PGPR-INDUCED GROWTH STIMULATION AND NUTRIENT ACQUISITION IN MAIZE: DO ROOT HAIRS MATTER?*

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Here we describe the effects of the well-characterized, commercial plant growth-promoting rhizobacteria (PGPR) strain *Pseudomonas* sp. DSMZ 13134 (Proradix®) on plant growth, root morphology, and nutrient acquisition of a maize mutant (rth2) with impaired root hair production as compared with the corresponding wild type line, to study the importance of root hairs for the interaction of the PGPR strain with the host plant. The study was conducted in rhizobox culture with a sand–soil mixture and moderate P supply. Root hair development of the mutant was clearly impaired, reflected by slower growth and limited elongation as compared with the wild type line. This defect was compensated by more intense root growth and fine root production of the mutant which was particularly expressed after inoculation with Proradix®. By contrast, PGPR inoculation had no effect on root hair length. The beneficial effects of Proradix® on root growth were reflected in higher shoot contents of the macronutrients P and K. Interestingly, negative effects on shoot accumulation of the micronutrients Zn and Cu were observed. These findings support proposed PGPR effects of this strain but also show limitations that may be explained by additional strain-specific properties. Possible implications of these findings are discussed.

Siderophores, *P. fluorescens*, rhizosphere, pyoverdine, root growth stimulation, mineral nutrients

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INTRODUCTION

Plant growth-promoting rhizobacteria (PGPRs) (Kloepper et al., 1980) are able to improve plant performance and nutrient acquisition by natural means to reduce the input of agrochemicals (Lucy et al., 2004; Richardson, Simpson, 2011). Formulated products are sold by various companies to act as bio-control agents against pathogens (Hol et al., 2013), as bio-fertilizers, increasing acquisition of sparingly available nutrients (Richardson et al., 2009), or as bio-stimulants, e.g. directly stimulating root growth (Lugtenberg, Kamilova, 2009). Nevertheless the mechanisms behind these proposed activities are complex and efficiency often depends on abiotic factors, such as soil pH, type of minerals, and climatic conditions as well as biotic factors, like bacterial rhizosphere competence or pathogen pressure (Benizri et al., 2001; Ortiz-Castro et al., 2009; Dutta, Podile, 2010) The genotype of the host plant determines root morphology, root exudation, and the mechanism for nutrient acquisition, factors that are crucial for compatibility with PGPR strains (Bais et al., 2006; Yang, 2016). Root hairs are important sites for water and nutrient uptake e.g. by largely increasing root surface but their contribution to root exudation and root colonization by rhizobacteria is not well understood (Neumann, Roemheld, 2002). Nevertheless, investigations on endophytic root colonizing *Pseudomonas* strains indicate a pivotal role of root hairs for root colonization (Prieto et al., 2011). In this context, mutants or genotypes affected in root hair production provide a tool to study the impact of root hairs on rhizosphere processes (Gahoonia

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et al., 1997). Wen, Schnable (1994) identified three root hairless (rth) maize mutants during a screen of mutants derived from a transposon stock. The rth2 mutant showed root hair length about $^1/_4$ to $^1/_5$ of the size of wild type maize plants but a still vigorous and healthy growth when grown in hydroponic systems with sufficient nutrient supply.

By comparing the rth2 mutant and the corresponding wild type, we wanted to investigate the significance of root hair development for the establishment and efficiency of host plant interactions with the commercially available PGPR strain *Pseudomonas* sp. (Proradix®), known for its bio-control potential (von Rad et al., 2005; Buddrus-Schiemann, 2008) and well-documented properties in plant growth promotion in different plant species, such as barley, tomato, and hybrid maize (Yusran et al., 2009; Frohlich et al., 2011; Nkebiwe et al., 2016; Thonar et al., 2017).

MATERIAL AND METHODS

Selection of plant material and bacterial strain

For plant growth experiments the roothairless 2 (rth2) mutant of the maize inbred line B73 was used. Rth2 seeds were provided by the working group of F. Hochholdinger (INRES, Bonn, Germany).

The commercial PGPR product Proradix® (SP Sourcon Padena GmbH, Tübingen, Germany) containing the Gram-negative bacterium *Pseudomonas* sp. DSMZ 13134 formulated as a freeze-dried product was used.

Seed sterilisation and pre-germination

Seeds were sterilized by soaking in 10% H_2O_2 for 2 min, washed, and then incubated overnight in aerated 10 mM $CaSO_4$ solution. For inoculation, one group of seeds was soaked in Proradix® suspension (10^9 cfu ml^{-1} suspension) for 1 min, and then pre-germinated separately wrapped in filter paper soaked with 10 mM $CaSO_4$ and incubated at 24°C in the dark. Three days after sowing (DAS) root hair morphology of the seedlings was observed. Because heterozygous rth2 mutants were used, seedlings with long root hairs were defined as control plants (wild type; W) in the following rhizobox experiment, seedlings with short root hairs were taken as rth2 mutants (M).

Growth conditions in rhizobox experiment

A silty-loam organic-farming soil (Experimental Station Kleinhohenheim, Stuttgart, Germany) was used for the experiment (pH = 6.9; available plant nutrients (mg kg^{-1} soil): CAL-P 60/Olsen-P 46; CAL-K 120; Cu (CAT) 2.5, Zn (CAT) 2.9, Mn (CAT) 130; 0.231% N; 2.92% humus; 1.86% carbonate; fertilization (mg kg^{-1} soil): N 130 as $Ca(NO_3)_2$; Mg 65 as $MgSO_4$; K 200 as K_2SO_4, and P 65 as $Ca(H_2PO_4)_2$. After fertilization the soil was mixed 2 : 1 with quartz sand. Rhizoboxes (35 × 10 × 2 cm) equipped with root observation windows were pre-filled with each 643 g soil–sand substrate. Three DAS one seedling was transferred into each rhizobox (Fig. 1) which was closed with a transparent Perspex lid. Two additional Proradix® inoculations were performed 7 and 14 DAS during watering of the rhizoboxes with a concentration of 6×10^9 cfu kg^{-1} soil. In total 20 plants from four treatments (u/W; u/M; Pr/W; Pr/M; Pr = Proradix®; u = untreated) with five replicates each were grown in a completely randomized design, in a growth chamber for 28 days (June 17th–July 15th 2015) with a 12 h light period (200 µmol m^{-2} s^{-1}) and a 28/23°C day/night temperature regime. A soil moisture level of 25% (w/w) was regularly adjusted gravimetrically with distilled water.

Root morphology

At 7, 14, and 21 DAS root length was determined by drawing the roots visible along the root observation window on transparent plastic foil and subsequent digitalization (Epson Expression 10000Xl, Epson,

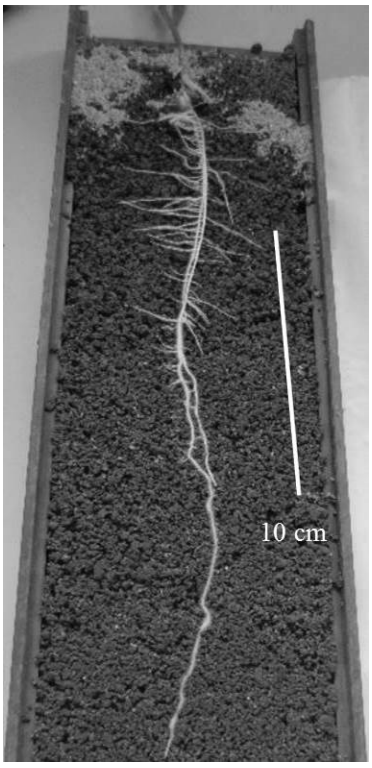

Fig. 1 Rhizobox with opened root observation window one week after sowing

USA) at a resolution of 400 dpi. At the same time pictures from the root hair zone were taken with a Stemi2000-C video macroscope equipped with the Axio Vision 3.1 software (Zeiss, Oberkochen, Germany) at a magnification of 12.5. From these pictures root hair length was determined by taking the average length of 10 root hairs per plant. After harvest the whole root was washed and scanned. All root scans from washed roots and drawings were analysed with the WinRhizo software (Regents Instruments Inc., Quebec, Canada).

Mineral analysis

For mineral nutrient analysis oven-dried (60°C) shoot material was ground to fine powder (Scheibenschwingmühle TS-100A; Sieb Technik GmbH, Mühlheim-Ruhr, Germany). For digestion of plant material, 0.2 g of shoot dry matter were incubated with 2.5 ml HNO_3, 2 ml H_2O_2, and 1 ml distilled water in a microwave (MLS Maxi 44; MLS GmbH, Leutkirch, Germany) at a maximum of 210°C and 1400 W for 65 min. Thereafter the solution was adjusted to 20 ml, destained with activated charcoal, and then filtered (paper filters 90 µm mesh size; MN 640 d, Macherey-Nagel, Düren, Germany). Mg, Mn, Zn, and Cu concentrations in the sample solutions were determined by atomic absorption spectroscopy (AAS) (ATI Unicam Solaar 939; Thermo Electron, Waltham, USA). Before measuring Mg concentrations, a buffer solution containing caesium chloride and lanthanum chloride (e.g. Merck, No. 116755) was added to the samples at a ratio 1 : 50 (1 part Cs/La-buffer + 49 parts of the sample solution) to eliminate interferences during the AAS analysis (Schinkel, 1984). Spectrophotometrical determination of orthophosphate was performed after addition of molybdate-vanadate reagent according to the method of Gericke, Kurmies (1952). Determination of K and Ca was conducted by flame photometry (ELEX 6361; Eppendorf, Hamburg, Germany).

Statistical analysis

For data analysis MS Excel 2010 (Microsoft Corporation) and SigmaPlot 11.0 (Systat Software Inc.) were used. One- and two-way ANOVA and subsequent Tukey's tests ($\alpha = 0.05$) were performed for pairwise comparison between treatments. All data were normally distributed and no outlier analysis was performed. In graphs means and standard deviation (SD) are given. Different letters indicate a statistically significant difference in means.

RESULTS

Root development

Although root length in drawings along the root window did not differ significantly among treatments, root length of Pr-treated plants 7, 14, and 21 DAS showed a trend for increased root length compared with the untreated control and root length of maize mutants was higher than that of the wild type plants (Fig. 2). The sequence of treatments according to root length changed between 14 and 21 DAS from Pr/M > Pr/W > u/M > u/W to Pr/M > u/M > Pr/W > u/W indicating that the influence of Pr was more expressed at the beginning of the culture period, while the mutant effect had more influence during later plant development. For whole root scanning after harvest, significant differences (one-way ANOVA) were found between Pr/M and u/W. When performing the two-way ANOVA, significant differences were recorded between Pr-treated plants and untreated plants, as well as between mutant and wild type plants. Additionally, Pr/M and Pr/W differed significantly, indicating an increased responsiveness of the mutant to the Pr treatment. A closer examination of the different root size classes revealed that the Pr treatment significantly increased only the fine root fraction (0–0.2 mm diameter), whereas the genotypic difference between mutant and wild type significantly influenced the classes 0–0.6 mm (Fig. 2). The classes 0.6–0.8 and > 0.8 did not significantly differ in any comparison.

Root hairs

Impairment of root hair development in rth2 mutants was recorded during the entire culture period. Root

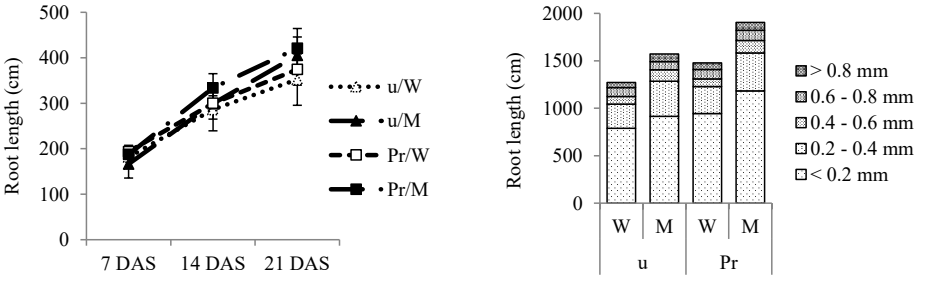

Fig. 2 Left: Root length from root window observations 7, 14, 21 DAS (cm); Right: Root length of different root diameter classes (cm); Wild type (W) or mutant (M) plants treated with Proradix® (Pr) or left untreated (u); Means + SD

hairs of mutants and wild type plants did not differ in their density but root hair length of the mutants reached only 20–25% as compared to the wild type plants three weeks after sowing (Fig. 3). Neither root hair length nor root hair density were influenced by the Pr treatment.

Plant habitus and biomass

Shoot dry biomass did not differ significantly among treatments but Pr treatments showed a trend for higher shoot biomass than the untreated controls (Fig. 4). Root dry weight and root to shoot ratio (R/S) of mutants were significantly higher than those of the wild type plants and reflected the results of root length analysis.

Macro- and micronutrients in shoots

The K and Ca status of shoots was in the sufficiency range. Mg, Cu, and Zn concentrations reached the deficiency thresholds (B e r g m a n n , 1993) and P and Mn deficiencies were recorded in all treatments. Mg shoot concentrations were significantly increased in the maize mutants (Fig. 5). Cu and Zn concentrations were decreased by Pr treatment (Fig. 6). By contrast, P and K contents were significantly increased in the Pr treated plants as compared to the untreated control when performing the two-way ANOVA. Mg contents were decreased in wild type plants. In contrast to K and P, Cu contents were significantly decreased by the Pr treatment. Ca, Zn, and Mn contents did not differ significantly among treatments.

DISCUSSION

Root hairs and their influence on nutrient acquisition

Root hairs are known to be important sites for water and nutrient uptake into the plant root (G i l r o y , J o n e s , 2000; M a r s c h n e r , R e n g e l , 2012),

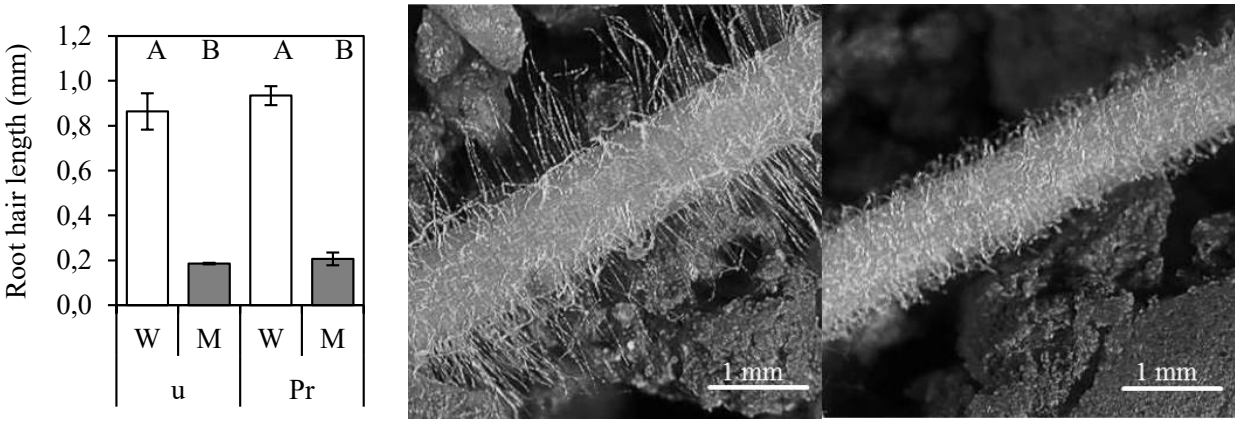

Fig. 3 Root hair length (RHL) of wild type (W) or mutant (M) plants treated with Proradix® (Pr) or left untreated (u) in mm 21 DAS; Means + SD; Different letters indicate significant difference in means (tukey's test, p<0.05); Picture show wild type (left) and rth2 mutant (right) roots 21 DAS

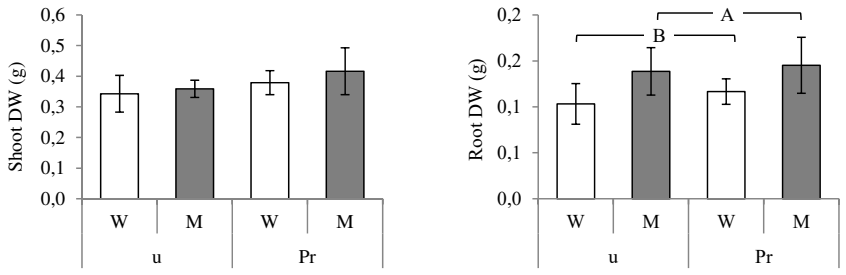

Fig. 4 Shoot (left) and root (right) dry weight (DW) in g at 28 DAS of wild type (W) or mutant (M) plants treated with Proradix® (Pr) or left untreated (u); Means + SD; Different letters indicate significant difference in means (tukey's test, p<0.05)

mainly due to an increased surface area and smaller radius and therefore higher absorption capacity (F o e h s e et al., 1991). Interestingly, in our experiments none of the investigated micro- and macronutrients was significantly reduced in mutant plants as compared to wild type plants. In contrast, in some cases total contents were even increased, especially for Mg. One probable explanation is the increased root growth, reflected by a significantly increased dry root weight and total root length found in this experiment in mutant plants, facilitating spatial acquisition of mineral nutrients. In various studies (F o e h s e et al., 1991; G a h o o n i a , N i e l s e n , 1997; G a h o o n i a et al., 1997), a strong correlation of root hair length and P uptake was found when different cultivars of cereals were compared but maize cultivars were not tested. Obviously, the mutant plants compensated for the impaired root hair formation by increased formation of fine roots as recently reported also for root hair mutants of barley (D o d d , D i a t l o f f , 2016).

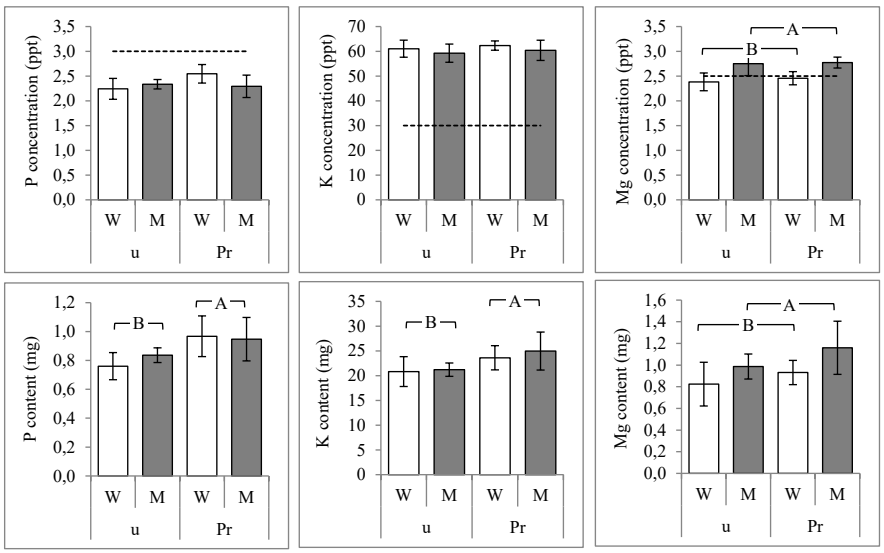

Fig. 5 Macronutrients in shoots of wild type (W) or mutant (M) plants treated with Proradix® (Pr) or left untreated (u); Upper row from left to right: P, K and Mg concentration (ppt); below from left to right: P, K and Mg content in mg shoot^{-1}. Dashed lines indicate lower threshold level after B e r g m a n n (1993); Means + SD; Different letters indicate significant difference in means (tukey's test, $p<0.05$)

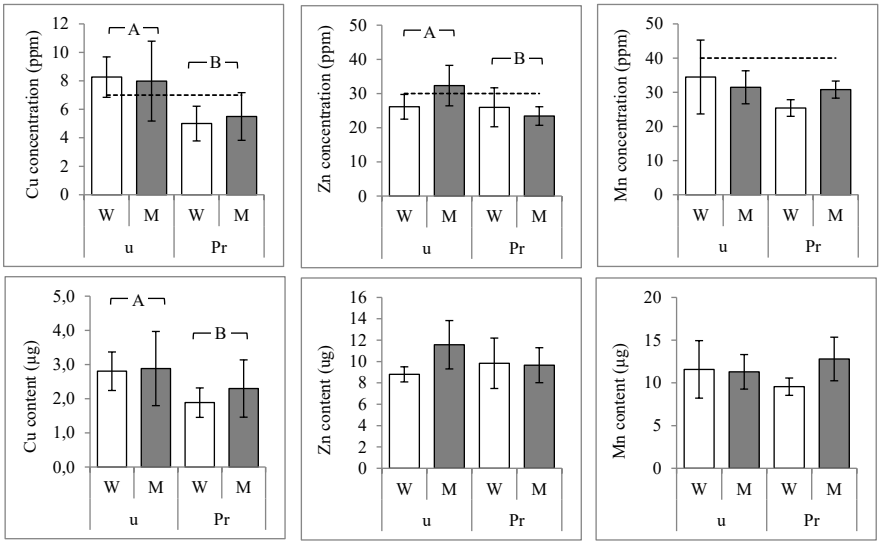

Fig. 6 Micronutrients in shoots of wild type (W) or mutant (M) plants treated with Proradix® (Pr) or left untreated (u); Upper row from left to right: Cu, Zn and Mn concentrations (ppm); below from left to right: Cu, Zn, and Mn contents (µg per shoot); dashed lines indicate lower threshold level after Bergmann (1993); Values are means + SD, A,B significant difference in means (tukey's test, $P < 0.05$)

Possible mode of action for growth stimulation by Proradix®

Pseudomonas fluorescens PGPR strains are proposed to act as bio-fertilizers that improve plant nutrient acquisition by stimulation of root growth and solubilization of minerals via chelators (such as pyoverdine siderophores), reductants, enzymes, and protons released into the surrounding environment (Richardson et al., 2009). In our experiment, Pr was able to promote particularly fine root development and, to a lesser extent, shoot growth. Total shoot contents of the macronutrients P and K were increased by Pr inoculation, while P shoot concentration was not changed, indicating that increased P uptake was immediately diluted by transformation into biomass production. P and K are nutrients known to be mainly transported to the root by diffusion (Marschner, Rengel, 2012). Therefore, an increased root length and formation of fine roots strongly increases the probability of their acquisition by plants. Promotion of root growth is one of the mechanisms proposed for PGPRs, often explained by their ability to produce auxins (Oberhansli et al., 1991; Buddrus-Schiemann, 2008) or reduce ethylene levels by production of the ACC (1-aminocyclopropane-1-carboxylic acid) deaminase, an enzyme that degrades the ethylene precursor ACC (Glick, 2014). However, in mutant plants, only Mg contents but not those of P and K were significantly increased. A possible explanation would be that the compensation effect by increased root elongation was only sufficient for increased uptake of the more easily available Mg, but not for sparingly soluble nutrients such as P and K. Alternative explanations for the increased P and K content in Pr treated plants would be a decrease of rhizosphere pH due to a release of protons or organic acids which are known to improve P availability by complexation of sesquioxides and may also influence K availability by cation exchange on clay minerals (Marschner, Rengel, 2012). Many publications report an increased solubility of Ca-phosphates by PGPRs when grown on artificial growth media (Rodriguez, Fraga, 1999; Froehlich et al., 2011; Richardson, Simpson, 2011; Fernandez et al., 2012). Additionally, some PGPRs, including the Pr strain (Yusran et al., 2009), are known as mycorrhiza helper bacteria that might improve mycorrhization of roots and therefore, uptake of P and K via mycorrhizal symbiosis (Barea et al., 2005; Frey-Klett et al., 2007).

Proradix® growth promotion and the influence of root hairs

Proradix® was preferentially colonizing root hairs when applied as seed treatment in barley (Buddrus-Schiemann et al., 2010). Therefore, the investigation of the root hair mutant offered the opportunity to study also the importance of root hair growth for the efficiency of Pr-mediated plant growth stimulation.

In our experiment the Pr treatments induced PGPR effects for mutant and wild type plants independent of their root hair development. Root growth promotion of Pr was even more pronounced in the mutant plants, reflected by significant differences between Pr/W and Pr/M, possibly due to a higher responsiveness of mutant plants to hormonal stimulation.

In a recent publication by Senga et al. (2017), they investigated root-hairless mutants from barley and found plant genotype specific changes in the soil microbial communities. Communities from root hairless mutants seemed to be less diverse and specific bacterial orders were found to discriminate between WT and mutant plants. Nevertheless, root hair growth had much less influence on microbial communities than soil type or soil compartment (rhizosphere vs bulk soil). Also the study by Pausch et al. (2016) showed that root exudation pattern and 'priming' of bacterial growth was not inhibited in root hairless barley mutants.

Limitations of PGPR application

Although our experiment could prove the efficiency of Pr for root growth stimulation and improved acquisition of macronutrients, the acquisition of the micronutrients Zn and Cu was reduced. Proradix® belongs to the *P. fluorescens* group, which is known to release the siderophores pyochelin, pseudobactin (Becker et al., 1985), and pyoverdine (Meyer, Abdallah, 1978). These siderophores are able to form chelate complexes with iron Zn and Cu (Haas, Defago, 2005; Brandel et al., 2012). Nevertheless, unlike phytosiderophores, released by *Poaceae* for Fe uptake, metal complexes with microbial siderophores seem to be a poor metal source for plants (Walter et al., 1994). Walter et al. (1994) also showed that a pyoverdine-producing *P. putida* strain could not improve P uptake in maize plants and that the application of a mixture of soil microbes even led to severe iron deficiency in maize plants grown in hydroponic systems possibly due to biodegradation of maize phytosiderophores. Becker et al. (1985) also reported a reduced iron uptake in maize plants and concluded that in well aerated soils, fluorescent pseudomonads could interfere with plant growth and functions by exacerbating iron starvation. Available Zn and Cu concentrations in our soil were comparatively low. However, Pr inoculation was performed with a relatively high cell density since previous reports (Buddrus-Schiemann et al., 2010) indicated that a certain inoculum density is necessary to establish the PGPR interaction in non-sterile substrates. Therefore, it is possible that PGPR application can exert negative effects on micronutrient

supply to plants due to competitive interactions in a dosage-dependent manner. Negative effects on the micronutrient status in lettuce plants have recently been observed also for the double application of *Serratia plymuthica* and *Pseudomonas jessenii*, whereas single applications increased the Zn and Mn status of plants (Windisch et al., 2017).

CONCLUSION

The present study aimed to investigate the importance of root hair development for plant growth promotion by PGPRs. The results showed that a maize mutants compensated impaired root hair development by an increased root length and root biomass production, without a reduction in shoot biomass and did not show reduced nutrient uptake under our experimental conditions. PGPR (Proradix®) inoculation improved fine root growth, especially in mutant plants, and increased phosphate and potassium contents regardless of the maize genotype. We conclude that efficiency of maize growth promotion by Pr does not necessarily depend on a normal root hair formation, provided that the lack of root hair development can be compensated by alternative fine root structures. Nevertheless, rth2 mutants do not completely lack root hair formation. Moreover it is also not clear in which way and how seriously exudation rates in the root hair zone might be influenced by the root hair impairment or if root colonization by Pr was affected. One last interesting finding was that Pr treatment reduced the contents of the micronutrients Zn and Cu. We hypothesized that the bacteria might compete with the maize plants for these micronutrients as previously reported also for other plant–microbial interactions.

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