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Potential of three microbial bio-effectors to promote maize growth and nutrient acquisition from alternative phosphorous fertilizers in contrasting soils

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Abstract

Background: Agricultural production is challenged by the limitation of non-renewable resources. Alternative fertilizers are promoted but they often have a lower availability of key macronutrients, especially phosphorus (P). Biological inoculants, the so-called bio-effectors (BEs), may be combined with these fertilizers to improve the nutrient use efficiency.

Methods: The goal of this study was to assess the potential of three BEs in combination with alternative fertilizers (e.g., composted manure, biogas digestate, green compost) to promote plant growth and nutrient uptake in soils typical for various European regions. Pot experiments were conducted in Czech Republic, Denmark, Germany, Italy, and Switzerland where the same variety of maize was grown in local soils deficient in P in combination with alternative fertilizers and the same set of BEs (*Trichoderma*, *Pseudomonas,* and *Bacillus* strains). Common guidelines for pot experiment implementation and performance were developed to allow data comparison, and soils were analyzed by the same laboratory.

Results: Efficiency of BEs to improve maize growth and nutrient uptake differed strongly according to soil properties and fertilizer combined. Promising results were mostly obtained with BEs in combination with organic fertilizers such as composted animal manures, fresh digestate of organic wastes, and sewage sludge. In only one experiment, the nutrient use efficiency of mineral recycling fertilizers was improved by BE inoculation.

Conclusions: These BE effects are to a large extent due to improved root growth and P mobilization via accelerated mineralization.

Keywords: Biofector, Bio-effector, Bio-inoculants, *Bacillus*, *Pseudomonas*, *Trichoderma*, Maize, Organic fertilizer, PGPR, Phosphorus, Recycling fertilizer

Background

To meet global demands for agricultural crops, farming systems in industrialized countries have undergone profound transformations. On the one hand, high application rates of synthetic fertilizers and manure together with the use of pesticides, irrigation, and short crop rotations have increased yields and have helped to reduce hunger in these countries [1]. On the other hand, modern high-input agriculture has created a multitude of environmental problems. In this context, groundwater pollution and the eutrophication of aquatic systems due to soil erosion and nutrient leaching and run-off from agricultural fields are of great concern [1, 2]. Additionally, the extensive use of synthetic

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fertilizers is contributing to climate change as fertilizer production and use cause significant greenhouse gas emissions [3]. Moreover, for some plant nutrients such as phosphorous (P), natural fixation processes in soil leads to a decrease in the efficiency of the fertilizers as only a fraction of the applied P fertilizer is taken up by plants. Depending on the soil pH and mineralogy, a considerable amount of P fertilizers gets either adsorbed to iron, aluminum oxides, or calcium and is no longer directly available to the plant [4]. Consequently, surplus fertilizer inputs are often maintained to meet the crop's demands. Finally, commonly used P fertilizers in modern agriculture are regarded as non-renewable resources with a limited supply, and the reserves are concentrated in a few countries around the globe [4, 5]. Hence, judicious use of the resource by effective recycling is required for long-term sustainability.

Today, increasing attention is being paid to the downside of high-input agricultural systems and much research is aimed at developing alternative ways to produce sufficient food in a sustainable and environmentally sound way. A number of different approaches have been investigated with the intention to reduce fertilizer inputs into agroecosystems, including breeding plant varieties with better P acquisition efficiency [6], fertilizer placement strategies [7], or application of soil organisms and natural extracts with plant growth-promoting potential [8–10]. These so-called bio-effectors (BEs) include plant growth-promoting rhizobacteria (PGPR) for instance *Pseudomonas* and *Bacillus* spp., as well plant-associated fungi, such as arbuscular mycorrhizal fungi (AMF) or *Trichoderma spp.* [11]. Moreover, BEs comprise non-living agricultural bio-stimulants (ABs) such as extraction products from macroalgae, compost, and plants [12]. The plant growth-promoting effect of BEs is based on different mechanisms, for example, the control of plant pathogens either by stimulation of the plant's defense mechanisms or by microbial antagonism [11, 13]. Some BEs induce plant growth by stimulating the plant's own hormone production such as auxins or cytokinins [13, 14], or by producing directly hormone-like compounds [15]. Regarding P acquisition, some BEs are associated with increased phosphatase activities in the soil [16–18] and have also shown the ability to liberate P from soil particles [9, 16, 19, 20] and from P-recycling fertilizers [21, 22]. Another important mechanism is based on the so-called "mycorrhiza helper" effect [23] which has been observed for many bacterial BEs [24]. This term refers to a stimulation of AMF which fulfill important functions regarding P uptake by plants.

Although BEs have shown the potential to promote plant growth, their effectiveness is influenced by numerous biotic and abiotic factors. Consequently, depending on the properties of the soil and the structure of the naturally occurring rhizosphere community, their plant growth-promoting effect has often shown limited reproducibility. As reported in Mosimann [25], soil properties such as pH can have impacts on the BEs' ability to exert plant growth-promoting effects. Moreover, the amount of total and plant available P seems to be a decisive factor for the performance of bacterial BEs [26, 27]. Also, competition with naturally occurring rhizosphere microorganisms can limit the survival of introduced BEs [28]. This indicates that more work is needed to unravel the conditions under which BEs can exert plant growth-promoting effects, in order to achieve more homogenous and predictable effects. With this new knowledge, specifically adapted BEs could be developed which might improve fertilizer use efficiency and decrease the dependence of agriculture on large external inputs of synthetic fertilizers.

Maize (*Zea mays*), a major crop globally, is particularly sensitive to low phosphorus availability [29] and has shown responsiveness to BE applications [30–32]. For this reason, maize was selected as the study crop to evaluate the potential of three BEs: the bacterial BEs *Pseudomonas* sp. DSMZ 13134 and *Bacillus amyloliquefaciens* FZB42 and the fungal BE *Trichoderma harzianum* T-22. We applied all BEs individually in combination with different organic and inorganic P fertilizers in order to study the impact of the different P sources on the BEs' ability to promote P acquisition and growth of maize. In order to shed more light on the impact of soil properties on the effectiveness of the applied BEs, we used six soils originating from different sites in Europe. We hypothesized that BE application improves plant P acquisition from soil and from recycling fertilizers, and that soil properties, e.g., pH and P levels (total P and plant available P), influence the effectiveness of the three BEs.

Methods
Overall experimental design
Maize growth (variety Colisée, KWS Saat, Germany) was investigated in pots using soils collected from six different sites (Table 1) and by applying a factorial design including the two following factors: P fertilizer (minimum 3 levels) and microbial BE application (2–4 levels). Table 2 gives an overview of the treatments applied to each soil.

Collection of soils and soil characterization
Topsoil was collected from the six different locations. The soils were characterized by one analytical lab (LA Chemie, University of Hohenheim, Germany) for pH (0.01% $CaCl_2$), texture, organic carbon content [33], and plant available soil P (CAL method for neutral and alkaline soils [33] and DL method for acidic soils [33]). Additional information regarding geographic origin of the

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Table 1 Soil characteristics

Soil-ID	Geographic origin	Management	Soil type	Texture			Soil pH	C org	Phosphorus
				Clay	Sand	Silt	(CaCl$_2$)		CAL/DL[d]
				(%)	(%)	(%)		(%)	(mg P kg^{-1})
1-Buus[a]	Buus (Switzerland)	Rotation: Lay rotation Fertilization: organic, low input Site type: farmer field site	Loamy soil	29.9	3.90	66.2	6.6	2.64	6.5
2-Castel[a]	Castel Volturno (Italy)	Rotation: Maize-clover Fertilization: mineral, low input Site type: farmer field site	Vertic Xerofluvent	36.5	19.0	44.5	7.3	1.34	19.2
3-Humpolec[a]	Humpolec (Czech Republic)	Rotation: P-WW-SB[c] Fertilization: mineral, low input Site type: experimental station	Cambisol Loamy sand	15.9	52.9	31.2	5.2	1.63	45.0/59.8
4-Lukavec[a]	Lukavec (Czech Republic)	Rotation: long-term protection area around field experiment Fertilization: none Site type: experimental station	Cambisol Loamy sand	14.0	56.0	30.0	5.4	1.5	48.0/49.8
5-Vörden[b]	Vörden (Germany)	Rotation: before 2009 grazing area for wildlife – since 2009 field forage cultivation Fertilization: low input (organic and mineral) Site type: farmer field site	Cambisol	12.0	41.1	46.9	5.0	0.72	11.4/9.4
6-Taastrup[a]	Taastrup (Denmark)	Rotation: Barley-barley Fertilization: no P fertilizer since 1964 Site type: long-term field trial	Sandy loam	13.4	55.4	31.2	5.8	1.09	15.3/17.9

[a] Data were recorded on the pure soil

[b] Data were recorded on the 2:1 soil: sand mixture used in the pot experiments

[c] *P* potato, *WW* winter wheat, *SB* spring barley

[d] P measured after double lactate (DL) extraction is also given for soil with pH < 6 (exception for soil 3)

soils, management of the sites, and soil type is given in Table 1. Most soils had low to very low levels of available P, which makes them ideal for testing the efficiency of BEs in improving P availability and/or P uptake of added fertilizers by maize. The pH was ranging from slightly acidic to medium alkaline.

BE treatments

The three BE strains tested (Table 3) were formulated within three commercial products, namely Trianum-P (Koppert, The Netherlands) containing *Trichoderma harzianum* strain T-22 (BE1), Proradix WP (Sourcon Padena, Germany) containing *Pseudomonas* sp. strain DSMZ 13134 (BE2), and Rhizo Vital 42 fl. (Abitep, Germany) containing *Bacillus amyloliquefaciens* strain FZB42 (BE3). The choice of these three BEs is based on their published ability to act as potential bio-stimulants and biofertilizers. The strain *Trichoderma harzianum* Rifai 1295-22 (T22) has demonstrated abilities to increase the solubilization of sparingly soluble calcium phosphates [34] and to promote plant growth in various plant species [35, 36] including maize [37]. The strain *Pseudomonas* DSMZ 13134 is an efficient root colonizer

[38] and has been shown to solubilize inorganic phosphate under in vitro conditions [39]. Moreover, it has recently shown the potential to improve plant growth and phosphorus acquisition when inoculated to maize [10, 25]. The gram-positive strain *Bacillus amyloliquefaciens* FZB42 is mainly studied for its biocontrol potential but other studies also indicate its ability to act as a biofertilizer [40, 41]. The *Trichoderma* strain was inoculated at a concentration of 2.5×10^4 spores per g of substrate and the two bacterial strains at a concentration of 2×10^6 cfu per g of substrate. A non-inoculated control (BE0) was included for every soil and in combination with every P treatment. The exact amount of inoculated cells or spores in the different products was checked in preliminary work where inoculation suspensions were plated on specific media and counted afterward. This has led to a standardized inoculation protocol that was later applied for all pot experiments.

P fertilization treatments

The three common P treatments were negative control without the addition of P fertilizer (P0), Triple Super Phosphate (TSP), and Rock Phosphate (RP). In most

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Table 2 Overview of the experimental design and setup at the different locations (1: Switzerland, 2: Italy, 3 and 4: Czech Republic, 5: Germany, 6: Denmark)

	1-Buus	2-Castel	3-Humpolec	4-Lukavec	5-Vörden	6-Taastrup
Factors tested (experimental design)						
Crop						
Maize (variety: Colisée)	x	x	x	x	x	x
Bio-effectors						
No bio-effectors (BE0)	x	x	x	x	x	x
BE1: Trianum-P	x	x	x	x		
BE2: Proradix	x	x	x	x	x	x
BE3: Rhizovital	x	x	x	x	x	
Fertilizers						
No P fertilizer (P0)	x	x	x	x	x	x
Triple Super phosphate	x	x	x	x	x	
Rock phosphate	x	x	x	x	x	
Local sewage sludge ashes					x	x
P-enriched BOF slag					x	
Fresh Digestate	x					
Local composts	x				x	
Composted cattle manure		x				
Composted horse manure		x				
Local sewage sludges					x	x
Experimental setup						
Amount of substrate per pot (kg-DW equivalent)	2.5	2.5	5	5	5	2.5
Number of plants per pot at harvest	1	1	3	3	1	1
Growth period (weeks)	8	8	15	15	8	6.5
Number of replicates	5	5	5	5	4	3
Soil to sand ratio	2:1	2:1	2:1	2:1	2:1	1:1
Growing facilities[a]	GC	GC	GH	GH	GH	GH

[a] GC means growth chamber and GH greenhouse

Table 3 Bio-effector strains applied

Product	Producer	Abbr.	Species	Type of organism	Formulation	Application rate (cfu g^{-1} soil)
Control	n.a.	BE0	n.a.	n.a.	n.a.	n.a.
Trianum-P	Koppert, The Netherlands	BE1	*Trichoderma harzianum*, strain T-22	Fungus	Powder	2.5×10^4
Proradix WP	Sourcon Padena, Germany	BE2	*Pseudomonas sp.* strain DSMZ 13134	Bacteria	Powder	2×10^6
Rhizovital 42 fl.	ABiTEP, Germany	BE3	*Bacillus amyloliquefaciens*, strain FZB42	Bacteria	Liquid	2×10^6

n.a. means not applicable

cases, a number of additional P treatments (Table 4) were investigated and consisted of organic fertilizers and/or recycled inorganic fertilizers. These fertilizers were locally available and previously studied for their properties by the individual groups. As a result, some soils were tested with organic fertilizers and others only with recycled inorganic fertilizers (in addition to TSP and RP). Except for the P0

treatment, all pots received P at a dose of 50 mg P per kg of dry substrate. The TSP (20% P) was provided by Landor in Switzerland and RP (12% P) by Herbert Molitor in Germany. The additional P treatments consisted of recycled inorganic fertilizers (sewage sludge ashes and P-enriched BOF (basic oxygen furnace) slags [42]) and organic fertilizers (fresh biogas digestate, mature compost from green

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Table 4 Main characteristics of the fertilizers used

Fertilizers	Abbr.	Inorg/org	Dry matter content (%)	Total P (mg P g^{-1})	Water extractable P (mg P g^{-1})	Total N (mg N g^{-1})	Ammonium (NH$_4^+$ − N) (mg N g^{-1})
Triple super phosphate	TSP	Inorg	100	201.0	201.0	0	0
Rock phosphate	RP	Inorg	100	115.4	0.22	0	0
Sewage sludge ashes (Germany)	SSA-DE	Inorg	100	103.5	0.79	n.d.	n.d.
Sewage sludge ashes (Denmark)	SSA-DK	Inorg	100	89	n.d.	n.d.	n.d.
P-enriched BOF slag (Germany)	LSD/SSA	Inorg	100	18.0	0.0	n.d.	n.d.
Fresh digestate (Switzerland)	FrDi	Org	48.0	4.36	0.130	14.6	0.61
Compost (Switzerland)	Comp-CH	Org	59.6	2.45	0.043	10.4	0.24
Compost (Germany)	Comp-DE	Org	57.2	1.42	0.67	n.d.	n.d.
Composted cattle manure (Italy)	C-MComp	Org	60.0	7.75	n.d.	22.7	n.d.
Composted horse manure (Italy)	H-MComp	Org	67.0	10.0	n.d.	14.2	n.d.
Sewage sludge (Germany)	SS-DE	Org	99	38.1	0.52	n.d.	n.d.
Sewage sludge (Denmark)	SS-DK	Org	23.0	30.4	1.5	n.d.	n.d.

n.d. means not determined

wastes, composted cow and horse manures, and sewage sludge). They were characterized by the local laboratories (Table 4) for their dry matter content, total P content, water extractable P, total potassium (K) and N content, and their ammonium N content.

Pot experiment setup, growing conditions, and harvest

After sieving, the soil was mixed with quartz sand (0.6–1.2 mm) in the ratio 2:1 or 1:1 (soil dry weight (DW)/sand DW; W/W). Each pot contained the equivalent of 2.5 kg or 5 kg DW of the experimental substrate (Table 2). All pots received N (100 mg N kg^{-1} substrate) and K (166 mg K kg^{-1} substrate) in form of calcium nitrate and Kalimagnesia, respectively. Where alternative P treatments were applied, N and K were measured and reduced from the basal dose of N and K (except for the German and the Danish trials where the addition of N and K was not reduced).

The N, K, and P fertilizers were added individually to every pot, and water addition was adjusted to reach 60–70% of the substrates water holding capacity (WHC). Three seeds were sown per pot, and the BE suspension was added in the seeding hole (5 ml per seeding hole = 15 ml per pot). BE suspensions were prepared under sterile conditions by diluting the products with 2.5 mM CaSO$_4$. The surface of the pots was then covered by a fine layer of quartz sand to avoid formation of surface crusts after watering.

The pots were transferred to growth chamber or greenhouse (see Table 2) and watered according to plant's need in order to keep the initial WHC (increased to 70–80% after two weeks). Thinning (including the root systems) was performed one week after sowing, leaving one plant in each pot. During the growth period, plant height was measured every second week and final harvest took place 8 weeks after sowing. Any deviation from this standard protocol (size pot, growing period, number of plant per pot, standard P fertilizers) is described in Table 2. Every treatment was replicated five times (or at minimum three times, see Table 2), and pots were randomly distributed in the growth chambers or greenhouses. The guidelines for establishing the pot experiments were common to all soils, and deviations to experimental setup are due to particular constraints of the different involved groups.

Plant analyses

At harvest, shoots were collected, dried (65 °C), and stored for further analyses. Root systems were washed from the substrate, and a subsample was kept (only in Swiss and Italian soils) to assess percentage root length colonization (RLC %) by arbuscular mycorrhizal fungi (AMF) following the protocol of Phillips and Hayman [43] and Brundrett et al. [44]. The remaining root was weighed and dried for total root DW assessment. Dried shoot material was milled for later determination of total N and P concentration. Shoot nitrogen concentration was measured using CHN analyzers, while shoot P concentration was determined spectrophotometrically or using flow injection analysis (Danish experiment) after acid extraction.

Data analyses and statistics

Residuals of the data (shoot DW, N and P content in shoot and RLC %) were tested for normality using the Shapiro–Wilk test. For every soil, a two-way ANOVA was performed to test the significance of each factor

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(P fertilizer and BE strain) and their interaction. Since this interaction was always significant and for the sake of the graphical representation (in Figs. 2, 3, 4, and 5), a one-way ANOVA was performed within each P treatment and for each soil to determine which BE treatment (BE1, BE2, or BE3) was significantly different from the un-inoculated controls (Tukey's test, $P < 0.05$). Due to differences in growing conditions (Table 2, lower part) leading to substantial differences in the aboveground biomass response among single experiments, we normalized the aboveground biomass, P, and N content relative to the negative control treatment (P0, BE0) in the following way (exemplified for biomass):

$$normalized\ biomass_{sample} = \frac{biomass_{sample}}{\overline{biomass_{control(P0,BE0)}}}$$

Furthermore, in order to quantify the relative effect of BE additions across soils and P fertilizer types, absolute values for shoot DW (g plant^{-1}) were converted to shoot biomass improvement (in %) for every BE (BE1, BE2, and BE3) calculated relative to the BE0 control. For this, we applied the following equation:

$$improvement_{\%}$$
$$= 100 \cdot \left(\frac{biomass_{sample(P_x,BE_y)} - \overline{biomass_{sample(P_x,BE_0)}}}{biomass_{sample(P_x,BE_0)}} \right)$$

A value of 0% means that the shoot biomass of maize inoculated with the BE strain in question did not improve shoot biomass as compared to the un-inoculated control (using the same P fertilizer), whereas a value below or above 0% indicates that the specific BE treatment led to a decrease or an increase in aboveground biomass, respectively. In order to investigate the relative effect of BEs across different experiments, we categorized the added P fertilizers as either organic or inorganic. Data from experiments conducted in the Czech Republic were not considered here as they only included inorganic fertilizers (TSP and RP). For each BE, we performed a separate two-way ANOVA on these data with "soil" and "fertilizer type" and their interaction as factors, in order to analyze if the effect of BE was dependent on soil and/or fertilizer type (Table 6).

For each fertilizer, the fertilizer efficiency (FE %) relative to TSP (positive control) was calculated as the mean efficiency measured in n replicated pots. The efficiency in individual pots was calculated as follows:

$$FE_{\%} = 100 \cdot \left(\frac{biomass_{sample} - \overline{biomass_{control(P0,BE0)}}}{biomass_{TSP} - \overline{biomass_{control(P0,BE0)}}} \right)$$

Finally, the P uptake efficiency (PUE %) from the different fertilizers was calculated as follows:

$$PUE_{\%} = 100 \cdot \left(\frac{P\ content_{sample} - \overline{P\ content_{control(P0,BE0)}}}{P\ content_{TSP} - \overline{P\ content_{control(P0,BE0)}}} \right)$$

Results

The aboveground biomass was highly variable across the experiments (Fig. 1). A two-way ANOVA on raw biomass data (Fig. 1) for the negative (P0) and positive controls (TSP) without addition of BE (BE0) yielded highly significant effects of soil ($P < 0.001$) and P fertilizer ($P < 0.001$), as well as a highly significant interaction between the two factors ($P < 0.001$). In five out of six soils, there was a significant positive effect of TSP addition on the aboveground biomass (Fig. 1, Tukey's test, $P < 0.05$). The Humpolec soil is the only soil not affected by TSP addition with regards to aboveground biomass production.

In absence of BE inoculation (BE0), plants responded differently to the addition of the various P fertilizers. This was true for normalized biomass (Fig. 2), normalized P content (Fig. 3), and normalized N content (Fig. 4). The most effective fertilizers were the P-enriched BOF slag (Vörden trial) and in general the composted material from green wastes (FrDi, Comp-CH, Comp-D (see Table 4 for abbr.)) and sewage sludges from Denmark (incinerated or not).

The following combinations of BE and P fertilizers resulted in a higher normalized shoot biomass (Fig. 2), when compared to the negative control treatment (BE0) receiving the same P fertilizer (treatments
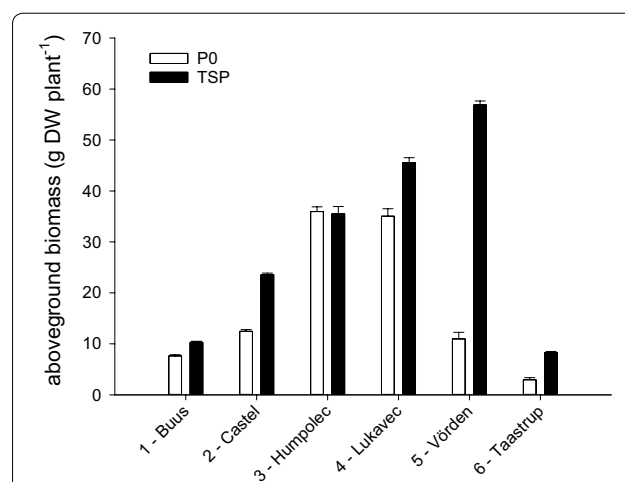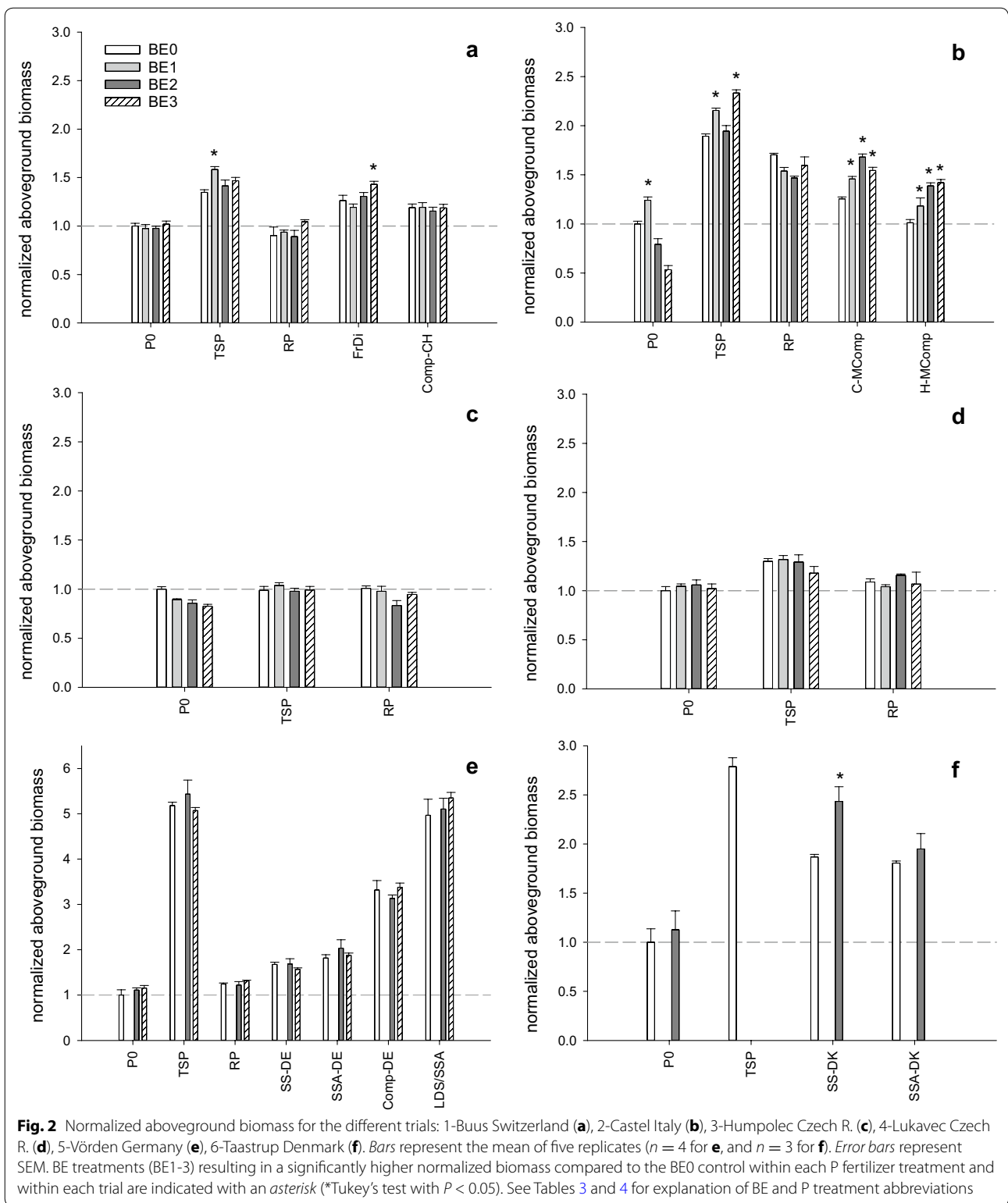

Fig. 1 Aboveground biomass of maize plant at harvest in the different trials: 1: Switzerland, 2: Italy, 3 and 4: Czech Republic, 5: Germany, 6: Denmark [without BE inoculation and either supplemented with Triple Super Phosphate (TSP) or not (P0)]. *Error bars* represent Standard Error of the Mean (SEM)

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Fig. 2 Normalized aboveground biomass for the different trials: 1-Buus Switzerland (**a**), 2-Castel Italy (**b**), 3-Humpolec Czech R. (**c**), 4-Lukavec Czech R. (**d**), 5-Vörden Germany (**e**), 6-Taastrup Denmark (**f**). *Bars* represent the mean of five replicates ($n = 4$ for **e**, and $n = 3$ for **f**). *Error bars* represent SEM. BE treatments (BE1-3) resulting in a significantly higher normalized biomass compared to the BE0 control within each P fertilizer treatment and within each trial are indicated with an *asterisk* (*Tukey's test with $P < 0.05$). See Tables 3 and 4 for explanation of BE and P treatment abbreviations

highlighted with an asterisk): BE1 combined with TSP in the Buus (Fig. 2a) and Castel soils (Fig. 2b); BE2 combined with sewage sludge in the Taastrup soil (Fig. 2f);

BE3 combined with fresh digestate and TSP in the Buus (Fig. 2a) and Castel (Fig. 2b) soils, respectively; and all BEs combined with composted animal manures
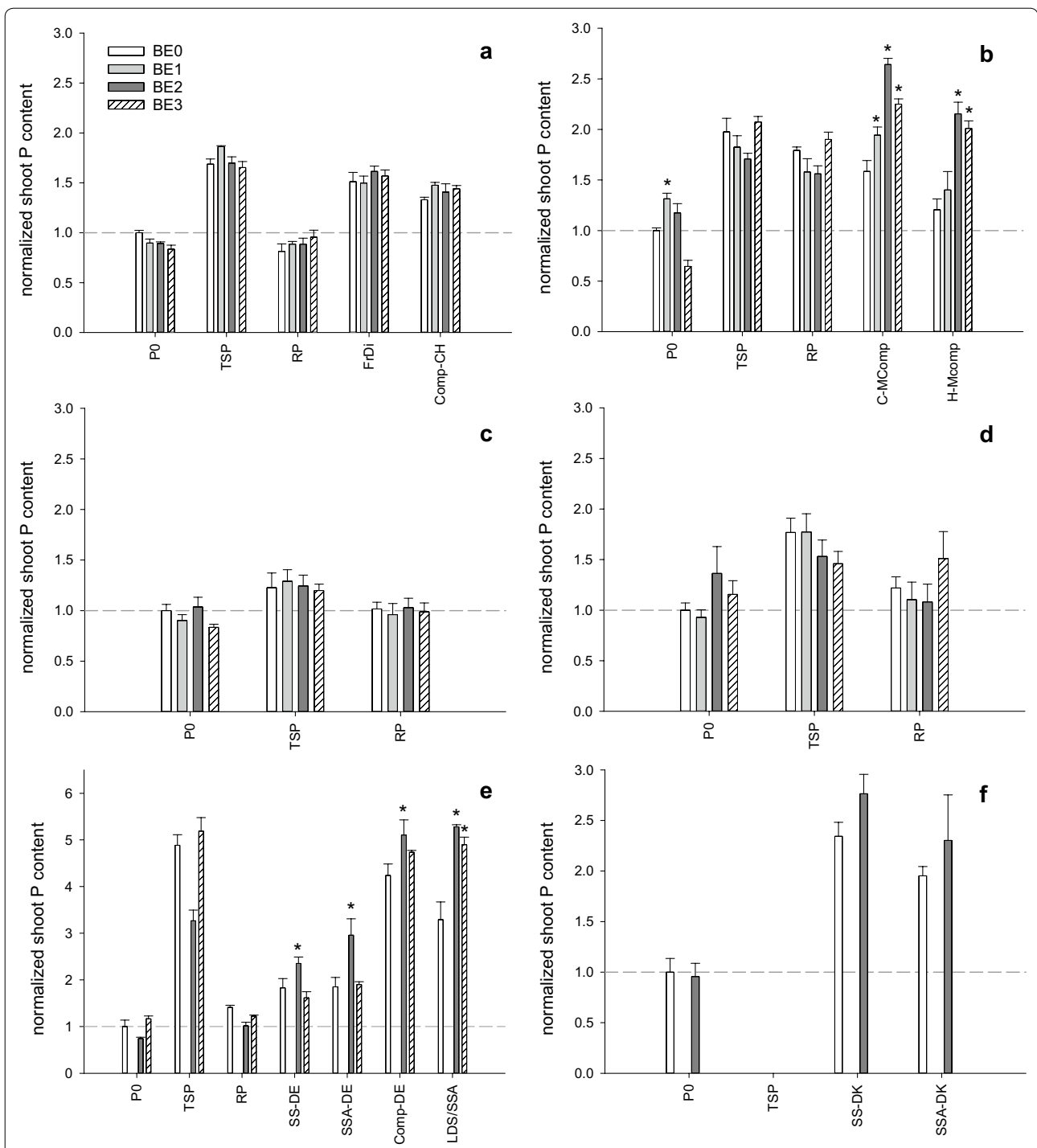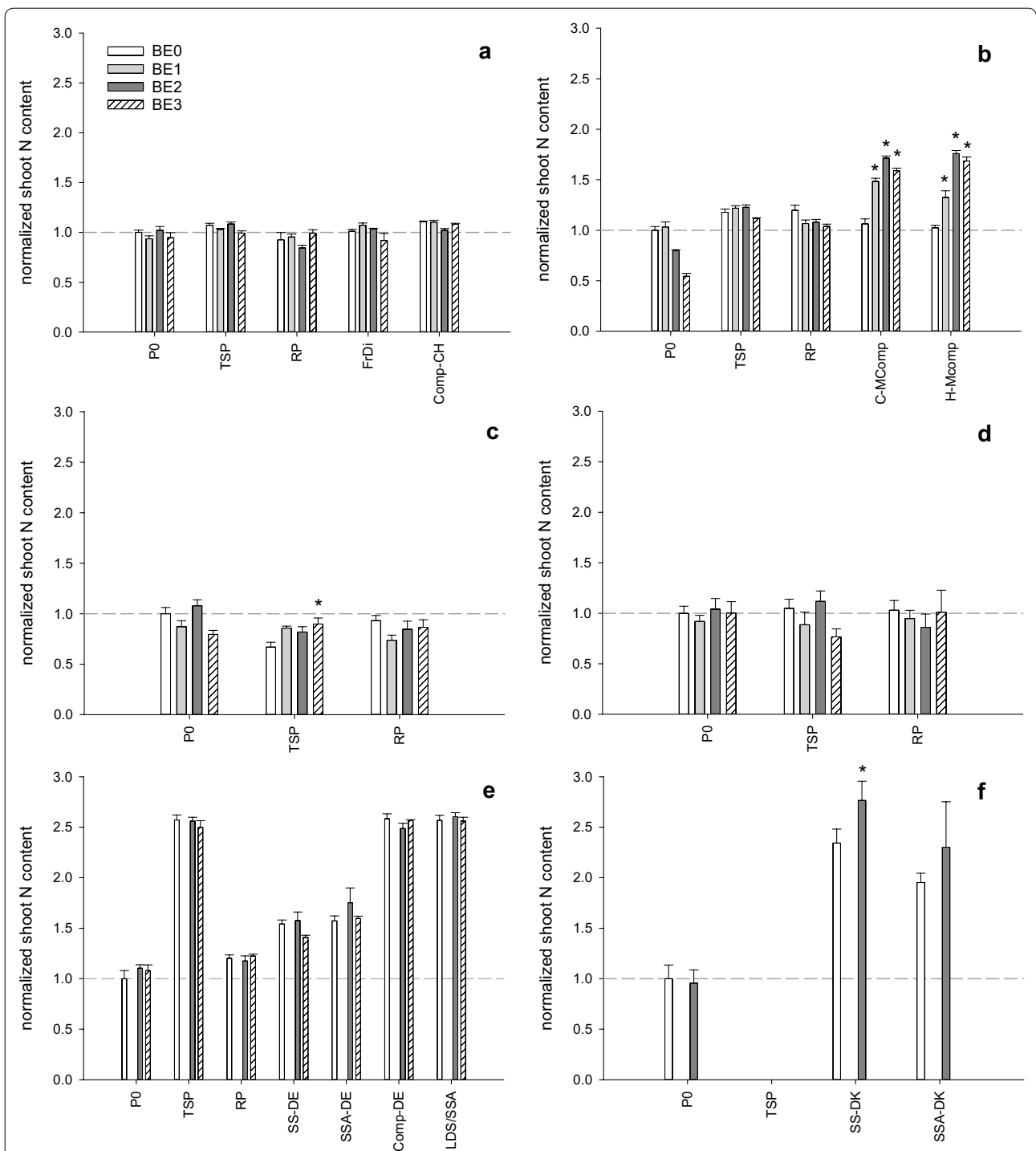
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Fig. 3 Normalized aboveground P content for the different trials: 1-Buus Switzerland (**a**), 2-Castel Italy (**b**), 3-Humpolec Czech R. (**c**), 4-Lukavec Czech R. (**d**), 5-Vörden Germany (**e**), 6-Taastrup Denmark (**f**). *Bars* represent the mean of five replicates (*n* = 4 for **e**, and *n* = 3 for **f**). *Error bars* represent SEM. BE treatments (BE1-3) resulting in a significantly higher normalized P content compared to the BE0 control within each P fertilizer treatment and within each trial are indicated with an *asterisk* (*Tukey's test with *P* < 0.05). See Tables 3 and 4 for explanation of BE and P treatment abbreviations

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Fig. 4 Normalized aboveground N content for the different trials: 1-Buus Switzerland (**a**), 2-Castel Italy (**b**), 3-Humpolec Czech R. (**c**), 4-Lukavec Czech R. (**d**), 5-Vörden Germany (**e**), 6-Taastrup Denmark (**f**). *Bars* represent the mean of five replicates (*n* = 4 for **e**, and *n* = 3 for (**f**). *Error bars* represent SEM. BE treatments (BE1-3) resulting in a significantly higher normalized N content compared to the BE0 control within each P fertilizer treatment and within each trial are indicated with an *asterisk* (*Tukey's test with *P* < 0.05). See Tables 3 and 4 for explanation of BE and P treatment abbreviations

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in the Castel soil (Fig. 2b). In the Humpolec, Lukavec, and Vörden soils, BE inoculation did not result in any increased normalized shoot biomass (Fig. 2 c–e).

Across the four experiments included in Table 6 (all except the two Czech experiments), BE inoculations induced an increase in shoot DW of 5–7.5%, as compared to un-inoculated controls (Table 6). For all three tested BEs, soil influenced the BE-derived increase in shoot biomass ($P < 0.01$). When looking across fertilizer types, the largest BE effects were observed in the Castel soil for BE1 (+12%), in the Taastrup soil for BE2 (+17%), and in the Buus soil for BE3 (8%). The factor "fertilizer type" significantly influenced the improvement in biomass when plants were inoculated with BE2 and BE3 (Table 6, $P < 0.0001$), but no such effect of "fertilizer type" was observed with BE1 (Table 6, $P > 0.05$). Furthermore, there was a significant interaction between "soil" and "fertilizer type" for all BE treatments (Table 6, $P < 0.0001$).

Across all soils, there was a significant positive effect of adding BE2 (+14%) and BE3 (+13%) when organic fertilizers were applied (Table 6, $P < 0.05$). For inorganic fertilizers, there was only a significant effect of BE3 (+7%, $P < 0.05$). When no fertilizer was added, none of the three BEs resulted in an improvement of the shoot biomass (Table 6, $P > 0.05$).

The normalized aboveground P content was significantly different between P fertilizers in all experiments (Fig. 3). In the Castel soil, inoculation with BE1 led to a significantly larger P content in the P0 and cow manure compost treatments, while inoculation with BE2 and BE3 resulted in an increased P content in both animal manure treatments (Fig. 3b). In the Vörden soil, inoculation with BE2 resulted in an increased P content when combined with any of the recycling fertilizers (Fig. 3e). In the remaining soils (Buus, Humpolec, Lukavec, and Taastrup), BE inoculation did not result in increased aboveground P contents. In the Castel soil, the higher P content due to BE2 addition (combined with cow and horse manures) is reflecting the significant increase in P concentration in the aboveground biomass (Table 7). BE3 increased P concentration only in the P0 treatment, while BE1 did not induce any P concentration increase. In the Vörden soil, where plants also accumulated more P in the aboveground biomass (Fig. 3e), P concentration was also higher (as compared to the corresponding BE0 control), but only when sewage sludge ashes were combined with BE2 and when P-enriched BOF slags were combined with BE2 or BE3.

The normalized aboveground N content (Fig. 4) showed less difference between P fertilization treatments than the normalized P content did (Fig. 3). Only in Vörden (Fig. 4e) and Taastrup (Fig. 4f) soils, a clear effect of P fertilizers on aboveground N content was observed.

In the Castel soil, the effect of P fertilization depended on BE addition since we only observed a significantly larger normalized aboveground N content when pots had been inoculated with BEs 1–3 (Fig. 4b). In the Taastrup soil, a significantly higher aboveground N content was observed in plants inoculated with BE2 and when sewage sludge was used as fertilizer. In the Humpolec soil, we found a significant increase in aboveground N content due to inoculation with BE3, when TSP was used as P fertilizer (Fig. 4c). However, the recorded N content (Fig. 4c) in this treatment (TSP/BE3) was numerically smaller than in the negative control (P0/BE0). In the remaining soils (Buus, Lukavec, and Vörden), BE inoculation did not result in increased aboveground N contents.

Intensity of RLC % by AMF was only measured in roots of plants growing in the Buus and Castel soils (Fig. 5). For un-inoculated treatments which were not amended with P fertilizer (BE0, P0), the two soils induced different
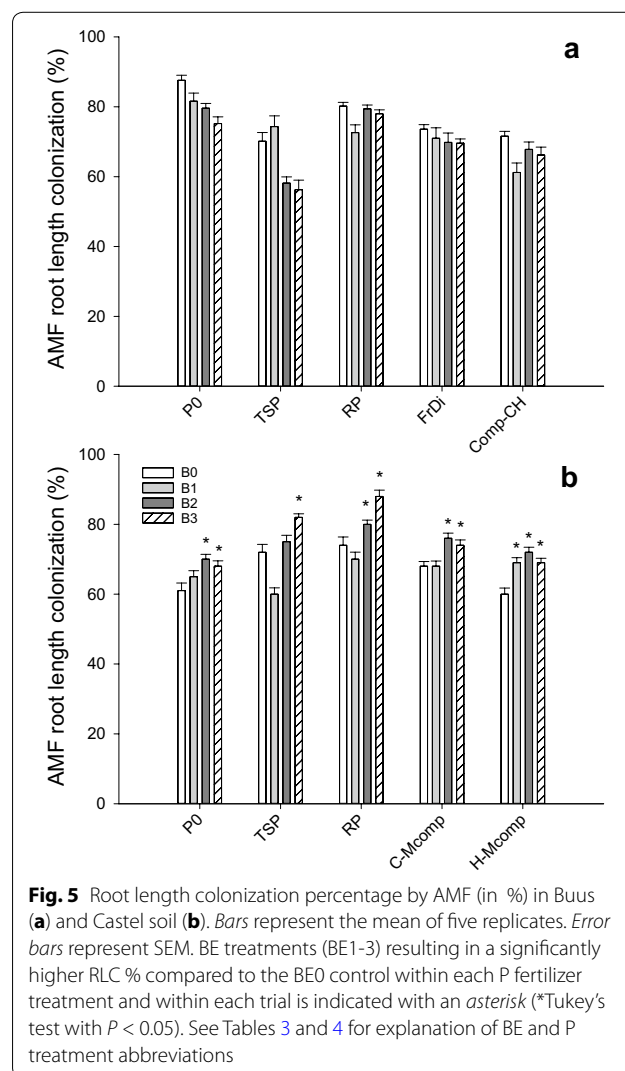
Fig. 5 Root length colonization percentage by AMF (in %) in Buus (**a**) and Castel soil (**b**). *Bars* represent the mean of five replicates. *Error bars* represent SEM. BE treatments (BE1-3) resulting in a significantly higher RLC % compared to the BE0 control within each P fertilizer treatment and within each trial is indicated with an *asterisk* (*Tukey's test with $P < 0.05$). See Tables 3 and 4 for explanation of BE and P treatment abbreviations

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levels of RLC % (87% for the Buus soil and 60% for the Castel soil). In the Buus soil, BE inoculation leads to either no change in RLC % or a decrease, especially for plants amended with TSP (Fig. 5a). In contrast, the roots from the Castel soil had significantly higher RLC % in BE-inoculated pots, especially for BE3 (independent of P fertilizer applied) and for the combination of BE2 and animal manures (Fig. 5b).

Discussion

Variable levels of soil fertility and harvested aboveground biomass (P0, BE0 and TSP, BE0)

Due to variation in the experimental setup (e.g., pot size and growth duration), different aboveground biomasses were harvested ranging from 4 to 36 g per plant in the negative control treatment (P0, BE0). The different increases due to TSP addition (TSP, BE0) indicated that soils had varying initial fertility levels (Figs. 1, 2) with the Czech soils (Humpolec and Lukavec) being the less depleted in P. Plants growing in the Vörden (DE), the Taastrup (DK), and, to a lesser extent, in the Castel (IT) soil, were obviously P limited (increase between 2- to fivefold due to TSP addition), while the plants in the Buus (CH) soil showed a moderate P increase (1.6-fold). The same response to TSP addition was observed with spring wheat with the Vörden and Taastrup soils [45], where a similar experimental setup was used. The Taastrup soil originates from a long-term field trial where no nutrients have been added for approximately 50 years [46], thereby explaining the response to application of easily available P sources. The Castel soil was taken from an alkaline site where native P is largely bound in calcium complexes. The Buus soil is from an organic field site where the plant available P fraction is indeed low (P CAL = 6.5 mg P/ kg), but with a large organic P fraction (data not shown) which likely contributed to P supply during the experiment causing a moderate response to TSP addition.

Variable P fertilizer effects (P, BE0)

We observed a range of responses due to the application of different P fertilizers. The calculated fertilizer efficiencies (Table 5) indicate that, in the absence of inoculated BEs, recycling fertilizers (organic or inorganic) were superior to the rock phosphate fertilizer (the Castel experiment was an exception). The fertilizer and P use efficiencies for sewage sludge, and sewage sludge ashes were quite similar within the same experiment but quite different between the two experiments where these products were included (16–22% for the Vörden experiment and 45–49% for the Taastrup experiment). This is in line with the data on water extractable P in the sludges where the level in the Danish sludge was three times higher than in the German sludge (Table 4). Furthermore, the

availability of P in sewage sludge ashes can be quite different depending on the processing of the sludge in the water treatment plant [47].The very high fertilizer efficiency recorded for the P-enriched BOF slag has been observed earlier in wheat [45] and maize [42].

Moderate effects in the absence of added P (P0, BE1-3)

When no P was added, the three BEs neither improved aboveground biomass nor P uptake. In one case (Castel-IT), there was even a reduced aboveground biomass due to the application of BE2 and BE3. The absence of response after BE application was observed in many other experiments [45, 48, 49] and in particular in soils characterized by extraordinary low levels of available P (20 mg kg^{-1} or lower) or that were limiting in other soil nutrients [10, 50, 51]. In contrast, other studies have shown that for maize and other crops, the application of BEs was more efficient in the absence of added P fertilizers [36–38]. However, in these cases, soil P availability was distinctly higher and P CAL ranged between 35 and 45 mg/kg.

The observed absence of plant responses to BE applications when soils are extremely low in available P could be explained by direct growth limitation of the host plants, which finally limits sufficient production of roots and root exudates to initiate beneficial plant–microbial interactions with indigenous or inoculated soil microbes in the rhizosphere. This phenomenon is well documented, e.g., for mycorrhizal associations frequently absent on extremely nutrient-impoverished soils [52, 53] or for the establishment of the Rhizobium symbiosis in leguminous plants on low P soils which is promoted by starter applications with soluble P [54]. Moreover, in the absence of significant soil P pools that can be mobilized by BEs, their presence will have no beneficial effect on plant P supply, or may even be in competition with plants by immobilizing P in microbial tissue.

BE effects depend on soil and fertilizer type

In the present study, it was observed that for the three tested BEs, the soil strongly modulated the observed BE effect (Table 6). Various studies have shown that soil pH, soil organic carbon content, soil microbial activity, and soil nutrient status are all influencing the success of BE application [16, 25, 55–57]. Our findings indicate that the most responsive soils to BE application (in combination with P fertilizers) were either alkaline (Castel-IT) or depleted in soil nutrients (Taastrup-DK and Vörden-DE for P acquisition), confirming the influence of soil pH and soil nutrient statues on the effectiveness of BEs. The type of fertilizer used along with BEs was also significantly influencing the magnitude of BE effects (Table 6). Since all tested fertilizers were different (with the exception of

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Table 5 Relative fertilizer efficiencies (FE) and P use efficiencies (PUE)

P fertilizer	Abbr.	1-Buus (Switzerland)		2-Castel Volturno (Italy)		3-Humpolec (Czech R.)		4-Lukavec (Czech R.)		5-Vörden (Germany)		6-Taastrup (Denmark)	
		FE	PUE	FE	PUE	FE	PUE	FE	PUE	FE	PUE	FE	PUE
		(% of TSP)	(% of TSP)	(% of TSP)	(% of TSP)	(% of TSP)	(% of TSP)	(% of TSP)	(% of TSP)	(% of TSP)	(% of TSP)	(% of TSP)	(% of TSP)
Negative control	P0	0bc	0c	0d	0d	0a	0a	0b	0b	0d	0c	0c	n.a.
Triple super phosphate	TSP	100a	100a	100a	100a	100a	100a	100a	100a	100a	100ab	100a	n.a.
Rock phosphate	RP	−28c	−27c	79b	81ab	−70.1a	7a	30b	28b	6cd	11 c	n.a.	n.a.
Fresh digestate	FrDi	76a	74ab	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Composts	Comp-CH, Comp-DE	54ab	48b	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	55b	84 ab	n.a.	n.a.
Composted cow manure	C-MComp	n.a.	n.a.	28c	60bc	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Composted horse manure	H-MComp	n.a.	n.a.	2d	21cd	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Sewage sludges	SS-DE, SS-DK	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	16cd	21c	49b	n.a.
Sewage sludge ashes	SSA-DE, SSA-DK	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	20cd	22c	45b	n.a.
P-enriched BOF slag	LSD/SSA	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	95 a	59b	n.a.	n.a.

The efficiencies are calculated for the BE0 treatments only. Efficiencies are calculated relative to TSP as the positive control (see "Methods" section for details). For each column, different letters after mean values represent significantly different means (Tukey's test, P < 0.05)

n.a. means not applicable

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Table 6 Shoot biomass improvement (%) of the different BE treatments relative to the un-inoculated control (BE0)

	Trianum-P (BE1)	Proradix (BE2)	Rhizovital (BE3)
Overall BE effect (across all treatments)	7.5%	5.2%	6.0%
Number of observations	50	87	78
BE effect in the different soils (across all fertilizers types)			
Buus (Switzerland)	2.7%b[a]	0.4%b[a]	8%a[a]
Castel (Italy)	12.3%a[a]	7.8%b[a]	6.7%a[a]
Vörden (Germany)	Not measured	3.4%ab[a]	3.6%b[a]
Taastrup (Denmark)	Not measured	17.0%a[a]	Not measured
BE effect with the different fertilizer types (across all soils)[b]			
No fertilizer added	10.8%a[a]	−1.2%b[a]	−11.5% b[a]
Organic fertilizer	6.9%a[a]	*14.0%a[a]*	*13.0% a[a]*
Inorganic fertilizer	6.4%a[a]	1.5%b[a]	7.3% a[a]
ANOVA on the effect of soil, fertilizer type and their interaction			
Soil (*p* value)	0.0002 (*df* = 1)	0.0070 (*df* = 3)	0.0054 (*df* = 2)
Fertilizer type (*p*-value)	ns (*df* = 2)	<0.0001 (*df* = 2)	<0.0001 (*df* = 2)
Soil x fertilizer type (*p*-value)	<0.0001 (*df* = 2)	<0.0001 (*df* = 6)	<0.0001 (*df* = 4)

df means degree of freedom

See "Methods" section for description of the calculations

[a] Levels not connected by the same letter are significantly different (Tukey's HSD test *P* < 0.05)

[b] Underlined combinations of BE and P fertilizer type indicate when BE addition significantly improved shoot biomass as compared to the un-inoculated control with the same fertilizer type (*t*-test with *P* < 0.05)

Table 7 Aboveground P concentration (mg/g)

	BE0	BE1	BE2	BE3
1-Buus				
Control P0	1.57a	1.45ab	1.44ab	1.29b
TSP	1.97a	1.85a	1.89a	1.77a
RP	1.42a	1.49a	1.63a	1.44a
FrDi	1.87ab	1.97a	1.94a	1.72b
Comp-CH	1.76a	1.95a	1.91a	1.91a
2-Castel Volturno				
Control P0	0.96c	1.01bc	1.40a	1.15b
TSP	1.00a	0.81b	0.84ab	0.85ab
RP	1.00a	0.98a	1.01a	1.15a
C-MComp	1.20b	1.27b	1.50a	1.39ab
H-MComp	1.13b	1.12b	1.48a	1.35ab
3-Humpolec				
Control P0	1.03a	1.03a	1.26a	1.04a
TSP	1.28a	1.27a	1.29a	1.25a
RP	1.03a	1.01a	1.26a	1.07a
4-Lukavec				
Control P0	0.88a	0.78a	1.10a	0.98a
TSP	1.19a	1.19a	1.04a	1.11a
RP	0.99a	0.92a	0.82a	1.21a
5-Vörden				
Control P0	1.12a	n.a.	0.76b	1.14a
TSP	1.06a	n.a.	0.68b	1.15a
RP	1.28a	n.a.	0.94b	1.05b
SS-DE	1.23ab	n.a.	1.58a	1.16b
SSA-DE	1.15b	n.a.	1.62a	1.14b
Comp-DE	1.45a	n.a.	1.84a	1.58a
LSD/SSA	0.75b	n.a.	1.17a	1.03a
6-Taastrup				
Control P0	1.99a	n.a.	1.71b	n.a.
SS-DK	2.49a	n.a.	2.28a	n.a.
SSA-DK	2.15a	n.a.	2.31a	n.a.

For each row, means which are followed by different letters are significantly different (Tukey's HSD test *P* < 0.05, performed within each P treatment and for each soil)

n.a. means not applicable

TSP and RP that were tested in almost all experiments), we categorized them in two types (organic or inorganic) in order to investigate if there were some more general trends in the interaction between P fertilizer applied and the effect of the three BEs across the experiments. Clearly, BE2 and BE3 (both bacterial) applied with organic fertilizers were the best combinations and in particular when these BEs were combined with composted animal manures (for BE2 and BE3) or with the Danish sewage sludge (for BE2). In the Vörden soil, sewage sludge was also combined with BE2. But here, BE2 failed to exert any effect on the obtained biomass, showing again the complexity of these processes and why the interaction between soil and fertilizer type was significant in the performed two-way ANOVA (Table 6). In two cases (Buus and Castel soils), BE1 (fungal) was able to increase the aboveground biomass when combined with TSP, indicating that the three BEs have likely different modes of

action (see below). The study of Lekfeldt et al. [45] indicates that, to some extent, these effects and interactions are also crop specific since they did not observe any positive effects of BE1-3 on plant growth or P uptake when wheat was fertilized with recycling fertilizers.

Possible modes of action

Several studies [27, 39, 58] have shown that, in in vitro conditions, many BEs are able to dissolve insoluble tricalcium phosphates by releasing protons and/or organic acids (e.g., gluconic acids), decreasing the pH of the growth medium and/or act as cation chelators (Al, Fe, and Ca) [59]. Nevertheless, in pot experiments where soil

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low in available P is used as the growth substrate, this solubilization ability was limited, as almost none of the BEs could increase plant P content in P0 and RP treatments, with the exception of BE1 in the alkaline Castel soil (P0 treatment,). This limited solubilization ability under in situ soil conditions was also observed in other studies [27, 45, 49] and may be explained by the soil buffering capacity that inhibits the BE-induced soil acidification [33] that otherwise would have favored solubilization of calcium phosphates from soil and/or RP. However, we observed that in the Vörden soil, the amendment of sewage sludge ashes or P-enriched BOF slags (both inorganic fertilizers containing P in calcium phosphates complexes) increased plant P uptake when they were inoculated with BE2 or BE3 (Fig. 3e). Since this was not associated with increased N content in shoot (Fig. 4e), we can exclude that the better acquisition of P is the result of improved root growth. Rather, it is possible that the higher P content and concentration (Fig. 3e and Table 7) measured in the shoots are indeed the result of P solubilization (from these fertilizers), induced by BEs and in particular BE2. Yet, it remains to be understood why the biomass of these plants was not larger than the BE0 control and why the same BE could not solubilize P when combined with rock phosphate in the same soil.

Inoculated plants with higher biomass, P, and N contents (in the Castel soil, BE1-3 with composted animal manures; or in the Taastrup soil, BE2 with sewage sludge) could result from BE-induced mineralization of organic P and N contained in the tested organic fertilizers. For P, several studies have shown that phosphatase activities (acid and alkaline) measured in soil can be increased upon inoculation with BEs [16–18, 60]. Although it was not measured in our experiments, we argue that increased mobilization of P from organic fertilizers via higher phosphatase activities is likely one of the major mechanisms behind the successful BE-organic fertilizer combinations observed in this study. Nevertheless, plants can also produce phosphatases, and therefore BEs may also have induced growth of more roots and these may in return have produced more of these enzymes. Indeed, the fact that BEs combined with TSP lead in some cases to higher aboveground biomass suggests that BEs can increase root growth and therefore the volume of soil explored for nutrient uptake. This mechanism has been described in several studies [61–64] and results from the direct or indirect production of root growth regulators such as auxin.

The volume of soil explored for nutrient uptake can also be increased via higher root length colonization percentage (RLC %) by AMF. In this study, the only roots analyzed for RLC % were from the experiments with the Buus and Castel soils. In the latter case, an increase in RLC % after BE inoculation was recorded with or without application of P fertilizers (Fig. 5b). This mycorrhizal boosting effect is known for several microorganisms [65–67] and in particular for BE2 [68]. In the Castel soil, the increased RLC % due to BE inoculation in combination with the P fertilization treatments P0, RP, and TSP did not necessarily translate into higher biomass or acquired P. This indicates that the mycorrhizal boosting effect of BE inoculation in that soil was of minor importance for plant P uptake and growth, although we do not know to which degree it contributed to the improvement in P uptake and growth for the plants inoculated with the three BEs and amended with the animal manures. In the Buus soil (Fig. 5a), RLC % was not increased after BE inoculation. This can be explained in part by the fact that roots were already highly colonized (almost 90%). But it also seems that in some combinations, BE inoculation had a negative impact on RLC % under these conditions, and this could be due to competition. Yet, the clear mechanisms remain to be explained.

Conclusion

We have observed that in our experiments, the most responsive soils for the three tested BEs were either alkaline or highly depleted in nutrients, and that a positive effect of the BEs was dependent on an input of P fertilizer under these conditions. Of all tested fertilizers, the combination of BE with organic fertilizers (in particular composted animal manures) was the most promising. Nevertheless, the combination of BE and organic fertilizers was not always improving biomass production or P uptake (e.g., Buus soil). The next challenge will be to understand what properties of organic fertilizers make them more compatible with a given BE and how this interacts with soil parameters, such as soil pH, soil microbial activities, organic matter content, and soil P and N status. Regarding the mechanisms, the results from this study suggest that the three BEs likely improved root growth (volume of soil explored for nutrients uptake) and P mobilization via accelerated mineralization, whereas chemical P solubilization likely took place in very few cases. Although higher mycorrhizal RLC% were observed upon inoculation with the three BEs in the alkaline soil of Castel, it remains to be determined if this was the principal mechanism contributing to improved P acquisition and aboveground biomass in that experiment.

Clarification of mechanisms together with new experiments with more diverse soils and characterization of BE-compatible fertilizers will give further insights about the window of conditions leading to successful use of BEs in agriculture. Nevertheless, our results show which kind of soils and fertilizers type is more responsive to BE inoculation.

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