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Sugarcane growth promotion by Kosakonia sp. ICB117 an endophytic and diazotrophic bacterium
Carolina Krebs Kleingesinds, Felipe Ibañez de Santi Ferrara, Eny Iochevet Segal Floh, Marcos Pereira Marinho Aidar and Heloiza Ramos Barbosa

Shelf life enhancement of plant growth promoting rhizobacteria using a simple formulation screening method
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Sugarcane growth promotion by *Kosakonia* sp. ICB117 an endophytic and diazotrophic bacterium

Carolina Krebs Kleingesinds¹,³*, Felipe Ibañez de Santi Ferrara¹, Eny Iochevet Segal Floh³, Marcos Pereira Marinho Aidar² and Heloiza Ramos Barbosa¹

¹Department of Microbiology, Institute of Biomedical Sciences, University of Sao Paulo. Av. Prof. Lineu Prestes, 1374 – Butantã, 05508-900, São Paulo, SP, Brasil.
³Department of Botany, Institute of Biology, Universidade of São Paulo, Rua do Matão, 277- Butantã, 05422-970, São Paulo, SP, Brasil.

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The present study investigates the strain *Kosakonia* sp. ICB117, an endophytic, N₂-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, CO₂ 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, CO₂ 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...
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 Dillenwijn, 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 N₂-fixing bacteria since they live inside plants for at least part of their life cycle and do not visibly harm the plant (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 (Bhattacherjee et al., 2008; Ferrara et al., 2012).

The genus *Kosakonia* was previously classified as *Enterobacter* 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 N₂-fixing *Kosakonia* sp. strain ICB117, on sugarcane growth (*Saccharum* sp.) and on some physiological parameters, such as C/N ratio and CO₂ 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, accession 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 NaHCO₃, 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 suspended 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). Electroporet 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)₂SO₄ 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 30 °C for 24 h, until the population reached 10⁷ 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;

**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:

KH₂PO₄ (1 mM); Ca(NO₃)₂ (5 mM); MgSO₄ (2 mM); KNO₃ (5 mM); micronutrients (5 mM); FeEDTA (10 mM); distilled water (q.s 1000 mL).

For the nutrient solution without nitrate, Ca(NO₃)₂ and KNO₃ 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 CO₂ 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 CO₂ 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 CO₂ 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 CO₂ 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.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Month 1</th>
<th>Month 2</th>
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<tr>
<td></td>
<td>C</td>
<td>B</td>
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<tr>
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<td>nt leaves**</td>
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<td>4.8±0.7</td>
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<td>root/shoot ratio</td>
<td>0.9±0.2</td>
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*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
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 CO₂ 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 ($A_{\text{max}}$, 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).

The 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 CO2 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 CO2 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.

ACKNOWLEDGEMENTS

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REFERENCES


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.
Shelf life enhancement of plant growth promoting rhizobacteria using a simple formulation screening method

Marina Teixeira Arriel-Elias¹,²#, Maythulene I. S. Oliveira²,³#, Valacia Lemes Silva-Lobo², Marta Cristina Corsi Filippi², Amadou H. Babana⁴, Edemilson Cardoso Conceição³ and Marcio Vinicius de C. B. Cortes²∗

¹Universidade Federal de Goiás, Escola de Agronomia, Avenida Esperança, s/n., Campus Samambaia, Goiânia, GO, Brazil.
²Embrapa Arroz e Feijão, Rodovia GO 462 Km 12, Santo Antônio de Goiás, GO, Brazil.
³Faculdade de Farmácia, Universidade Federal de Goiás, Rua 240 s/n, Setor Leste Universitário, Goiânia, GO, Brazil.
⁴Faculty of Sciences and Techniques, University of Sciences Techniques and Technology of Bamako, BP E3206, Bamako, Mali.

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Plant growth promoting rhizobacteria (PGPR) are a specific group of bacteria interacting beneficially with plants. Among the known PGPRs, the species Pseudomonas fluorescens and Burkholderia pyrrocinia have been highlighted in both growth promotion and control of rice diseases. Ensuring the stability of the microorganism during production, formulation, distribution and storage has been a challenge for these species. In this context, the objective of this work was to develop liquid formulations, through a simplified process, that allows increase in the shelf life of these rhizobacteria for commercial application. Both bacteria were tested in 32 formulations under two storage temperature conditions: 8 and 28°C, resulting in 64 treatments for each species, which were evaluated for 180 days. Combinations of the adjuvants: molasses, glycerol, NaCl, PVP, MgSO₄, K₂HPO₄ and yeast extract were evaluated. Formulations containing molasses, stored at 8°C, were considered the most efficient in maintaining microbial viability. The method used was considered efficient to select three formulations that allowed maintenance of the concentration of viable cells of P. fluorescens and B. pyrrocinia in 10⁸ cfu.mL⁻¹, for at least 90 and 150 days, respectively, not interfering with bacterial action potential.

Key words: Pseudomonas fluorescens, Burkholderia pyrrocinia, plant growth promoting rhizobacteria, shelf life, liquid formulations.

INTRODUCTION

Positive effects of plant growth promoting rhizobacteria (PGPR) are directly related to nutritional issues, stress...
tolerance and disease control (Zhou et al., 2015; Selvaraj et al., 2014). Among the known PGPRs are the species of the genus, *Pseudomonas* and *Burkholderia* that have been described (Sundaramoorthy et al., 2013). Excellent results have been obtained in the use of the Brazilian strains, *Pseudomonas fluorescens* BRM 32111 and *Burkholderia pyrocina* BRM 32113 in experiments performed *in vivo* and *in vitro*, including field trials, aiming at both the promotion of growth and the control of rice diseases. The data show suppression of leaf blast and panicle by up to 60 and 33%, respectively, promoting root growth by up to 87% and increasing up to 20% in field productivity (Sousa et al., 2017; Sperandio et al., 2017).

Shakih and Sayyed (2015) affirm that commercial success of a bioprodut in suppressing diseases or enhancing plant growth depends on availability and shelf life. However, ensuring the stability of the microorganism during production, distribution and storage is a general challenge for several species (Leggett et al., 2011). In this sense, PGPRs characterized as Gram negative, as is the case of *P. fluorescens* and *B. pyrocina*, non-producing resistance structures (spores), are an even greater challenge. Because of their structure and cellular composition, these bacteria have a reduced shelf life. The strategy adopted in these cases, in general, is to produce a larger number of cells, whose population is reduced throughout the storage period, but still has a number of viable cells necessary for its action as PGPR to be complete (Tabassum et al., 2017).

Another strategy aiming at the shelf life increase of these microorganisms would be the development of new formulations. Liquid formulations should be considered in this aspect, because unlike solid formulations, they allow addition of sufficient amount of nutrient and cell protectants, improving bacteria shelf life (Brar et al., 2012). About 80% of biological products containing *Azospirillum* sp., a PGPR available for commercialization in South America, uses liquid carriers for the formulation of these biological products with more frequent shelf life of the registered products being 6 months (Cassan et al., 2016).

However, Slininger et al. (2013) reported that the availability of information related to the methodologies of production and formulation of microorganisms with agricultural application is scarce, since the details of related processes are kept as intellectual property of the few companies that commercialize these types of products. This is still a reality, since it is a field of research with several opportunities for new discoveries.

In this context, the objective of this work was to develop liquid formulations, through a simplified process, to increase the shelf life of the rhizobacteria, *P. fluorescens* BRM 32111 and *B. pyrocina* BRM 32113. The result of this work will facilitate analysis of the viability of the development of a product based on such bacteria for future commercialization, as a growth promoter and/or biocontrol agent for rice diseases.

### MATERIALS AND METHODS

#### Microorganisms

Bacteria, *Pseudomonas fluorescens* BRM 32111, *B. pyrocina* BRM 32113 and the fungi *Magnaporthe oryzae* BRM 31295, all belonging to the Embrapa Microbial Collection were used in this work. Bacterial and fungal strains were preserved by the Castellani (fungi or bacteria water suspension stored at 8°C) and ultra-freeze methods (-80°C), respectively, until their use. The growth of the bacterial isolates was conducted by scattering them in Petri dishes containing nutrient agar (NA), which were incubated for 48 h at 28°C. The fungus was grown in Potato Dextrose Agar (PDA), incubated at 24°C for 15 days. Petri dishes containing the bacteria and fungus were stored at 8°C until application.

#### Bacteria biomass production

Bacterial strains were cultured separately in 500 mL Erlemeyers flasks containing 100 mL of nutrient broth (NB) and incubated under constant shaking at 150 rpm for 48 h at 28 ± 2°C. These conditions were necessary to ensure that both bacteria reached the stationary phase of their respective growth curves at the moment of their incorporation in the formulations.

#### Evaluation of adjuvants formulation phytotoxic effect

Prior to preparation of bacterial formulations, adjuvants (components of formulations) were tested individually to evaluate their phytotoxic effects against rice. Detached leaves of BRS Primavera rice cultivar 21 days after planting were sprayed with the different adjuvants (Table 1), separately, at the final concentrations as described below. The pulverized leaves were kept in a humid chamber and incubated at 25 ± 2°C under constant common light. Visual evaluations of phytotoxicity, characterized by yellowish/whitish spots (chlorotic lesions), or any other change in leaf surface in relation to the control, were performed daily for seven days based on Sakthivel et al. (2002). The control of the experiment was represented by spraying leaves with sterilized distilled water.

#### Preparation of formulations

Bacterial formulations (bacteria plus specific adjuvant set) were assembled into sterile capped concave 96-well microplates. The general composition of which was 60 μL of the bacterial inoculum (bacteria biomass in stationary phase as described previously) and 90 μL of the adjuvant combination resulting in a final volume of 150 μL formulation. Adjuvants used and respective final concentrations were: molasses 1%, glycerol 1%, K₂HPO₄ 0.05%, PVP 0.1%, NaCl 0.01%, yeast extract 0.1% and MgSO₄ 0.02%, as described in Table 1. The use of these adjuvants was based on Parzianello (2012). Each component of the formulation was pre-sterilized. The control treatment (formulation without bacteria) was 60 μL of the bacterial inoculum and 90 μL of 0.85% saline, resulting in 150 μL final volume. The microplates containing the bacterial formulations and the controls were sealed with plastic film, avoiding contaminations and high loss of humidity, and stored at 8 ± 2°C (simulating storage at cold chamber) and 28 ± 2°C (simulating storage at room temperature).

#### Shelf life evaluation period

Shelf life of bacterial formulations at two storage conditions was...
evaluated. The evaluations were carried out at 10, 20, 30, 60, 90, 120, 150 and 180 days for *P. fluorescens* and at 7, 14, 20, 30, 60, 90, 120, 150 and 180 days for *B. pyrrocinia*. Different periods of evaluation were defined for each species. The reason was to better understand *B. pyrrocinia* behavior in early periods of storage. *P. fluorescens* storage behavior was already known.

**Shelf life evaluation method**

Evaluations were performed by comparing viability of both bacteria using growth kinetics parameters of the two bacteria in the different formulations. At the end of each storage period, the contents of the microplates were homogenized on a suitable shaker for 15 min at 7000 rpm. Then, 10 μl of each formulation were transferred to a new, top coated sterile capped plate containing 140 μl of culture defined medium: glucose 0.1%, NH₄Cl 0.05%, K₃HPO₄ 0.01%, NaCl 0.05%, MgSO₄ 0.002, in sterile distilled water and pH adjusted to 7.0. Microplates were maintained under constant stirring on microplate shakers at 7000 rpm for 24 h at 28 ± 2°C. The conditions and incubation period were defined in a preliminary test (data not shown), ensuring that the bacteria present in the formulations were in the exponential phase (log) of the growth curve. After the exact 24 h of incubation, optical density (OD) measures were taken in absorbance in an Epoch Microplate Reader (Biotek®) with the data collected by the Gen5 software (Biotek, Vermont, USA). The readings were performed at the wavelength of 620 nm. The kinetics of bacterial growth (microbial activity) was defined in "abs.h⁻¹" (calculated = ΔAbs/24). Only values of microbial activity greater or equal to 0.02 abs.h⁻¹ were acceptable to consider a bacteria formulation effective. The assay was conducted in a completely randomized design in three replicates. The best bacterial formulation, for each storage temperature, storage period and species under analysis, were evaluated by direct plate counting using nutrient agar through the serial dilution methodology, with results expressed in cfu.mL⁻¹. These evaluations were performed at

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<th>NaCl 0.01%</th>
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"+1" Means contains adjuvant and "-1" means does not contain adjuvant.
the end of each previously described period, together with the control for comparison purposes. The methodology was based on Slininger and Schisler (2013) with several modifications previously described.

Impact of formulation on antagonistic bacterial capacity

*M. oryzae* isolate was previously grown in a Petri dish containing PDA. After seven days under incubation at 24°C, 5 mm diameter mycelial discs were transferred to new 90 mm diameter Petri dishes containing PDA culture medium and positioned in the center of the plates. The best bacterial formulations, with cell concentration at approximately 1.0 x 10⁶ cfu mL⁻¹ were applied around the mycelial disc, forming a square with 4 cm sides. The unformulated bacteria, in the same concentration, were applied as described above, being a control treatment. The absolute control was considered a PDA plate only containing the mycelial disc, in the absence of the bacteria. The plates were incubated under light and constant temperature of 24°C for seven days. At the end of the incubation period, the diameters of the colonies were measured with the aid of a millimeter ruler. The intensity of the antagonism was evaluated by comparing the percentage of reduction of the means of the colonies areas in the different treatments (Filippi et al., 2011). The assay was conducted in a completely randomized design in three replicates.

Bacterial formulations against rice blast– plants cultivation

Seeds of the cultivar, BRS Primavera were sown in plastic vessels with 500 g of soil fertilized with NPK (5 g of 5-30-15 + Zn). Cover fertilization was performed twenty days after sowing with 3 g of ammonium sulfate. The seeds were previously disinfested with 70% alcohol and sodium hypochlorite.

Bacterial formulations against rice blast– treatments

Two completely randomized trials (DIC) were performed. Experiment 1 (E1) involving *B. pyrocinia* was composed of six treatments in five replicates. The treatments consisted of: T1: Bacterial formulation F11 + *M. oryzae*; T2: Bacterial formulation F32 + *M. oryzae*; T3: Formulation F11 without bacteria + *M. oryzae*; T4: Formulation F32 without bacteria + *M. oryzae*; T5: *B. pyrocinia* non-formulated + *M. oryzae*; T6: *M. oryzae*. Experiment 2 (E2) involving *P. fluorescens* was composed of six treatments in five replicates. The treatments consisted of: T1: Bacterial formulation F11 + *M. oryzae*; T2: Bacterial formulation F20 + *M. oryzae*; T3: Formulation F11 without bacteria + *M. oryzae*; T4: Formulation F20 without bacteria + *M. oryzae*; T5: *P. fluorescens* non-formulated + *M. oryzae*; T6: *M. oryzae*. A bacterium, formulated or not, as well as the formulations without bacteria (combined adjuvants) were applied as follows: E1, microbiolization of the seeds before planting and application of the formulations (or free bacteria) were carried out at seven and fourteen days after planting. E2, microbiolization of the seeds before planting and application of the formulations (or free bacteria) were carried out by spraying at seven and fourteen days after planting. The microbiolization of the seeds was performed by immersion and constant agitation of the seeds together with the formulated or free bacteria in a shaker table at 150 rpm for 24 h, added to a 24 h drying period at 25°C (Filippi et al., 2011). The irrigation and spraying applications were carried out by spraying at seven and fourteen days after inoculation with a severity scale (0, 0.5, 1, 2, 4, 8, 16, 32 64 and 82%) according to Notteghem (1981), determining the percentage of leaf area affected by the disease.

Statistical analysis

For impact of each adjuvant and storage conditions on shelf life, principal components analysis was performed using the licensed Action® software. For shelf life and impact of formulation on bacteria activity, data were analyzed using the Tukey’s test with 95% confidence, using the licensed Action® software. For blast disease, data were analyzed by comparing the means using the Tukey’s test at 5% of significance using the SPSS software, version 2.1.

RESULTS AND DISCUSSION

Evaluation of adjuvant phytotoxicity in rice plants

Adjuvants individually tested negative for phytotoxic effect on detached rice leaves on a visual evaluation (Figure 1). Different treatments did not alter the characteristics of leaves in a period of one week of evaluation, mainly in relation to the appearance of yellowish, whitish spots, necrosis or any other alteration of the leaf surface as compared to the control. *In vivo* tests under greenhouse conditions were also performed with the formulations without bacteria. The results confirmed that formulations, in the absence or presence of bacteria, did not generate direct toxicity reactions in rice plants.

Shelf life evaluation of bacterial formulations

Data shows that viability of the bacterial strains studied stored under refrigeration (8°C) or room temperature (28°C), in the absence of stabilizing formulations, rapidly reduced (Figure 2). The *P. fluorescens* strain reached values close to 0% viability in only ten days under both storage conditions (Figures 2a and b), while the *B. pyrocinia* strain reached 0% viability at 90 days at room temperature (Figures 2c and d). This low viability (poor shelf life) is the first factor that impairs its commercial application, overlapping the potential of action of these two PGPRs as described by Berg (2009). In this sense, different combinations of adjuvants (Table 1), presenting different functions, associated with the two storage conditions described (8 and 28°C) were analyzed for two

Bacterial formulations against rice blast– pathogen inoculation and disease evaluation

*M. oryzae* conidial suspension produced in oat agar was adjusted to 3.0 x 10⁸ conidia mL⁻¹. At 21 days of cultivation, the plants kept in cages coated with clear plastic were sprayed with 30 mL per cage of the conidia suspension by a pressure pump and a spray gun, with pressure of 0.001 kg/cm. The plants were kept in greenhouses with temperatures ranging from 25 to 28°C and relative humidity above 80%. The evaluation of leaf blast severity was performed eight days after inoculation using a severity scale (0, 0.5, 1, 2, 4, 8, 16, 32 64 and 82%) according to Notteghem (1981), determining the percentage of leaf area affected by the disease.
strains. In general, Figure 2 shows that in both species, when formulated, cell viability is increased, regardless of the form of storage. By evaluating strains separately, the most unstable bacteria, *P. fluorescens* (Figure 2a and b)
was able to maintain its cell concentration at approximately $10^8$ cfu.mL$^{-1}$ for up to 90 days at 8°C and 60 days at 28°C, under formulation. For the bacteria, B. pyrocinia, the cell concentration was maintained at approximately $10^3$ cfu.mL$^{-1}$ for at least 150 days, stored at 8°C and $10^8$ cfu.mL$^{-1}$, also for 150 days when stored at 28°C, under formulation. These results are similar to that of Taurian et al. (2010). Sousa et al. (2017) and Filippi et al. (2011) used both PGPRs in a concentration of $10^8$ cfu.mL$^{-1}$ in their studies and showed the results of characterization of the strains application. Thus, the formulations tested appear to be sufficient for increasing the shelf life of the studied bacteria.

It is noteworthy that in order to obtain the results presented previously, 64 treatments for each PGPR were analyzed. The establishment of a 0.02 Abs.h$^{-1}$ cutoff line of microbial activity for the selection of the most efficient treatments (bacteria formulations), during the 180 days of evaluation, was fundamental for a practical analysis of the data. Figure 3 shows only the formulations that reached the established cut line at some points in the evaluation. For each treatment (bacterial formulation), a gradual increase of the microbial activity is observed due to increase in the number of cells, reaching a maximum point until its decrease. This fact is explained by the time of adaptation and growth of the microbial cells, even if stored without agitation or at temperatures different from those considered optimal. Because their metabolism is not destroyed, the cells continue to consume the nutrients from the formulations, even slowly. When these

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**Table 1. Combination of adjuvants used in composition of the formulations and their final concentrations.**

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"+1" Means contains adjuvant and "-1" means does not contain adjuvant.
Figure 3. Viability assessment of *P. fluorescens* BRM 32111 or *B. pyrrocinia* BRM 32113, incorporated in the different formulations under different storage conditions. The data are presented in Abs.$h^{-1}$ values in OD measurement (620 nm) performed after 24 h of incubation. (A) *P. fluorescens* BRM 32111 at 8°C, (B) *P. fluorescens* BRM 32111 at 28°C, (C) *B. pyrrocinia* BRM 32113 at 8°C and (D) *B. pyrrocinia* BRM 32113 at 28°C.

are depleted, cellular activity declines as a result of cell death. Sipahutara et al. (2018) studied on a 20 week shelf life experiment using adjuvants PVP and molasses combined in bacterial strain *P. fluorescens* MC46 formulation, showing similar microbial activity pattern with the present work.

From 32 formulations tested for each bacterial species, 16 were highlighted (Figure 3). Among these, the 13 containing molasses (formulations F17 to F32) were the most efficient to the detriment of the three containing glycerol (formulations F1 to F16). This fact is related to the nutritional value of molasses in comparison with glycerol, as will be described later. For the subsequent tests, three formulations were selected. Among these, formulation F11, containing glycerol was chosen, although it is not generally one of the most efficient, it contains glycerol and presents relative efficiency for the two bacterial species, allowing a more complete study on the adjuvants.

Individual impact of adjuvants and storage conditions on the stability of bacterial formulations

For the development of the formulations, seven adjuvants were used at defined concentrations (Table 1), considered as inert or beneficial in relation to the bacteria under study and inert to rice plants. These adjuvants were chosen because they exhibited one or more specific properties, among them (1) molasses, glycerol and yeast extract: nutrient sources and protection against desiccation, (2) $\text{K}_2\text{HPO}_4$: buffer, (3) $\text{NaCl}$: osmotic protector or stabilizers, (4) $\text{MgSO}_4$: micronutrients and (5) polyvinylpirrolidone (PVP): desiccation protector. Molasses can act as an adhesive/dispersant (Bashan et al., 2014). For the analysis of the impact of each of these components on the stability of the formulated bacteria, a multivariate analysis of the main components was performed. As shown in Figure 4, the first major component (PC1) accounts for 93.8% of the total...
Figure 4. Principal components analysis (PCA) of the individual impact of adjuvants (glycerol, molasses, NaCl, PVP, MgSO₄ and yeast extract) and storage conditions (8 and 28°C) on the stability of bacterial formulations. Vectors correspond to microbial activity (shelf life) of *P. fluorescens* and *B. pyrocinia*.

variation. The graphical information shows that the values of the vectors, representing the stability of both formulated bacteria, are negatively high for PC1, meaning that the higher the score of these variables, the lower the PC1 score. In this way, the first major component (PC1) can be understood as a global index of bacterial stability. Based on this premise, the lowest PC1 score indicates that the bacterial stability index is higher. Therefore, the lower the score of this component, the greater the stability. According to the score table obtained in this analysis, the adjuvants that had a positive impact on the stability of the formulations are molasses and the low storage temperature (8°C). In contrast, the use of glycerol and the higher storage temperature (28°C) appear to negatively interfere with the stability of the bacteria.

It was already expected that the lower storage temperature would positively influence the stability of the bacterial formulation over the higher one. At low temperatures, the microorganisms reduce their metabolic rate in such a way that their viability stays in a prolonged way. However, it is important to note that these results only show the impact of the storage conditions on the stability of the formulations, not meaning that there is no formulation capable of minimally maintaining the viability of the bacteria at the temperature of 28°C, as previously noted.

The same was observed regarding the use of molasses or glycerol as nutrient sources. As a material with higher nutritive content, it was also expected that the molasses would stand out for the stabilization of the bacterial formulations in relation to the glycerol. The first has approximately 48% of sugars (g.g⁻¹), 0.6% of nitrogen (g.mL⁻¹) and potassium and phosphorus salts that help maintain cell viability due to its high nutrient content. Glycerol in turn, has in its composition, in addition to the main molecule, some salts in low concentration that are considered contaminants, being less nutritious than molasses.

The other components of the formulations: NaCl, PVP, MgSO₄ and yeast extract according to the PCA analysis did not significantly impact the stability of the bacterial formulations. However, for conclusive data on this information, other studies should be performed, since in this case, the action of each of the components is related only to bacterial stability, but other effects not studied may be expressed.

Impact of formulation on antagonistic bacterial capacity

Figure 5 and Tables 2 and 3 show that the bacterial strains of *P. fluorescens* non-formulated and in formulation F11 (F11 + *P. fluorescens*) and formulation F20 (F20 + *P. fluorescens*) also inhibited growth of the colonies of *M. oryzae* in the Petri dish (*in vitro*) pairing test by approximately 90%. The same occurred with the
Figure 5. Evaluation of the interference of formulations determined as more efficient in the capacity of bacterial antagonism. The images are related to the match between *M. oryzae* fungus and the bacteria *P. fluorescens* and *B. pyrrocinia*, respectively, formulated or not. (A) non-formulated *P. fluorescens*, (B) Formulation 11 + *P. fluorescens*, (C) Formulation 20 + *P. fluorescens*, (D) Formulation 11 without *P. fluorescens*, (I) *B. pyrrocinia*, (I) *B. pyrrocinia* non-formulated, (J) Formulation 11 without *B. pyrrocinia*, (K) Formulation 32 without *B. pyrrocinia*, (L) Water (control).
bacterial strain of *B. pyrrocinia* and its incorporation in the formulation F11 (F11 + *B. pyrrocinia*) and F32 (F32 + *B. pyrrocinia*), which in this case is about 60%. This confirms that the formulations analyzed do not interfere with the antagonistic capacity of the bacterial strains against the phytopathogen *M. oryzae*, the causative agent of the rice blast.

**In vivo** evaluation of the bacteria formulated in the blast control

The results obtained were considered significant in the two experiments performed in vivo in a controlled environment (greenhouse). The control, formulation without bacteria, presented an average percentage of leaf blast severity of approximately 22 and 45% in experiments 1 and 2, respectively. Typical lesions of the disease were observed: grayish center and brown edges that coalesced over time. However, the formulation F20, in the absence of the *P. fluorescens* bacterium, showed a slight increase in disease severity, which is compensated when the bacterium is inserted into the formulation. In the case of the F11 or F32 formulations, these formulations besides stabilizing the microbial cells, significantly contribute to reduction of severity of the disease. The reasons for the phenomenon are unknown but are probably linked to the stimulus to the plant defense system or to some physical protection, preventing the full establishment of the pathogen.

All treatments containing the bacteria *P. fluorescens* and *B. pyrrocinia* were able to significantly suppress the severity of leaf blast regardless of whether they were formulated or not. There was no statistical difference between the suppression intensities of the disease when comparing the results obtained with the unformulated and formulated bacteria. *P. fluorescens* formulated was able to suppress the leaf blast by approximately 4 times, whereas *B. pyrrocinia* was able to suppress the disease by approximately 45 times (Figure 6). In both experiments, treatments containing bacteria (formulated or not) showed few lesions of small size.

Experiment 1 shows treatments containing the components of the formulations F11 and F20, in the absence of *P. fluorescens* bacterium, had no significant effect on the reduction of blast severity. In contrast, the formulation F20 provided a slight but significant increase in leaf blast severity. This phenomenon may have occurred due to its highly nutritive composition that may have helped the development of the pathogen. Experiment 2 shows formulations F11 and F32, differently from experiment 1, added for reduction of leaf blast significantly. These treatments presented typical open lesions, in smaller size and number in relation to the

### Table 2. Reduction of mycelial growth of *M. oryzae* when paired with formulations with (+) or without (−) the bacterium *P. fluorescens*, after 7 days of growth in BDA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reduction of colony area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F11 + <em>P. fluorescens</em></td>
<td>90.850&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F20 + <em>P. fluorescens</em></td>
<td>90.586&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>P. fluorescens</em></td>
<td>91.508&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F11 - <em>P. fluorescens</em></td>
<td>3.438&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>F20 - <em>P. fluorescens</em></td>
<td>0.128&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Means followed by the same letter do not differ statistically from each other by the Tukey’s test (p <0.05).

### Table 3. Reduction of mycelial growth of *M. oryzae* when paired with the formulations with (+) or without (−) *B. pyrrocinia* after 7 days of growth in BDA. Means followed by the same letter do not differ statistically from each other by the Tukey’s test (p <0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Reduction of colony area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F11 + <em>B. pyrrocinia</em></td>
<td>54.333&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F32 + <em>B. pyrrocinia</em></td>
<td>68.824&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>B. pyrrocinia</em></td>
<td>65.015&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>F11 - <em>B. pyrrocinia</em></td>
<td>0.605&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>F32 - <em>B. pyrrocinia</em></td>
<td>0.832&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>0.000&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>
Figure 6. Control of rice blast using bacterial formulations selected because they have the greatest capacity to increase shelf life. Results presented as leaf blast severity (LBS) in percentage of affected area measured seven days after inoculation with *M. oryzae*. (A) Experiment 1: Formulation F11 and F20 with (+) or without (−) the bacterium *P. fluorescens* and (B) Experiment 2: Formulations F11 and F32 with (+) or without (−) the bacterium *B. pyrrocinia*. The control corresponds to the untreated rice plant with the bioagent, only inoculated. Means followed by the same letter do not differ statistically from each other by the Tukey’s test (p <0.05).

control. They also presented small brown scores (pin-like lesions) that did not evolve and did not coalesce with the passage of time.

It is noteworthy that in this experiment, the formulation F11 (without bacteria) reduced the severity of the disease, whereas in experiment 1, the same formulation (without bacteria) stimulated the increase of disease severity, even though in this last case, this occurred with little intensity. A possible explanation for this fact is that in experiment 2, the values of disease severity were much more intense than in experiment 1. Therefore, the application of any bacteria formulation tested would have an effect on the reduction of the disease.

Conclusions

The method used in this work was efficient to identify formulations that allowed increase in the shelf life of the rhizobacteria, *P. fluorescens* BRM 32111 and *B. pyrrocinia* BRM 32113 without deleterious impact on the beneficial activities, either for the plant or PGPR itself.
Using bacterial formulations F11, F20 or F32, regardless of the storage conditions, it was possible to significantly increase the shelf life of the bacteria, thereby allowing them to be stored in the medium and a long term.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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