandeler et al. (2008). Within bacteria, PLFAs were grouped into Gram-positive ($gram^+$), represented by i15:0, a15:0, i16:0, and Gram-negative ($gram^-$), specified by cy17:0 and cy19:0. Total bacterial PLFAs were calculated by the sum of $gram^+$ and $gram^-$ plus 16:1ω7. Fungal PLFA was represented by 18:2ω6,9.

Enzyme assays

Soil in situ zymography

Soil in situ zymography uses membranes coated with methylumbelliferyl (MUF)-substrates which become fluorescent during enzyme cleavage, yielding information about the distribution of exoenzymes in soil. Distributions of alkaline and acid phosphomonoesterase (EC 3.1.3) in the rhizosphere were analysed by soil in situ zymography using an approach similar to that described in Spohn and Kuzyakov (2014). All replicates were analysed by zymography at intervals of seven days, starting 18 days after sowing. MUF phosphate (4-MUF, Sigma-Aldrich, St. Louis, USA) was used as substrate; a 12 m*M* solution was prepared and used to coat polyamide membranes, with diameter 14.2 cm, and pore size 0.45 μm (Sartorius, Göttingen, Germany). Substrate solution was prepared using modified universal buffer (MUB) adjusted to pH 11 for alkaline PA, and pH 6.5 for acid PA. Coated membranes were laid flat onto opened rhizoboxes which were separated from soil particles by an underlying layer of fresh 1% agarose gel (1 mm thick). Soil zymography was performed for each enzyme separately on the same rhizobox; first, acid PA was evaluated due to its affinity with the soil's pH of 7.4; second, alkaline PA was assayed. This order was maintained throughout the experiment. The possible loss of alkaline phosphatases by diffusion into the agarose gel or membrane during measurement of the acid PA cannot be excluded. In contrast to Spohn and Kuzyakov (2014), an incubation time of 35 min, adjusted to achieve the best practical contrast obtained by imaging, was used. Incubations were performed at a constant temperature of 20 °C; membranes were covered by aluminium foil to minimize liquid loss during incubation time. After incubation, membranes were placed on an epi-UV-desk (Desaga, Sarstedt, Nümbrecht, Germany) in the dark, and viewed at 360 nm wavelength. After being photographed

with a digital camera (D60, Nikon, Tokyo, Japan) image processing and analysis of the zymograms were done using the open source software ImageJ. Digital images were transformed to 8-bit and multiplied by a factor of 1.25 to enhance the contrast. Images were transformed into false colors to create a color representation of enzyme activity, as given in Fig. S2. Calculation of enzyme activity was based on a linear function using a calibration curve fitted to different concentrations of 4-methylumbelliferone (0, 35, 70, 130, 200, 240 μM). Image processing of calibration zymograms was adapted to the modifications made with the soil zymograms. Calculation of enzyme activity was based on mean gray values obtained for each concentration in the calibration curve. As there was no distinct separation observed in enzyme activity between root and surrounding soil, the mean activity of the total incubated area was calculated.

Analyses of enzyme activities in bulk after final harvest

In addition to regularly conducted soil in situ zymography during the growth period, samples from the harvested bulk soil were analysed for potential alkaline and acid phosphatase (EC 3.1.3) activity using MUF substrates (4-MUF; Sigma-Aldrich, St. Louis, USA) according to Marx et al. (2001). The assay followed the method described in Poll et al. (2006) with an alteration; MUB instead of 2-(N-morpholino)ethanesulfonic acid (MES) was used to ensure comparability with the results obtained by the zymograms. Alkaline phosphatase was measured at pH 11, acid phosphatase at pH 6.5. Contrary to the findings of Niemi and Vepsäläinen (2005), pre-tests of this study demonstrated that the stability of 4-MUF phosphate in alkaline pH ranges (pH 8–12) is constant over time (2 h) when MUB instead of MES buffer is used. The activities of three enzymes involved in the C and N cycle were also measured using fluorescent MUF substrates (4-MUF; Sigma–Aldrich, St. Louis, USA): β-d-glucosidase (EC 3.2.1.21), β-xylosidase (EC 3.2.1.37) and β-N-acetylglucosaminidase (EC 3.2.1.52) according to Marx et al. (2001). Enzyme activity was measured in autoclaved MES buffer (pH 6.1).

Microbial-bound C and P

To determine microbial biomass C (C_{mic}) the chloroform fumigation extraction method (Vance et al. 1987) according to Mackie et al. (2015) was used. C_{mic} was calculated using *keC* 0.45 as extraction factor (Joergensen 1996). The estimation of microbial biomass P (P_{mic}) was done by liquid fumigation extraction with anion-exchange resin membranes (Kouno et al. 2002) using hexanol instead of liquid chloroform (Bünemann et al. 2004). A fresh weight of soil corresponding to 2 g dry matter was used for fumigated and non-fumigated subsamples of each sample. Pre-tests of this study indicated that the observed variability in P adsorption behaviour of soil depended on total P concentration in the soil solution to be analysed. Thus, the use of identical soil weights in all subsamples is a prerequisite to obtain an accurate correction factor for P retained by soil after fumigation. Fumigation and extraction were performed according to Bünemann et al. (2004). Extracted P was mixed with Murphy and Riley color reagent (Murphy and Riley 1962) and H_2O_{deion} in a ratio of 1:1:4 (*v*/v), respectively. P concentration was photometrically measured at 710 nm using a microplate absorption reader (ELx808; BioTek Instruments Inc., Winooski, VT, USA). To determine the amount of P retained by soil particles and complexation after fumigation incubation, a defined P concentration (K_2HPO_4), which was equal to the measured P concentrations in fumigated subsamples (μg P g^{-1}) was added to additional non-fumigated but otherwise identically treated subsamples. The ratio of recovered P to added P was used to calculate the P_{mic} concentration as follows:

$$P_{mic}\left[\mu g\ g^{-1}\right] = \frac{\left(P_{fumigated}\left[\mu g\ g^{-1}\right] - P_{non\text{-}fumigated}\left[\mu g\ g^{-1}\right]\right)}{\left(P_{recovered}\left[\mu g\ g^{-1}\right] / P_{added}\left[\mu g\ g^{-1}\right]\right)}$$

Given values of water-extractable soil P (P_{H2O}) corresponds to the P concentration (μg P g^{-1}) determined in the non-fumigated subsamples. However, as the used anion-exchange resin membranes compete for P adsorption by soil particles, it cannot be assumed that given P_{H2O} values completely represent the plant available P fraction.

Mineral N

To determine the concentrations of ammonium (NH_4^+) and nitrate (NO_3^-) in soil, undiluted (0.5 *M* K_2SO_4) soil extracts from non-fumigated samples used for C_{mic} determination were colorimetrically measured on an Autoanalyzer III (Bran + Luebbe, Norderstedt, Germany).

Statistical analyses

Differences between the treatments were statistically analysed as follows: Homogeneity of variance was tested by the Levene-test. Significance of differences was tested by ANOVA followed by the Tukey HSD-test, where $p < 0.05$ was considered as the threshold value for significance. In cases of variance heterogeneity, the Games-Howell-test was used for pairwise comparison, where $p < 0.05$ was also considered as significant. Statistical analyses were performed using SPSS Statistics 22 (IBM 2013).

Results

Plant growth and soil nutrients

In comparison to the bacterial mix treatment, inoculations with RU47 or HT-RU47 cells resulted in significantly enhanced plant growth, as shown by higher stem diameter, leaf number (Table 2), and shoot biomass (Fig. 1). Furthermore, we observed trends of increased shoot height and leaf area (Table 2). Symptoms of P deficiency (violet discoloration on the leaves) were less obvious in plants receiving both RU47 treatments than in the bacterial mix treatment but were not significantly different from the bacterial mix (Table 2). In comparison with the bacterial mix treatment, both RU47 treatments revealed higher P uptakes (Fig. 1). Plants of the treatments P-fertilized, and bacterial mix had concentrations of about 4 g P kg^{-1}, which represented an adequate P supply for tomato plants before flowering. Plants inoculated with HT-RU47 cells had an optimal concentration of 6 g P kg^{-1}, whereas plants inoculated with RU47 exhibited a remarkably low P tissue concentration of 2 g P kg^{-1} but this was an improvement in absolute uptake compared to the bacterial mix (Fig. 1, Table S2). In measurements of water-extractable P (P_{H2O}) in soil, bulk soil samples of both RU47 treatments had a 2.3-fold higher P concentration than samples inoculated with the bacterial mix, and about one fourth of the P concentration measured in the optimally P-fertilized non-inoculation treatment (Table 2). No significant treatment effects on NH_4^+ and NO_3^- concentrations in bulk soil were observed (Table 2). However, NO_3^- concentrations were negatively correlated with shoot biomass (Pearson's $r = -0.71$; $p < 0.05$).

Enzyme activities involved in P, C, and N cycling

Soil in situ zymography has revealed that the addition of viable RU47 significantly increased alkaline PA in the rhizosphere of tomato on days 25–26 and 31–32 after sowing (Fig. 2a). Alkaline PA in the rhizosphere of plants inoculated with viable RU47 increased significantly over time, with highest activity on days 25–26 after sowing, whereas the temporal pattern was stable in the HT-RU47 treatment (Fig. 2a). Based on results found by zymography, the activity of acid phosphatase was marginally less than that detected for alkaline PA (Fig. 2a, b). Acid PA increased slightly over time; significant increases of 21% (bacterial mix) and 15% (RU47) could be observed in the treatments to which living bacteria were added (Fig. 2b). As expected, acid PA in the rhizosphere was positively correlated with plant properties (e.g. shoot height, Pearson's $r = 0.60$; $p < 0.001$). Potential alkaline and acid PA in homogeneous bulk soil samples after final harvest indicated highest activities in both RU47 treatments (Table 2). RU47 inoculation did not influence enzyme activities involved in C and N cycling in bulk soil (Table 2). Nevertheless, the bacterial mix treatment stimulated activities of the mainly fungus-derived β-xylosidase by more than 100% compared with the average β-xylosidase activity observed in all other treatments (Table 2).

Microbial biomass

Both RU47 treatments did not influence microbial C content of the bulk soil (Fig. 3). Microbial biomass P was almost equal in all inoculation treatments and significantly higher than values detected in the P-fertilized treatment (Fig. 3). Thus the calculated atomic C:P ratio of 305 in microbial biomass of the P-fertilized treatment was much higher (4.4 times) as compared to the average C:P ratios of all other treatments (Table 2).

PLFA

The addition of RU47 did not result in significant shifts in microbial groups representation based on PLFA patterns (Table 2). However, while bulk soils of the P-fertilized and bacterial mix treatments exhibited identical PLFA patterns, abundances of bacterial PLFAs were higher in bulk soil inoculated with HT-RU47 or RU47 cells by 11 and 7%, respectively (Table 2). Abundances

Table 2 Summarized plant and soil properties of tomato plants under one optimally P-fertilized, non-inoculation (P-fertilized) and three inoculation treatments using unselectively cultivated soil bacteria (bacterial mix), heat treated RU47 (HT-RU47), or viable RU47 (RU47) cells, recorded 36 and 39 days after sowing (DAS)

		P-fertilized		Bacterial mix		HT-RU47		RU47	
	Unit	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Plant properties (36 DAS)									
Shoot height	[cm]	33.8 ^{a}	± 0.6	17.2 ^{b}	± 2.8	27.1 ^{a}	± 0.7	26.4 ^{ab}	± 1.8
Stem diameter	[cm]	0.5 ^{a}	± 0.0	0.3 ^{b}	± 0.0	0.5 ^{a}	± 0.0	0.5 ^{a}	± 0.0
Leaf number	–	5.5 ^{a}	± 0.3	3.3 ^{b}	± 0.3	5.0 ^{a}	± 0.0	4.8 ^{a}	± 0.3
Leaf area	[cm^2]	775.6 ^{a}	± 11.3	205.4 ^{b}	± 84.2	570.6 ^{ab}	± 34.8	567.8 ^{ab}	± 101.7
Violet discolouration	–	0.0 ^{b}	± 0.0	2.5 ^{a}	± 0.5	0.8 ^{ab}	± 0.5	1.0 ^{a}	± 0.0
Soil properties (39 DAS)									
pH ($CaCl_2$)	–	7.2 ^{b}	± 0.0	7.5 ^{a}	± 0.0	7.5 ^{a}	± 0.0	7.5 ^{a}	± 0.0
P_{H2O}	[mg kg^{-1}]	100.5 ^{a}	± 5.5	9.6 ^{b}	± 5.1	22.4 ^{b}	± 2.0	22.6 ^{b}	± 1.0
Molar microbial C:P ratio	–	305.1	± 92.1	55.1	± 16.1	80.4	± 8.5	73.7	± 7.2
NH_4^+	[mg kg^{-1}]	1.9	± 0.6	2.1	± 0.4	1.5	± 0.2	1.1	± 0.1
NO_3^-	[mg kg^{-1}]	141.7	± 4.0	159.5	± 3.5	142.5	± 5.4	150.6	± 4.5
$Gram^+$ PLFAs	[nmol FAME g^{-1}]	8.0	± 0.3	8.0	± 0.2	8.9	± 0.8	8.6	± 0.3
$Gram^-$ PLFAs	[nmol FAME g^{-1}]	1.2	± 0.0	1.2	± 0.0	1.3	± 0.1	1.3	± 0.1
Bacterial PLFAs	[nmol FAME g^{-1}]	14.2	± 0.5	14.2	± 0.3	15.7	± 1.4	15.2	± 0.6
Fungal PLFA	[nmol FAME g^{-1}]	0.4	± 0.0	0.4	± 0.0	0.4	± 0.1	0.4	± 0.0
Acid phosphomonoesterase	[nmol g^{-1} h^{-1}]	73.8	± 20.1	73.7	± 28.7	105.5	± 10.3	114.6	± 13.9
Alkaline phosphomonoesterase	[nmol g^{-1} h^{-1}]	475.9	± 33.5	503.8	± 38.2	518.5	± 31.1	584.1	± 19.0
ß-glucosidase	[nmol g^{-1} h^{-1}]	188.7	± 10.5	181.9	± 4.0	187.5	± 12.6	196.0	± 9.4
N-acetyl-ß-glucosaminidase	[nmol g^{-1} h^{-1}]	63.3	± 9.3	45.1	± 3.9	41.4	± 3.1	43.4	± 1.6
ß-xylosidase	[nmol g^{-1} h^{-1}]	13.2	± 3.2	34.1	± 17.6	17.9	± 1.3	17.5	± 1.4

Values are presented as mean ± standard error (SE) of four replicates. Significant differences (Tukey-HSD/Games-Howell, $p < 0.05$) between the treatments are marked by lowercase letters. Please note the different time points of plant observation and soil sampling. Percentage of violet discolouration on the undersides of the leaves is based on total leaf area, coded as follows: 0% = 0, > 0–25% = 1, > 25–50% = 2, > 50–75% = 3, > 75–100% = 4

of PLFAs representing $gram^+$ bacteria were higher by about 9% in treatments using both HT-RU47 and viable RU47 compared to the control and bacterial mix treatments (Table 2).

Amplicon sequencing

RDA demonstrated clear effect of RU47 inoculation along the first axis explaining about 24% of the variance, positively correlating with microbial biomass (total PLFA) and PA, which, in turn were positively correlated with plant's P uptake, despite weak linkage to shoot biomass (Fig. 4). However, a negative correlation between RU47 and N pool in bulk soil was determined (Fig. 4). Second component clearly segregated clusters of the P-fertilized, non-inoculation treatment from those of the bacterial mix treatment (about 19% of the variance; Fig. 4). While OTU cluster of the P-fertilized treatment was positively correlated with plant's P uptake and shoot biomass, this treatment was mainly characterized by molar C:P ratio in microbial biomass as well as activities of soil enzymes involved in the C cycle (β-D-glucosidase, β-D-xylosidase; Fig. 4). Bacterial mix cluster showed highly positive correlation with the leaf discoloration, N pool, and pH, while plant's P uptake, shoot biomass, and molar C:P ratio determined in bulk soil's microbial biomass were correlated negatively (Fig. 4).

Amplicon sequences provided insights into the phylogenetic composition of the prokaryotic community in tomato rhizosphere DNA of the four different treatments from plants sampled 39 days after sowing. In terms of alpha-diversity, a clear and significant differences

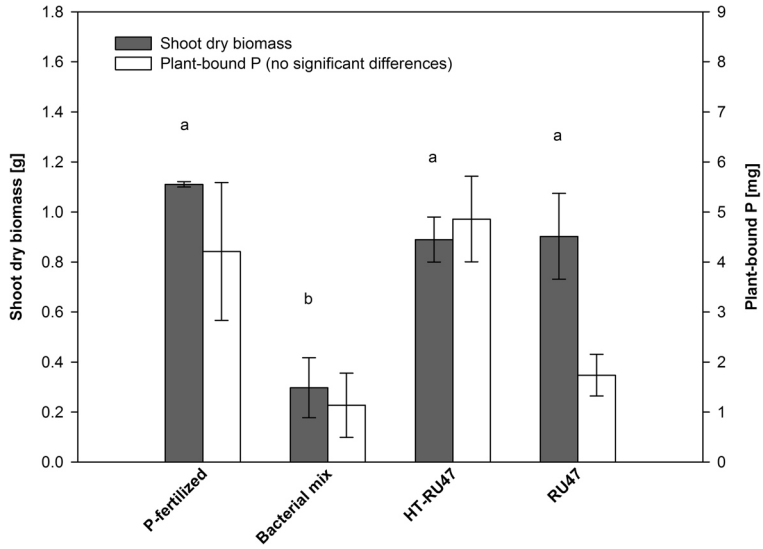

Fig. 1 Tomato shoot biomass (dry weight) and plant-bound P of one optimally P-fertilized, non-inoculation (P-fertilized) and three inoculation treatments using unselectively cultivated soil bacteria (bacterial mix), heat treated RU47 (HT-RU47), or viable RU47 (RU47) cells. Data were recorded 39 days after sowing. Error bars indicate standard error ($n = 4$); significant differences (Tukey-HSD, $p < 0.05$) between the treatments are designated by lowercase letters

($p < 0.05$) were observed between samples treated with dead/alive RU47 which had lower evenness (Shannon index: HT-RU47 = 3.66 ± 0.34 and RU47 = 3.89 ± 0.10) and richness (HT-RU47 = 649 ± 72 and RU47 = 683 ± 46) as opposed to P-fertilized/bacterial mix samples (Shannon index: P-fertilized = 4.98 ± 0.19 and bacterial mix = 5.18 ± 0.09; Richness: P-fertilized = 884 ± 21 and Bacterial mix = 860 ± 17). The HT-RU47 and the RU47 treatments had significantly higher relative abundance of *Proteobacteria*. In particular *Gammaproteobacteria* were strikingly increased in relative abundance compared to the P-fertilized and bacterial mix treatments (Table 3). In both HT-RU47 and RU47 treatments *Pseudomonas* was significantly increased in relative abundance. Interestingly, in the HT-RU47 treatment the sequences were distinct from those related to RU47. Sequence comparison with the recently available RU47 genome sequence showed that two OTU were likely RU47 derived due to 16S operon heterogeneities (Fig. S3). Although at the phylum level for *Firmicutes* there were no significant differences observed, the relative abundance of *Bacilli* was significantly lower in the HT-RU47 and RU47 treatments. *Bacteroidetes* (*Cytophagia*, *Sphingobacteria*) were significantly higher in the P-fertilized treatment. The less abundant phyla *Gemmatimonadets*, *Nitrospirae*, *Chloroflexi*, *Planctomycetes* and *Verrucomicrobia* had a significantly lower relative abundance in the HT-RU47 and the RU47 treatments compared to the P-fertilized and bacterial mix treatments (Table 3). Heatmap demonstrated that each treatment displayed distinct dominant OTUs significantly responding between treatments in term of abundance (Fig. 5). The heat map shows that RU47 related OTUs were dominant members of the tomato rhizosphere only in the RU47 treatment. Interestingly in the HT-RU47 treatments these OTU were not detected but instead OTU with sequence similarity to different *Pseudomonas* species were dominant. OTUs affiliated to *Clostridium*, *Lysobacter* and *Tumebacillus* showed a higher abundance in the bacterial mix treatments. Numerous dominant OTUs (18) were observed in the P-fertilized treatment that were affiliated to diverse range of genera belonging to different phyla (Fig. 5). Samples of the bacterial mix treatment had 4 OTUs with higher abundance, partly overlapping with those of the P-fertilized treatment, mostly belonging to *Firmicutes* and *Gammaproteobacteria* (Fig. 5). Furthermore, in RU47 treatment OTUs belonging to *Rubrobacter* sp. and *Terrimonas* sp. were found (Fig. 5).

Discussion

Plant growth

In tomato plants inoculated with RU47, not only stem diameter and leaf number, but also 3-fold higher shoot biomass was observed in comparison to plants which were inoculated with the bacterial mix (Table 2, Fig. 1). Therefore, the present study demonstrates the actual plant growth promoting activity of this particular inoculant, with similar results compared to previous studies.

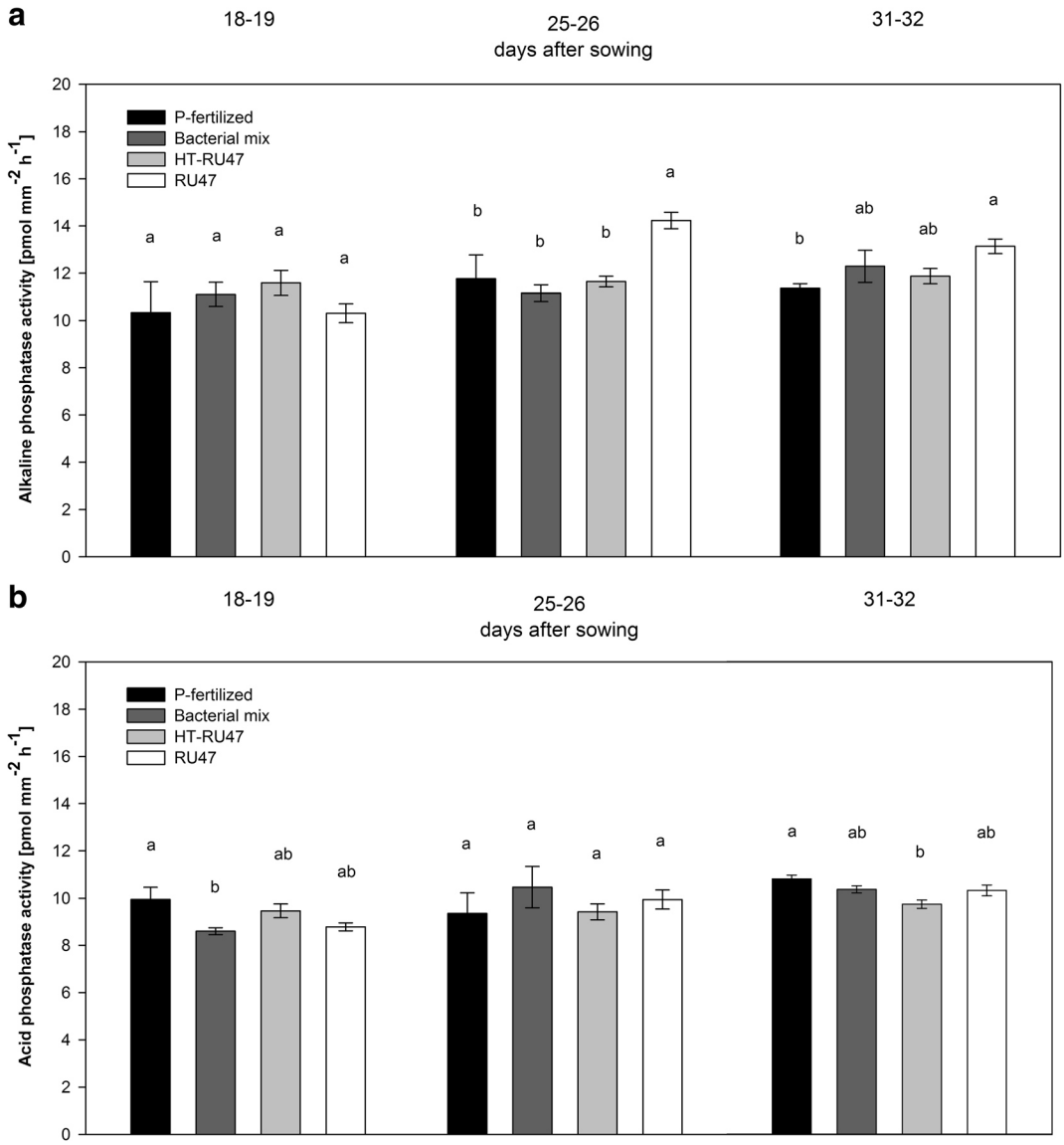

Fig. 2 **a** Alkaline phosphomonoesterase activity during different growth stages of tomato plants. The experiment comprised one optimally P-fertilized, non-inoculation (P-fertilized) and three inoculation treatments using unselectively cultivated soil bacteria (bacterial mix), heat treated RU47 (HT-RU47), or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4). Letters indicate significant differences (Tukey-HSD, $p < 0.05$) between the treatments, tested individually for each growth stage. **b** Acid phosphomonoesterase activity during different growth stages of tomato plants. The experiment comprised one optimally P-fertilized, non-inoculation (P-fertilized) and three inoculation treatments using unselectively cultivated soil bacteria (bacterial mix), heat treated RU47 (HT-RU47), or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4). Letters indicate significant differences (Tukey-HSD, $p < 0.05$) between the treatments tested individually for each growth stage

For instance, Kim et al. (1997) observed a 2-fold higher plant biomass in 35 day-old tomato plants inoculated with *Enterobacter agglomerans* cells compared to the not inoculated control. However, in our study, a growth-promoting effect was also observed in tomato plants inoculated with HT-RU47 cells (Fig. 1, Table 2). The difference between the treatments RU47 and HT-RU47 makes it possible to estimate whether potential plant growth promotion is a result of direct or indirect mechanisms. Direct mechanisms can include, for example, the production of phosphatases by RU47 resulting in an improved supply of P by plants. Indirect mechanisms include the release of cell-derived phytohormones or other compounds which may stimulate and/or facilitate

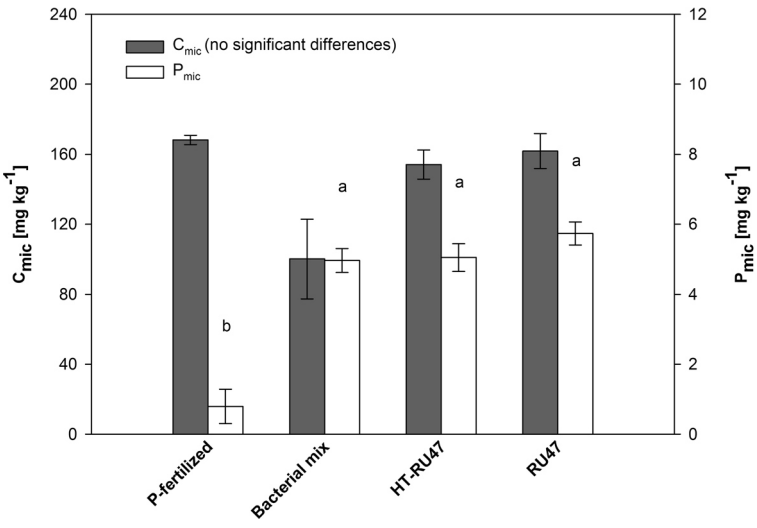

Fig. 3 Microbial-bound carbon (C_{mic}) and phosphorus (P_{mic}) in bulk soil of tomato plants harvested 39 days after sowing. The experiment comprised one optimally P-fertilized, non-inoculation (P-fertilized) and three inoculation treatments using unselectively cultivated soil bacteria (bacterial mix), heat treated RU47 (HT-RU47), or viable RU47 (RU47) cells. Error bars indicate standard error (n = 4); significant differences (Tukey-HSD, $p < 0.05$) between the treatments are designated by lowercase letters

establishment of indigenous microbes and their activity in soil. These indirect mechanisms are discussed in more detail below.

Tracing RU47

The strain RU47 was originally isolated from a soil which had previously been reported as suppressive to phytopathogenic fungi (Adesina et al. 2007). Amplicon sequencing of 16S rRNA gene from TC-DNA, which was extracted from the rhizosphere, revealed clear taxonomic segregation between P-fertilized, bacterial mix, and both treatments where RU47 cells were added (Figs. 4 and 5, Table 3). Furthermore, as strain RU47 displays heterogeneity in its own 16S sequence due to multiple genomic copies ($n = 6$), several OTUs were identified matching the RU47 variants (Fig. S3). Based on amplicon sequencing data cross-contaminations

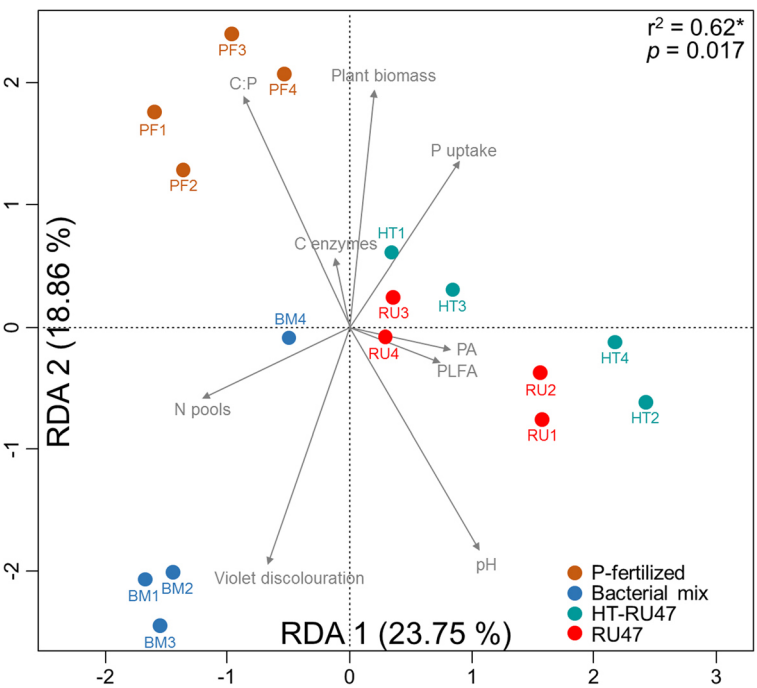

Fig. 4 Redundancy analysis (RDA) applied on whole prokaryotic communities obtained from 16S amplicon sequencing in the rhizosphere of one optimally P-fertilized, non-inoculation (P-fertilized) and three inoculation treatments using unselectively cultivated soil bacteria (bacterial mix), heat treated RU47 (HT-RU47), or viable RU47 (RU47) cells of tomato plants 39 days after sowing

Table 3 Phylogenetic composition of the prokaryotic community in tomato rhizosphere DNA of one optimally P-fertilized, non-inoculation (P-fertilized) and three inoculation treatments (bacterial mix of soil bacterial isolates; heat treated RU47 [HT-RU47], or viable RU47 [RU47] cells) from plants harvested 39 days after sowing

	P-fertilized		Bacterial mix		HT-RU47		RU47	
Phylum/class	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Proteobacteria	40.7^{b}	± 4.4	36.8^{b}	± 4.1	62.4^{a}	± 5.6	58.7^{a}	± 4.6
Alphaproteobacteria	8.8^{a}	± 0.6	9.6^{a}	± 1.0	4.0^{b}	± 0.9	5.3^{b}	± 0.4
Betaproteobacteria	1.5^{a}	± 0.1	1.1^{ab}	± 0.2	1.2^{ab}	± 0.2	0.9^{b}	± 0.1
Deltaproteobacteria	0.5^{a}	± 0.1	0.4^{a}	± 0.1	0.2^{b}	± 0.1	0.2^{b}	± >0.01
Gammaproteobacteria	29.9^{b}	± 4.9	25.6^{b}	± 4.8	56.9^{a}	± 6.6	52.4^{a}	± 4.1
Unclassified	>0.01	± >0.01	nd		>0.01	± >0.01	nd	
Firmicutes	26.7	± 3.3	25.2	± 4.3	19.2	± 3.0	17.8	± 6.4
Bacilli	5.2^{a}	± 0.7	3.9^{a}	± 0.7	1.7^{b}	± 0.3	1.7^{b}	± 0.2
Clostridia	21.5	± 3.4	20.3	± 3.6	18.0	± 3.0	16.1	± 6.4
Unclassified	>0.01	± >0.01	0.9	± 0.9	nd		nd	
Actinobacteria	14.6^{b}	± 1.2	22.9^{a}	± 2.0	9.9^{c}	± 2.7	14.9^{b}	± 0.9
Bacteroidetes	8.6^{a}	± 0.9	4.0^{b}	± 0.3	4.3^{b}	± 1.3	4.3^{b}	± 0.6
Bacteroidia	1.2^{a}	± 0.2	1.4^{a}	± 0.3	0.6^{b}	± 0.1	0.6^{b}	± 0.1
Cytophagia	0.4^{a}	± >0.01	0.2^{b}	± 0.1	0.2^{b}	± 0.1	0.2^{b}	± >0.01
Flavobacteriia	0.6	± 0.1	0.6	± 0.2	0.5	± 0.2	0.5	± 0.1
Sphingobacteriia	6.4^{a}	± 0.9	1.8^{c}	± 0.4	3.0^{bc}	± 0.9	3.0^{b}	± 0.4
Gemmatimonadetes	3.3^{a}	± 0.5	3.8^{a}	± 0.6	1.2^{b}	± 0.4	1.4^{b}	± 0.3
Nitrospirae	1.5^{a}	± 0.2	1.9^{a}	± 0.3	0.6^{b}	± 0.2	0.8^{b}	± 0.1
Chloroflexi	1.4^{a}	± 0.2	1.8^{a}	± 0.3	0.5^{b}	± 0.2	0.7^{b}	± 0.1
Anaerolineae	0.3^{a}	± 0.1	0.4^{a}	± 0.1	0.1^{b}	± >0.01	0.2^{b}	± >0.01
Caldilineae	0.4^{a}	± 0.1	0.5^{a}	± 0.1	0.2^{b}	± >0.01	0.2^{b}	± >0.01
Chloroflexia	0.2^{a}	± >0.01	0.3^{a}	± 0.1	0.1^{b}	± >0.01	0.1^{b}	± >0.01
Dehalococcoidia	0.1	± >0.01	0.1	± >0.01	>0.01	± >0.01	0.0	± >0.01
Ktedonobacteria	>0.01	± >0.01	nd		nd		nd	
Thermomicrobia	0.4^{b}	± >0.01	0.5^{a}	± >0.01	0.2^{c}	± >0.01	0.2^{c}	± >0.01
Planctomycetes	0.8^{a}	± 0.1	0.7^{a}	± 0.1	0.3^{b}	± 0.1	0.3^{b}	± 0.1
Phycisphaerae	>0.01	± >0.01	>0.01	± >0.01	>0.01	± >0.01	>0.01	± >0.01
Planctomycetia	0.8^{a}	± 0.1	0.7^{a}	± 0.1	0.3^{b}	± 0.1	0.3^{b}	± 0.1
Verrucomicrobia	0.6^{a}	± 0.1	0.7^{a}	± 0.1	0.2^{b}	± 0.1	0.2^{b}	± >0.01
Opitutae	>0.01	± >0.01	nd		nd		nd	
Verrucomicrobiae	0.6^{a}	± 0.1	0.7^{a}	± 0.1	0.2^{b}	± 0.1	0.3^{b}	± >0.01
Ignavibacteriae	0.1^{a}	± >0.01	0.2^{a}	± >0.01	0.1^{b}	± >0.01	0.1^{b}	± >0.01
Unclassified Bacteria	1.8^{a}	± 0.3	2.1^{a}	± 0.4	0.8^{b}	± 0.2	0.9^{b}	± 0.1

Values are presented as mean relative abundance ± standard error (SE) of phylogenetic groups at the phylum and class levels. Statistical significances between the treatments inferred by ANOVA with false discovery rate post-hoc multiple correction test (FDR, $p < 0.05$) are marked by lowercase letters. Nd stands for 'not detected'

between the treatments can be excluded (Figs. 4 and 5, Fig. S3). RU47 was significantly more abundant in rhizosphere of treatments with RU47 cells than in all other treatments, especially in comparison with HT-RU47 (Fig. 5). This finding confirms the high rhizosphere competence of RU47 as already reported by Adesina et al. (2009) and Schreiter et al. (2014b). Both studies investigated the ability of RU47 to colonize the

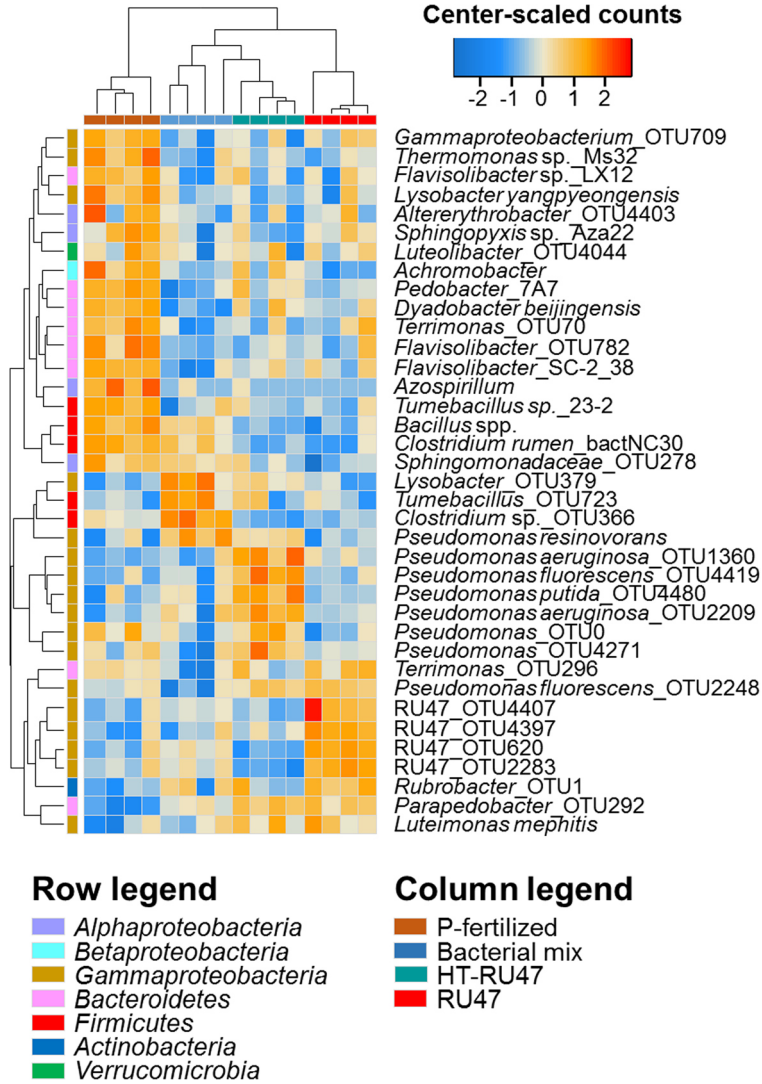

Fig. 5 Generalized heatmap of dominant responders in the rhizosphere of one optimally P-fertilized, non-inoculation (P-fertilized) and three inoculation treatments using unselectively cultivated soil bacteria (bacterial mix), heat treated RU47 (HT-RU47), or viable RU47 (RU47) cells of tomato plants 39 days after sowing. Only OTUs with relative abundance >0.1% and significantly responding are displayed (nbGLM, likelihood ratio test, $p < 0.05$)

rhizosphere of lettuce either in growth chambers or under field conditions. However, in these studies, cultivation-dependent methods (i.e. selective plating/re-cultivating the inoculum) were used. The RU47 is a spontaneous rifampicin-resistant mutant; this makes sensitive and specific detection of RU47 in rhizosphere and bulk soil possible using selective plating. Unfortunately, selective plating was not used in the present experiment. Commercial as well as non-commercial PMB strains, including RU47, were originally isolated from indigenous microbial communities associated with soils and plants. In contrast to genetically modified strains, specific and sensitive monitoring of naturally occurring strains is more difficult. The literature, though sparse, suggests that survival of inoculants such as PMB is difficult to track and that the inoculants exhibit great temporal and spatial dependency. A temporal decrease in abundance has frequently been reported (Kim et al. 1997; Dey et al. 2004; Hameeda et al. 2008; Meyer et al. 2017). For instance, Meyer et al. (2017) documented a loss of more than 99% of the inoculated *Pseudomonas protegens* CHA0 cells within 40 days. Kim et al. (1997) determined that *Enterobacter agglomerans* found in the rhizosphere of non-inoculated tomato plants (35 days after sowing) corresponded to almost 50% of the abundance in the inoculation treatment.

Improved P supply

Data on effects of PMB addition on plant growth and P uptake are rare and somewhat inconsistent. However,

Egamberdiyeva (2007) and Kumar et al. (2013) demonstrated improved P uptake in maize and mustard respectively due to addition of single PMB strains. Although not significant, these findings are consistent with the results of our study, which showed higher P accumulation in tomato plants inoculated with HT-RU47 and viable RU47 (Fig. 1). In evaluating plant P uptake, P tissue concentration is the meaningful value because differences resulting from variations in plant growth are excluded. Variations in plant growth may therefore explain the adequate P tissue concentration of 0.4% (Table S2) that was observed not only in the optimally P-fertilized treatment but also in the plants inoculated with a bacterial mix. In the bacterial mix treatment, the lowest amounts of available P in the soils (from small starter P fertilization at the beginning of the experiment) were taken up by the plants in comparison to the other treatments, and this P was not enough to maintain growth (Fig. 1). The previously incorporated P was concentrated in the small biomass, resulting in apparent adequate initial P tissue concentration of 0.4%, but this was a concentration effect relative to low tissue biomass. As plants grew, the initially adsorbed P was no longer available, and these plants then exhibited P deprivation, as indicated by violet discoloration of leaves (Table 2, Table S2). Plants inoculated with RU47 had a P tissue concentration of 0.2% (Table S2), which is in close agreement with the data reported by Kim et al. (1997) but may also indicate competition for available P between added bacteria and plant. This assumption is supported by the optimal P tissue concentration of 0.6% found in plants which were inoculated with HT-RU47 cells. In these plants competition was reduced, while highest P_{mic} values were determined in soil of the viable RU47 treatment, a condition in which competition between plants and bacteria is expected to be highest. An improved P supply by the addition of RU47 or HT-RU47 was also observed by a 2-fold higher P_{H2O} concentration compared to the bacterial mix treatment (Table 2). However, a fertilizing effect due to addition of HT-RU47 cells can be excluded since N and P concentrations in cell suspension ($OD_{600} = 1$) were determined as 41.5 and 0.9 μg mL^{-1}, respectively, corresponding to a total N and P addition of less than 0.6 mg kg^{-1} (data not shown). These values are negligible in comparison to the initial slight P fertilization (50 mg kg^{-1}) and the optimal fertilized control (200 mg kg^{-1}).

Improved P mobilization in soil

An improved P supply by PMB, including some *Pseudomonas* strains, has been reported in several studies (for overviews, see Rodríguez and Fraga 1999; Khan et al. 2007; Harvey et al. 2009). For instance, Malboobi et al. (2009) documented effective mobilization of inorganic and organic phosphate compounds by *Pseudomonas putida* P13 in culture media. Similar findings were reported by Pastor et al. (2012). They observed growth stimulation of tomato seedlings by the addition of *P. putida* PCI2 and were able to identify this strain as positive for PA and highly effective for solubilizing Al- and Ca-bound phosphates. In our study, inoculations with RU47 resulted in increased alkaline phosphomonoesterase activity in the rhizosphere of tomato plants (Fig. 2a). Moreover, enzyme activity measured by zymography increased from 18 to 19 to 25–26 days after sowing, likely due to increasing bacterial colonization and P depletion. In contrast, alkaline PA in the rhizosphere inoculated with HT-RU47 remained stable (Fig. 2a). These findings suggest increased P mineralization by microbial phosphatases produced by viable RU47. In general, zymography revealed similar activity levels for alkaline and acid PA, which is in accordance with Spohn et al. (2015). They determined the PA in the rhizosphere of barley grown under low and adequate P soil conditions and observed a similarity of approximately 90% between alkaline and acid PA. In contrast to the experiment of Spohn et al. (2015), we observed no distinct separation between roots and surrounding soil (Fig. S2) and also generally lower PA (Fig. 2a, b). This may be attributable to the comparatively fine roots of tomato plants as compared to barley. Lower enzyme activity levels in comparison to the values of Spohn et al. (2015) may have been due to the addition of quartz sand in the present experiment.

PA data determined by soil in situ zymography indicated the spatial and temporal distribution of enzyme activity in the rhizosphere (soil area) during different growth stages of the tomato plants. Enzyme analyses performed after final harvest enabled us to gain additional information about the potential PA in bulk soil (soil body) at a single time point. Measured highest alkaline and acid PA values in soil inoculated with RU47 after final harvest agreed with our soil zymography results (Table 2, Fig. 2a, b). In comparison to the bacterial mix treatment, alkaline PA increased by 16% (Table 2). These results are in agreement with those

obtained by Kaur and Reddy (2014), who documented increases in alkaline PA of 31% due to the addition of *Pseudomonas plecoglossicida* in soil of wheat plants. These findings reinforce the evidence for improved P mineralization by the addition of RU47. Nevertheless, bulk soil inoculated with HT-RU47 cells also revealed increased PA (Table 2), despite the effectively killing of RU47 by HT and the denaturation of phosphatases. By taking into account that increased PA in HT-RU47 treatment was observed only once and perhaps temporary, this observation allows us to speculate that the addition of bacterial residues (HT-RU47 cells) and thus a supply of fresh organic matter (FOM) may enhanced growth and activity of previously of bacterial populations, especially *Pseudomonads* (Fig. 5), also known as the priming effect (Bingeman et al. 1953; Fontaine et al. 2003).

Interactions with indigenous soil microbes and hormone-derived effects

An initially conducted denaturing gradient gel electrophoresis (DGGE) analysis of 16S rRNA gene amplified from TC-DNA revealed no RU47-related band in the HT-RU47 treatment, but dominant bands which were absent or less intense in all other treatments (Fig. S4). Furthermore, high d-values (permutation test) indicating large differences were observed between the fingerprints of the HT-RU47 and viable RU47 (43.2), and between the bacterial mix and HT-RU47 (28.4; Table S3). This first indication of changed bacterial community composition, especially when HT-RU47 cells were added, was supported by the amplicon sequencing showing less present RU47-specific OTUs in HT-RU47 treatment, as well as the presence of 8 OTUs affiliated to other different *Pseudomonas* (Fig. 5). We assume, therefore, that inoculation of HT-RU47 cells, and thus the addition of FOM in the form of bacterial residues to soil, resulted in a priming effect that enhanced bacterial populations, especially belonging to *Gammaproteobacteria* (Fig. 5). This, in turn, resulted in the growth of bacterial populations responding to the C spike accompanied by increased microbial PA in bulk soil, resulting in the observed improved P supply for the tomato plants in this treatment (Table 2). This assumption is confirmed by data of RDA that revealed a highly positive correlation between HT-RU47 and the variables plant's P uptake and PA (Fig. 4). However, despite finding no strong evidence of enhancement in microbial C degradation via RDA, phylogenetic variations within the genus *Pseudomonas* dominating bacterial diversity in HT-RU47 might have disguised a positive correlation between HT-RU47 and enzymes involved in the C cycle (Fig. 4, Fig. S3). On the other hand, RDA revealed positive correlations between the P-fertilized treatment and the microbial biomass C:P ratio as well as activity of carbohydrate degrading enzymes (Fig. 4). Phylogenetic diversity of this treatment was mainly characterised by, inter alia, *Bacteroidetes*, *Alpha-* and *Betaproteobacteria*, which are identified to follow the copiotrophic nutritional strategy (Fierer et al. 2007; Fig. 5). Activities of carbohydrate degrading enzymes might explain the negative correlation between this treatment and soil's pH, as higher C degradation activity may increase soil acidity via organic acid release (e.g. acetate; Fig. 4). The absence of positive correlation between P-fertilized and the PA is well explainable by soil's initially high P fertilization (Fig. 4). Although we found no significant effects on PLFA patterns, bulk soil inoculated with RU47 cells showed a minor increase in bacterial abundance, especially of gram$^+$ bacteria (Table 2) likely indicating microbiome shift as a result of RU47 inoculation, a finding also reported by Schreiter et al. (2014a). Amplicon sequencing revealed strikingly different taxonomic affiliation of the dominant genera and showed that in the HT-RU47 treatments OTUs affiliated to *Pseudomonas* were dominant which were not detected in the other treatments and were clearly distinct from RU47 (Fig. 5). This *Pseudomonas* population might have contributed to the increased microbial PA and improved P supply determined in bulk soils of both RU47 treatments. An increase in abundance of indigenous PMB after the application of specific PMB strains has also been reported by Sundara et al. (2002) and Canbolat et al. (2006). Supporting this assumption, heatmap showed OTUs that seemed to have been facilitated by RU47 regardless of its viability, including *Parapedobacter* sp., *Luteimonas mephitis*, and *Pseudomonas fluorescens*, the latter, at least, include several well studied plant growth-promoting bacteria strains (e.g. McGrath et al. 1995; Park et al. 2015). To solve this open question, functional gene analyses of phosphomonoesterases could clarify identities of the main producers of different phosphomonoesterases and should be considered in future studies. Plant growth

promotion can be strongly influenced by modulation of the phytohormone level of the plant. Several studies have shown that many soil bacteria, including *Pseudomonas*, are able to synthesize phytohormones or the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, affecting the plant's hormonal balance and thus its growth and response to stress, ensuring the bacteria's supply of C resources from root exudation (for review see Tsavkelova et al. 2006; Glick 2012). Hence, the plant growth promotion observed in both RU47 treatments was likely due to a hormone-mediated effect. Rajkumar and Freitas (2008) reported a strong effect of ACC deaminase production by *P. jessenii* M6; this was also detected for RU47 (Eltlbany et al., under review). But, due to enzyme inactivation in the HT-RU47 treatment, an ACC deaminase effect was negligible here. However, it is known that phytohormones such as auxins and cytokinins remain stable after heating to 121 °C (Murashige and Skoog 1962; Kumar 2009). Although, it is entirely possible that co-extracted thermally stable phytohormones produced by RU47 before they were killed were added through inoculation of HT-RU47 cells it is more likely that the addition of HT-RU47 cells promoted indigenous, phytohormones synthesizing soil microorganisms, including especially bacterial genus belonging to *Pseudomonas* as indicated by the amplicon sequencing analysis (Fig. 5; for review see Tsavkelova et al. 2006; Glick 2012). Taken together, the improved P supply in plants inoculated with HT-RU47 or viable RU47 may have been due to phytohormones, stimulating root growth and activity, and improving P acquisition in soil.

Conclusion

This study demonstrated that addition of RU47 improves the P supply and subsequent growth of tomato plants under P-limited growing conditions. Furthermore, it indicated enhanced production of alkaline phosphatase in the RU47 treatments. This is the first study to compare the effects of adding viable and dead RU47 cells to plants and soil. In both treatments higher P uptake and plant growth promotion were observed. The plant growth-promoting effect was likely caused by increased PA in the rhizosphere of tomato amended with viable RU47. In the HT-RU47 treatment, the bacterial populations which proliferated in response to the added resource may have contributed to improved P supply and growth promotion via other mechanisms. Thus, the use of RU47 offers a promising approach for more efficient P fertilization in agriculture. In contrast to our hypothesis that the colonization of RU47 leads to spatially distinct zones of increased PA in the rhizosphere, no clear differences in rhizosphere and bulk soil were found. This was likely due to the fine roots of tomato plants and homogeneously distributed enzyme activity of the topsoil used in the treatments. We found no significant effects of RU47 on soil microbial community structure as determined by PLFAs, but we detected significant shifts in bacterial composition of the rhizosphere using 16S amplicon sequencing. Our study shows that RU47 increases microbial PA in soil with low P availability and leads to growth promotion of tomato plants.

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Publications and Presentations

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Reinhardt D, Jansen G, Seddig S, Eichler-Löbermann B (2013) Temperature stress during flowering time affects yield and quality parameters of waxy barley. Applied Agricultural and Forestry Research 63: 79-84

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Presentations

Poster presentations

Reinhardt D, Spohn M, Kandeler E (2014) Bio-effectors might stimulate P mobilization in the rhizosphere of tomato and maize. Annual meeting of the BIOFECTOR project, Naples, Italy

Reinhardt D, Spohn M, Marhan S, Eltlbany N, Smalla K, Kandeler E (2015) No matter whether dead or alive? An investigation of bio-effector application in tomato and maize experiments under phosphorus limited soil conditions. Rhizosphere 4 congress, Maastricht, the Netherlands

Reinhardt D, Spohn M, Marhan S, Eltlbany N, Smalla K, Kandeler E (2015) No matter whether dead or alive? – Application of *Pseudomonas jessenii* RU47 in tomato and maize experiments under phosphorus limited soil conditions. Annual meeting of the BIOFECTOR project, Budapest, Hungary

Reinhardt D, Spohn M, Marhan S, Eltlbany N, Smalla K, Kandeler E (2015) No matter whether dead or alive? An investigation of bio-effector application in tomato and maize experiments under phosphorus limited soil conditions. Ecology of soil Microorganisms, Prague, Czech Republic

Nassal D, Marhan S, Kandeler E (2016) Pseudomonads also like it well-fertilized? A rhizobox experiment proving the plant growth-promoting effect of *Pseudomonas jessenii* RU47 under varying P supplied soil conditions. Annual meeting of the BIOFECTOR project, Prague, Czech Republic

Nassal D, Spohn M, Marhan S, Eltlbany N, Smalla K, Kandeler E (2016) Short-term effects of *Pseudomonas jessenii* RU47 on C and P cycle in soil of maize plants. Annual meeting of the BIOFECTOR project, Prague, Czech Republic

Oral presentations

Reinhardt D, Spohn M, Kandeler E (2014) Do bio-effectors affect temporal and spatial enzyme activities? A rhizobox approach using soil *in situ* zymography. Wissenschaftliches Kolloquium, Julius Kühn-Institut, Braunschweig, Germany

Reinhardt D, Spohn M, Marhan S, Eltlbany N, Smalla K, Kandeler E (2015) Egal ob tot oder lebendig? Bio-Effektoren im Pflanzenversuch unter phosphorlimitierten Bodenbedingungen. Jahrestagung der Deutschen Bodenkundlichen Gesellschaft, Munich, Germany

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