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Sugarcane growth promotion by *Kosakonia* sp. ICB117 an endophytic and diazotrophic bacterium

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The present study investigates the strain *Kosakonia* sp. ICB117, an endophytic, N_2-fixing bacterium that belongs to a genus recently described. The bacteria were isolated from sugarcane stalks (*Saccharum* sp. variety SP791011) and inoculated into other sugarcane plants of the same variety. The effect of inoculation on sugarcane growth was then studied in the presence or absence of nitrate supplementation (10 mM). The following plant growth parameters were analyzed: biomass, plant height and number of leaves. Furthermore, CO_2 assimilation and the C and N content of plants were also determined, as was the size of the endophytic bacteria population resulting from the inoculation. The findings showed that inoculation with bacteria (both with or without additional nitrate) led to an increase of plant biomass, CO_2 assimilation, total C and N in the roots, and the number of leaves. In addition, the polyamine putrescine and indole-3-acetic acid were actively released by the bacterium in *in vitro* assays and might be released in internal plant tissues as well, resulting in plant growth promotion. In conclusion, inoculation of sugarcane with *Kosakonia* sp. ICB117 increased the efficiency of the plant's metabolism.

Key words: Biological N, N-fixation, *Saccharum* sp., growth parameters, photosynthesis, nitrate supplementation, Brazil.

INTRODUCTION

Brazil has ample available land and a favorable climate for sugarcane growth. Both factors contribute to Brazilian's position as the world's largest sugar producer and exporter, and an important producer and consumer of ethanol (Adami et al., 2012; Bentivoglio et al., 2016).

Sugarcane production in Brazil has more than doubled between 2000 and 2013 but that increase resulted from an expansion of the sugarcane producing areas and not from an increase in crop yields (Marin et al., 2016). Therefore, it is important to increase sugarcane

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productivity and minimize their contribution to environmental degradation. The development of suitable expansion models for this crop, and an increase in technology investment, are some of the means that may lead to increasing sugarcane yields (Sparovek et al., 2009).

Nitrogen fertilizer application in sugarcane is an important issue because a deficiency in nitrogen greatly affects crop growth and production (Meinzer and Zhu, 1998), although this element constitutes only 1% of the total dry mass of a mature sugarcane plant (Van Dillewijn, 1952). Even with nitrogen fertilizer application, the uninterrupted growth of sugarcane might deplete the nitrogen in the soil, causing a possible decrease in plant yield. However, that decrease has not been verified in Brazil, suggesting the possibility that sugarcane benefits from a significant contribution of nitrogen through biological nitrogen fixation (Baldani et al., 2002). These observations led to an increase in the number of studies analyzing the effects of inoculation of diazotrophic bacteria into sugarcane plants.

There is a special interest in studying endophytic N_2-fixing bacteria since they live inside plants for at least part of their life cycle and do not visibly harm the host (Hallmann et al., 1997; Hardoim et al., 2008; Ryan et al., 2008). Studies about the interaction of endophytes with their host plants are important to understand the ecological relevance of this relationship (Hardoim et al., 2008). In addition to the possibility of making fixed nitrogen available to the plant, either through cell death or active release of nitrogenous compounds, diazotrophic bacteria can act as biocontrol agents and can release phytohormones such as indole-3-acetic acid, cytokinins and gibberellins (Bhattacharjee et al., 2008; Ferrara et al., 2012).

The genus *Kosakonia* was previously classified as *Enterobacte,r* and just recently was separated as a new genus (Brady et al., 2013). After that, a strain of endophytic diazotrophic bacteria isolated from sugarcane plants was described as *Kosakonia sacchari* (Chen et al., 2014). *K. radicincitans* was isolated from wheat and it too promoted the growth of other plants, such as accelerating the flowering and ripening of tomato and making its fruits more tasteful (Berger et al., 2017).

Understanding the effects of inoculating sugarcane plants with diazotrophic bacteria can serve as a foundation for studies seeking to improve sugarcane yields. *Kosakonia,* as a recently described genus, becomes a very good candidate to be used in similar studies. The hypothesis of the present study is that *Kosakonia* sp. ICB117, an endophytic N-fixing bacteria, can release nitrogenous substances produced using fixed-N and improve plant growth parameters of the inoculated sugarcane plants.

Therefore, the present study analyzed the influence of the endophytic N_2-fixing *Kosakonia* sp. strain ICB117, on sugarcane growth (*Saccharum* sp.) and on some physiological parameters, such as C/N ratio and CO_2 assimilation. The studies were performed with and without nitrate (10 mM) supplementation.

MATERIALS AND METHODS

Biological material

The strain, ICB117, previously isolated from a surface-disinfected sugarcane stalk in nitrogen free medium and identified as *Kosakonia* sp. (GenBank, access number HQ413276) at the Laboratory of Physiology of Microorganisms, Biomedical Sciences Institute – University of São Paulo, was submitted to physiological tests and inoculated into sugarcane plants (Supplementary Figure 1). Sugarcane plantlets (*Saccharum* sp.) of SP79-1011 variety were used for bacterial inoculation.

Bacterial growth curve, nitrogenase activity and detection of N-compounds

Three replicates of *Kosakonia* sp. ICB 117 cultures were grown in MS medium (Murashige and Skoog, 1962) deprived of reduced N, as described by Ferrara et al. (2012). For IAA detection, 0.5 $g.L^{-1}$ tryptophan was added to the medium as a precursor. Bacterial cultures were incubated unshaken at 30°C. After 5, 24, 100 and 200 h, aliquots were taken to perform the following analyses. The growth curve was drawn by counting the colony forming units (CFU) using the drop method described by Barbosa et al. (1995). The nitrogenase activity was measured by acetylene reduction assay (Anderson et al., 2004). Analyses of amino acids and IAA were performed in the filtered supernatants by reverse phase high-performance liquid chromatography (HPLC) with a C18 column, (Shimadzu Shim-pack CLC ODS) and a fluorescence detector according to Ferrara et al. (2012). Soluble polyamine concentrations in the culture medium were analyzed using methods described by Silveira et al. (2004) adapted to bacterial cultures. Polyamines were derivatized by mixing 40 µL of the bacterial supernatant to 100 µL of dansyl chloride (5 $mg.mL^{-1}$), 50 µL of a saturated solution of $NaHCO_3$, and 20 µL of 1,7-diamineheptane. Flasks containing the mix were incubated for 50 min at 70°C. Twenty five microliters of proline were added to convert the dansyl chloride into dansyl-proline after 30 min of incubation at room temperature. Polyamines were partitioned using 200 µL of toluene. A sample of 175 µL of the non-polar phase was collected and then dried under a nitrogen blow and resuspended in 175 µL of acetonitrile. Polyamine determination was performed using high performance liquid chromatography (HPLC) in a reverse phase C18 column (Shimadzu Shim-pack CLC ODS) and fluorescence detector (excitation 340 nm; emission 510 nm). Peak areas and retention times were compared to standards to calculate the polyamines concentrations. Losses were measured by comparing the initial and final concentrations of 1.7-diamineheptane.

Inoculation of *Kosakonia* ICB 117 into sugarcane plants

Bacterial transformation

Kosakonia sp. ICB117 was transformed by electroporation with plasmid pWM1007 (Miller et al., 2000). The plasmid contained a GFP gene and a kanamycin resistance gene, and was provided by the United States Department of Agriculture (USDA). Electro competent cells were obtained following the procedure described by Ausubel et al. 1995). Electroporation was performed using the BioRad Gene Pulser and parameters: 200 Ω, 25 µF e 1,8 KV.

Bacterial growth

Kosakonia sp. ICB117 containing plasmid pWM1007 was cultivated in 100 mL of NFb medium (Döbereiner, 1980) supplemented with 0.134 g of $(NH4)_2SO_4$ and incubated at 30°C for 16 h at 200 rpm. The bacterial culture was transferred to 900 mL of N-free NFb medium and was incubated unshaken at 30ºC for 24 h, until the population reached 10^8 CFU.mL^{-1}. The culture was centrifuged at 4°C for 20 min at 5000 *g*. The pellet was re-suspended in 1000 mL of sterile distilled water. That suspension was used for inoculation in sugarcane plants.

Inoculation procedure

The plants were not watered on the day before inoculation. For inoculation, roots were washed with tap water and small cuts were made at their tips. Plants were divided in two groups. Roots of the inoculated group were immersed in 4 L of bacterial suspension. Roots of non-inoculated plants were immersed in 4 L of sterile distilled water. Roots of both groups remained immersed for 2 hours. Afterwards, sugarcane shoots were planted individually in pots (7 L) with vermiculite and sand (2:1) as substrate. Plants were uniformly distributed by size into four groups, each receiving a different treatment:

(1) Control (C) – no bacteria and no nitrate
(2) Bacteria (B) – bacterial inoculation and no nitrate
(3) Nitrate (N) – no bacteria with nitrate supplementation;
(4) Bacteria + nitrate (BN): bacterial inoculation and nitrate supplementation.

Plant growth conditions

Once a week, 200 mL of a nutrient solution based on the Hoagland solution (Hoagland and Arnon, 1950), with or without nitrate 10 mM (following the experimental protocol depicted above) were added to each plant pot. The nutrient solution with nitrate was composed of:

KH_2PO_4 (1 mM); $Ca(NO_3)_2$ (5 mM); $MgSO_4$ (2 mM); KNO_3 (5 mM); micronutrients (5 mM); FeEDTA (10 mM); distilled water (q.s 1000 mL).

For the nutrient solution without nitrate, $Ca(NO_3)_2$ and KNO_3 were substituted by $Ca(Cl)_2$ and KCl, respectively. Plants were kept for two months in a greenhouse.

Plant sampling and analyses of growth and metabolism

Plant material was sampled once a month for two months, and the following parameters were analyzed: growth, C/N content of leaves and roots, photosynthetic CO_2 assimilation and estimation of the *Kosakonia* sp. ICB117 endophytic population. Height, number of leaves and dry mass were measured. In order to determine shoot and root dry mass. Plant material was dried in an oven at 50 °C until a constant mass was achieved. Roots and leaves were dried at 50°C and ground. C and N concentrations were determined by an elemental analyzer (Carlo Erba EA 1110 CHNS, CE Instruments) and a mass spectrometer (Delta Plus, ThermoQuest-Finnigan) in CENA/ESALQ/USP Isotopic Laboratory of Ecology. Approximately 2.6 mg of the dry mass were used for each analysis. Results are reported in percentages.

Measurements of net CO_2 assimilation rate (A) were made with the first leaf totally expanded, in the morning, using an infrared gas analyzer (LI-6400; Li-Cor, Lincoln, NE, USA and Ciras2). Light response curves were obtained in order to identify maximum photosynthesis (A max) and photosynthetically active radiation on photosynthesis saturation (PARsat), using an artificial light chamber and a CO_2 cylinder. Light response curves (A X PAR) were obtained according to the variation of PAR from 0 to 1250 µmol photons m^{-2} s^{-1} in a decreasing manner. The CO_2 concentration was maintained at 380 ppm and leaf temperature was maintained between 22 and 30°C.

Estimation of the endophytic Kosakonia sp. ICB117 population

Roots and shoots were washed in running tap water and 3 g of each were externally disinfected according to Araújo et al. (2002). As a control of the disinfection process, samples of shoots and roots which had been cut had their cut tips sealed with paraffin and were submitted to the same process of disinfection. The controls were incubated in a nutrient broth for 24 h at 30 °C. If any bacterial growth was detected, samples were discarded (Pariona-Llanos et al., 2010). The disinfected material was macerated in 6 mL of distilled sterile water with sterile mortar and pestle and 100 µL of the extract obtained were plated in LB medium (Sambrook and Russell, 2001) containing kanamycin (50 µg mL^{-1}) and nystatin (30 µg mL^{-1}). Plates were incubated at 30°C for 48 h. The CFU expressing green fluorescence were counted and the *Kosakonia* sp. ICB117 population density in roots and shoots was estimated (CFU.g^{-1}). Fluorescence was observed through a transilluminator M-20 Cambridge – UK.

Statistical analysis

Plant height data were submitted to a two-way analysis of variance (ANOVA) and one repeated measure, followed by Bonferroni test. Dry mass data were submitted to a three-way ANOVA. For other variables, a two-way ANOVA was used, followed by the Tukey test. The significance level was set at 5%.

RESULTS

Bacterial growth profile, nitrogenase activity and detection of N-compounds

The growth curve of *Kosakonia* sp. ICB117 shows that its population peaked after 24 h (Figure 1A). Nitrogenase activity was detected only during the exponential phase, with maximum values of 23.02 fmol.CFU^{-1}.h^{-1} (Figure 1A). All compounds studied in the present work were detected in the culture medium of *Kosakonia* sp. ICB117. Four different amino acids Ala, Asp, Trp and Val were detected after 200 h at the concentration of 0.02, 0.06, 0.01, and 0.04 µg.mL^{-1}, respectively. Putrescine was the only polyamine detected during the exponential phase, reaching 0.76 µg.mL^{-1} at 100 h. IAA was detected from 5 h onwards, reaching the concentration of 6.40 µg.mL^{-1} at 100 h.

Effects of *Kosakonia* ICB 117 inoculation into sugarcane plants

Plant height was not significantly influenced by the

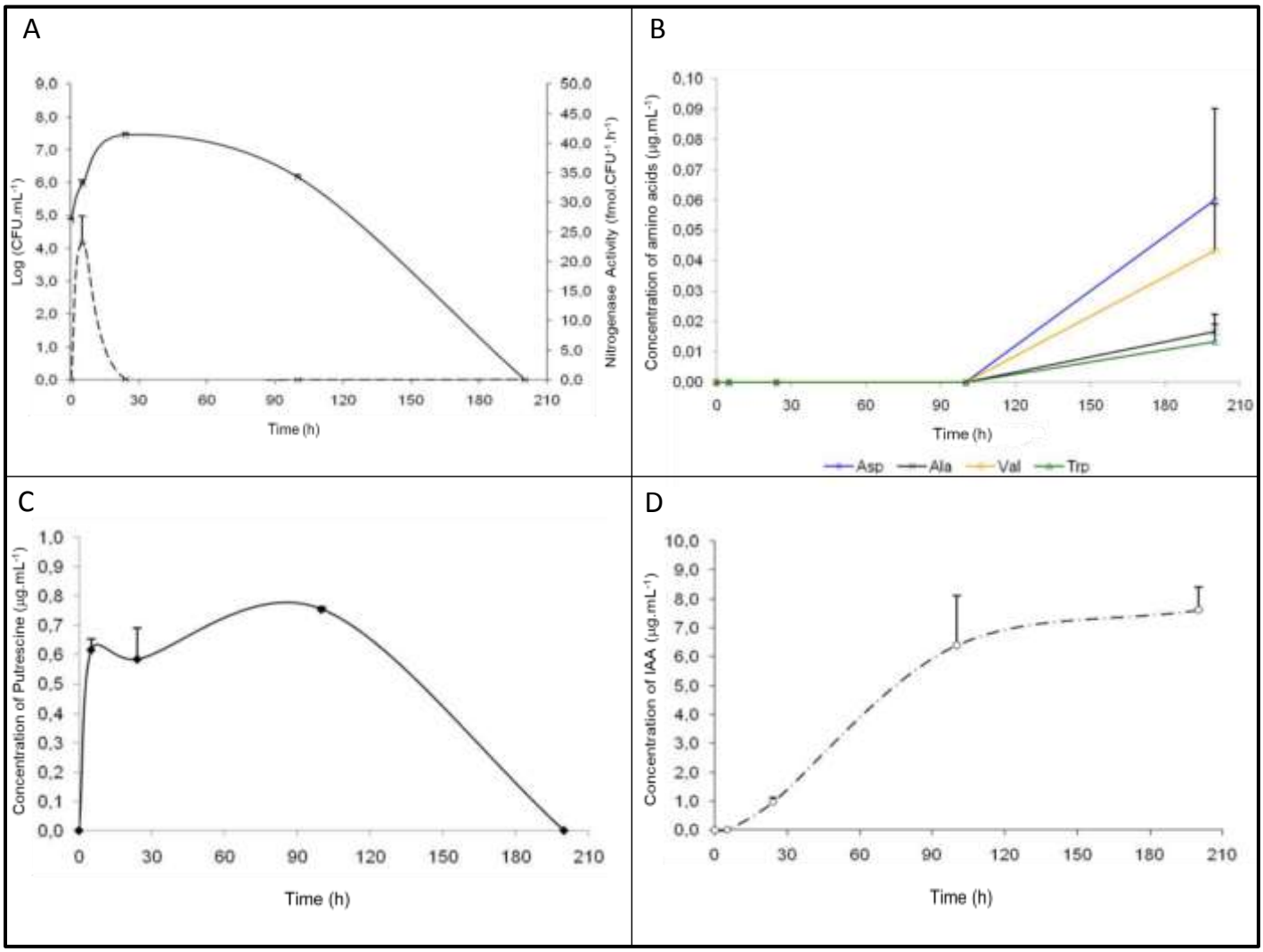

Figure 1. Growth curve of *Kosakonia* sp. ICB117 and their products released over time (h) in modified MS medium. A: growth (—) and nitrogenase activity (---); B: concentration of the four amino acids released; C: concentration of putrescine; D: concentration of IAA released.

Table 1. Average values and standard deviation of growth parameters assessed in plants submitted to the different treatments, after one and two months of growth. Control (C) – no bacteria inoculation and no nitrate supplementation; Bacteria (B) – inoculation with *Kosakonia* sp. ICB117 and no nitrate supplementation; Nitrate (N) – no bacterial inoculation with nitrate supplementation (10 mM); and Bacteria + Nitrate (BN) – bacterial inoculation plus nitrate supplementation. For each parameter at least four samples were analyzed.

Parameter	Month 1				Month 2			
	C	B	N	BN	C	B	N	BN
height (cm)*	85.0±11.9	92.1±9.2	114.8±14.3	119.3±12.0	98.3±9.3	102.8±6.5	152.8±12.8	154.3±13.6
nº leaves**	4.3±0.5	4.8±0.7	5.4±0.6	5.5±0.5	5.0±0.0	6.3±0.5	7.0±0.0	7.0±0.0
root/shoot ratio	0.9±0.2	1.6±0.5	0.9±0.2	1.1±0.4	0.7±0.2	1.4±0.6	0.5±0.1	0.9±0.3

*Effect of nitrate and effect of time, ANOVA ($p<0.001$). ** ANOVA only in month 1: Effect of bacteria ($p=0.025$) and effect of nitrate ($p<0.001$) ($n=15$).

presence of ICB117 (Table 1), as opposed of the effect of nitrate. The number of leaves increased in the first month on both inoculated plants ($p=0.025$) and plants treated with nitrate ($p<0.001$). The number of leaves did not vary

Figure 2. Roots of plants submitted to different treatments after two months. Control (C); Control (C) – no bacteria inoculation and no nitrate supplementation; Bacteria (B) – inoculation with *Kosakonia* sp. ICB117 and no nitrate supplementation; Nitrate (N) – no bacterial inoculation with nitrate supplementation (10 mM); and Bacteria + Nitrate (BN) – bacterial inoculation plus nitrate supplementation.

after two months. Inoculations with ICB117 induced the plant root thickening (Figure 2) and led to an increase in the root dry mass of plants of up to 114.5% in the B group and of up to 92.3% the BN group after two months. Unlike root dry mass, shoot dry mass did not show significant differences among inoculated and non-inoculated plants. On the opposite, supplementation with nitrate did not significantly change root dry mass (Figure 3), but plants supplemented with nitrate (groups N and BN) showed larger shoots dry mass than plants without nitrate (groups C and B) in the second month, ($p<0.05$) (Figure 3). The presence of ICB117 increased total dry mass of plants ($p=0.005$) throughout the experiment while nitrate led to an increase in total dry mass only in the second month ($p<0.05$) (Figure 3). Total nitrogen and carbon contents of roots increased ($p=0.009$ and 0.005, respectively), after two months of inoculation. Shoots, however, did not present the same result. Nitrate supplemented groups (N and BN) increased total N content of both shoots ($p<0.001$) and roots ($p=0.031$), but increased the total C content of shoots only ($p=0.002$) (Figure 4). Both, the inoculation ($p=0.016$) and nitrate supplementation ($p<0.001$) increased photosynthetic CO_2 assimilation (Figure 5).

Estimation of bacterial population

Group B presented a larger endophytic bacterial population than group BN ($p<0.001$) throughout the experiment. For both groups, a larger number of $CFU.mL^{-1}$ of ICB117 was isolated in the first month than in the second month ($p<0.001$) (Figure 6). CFU expressing GFP were not detected in non-inoculated plants (groups C and N) (Table 1).

DISCUSSION

Among the growth parameters analyzed, root dry mass was the most affected by the inoculation with ICB117. The positive effects of these bacteria on the plant are consistent with literature data (Dobbelaere et al., 2003; Mantelin et al., 2006) suggesting that the stimuli to plant growth are always correlated with remarkable changes in root morphology, such as increased length of lateral roots and number of hairs (Mantelin and Touraine, 2004; Vacheron et al., 2013).

Several authors attribute the increase in root development in inoculated plants to the release of auxin by bacteria (Bhattacharjee et al., 2008; Dobbelaere et al., 2003; Mantelin and Touraine, 2004). Knowing that *Kosakonia* sp. ICB117 can release IAA *in vitro*, one might suggest that the bacterium released this phytohormone into plant roots, thus causing the observed increase in root dry mass. However, it is also possible that the

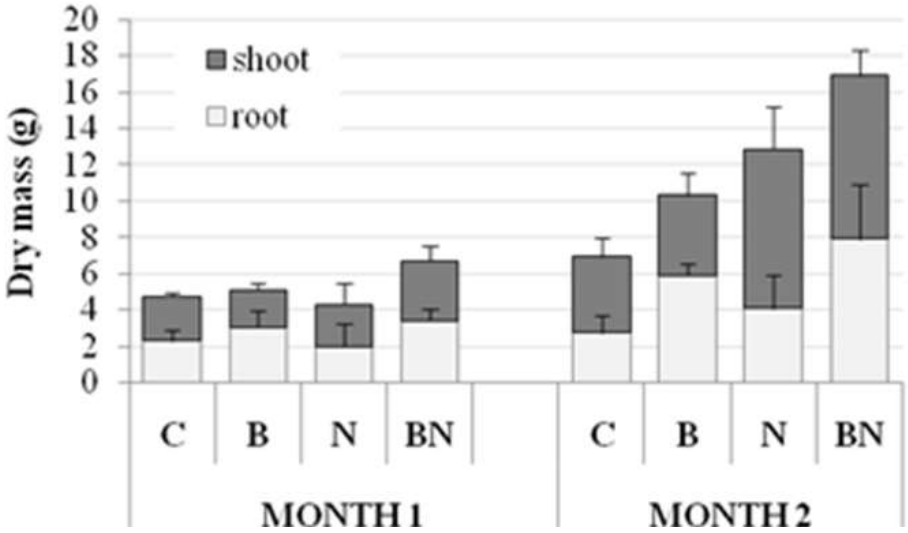

Figure 3. Average values and standard deviation (n=4) of dry mass of root and shoot of plants submitted to different treatments after the first and second months. Control (C) – no bacteria inoculation and no nitrate supplementation; Bacteria (B) – inoculation with *Kosakonia* sp. ICB117 and no nitrate supplementation; Nitrate (N) – no bacterial inoculation with nitrate supplementation (10 mM); and Bacteria + Nitrate (BN) – bacterial inoculation plus nitrate supplementation; Root dry mass: effect of bacteria, ANOVA ($p<0.001$) and effect of time ($p<0.001$); Shoot dry mass: Interaction effect of nitrate and time: month 2 – with nitrate > without nitrate. Month 2 > month 1 (Tukey test, $p<0.05$). Total dry mass - effect of bacteria, ANOVA ($p=0.005$) and interaction effect of nitrate and time: month 2 – with nitrate > without nitrate. Month 2 > month 1 (Tukey test, $p<0.05$).

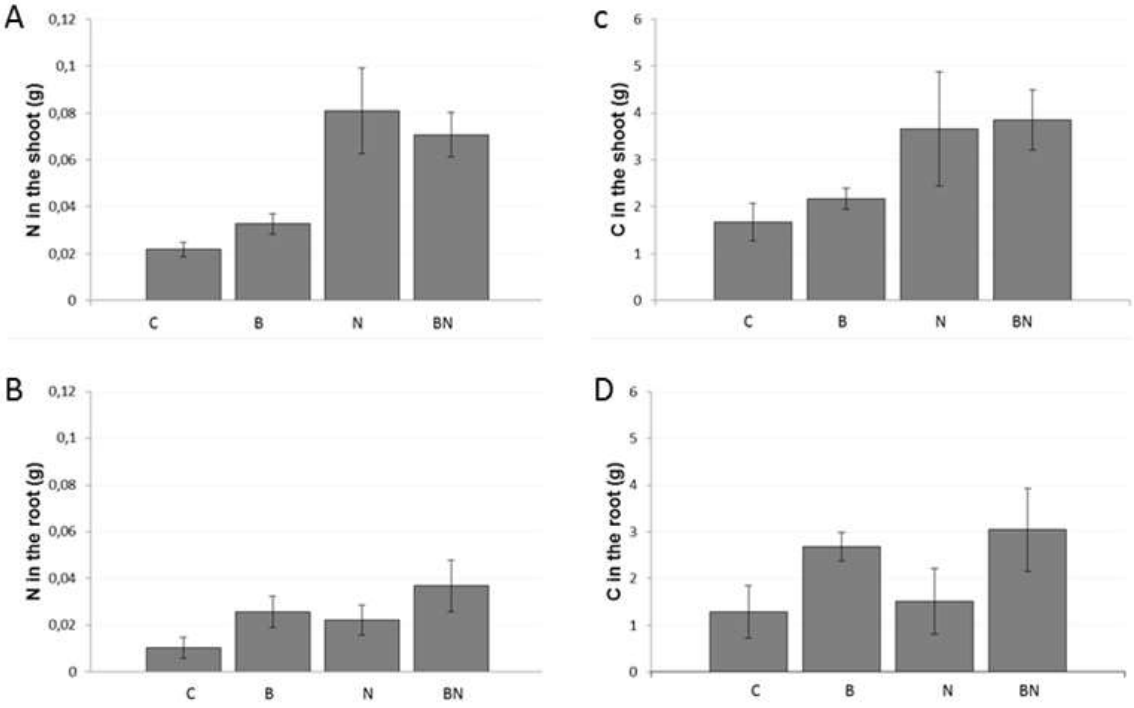

Figure 4. Average values (n=3) of total nitrogen (g) and total carbon (g) in shoot (A,C) and root (B,D) at the second month. Error bars are standard deviation. A: effect of nitrate ANOVA $p<0.001$; B: effect of bacteria ANOVA $p=0.009$ and effect of nitrate ANOVA $p=0.031$; C: Effect of nitrate ANOVA $p=0.002$; D: Effect of bacteria ANOVA $p=0.005$.

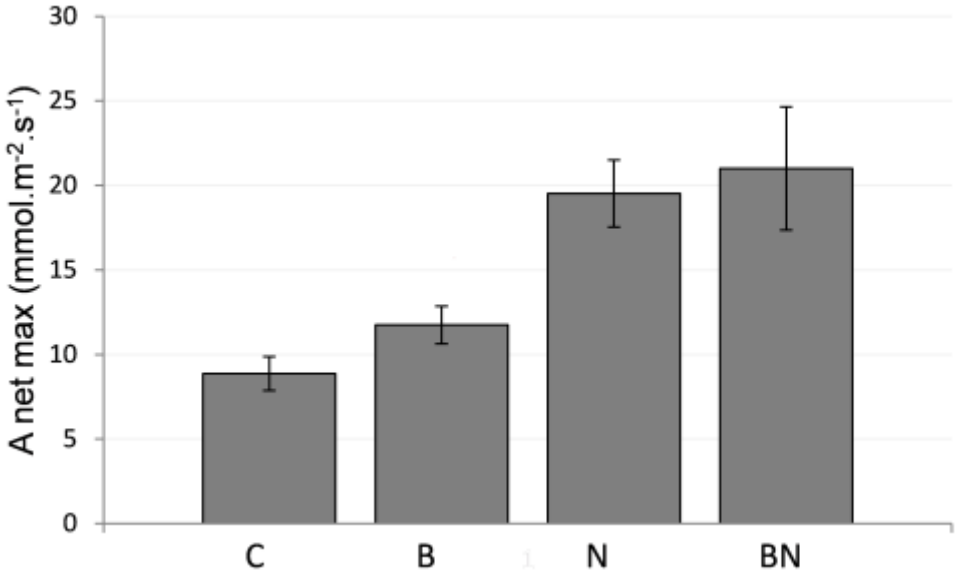

Figure 5. Average values (after two months) of net CO_2 assimilation rate (A max, n=4) for plants submitted to the different treatments: Control (C) – no bacteria inoculation and no nitrate supplementation; Bacteria (B) – inoculation with *Kosakonia* sp. ICB117 and no nitrate supplementation; Nitrate (N) – no bacterial inoculation with nitrate supplementation (10 mM); and Bacteria + Nitrate (BN) – bacterial inoculation and nitrate supplementation. Error bars are standard deviation. Effect of bacteria, ANOVA (p=0.016) and effect of nitrate, ANOVA (p<0.001).

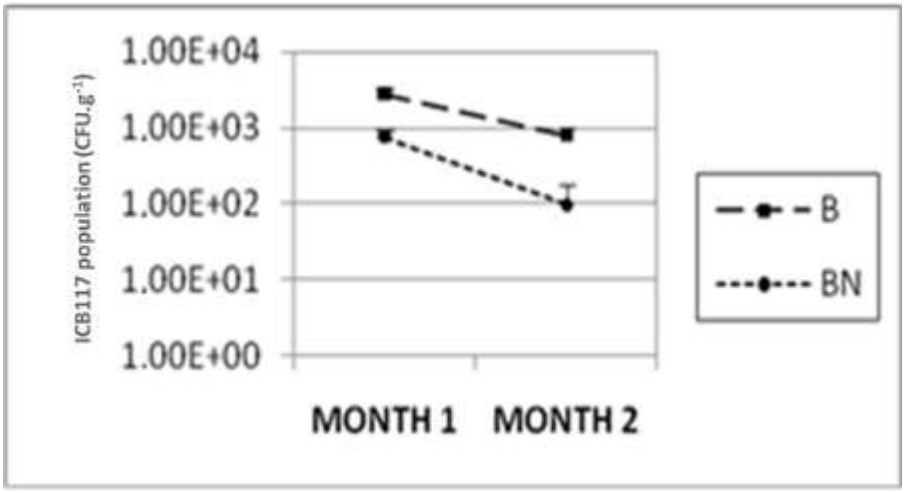

Figure 6. Average values and standard deviation (n=3) of the population of *Kosakonia* sp. ICB117 measured by the number of colony forming units expressing green fluorescence protein per gram of fresh tissue. Effect of nitrate, ANOVA (p<0.001) and effect of time, ANOVA (p<0.001).

presence of the bacterium may have stimulated IAA production by the plant itself (Carvalho et al., 2014).

The release of amino acids into the culture medium apparently results from cell death, as the ICB117 population is dead after 200 h, the time when amino acids were detected. On the other hand, putrescine was actively released by the bacterium, mainly in the exponential phase of growth. No nitrogen was used to supplement the culture medium where these features were studied, indicating that the molecules released were synthesized with nitrogen obtained through the reduction of atmospheric N_2. Nitrogen is one of the main limiting

factors to plant growth (Agren et al., 2012) and the results of the present study indicate that both N-containing molecules, amino acids and mainly the actively excreted polyamines, could be a significant source of this element if ICB117 is able to fix nitrogen and release these compounds into either the rhizosphere or inside the plant. The increment in root dry mass was remarkable in the BN group. Nitrate was supplemented at a concentration of 10 mM. That concentration might be too high for sugarcane but it was also shown that the preference for different nitrogen forms and concentrations depends on plant variety and conditions to which plants are subjected (*in vitro*, in pots or in the field; Hajari et al., 2014; Hajari et al., 2015). Hajari et al. (2014) showed that *in vitro* sugarcane plants submitted to different N forms and concentrations in general exhibited a higher affinity for nitrate but a higher uptake rate for ammonia (higher Vmax). Generally, *in vitro* plants accumulated more biomass when grown in a culture medium with nitrate than with ammonia (Hajari et al., 2015). In the present study, it was not observed that 10 mM nitrate was harmful to plants. Despite the apparent decline in the number of roots in Group N, there was no significant decrease in the biomass of either roots or shoots. An increase in root surface enables the plant to increase soil exploration. Thus, inoculation with *Kosakonia* sp. ICB117 provided the plant with greater potential for nutrient uptake. The increase in nutrient uptake of plants leads to an improved performance in CO_2 fixation through photosynthesis. Considering that in sugarcane a higher input of carbon through photosynthesis leads to a greater accumulation of sucrose in the stem (Souza et al., 2008), it is possible to infer that, in later stages of plant development, sugar accumulation would probably be greater in inoculated plants than in non-inoculated plants. Moreover, the increase in the number of leaves caused by the bacterium represents a larger photosynthetic area for the plant. Although this increase amounted to only one leaf, it is an interesting finding since it is beneficial for sugarcane to produce many leaves at early growth stages (Bonett, 1998). A greater number of leaves enhances leaf area development that will maximize capture of radiation (Bonett, 1998; Robertson et al., 1998), with increase in carbon fixation and subsequent sucrose accumulation. In addition, higher number of leaves per stalk will generate a larger number of internodes which are used for sugarcane propagation (Bonnet, 1998).

Kosakonia sp. ICB117 expressing the green fluorescence was isolated from disinfected roots only, the plant organ where the effects of the inoculation were mainly observed. The fact that this bacterium was not found in stems stalks or leaves indicates that ICB117 adapted itself to the internal niche of roots rather than shoots. The presence of bacteria in different plant organs is an indication of their ability to be adapted to specific ecological niches (Lee et al., 2013). It is possible that ICB117 colonized intercellular spaces of cortical tissues as endophytic bacteria usually remain in the roots (Bacon and Hinton, 2006).

A higher population density of *Kosakonia* sp. ICB117 was found in plants inoculated with the bacterium and not treated with nitrate (Group N), than in plants inoculated and treated with nitrate (Group BN). This observation is consistent with literature reports which indicate that high concentrations of N fertilizers result in a decrease of diazotrophic microbial populations inside plants (Boddey et al., 2006). This effect is caused by the formation of long, pleomorphic, immobile cells when high concentrations of nitrogen sources are applied (Muñoz-Rojas and Caballero-Mellado, 2003). Although the graphic shows a declining trend, it would take a much longer experiment to show that the bacteria were being eliminated from the plant.

In conclusion, *Kosakonia* sp. ICB117 was able to produce and release nitrogenous compounds, including the phytohormone IAA. In addition, inoculation with this strain indicated that plants became more efficient, increasing their biomass, total C and N concentrations in roots, the number of leaves and the rate of CO_2 assimilation. Even with nitrate supplementation, inoculation significantly helps plant growth, which shows that the use of conventional fertilization does not impair its use as inoculant. This study shows that this strain is a very good candidate for field trials in order to study its capability as a biological fertilizer.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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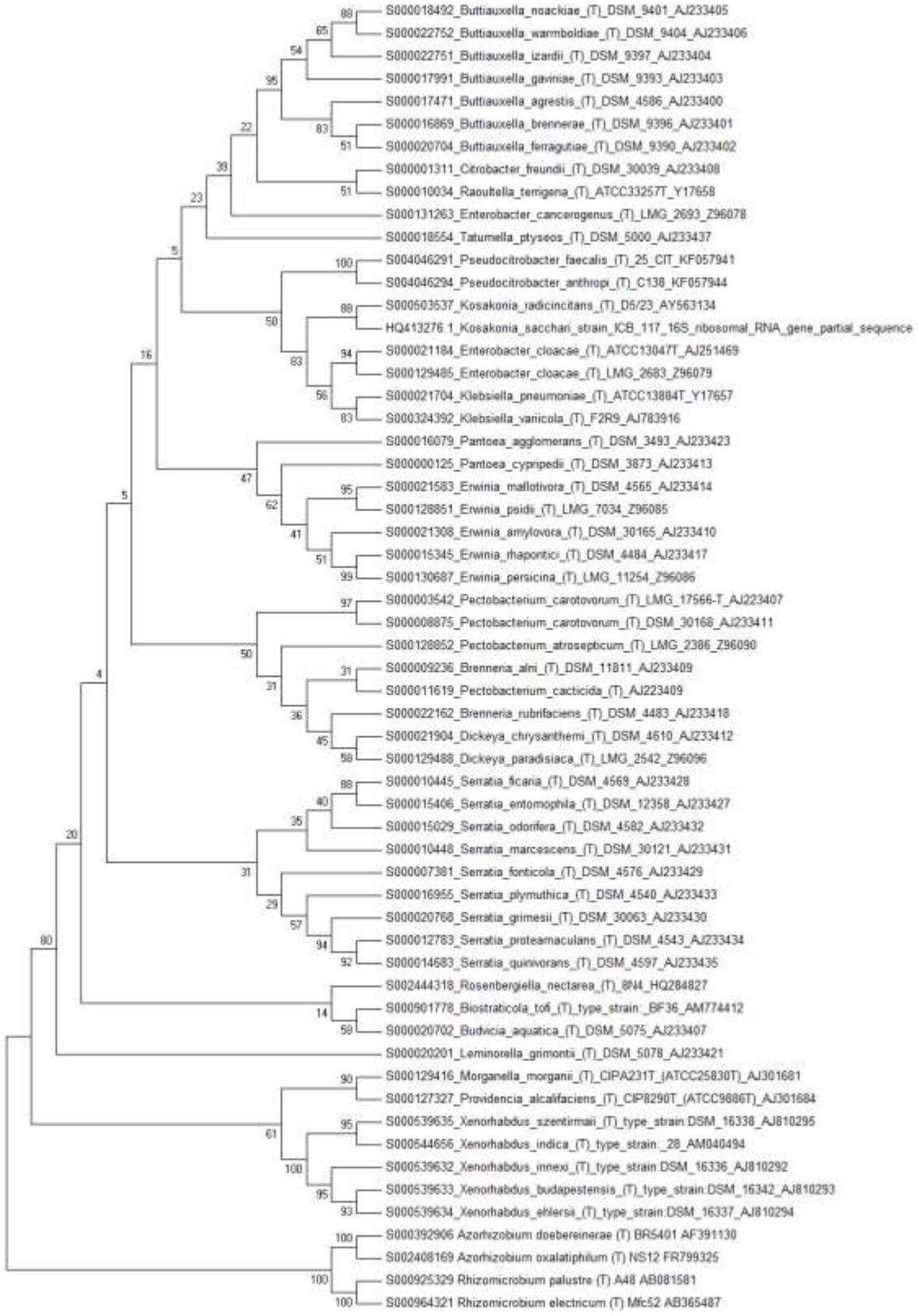

Supplementary Figure 1. Phenetic tree based on 16S RNA of *Kosakonia* sp. ICB117 and 57 type strains of the Enterobacteriaceae family. The sequences were aligned using the Muscle program and were constructed with the neighbor-joining algorithm in MEGA 7.0.21 software. Bootstrap values are shown in the nodes.