

Plant growth-promoting potential of *Bacillus* species isolated from the rhizosphere of crops in Sudan and Saudi Arabia

Elsorra Elamin Idris^{a,b,*}, Michael Hemkemeyer^{c,d}, Florian Wichern^c

^aDepartment of Botany and Agricultural Biotechnology, University of Khartoum, Sudan

^bThe Faculty of Science and Arts, Al-Ula-Branch, Taibah University, Kingdom of Saudi Arabia

^cSoil Science and Plant Nutrition, Sustainable Food Systems Research Centre, Rhine-Waal University of Applied Sciences, Germany

^dSoil- and Groundwater-Management, University of Wuppertal, Germany

Abstract

There is an increasing interest in using plant growth-promoting rhizobacteria as alternatives for fertilisers and pesticides in sustainable agriculture. In this study, rhizosphere soil samples from 15 crop species in Sudan and Saudi Arabia were extracted, and 113 rhizobacterial isolates were obtained. Out of eight *Bacillus* isolates, seven were identified as members of the *Bacillus cereus* group, which is mainly differentiated by their plasmid-driven phenotypes. The eight strains were tested for their plant growth stimulatory effects on maize (*Zea mays* L.) and wheat (*Triticum aestivum* L.) using a model biotest under controlled environmental conditions in a growth chamber. Depending on application form, i.e. viable cells or their culture supernatant, and applied concentrations, six isolates stimulated maize plant growth. Similarly, six isolates enhanced wheat growth, but the influence of the single isolates differed between and within plant species, indicating plant-specific responses. Furthermore, diversity of rhizospheric members of the *B. cereus* group is highlighted, as all seven isolates differed in terms of colony traits, capacity to produce indole-3-acetic acid, and response by the maize and wheat plants. Overall, this study indicates the potential of plant growth-promoting *Bacillus* strains for commercial application promoting further investigation in soil and under field applications.

Keywords: *Bacillus cereus* group, indole-3-acetic acid, maize and wheat crops, plant growth-promoting rhizobacteria, rhizosphere-microbe interaction

1 Introduction

The rising global human population, coupled with the continuous degradation of land and soil resources, place a great threat to food production. Resource poor farmers, especially those affected by the effects of climate change such as droughts, heatwaves and flooding, cannot afford most of the means of modern agriculture, such as synthetic fertiliser, pesticides and modern mechanisation. Hence, there is a need to seek alternative means to improve agricultural production systems. Synthetic fertilisers are employed as inputs to boost productivity, however their use is undermined due to their negative impacts on the soil such as increasing soil acidity (Turan *et al.*, 2007), reduction of beneficial soil microbial populations, and interference with plant growth (Asuming-Brempong & Aferi, 2014). Additionally, the growing costs

of synthetic fertilisers and increasing demand for chemical-free foods necessitate the search for effective alternatives to boost food production.

Bio-fertilisers are currently being considered as effective and environmentally safe alternatives to synthetic fertilisers (Emmert & Handelsman, 1999; Gerhardson, 2002). These include plant growth-promoting rhizobacteria (PGPR), which in recent times have found commercial utilisation in developing countries (Khan *et al.*, 2018). These bacteria increase plant growth (Illmer & Schinner, 1992; Glick *et al.*, 1999; Liu *et al.*, 2007; Tripura *et al.*, 2007; El-Sayed *et al.*, 2014) and also protect the plant from soil-borne diseases and suppress disease incidence in plants (Kloepper *et al.*, 1980). PGPR exert their influence on plants directly or indirectly through other rhizospheric species via the production of diverse extracellular molecules for communication and defence (Rosier *et al.*, 2018). These extracellular mo-

* Corresponding author: elsorraidris@yahoo.com

lecules can directly affect plant genetic and metabolic pathways and, thus, leading to enhanced growth, increased resistance to pathogens, and tolerance in plants (Rosier *et al.*, 2018).

Plants may contribute to shape their microbiome, while bacteria in the rhizosphere receive metabolites secreted from the plant roots that are used as nutrients (Lugtenberg & Kamilova, 2009). Thereby, these root exudates lead to an increase in microbial abundances and activities in the rhizosphere compared to bulk soil (Kuzyakov & Blagodatskaya, 2015). The selection of rhizospheric bacteria depends on their ecological niche and the rhizospheric processes derived from the interplay with plants leading to specific quantities and qualities of ions, primary and secondary metabolites including mucilage and exoenzymes along with altered redox conditions (Dutta *et al.*, 2013).

Previous research indicated a natural formulation advantage of Gram-positive PGPR of the genus *Bacillus* over their Gram-negative counterparts. The endospores formation offers a biological solution to the formulation problem due to their heat and desiccation resistance, which enhances their ability to survive hard conditions (Emmert & Handelsman, 1999; Logan & De Vos, 2009). Insufficient insight in the basic molecular principles of PGPR's actions, besides farmers' lack of knowledge on how to obtain successful formulations, have hindered commercial use. In recent years "omic"-approaches have revealed specific microbial metabolites, genes, and taxa down to species and even strain-level which benefit plant hosts and even influence their traits (Gutiérrez-Mañero *et al.*, 2001; Idris *et al.*, 2002; Idris *et al.*, 2004; Friesen *et al.*, 2011; Paterson *et al.*, 2017). In this context, *Bacillus amyloliquefaciens* subsp. *plantarum* FZB42 has become a Gram-positive model organism for investigating plant-PGPR interactions (Chowdhury *et al.*, 2015). Agribusiness companies are showing increasing interest in developing applications of PGPR and developing combinations of different species that best fit specific crop species and their habitat conditions (Dessaux *et al.*, 2016).

Based on these observations, the objective of the present study is to identify *Bacillus* isolates from the rhizosphere of various plants from Sudan and Saudi Arabian farms, aiming to assess plant-growth-promotion contributing potential.

2 Materials and methods

2.1 Rhizosphere soil sampling and study sites

Fifteen crops relevant to Sudan and Saudi Arabia were sampled in their vegetative growth stage in May 2019 (Table 1). Soil adhering to and between roots, i.e. the

rhizosphere, was collected aseptically within 0.5–1.0 cm depth and stored in plastic bags at 4 °C until further processing. The Sudanese samples were collected from the University of Khartoum top farm (Shambat area), Khartoum State. The soils are clay-textured with 58.3 % clay, 22.6 % silt and 19.1 % sand), non-saline (electrical conductivity EC <4 dS m⁻¹), non-sodic (sodium adsorption ratio (SAR) <8 meq L⁻¹), and calcareous (CaCO₃ >5 %). Soil pH and bulk density ranged between 7.4–8.0 and 1.5–1.9 g cm⁻³, respectively. The location has a mean annual temperature of 29.9 °C, and 121 mm precipitation (Ibrahim *et al.*, 2013). In Saudi Arabia the soils were collected from Al-Ula, Medina Province. The soil in Al-Ula is sandy-textured with 5.0 % silt and 6.1 % clay, with 3.8 % CaCO₃ and 0.1 % organic matter. This location has a mean annual temperature of 24.3 °C and a mean annual precipitation of 0.00 mm (maximum 0.60 mm) (Al-Omran *et al.*, 2019). Therefore, Al-Ula soil strongly depends on irrigation and shows pH 7.6, EC=0.85 dS m⁻¹, and SAR=3.4 meq L⁻¹ (Al-Omran *et al.*, 2019).

2.2 Isolation of Gram-positive non-phosphate solubilising bacteria

Spreading and pour plate viable count methods were used for bacterial isolation and culture. 0.1 g of rhizosphere soil sample was suspended in 1 mL sterile distilled water under aseptic conditions. Serial dilutions (10⁻²–10⁻⁶) were spread on Lytic Broth agar (10.0 g L⁻¹ peptone, 5.0 g L⁻¹ yeast extract, 10.0 g L⁻¹ NaCl, 15.0 g L⁻¹ agar, pH 6.8–7.0) and nutrient agar (5.0 g L⁻¹ tryptic peptone, 3.0 g L⁻¹ yeast extract, 10.0 mg L⁻¹ MnSO₄ · H₂O, 15.0 g L⁻¹ agar, pH 6.8–7.0). In addition, 0.1 mL of serial dilutions (10⁻²–10⁻⁴) were used in the pour plate method (Mudili, 2007) with 18 mL agar. Plates were incubated at 37 °C overnight. The number of colony forming units (CFU) were counted using a simple colony counter (Table 1). From each plate, one of the colonies, was selected and streaked on nutrient agar plates for purification. Isolates were screened visually (colony traits), microscopically (cell shape), and for Gram staining using the Gram staining kit (Carl Roth, Karlsruhe, Germany) according to the manufacturer's instructions. Gram-positive bacteria were further selected for the ability to solubilise phosphate using National Botanical Research Institute Phosphate (NBRIP) growth medium, which is an optimisation of the Pikovskaya medium (Pikovskaya, 1948), consisting of 10.0 g L⁻¹ D-glucose, 5.0 g L⁻¹ Ca₃(PO₄)₂, 0.1 g L⁻¹ (NH₄)₂SO₄, 0.25 g L⁻¹ MgSO₄ · 7H₂O, 0.2 g L⁻¹ KCl, 2.0 g L⁻¹ agar, and pH 7 (Nautiyal, 1999). Ca₃(PO₄)₂ was autoclaved separately and added to the NBRIP medium at a ratio of 1:4 immediately before pouring the plates. Isolates were incubated

Table 1: Collected samples from various Sudanese and Saudi plants and crops' rhizosphere.

Plant species	Origin	CFU* g ⁻¹ soil	Isolates number	Isolates selected
<i>Allium cepa</i> L.	Sudan	1.4×10 ⁴	2	
<i>Capsicum annuum</i> L.	Saudi Arabia	1.0×10 ³	13	E3-rp10, E4-rp2
<i>Citrus x sinensis</i> (L.) Osbeck	Saudi Arabia	1.3×10 ³	6	E8-or3
<i>Cyamopsis tetragonoloba</i> (L.) Taub.	Sudan	5.2×10 ⁴	5	
<i>Eruca vesicaria</i> (L.) Cav.	Saudi Arabia	5.3×10 ⁴	6	E7-e3
<i>Helianthus annuus</i> L.	Sudan	3.3×10 ⁴	7	
<i>Mangifera indica</i> L.	Saudi Arabia	5.0×10 ²	4	E1-m1, E2-m2
<i>Portulaca oleracea</i> L.	Sudan	7.7×10 ⁴	4	
<i>Punica granatum</i> L.	Saudi Arabia	1.2×10 ³	6	E5-pu2
<i>Raphanus raphanistrum</i> L.	Sudan	7.2×10 ⁴	6	
<i>Solanum lycopersicum</i> L.	Sudan	1.9×10 ⁴	20	
<i>Sorghum bicolor</i> (L.) Moench	Sudan	6.7×10 ⁴	9	E6-s8
<i>Vicia faba</i> L.	Sudan	1.2×10 ⁵	7	
<i>Vigna unguiculata</i> (L.) Walp.	Sudan	1.1×10 ⁵	3	
<i>Zea mays</i> L.	Sudan	5.4×10 ⁴	15	

*Colony Forming Units.

at 30 °C for 10–12 days and examined for clear halo zones around the colonies, indicating phosphate solubilisation.

2.3 Sequencing of selected isolates' 16S rRNA genes

DNA of overnight cultures grown in lytic broth medium was extracted with the FastDNA® Spin Kit using the FastPrep®-24 Instrument (both MP Biomedicals, Santa Ana, CA) according to the manufacturer's instructions. Partial 16S rRNA genes were amplified using the primers S-D-Bact-0008-a-S-16 and S-D-Bact-1492-a-A-16 (i.e. GM3F and GM4R; Muyzer *et al.*, 1995). The 50 µL reaction mixture contained 0.5 µM of each primer, 0.2 µM of each dNTP (Carl Roth, Karlsruhe, Germany), 1x buffer, 0.025 U µL⁻¹ Hot-StarTaq DNA polymerase (both Qiagen, Hilden, Germany), and 2.0 µL 100-fold diluted template. Amplification started with initial denaturation at 95 °C for 1.5 min, followed by 12 cycles of denaturation at 95 °C for 60 s, annealing at 49 °C for 60 s, and elongation at 72 °C for 1.5 min. This was followed by 23 cycles maintaining the same conditions except for a lower annealing temperature of 44 °C, and ended with a final elongation at 72 °C for 10 min. All amplifications were conducted in duplicates, which were subsequently pooled and analysed on 1.5 % agarose gel. The pooled amplicants were purified using the Hi Yield® Gel/PCR DNA Fragments Extraction Kit (Süd-Laborbedarf GmbH, Gauting, Germany) according to the manufacturer's instructions and eluted in 50 µL elution buffer. DNA concentrations were determined in a BioSpectrometer® basic (Eppendorf, Hamburg, Germany) and diluted to 25 ng µL⁻¹ in PCR-grade water. Each sample was mixed with either S-D-Bact-0008-a-S-

16 or S-D-Bact-1492-a-A-16 and sent to Eurofins Genomics Sequencing GmbH (Köln, Germany) for LightRun sequencing. Sequences obtained with the reverse primer were converted into the reverse complement and merged with the according sequence obtained by the forward primer using MEGA7 (Kumar *et al.*, 2016). The same software was also used for alignment using the ClustalW approach (Larkin *et al.*, 2007). Finally, the construction of a Maximum Likelihood tree was based on the Kimura 2-parameter (Kimura, 1980) and 500-fold bootstrapping (Felsenstein, 1985). Selection of reference sequences of type strains was based on results obtained from the "Sequence Match" tool of the Ribosomal Database Project (Cole *et al.*, 2014). A sequence of *Clostridium tetani* was chosen as root.

2.4 Preparation of culture supernatants and determination of indole-3-acetic acid (IAA)

The ability to produce the plant growth-promoting substance IAA was determined using the method of Sarwar & Kremer (1995) modified by Idris *et al.* (2004). Isolates were pre-cultured in 10 mL glucose nutrient broth (GNB), containing 22.5 g L⁻¹ pancreatic peptone, 1.0 g L⁻¹ D-glucose, 2.0 g L⁻¹ K₂HPO₄, and 3.0 g L⁻¹ NaCl with an overall medium pH of 6.0, and incubated at 30 °C / 200 rpm overnight. The pre-culture was diluted 10-fold in 20 mL phosphate buffer saline and was incubated at 30 °C / 200 rpm until the optical density (OD₆₀₀) reached 1.0. Landy medium (Landy *et al.*, 1948) containing 5.0 g L⁻¹ L-glutamic acid, 0.25 g L⁻¹ MgSO₄, 0.25 g L⁻¹ KCl, 0.5 g L⁻¹ K₂HPO₄, 0.15 g L⁻¹ Fe₂(SO₄)₃ · 6H₂O, 5.0 mg L⁻¹ MnSO₄ · H₂O, 0.16

Table 2: Hydroponic system biotests: Effect of selected Gram-positive isolates on stimulatory plant growth of maize and wheat through the addition of culture supernatants (CF), and cells/spores.

Biotest nr.	Materials		Application of bacterial culture supernatant and fresh cells				
			Isolate Supernatant/CF		Cells/Spores		
	Plant	Isolate/s	%	(v/v)	%	(v/v)	(CFU [†] mL ⁻¹)
I	Maize	E1–E7*	0.1	1:1000 (20.0 µl)	0.1	1:1000 (20.0 µl)	20×10 ⁶
II	Maize	E1–E8*	0.05	1:2000 (10.0 µl)	0.05	1:2000 (10.0 µl)	10×10 ⁶
		E1-m1, E4-rp2, E5-pu2	0.025	1:4000 (5.0 µl)			
III	Wheat	E1–E8*	0.025	1:4000 (5.0 µl)	0.01	1:10000 (2.0 µl)	2×10 ⁶
		E1-m1, E3-rp10, E4-rp2	0.013	1:10000 (2.5 µl)			

* all selected isolates; † Colony Forming Units

g L⁻¹ CuSO₄ · 5H₂O, and 20.0 g L⁻¹ D-glucose was prepared. Glutamic acid and glucose were filter sterilised and added immediately before inoculation. Pre-cultured isolates in the phosphate buffer saline and distilled H₂O serving as control, respectively, were mixed with Landy medium at a ratio of 1:10 (v/v). These were incubated at 22 °C in the dark (flasks covered with aluminium foils), and limited aeration (75 rpm) for 72 hours. Cultures were then centrifuged at 14,000 × g and 4 °C for 30 min and supernatants were stored at –20 °C until further processing. Salkowski reagent containing 0.5 M FeCl₃ and 35 % HClO₄ at a ratio of 1:50 (v/v) (Gordon & Weber, 1951) was added to the supernatant at a ratio of 1:3 (v/v). The mixture was gently vortexed, incubated at 23 °C in the dark (covered with aluminium foil) for 25–30 minutes, and its absorbance was measured at 530 nm wavelength using a spectrophotometer.

2.5 Biotests on plant growth promotion of isolates and culture supernatant

Biotests were conducted using maize (*Zea mays* L. variety Ricardinio) and wheat (*Triticum aestivum* L. variety Maddox-2). Seeds kindly provided by KWS SAAT SE (Einbeck, Germany) were surface-sterilised in 3 % NaClO for 30 min and subsequently rinsed three times with sterile H₂O for 7 min. The seeds germinated under sterile conditions on moistened filter papers at 30 °C. Four-day-old seedlings were transferred to sterile test tubes (one seedling per tube, 16 cm × 1.5 cm) containing 20 mL of plant nutrient solution according to Göhler & Drews (1986) (Supplementary Table S1). Seedlings were held in the centre of the tube just above the solution with Niscofilm having a hole of 2.0 mm in diameter. The nutrient solution was either used as an untreated control or added with culture supernatants or fresh living cells from the selected isolates. Supernatants or cells were added two days after transfer of the seedlings, i.e. the seedlings were then six days old. Fresh growing cells were produced in nutrient broth at 37 °C at 200 rpm on the

day of application by addition of 20 µL of a cell density of 10⁹ CFU mL⁻¹, i.e. 0.1 % (v/v). Each treatment was replicated six times. Plants were kept in a growth chamber at 28 °C and 26 °C for maize and wheat, respectively, under 16 light hours, and 60 % relative humidity for two weeks. Plants were irrigated with sterilised tap water to compensate for loss of the nutrient solution by plant consumption. After two weeks plants were harvested, root length was measured, and shoots and roots were separated and dried at 60 °C for 72 hours.

2.6 Statistical analyses

For statistical analysis, a two-way analysis of variance based on Type II sums of squares (Langsrud, 2003) was performed in R (R Core Team, 2023) using the car package (Fox & Weisberg, 2019), followed by model simplification. A Box-Cox transformation was applied to the data using MASS (Venables & Ripley, 2002). If residuals were still non-parametric or heteroscedastic, the Scheirer-Ray-Hare test from the package rcompanion (Mangiafico, 2023) was used. Post hoc comparisons were conducted, using estimated marginal means (emmeans; Lenth, 2023) and Dunn's test using FSA (Ogle *et al.*, 2023), and they were supported by the cld- (multcomp; Hothorn *et al.*, 2008) and cldList-command (rcompanion), respectively. Figures were prepared with ggplot2 (Wickham, 2016) in combination with scales (Wickham *et al.*, 2023) and patchwork (Pedersen, 2023).

3 Results

3.1 Characterisation of isolated Gram-positive rhizobacteria

Fifteen crop species from the Sudan and Saudi Arabia were screened for Gram-positive plant growth-promoting rhizobacteria. In total, 113 isolates were obtained, of which

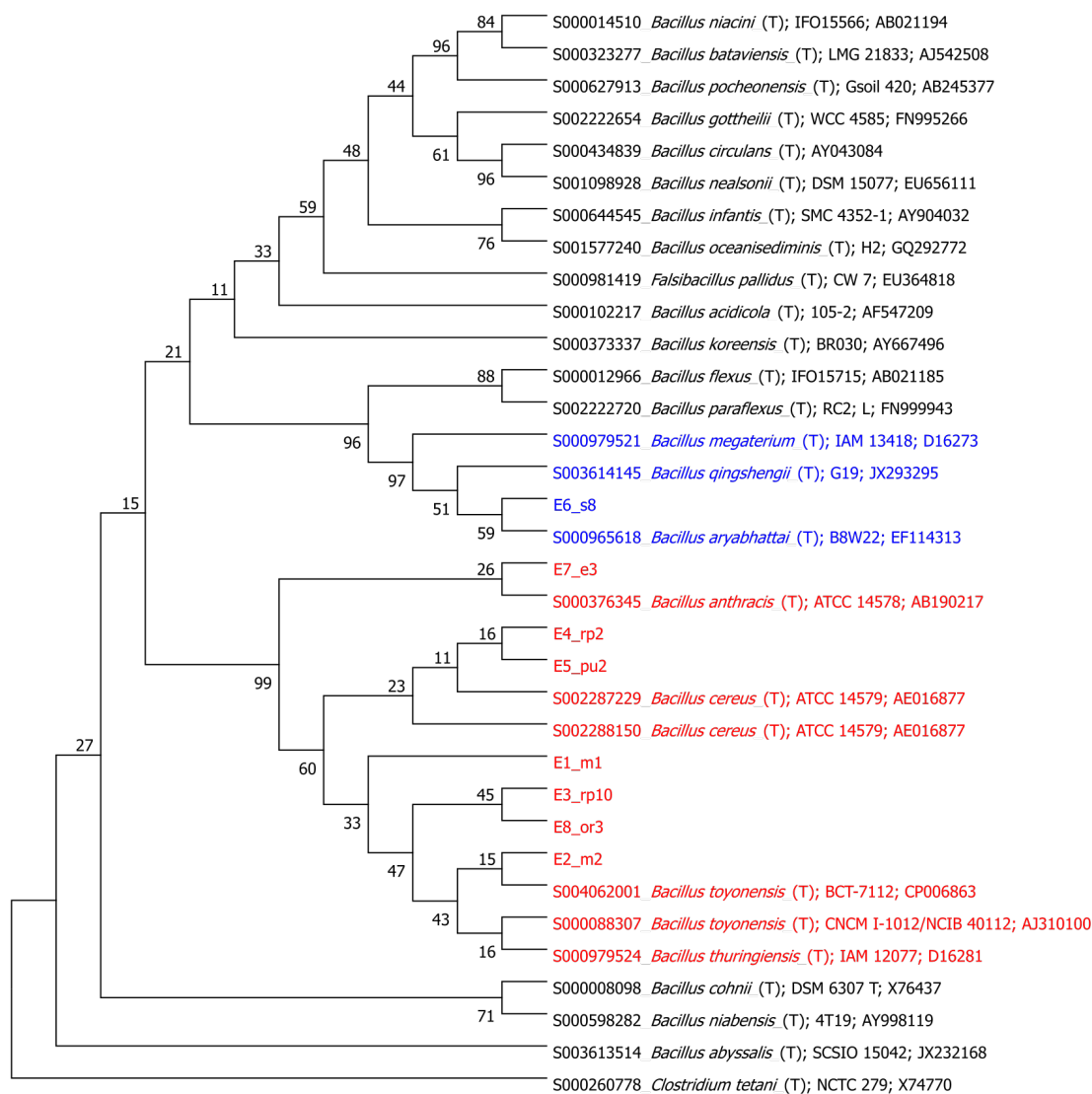


Fig. 1: Maximum likelihood tree of selected isolates. Closest type strains are highlighted in blue and red, respectively.

nine stained Gram-positive. One isolate derived from *Solanum lycopersicum*-rhizosphere had coccus-shaped cells and was able to solubilise phosphate, but was excluded from further plant biotests due to the focus on *Bacilli*. The remaining eight isolates were rod-shaped and tested negative for the phosphate solubilisation. These isolates were derived from six crop species, and, with the exception of isolates E6-s8 and E7-e3, were mostly found in Saudi Arabian soils containing low CFU counts (10^4 g⁻¹) (Table 1). Sequencing of 16S rRNA genes identified seven isolates as members of the *Bacillus cereus* group with E7-e3, which was obtained from the rhizosphere of *Eruca vesicaria*, clustering differently from the other six isolates (Fig. 1). Isolate E6-s8 obtained from Sudanese *Sorghum bicolor*-rhizosphere clustered together with *B. aryabhattai* and *B. qingshengii*

close to *B. megaterium*. However, all eight isolates differed in colony characteristics and in their cell shape, with some forming single rods while others formed chains (Table 3). Furthermore, the isolates showed a significant variation in the IAA production, ranging from undetectable level of E2-m2 and E7-e3 to 18 μ g mL⁻¹ in E1-m1 and E3-rp10 (Table 3).

3.2 Effect of *Bacillus* isolates on maize growth in a hydroponic system

To analyse the growth-promoting potential of Gram-positive rhizobacteria, the growing solution of maize plants was supplemented with 0.1 % separated culture supernatants, including indole-3-acetic acid (IAA), or 0.1 % cells,

Table 3: Traits of Gram-positive rhizosphere isolates grown on nutrient agar.

Isolate	Colony				Cell shape	IAA* ($\mu\text{g mL}^{-1}$)
	form	elevation	surface	colour		
E1-m1	irregular	raised	tough	creamy	rod	18.39
E2-m2	irregular	raised	tough	whitish-creamy	rod in chain	0.00
E3-rp10	irregular	raised	slimy	whitish	rod in chain	17.87
E4-rp2	irregular	flat	slimy	brownish	rod	1.75
E5-pu2	irregular	flat	slimy	whitish-creamy	rod	1.16
E6-s8	regular	flat	slimy/shine	whitish	rod	0.52
E7-e3	circular	raised	slime/shine	whitish-creamy	rod in chain	0.00
E8-or3	irregular	raised	slimy/shine	whitish	rod in chain	1.75

*Indole-3-acetic acid.

equivalent to 20×10^6 CFU, of seven selected isolates (Biotest I, Table 2; E8-or3 was not included). Due to negative effects of E1-m1 culture supernatant (i.e. the isolate producing highest IAA concentration) and other isolates that showed no effect on plant growth, a second test was performed with halved concentration for all eight isolates (i.e. 0.05 % supernatant and 10×10^6 CFU, respectively), while, in addition, supernatants of isolates E1-m1, E4-rp2, and E5-pu2 were included at 0.025 % (Biotest II, Table 2).

At low concentrations of culture supernatants and cells root length was significantly increased by 10–19 % by five (E1-m1, E2-m2, E3-rp10, E5-pu2, E7-e3) of the eight isolates ($F=9.74$, $p < 0.001$; Fig. 2.A.) without differences between the form of application ($F=2.29$, $p = 0.107$). The other three isolates had no effect compared to the control. In the case of higher concentrations, the low power of the Dunn test was hardly able to discern the obvious increase by ~40 % by five isolates ($H=35.08$, $p < 0.001$), which were not congruent to the case of low concentrations (Fig. 2.B.). While both application forms of E2-m2 and E3-rp10 increased root length, cells of E1-m1 and E5-pu2 and supernatants of E4-rp2 increased root length, in contrast to supernatants and cells, respectively. Especially, supernatants of E1-m1 and E5-pu2 and cells of E6-s8 even decreased root lengths at high concentrations by up to 44 %. Even stronger effects were found for root dry mass. Except for cells of E4-rp2 and supernatants of E6-s8, dry mass increased by 22–56 % at low concentrations ($F=6.88$, $p < 0.001$; Fig. 2.C.). At high concentrations increases by five isolates including cells of E5-p2 ranged 20–43 % and again supernatants of E5-pu2 and cells of E6-s8, but also cells of E7-e3 reduced dry mass by 20–30 % ($F=32.11$, $p < 0.001$; Fig. 2.D.). These stronger effects were also reflected in the root length-to-dry mass ratios, as except for cells of E4-rp2 and E5-pu2 and supernatant of E6-s8, these ratios were 18–28 % lower than the control at low concentrations ($F=4.45$, $p < 0.001$; Supple-

mentary Fig. S1.A.) suggesting an impact on root diameter. However, at high concentrations only products by E1-m1 and E5-pu2 led to ~30 % significantly lower ratios ($F=12.62$, $p < 0.001$; Fig. S1.B.).

In general, the patterns of shoot and total dry mass were similar to those of root dry mass (Supplementary Figs. S1.C. and S1.F.). However, at low concentrations, the shoot-to-root ratio decreased from 1.2 to ~1.0 for all application forms ($H=4.20$, $p = 0.018$; Fig. 2.E.) independently of the isolate ($H=1.90$, $p = 0.076$). At high concentrations, a decrease from 1.1 to 0.9 was only found for cells of E1-m1 ($F=10.57$, $p < 0.001$; Fig. 2.F.), while the shoot-to-root ratio increased to 1.4 for cells of E7-e3. However, this latter case was not indicated as significant by the post hoc test.

3.3 Effect of *Bacillus* isolates on wheat growth in a hydroponic system

To analyse the growth-promoting potential of eight *Bacillus* isolates, the growing solution of wheat plants was supplemented with 0.025 % culture supernatant (from all selected isolates) and 0.013 % (from isolates E1-m1, E3-rp10, and E4-rp2), or 0.01 % fresh growing cells (equivalent to 2×10^6 CFU) of each selected isolate (Table 2).

All eight isolates were able to increase root length by 13–25 % in at least one application form ($F=11.38$, $p < 0.001$; Fig. 3.A.). Exceptions were culture supernatants of E2-m2 (0.025 %) and E4-rp2 (0.013 %) as well as cells of E1-m1 and E4-rp2. Isolates E2-m2 and E4-rp2 were also the only ones which did not significantly increase root dry mass by 44–62 % ($H=56.47$, $p < 0.001$; Fig. 3.B.) independently of the application form ($H=2.80$, $p = 0.247$). This phenomenon is probably mainly related to culture supernatant of E2-m2 and cells of E4-rp2, which also showed no significant differences for root length-to-dry weight ratios and shoot or total dry mass, which in the other cases were decreased and increased, respectively (Supplementary Fig. S2).

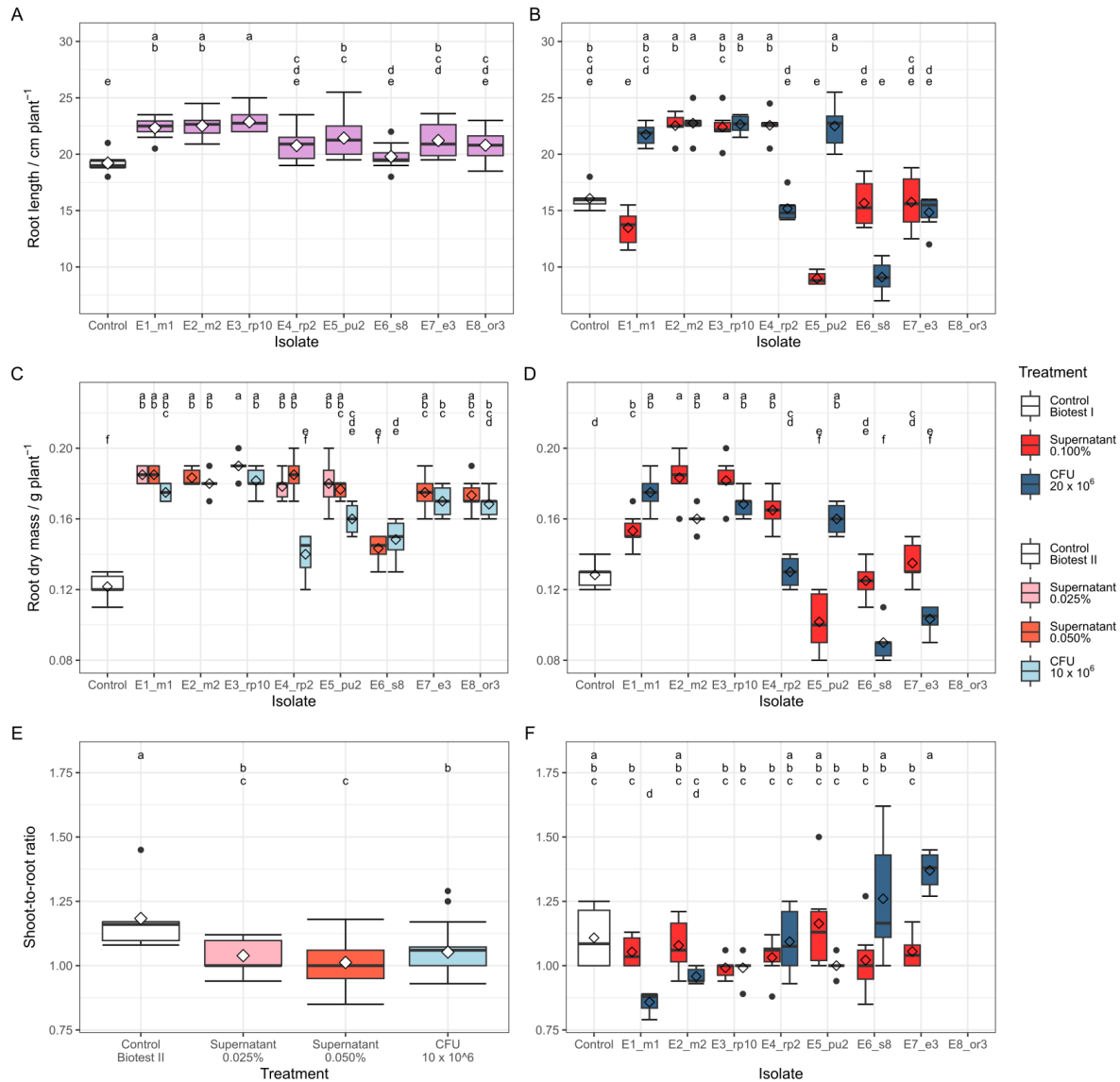


Fig. 2: Effect of bacterial supernatants and growing cells of *Bacillus* isolates on maize plants' root length (A, B), root dry mass (C, D), and shoot-to-root ratios (E, F) at low (biotest II, left panels) and high (biotest I, right panels) concentrations. Diamonds indicate mean values of six replicates; letters indicate differences between treatments.

The decrease in root length-to-dry weight ratios indicated a stronger effect of weight gain rather than length increase and, thus, suggests an impact on root width. Shoot-to-root ratios were decreased by six isolates in at least one application form from 2.4 to 1.9–2.0 ($F=6.89$, $p < 0.001$; Fig. 3.C.) with E2-m2 and E3-rp10 being restricted to cells and 0.013 % supernatant, respectively.

4 Discussion

This study aimed to isolate and identify plant growth-promoting Bacilli showing potential to be applied as bio-

stimulants for crops. Out of 113 bacterial strains isolated from crops relevant to Sudanese and Saudi agriculture, eight strains shared the trait of being Gram-positive without solubilising phosphorus.

Seven of the isolates were identified as members of the *Bacillus cereus* group. This group includes several *Bacillus* species with closely related phylogeny indiscernible by 16S rRNA genes and is best known for its pathogenic strains like *B. anthracis*, *B. cereus*, and *B. thuringiensis* (Ehling-Schulz et al., 2019). However, some strains have also been reported to promote plant growth and to suppress plant diseases (Halverson et al., 1993; Dutta et al., 2013; Hoornstra et al.,

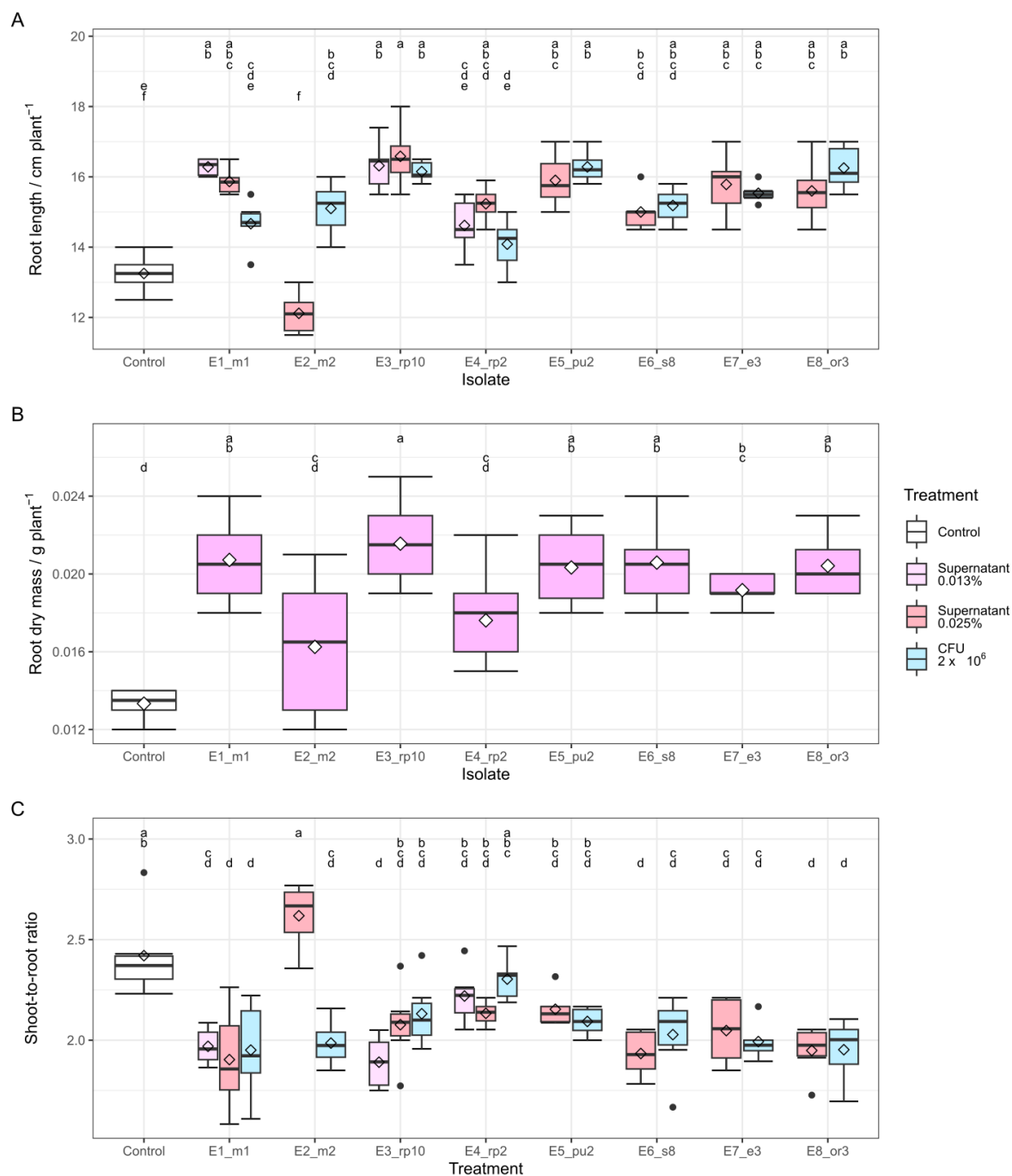


Fig. 3: Effect of bacterial supernatants and growing cells of *Bacillus* isolates on wheat plants' root length (A), root dry mass (B), and shoot-to-root ratios (C). Diamonds indicate mean values of six replicates; letters indicate differences between treatments.

2013; Ehling-Schulz *et al.*, 2015;). The seven isolates were derived from five of the sampled crops, i.e. *Capsicum annum*, *Citrus x sinensis*, *Eruca vesicaria*, *Mangifera indica*, and *Punica granatum*. These crops grew in a sandy soil in Saudi Arabia. As the numbers of colony forming units in these rhizosphere samples were lower than in the other, the seven isolates appear to belong to the most dominant cultiv-

able species in the respective crop rhizospheres. Their ability to form endospores (Logan & De Vos, 2009) enhances their ability to survive in the arid conditions of the Medina region of Saudi Arabia. Main differentiation between *B. cereus* group members is the phenotype often related to plasmid content (Ehling-Schulz *et al.*, 2019). All seven isolates including those originating from the same plant spe-

cies (E1-m1 and E2-m2 from *M. indica*; E3-rp10 and E4-rp2 from *C. annuum*) differed from each other in terms of colony traits, formation of cell chains, capacity to produce IAA, and response by maize and wheat plants. IAA production was observed in a *B. cereus* strain before (Wagi & Ahmed, 2019). But as no further attempts were made characterising the isolates, designation to species is not possible. However, as no isolate showed rhizoid colonies, *B. mycoides* and *B. pseudomycoides* can be precluded (Logan & De Vos, 2009).

The only other Gram-positive non-phosphorus solubilising strain obtained, i.e. E6-s8, was also identified as *Bacillus* species but due to the low bootstrapping values (<60) more than one species appears as possible close relative. This isolate was the only one which derived from the Sudanese clay soil and originated from *Sorghum bicolor* rhizosphere. Its traits determined, however, were mostly within the range of those of the *B. cereus* group isolates.

In maize, compared to the control, the different isolates showed various stimulatory effects in terms of plant root length and dry weights. This could be due to different plant-growth-promoting substances of different types and concentrations produced by the *Bacillus* isolates, as shown in the IAA production by these isolates. Interestingly, at high concentrations, i.e. 0.1 % culture supernatant or 0.1 % cells (20×10^6 CFU), some isolates showed either no positive effect (culture supernatants of E1-m1, E6-s8, E7-e3 and cells of E4-rp2) or even negatively affected plant development (culture supernatants of E5-pu2 and cells of E6-s8 and E7-e3). At two-fold diluted concentrations the adverse effects were not only reduced, but plants performed better in terms of dry mass. In contrast, root length, in positive cases, was two to four times higher at elevated concentrations than at lower ones. Thus, application concentrations appear to play an important role in the effects on plant development. However, further two-fold dilution, i.e. 0.025 vs. 0.05 % (or 0.013 vs. 0.025 % in case of wheat), had no significant effect.

Furthermore, depending on the isolate the form of application, i.e. culture supernatant vs. cells, especially at high concentrations, could result in significant differences as demonstrated for five and one isolates in case of maize and wheat, respectively. While culture supernatants of E4-rp2, E6-s8, and E7-e3 affected roots less positively or negatively, for isolates E1-m1, E5-pu2, and, for wheat, E2-m2 this was true for application as cells. It should be considered that culture supernatants affected growth immediately from the time of addition, while cells added need time to establish a symbiotic relationship with plant roots and then could start affect-

ing or secreting plant growth-promoting (PGP)-substances that promote the plant growth and development.

Overall, compared to the control the isolates showed positive effects on growth of maize plants at least at low concentrations, except for specific application forms of isolates E1-m1 high concentration supernatant, E5-pu2, and cells of E4-rp2 and E6-s8. Generally, this was also true for wheat, but the exceptions were E2-m2 and E4-rp2 and, considering root length, the better suited application form of E1-m1 and E3-rp10, was reversed. Accordingly, the response towards the different isolates was plant species-specific. Though it should be considered that wheat plants received four- to ten-fold diluted concentrations compared to maize plants. The isolates originated from rhizospheres of plant species other than the tested ones. Nevertheless, except for *M. indica*-derived E4-rp2, all isolates obtained from the subtropics rhizosphere soils, though plant species-specific and to different extents in terms of application form and concentration, were able to enhance growth of maize and wheat varieties common for Central Europe.

For PGPR species to be effective, they must successfully colonise roots at sufficient population densities to produce the beneficial effects. Plants and microorganisms can communicate with each other and plants respond to the presence of an organism by changing the composition of their root exudates. This initiates changes in the bacterial surface chemical composition, which may contribute to successful colonisation, as has been demonstrated for a *B. cereus* strain (Dutta et al., 2013). There are several hypothesised mechanisms by which PGPR subsequently stimulate plant growth, including phosphate solubilisation, enhanced mineral nutrient uptake, suppression of phytopathogens, and production of phytohormones (Lalande et al., 1989; Glick, 1995; Bowen & Rovira, 1999).

A direct effect of different IAA production capacities of the isolates on plant growth performance did not become clear, as, for instance, for maize isolates E1-m1 and E3-rp10, which had highest production capacities, were not significantly different from or even performed worse than E2-m2, for which no IAA production was detected. The promotion effect by E2-m2 can be explained by production of other unknown plant-growth-promoting substances. The negative effect on plant roots length by supernatant from E1-m1, disappeared in biotest II, by applying a lower concentration (0.05 %, 0.025 %), that confirmed presence of high concentration of IAA, or other PGP-substances, in the culture supernatant.

In the case of wheat, the culture supernatant of E2-m2 did not increase growth, unlike most other isolates, including E7-e3, which also did not produce detectable IAA. This

can again be explained by the fact that PGP-substances differ in type and concentration affecting wheat and maize plant growth differently. Furthermore, microbe-plant interactions can be affected by the plant species, due to differences in plant anatomy, physiology, and types of roots exudates (Lugtenberg & Kamilova, 2009). Thus, possible positive effects of E2-m2 PGP-substances occurred in the case of maize, but not wheat plants growth, were either concealed or even counteracted by other substances, e.g. toxic ones, or cell behaviours. Nevertheless, this study demonstrated that rhizosphere is a rich source of bacteria able to produce IAA as also shown for other rhizobacteria of the genus *Bacillus* (Idris et al., 2004; Idris et al., 2007). Furthermore, this study confirms results of other research studies (Gutiérrez-Mañero et al., 2001; Idris et al., 2004; Idris et al., 2007) on PGPR, showing that the effects of different crops' rhizosphere isolates depend on the plant species, as well as the application forms and concentrations of the isolates producing different PGP substances in various concentrations.

5 Conclusions

This study demonstrates that the *B. cereus* group together with other *Bacillus* species isolated from different crops, harbours relevant PGPR capable of supporting members of the Poaceae family. Plant responses, however, varied among isolates, and growth promotion was influenced by the form of application, i.e. living cells or culture supernatants. These findings highlight both the specificity of plant-microbe interactions and the diversity of *Bacillus* strains within rhizospheres. As this study was based on hydroponic batch experiments only, field performance cannot be extrapolated. However, this study indicates the potential application of *Bacillus* species as IAA producers and PGPR, warranting further investigation of their applicability in field conditions for sustainable crop production.

Supplement

The supplement related to this article is available online on the same landing page at: <https://doi.org/10.17170/kobra-2025081111375>.

Author contributions

Elsorra Idris: Funding acquisition (lead); Conceptualisation (lead); Investigation (lead); Formal analysis (supporting); Visualisation (equal); Writing – original draft (lead); Writing – review & editing (supporting); Michael Hemkemeyer: Investigation (supporting); Formal analysis (lead); Visualisation (equal); Writing – review & editing (lead); Florian

Wichern: Funding acquisition (supporting), Conceptualisation (supporting); Writing – review & editing (supporting).

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Conflict of interest

The authors report no declarations of interest.

References

- Al-Omran, A., Eid, S., & Alshammari, F. (2019). Crop water requirements of date palm based on actual applied water and Penman-Monteith calculations in Saudi Arabia. *Applied Water Science*, 9(4), 69. doi: 10.1007/s13201-019-0936-6.
- Asuming-Brempong, S., & Aferi, N. K. (2014). Isolation of phosphate solubilizing bacteria from tropical soil. *Global Advanced Research Journal of Agricultural Science*, 3(1), 8–15.
- Bowen, G. D., & Rovira, A. D. (1999). The rhizosphere and its management to improve plant growth. In D. L. Sparks (Ed.), *Advances in Agronomy* (pp. 1–102). Academic Press.
- Chowdhury, S. P., Hartmann, A., Gao, X., & Borriess, R. (2015). Biocontrol mechanism by root-associated *Bacillus amyloliquefaciens* FZB42 – a review. *Frontiers in Microbiology*, 6, 780. doi: 10.3389/fmicb.2015.00780.
- Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., Brown, C. T., Porras-Alfaro, A., Kuske, C. R., & Tiedje, J. M. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research*, 42(D1), D633–D642. doi: 10.1093/nar/gkt1244.
- Dessaux, Y., Grandclément, C., & Faure, D. (2016). Engineering the rhizosphere. *Trends in Plant Science*, 21(3), 266–278. doi: 10.1016/j.tplants.2016.01.002.

- Dutta, S., Rani, T. S., & Podile, A. R. (2013). Root exudate-induced alterations in *Bacillus cereus* cell wall contribute to root colonization and plant growth promotion. *PLOS ONE*, 8(10), e78369. doi: 10.1371/journal.pone.0078369.
- Ehling-Schulz, M., Frenzel, E., & Gohar, M. (2015). Food–bacteria interplay: pathometabolism of emetic *Bacillus cereus*. *Frontiers in Microbiology*, 6, 704. doi: 10.3389/fmicb.2015.00704.
- Ehling-Schulz, M., Lereclus, D., Koehler, T. M., Fischetti, V. A., Novick, R. P., Ferretti, J. J., Portnoy, D. A., Braunstein, M., & Rood, J. I. (2019). The *Bacillus cereus* group: *Bacillus* species with pathogenic potential. *Microbiology Spectrum*, 7(3), GPP3–0032–2018. doi: 10.1128/microbiolspec.GPP3–0032–2018.
- Emmert, E. A. B., & Handelsman, J. (1999). Biocontrol of plant disease: a (Gram-) positive perspective. *FEMS Microbiology Letters*, 171(1), 1–9. doi: 10.1111/j.1574-6968.1999.tb13405.x.
- Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution*, 39(4), 783–791. doi: 10.1111/j.1558-5646.1985.tb00420.x.
- Fox, J., & Weisberg, S. (2019). *An R Companion to Applied Regression*. (3rd ed.). Thousand Oaks, CA: Sage.
- Friesen, M. L., Porter, S. S., Stark, S. C., von Wettberg, E. J., Sachs, J. L., & Martinez-Romero, E. (2011). Microbially mediated plant functional traits. *Annual Review of Ecology, Evolution, and Systematics*, 42(1), 23–46. doi: 10.1146/annurev-ecolsys-102710-145039.
- Gerhardson, B. (2002). Biological substitutes for pesticides. *Trends in Biotechnology*, 20(8), 338–343. doi: 10.1016/S0167-7799(02)02021-8.
- Göhler, F., & Drews, M. (1986). Sink-source-Beziehungen bei Gewächshaustomate und -gurke im NFT-Verfahren. *Archiv für Gartenbau*, 34(2), 109–117.
- Glick, B. R. (1995). The enhancement of plant growth by free-living bacteria. *Canadian Journal of Microbiology*, 41(2), 109–117. doi: 10.1139/m95-015.
- Gordon, S. A., & Weber, R. P. (1951). Colorimetric estimation of indoleacetic acid. *Plant Physiology*, 26(1), 192–195. doi: 10.1104/pp.26.1.192.
- Gutiérrez-Mañero, F. J., Ramos-Solano, B., Probanza, A., Mehrouachi, J., Tadeo, F. R., & Talon, M. (2001). The plant-growth-promoting rhizobacteria *Bacillus pumilus* and *Bacillus licheniformis* produce high amounts of physiologically active gibberellins. *Physiologia Plantarum*, 111(2), 206–211. doi: 10.1034/j.1399-3054.2001.1110211.x.
- Halverson, L. J., Clayton, M. K., & Handelsman, J. (1993). Variable stability of antibiotic-resistance markers in *Bacillus cereus* UW85 in the soybean rhizosphere in the field. *Molecular Ecology*, 2(2), 65–78. doi: 10.1111/j.1365-294X.1993.tb00001.x.
- Hoornstra, D., Andersson, M. A., Teplova, V. V., Mikkola, R., Uotila, L. M., Andersson, L. C., Roivainen, M., Gahmberg, C. G., & Salkinoja-Salonen, M. S. (2013). Potato crop as a source of emetic *Bacillus cereus* and cereulide-induced mammalian cell toxicity. *Applied and Environmental Microbiology*, 79(12), 3534–3543. doi: 10.1128/AEM.00201-13.
- Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometrical Journal*, 50(3), 346–363. doi: 10.1002/bimj.200810425.
- Ibrahim, M. M. M., Dafalla, M. S., Elhag, A. M. H., & Ibrahim, S. I. (2013). Land evaluation of University of Khartoum top farm using remote sensing and geographical information system. *International Journal of Scientific and Research Publications*, 3(2), 1–6.
- Idris, E. E., Bochow, H., Ross, H., & Borriss, R. (2004). Use of *Bacillus subtilis* as biocontrol agent. VI. Phytohormone-like action of culture filtrates prepared from plant growth-promoting *Bacillus amyloliquefaciens* FZB24, FZB42, FZB45 and *Bacillus subtilis* FZB37. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz / Journal of Plant Diseases and Protection*, 111(6), 583–597.
- Idris, E. E., Iglesias, D. J., Talon, M., & Borriss, R. (2007). Tryptophan-dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. *Molecular Plant-Microbe Interactions*, 20(6), 619–626. doi: 10.1094/mpmi-20-6-0619.
- Idris, E. E., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R., Bochow, H., Richter, T., & Borriss, R. (2002). Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB45 contributes to its plant-growth-promoting effect. *Microbiology*, 148(7), 2097–2109. doi: 10.1099/00221287-148-7-2097.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*, 16(2), 111–120. doi: 10.1007/Bf01731581.
- Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Molecular Biology and Evolution*, 33(7), 1870–1874. doi: 10.1093/molbev/msw054.

- Kuzyakov, Y., & Blagodatskaya, E. (2015). Microbial hot-spots and hot moments in soil: Concept & review. *Soil Biology and Biochemistry*, 83, 184–199. doi: 10.1016/j.soilbio.2015.01.025.
- Lalande, R., Bissonnette, N., Coutlée, D., & Antoun, H. (1989). Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. *Plant and Soil*, 115(1), 7–11. doi: 10.1007/BF02220688.
- Landy, M., Warren, G. H., Rosenman, S. B., & Colio, L. G. (1948). Bacillomycin: An antibiotic from *Bacillus subtilis* active against pathogenic fungi. *Proceedings of the Society for Experimental Biology and Medicine*, 67(4), 539–541. doi: 10.3181/00379727-67-16367.
- Langsrud, Ø. (2003). ANOVA for unbalanced data: Use type II instead of type III sums of squares. *Statistics and Computing*, 13(2), 163–167. doi: 10.1023/A:1023260610025.
- Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J., & Higgins, D. G. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics*, 23(21), 2947–2948. doi: 10.1093/bioinformatics/btm404.
- Lenth, R. (2023). emmeans: Estimated Marginal Means, aka Least-Squares Means. <https://CRAN.R-project.org/package=emmeans>. R package version 1.8.9.
- Logan, N. A., & De Vos, P. (2009). Genus I. *Bacillus* Cohn 1872, 174AL. In P. De Vos, G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K.-H. Schleifer, & W. B. Whitman (Eds.), *Bergey's Manual of Systematic Bacteriology - Second Edition - Volume Three - The Firmicutes* (pp. 21–128). Dordrecht, Heidelberg, London, New York: Springer.
- Lugtenberg, B., & Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology*, 63(1), 541–556. doi: 10.1146/annurev.micro.62.081307.162918.
- Mangiafico, S. (2023). rcompanion: Functions to Support Extension Education Program Evaluation. <https://CRAN.R-project.org/package=rcompanion>. R package version 2.4.34.
- Mudili, J. (2007). *Introductory Practical Microbiology*. Oxford: Alpha Science International.
- Muyzer, G., Teske, A., Wirsén, C. O., & Jannasch, H. W. (1995). Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Archives of Microbiology*, 164(3), 165–172. doi: 10.1007/BF02529967.
- Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiology Letters*, 170(1), 265–270. doi: 10.1111/j.1574-6968.1999.tb13383.x.
- Ogle, D. H., Doll, J. C., Wheeler, P., & Dinno, A. (2023). FSA: Simple Fisheries Stock Assessment Methods. <https://CRAN.R-project.org/package=FSA>. R package version 0.9.5.
- Paterson, J., Jahanshah, G., Li, Y., Wang, Q., Mehnaz, S., & Gross, H. (2017). The contribution of genome mining strategies to the understanding of active principles of PGPR strains. *FEMS Microbiology Ecology*, 93(3), fiw249. doi: 10.1093/femsec/fiw249.
- Pedersen, T. L. (2023). patchwork: The composer of plots. <https://CRAN.R-project.org/package=patchwork>. R package version 1.1.3.
- Pikovskaya, R. I. (1948). [Mobilization of phosphorus in soil in connection with vital activity of some microbial species]. *Mikrobiologiya*, 17, 362–370.
- R Core Team (2023). R: A language and environment for statistical computing.
- Rosier, A., Medeiros, F. H. V., & Bais, H. P. (2018). Defining plant growth promoting rhizobacteria molecular and biochemical networks in beneficial plant-microbe interactions. *Plant and Soil*, 428(1), 35–55. doi: 10.1007/s11104-018-3679-5.
- Sarwar, M., & Kremer, R. J. (1995). Determination of bacterially derived auxins using a microplate method. *Letters in Applied Microbiology*, 20(5), 282–285. doi: 10.1111/j.1472-765X.1995.tb00446.x.
- Turan, M., Ataoglu, N., & Sahin, F. (2007). Effects of *Bacillus* FS-3 on growth of tomato (*Lycopersicon esculentum* L.) plants and availability of phosphorus in soil. *Plant Soil and Environment*, 53(2), 58–64. doi: 10.17221/2297-pse.
- Venables, W. N., & Ripley, B. D. (2002). *Modern Applied Statistics with S*. (4th ed.). New York: Springer.
- Wagi, S., & Ahmed, A. (2019). *Bacillus* spp.: potent microfactories of bacterial IAA. *PeerJ*, 7, e7258. doi: 10.7717/peerj.7258.
- Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York: Springer.
- Wickham, H., Pedersen, T. L., & Seidel, D. (2023). scales: Scale functions for visualization. <https://CRAN.R-project.org/package=scales>. R package version 1.3.0.