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

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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, $MgSO_4$, K_2HPO_4 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 10^8 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 pyrrocinia* 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 bioproduct 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. pyrrocinia*, 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. pyrrocinia* 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, *P.seudomonas fluorescens* BRM 32111, *B. pyrrocinia* 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-freezing 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%, K_2HPO_4 0.05%, PVP 0.1%, NaCl 0.01%, yeast extract 0.1% and $MgSO_4$ 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 (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

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

Combinations/ formulations	Molasses 1%	Glycerol 1%	K_2HPO_4 0.05%	PVP 0.1%	NaCl 0.01%	Yeast extract 0.1%	$MgSO_4$ 0.02%
F1	-1	+1	+1	+1	+1	+1	+1
F2	-1	+1	+1	+1	+1	+1	-1
F3	-1	+1	+1	+1	+1	-1	+1
F4	-1	+1	+1	+1	+1	-1	-1
F5	-1	+1	+1	+1	-1	+1	+1
F6	-1	+1	+1	+1	-1	+1	-1
F7	-1	+1	+1	+1	-1	-1	+1
F8	-1	+1	+1	+1	-1	-1	-1
F9	-1	+1	+1	-1	+1	+1	+1
F10	-1	+1	+1	-1	+1	+1	-1
F11	-1	+1	+1	-1	+1	-1	+1
F12	-1	+1	+1	-1	+1	-1	-1
F13	-1	+1	+1	-1	-1	+1	+1
F14	-1	+1	+1	-1	-1	+1	-1
F15	-1	+1	+1	-1	-1	-1	+1
F16	-1	+1	+1	-1	-1	-1	-1
F17	+1	-1	+1	+1	+1	+1	+1
F18	+1	-1	+1	+1	+1	+1	-1
F19	+1	-1	+1	+1	+1	-1	+1
F20	+1	-1	+1	+1	+1	-1	-1
F21	+1	-1	+1	+1	-1	+1	+1
F22	+1	-1	+1	+1	-1	+1	-1
F23	+1	-1	+1	+1	-1	-1	+1
F24	+1	-1	+1	+1	-1	-1	-1
F25	+1	-1	+1	-1	+1	+1	+1
F26	+1	-1	+1	-1	+1	+1	-1
F27	+1	-1	+1	-1	+1	-1	+1
F28	+1	-1	+1	-1	+1	-1	-1
F29	+1	-1	+1	-1	-1	+1	+1
F30	+1	-1	+1	-1	-1	+1	-1
F31	+1	-1	+1	-1	-1	-1	+1
F32	+1	-1	+1	-1	-1	-1	-1

"+1" Means contains adjuvant and "-1" means does not contain adjuvant.

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%, NH_4Cl 0.05%, K_2HPO_4 0.01%, NaCl 0.05%, $MgSO_4$ 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

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 1.0×10^8 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. pyrrocinia* 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. pyrrocinia* 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 in a volume of 30 mL per vessel, with formulations containing at least 1.0×10^8 cfu.mL^{-1} and free bacteria in a concentration adjusted to this same value.

Bacterial formulations against rice blast– pathogen inoculation and disease evaluation

M. oryzae conidial suspension produced in oat agar was adjusted to 3.0×10^5 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.

Statistics 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. pyrrocinia* 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
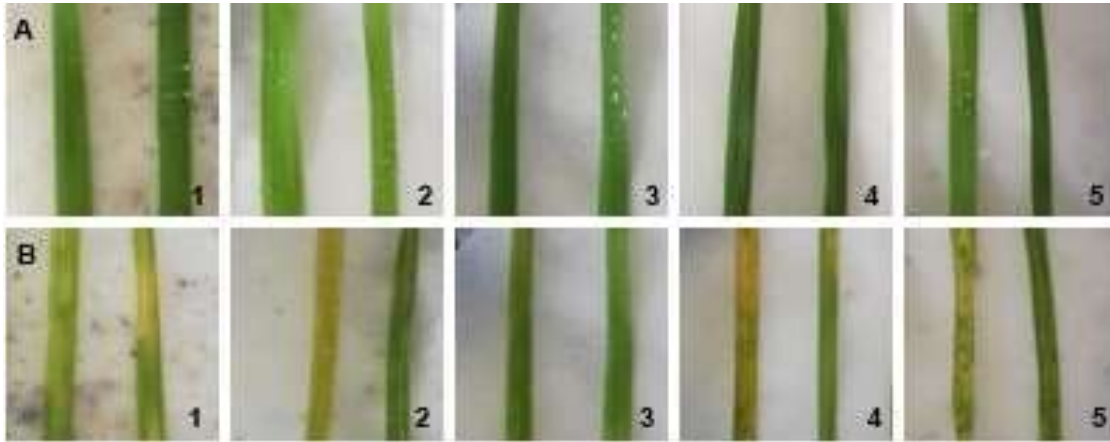
Figure 1. Evaluation of phytotoxicity in rice (leaves) with adjuvants used in the different formulations. (A) First day of evaluation, (B) last day of evaluation (seven days after spraying), (1) glycerol, (2) molasses, (3) PVP, (4) yeast extract and (5) water.
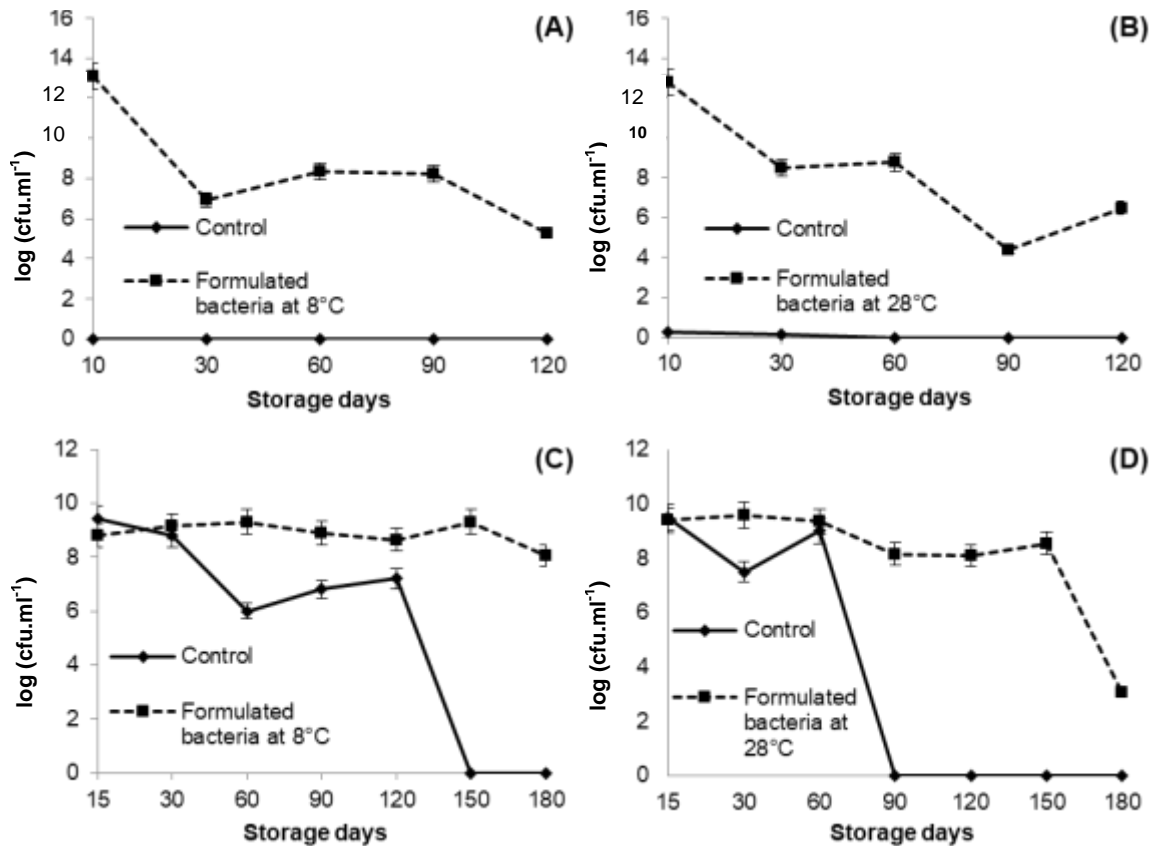
Figure 2. Viability assessment of *P. fluorescens* BRM 32111 or *B. pyrrocinia* BRM 32113, in the best bacterial formulations, under different storage conditions in different periods. Count expressed in logarithm on base ten of the count of colonies in cfu.mL^{-1}. (A) *P. fluorescens* BRM 32111 at 8°C, (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. Control refers to the unformulated bacterial suspension in the same initial concentration of viable cells present in the formulations, maintained under the same storage conditions.

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)

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

Combinations/ formulations	Molasses 1%	Glycerol 1%	K_2HPO_4 0.05%	PVP 0.1%	NaCl 0.01%	Yeast extract 0.1%	$MgSO_4$ 0.02%
F1	-1	+1	+1	+1	+1	+1	+1
F2	-1	+1	+1	+1	+1	+1	-1
F3	-1	+1	+1	+1	+1	-1	+1
F4	-1	+1	+1	+1	+1	-1	-1
F5	-1	+1	+1	+1	-1	+1	+1
F6	-1	+1	+1	+1	-1	+1	-1
F7	-1	+1	+1	+1	-1	-1	+1
F8	-1	+1	+1	+1	-1	-1	-1
F9	-1	+1	+1	-1	+1	+1	+1
F10	-1	+1	+1	-1	+1	+1	-1
F11	-1	+1	+1	-1	+1	-1	+1
F12	-1	+1	+1	-1	+1	-1	-1
F13	-1	+1	+1	-1	-1	+1	+1
F14	-1	+1	+1	-1	-1	+1	-1
F15	-1	+1	+1	-1	-1	-1	+1
F16	-1	+1	+1	-1	-1	-1	-1
F17	+1	-1	+1	+1	+1	+1	+1
F18	+1	-1	+1	+1	+1	+1	-1
F19	+1	-1	+1	+1	+1	-1	+1
F20	+1	-1	+1	+1	+1	-1	-1
F21	+1	-1	+1	+1	-1	+1	+1
F22	+1	-1	+1	+1	-1	+1	-1
F23	+1	-1	+1	+1	-1	-1	+1
F24	+1	-1	+1	+1	-1	-1	-1
F25	+1	-1	+1	-1	+1	+1	+1
F26	+1	-1	+1	-1	+1	+1	-1
F27	+1	-1	+1	-1	+1	-1	+1
F28	+1	-1	+1	-1	+1	-1	-1
F29	+1	-1	+1	-1	-1	+1	+1
F30	+1	-1	+1	-1	-1	+1	-1
F31	+1	-1	+1	-1	-1	-1	+1
F32	+1	-1	+1	-1	-1	-1	-1

"+1" Means contains adjuvant and "-1" means does not contain adjuvant.

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. pyrrocinia*, the cell concentration was maintained at approximately 10^9 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
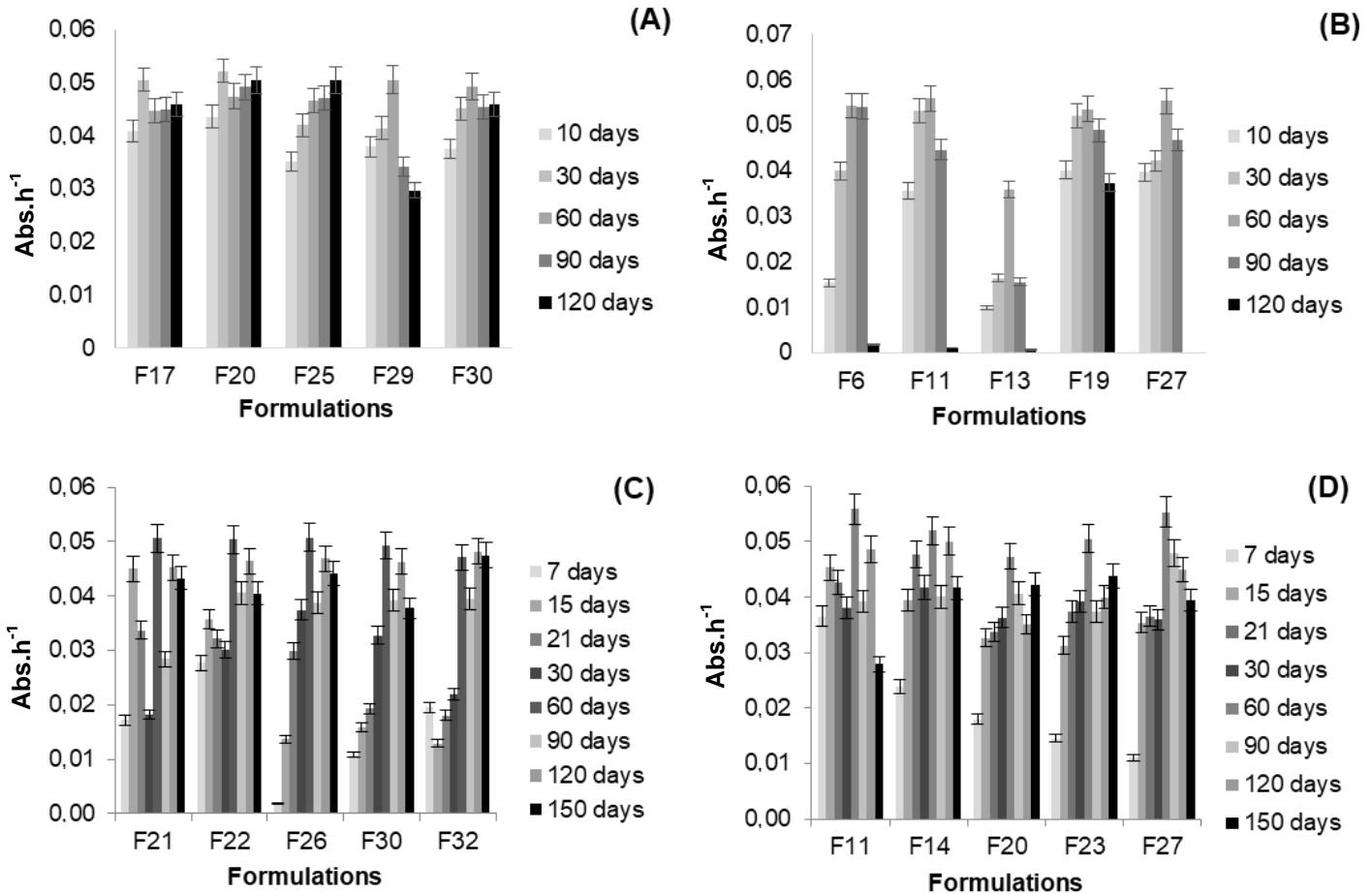
Figure 3. Viability assessment of *P. fluorescens* BRM 32111 or *B. pyrrocinia* BRM 32113, incorporated in the different formulations under different storage conditions. The data are presented in Abs.h^{-1} values in OD measurement (620 nm) performed after 24 h of incubation. (A) *P. fluorescens* BRM 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) K_2HPO_4: buffer, (3) NaCl: osmotic protector or stabilizers, (4) $MgSO_4$: 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
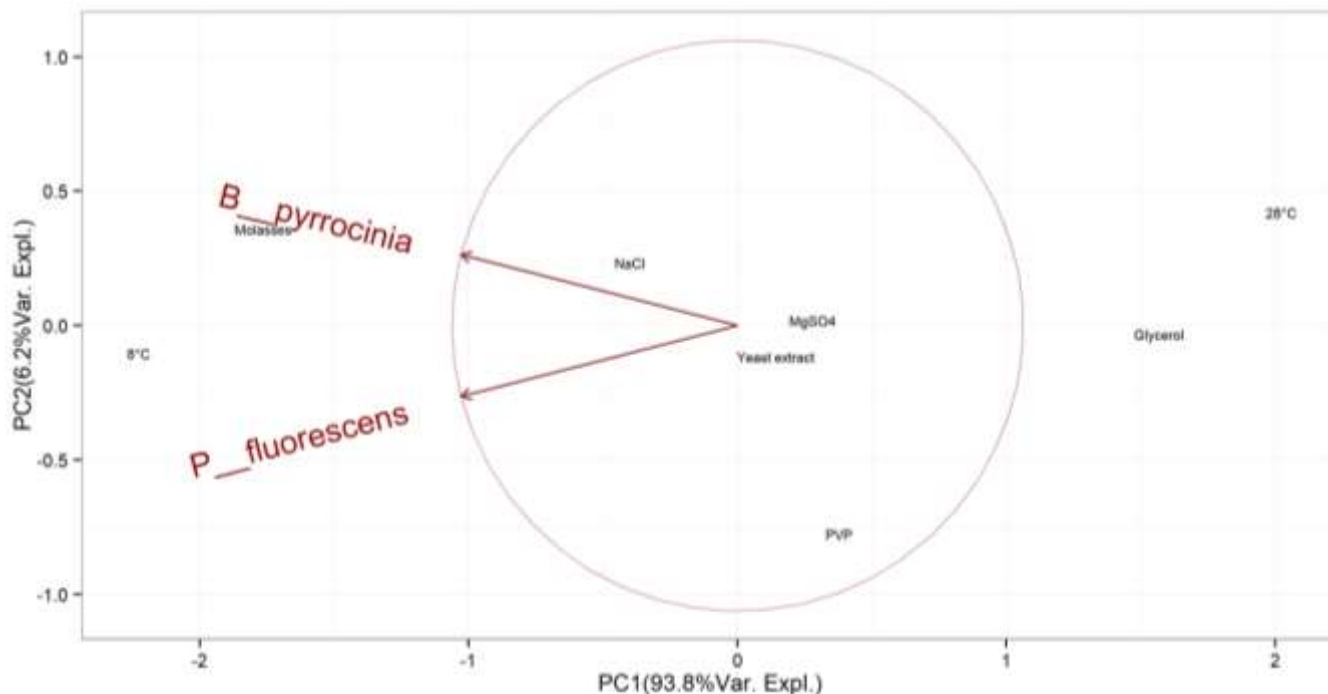
Figure 4. Principal components analysis (PCA) of the individual impact of adjuvants (glycerol, molasses, NaCl, PVP, MgSO₄ and yeast extract) and storage conditions (8 and 28°C) on the stability of bacterial formulations. Vectors correspond to microbial activity (shelf life) of *P. fluorescens* and *B. pyrrocinia.*

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 impacts 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
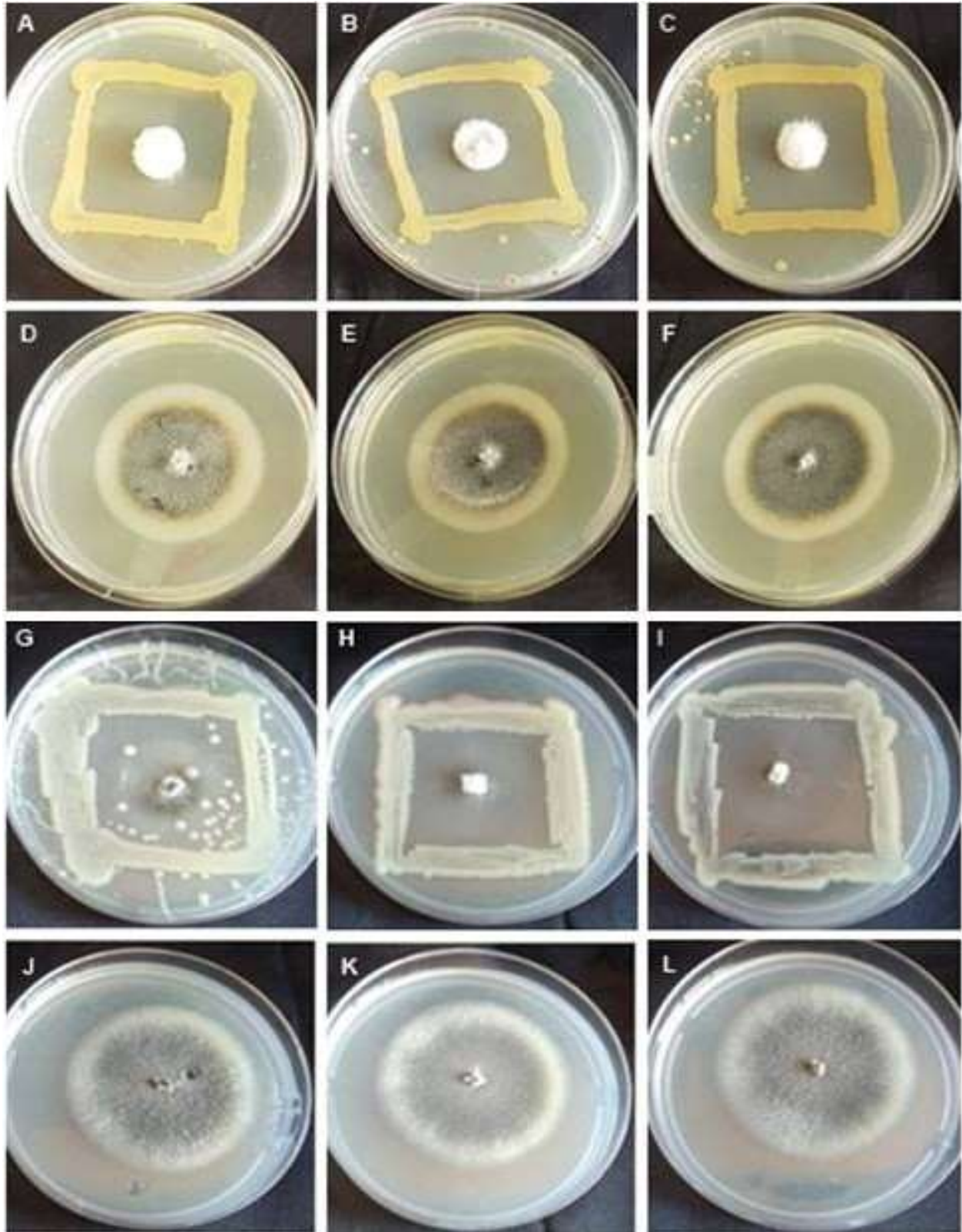
Figure 5. Evaluation of the interference of formulations determined as more efficient in the capacity of bacterial antagonism. The images are related to the match between *M. oryzae* fungus and the bacteria *P. fluorescens* and *B. pyrrocinia*, respectively, formulated or not. (A) non-formulated *P. 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).

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.

Treatment	Reduction of colony area (%)
F11 + *P. fluorescens*	90.850[a]
F20 + *P. fluorescens*	90.586[a]
P. fluorescens	91.508[a]
F11 - *P. fluorescens*	3.438[b]
F20 - *P. fluorescens*	0.128[b]
Control	0.000[b]

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).

Treatment	Reduction of colony area (%)
F11 + *B. pyrrocinia*	54.333[a]
F32 + *B. pyrrocinia*	68.824[a]
B. pyrrocinia	65.015[a]
F11 - *B. pyrrocinia*	0.605[b]
F32 - *B. pyrrocinia*	0.832[b]
Control	0.000[b]

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
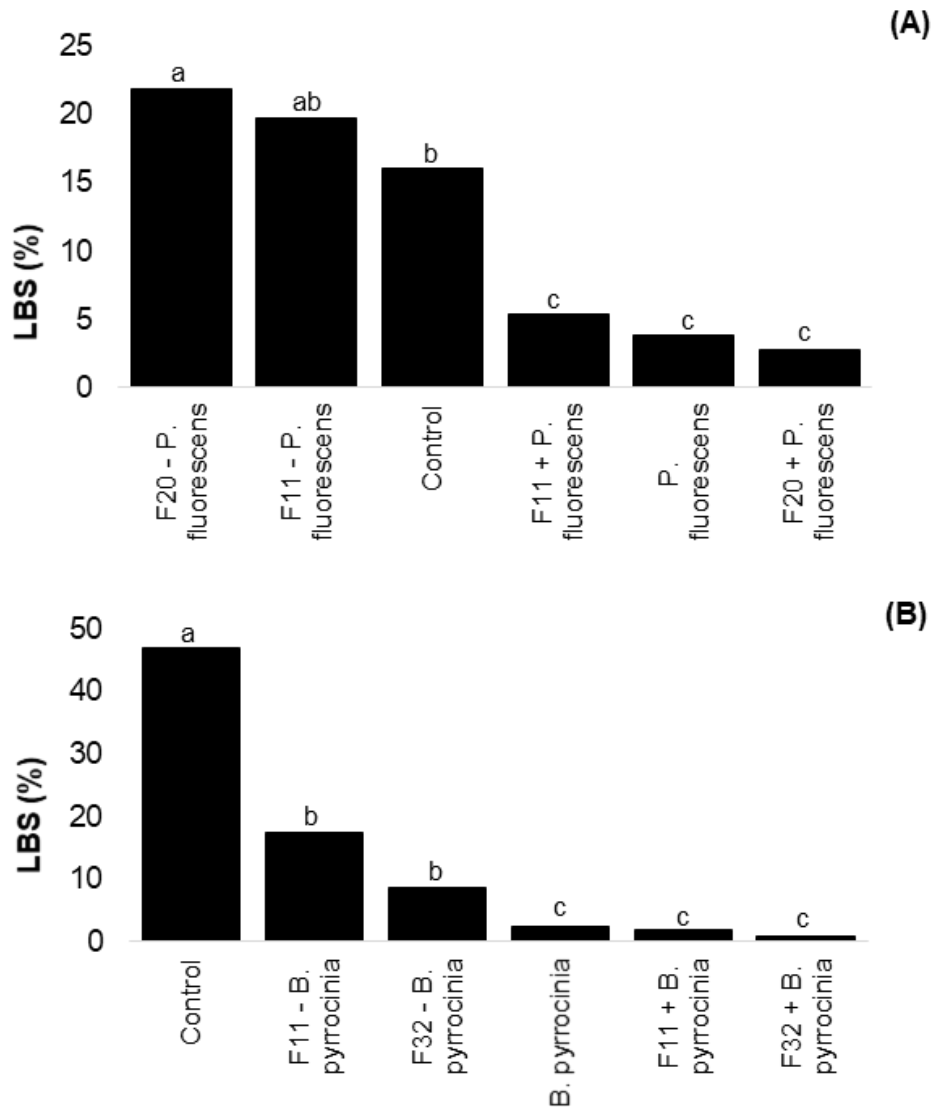
Figure 6. Control of rice blast using bacterial formulations selected because they have the greatest capacity to increase shelf life. Results presented as leaf blast severity (LBS) in percentage of affected area measured seven days after inoculation with *M. oryzae*. (A) Experiment 1: Formulation F11 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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