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Beneficial effects of inoculation of growth-promoting bacteria in strawberry

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ABSTRACT

Plant growth-promoting bacteria have been highlighted by their potential for application in plant production, allowing the reduction of the use of fertilizers and pesticides, which is due to the ability to stimulate the growth of plants by nitrogen-fixation and production of phytohormones, such as indole-3-acetic acid (IAA). The objective of this study was to verify the potential of plant growth promotion of 25 wild isolates from the Agricultural Microbiology Culture Collection of the Federal University of Lavras (CCMA-UFLA) through the evaluation of the biological nitrogen-fixation capacity and the production of IAA. In addition, the growth of three selected strains inoculated on roots of strawberry seedlings in greenhouse conditions was evaluated. The experiment was conducted in a completely randomized design (CRD), with an 8 × 2 factorial schemes involving eight combinations of bacteria: alone, in pairs and threes, plus the control without inoculation. Two fertilizer levels were used (0% and 50% of nitrogen), totaling 16 treatments with eight replicates each. After 75 days, variables such as root length, root dry weight, aerial part length, aerial part dry weight, leaf number, total dry mass and ultrastructural analysis of the inoculated and uninoculated roots, were evaluated. The results showed that the strawberry crop responded positively to inoculation with the three bacteria combined *Azospirillum brasilense* (Ab-V5) + *Burkholderia cepacia* (CCMA 0056) + *Enterobacter cloacae* (CCMA 1285) compared to the uninoculated controls. More expressive responses in terms of plant growth were observed in relation to the combined inoculation of the three bacterial strains plus fertilizer application with 50% of nitrogen.

1. Introduction

The strawberry (*Fragaria × ananassa,* Duch.) belongs to the Rosaceae family, subfamily *Maloideae*. The fruit stands out for its organoleptic and nutraceutical properties (Taco, 2011) and is appreciated around the world, with a large consumer market in the world's main economies. This species is cultivated in different parts of the world, in both tropical and subtropical areas, but its production is more pronounced in temperate regions, with the largest producers being the United States, Spain, China, Mexico, Poland and Japan (Pereira et al., 2012; FAO, 2014). Brazil, despite not being among the world largest producers and exporters, has been notable for presenting natural conditions favorable to strawberry production in most months of the year (Agrianual, 2008; Antunes and Reisser, 2007).

However, in most of the strawberry producing countries in the world, cultivation is generally carried out intensively with high consumption of agrochemicals, which, although contributing to the success of the crop, can cause several impacts on the environment (Delaporte-Quintana et al., 2017). Consequently, the search for environmentally-friendly forms of cultivation becomes essential from the point of view of public health, in order to maintain high productivity.

In this context, organic production presents itself as a potential alternative to the current strawberry production system. In this system, the use of high solubility synthetic fertilizers, pesticides, growth regulators and feed additives are prohibited, giving space to strategies, such as crop rotation, crop residues, green manures, mechanical cultivation, integrated pest and disease management, and biofertilizers, among other methodologies (Esitken et al., 2010). In organic production, a great part of attention is focused on the soil-plant system, especially the rhizosphere. Rhizosphere is a microzone at the root-soil interface that is under the influence of the plant and its exudates and is characterized by a large variety of microorganisms and their intense

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metabolic activity. Among the microorganisms present in the rhizosphere, some are able to form symbiotic or non-symbiotic associations with plants, so-called plant growth-promoting bacteria (PGPB) (Ahmad et al., 2008; Compant et al., 2010; Moreira and Siqueira, 2006; Oliveira et al., 2014).

These PGPB can be defined as a group of plant-beneficial microorganisms due to the mutual interactions they carry out with plants and might increase plant productivity (Oliveira et al., 2014; Pereira et al., 2012). The bacteria are of potential interest for application in agriculture, such as biofertilizers, pesticides, in phytoremediation (Compant et al., 2010) and in the production of inoculants to promote plant growth and biocontrol (Lacava and Azevedo, 2014; Quecine et al., 2014). Some PGPBs might present more than one mechanism that stimulates plant growth (Ahmad et al., 2008), which includes nitrogen fixation, synthesis of phytohormones, such as indole-3-acetic acid (IAA), and organic acids, production of HCN, enzyme release (soil dehydrogenase, phosphatase and nitrogenase), antagonism to pathogenic fungi working as biocontrol agents, production of siderophores, increased solubilization of phosphates and induction of systemic resistance (Barriuso et al., 2008; Santoyo et al., 2016).

Increase in productivity in the field has been reported in response to the use of PGPBs; their effects are mainly on the increase of leaf area, diameter of pseudocaule, number of leaves and dry matter, with consequent reduction on the acclimatization period and greater survival of the seedlings after the transplant (Faleiro et al., 2011). The use of different beneficial microorganisms, such as *Pseudomonas*, *Bacillus*, and *Azospirillum*, has been described as growth promotion agents with the increase of plant biomass and yield of fruits (Esitken et al., 2010; Erturk et al., 2012). Moreover, PGPB has showed considered effect on the pathogens *Colletotrichum acutatum, Macrophomina phaseolina* and *Fusarium solani* in strawberry plants (Pastrana et al., 2016; Tortora et al., 2011).

Considering the economic and social importance of strawberry cultivation and its high nutritional requirement, including high nitrogen requirement, it is expected that the inoculation of microorganisms may contribute to the growth of strawberry plants through the colonization of their root system. Therefore, the objective of this study was to select wild strains from the bacteria belonging to the Agricultural Microbiology Culture Collection of the Federal University of Lavras (CCMA-UFLA) using *in vitro* tests of biological nitrogen-fixation capacity and production of IAA, as well as to evaluate the potential of the isolates obtained for the promotion of strawberry growth by determining growth and root colonization variables.

2. Materials and methods

2.1. Strains used

The 22 bacterial strains isolated from the soil, coffee fermentation, coffee residual processing water and indigenous beverage were used. These strains belong to the Agricultural Microbiology Culture Collection (CCMA-UFLA http://www.ccma.dbi.ufla.br/pt/), Department of Biology, Federal University of Lavras (UFLA), Lavras, Minas Gerais, Brazil and three strains (UNIFENAS 100-13, UNIFENAS 100-39 and Ab-V5 provided by EMBRAPA Londrina, Paraná, Brazil) by the José of RosárioVellano University (UNIFENAS), Alfenas, Minas Gerais, Brazil. For the initial tests, 25 strains were selected according to genera and species, described in the literature as plant growth-promoting bacteria (Table 1). Strains maintained at −80 °C were reactivated in nutrient broth for 48 h at 30 °C.

2.2. Evaluation of biological nitrogen-fixation capacity and IAA production capacity

The methodology used for determining nitrogen fixation was according to Döbereiner et al. (1995) for the NFb medium and Baldani et al. (2000) for the JMV medium, both semi-solid media; the experiments were conducted in triplicate. The strain Ab-V5 was used as the control for the NFb medium (Pedrinho et al., 2010; Szilagyi-Zecchin et al., 2015). For the JMV culture medium, the strain UNIFENAS 100-13 was used as a positive control. Typical aerobic film formation near the surface of the medium was considered a positive result, indicating that nitrogen fixation had occurred, since there was a reduction of the atmospheric nitrogen in ammonia.

The production of auxin was evaluated using the methodology adapted from Gordon and Weber (1951) and Loaces et al. (2011). For inoculum standardization, strains were initially transferred to nutrient broth for 48 h and incubated at 30 °C. The OD was then adjusted to optical density of 0.5 (10^7–10^8 CFU mL^{-1}). Afterwards, strains were transferred (5% v/v) to the nutrient broth medium amended with tryptophan (100 μg mL^{-1}) and incubated at 30 °C in the dark for 72 h. Culture supernatants were recovered after centrifugation at 12,000 × g for 5 min. Auxin production was determined by mixing 1 mL of the supernatant with 1 mL of Salkowski's Reagent (1.875 g of $FeCl_3{\cdot}6H_2O$, 100 mL of H_2O, and 150 mL of H_2SO_4). The mixture was incubated at 30 °C for 15 min in the dark. The phytohormone was quantified in a spectrophotometer at 530 nm. The concentrations, in μgmL^{-1}, were calculated from a standard curve constructed with pure IAA. Red-pink coloration of the samples was used as indicative of auxin production.

2.3. Design and treatments

In order to evaluate the capacity to promote plant growth in strawberry's, a greenhouse experiment was carried out with seedlings of the same plant species (cv *Aromas*) cultivated in a Dystrophic Red Latosol (EMBRAPA, 2006). Samples taken from the 0–20 cm soil layer showed the following chemical characteristics: pH in water = 5.0; P = 0.634 mg dm^{-3}; K = 41.79 mg dm^{-3}; Na = 6.19 mg dm^{-3}; Ca = 0.40 cmol dm^{-3}; Mg = 0.20 cmol dm^{-3}; H + Al = 8.60 cmol dm^{-3}; T = 9.31 cmol dm^{-3}; V = 7.60%; organic matter = 35 g kg^{-1}; Zn = 0.86 mg dm^{-3}; Fe = 181.16 mg dm^{-3}; Mn = 6.55 mg dm^{-3}; Cu = 1.76 mg dm^{-3}; B = 0.27 mg dm^{-3}; S = 4.72 mg dm^{-3}; clay = 660 g kg^{-1}; silt = 80 g kg^{-1}; sand = 260 g kg^{-1} and total nitrogen = 2.4 g kg^{-1}. Soil chemical analysis was carried out at the Soil Physics Laboratory of the Department of Soil Science, UFLA, Lavras, Minas Gerais, Brazil.

The soil was collected, sieved and dried. After drying soil samples, liming requirement was determined using the base saturation method, and calcium carbonate and magnesium carbonate added accordingly. After liming, the soil was autoclaved twice every other day at 121 °C for 1 h and 30 min. The soil was sterilized to eliminate the native mycorrhizal fungi and other spore forming microorganisms, according to the methodology proposed by Vilela et al. (2012). The microbial composition was checked after sterilization. Fertilizing of the soil was performed according to the recommendations of Malavolta (1980), added to the solution micronutrients (2.17 g H_3BO_3, 5 g $CuSO_4.5H_2O$, 5.67 g $MgSO_4.H_2O$, 0.167 g $(NH_4)6Mo_7O_{24}.4H_2O$, 17.17 g $ZnSO_4.7H_2O$ dissolved in 2 L distilled water); 11.5 g/vase of Ca(H_2 PO_4) $2H_2O$ + $CaSO_4$; 0.34 g/vase of K_2SO_4 and urea (CH_4N_2O) at 0% and 50% of the recommended requirements for the culture. Fertilizing with potassium sulfate and urea were carried out biweekly until the 75th day of the experiment. Strawberry seedlings with roots were used, with approximately one month of growth.

The experiment was conducted in a completely randomised design, with a 8 × 2 factorial scheme, involving eight combinations of bacteria and two levels of nitrogen fertilizer application (0 and 50%). The bacteria, *Azospirillum brasilense* (Ab-V5), *Burkholderia cepacia* (CCMA 0056), and *Enterobacter cloacae* (CCMA 1285), were inoculated in pairs, in threes and alone. The control consisted of two sets: eight not inoculated plants containing 0% nitrogen and eight not inoculated plants containing 50% nitrogen. A total of 16 treatments were used, with eight plants inoculated for each treatment. The different treatments used in the experiment were described in Table 2.

Table 1
Strains belonging to the CCMA used in the experiment.

Code	Genera and species	Geographical origin in Brazil	Substrate/host
CCMA 0010	*Bacillus subtilis subsp. Subtilis*	Xingú Park-MT	Indigenous drink (Caxiri)
CCMA 0054	*Bacillus subtilis*	Passos-MG	Fruit (Cerrado) (Pimenta-do-Mato)
CCMA 0085	*Bacillus subtilis*	Arcos-MG	Fruit (Cerrado) (Marolo)
CCMA 0088	*Bradyrhizobium japonicum*	Arcos-MG	Fruit (Cerrado) (Marolo)
CCMA 0101	*Burkholderia cenocepacia*	Arcos-MG	Fruit (Cerrado) (Marolinho)
CCMA 0401	*Bacillus subtilis*	Indian tribe-MT	Indigenous drink (Chicha)
CCMA 0549	*Bacillus subtilis*	Xingú Park-MT	Indigenous drink (Caxiri)
CCMA 0087	*Bacillus subtilis*	Arcos-MG	Fruit (Cerrado) (Marolo)
CCMA 0082	*Burkholderia phenazinium*	Arcos-MG	Fruit (Cerrado) (Ananás)
CCMA 0113	*Burkholderia multivorans*	Luminárias-MG	Fruit (Cerrado) (Murici-do-Cerrado)
CCMA 0448	*Paenibacillus amylolyticus*	Lavras-MG	Coffee bean (wet)
CCMA 0056	*Burkholderia cepacia*	Passos, MG	Fruit (Cerrado) (Pimenta-do-Mato)
CCMA 0551	*Bacillus subtilis*	Arcos-MG, Brazil	Soil from Cerrado (dry season)
CCMA 0550	*Bacillus subtilis*	Passos-MG, Brazil	Soil from Cerrado (wet season)
CCMA 0083	*Burkholderia sordicola*	Arcos-MG, Brazil	Fruit (Cerrado) (Ananás)
CCMA 0457	*Bacillus amyloliquefacien*	Lavras-MG, Brazil	Coffee bean (wet)
CCMA 1269	*Paenibacillus illinoisensis*	Patrocínio-MG, Brazil	Coffee fermentation water
CCMA 1254	*Lysinibacillus fusiformis*	Patrocínio-MG, Brazil	Coffee fermentation water
CCMA 1285	*Enterobacter cloacae*	Patrocínio-MG, Brazil	Coffee fermentation water
CCMA 1286	*Bacillus pumilus*	Patrocínio-MG, Brazil	Coffee fermentation water
CCMA 1287	*Bacillus subtilis*	Patrocínio-MG, Brazil	Coffee fermentation water
CCMA 1288	*Lysinibacillus amylolyticos*	Patrocínio-MG, Brazil	Coffee fermentation water
Ab-V5	*Azospirillum brasilense*	EMBRAPA Londrina-PR, Brazil	Fukami et al. (2018)
UNIFENAS 100-13	*Burkholderia cenocepacia*	Alfenas-MG, Brazil	*Brachiaria brizantha* cv. Marandu Rhizosphere
UNIFENAS 100-39	*Burkholderia cenocepacia*	Alfenas-MG, Brazil	*Brachiaria brizantha* cv. Marandu Rhizosphere

2.4. Inoculant preparation and strawberry growth assessments

The three selected strains were prepared separately, grown in nutrient broth medium at 30 °C until reaching a concentration of 1×10^9 CFU mL^{-1}. After incubation, the cells were centrifuged at 9000 × g for 10 min and washed twice with phosphate buffer (pH 7.0) to remove any residue from the culture medium that could interfere with growth on strawberry plants. mL^{-1} aliquots of the bacterial suspensions (10^9 CFU mL^{-1}) were inoculated into the roots according to each treatment. The plants were watered with sterile distilled water. The vessels were randomly arranged and kept in a greenhouse.

Inoculations were performed biweekly with a total of five inoculations (0, 15, 30, 45 and 60 days). After a period of 75 days, the following characteristics of plant development and growth were evaluated: root length and aerial part length (cm) root, aerial part and total dry weight (g). Weights were recorded using a precision analytical balance with four decimal places. Total dry weight (g) was determined as the sum of the root dry weight and aerial part dry weight. The plants were stored in paper bags and labelled. Afterward, they were dried in an oven with forced air circulation at 60 °C until constant weight was achieved. The weight was measured at three different time-points (24, 48 and 72 h). Plant diameter was also measured at 0, 15, 30, 45, 60 and 75 days using a digital pachymeter stain less steel Lee Tools 682132, 150 mm capacity, 0.01 mm / 0.0005 resolution, +/- 0.03 mm / 0.001 accuracy, considering the extremities of two opposite leaves that presented the largest dimension. The results were submitted to analysis of variance using the R Studio software. The mean values were compared using the Scott-Knott test at a 5% probability level. The analysis of variance for plant diameter was performed employing a mixed model in a time subdivided plot scheme using R software.

2.5. Colonization of the root system - scanning electron microscopy

Samples of strawberry roots from all treatments were used for the analysis of scanning electron microscopy. Root samples were cut by hand with a scalpel and pre-fixed in Karnovsky fixative (glutaraldehyde 2.5%, paraformaldehyde 2.0% in sodium cacodylate buffer 0.05 M and $CaCl_2$ 0.001 M; pH 7.2), and fixed for at least 24 h at 4 °C.

Analysis was conducted according to coded protocol of Bozzola and Russel (1999). The pre-fixed roots samples were removed from the

Table 2
Treatments used in the experiment, strains and nitrogen concentration.

Treatments	Code	Method
Control 1	C1	Control (no bacteria) and 0% nitrogen
Control 2	C2	Control (no bacteria) and 50% nitrogen
Treatment 3	T3	*Azospirillum brasilense* (Ab-V5), and 0% nitrogen
Treatment 4	T4	*Burkholderia cepacia* (CCMA 0056) and 0% nitrogen
Treatment 5	T5	*Enterobacter cloacae* (CCMA 1285) and 0% nitrogen
Treatment 6	T6	*Azospirillum brasilense* (Ab-V5) + *Burkholderia cepacia* (CCMA 0056) and 0% nitrogen
Treatment 7	T7	*Azospirillum brasilense* (Ab-V5) + *Enterobacter cloacae* (CCMA 1285) and 0% nitrogen
Treatment 8	T8	*Burkholderia cepacia* (CCMA 0056) + *Enterobacter cloacae* (CCMA 1285) and 0% nitrogen
Treatment 9	T9	*Azospirillum brasilense* (Ab-V5) + *Burkholderia cepacia* (CCMA 0056) + *Enterobacter cloacae* (CCMA 1285) and 0% nitrogen
Treatment 10	T10	*Azospirillum brasilense* (Ab-V5) and 50% of nitrogen
Treatment 11	T11	*Burkholderia cepacia* (CCMA 0056) and 50% nitrogen
Treatment 12	T12	*Enterobacter cloacae* (CCMA 1285) and 50% nitrogen
Treatment 13	T13	*Azospirillum brasilense* (Ab-V5) + *Burkholderia cepacia* (CCMA 0056) and 50% nitrogen
Treatment 14	T14	*Azospirillum brasilense* (Ab-V5) + *Burkholderia cepacia* (CCMA 0056) and 50% nitrogen
Treatment 15	T15	*Burkholderia cepacia* (CCMA 0056) + *Enterobacter cloacae* (CCMA 1285) and 50% nitrogen
Treatment 16	T16	*Azospirillum brasilense* (Ab-V5) + *Burkholderia cepacia* (CCMA 0056) + *Enterobacter cloacae* (CCMA 1285) and 50% nitrogen

Table 3
Auxin production (μg mL^{-1}) and nitrogen fixation capacity (+/-) of CCMA-UFLA and UNIFENAS bacteria strains.

CODE	GENERA AND SPECIES	IAA (μg mL^{-1})	Nitrogen-fixation	
			NFb	JMV
CCMA 0088	*Bradyrhizobium japonicum*	3.698 ± 0.26 a	+	–
Ab-V5	*Azospirillum brasilense*	3.488 ± 2.01 a	+	–
CCMA 0101	*Burkholderia cenocepacia*	3.312 ± 0.21 a	+	–
CCMA 0056	*Burkholderia cepacia*	3.105 ± 0.42 a	+	–
CCMA 1285	*Enterobacter cloacae*	2.869 ± 0.32 a	+	–
CCMA 1269	*Paenibacillus illinoisensis*	2.463 ± 1.92 b	–	+
UNIFENAS 100-13	*Burkholderia cenocepacia*	2.363 ± 0.35 b	+	+
CCMA 0401	*Bacillus subtilis*	2.210 ± 0.99 b	+	–
CCMA 1288	*Lysinibacillus amylolyticos*	2.065 ± 1.15 b	+	–
CCMA 0551	*Bacillus subtilis*	2.017 ± 1.44 b	+	–
CCMA 0549	*Bacillus subtilis*	1.824 ± 0.75 b	+	–
CCMA 0113	*Burkholderia multivorans*	1.268 ± 0.23 c	+	–
CCMA 0082	*Burkholderia phenazinium*	1.188 ± 0.20 c	+	–
CCMA 0085	*Bacillus subtilis*	1.114 ± 0.88 c	+	–
CCMA 1287	*Bacillus subtilis*	1.074 ± 0.71 c	+	–
CCMA 0448	*Paenibacillus amylolyticos*	1.074 ± 0.61 c	–	–
CCMA 0083	*Burkholderia sordicola*	1.006 ± 0.61 c	+	–
CCMA 0054	*Bacillus subtilis*	0.947 ± 1.04 c	+	–
CCMA 0010	*Bacillus subtilis* subsp.	0.796 ± 0.20 c	+	–
CCMA 0457	*Bacillus amyloliquefaciens*	0.779 ± 0.26 c	+	–
UNIFENAS 100-39	*Burkholderia cenocepacia*	0.714 ± 0.36 c	+	+
CCMA 0550	*Bacillus subtilis*	0.626 ± 0.09 c	+	–
CCMA 1286	*Bacillus pumilus*	0.597 ± 0.18 c	+	–
CCMA 1254	*Lysinibacillus fusiformis*	0.561 ± 0.12 c	+	–
CCMA 0087	*Bacillus subtilis*	0.387 ± 0.28 c	+	–

Karnovsky fixative, added to glycerol for a period of 30 min and, afterward, sections of roots were cut in the liquid nitrogen. The samples were then washed in distilled water three times and dehydrated in acetone gradient (25%, 50%, 75%, 90%, once and 100%, three times). Thereafter, cacodylate buffer (0.05 M) was added and allowed to stand for 10 min. This operation was repeated twice. Subsequently, the dehydration procedure was performed using the same acetone gradients used for the root samples.

After dehydration, the roots were taken to the Balzers CPD 030 critical-point dryer device in order to replace the acetone with CO_2 and to complement the drying. The samples were mounted on aluminum supports and taken to the Balzers SCD 050 evaporator for examination using the LEO EVO 40 scanning electron microscope of the Department of Plant Pathology, UFLA, Lavras, Minas Gerais, Brazil. Photographs were edited using the Corel Draw X7 graphics program.

3. Results and discussion

3.1. Selection of bacteria

Results in Table 3 showed the test for the 25 strains from the CCMA-UFLA and UNIFENAS. The strains showed production of IAA ranging from 0.387 to 3.698 μg mL^{-1} statistically divided in three groups (higher, intermediate and lower IAA production). The best results were observed for *Bradyrhizobium japonicum* (CCMA 0088), *Azospirillum brasilense* (Ab-V5) *Burkholderia cenocepacia* (CCMA 0101), *Burkholderia cepacia* (CCMA 0056) and *Enterobacter cloacae* (CCMA 1285) strains, producing IAA at 3.698, 3.488, 3.312, 3.105 and 2.869 μg mL^{-1}, respectively. IAA production for the second group ranged from 2.463 to 1.824 μg mL^{-1}, while the lower IAA contents ranged from 1.268 to 0.387 μg mL^{-1} in the third group. Other authors also found diverse values for IAA production, showing that different bacterial strains have different ability to produce this phytohormone (Chaves et al., 2015; Pedrinho et al., 2010).

The ability to produce IAA is dependent on the metabolites produced by the bacteria, the capacity of bacterial culture (Cassán et al., 2014), the condition of the culture, the stage of its growth and availability of substrate (Spaepen, 2015), justifying the variability in the IAA production. Data presented by Dias et al. (2009) reported the production of IAA by *Bacillus megaterium* and *Bacillus subtilis*, endophytic in strawberries, and found values less than 5 μg mL^{-1}, similar to this study, even with production of low concentrations of IAA, inoculation of strawberry with this species of bacteria promoted plant growth.

The production of IAA by *Enterobacter cloacae* (CCMA 1285) strain was 2.869 μg mL^{-1}, which was higher than that reported by George et al. (2013) with a production of 2.40 μg mL^{-1} by bacteria of the same genus. It was verified that the *Bradyrhizobium japonicum* (CCMA 0088) and *Burkholderia cenocepacia* (CCMA 0101) strains produced higher IAA production compared to *Azospirillum brasilense* (Ab-V5) (reference in the literature as PGPR according to Pedrinho et al., 2010 and Szilagyi-Zecchin et al., 2015), indicating its potential as an inoculant in future studies. The IAA production reported by Pereira et al. (2012) for the genus *Bacillus* varied from 1.36 to 19.42 μg mL^{-1}, IAA concentrations reported here from bacteria of the same genus were in this range (0.387–2.210 μg mL^{-1}). The auxin production can vary among strains also due to the influence of other factors such as average conditions of pH, availability of substrates, presence of organic acids, metals and microorganism growth phase (Jha et al., 2012; Martínez-Viveros et al., 2010).

All strains, except *Paenibacillus amylolyticos* (CCMA 0448) and *Paenibacillus illinoisensis* (CCMA1269), were capable of fixing nitrogen in a free-living form in NFb medium. However, when these strains were tested in JMV medium, only *Burkholderia cenocepacia* (UNIFENAS 100-13), *Burkholderia cenocepacia* (UNIFENAS 100-39) and *Paenibacillus illinoisensis* (CCMA 1269) strains gave positive results. This difference might be due to the specificity of both tests, as FNb is a more generalist test, while JMV is less generalist and more specific to *Burkholderia sp*. Several authors reported genes *Bradyrhizobium*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Pseudomonas* (Vessey, 2003; Bhattacharyya and Jha, 2011) as nitrogen fixing bacteria. The contribution of these bacteria is not only limited to the supply of nitrogen to the plant but also to phytonutrient synthesis, phytopathogen antagonism, phosphate solubilization, among other functions. Moreover, the use of nitrogen-fixing bacteria in agriculture can contribute to increase productivity reducing the use of synthetic ammonia decreasing the pollution of rivers and lakes (Botta et al., 2013).

From these results presented in Table 3, the following three strains were selected for the second part of this work: *Azospirillum brasilense* (Ab-V5), *Burkholderia cepacia* (CCMA 0056) and *Enterobacter cloacae* (CCMA 1285).

Means followed by the same letter do not differ significantly by the Scott-Knott test at the 5% level of significance. Means of duplicated assays are followed by the standard deviation. Nitrogen-fixation: indicated by the presence of a growth film in semi-solid medium without nitrogen, NFb and JMV, as (+) positive and (-) negative.

3.2. Evaluation of strawberry growth

There was a significant effect regarding the inoculation with bacteria on the aerial part, but nitrogen and the interaction between bacteria and nitrogen fertilizer was not significant (Fig. 1A). Higher aerial part length was found with the inoculation of *Enterobacter cloacae*-CCMA 1285 (21.83 cm) and the combinations *Azospirillum brasilense*-Ab-v5 + *Burkholderia* cepacia-CCMA 0056 (22.20 cm); *Azospirillum*

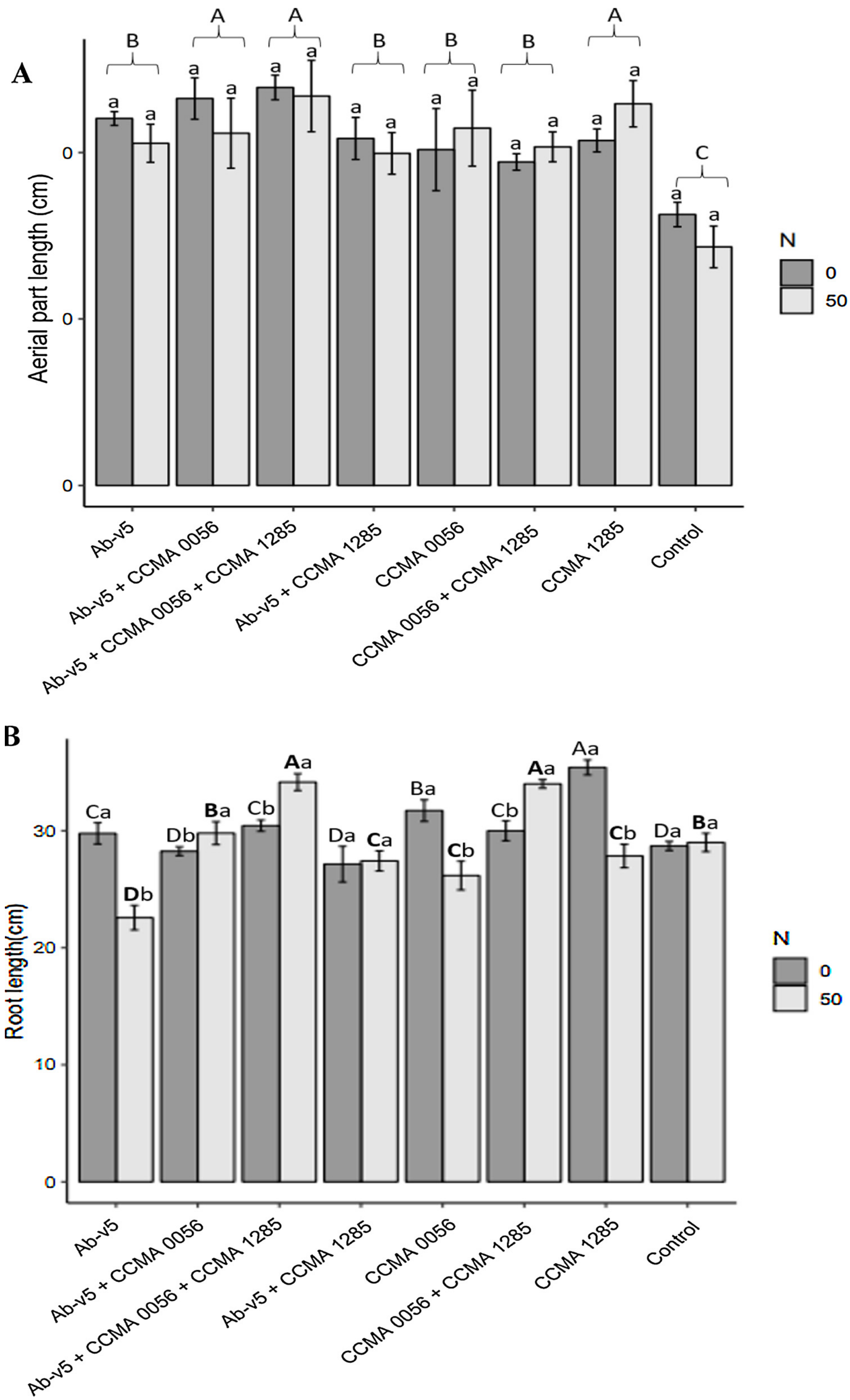

Fig. 1. (A) Aerial part and (B) root length of strawberry plants inoculated with *Azospirillum brasilense* (Ab-V5), *Burkholderia cepacia*-CCMA 0056, *Enterobacter cloacae*-CCMA 1285, their combination and Control (no bacteria). Means followed by the same lower case or capital letters do not differ significantly by the Scott-Knott test at 5% of significance. 1(A) Capital letter indicates the difference between the strains; lower case letter indicates the difference between the concentrations of nitrogen for each strain. 1(B) Capital letter indicate the difference between the strains at 0% N; Bold capital letter indicate the difference between the strains at 50% N; lower case letter indicates the difference between the concentrations of nitrogen for each strain.

brasilense-Ab-v5 + *Burkholderia cepacia*- CCMA 0056 + *Enterobacter cloacae*-CCMA 1285 (23.65 cm), while uninoculated plants had lower aerial part length (15.30 cm). Results suggest that bacteria inoculation stimulated plant growth, once their length was higher compared to the control treatment, with an increase of 30, 31 and 35%, respectively. Aerial part length did not differ regarding the fertilization with nitrogen, which can be a promising result, once it suggests that bacteria inoculation on the cultivar Aromas can mitigate the need for nitrogen fertilization, reducing production costs. This nutrient is required in the order of 100 kg ha^{-1} along with phosphorus and potassium, while calcium, magnesium and sulfur are required in the order of 10 kg ha^{-1} and the micronutrients in the order of g ha^{-1} (Barker and Pilbeam, 2015).

The interaction between bacteria and nitrogen was significant for root length. In the absence of nitrogen *Enterobacter cloacae*-CCMA 1285 (35.40 ± 0.63 cm) led to higher root length than the others treatments. *Azospirillum brasilense*-Ab-v5 + *Enterobacter cloacae*-CCMA 1285 (27.14 ± 1.52 cm), *Azospirillum brasilense*-Ab-v5 + *Burkholderia cepacia*-CCMA 0056 (28.25 ± 0.37 cm) and control treatments showed lower root lengths. On the other hand, when nitrogen was added to the soil, higher root length was found in the treatments inoculated with *Azospirillum brasilense*-Ab-v5 + *Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285 (34.15 ± 0.72 cm) and *Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285 (34.00 ± 0.36 cm). Inoculation with *Azospirillum brasilense*-Ab-v5 presented the lowest root length (22.57 ± 1.05 cm).

Regarding the nitrogen levels for each bacteria, no difference was found between the nitrogen concentrations for root length in the control treatment (N0: 28.69 ± 0.38; N50: 28.99 ± 0.78 cm) and when *Azospirillum brasilense*-Ab-v5 + *Enterobacter cloacae*-CCMA 1285 (N0: 27.14 ± 1.52; N50: 27.42 ± 0.85 cm) was inoculated. The isolated application of the bacteria (*Azospirillum brasilense*-Ab-v5; *Burkholderia cepacia*-CCMA 0056 and *Enterobacter cloacae*-CCMA 1285) led to lower root growth when nitrogen was added to the soil, being 24, 17 and 21% lower than in the absence of this mineral, respectively. In contrast, the combination between the bacteria increased root length when soil was fertilized with nitrogen, excepting *Azospirillum brasilense*-Ab-v5 + *Enterobacter cloacae*-CCMA 1285, with an increase of 5% (*Azospirillum brasilense*-Ab-v5 + *Burkholderia cepacia*-CCMA 0056), 12% (*Azospirillum brasilense*-Ab-v5 + *Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285) and 13% (*Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285).

Similar findings were reported by Deepa et al. (2010) with the inoculation of *Enterobacter cloacae* in cowpea, in which they demonstrated a positive effect in the inoculated plants, with considerable influence on the length of the root and aerial part in comparison with the uninoculated control, besides a significant increase in the biomass of the plant root and aerial part, and an increase in the number of roots.

According to Zhao et al. (2014), the inoculation of corn with *Burkholderia cepacia* bacteria, promoted an increase in the leaf area and the average length and dry weight of the root and aerial part in relation to the uninoculated control. Studying inoculation with the bacterium *Azospirillum brasilense*, this being isolated from different strawberry tissues, Pedraza et al. (2010) reported root lengths ranging from 13.6–19.4 cm after 54 days of inoculation; in this same study, plants inoculated with *Azospirillum brasilense* (isolated from internal strawberry stolon tissues of the cv. *Milsei*) showed greater root lengths than the uninoculated control.

The difference in results regarding the absence or presence of nitrogen fertilizer (50% of the crop requirement) was not significant in this study for the first parameter (shoot length). Therefore, it is possible to suggest that the lengths of the aerial part and root were directly influenced by the inoculations with the bacteria. Despite the inoculation with bacteria showed neutral or negative effect on root length for some of the treatments, the growth of the aerial part was positively influenced for all treatments inoculated with bacteria. This growth suggests the presence of higher leaf area, organ responsible for the metabolism of photoassimilates, increasing the photoassimilates amount directed to the fruits (Syed-Ab-Rahman et al., 2018; Xie et al., 2018). Moreover, this may be a result of the capacity of each bacterial species to produce IAA, since this phytohormone is proposed to be involved in the promotion of growth and development of the roots, increasing the absorption of nutrients (Deepa et al., 2010; Khalid et al., 2004) and, consequently, causing an increase in plant size.

There was a significant difference between bacteria inoculation and use of nitrogen fertilizer for dry weight of shoots, roots, and total (Fig. 2). Aerial dry weight varied from 7.83 g to 14.64 g in the absence of nitrogen fertilizer (Fig. 2A). The highest value was found (14.65 ± 1.93 g) when the combination of *Azospirillum brasilense* (Ab-V5) + *Burkholderia cepacia* (CCMA 0056) was inoculated, indicating an

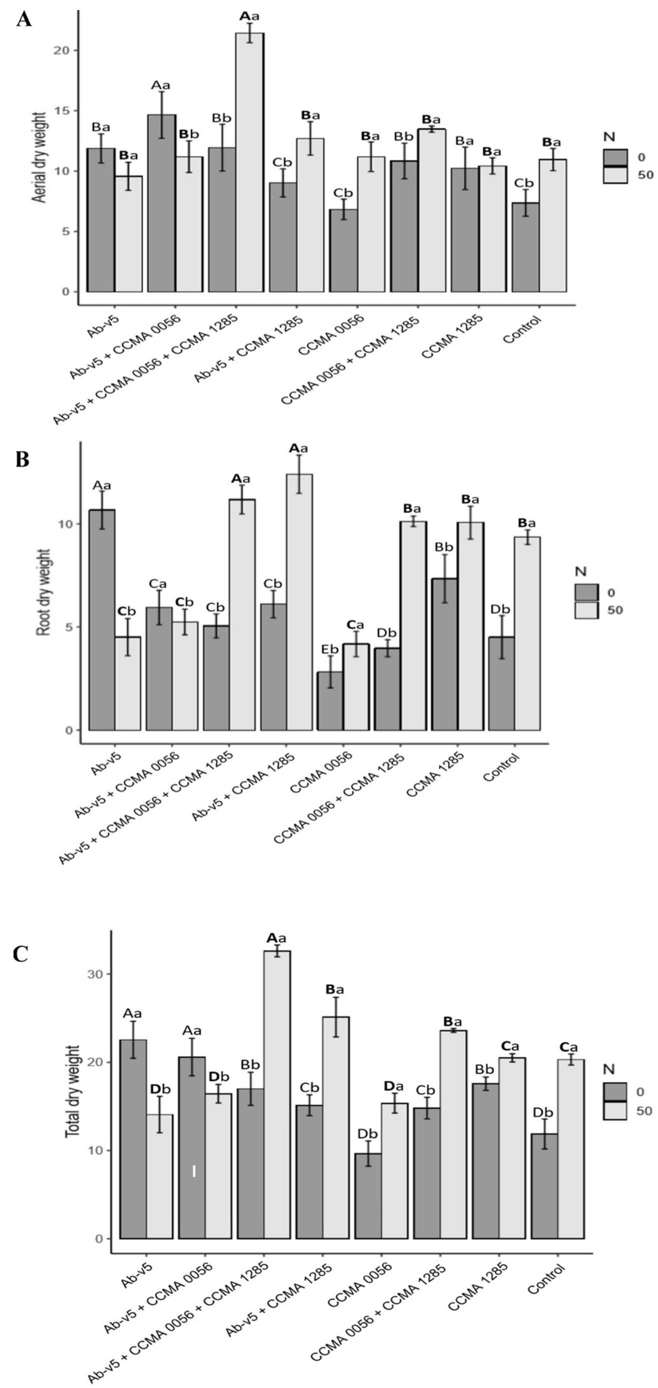

Fig. 2. Growth parameters of strawberry plants inoculated with *Azospirillum brasilense*-Ab-V5, *Burkholderia cepacia*-CCMA 0056, *Enterobacter cloacae*-CCMA 1285, their combination and Control (no bacteria) after 75 days of growth in a greenhouse. (A) aerial part dry weight (g); (B) root dry weight (g); and (C) total dry weight (g). Means from 8 replicates of duplicated assays are followed by the standard deviation Means followed by the same lower case or capital letters do not differ significantly by the Scott-Knott test at 5% of significance. Capital letter indicate the difference between the strains at 0% N; Bold capital letter indicate the difference between the strains at 50% N; lower case letter indicates the difference between the concentrations of nitrogen for each strain.

increase of 82% in relation to control without nitrogen. Treatments inoculated with *Burkholderia cepacia*-CCMA 0056 (7.84 ± 2.51 g), *Enterobacter cloacae*-CCMA 1285 (10.23 ± 1.76 g), co-inoculation *Azospirillum brasilense*-Ab-V5 + *Enterobacter cloacae*-CCMA 1285 (9.03 ± 1.17 g) and *Burkholderia cepacia*-CCMA 0056 + *Enterobacter* *cloacae*-CCMA 1285 (10.18 ± 2.29 g) did not differ from the control (8.04 ± 2.12 g). When 50% of nitrogen fertilizer was applied, the dry weight of the aerial part varied from 9.57 g to 21.44 g, being the highest value (21.44 ± 0.81 g) observed with the combination of the three bacteria, *Azospirillum brasilense*-Ab-V5 + *Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285, which represents 96% higher than the control with nitrogen (10.96 ± 0.91 g).

Regarding to the roots dry weight (Fig. 2B), the weight varied from 2.81 g to 10.67 g without nitrogen fertilizer. Highest weight was obtained with the inoculation of *Azospirillum brasilense*-Ab-V5 (10.67 ± 0.92 g); application of *Burkholderia* cepacia-CCMA 0056 (2.81 ± 0.77 g), *Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285 (4.30 ± 0.98 g) and *Azospirillum brasilense*-Ab-V5 + *Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285 (5.05 ± 0.58 g) did not differ from the control (4.50 ± 1.05 g). Using 50% of nitrogen fertilizer, the root dry weight varied from 4.17 g to 12.41 g, being the co-inoculation with *Azospirillum brasilense*-Ab-V5 + *Enterobacter cloacae*-CCMA 1285 (12.41 ± 0.93 g) superior than the other treatments, with an increase of 32% in relation to the control (9,35 ± 0.35 g).

The addition of the bacteria was differentiated, especially when co-inoculation of the strains was tested. The same was observed in an investigation by Botta et al. (2013), with inoculation of four different bacteria species (*Azospirillum brasilense*, *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae* and *Burkholderia ambifaria*) in tomato plants. The researchers in this study obtained positive responses compared to the uninoculated controls, with significant increase in root length, number of lateral roots, number of leaves and aerial parts. Frequently, co-inoculation increased the growth and yield, compared to a single inoculation, therefore, plants are a more balanced nutrition, increase the availability of minerals and nutrients and improves the economy of nitrogen and phosphorus.

The total dry weight (Fig. 2C) showed a significant difference between treatments with inoculation of bacteria and use of nitrogen fertilizer. In the absence of nitrogen, the total dry weight varied from 9.65 to 22.55 g, the higher value occurring mainly with treatment with the bacterium *Azospirillum brasilense* (Ab-V5) (22.55 ± 4.03 g), and the control had an average value of 12.54 ± 2.86 g, indicating an increase of 79.82%. Only treatment inoculated with *Burkholderia cepacia*-CCMA 0056 (9.65 ± 1.42 g) did not differ from the control. The other treatments had a positive growth result in relation to the control. With 50% nitrogen fertilizer, the total dry weight ranged from 14.07–32.62 g, the highest value occurring with the combination of the three bacteria (*Azospirillum brasilense*-Ab-V5 + *Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285 (32.62 ± 0.67 g), being 60.61% higher than the control (20.31 ± 0.62 g). Moreover, the application of *Azospirillum brasilense*-Ab-V5 + *Enterobacter cloacae*-CCMA 1285 (25.12 ± 2.24 g) and the co-inoculation with *Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285 (23.61 ± 0.21 g) were also higher than the control. The isolated application of CCMA 1285 did not differ from the control. On the other hand, the isolates Ab-v5, Ab-v5 + CCMA 0056 and CCMA 0056 led to lower total dry weight compared to control. These results suggest that CCMA 1285 can positively influence the interaction between the isolates leading to higher dry weight of the plants.

When the inoculants were associated with 50% nitrogen fertilizer, in general, there were significant additions to the total dry weight. Each bacterium and its combination responded differently to the addition of nitrogen, with emphasis on the inoculation of the three bacteria together, *Azospirillum brasilense*-Ab-V5 + *Burkholderia cepacia*-CCMA 0056 + *Enterobacter cloacae*-CCMA 1285, plus 50% nitrogen fertilizer, which caused an increase of 160.13% (12.54 g–32.62 g) in relation to the control without nitrogen.

In general, the use of 50% nitrogen fertilizer was better for biomass accumulation due to a significant increase in the aerial part, root and total dry weight, except for Av-b5 and Av-b5 + CCMA 0056. The results obtained in this work were better compared to the studies in Pereira et al. (2012) also studying inoculation with bacteria in strawberry plants. The researchers found values for the aerial part dry weigth between 1.21 and 2.33 g, and for the root between 0.86 and 2.45 g, both without adubation with nitrogen, but with evaluation of the plants with 90 days of age in growth, in which the highest values were obtained with the inoculation of *B. subtilis* and *Enterobacter* sp. compared with the control plants. In this work, the plants evaluation were realized in 75 days of growth, and the aerial part dry weight between 7.83 and 14.64 g, and the root between 3.15 and 10.00 g, both without adubation of nitrogen, where the best value obtained is from of the lonely bacterium *Azospirillum brasilense*-Ab-V5 and from the combination of the bacteria *Azospirillum brasilense*-Ab-V5 + *Burkholderia cepacia*-CCMA 0056.

Also, to the strawberry crop, the use of different beneficial microorganisms, such as *Pseudomonas*, *Bacillus*, and *Azospirillum*, has been reported and it has been shown that they were able to increase the production of vegetal biomass and fruit (Erturk et al., 2012; Esitken et al., 2010; Salazar et al., 2012).In addition, the species *Burkholderia* has been reported in terms of their beneficial effects, such as biomass increase in sugarcane (Oliveira et al., 2006) and grain yield in maize and rice (Estrada et al., 2013; Hernández-Rodríguez et al., 2010).

The interaction between bacteria strains and nitrogen fertilizer was significant regarding the crown diameter of the plants over the inoculation period. Fig. 3 shows that the inoculation with bacteria lead to higher crown diameter with or without nitrogen application compared to uninoculated plants. However, nitrogen application reduced the efficiency of *Azospirillum brasilense*-Ab-V5, leading to smaller crown diameter compared to the others treatments with bacteria. Although the difference between the strains were not significant, the tendency line suggests that inoculation with the three bacteria showed higher crown diameter at 75th day. Despite the higher crown diameter find for inoculated plants (Fig. 3), it does not necessarily imply higher aerial dry matter accumulation (Fig. 2A), once greater diameters are related not only with the leaf area but also with the length of the petiole.

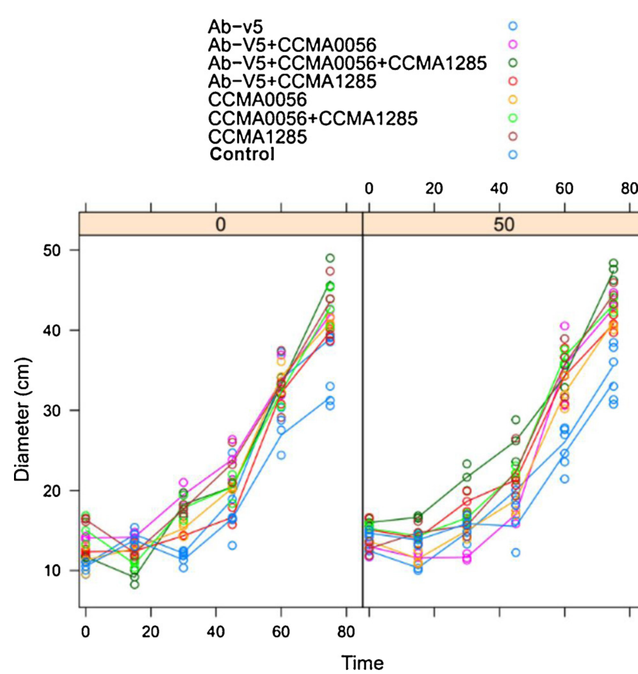

Fig. 3. Average crown diameter throughout the inoculation time (bacterium × time) with and without nitrogen fertilizer. *Azospirillum brasilense* (Ab-V5), *Burkholderia cepacia* (CCMA 0056) and *Enterobacter cloacae* (CCMA 1285), CONTROL (no bacteria).

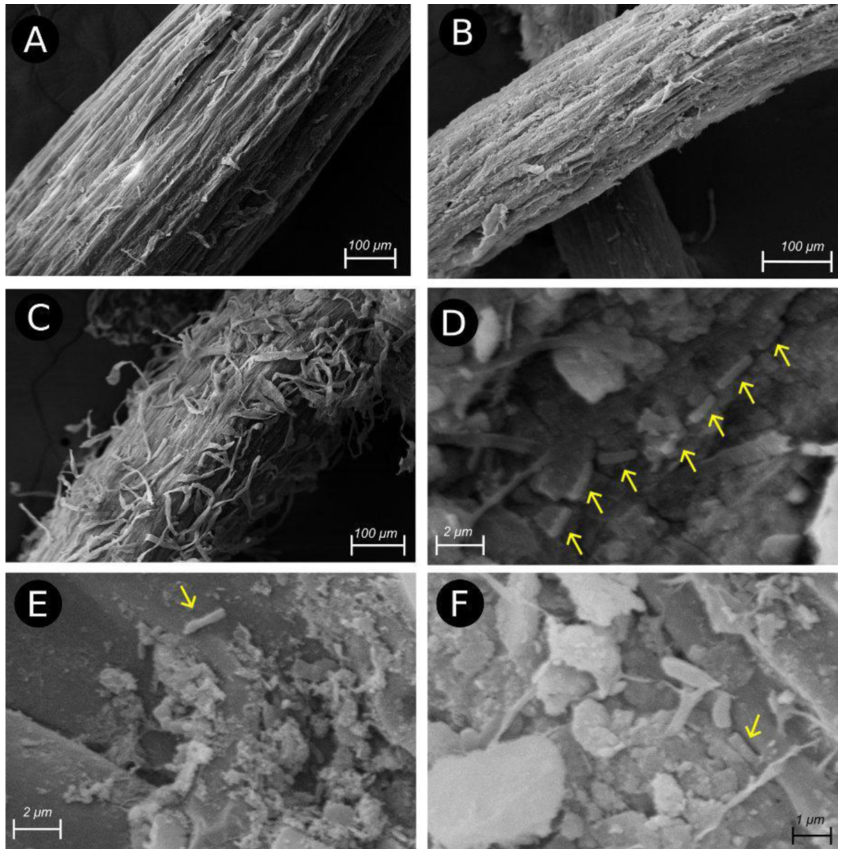

Fig. 4. Images obtained by scanning electron microscopy of the inoculated and uninoculated strawberry roots. (A) Control 1; (B) Control 2; (C) Treatment 8; (D) Treatment 12; (E) Treatment 15 and (F) Treatment 14.

3.3. Analysis of ultrastructure by scanning electron microscopy

Fig. 4 shows the roots of strawberry plants, bacteria and hairy roots formation with treatments and in control plants. In general, all the inoculated plants showed different aspects related to the hairy roots formation and the colonization of bacteria on the root surface, with some areas containing a low density of bacteria randomly associated or dispersed, plus zones of bacterial aggregates (Fig. 4D, E, and F).

Controls showed lower proliferation of hairy roots compared to inoculated plants which had higher hairy roots formation (Figs. 4C), as also reported in other studies by Guerrero-Molina et al. (2012) and Pedraza et al. (2010). The root hair is important because it increases the volume of soil substrate to be explored, besides allowing greater absorption of water and nutrients and other processes, due to the increased root surface area, thus helping to further develop the plant (Reis et al., 2008).

A fundamental characteristic of the beneficial bacteria in plants is the efficient colonization of the roots (Compant et al., 2005), as shown in images D, E and F. After the initial colonization step on the root surfaces, bacteria are able to colonize roots internally (Luna et al., 2010). This colonization can occur as follows: (1) through lateral root emergence sites (Botta et al., 2013); (2) through the root hairs (Fig. 4C); and (3) through cells of the root apical meristem (Hurek et al., 1994).

4. Conclusion

The present study is a pioneer in the evaluation of CCMA-UFLA and UNIFENAS wild isolates in relation to plant-growth promoting capacity, demonstrating the potential of these microorganisms for use as strawberry inoculants. The strawberry crop significantly responded to the inoculation of the strains *Azospirillum brasilense* (Ab-V5), *Burkholderia cepacia* (CCMA 0056) and *Enterobacter cloacae* (CCMA 1285), and the best results were observed when the plants were inoculated with the combination of the three bacteria together (*Azospirillum brasilense (*Ab-V5*)*, *Burkholderia cepacia* (CCMA 0056) and *Enterobacter cloacae* (CCMA 1285)) plus 50% nitrogen fertilizer. Therefore, it is possible to infer that co-inoculation of the strains with the use of nitrogen fertilizer is better for the strawberry crop, resulting in better plant growth responses. With these results the knowledge about the interactions of the plant-bacterial system were increased. Potential alternative practices that stimulate strawberry productivity in order to reduce traditional agricultural practices that harm biodiversity and the environment were proposed.

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