http://doi.org/10.1590/1678-992X-2021-0102

ISSN 1678-992X

SCIENTIA AGRICOLA

Research Article

Soybean extracts can improve plant development

André May[1]*, Evandro Henrique Figueiredo Moura da Silva[2], Nilson Aparecido Vieira Junior[2], Elke Simoni Dias Vilela[1], Michelli de Souza dos Santos[1], Luciana Fontes Coelho[1], Alexandre Pedrinho[3], Bruna Durante Batista[4], Ronaldo da Silva Viana[5]

[1]Embrapa Meio Ambiente, Rod. SP 340, km 127 – 13918-110 – Jaguariúna, SP – Brasil.
[2]Universidade de São Paulo/ESALQ – Depto. de Engenharia de Biossistemas, C.P. 09 – 13418-900 – Piracicaba, SP – Brasil.
[3]Universidade de São Paulo/ESALQ – Depto. de Ciência do Solo, Lab. de Microbiologia do Solo, C.P. 09 – 13418-900 – Piracicaba, SP – Brasil.
[4]Western Sydney University/Hawkesbury Institute for the Environment, Richmond NSW 2753 – Sydney – Australia.
[5]Universidade Estadual Paulista "Júlio de Mesquita Filho"/FCAT – Depto. de Produção Vegetal – Campus Dracena, Rod. Comandante João Ribeiro de Barros, km 651 – Bairro das Antas – 17900-000 – Dracena, SP – Brasil.
*Corresponding author <andre.may@embrapa.br>

Edited by: Fernando Dini Andreote

Received April 16, 2021
Accepted November 12, 2021

ABSTRACT: Microbial biodiversity of an environment can contribute to plant growth and increase crop yield. Plant extracts from soybean (*Glycine max* (L.) Merrill) were investigated on soybean plants grown after inoculation with these extracts. Soil samples were collected from two important Brazilian soybean-growing regions to produce the extracts used in the experiments. The extracts were produced with material collected from aboveground biomass and rhizosphere of soybean plants cultivated in a controlled greenhouse (phase 1). The extracts produced in phase 1 were applied in a sequential experiment (phase 2). Phase 2 was conducted to examine the plant microbiome after the microbial alteration process in the greenhouse through seed inoculation with the extracts produced previously. Samples of aboveground biomass were collected to determine root dry matter and crop yield. Bacterial 16S rRNA sequences were processed to determine the final microbial content of soybean. The inoculated treatments had lower species diversity; however, the phyla Firmicutes and Bacteroidetes were more abundant in the treatments than in the non-inoculated treatment. The soybean plant stem in the inoculated treatment also had a positive response to enrichment of the bacterial classes Betaproteobacteria, Bacilli and Flavobacteria. Inoculation affected the microbial composition of soybean plants. The alteration of microbiome changes revealed differences for crop yield between the inoculated and non-inoculated treatments, with up to 93.5 % higher crop yields per plant according to the extract applied.

Keywords: microorganisms, sustainable agriculture, enzymes, microbiome

Introduction

The practices of sustainable agriculture can ensure food security for the next decades (Garnett and Godfray, 2012). Plant-associated microbiomes have been of great interest for their potential to improve crop yield through the application of optimized environmental-friendly microbial biofertilizers (Kour et al., 2020) and biopesticides (Pavela and Benelli, 2016). Therefore, integrating crop management and the environmental microbiome has become to increase crop yields.

The use of microbial inoculants for sustainable agriculture has grown in many regions worldwide (Hassen et al., 2016). Accordingly, the use of microbes as an agricultural input is able to replace several inputs of high environmental impact (Kour et al., 2020). Plant growth-promoting microbes play a significant role in the dynamics of various processes, such as OM decomposition (Pérez-Valera et al., 2020), availability of several nutrients (Babalola and Glick, 2012), and decrease in plant stress (May et al., 2019).

Inoculants with only one microorganism have difficulty to stablish, as it is not adapted to environmental conditions. Therefore, studies report on advantages in using the microbiome, that is, the indigenous (native) community, to improve plant resistance to biotic/abiotic stresses, demonstrating that these strains are adapted to the plant environment and can increase the chances of the inoculum survive, favoring plant growth (Marulanda et al., 2009; Banerjee et al., 2017; May et al., 2021).

Microorganisms colonize all plant parts, which leads to their constant interaction. However, regions closer to the root show greater variability of microbial species (Peiffer et al., 2013), with great competition in the rhizosphere and changes in the environmental conditions of selection, according to plant species (Haichar et al., 2008), genotype (Lundberg et al., 2012), and pedological characteristics (Tkacz et al., 2015).

The plant microbiome can be beneficial or detrimental to its health, depending on its ecological niche. The manipulation of the plant microbiome allows inhibiting the occurrence of plant diseases (Andrews, 1992) and increase productivity (Bakker et al., 2012) by reducing the use of chemical inputs (Adesemoye et al., 2009).

In this study, we hypothesized that the use of inoculum with microbiological content from extract of plants of the same species can change crop yield, based on the concept debated by May et al. (2021). Therefore, we evaluated the response of soybean (*Glycine max* (L.) Merrill) to seed inoculation with extracts made from soybean grown in the previous season. The specific objective was to investigate whether seed inoculation can change plant microbial diversity and if the use of plant extract from aboveground biomass applied to soybean seeds increases crop yield.

Materials and Methods

Phase 1: Building the clonal garden

Soil was collected from Itapetininga (S-IT), state of São Paulo (SP) (23°35′30′′ S, 48°03′11′′W, altitude of 670 m), and Castro (S-CA), state of Paraná (PR) (24°47′28′′ S, 50°00′43′′W, altitude of 999 m), Brazil, after the harvest of soybean to build a clonal garden to cultivate soybean plants (Table 1). Soybean plants are defined here as plants cultivated with environmental control in pots with no incidence of diseases or pests during the developmental cycle. The aim was to produce fresh mass from these plants to be used in phase 2 of the study. In each soil collection, the fields were selected according to their soybean yield history by choosing the site with the highest crop yield (> 4.5 Mg ha^{-1}) of each farm.

According to previous crop management reports, the areas were free of phytosanitary problems. The S-IT soil was collected from a no-tillage area established 17 years before, in a soybean-maize (*G. max* L.- *Zea mays* L.) succession system, whereas S-CA was collected from a farm that adopted no-tillage for more than 40 years, with various species in crop rotation (e.g., soybean, maize, wheat, and oats - *G. max* L., *Z. mays* L., *Triticum* spp. L., *Avena sativa* L.) and annual application of organic wastes, such as pig manure.

In total, 35 soil samples were collected in a zigzag pattern from a 10-ha site, in both S-IT and S-CA. Each sample corresponded to approximately 3 kg of soil from the 0 – 0.20 m soil layer. Before collection, the mulch above the soil layer was discarded. The samples of each soil were then mixed to obtain homogeneous soil to fill plastic pots (0.20 m high × 0.16 m wide) with approximately 5 kg of soil per pot for building the clonal garden in Jaguariúna, SP, Brazil (22°43′06′′ S, 47°01′09′′ W, altitude of 570 m). For soil collection, we used agricultural tools, such as a hoe and a shovel, sanitized in running water at each new sampling point.

In phase 1, the soybean was cultivated in pots for maximum environmental control of each plant individually for each soil type collected from the production areas. The pots were used to sow soybean in two phases: (I) production of extracts; and (II) inoculation of soybean seeds using the extract produced in (I). The procedures are detailed in the subsequent sections. The irrigation management consisted of frequent irrigation with a drip system to meet water requirements of the crop.

Phase 1: Production of extracts

Soybean cultivar M5917 IPRO (*G. max* L.) was sown on 7 July 2018 using three seeds per pot, which were inoculated with *Bradyrhizobium japonicum* (10^9 viable cells kg of seed^{-1}) in a clonal garden in Jaguariúna, SP, Brazil. The experiment was conducted in pots filled with both soil types (S-IT and S-CA) in a completely randomized design. The greenhouse was controlled with the temperature set at 28 °C (day) and 19 °C (night) and a 12-h photoperiod until the beginning of flowering (R1 stage), when the soybean plants were collected to make the extracts. The extracts were produced with material collected from the aboveground biomass (I-1) and rhizosphere (I-2) of soybean.

To produce I-1, the collected materials were cleaned by immersion in a solution of 2 % sodium hypochlorite, 0.1 % polyethylene glycol sorbitan monolaurate, and in sterile water. In each solution, the plants were shaken for about 10 s. The materials collected were then ground in a knife mill to obtain particles of 0.5 cm in length. Subsequently, a 1 % PVP stabilizing solution was applied to the ground mass. The materials were then pressed in a hydraulic press at a pressure of 15 t cm^{-2} and the fluid resulting from pressing was centrifuged at 7,000 rpm for 10 min to collect the microbial precipitate. For I-2, the material was washed with potassium phosphate buffer (pH 7).

Afterward, the material was filtered in a 100-mesh sieve for the separation and disposal of the solid phase. The fluid resulting from the filtering process was centrifuged at 5,000 rpm for 10 min to collect the microbial precipitate. Both microbial precipitates were stored in a cryoprotectant solution (20 % glycerol) at –80 °C for further use. After these processes, the produced and frozen extracts were lyophilized for later use.

Table 1 – Description of the sites where the soils were collected in Itapetininga, SP, and Castro, PR, Brazil.

Site	Soil collection date	Location	Soil class	Texture	Climate	Average temperature and precipitation
Itapetininga (S-IT)	2019	23°30′ S 48°02′ W 670 m a.s.l.	Oxisol	Clayey	Cfa	18.9 °C
						1175 mm
Castro (S-CA)	2019	22°43′ S 47°38′ W 988 m a.s.l.	Inceptisol	Clayey	Cfb	16.8 °C
						1553 mm

Chemical analysis (0 – 20 cm)															
Soil	OM	P	K	Ca	Mg	H + Al	S.B.	C.T.C.	V%	B	Cu	Fe	Mn	Zn	pH
	g dm^{-3}	mg dm^{-3}	———	———	mmol$_c$ dm^{-3}	———	———	———	%	———	———	mg dm^{-3}	———	———	
1	69	162	5	132	63	18	200	218	92	0.3	2	22	30	3	6.8
2	86	102	5	48	18	77	71	148	48	0.3	8	80	20	16	5.4

Soil 1 = IT soil for inoculant production in phase 1 and soil for phase 2 experiment; Soil 2 = C soil for inoculant production in phase 1; OM = organic matter; C.T.C. = Cationic Exchange Capability; V% = base saturation; S.B. = Sum of bases.

Phase 2: Extract application, growth conditions of potted plants and assessment

In the second phase of this experiment, soybean cultivar M5917 IPRO was sown on 19 Oct 2018 using three seeds per pot, which were inoculated in a greenhouse located in Jaguariúna, SP, Brazil. Each pot (0.2 m high × 0.16 m wide) was filled with approximately 5 kg of soil content from S-IT. The experiment was conducted to measure the enrichment of the microbiome after the microbial alteration process in the greenhouse through seed inoculation. The following five treatments were evaluated: T1 – extract inoculant I-1, using S-IT soil in phase 1; T2 – extract inoculant I-1, using S-CA soil in phase 1; T3 – extract inoculant I-2, using S-IT soil in phase 1; T4 – extract inoculant I-2, using S-CA soil in phase 1, and T5 – no inoculation. Each treatment was replicated seven times, totaling 35 experimental plots, in a completely randomized block design.

The extracts were applied before sowing, using 10 mL kg^{-1} of seeds for I-1 and I-2. The experiment was conducted without any stress or factors that hinder plant development. Samples of aboveground biomass were collected, and the root dry matter was determined between stages R5 and R6 (Fehr and Caviness, 1977). Then, the material was oven-dried to constant weight at 40 °C and weighed. Crop yield was determined in the final season, when the seeds reached 21 % water. Enzymatic and metataxonomic analyses were carried out to evaluate the final microbial content of soybean.

Fluorescein diacetate, β-glycosidase, acid phosphatase, arylsulfatase and urease were measured enzymatically. To determine the activity of these enzymes, soil samples from all treatments were collected near the rhizosphere of the plants, approximately 1 cm around the roots, at the R1 stage (Fehr and Caviness, 1977). The same procedure was followed for peroxidase by collecting leaves from the plants analyzed.

The β-glicosidase (Black et al., 1965; Eivazi and Tabatabai, 1988), acid phosphatase (Tabatabai and Bremner, 1969; Eivazi and Tabatabai, 1977), and arylsulfatase (Tabatabai and Bremner, 1970) activities were determined after 1 h of incubation at 37 °C with the specific substrates p-nitrophenyl-β-D-glucopyranoside, p- nitrophenol-phosphate and p-nitrophenyl sulfate. A standard curve was prepared using the p-nitrophenol reagent and results were expressed in nitrophenol g $soil^{-1}$ h^{-1}. The dosage of fluorescein diacetate (FDA) was determined by the hydrolysis method, following Ghini et al. (1998).

After developing a standard curve of hydrolyzed FDA at 100 °C, the activities were determined in µg hydrolyzed FDA mL $extract^{-1}$ g dry $soil^{-1}$ min^{-1}. Urease was determined by the approach proposed by Tabatabai and Bremner (1995), which is based on determining the ammonium released after incubating the soil with urea for 2 h at 37 °C. The amount of ammonia produced was determined by distillation and titration, and the activity was described in µg ammonia g dry $soil^{-1}$ h^{-1} (Tedesco et al., 1985).

The extract used for the analysis of the peroxidase enzyme activity was prepared by crushing a 1-g sample of soybean leaf in a mortar with liquid nitrogen. Afterward, 7 mL of 50 mM sodium phosphate buffer solution (pH 6.5) with 1 % (w v^{-1}) polyvinylpyrrolidone (PVP) and 1 mM phenylmethylsulfonyl fluoride (PMSF) were added. The solution was then centrifuged at 3500 rpm at 4 °C for 20 min and the supernatants were collected and stored in a freezer (–80 °C) until the analysis.

Peroxidase activity was determined by the reaction of 10 µL extract, 70 µL 0.01 mol sodium phosphate buffer L^{-1} (pH 6.5), 70 µL hydrogen peroxide, 3 mmol L^{-1} and 70 µL guaiacol 15 mmol L^{-1}, with absorbance reading at 470 nm using the Magellan software in 20 cycles of 30 s at 30 °C (Hammerschmidt et al.,1982). The results were expressed in units of peroxidase mg^{-1} leaf tissue min^{-1}, and one unit was defined as a 0.01 increase in absorbance per min of reaction per milligram of tissue (Halfeld-Vieira et al., 2006).

The analysis of variance (ANOVA) was performed and the means were compared by the Tukey test at 5 % significance for the data set (ground biomass, root dry matter, crop yield, and soil enzymes), using the R software (version 1.2.5001, 2007).

Phase 2: DNA extraction and 16S rRNA sequencing

Total DNA was extracted from the stems of the recipient plants. The DNeasy Power Lyzer PowerSoil DNA Isolation Kit was used for rhizospheric soil and the DNeasy Mini Plant kit for stem DNA extraction, following the manufacturer's instructions. DNA quality and quantity were assessed using Quibit NanoDrop 1000 spectrophotometry and 1 % sodium boric acid agarose gel electrophoresis (Brody and Kern, 2004).

For the taxonomic profiling, amplicon-sequencing was performed targeting the V3-V4 (Fehr and Caviness, 1977) region of the 16S rRNA gene of bacteria. The DNA sample libraries (under the five treatments for the shoots, in three replicates) were prepared using Miseq Reagent Kit v3, following the manufacturer's instructions for Illumina MiSeq platform (2 × 250 bp paired-end).

The V3-V4 region of the bacterial 16S rRNA gene was amplified with region-specific primers (515F/806R) (Caporaso et al., 2010). Each 25-µL PCR reaction contained 12.25 µL nuclease-free water, 5.0 µL of buffer solution 5 × (2 Mm $MgCl_2$), 0.75 µL of dNTP solution (10 mM), 0.75 µL of each primer (515 YF 40 µM and 806 R 10 µM), 1.0 unit of Platinum Taq polymerase High Fidelity at a concentration of 0.5 µL and 2.0 µL of template DNA. In addition, a control reaction was performed by adding water rather than DNA. The PCR reaction conditions were as follows: 95 °C for 3 min, 35 cycles at 98 °C for 20 s, 55 °C for 20 s, 72 °C for 30 s, and a final extension of 3 min at 72 °C.

After indexing, the Polimerase Chain Reaction (PCR) products were cleaned using Agencourt AMPure XP – PCR purification beads, according to the manufacturer's manual and quantified using the dsDNA BR assay kit on a Qubit 2.0 fluorometer. Once quantified, equimolar concentrations of each library were pooled into a single tube. After quantification, the molarity of the pool was determined and diluted to 2 nM, denatured and then diluted to a final concentration of 8.0 pM with a 20 % PhiX spike for loading into the Illumina MiSeq sequencing machine.

Phase 2: Data processing and analysis

The bacterial 16S rRNA sequences were processed using QIIME 2 (version 2017.11). First, the sequences were demultiplexed and the quality control was carried out in DADA2 (Callahan et al., 2016), using the consensus method to remove any remaining chimeric and low-quality sequences. Afterward, singletons, doubletons, chloroplast and mitochondria sequences were removed for further analyses. The taxonomic affiliation was performed at 99 % similarity using SILVA database (version 132) (Quast et al., 2013) and the matrices generated were used for the statistical analyses.

To compare the bacterial community structure between treatments, the Principal Component Analysis (PCA) was performed using Canoco 4.5 software, as described by Ter Braak and Smilauer (2002). Then, bacterial structures were clustered into operational taxonomic units (OTUs) by species biodiversity. To compare the differential abundance of bacteria between treatments, the OTU table was used as input in STAMP software (Parks and Beiko, 2010). The diversity indices for each sample were calculated using the Shannon's diversity index based on the OTU table using PAST (Paleontological Statistics Software, version 3) (Hammer et al., 2001) and compared using the Tukey HSD test. *P*-values were calculated based on two-sided Welch's t-test and corrected using the Benjamini-Hochberg FDR procedure.

To compare the bacterial community in stem and rhizosphere, the OTU data were transformed into relative proportions, and significant differences in the bacterial community structure were assessed by the Permutational Analysis of Variance (PERMANOVA), following the methodology described by Anderson, 2017, using PAST (Paleontological Statistics Software, version 3) (Hammer et al., 2001).

Table 2 – Mean values of above-ground biomass, root and grain dry matter under treatments.

Treatment	Above-ground	Root	Grain
	g per plant		
T1 (I-1, S-IT)	9.53 a	0.93 a	8.3 a
T2 (I-1, S-CA)	11.27 a	1.05 a	6.5 ab
T3 (I-2, S-IT)	9.37 a	0.62 a	8.9 a
T4 (I-2, S-CA)	10.08 a	0.99 a	7.8 a
T5 (non-inoculated)	9.68 a	0.85 a	4.6 b

Lowercase letters compare treatments by Tukey's test ($p < 0.05$).

Results

Crop yield differed between the inoculated and non-inoculated treatments. The soybean crop yield means differed between the non-inoculated treatments and inoculated treatments T1, T3, and T4. There was no difference between the studied treatments for aboveground biomass or root dry matter (Table 2). The bacterial consortia application could increase the abundance of beneficial microorganisms, with a positive effect on plant development, according to May et al. (2021).

Urease was higher in treatment T4 than in the control (T5) (Table 3). Peroxidase was higher in the control than in the other treatments.

Although the PERMANOVA analysis did not reveal differences for the bacterial community in the stem (p = 0.7192) (Figure 1) and rhizosphere (p = 0.1466) (Figure 2), unsupervised clustering using principal coordinate analysis of distance matrices indicated that seed inoculation explains the variation in our data set, with a 48 % differentiation of the plants inoculated with I-1 and I-2 in both soil treatments S-IT and S-CA, in relation to the control (Figure 1A).

There were changes in the microbial community composition of the soybean stem (Figure 1B), with the inoculated treatments showing lower OTUs than the non-inoculated treatment. The inoculated soybean treatments

Table 3 – Soil and leaf enzymatic activity using extracts produced with material collected from the above-ground biomass (I-1) and rhizosphere of soybean (I-2) combined with soil collected from Itapetininga (S-IT) and Castro (S-CA): T1 – I-1 with S-IT; T2 – I-1 with S-CA; T3 – I-2 with S-IT; T4 – I-2 with S-CA, and T5 – no inoculation.

Variable	T1	T2	T3	T4	T5
FDA (μg FDA hydrolyzate mL extract $^{-1}$ g dry soil^{-1} min^{-1})	2.2 a	2.2 a	2.1 a	2.1 a	2.4 a
β-glicosidase (μg p-nitrophenol g dry soil^{-1} h^{-1})	84.2 a	91.6 a	80.3 a	84.7 a	94.6 a
Phosphatase (μg p-nitrophenol g dry soil^{-1} h^{-1})	484.0 a	444.2 ab	395.7 b	409.4 ab	416.2 ab
Arylsulfatase (μg p-nitrophenol g dry soil^{-1} h^{-1})	116.8 a	103.6 a	110.5 a	120.4 a	121.7 a
Urease (μg ammonia g dry soil^{-1} h^{-1})	38.9 b	37.6 b	37.7 b	47.4 a	35.8 b
Peroxidase (mg^{-1} leaf tissue min^{-1})	5.8 b	4.9 b	4.6 b	5.4 b	13.1 a

Means followed by different letters in the row differ significantly according to Tukey's test at 5 % probability.

exhibited a substantial decrease in microbial diversity when compared with the non-inoculated treatment. Inoculation with I-1 reduced microbial diversity in S-IT, whereas the plants inoculated with I-2 had reduced microbial diversity in S-CA when compared to the non-inoculated treatment.

The first two principal components (PCs) explained 71.16 % of the variance. Overall, the treatments with inoculation of the aboveground biomass from Itapetininga and Castro were grouped together. Likewise, the treatments with inoculation of soil rhizosphere from Itapetininga and Castro were also grouped together. Root samples tended to cluster with shoots (Figure 2A). Changes occurred in the composition of the microbial community of the soybean stem (Figure 2B), and treatments inoculated with shoot extracts from soybean plants had lower OTUs than treatments inoculated with rhizosphere extracts from soybean plants.

The inoculated treatments showed decreased OTUs and species diversity, the results provided compelling evidence that inoculation affected the microbial composition of the soybean plants. The microbiome of the soybean plants was changed through seed inoculation with I-1 and I-2, with an increase in abundance of certain classes and phyla (Figure 3A). Our findings showed 20 phyla across treatments, with only five phyla reaching a relative frequency greater than 1 %, namely Actinobacteria, Proteobacteria, Cyanobacteria,

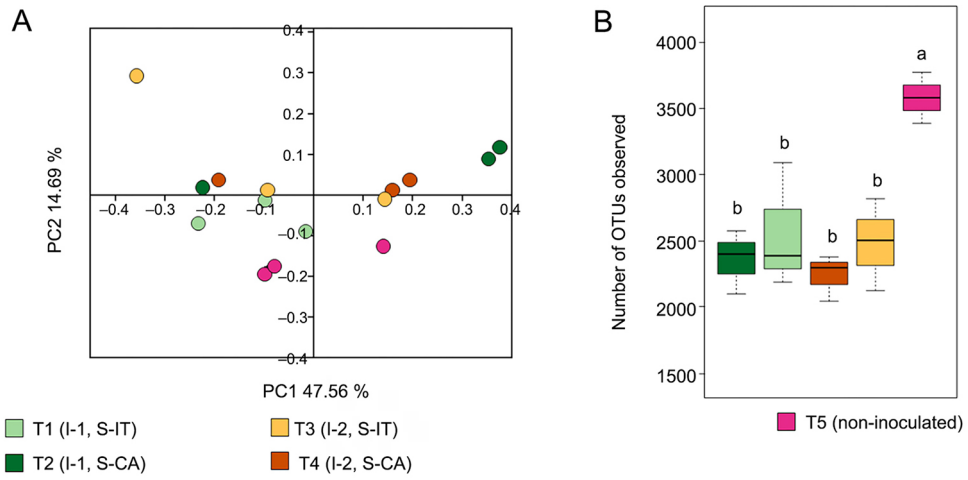

Figure 1 – Principal coordinate analysis of unweighted distances for the microbial community composition of soybean stem using extracts produced with material collected from the aboveground biomass (I-1) and rhizosphere (I-2) of soybean combined with soil collected from Itapetininga (S-IT) and Castro (S-CA). PC = principal coordinate (A); number of observed OTUs on the soybean stem in all treatments (B). Boxplots; the horizontal lines composing the box from top to bottom represent the 3rd quartile, the median, and the 1st quartile, respectively.

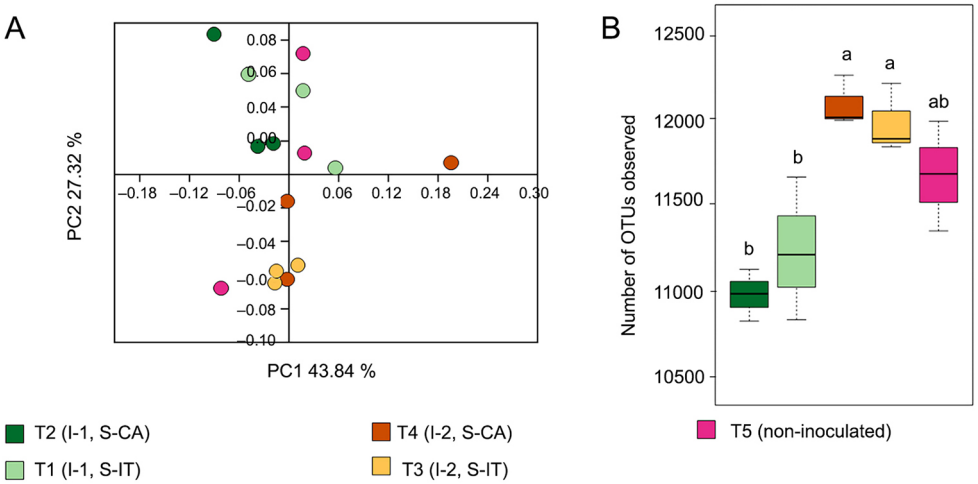

Figure 2 – Principal coordinate analysis of unweighted distances for the microbial community composition of soybean rhizosphere using extracts produced with material collected from the aboveground biomass (I-1) and rhizosphere (I-2) of soybean combined with soil collected from Itapetininga (S-IT) and Castro (S-CA). PC = principal coordinate (A); number of observed OTUs on the soybean stem in all treatments (B). Boxplots; the horizontal lines composing the box from top to bottom represent the 3rd quartile, the median, and the 1st quartile, respectively.

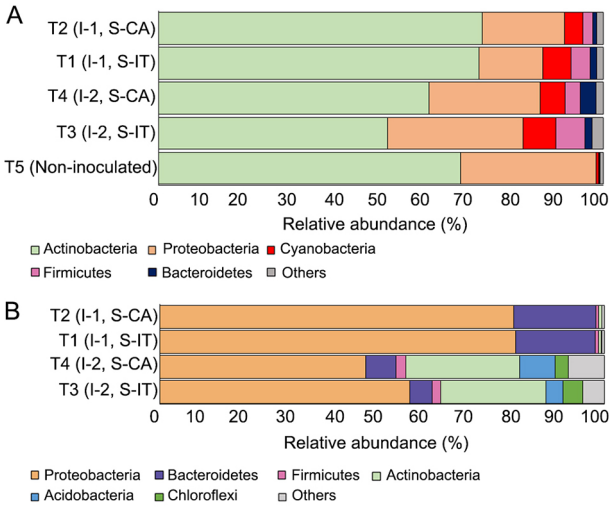

Figure 3 – Analysis of the phylogenetic distribution of bacteria. Relative abundance for each phylum under treatments (A); relative abundance (RA %) in extracts produced with material collected from the aboveground biomass (I-1) and rhizosphere (I-2) of soybean in both soil conditions from Itapetininga, SP (S-IT), and Castro, PR (S-CA) (B).

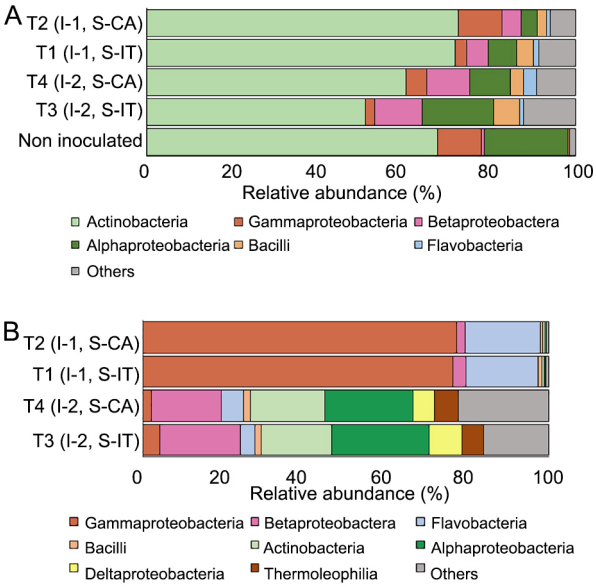

Figure 4 – Analysis of the distribution of bacterial classes. Relative abundance for each class under treatments (A); relative abundance (RA %) of the extracts produced with material collected from the aboveground biomass (I-1) and rhizosphere (I-2) of soybean in both soil conditions from Itapetininga, SP (S-IT), and Castro, PR (S-CA) (B).

Firmicutes, and Bacteroides. For all treatments, the phylum Actinobacteria had the highest relative abundance (50 to 70 %), followed by Proteobacteria (10 – 20 %).

The phyla Firmicutes and Bacteroidetes were more abundant in the inoculated than in the non-inoculated treatment, which may indicate an alteration of the microbiome despite inoculation procedures. There was an indirect effect of the extract on the phylum Cyanobacteria. Although more abundant in the inoculated soybean treatment, this phylum was not detected in I-1 or I-2 (Figure 3B) and the substances in the extracts possibly stimulate the development of these bacteria.

Actinobacteria dominated the collection of isolates from soybean stems, comprising a relative abundance of 50 to 70 % of the total isolates (Figure 4A). There was an enrichment of the bacterial classes Betaproteobacteria, Bacilli, and Flavobacteria on the soybean stems in T1, T2, T3, and T4 compared to the non-inoculated treatment (T5). These three bacterial classes were present in I-1 and I-2 (Figure 4B), showing evidence of microbiome modification by seed inoculation.

Discussion

Crop yield was greatly affected by the use of inoculants compared to the control with an increase of 71.2 % on average of yield of the inoculated treatments in relation to the non-inoculated control and 93.5 % of production increase of treatment 3 related to the control (Table 2).

Peroxidase is an enzyme made by the plant that has an antioxidant effect, which contributes to reducing oxygen-reactive pathogenic microorganisms (Pheomphun et al., 2019). The reduction in peroxidase in the soybean leaves treated with the extracts (Table 3) may be attributed to the reduction of biotic or abiotic stress (Nechet et al., 2017) in response to the presence of beneficial microorganisms in the extracts applied to the seeds, possibly affecting the productive expression of plants.

Actinobacteria was the most abundant phylum in the soybean rhizosphere. This group produces secondary antimicrobial metabolites, which are responsible for the decomposition of organic materials, that is, organic matter rotation and the carbon cycle (Ventura et al., 2007). The soybean rhizosphere harbors several phylum of Proteobacteria, which are responsible for promoting plant growth. One of them is Gammaproteobacteria (García-Salamanca et al., 2013), which survive for long periods and colonize the shoots of different plant species due to their ability to adapt to stress conditions (Mechaly et al., 2018). Gammaproteobacteria contribute to plant growth and suppression of plant tissue colonization/infection by pathogens, such as the genera *Pseudomonas*, *Xanthomonas* and *Enterobacter*, which can also produce indole-3-acetic acid (IAA) (Rasche et al., 2006).

The class Alphaproteobacteria showed significant enrichment in the soybean rhizosphere over five years (Mendes et al., 2015). Mendes et al. (2015) suggest that soybean plants selects a specific microbial community that is capable of bringing benefits to the plant, such as growth promotion and nutrition. Soybean plants treated with the different extracts had microbial communities distinct from the plants that did not receive extracts. The most abundant phyla in the inoculated treatments were Firmicutes and Bacteroidetes.

Bacteroidetes are particularly found in natural polymer biodegradation processes in soils (Pinhassi et al., 2004). In a study with soybean crop remnants, Lian et al. (2019) found that the presence of soybean residues increased the abundance of the phyla Bacteroidetes, Actinobacteria and Firmicutes. In the study of Mendes et al. (2015), in soils cultivated with soybean, the phylum Bacteroidetes was found at large amounts. Interestingly, this phylum includes plant growth-promoting bacteria (Soltani et al., 2010). The phylum Bacteroidetes is also known to promote plant growth through the production of auxins (Wolińska et al., 2017). Bacteroidetes have gram-negative bacteria, which compose several microorganisms relevant to the soil during the cycling of nutrients, such as C, N, and S (Shi et al., 2011).

The higher incidence of Bacteroidetes in extract I-2 from S-CA (T4) (Figure 3) may have influenced N release into the soil due to the greater urease activity (Table 3). In studies on rhizospheric soil enzymes, Yi et al. (2018) observed that a higher rate of urease allowed N increase in the plant, which correlated with the protein content of the grain. Thus, the phylum Bacteroidetes may have increased crop yield in the treatments with the extracts, since these bacteria can improve plant growth by providing or triggering the organization of growth-regulating substances by the plant itself and ultimately producing or changing the absorption of essential nutrients (Ahmad et al., 2008).

In the treatments that received the extracts, the phylum Firmicutes also occurred at a higher frequency. This phylum usually inhabits the rhizosphere, stems, and leaves of soybean (Ikeda et al., 2011) and are thus important for the control of plant pathogens (Bulgari et al., 2011).

The soybean plants inoculated in our study had a greater expression of the classes Bacilli and Flavobacteria, which belong to the phyla Firmicutes and Bacteroidetes, respectively. These phyla were possibly transferred from the extracts applied to the soybean seeds, increasing crop yield, since the aforementioned classes are known to have genera of plant growth-promoting bacteria.

Endophytic bacteria of the genus Bacillus belonging to the class Bacilli enhanced the development of sugarcane (*Saccharum officinarum* L.) seedlings (Silva et al., 2015), reduced phytopathogenic genera in soybean (Bezerra et al., 2013), and increased shoot dry matter in soybean (Chagas et al., 2017). In addition, there was an enrichment of the phylum Cyanobacteria in the treatments with seed inoculation. This phylum can be found in soils, in fresh and salt water, or even in areas with extremely high temperatures and/or very arid. Most species of this phylum have great nitrogen-fixing and photosynthetic abilities through changes in plant metabolism (Bocchi and Malgioglio, 2010) thus influencing crop yield, as observed in our results.

Sustainable management techniques that use seed inoculation methods containing the microbiome can contribute to the development of a less environmentally impactful agriculture, while ensuring high crop yield. To this end, further research should elucidate whether the species of microorganisms in the soybean extracts are specific for soybean genome to allow generating inoculants containing a specific microbial community for each associated plant genomic group.

Conclusions

The plant extract from the aboveground biomass of soybean plants can be applied to seeds of the same species. The use of soybean plant extract from the aboveground biomass applied to soybean seeds increased crop yield and changed the peroxidase enzyme tendency of the plants. The application of soybean plant extracts at sowing changed the soybean plant microbiome.

Acknowledgments

This research was supported by the São Paulo Research Foundation (FAPESP 2017/15556-5) (SISGEN ADCB4D2).

Authors' Contributions

Conceptualization: May, A.; Silva, E.H.F.M. **Data acquisition**: May, A.; Batista, B.D.; Vilela, E.S.D; Santos, M.S. **Data analysis**: Batista, B.D.; Vilela, E.S.D.; Santos, M.S.; Silva, E.H.F.M.; Coelho, L.F. **Design of methodology**: May, A.; Silva, E.H.F.M.; Batista, B.D.; Vilela, E.S.D. **Software development**: May, A.; Silva, E.H.F.M.; Batista, B.D.; Vilela, E.S.D. **Writing and editing**: May, A.; Silva, E.H.F.M.; Santos, M.S.; Batista, B.D.; Vilela, E.S.D.; Vieira Junior, N.A.; Viana, R.S.; Coelho, L.F.; Pedrinho, A.

References

Adesemoye, A.O.; Torbert, H.A.; Kloepper, J.W. 2009. Plant growth-promoting rhizobacteria allow reduced application rates of chemical fertilizers. Microbial Ecology 58: 921–929. https://doi.org/10.1007/s00248-009-9531-y

Ahmad, F.; Ahmad, I.; Khan, M.S. 2008. Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiological Research 163: 173–181. https://doi.org/10.1016/j.micres.2006.04.001

Anderson, M.J. 2017. Permutational multivariate analysis of variance (PERMANOVA). p. 1-15. In: Balakrishnan, N.; Colton, T.; Everitt, B.; Piegorsch, W.; Ruggeri, F.; Teugels, J.L., eds. Wiley StatsRef: statistics reference online. Wiley, Hoboken, NJ, USA. https://doi.org/10.1002/9781118445112.stat07841

Andrews, J.H. 1992. Biological control in the phyllosphere. Annual Review of Phytopathology 30: 603–635. https://doi.org/10.1146/annurev.py.30.090192.003131

Babalola, O.; Glick, B. 2012. The use of microbial inoculants in African agriculture: current practice and future prospects. Journal of Food, Agriculture and Environment 10: 540–549. https://doi.org/10.1234/4.2012.3447

Bakker, M.G.; Manter, D.K.; Sheflin, A.M.; Weir, T. L.; Vivanco, J.M. 2012. Harnessing the rhizosphere microbiome through plant breeding and agricultural management. Plant and Soil 360: 1–13. https://doi.org/10.1007/s11104-012-1361-x

Banerjee, A.; Bareh, D.A.; Joshi, S. 2017. Native microorganisms as potent bioinoculants for plant growth promotion in shifting agriculture (Jhum) systems. Journal of Soil Science and Plant Nutrition 17: 127–140. https://doi.org/10.4067/S0718-95162017005000010

Bezerra, G.A.; Macedo, D.A.; Nascimento, I.O.; Sousa, T.P.; Costa, N.B.; Sousa, L.F.R. A. 2013. Use of Bacillus spp. in the control pathogens in soybean seed variety BRS valuable RR. Revista Agroecossistemas 5: 68-73 (in Portuguese, with abstract in English).

Black, C.A.; Evans, D.D.; White, J.L.; Ensminger, L.E; Clark, F.E., eds. 1965. Methods of Soil Analysis: Chemical and Microbiological Properties. Part 2. ASA, Madison, MI, USA.

Bocchi, S.; Malgioglio, A. 2010. Azolla-anabaena as a biofertilizer for rice paddy fields in the Po Valley, a temperate rice area in northern Italy. International Journal of Agronomy 2010: 1-5. https://doi.org/10.1155/2010/152158

Brody, J.R.; Kern, S.E. 2004. History and principles of conductive media for standard DNA electrophoresis. Analytical Biochemistry 333: 1–13. https://doi.org/10.1016/j.ab.2004.05.054

Bulgari, D.; Casati, P.; Paola, C.; Daffonchio, D.; Quaglino, F.; Brusetti, L.; Bianco, P. 2011. Restructuring of endophytic bacterial communities in grapevine yellows-diseased and recovered vitis vinifera L. plants. Applied and Environmental Microbiology 77: 5018–5022. https://doi.org/10.1128/AEM.00051-11

Callahan, B.J.; McMurdie, P.J.; Rosen, M.J.; Han, A.W.; Johnson, A.J.A.; Holmes, S. P. 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nature Methods 13: 581–583. https://doi.org/10.1038/nmeth.3869

Caporaso, J.G.; Lauber, C.L.; Walters, W.A.; Berg-lyons, D.; Lozupone, C.A.; Turnbaugh, P.J.; Fierer, N.; Knight, R. 2010. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences of the United States of America 108: 4516-4522. https://doi.org/10.1073/pnas.1000080107

Chagas, L.F.B.; Martins, A.L.L.; Carvalho Filho, M.R.; Miller, L.O.; Oliveira, J.C.; Chagas Junior, A.F. 2017. Bacillussubtilis and Trichoderma sp. in biomass increase in soybean, beans, cowpea, corn and rice plants. Revista Agri-Environmental Sciences 3: 10–18 (in Portuguese, with abstract in English).

Eivazi, F.; Tabatabai, M.A. 1988. Glucosidases and galactosidases in soils. Soil Biology and Biochemistry 20: 601–606. https://doi.org/10.1016/0038-0717(88)90141-1

Eivazi, F.; Tabatabai, M.A. 1977. Phosphatases in soils. Soil Biology and Biochemistry 9: 167–172. https://doi.org/10.1016/0038-0717(77)90070-0

Fehr, W.R.; Caviness, C.E. 1977. Stages of Soybean Development. Iowa State University, IA, USA.

García-Salamanca, A.; Molina-Henares, M.A.; van Dillewijn, P.; Solano, J.; Pizarro-Tobías, P.; Roca, A.; Duque, E.; Ramos, J.L. 2013. Bacterial diversity in the rhizosphere of maize and the surrounding carbonate-rich bulk soil. Microbial Biotechnology 6: 36–44. https://doi.org/10.1111/j.1751-7915.2012.00358.x

Garnett, T.; Godfray, C.J. 2012. Sustainable Intensification in Agriculture: Navigating a Course through Competing Food System Priorities. University of Oxford, Oxford, UK.

Ghini, R.; Mendes, M.D.L.; Bettiol, W. 1998. Rate of hydrolysis of fluorescein diacetate (FDA) as indicator of microbial activity and soil suppressiveness to Rhizoctonia solani. Summa Phytopathologica 24: 238-242 (in Portuguese, with abstract in English).

Haichar, F.Z.; Marol, C.; Berge, O.; Rangel-Castro, J.I.; Prosser, J.I.; Balesdent, J.; Heulin, T.; 2 Achouak, W. 2008. Plant host habitat and root exudates shape soil bacterial community structure. The ISME Journal 2: 1221–1230. https://doi.org/10.1038/ismej.2008.80

Halfeld-Vieira, B.A.; Vieira Júnior, J.R.; Romeiro, R.S.; Silva, H.S.A., Baracat-Pereira, M.C. 2006. Induction of systemic resistance in tomato by the autochthonous phylloplane resident Bacillus cereus. Pesquisa Agropecuária Brasileira 41: 1247–1252. https://doi.org/10.1590/S0100-204X2006000800006

Hammer, O.; Harper, D.; Ryan, P. 2001. PAST: paleontological statistics software package for education and data analysis. Palaeontologia Electronica 4: 1–9.

Hammerschmidt, R.; Nuckles, E.M.; Kuć, J. 1982. Association of enhanced peroxidase activity with induced systemic resistance of cucumber to Colletotrichum lagenarium. Physiological Plant Pathology 20: 73–82. https://doi.org/10.1016/0048-4059(82)90025-X

Hassen, A.; Bopape, F.; Sanger, L.K. 2016. Microbial inoculants as agents of growth promotion and abiotic stress tolerance in plants. p. 23–36. In: Singh, D.P.; Singh, H. B.; Prabha, R. Microbial inoculants in sustainable agricultural. Springer, Basel, Switzerland. https://doi.org/10.1007/978-81-322-2647-5_2

Ikeda, S.; Anda, M.; Inaba, S.; Eda, S.; Sato, S.; Sasaki, K.; Tabata, S.; Mitsui, H.; Sato, T.; Shinano, T.; Minamisawa, K. 2011. Autoregulation of nodulation Interferes with impacts of nitrogen fertilization levels on the leaf-associated bacterial community in soybeans. Applied and Environmental Microbiology 77: 1973–1980. https://doi.org/10.1128/AEM.02567-10

Kour, D.; Rana, K.L.; Yadav, A.N.; Yadav, N.; Kumar, M.; Kumar, V.; Vyas, P.; Dhaliwal, H.S.; Saxena, A.K. 2020. Microbial biofertilizers: bioresources and eco-friendly technologies for agricultural and environmental sustainability. Biocatalysis and Agricultural Biotechnology 23: 1-11. https://doi.org/10.1016/j.bcab.2019.101487

Lian, T.-X.; Yu, Z.; Li, Y.; Jin, J.; Liu, X.; Tang, C.; Franks, A.; Liu, J.; Liu, J. 2019. The shift of bacterial community composition magnifies over time in response to different sources of soybean residues. Applied Soil Ecology 136: 163-167. https://doi.org/10.1016/j.apsoil.2019.01.001

Lundberg, D.S.; Lebeis, S.L.; Paredes, S.H.; Yourstone, S.; Gehring, J.; Malfatti, S.; Tremblay, J.; Engelbrektson, A.; Kunin, V.; Del Rio, T.G.; Edgar, R.C.; Eickhorst, T.; Ley, R.E.; Hugenholtz, P.; Tringe, S.G.; Dangl, J.L. 2012. Defining the core Arabidopsis thaliana root microbiome. Nature 488: 86–90. https://doi.org/10.1038/nature11237

Marulanda, A.; Barea, J.M.; Azcón, R. 2009. Stimulation of plant growth and drought tolerance by native microorganisms (AM fungi and bacteria) from dry environments: mechanisms related to bacterial effectiveness. Journal of Plant Growth Regulation 28: 115–124. https://doi:10.1007/s00344-009-9079-6

May, A.; Moreira, B.R.A.; Mascarin, G.M.; Viana, R.S.; Santos, M.S.; Silva, E.H.F.M.; Ramos, N.P.; Melo, I.S. 2019. Induction of drought tolerance by inoculation of Bacillus aryabhattai on sugarcane seedlings. Científica 47: 400–410. http://dx.doi.org/10.15361/1984-5529.2019v47n4p400-410

May, A.; Coelho, L.F.; Pedrinho, A.; Batista, B.D.; Mendes, L.W.; Mendes, R.; Morandi, M.A.B.; Barth, G.; Viana, R.S.; Vilela, E.S.D. 2021. The use of indigenous bacterial community as inoculant for plant growth promotion in soybean cultivation. Archives of Agronomy and Soil Science. https://doi.org./10.1080/03650340.2021.1964017

Mechaly, A.E.; Haouz, A.; Sassoon, N.; Buschiazzo, A.; Betton, J.-M.; Alzari, P.M. 2018. Conformational plasticity of the response regulator CpxR, a key player in Gammaproteobacteria virulence and drug-resistance. Journal of Structural Biology 204: 165–171. https://doi.org/10.1016/j.jsb.2018.08.001

Mendes, L.W.; Tsai, S.M.; Navarrete, A.A.; Hollander, M.; van Veen, J.A.; Kuramae, E. E. 2015. Soil-borne microbiome: linking diversity to function. Microbial Ecology 70: 255–265. https://doi.org/10.1007/s00248-014-0559-2

Nechet, K.L.; Vilela, E.S.D.; Heck, D.W.; Terao, D.; Halfeld-Vieira, B.A. 2017. Effect of increased UV-B radiation on biological control of the gray mold by Clonostachys rosea and on the expression of strawberry defense-related enzymes. Australasian Plant Pathology 46: 107–113. https://doi.org/10.1007/s13313-017-0467-2

Parks, D.H.; Beiko, R.G. 2010. Identifying biologically relevant differences between metagenomic communities. Bioinformatics 26: 715–721. https://doi.org/10.1093/bioinformatics/btq041

Pavela, R.; Benelli, G. 2016. Essential oils as ecofriendly biopesticides? Challenges and constraints. Trends in Plant Science 21: 1000–1007. https://doi.org/10.1016/j.tplants.2016.10.005

Peiffer, J.A.; Spor, A.; Koren, O.; Jin, Z.; Tringe, S.G.; Dangl, J.L.; Buckler, E.S.; Ley, R.E. 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proceedings of the National Academy of Sciences of the United States of America 110: 6548–6553. https://doi.org/10.1073/pnas.1302837110

Pérez-Valera, E.; Verdú, M.; Navarro Cano, J.; Goberna, M. 2020. Soil microbiome drives the recovery of ecosystem functions after fire. Soil Biology and Biochemistry 149: 1-7. https://doi.org/10.1016/j.soilbio.2020.107948

Pheomphun, P.; Treesubsuntorn, C.; Thiravetyan, P. 2019. Contribution of Bacillus cereus ERBP in ozone detoxification by Zamioculcas zamiifolia plants: effect of ascorbate peroxidase, catalase and total flavonoid contents for ozone detoxification. Ecotoxicology and Environmental Safety 171: 805–812. https://doi.org/10.1016/j.ecoenv.2019.01.028

Pinhassi, J.; Montserrat Sala, M.; Havskum, H.; Peters, F.; Guadayol, O.; Malits, A.; Marrasé, C. 2004. Changes in bacterioplankton composition under different phytoplankton regimens. Applied and Environmental Microbiology 70: 6753–6766. https://doi.org/10.1128/AEM.70.11.6753-6766.2004

Quast, C.; Pruesse, E.; Yilmaz, P.; Gerken, J.; Schweer, T.; Yarza, P.; Peplies, J.; Glöckner, F. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research 41: 590–596. https://doi.org/10.1093/nar/gks1219

Rasche, F.; Marco-Noales, E.; Velvis, H.; van Overbeek, L.S.; López, M.M.; van Elsas, J.D.; Sessitsch, A. 2006. Structural characteristics and plant-beneficial effects of bacteria colonizing the shoots of field grown conventional and genetically modified T4-lysozyme producing potatoes. Plant and Soil 289: 123–140. https://doi.org/10.1007/s11104-006-9103-6

Shi, S.; Richardson, A.E.; O'Callaghan, M.; DeAngelis, K.M.; Jones, E.E.; Stewart, A.; Firestone, M.K.; Condron, L.M. 2011. Effects of selected root exudate components on soil bacterial communities. FEMS Microbiology Ecology 77: 600–610. https://doi.org/10.1111/j.1574-6941.2011.01150.x

Silva, J.M.; Santos, T.M.C.; Albuquerque, L.S.; Montaldo, Y.C.; Oliveira, J.U.L.; Silva, S.G.M.; Nascimento, M.S.; Teixeira, R.R.O. 2015. Potential of the endophytic bacteria (Herbaspirillum spp. and Bacillus spp.) to promote sugarcane growth. Australian Journal of Crop Science 9: 754–760.

Soltani, A.A.; Khavazi, K.; Rahmani, H.; Omidvari, M.; Abbaszadeh Dahaji, P.; Hamed, M. 2010. Plant growth promoting characteristics in some flavobacterium spp. isolated from soils of Iran. The Journal of Agricultural Science 2: 106-115. https://doi.org/10.5539/jas.v2n4p106

Tabatabai, M.A.; Bremner, J.M. 1995. Estimation of urease activity. p. 317-318. In: Alef, K.; Nannipieri, P., eds. Methods in applied soil microbiology and biochemistry. Academic Press, London, UK.

Tabatabai, M.A.; Bremner, M.A.J. 1970. Arylsulfatase activity of soils. Soil Science Society of America Proceedings 34: 225–229. https://doi.org/10.2136/sssaj1970.03615995003400020016x

Tabatabai, M.A.; Bremner, J.M. 1969. Use of p-nitrophenyl phosphate for assay of soil phosphatase activity. Soil Biology and Biochemistry 1: 301–307. https://doi.org/10.1016/0038-0717(69)90012-1

Tedesco, M.J.; Volkweiss, S.J.; Bohnen, H. 1985. Soil and Other Materials Analysis = Análises de Solos, Plantas e Outros Materiais. UFRGS-Faculdade de Agronomia, Porto Alegre, RS, Brazil. (Boletim Técnico, 5) (in Portuguese).

Ter Braak, C.J.F.; Smilauer, P. 2002. CANOCO Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (version 4.5). Canoco, Ithaca, NY, USA.

Tkacz, A.; Cheema, J.; Chandra, G.; Grant, A.; Poole, P.S. 2015. Stability and succession of the rhizosphere microbiota depends upon plant type and soil composition. The ISME Journal 9: 2349–2359. https://doi.org/10.1038/ismej.2015.41

Ventura, M.; Canchaya, C.; Tauch, A.; Chandra, G.; Fitzgerald, G.F.; Chater, K.F.; van Sinderen, D. 2007. Genomics of actinobacteria: tracing the evolutionary history of an ancient phylum. Microbiology and Molecular Biology Reviews 71: 495–548. https://doi.org/10.1128/MMBR.00005-07

Wolińska, A.; Kuźniar, A.; Zielenkiewicz, U.; Izak, D.; Anna, S.-N.; Banach, A.; Blaszczyk, M. 2017. Bacteroidetes as a sensitive biological indicator of agricultural soil usage revealed by a culture-independent approach. Applied Soil Ecology 119: 128–137. https://doi.org/10.1016/j.apsoil.2017.06.009

Yi, X.; Yuan, J.; Zhu, Y.; Yi, X.; Zhao, Q.; Fang, K.; Cao, L. 2018. Comparison of the abundance and community structure of N-cycling bacteria in paddy rhizosphere soil under different rice cultivation patterns. International Journal of Molecular Science 19: 1-19. https://doi.org/10.3390/ijms19123772