Biofungicide Formulation Based on *Bacillus velezensis* EM-A8 for Control of Maize Foliar Diseases

Formulación de biofungicida a base de *Bacillus velezensis* EM-A8 para el control de enfermedades foliares en maíz

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ABSTRACT

The aim was to evaluate inoculum production of *Bacillus velezensis* EM-A8, a native bacterium of maize phyllosphere, antagonist to foliar pathogens *Exserohilum turcicum* and *Puccinia sorghi*. Six economic media were tested: 1) Nutrient Broth (8 $g.L^{-1}$); 2) Whole soybean flour (40 $g.L^{-1}$) + sucrose (20 $g.L^{-1}$); 3) Whole soybean flour (10 $g.L^{-1}$) + molasses (20 $g.L^{-1}$), 4) Whole soybean flour (40 $g.L^{-1}$) + molasses (10 $g.L^{-1}$); 5) Yeast extract (10 $g.L^{-1}$) + molasses (5 $g.L^{-1}$) and 6) Yeast extract (10 $g.L^{-1}$) + sucrose (5 $g.L^{-1}$). Growth was determined spectrophotometrically at 620 nm and viability was estimated. M2 showed the shortest generation time (*g* 1.22 h). M3 and M5 were selected for efficiency and a_w was modified with glycerol at 0.97. Inoculums were stored under refrigeration (5°C) and room temperature (20-25°C) for 8 months. At 5°C the viability of the antagonist at 3.3-4.5 log CFU ml^{-1} was significantly lower than at room temperature (6-6.8 log CFU ml^{-1}), where M5 showed the highest stability. The bioformulation of *B. velezensis* EM-A8 in M5 at a_w 0.97 and stored at room temperature will allow successful control of maize foliar diseases.

Keywords
biological control • formulation • growth medium • Northern leaf blight

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Resumen

El objetivo fue evaluar la producción de inóculo de *Bacillus velezensis* EM-A8, bacteria nativa de la filosfera de maíz, antagonista de los patógenos foliares *Exserohilum turcicum* y *Puccinia sorghi*. Se probaron seis medios económicos: M1) Caldo Nutritivo (8 $g.L^{-1}$); M2) Harina integral de soja (40 $g.L^{-1}$) + sacarosa (20 $g.L^{-1}$); M3) Harina integral de soja (10 $g.L^{-1}$) + melaza (20 $g.L^{-1}$), M4) Harina integral de soja (40 $g.L^{-1}$) + melaza (10 $g.L^{-1}$); M5) Extracto de levadura (10 $g.L^{-1}$) + melaza (5 $g.L^{-1}$); M6) Extracto de levadura (10 $g.L^{-1}$) + sacarosa (5 $g.L^{-1}$). El crecimiento se determinó espectrofotométricamente a 620 nm y se estimó la viabilidad. M2 mostró el menor tiempo de generación (*g* 1.22 h). Por eficiencia se seleccionaron M3 y M5. Se modificó a_W a 0,97 con glicerol. Los inóculos se almacenaron en refrigeración (5°C) y a temperatura ambiente (20-25°C), durante 8 meses. A 5°C la viabilidad del antagonista se mantuvo en 3.3-4.5 log UFC ml^{-1}, significativamente menor que a temperatura ambiente (6-6.8 log UFC ml^{-1}), donde M5 mostró la mayor estabilidad. El bioformulado de *B. velezensis* EM-A8 en M5, a_W 0,97 y almacenado a temperatura ambiente, nos permitirá avanzar en el control exitoso de enfermedades foliares en maíz.

Palabras clave
control biológico • formulación • medio de crecimiento • tizón foliar del norte

Introduction

Currently, there is a strong need for environment, human and animal health-friendly alternatives to chemical compounds in agriculture. For this, previous studies in our lab selected epiphytic bacteria of maize phyllosphere with antagonist capacity against the foliar diseases Northern leaf blight produced by *Exserohilum turcicum* and Common rust produced by *Puccinia sorghi* (15, 17). Foliar applications on maize plants controlled both diseases (17). Additionally, we determined mechanisms of action and tolerance to environmental stress (18) and *in vitro* biofilm formation of the biocontrol agent (9).

Biological control is defined as the reduction of a pathogenic agent that causes a disease, through the action of live microorganisms. Practically, this control uses antagonist agents generally isolated from fruit or plant surfaces. These microorganisms, when stimulated *in situ* or artificially reintroduced into environments with the pathogens to be controlled, reduce or suppress disease development (2). A successful biocontrol product depends on the formulation process of a product composed of a biocontrol agent and ingredients to improve survival and product effectiveness (23). The formulation process can affect biocontrol efficiency, extend life, ease handling, raise compatibility with agricultural equipment and practices, and lower production costs. Formulations with high density of microorganisms and greater survival during storage are key for effective biocontrol development. For a biological product to be competitively commercialised, its shelf life should last from 6 months to a year (11, 23). Storage and packaging conditions affect product viability. Temperature modulates bacterial survival during storage (23).

The development of an economic culture medium allowing a large amount of a microbial agent at a low price must maintain control efficacy (23, 26). Low-cost medium components must provide enough energy for biosynthesis and cell maintenance (20). Numerous amendments have been utilised in experimental and commercial formulations of *Bacillus* and other biocontrol agents. Generally, amendments can be grouped as either carriers (fillers, extenders) or those improving chemical, physical, or nutritional properties (19). On the other hand, waste products from soy-based food processing are considered excellent substrates for industrial production of beneficial bacteria and metabolites. Soy waste provides high percentages of proteins (40%), carbohydrates (35%), vitamins and minerals. Soy by-products such as defatted flours are used for industrial production of beneficial bacteria and their metabolites including *Bacillus subtilis* (13, 23).

Also, molasses is a by-product of sugar cane and sugar beet. Given its low cost and contents of sugars (sucrose in 50%), nitrogen, B vitamins and minerals like iron, phosphorus, potassium, zinc, sodium, copper and magnesium, molasses constitutes one

of the most used carbon sources for industrial production of microorganisms (23, 27). Medium growths with molasses and other nitrogen sources provide the nutrients and energy necessary for a rapid cellular increase and maintenance (14, 26). Various nitrogen sources such as peptone, significantly affect the production of antifungal substances in *B. subtilis* (21, 23). Microorganisms for biocontrol are subjected to low a_w causing water stress and synthesis of compatible solutes like polyhydroxyalcohols, carbohydrates or amino acids (23).

Formulation of microbial products can be liquid (aqueous suspensions and flowables) or dry (wettable powders, dust and granules) (19). Lyophilization can maintain viability over twenty years and under no special temperature conditions (10, 25). However, post-lyophilization viability varies depending on numerous factors. This study aimed to obtain an efficient and low-cost formulation of *Bacillus velezensis* EM-A8, able to maintain high cell viability during storage, and evaluating different conditions. This *in vitro* study would provide a reliable basis for further industrial scaling.

Materials and Methods

Biocontrol Agent

Antagonist bacteria were isolated from maize leaves with disease lesions from fields of different sites in Córdoba province, Argentina. Antagonistic ability was evaluated *in vitro* and potential control agents were selected (15). These isolates were identified and deposited in the culture collection of the Department of Microbiology and Immunology, Universidad Nacional de Río Cuarto. *B. velezensis* EM-A8 Genbank accession number KY694464.1 was selected as the more effective strain in biological control of leaf blight caused by *E. turcicum* and common rust caused by *P. sorghi* (16, 17, 18).

Inoculum Preparation

B. velezensis EM-A8 inoculum was prepared from cultures grown on nutrient broth (NB) for 24 h at 140 rev min^{-1} and 25°C up to the exponential phase. Serial dilutions were performed and plated on nutrient agar (NA) to evaluate cell viability and count of colony-forming units per ml (CFU ml^{-1}).

Growth Media and Culture Conditions

The following low-cost media were used to increase viable cells of *B. velezensis* EM-A8:
1) Nutrient Broth (8 g L^{-1}) (control) (M1).
2) Whole soy flour (40 g L^{-1}) + sucrose (20 g L^{-1}) (26) (M2).
3) Whole soy flour (10 g L^{-1}) + molasses (20 g L^{-1}) (14) (M3).
4) Whole soy flour (40 g L^{-1}) + molasses (10 g L^{-1}) (26) (M4).
5) Yeast extract (10 g L^{-1}) + molasses (5 g L^{-1}) (26) (M5).
6) Yeast extract (10 g L^{-1}) + sucrose (5 g L^{-1}) (6) (M6).

All media were prepared in 250 ml flasks with 50 ml of each tested medium with 0.97 water activity (a_w) adjusted by glycerol addition (7) and autoclaved. Flasks were inoculated with 100 µl of fresh inoculum suspension of *B. velezensis* EM-A8, with an initial inoculum of 3 x 10^8 CFU ml^{-1} and incubated at 25°C under orbital agitation at 140 rev min^{-1} for 32 h.

Growth in each treatment was determined spectrophotometrically at 620 nm. Cell viability was estimated using the surface-plated method at 5, 9, 24 and 32 hours. For this, serial decimal dilutions until 10^{-9} were performed in NB. An aliquot of 0.1 ml of each dilution was spread on the surface of NA. Plates were incubated in darkness at 25°C for 24 h. Total number of viable bacteria was expressed as mean colony-forming units developed per ml of medium (CFU ml^{-1}). The experiments were replicated three times for each treatment and the assay was repeated twice. Growth parameters *g* (generation time) and *k* (constant growth rate) were calculated by linear regression of the exponential growth phase.

Viability of *B. velezensis* EM-A8 at Two Different Storage Conditions

The viability evaluation of two storage conditions was carried out for two media (M3 and M5). For this, 250 ml of each media inoculated with *B. velezensis* EM-A8 were stored at room temperature (20-25°C) and in cool storage (5°C) for 8 months (240 days). Antagonist viability was determined by plate count at different times. Sample dilutions were made in NB and spread-plated onto NA. Plates were incubated at 25°C for 24 h and the viable count was expressed as colony-forming units per ml (CFU ml^{-1}). The experiments were conducted in triplicates.

Viability of *B. velezensis* EM-A8 Under Lactose-Added

The effect of adding a post-incubation protector such as lactose was evaluated for the same media. For this assay, M1 was used as control and M3 and M5 were supplemented with 5% lactose post-incubation of *B. velezensis* EM-A8. Media were stored at room temperature and cool storage. Bacterial viability was monitored after 5 months of storage (150 days) and estimated using a surface-plated method, as previously mentioned.

Lyophilized Formulation

Since the addition of glycerol does not allow freeze-drying, flasks with M5 and not modified a_w, were inoculated with a suspension of 0.1 ml of *B. velezensis* EM-A8, incubated in an orbital shaker at 140 rev min^{-1} and 25°C for 24 h the number of colony forming units per millilitre (CFU ml^{-1}) was calculated. In order to protect cells from temperature, 5% lactose was added before lyophilization (1). The conical flasks containing inoculum were frozen directly at -20°C for 12 h and then at -80°C for 4 h. These were then connected to a freeze-dryer operating at a chamber pressure of 50.05 mbar and -45°C for 48 h.

A week later, viability of lyophilized M5 was determined. For this, 0.2 g and 1 g were rehydrated with 9 ml of NB, incubated for 1 h at room temperature and homogenised with a vortex mixer. Serial dilutions were spread-plated on the surface of NA plates. These plates were incubated at 25°C for 24 h and viable populations were counted. Survival levels were expressed as number of CFU ml^{-1} (15). The experiments were conducted in triplicates.

Statistical Analysis

ANOVA test was made for growth parameters and viable counts using InfoStat version 2012 (8). Means were compared according to the DGC test ($p > 0.05$).

Results

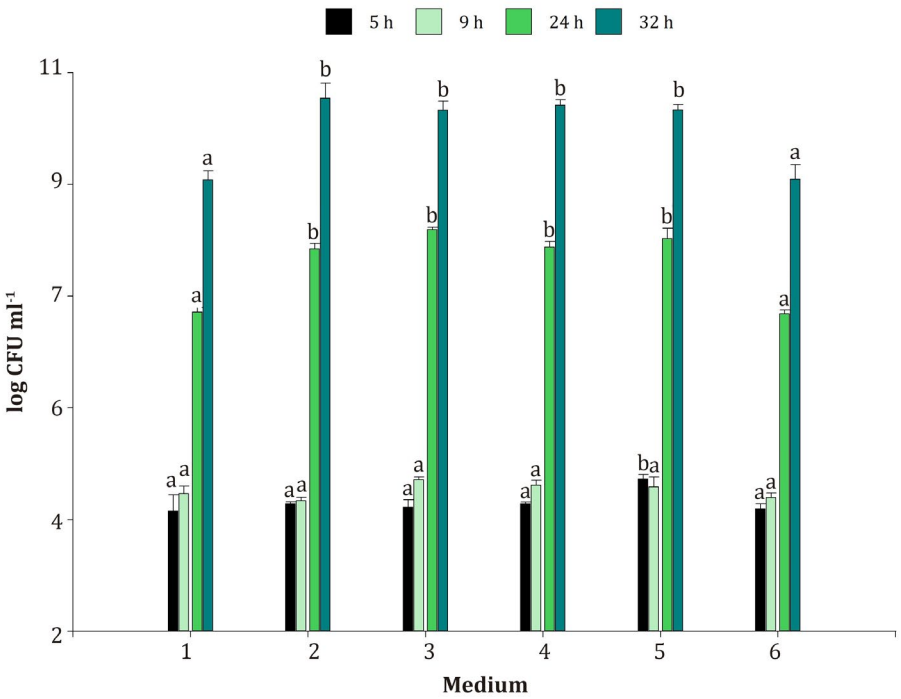
***B. velezensis* EM-A8 Growth on Low-Cost Media**

Growth data at different media and time incubation were analysed by ANOVA. All media provided high *B. velezensis* EM-A8 growth. After 9 h of incubation, no significant differences ($p<0.05$) were observed between media and time. The lowest count (4 log CFU ml^{-1}) was determined at 5 h. M5 presented a significantly higher count (f: 2,22; gl: 5; p-value 0,1192).

Significant differences were observed for *B. velezensis* EM-A8 level between culture media at 24 h (f: 40.3; gl:5: p-value <0.0001) and 32 h of incubation (f: 12.95; gl: 5; p-value 0.0002). Growth was significantly higher in M2, M3, M4 and M5 with similar counts (10.4-10.7 log CFU ml^{-1}), while the lowest count corresponded to control media (Ml) and M6 (9.3-9.7 log CFU ml^{-1}) (figure 1, page 5).

Growth parameters significantly differed among mediums (table 1, page 5). *B. velezensis* EM-A8 had the shortest generation time (*g*) in M2, followed by M3, M4, M5, M6 and M1 (control). Besides, the growth rate constant *(k)* was greater for M2, M3 and M4 with an average value of 0.55 and no significant differences among the three media ($p<0.05$). However, M1, M5 and M6 showed significant differences for *k*.

Culture media selection discarded M2 for its high soy flour content (40 g L^{-1}) and considering that M3 showed comparable growth with less soy flour content (10 g L^{-1}). We also selected M5 for growth parameters obtained in a very different media composition (yeast extract and molasses).



Different letters indicate significant differences for each medium at different incubation times according to the DGC test (P<0.05).

Datos con la misma letra no son significativamente diferentes para cada medio a diferentes tiempos de incubación según el test DGC (P<0,05).

Figure 1. Incubation of *B. velezensis* EM-A8 in different low-cost media for 32 h.

Figura 1. Incubación de *B. velezensis* EM-A8 en diferentes medios de bajo costo durante 32 h.

g: generation time.

k: constant growth rate.

Data with the same letter are not significantly different according to the DGC test (P<0.05).

g: tiempo de generación.

k: constante de la velocidad de crecimiento.

Los datos en una misma letra no son significativamente diferentes según la prueba DGC (P<0,05).

Table 1. Influence of growth media on growth parameters of *B. velezensis* EM-A8.

Tabla 1. Influencia de los medios de crecimiento en los parámetros de crecimiento de *B. velezensis* EM-A8

Media growth parameters						
	M1	**M2**	**M3**	**M4**	**M5**	**M6**
g (h)	1.52 *a*	1.22 *e*	1.26 *d*	1.27 *d*	1.36 *c*	1.43 *b*
k (h^{-1})	0.45 *D*	0.56 *A*	0.55 *A*	0.54 *A*	0.50 *B*	0.48 *C*

Viability of *B. velezensis* EM-A8 in Storage

Figure 2 (page 6), shows *B. velezensis* EM-A8 viability in M1, M3 and M5 under two different storage conditions. After 240 days of storage, viability was significantly lower in the three-growth media stored under refrigeration (5°C), showing a count average of 3.3-4.5 log CFU ml^{-1}, while viability in growth media stored at room temperature (20°C) was 6-6.8 log CFU ml^{-1}. Growth media 5 showed smaller variations in *B. velezensis* EM-A8 counts throughout storage time at room temperature.

Viability of *B. velezensis* EM-A8 Stored with Added Lactose

Lactose effect of *B. velezensis* EM-A8 viability during storage was determined in M5 at 150 days of incubation. Initial strain population was approximately 10 log (figure 3, page 6). However, after incubation, the population decreased to 4-4,5 log CFU ml^{-1} under refrigerated conditions with and without added lactose; and at room temperature at the end of the experiment, decreasing from 10 to 6 log CFU ml^{-1} with and without lactose addition.

1R (M1 with refrigeration), 3R (M3 with refrigeration), 5R (M5 with refrigeration), 1A (M1roomtemperature),3A (M3 room temperature), 5A (M5 room temperature).

1R (M1 con refrigeración), 3R (M3 con refrigeración), 5R (M5 con refrigeración), 1A (M1 temperatura ambiente), 3A (M3 temperatura ambiente), 5A (M5 temperatura ambiente).

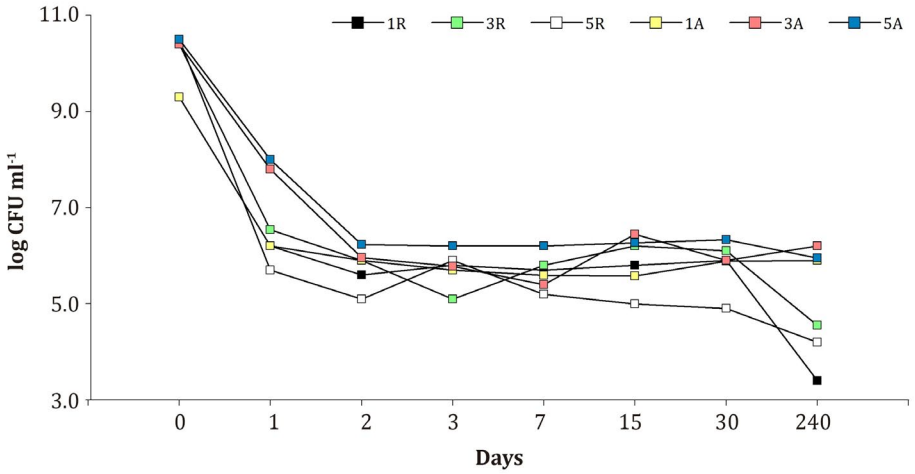


Figure 2. *B. velezensis* EM-A8 viability (CFU ml^{-1}) during storage with refrigeration (R) and room temperature (A) for 240 days. Media 1, 3 y 5.

Figura 2. Viabilidad de *B. velezensis* EM-A8 (UFC ml^{-1}) durante el almacenamiento con refrigeración (R) y temperatura ambiente (A) durante 240 días. Medios 1. 3 y 5.

Bars represent the average of three independent replications and different letters indicate significant differences (P>0.05) according to the DGC test.

Cada barra representa el promedio de tres replicaciones independientes y letras diferentes indican diferencias significativas (P>0,05) según la prueba DGC.

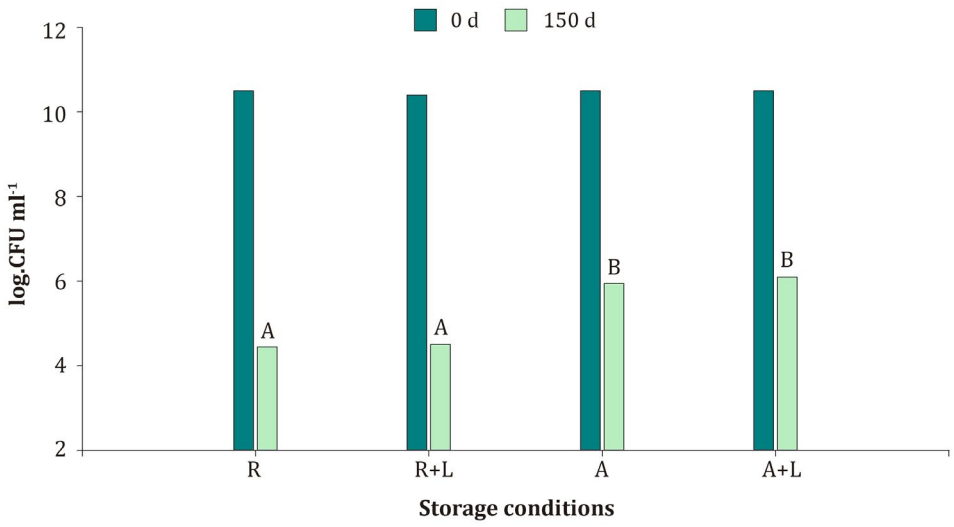


Figure 3. Viability of *B. velezensis* EM-A8 (CFU ml^{-1}) during storage under refrigeration (R) and room temperature (A), with and without lactose, at zero and 150 days.

Figura 3. Viabilidad de *B. velezensis* EM-A8 (UFC ml^{-1}) durante el almacenamiento en refrigeración (R) y temperatura ambiente (A), con adición de lactosa y sin lactosa, a cero y a los 150 días después.

Lyophilized Formulation

The initial inoculum of *B. velezensis* EM-A8, in M3 and M5 showed 5.6 x 10^9 CFU ml^{-1} before freezing. After lyophilization, each gram of rehydrated powder had 2 x 10^6 CFU ml^{-1}, indicating a significant loss of *B. velezensis* EM-A8 viability. Furthermore, the volume of liquid formulation to obtain one gram of lyophilized powder was excessive and impractical.

Discussion

The biological control of plant pathogens is an effective and eco-friendly method to manage diseases, preserving nature and human health from chemical pesticides (1). Biofungicides curb and control fungal plant pathogens by inoculating microbiota in or onto the plant. Several complex interactions among pathogen-biocontrol agents, biocontrol agent-plant, and environment play a significant role in disease control (3). In this sense, formulation determines biofungicide efficacy. Our study showed that commercial products and by-products in different media provide high growth and shelf life of *B. velezensis* EM-A8. The chosen sources of nitrogen and carbon (yeast extract, whole soy flour, sucrose, molasses) are widely described for producing beneficial microorganisms, including *B. velezensis* EM-A8 (6, 23). After one day of incubation in the studied media, cell count was 9 log. For M2, M3, M4 and M5, cell count increased by one logarithm after 32 hours, showing that *B. velezensis* EM-A8 population increased in one day of incubation and all media.

On the other hand, growth rate of *B. velezensis* EM-A8 was significantly different between M5 and M6, being higher with molasses as sugar source. Molasses concentration did not negatively affect *B. velezensis* EM-A8 growth. M3 and M4 show similar growth, even with twice the concentration of molasses in M3. However, some authors observed that high concentrations of molasses (20 and 40 g L^{-1}) did not support *B. subtilis* CPA-8 growth (26). Similar results were found by Costa *et al.* (2001) for *P. agglomerans* CPA-2, where increased molasses concentration (40 g L^{-1}) did not improve production, probably given by high toxic concentrations (6).

Regarding whole soy flour concentration, *B. velezensis* EM-A8 showed similar growth in M2 and M4 with 40 g L^{-1} and M3 with 10 g L^{-1} suggesting no influence over growth. The high concentrations of *B. velezensis* EM-A8 obtained in the whole soy flour media compared to yeast extract media showed that this carbon source promotes bacterial growth influenced by the capacity of microorganisms to use available nitrogen, similar to what Costa *et al.* (2002) and Yáñez-Mendizábal *et al.* (2012) found. However, media with whole soy flour became opaque, with lumps and debris from soy seed, complicating viability controls. On the contrary, selected media with yeast extract as nitrogen source were translucent and without lumps.

Storage temperature and media composition significantly affected *B. velezensis* EM-A8 viability, which markedly decreased in both storage conditions. After two days, the count was 4 log less than initial count. However, in both growth media (3 and 5), as well as in the control, viability remained stable throughout 8 months of storage. In all media *B. velezensis* EM-A8 cell viability showed significant difference in cold storage. In M3 and M5, viability decreased to 4 log while in the control, it decreased to 3 log. In contrast, Yáñez-Mendizabal *et al.* (2012) showed that all formulations of *B. subtilis* CPA-8 during 6 months of storage in cold and at room temperature had the same behaviour, viable cells maintained or slightly decreased around 0.2 - 0.3 log.

Contrarily, other authors demonstrated greater stability and higher microbial survival in cold-stored samples compared to those stored at room temperature (4). Moreover, it was observed that in-storage protective lactose failed to reverse viability loss of *B. velezensis* EM-A8, both in refrigeration and at room temperature. Lactose additions did not reverse viability loss, unlike previously seen (23). On the other hand, unlike other investigations (6, 10, 25), lyophilization was discarded as conservation and storage method given the amount of powder needed for later foliar application.

The viability loss of the microorganisms after the formulation and during the storage and distribution is a regular problem (23). All three liquid formulations stored at room temperature maintained stable viability in 6 log for 8 months. Practically, this finding determines such a convenient storage condition as room temperature is preferable to refrigerated storage. Formulation, production and stabilization of the biocontrol agent determines the final efficacy of a *Bacillus*-based product (19). An ideal formulation is not toxic to the host plant, easy to handle, has long shelf life, is compatible with other agrochemicals, cost-effective and stable. Formulations should work under different environmental conditions, providing reliable control of plant diseases (12). Moreover, the ability of biocontrol agents to control foliar diseases is largely affected by environmental fluctuation (12). *B. velezensis* EM-A8 is native to the phyllosphere with the advantage of being adapted to such conditions.

Agroecological management strategies would not only help in optimal recycling of nutrients and organic matter turnover, closed energy flows, and water and soil conservation but also would help in balancing pest-natural enemy populations (24). Diverse biocontrol agents have successfully contributed to the sustainable management of phytopathogens and various foliar diseases can be effectively controlled by spray application of bacterial and fungal antagonists. *Bacillus* strains are gaining enormous attention due to their ability to effectively cause disease suppression (1).

Conclusions

The liquid formulation developed from *B. velezensis* EM-A8 in growth medium with yeast extract and molasses, modified a_w with glycerol at 0.97 and stored at room temperature is a promising product and an important step towards the successful control of foliar diseases in maize.

References

1. Babbal, A.; Khasa, Y. 2017. Microbes as biocontrol agents. In: Probiotics and Plant Health. Kumas, V. (Eds). Springer. 507-551.
2. Barkai-Golan, R. 2001. Postharvest diseases of fruits and vegetables. Development and control. Elsevier, Amsterdam, The Netherlands.
3. Bonaterra, A.; Badosa, E.; Daranas, N.; Francés, J.; Roselló, G.; Montesinos, E. 2022. Bacteria as biological control agents of plant diseases. Microorganisms. 10: 1759.
4. Boza, Y.; Barbin, D.; Scamparini, A. 2004. Effect of spray drying on the quality of encapsulated cells of *Beijerinckia* sp. Process Biochemistry. 39: 1275-1284.
5. Costa, E.; Teixidó, N.; Usall, J.; Atarés, E.; Viñas, I. 2001. Production of the biocontrol agent *Pantoea agglomerans* strain CPA-2 using commercial products and byproducts. Applied Microbiology and Biotechnology. 56: 367-371.
6. Costa, E.; Teixidó, N.; Usall, J.; Atarés, E.; Viñas, I. 2002. The effect of nitrogen and carbon sources on growth of the biocontrol agent *Pantoea agglomerans* strain CPA-2. Letters in Applied Microbiology. 35: 117-120.
7. Dallyl, H.; Fox, A. 1980. Spoilage of material of reduced water activity by xerophilic fungi. Technical Series 15. London: Academic Press. 219-239.
8. Di Rienzo, J.; Casanoves, F.; Balzarini, M.; Gonzalez, L.; Tablada, M.; Robledo, C. 2012. InfoStat versión. Argentina: GrupoInfoStat, FCA, Universidad Nacional de Córdoba. http://www.infostat.com.ar
9. Fessia, A.; Sartori, M.; García, D.; Fernández, L.; Ponzio, R.; Barros, G.; Nesci, A. 2022. *In vitro* studies of biofilm-forming *Bacillus* strains, biocontrol agents isolated from the maize phyllosphere. Biofilm 4, 100097.
10. Fonseca, F.; Cenard, S.; Passot, S. 2015. Freeze-drying of lactic acid bacteria. In: Cryopreservation and Freeze-Drying Protocols. Methods in Molecular Biology, vol 1257. Wolkers, W.; Oldenhof, H. (eds) Springer, New York,
11. Kinay, P.; Yildiz, M. 2008. The shelf life and effectiveness of granular formulations of *Metschnikowia pulcherrima* and *Pichia guilliermondii* yeast isolates that control postharvest decay of citrus fruit. Biological Control. 45: 433-440.
12. Kumar, S.; Thakur, M.; Rani, A. 2014. Trichoderma: Mass production, formulation, quality control, delivery and its scope in commercialization in India for the management of plant diseases. African Journal of Agricultural Research. 9(53): 3838-3852.
13. Reis, F; Servulo, E.; De Franca, F. 2004. Lipopeptide surfactant production by *Bacillus subtilis* grown on low-cost raw materials. Applied Biochemistry and Biotechnology. 113: 899-912.
14. Sartori, M.; Nesci, A.; Etcheverry, M. 2012. Production of *Fusarium verticillioides* biocontrol agents, *Bacillus amyloliquefaciens* and *Microbacterium oleovorans*, using different growth media: evaluation of biomass and viability after freeze-drying. Food Additives and Contaminants (A). 29(2): 287-292.
15. Sartori, M.; Nesci, A.; Formento, A.; Etcheverry, M. 2015. Selection of potential biological control of *Exserohilum turcicum* with epiphytic microorganisms from maize. Revista Argentina de Microbiología. 47(1): 62-71.
16. Sartori, M.; Nesci, A.; Garcia, J.; Passone, M.; Montemarani, A.; Etcheverry M. 2017a. Efficacy of epiphytic bacteria to prevent northern leaf blight caused by *Exserohilum turcicum* in maize. Revista Argentina de Microbiología. 49(1): 75-82.
17. Sartori, M.; Nesci, A.; Montemarani, A.; Barros, G.; Garcia, J.; Etcheverry M. 2017b. Preliminary evaluation of biocontrol agents against maize pathogens *Exserohilum turcicum* and *Puccinia sorghi* in field assays. Agricultural Sciences. 8: 1003-1013.

18. Sartori, M.; Bonacci, M.; Barra, P.; Fessia, A.; Etcheverry, M.; Nesci, A.; Barros, G. 2020. Studies on possible modes of action and tolerance to environmental stress conditions of different biocontrol agents of foliar diseases in maize. Agricultural Sciences. 11: 552-566.
19. Schisler, D.; Slininger, P.; Behle, R.; Jackson, M. 2004. Formulation of *Bacillus* spp. for biological control of plant diseases. Phytopathology. 94: 1267-1271.
20. Stanbury, P.; Whitaker, A.; Hall, S. 1995. Media for industrial fermentations. In: Principles of fermentation technology. Pergamon Press, Oxford. 93-121.
21. Tabbene, O.; Slimene, I.; Djebali, K.; Mangoni, M.; Urdaci M.; Limam F. 2009. Optimization of medium composition for the production of antimicrobial activity by *Bacillus subtilis* B38. Biotechnology Progress. 25: 1266-1274.
22. Teixidó, N.; Cañamás, T. P.; Abadias, M.; Usall, J; Solsona, C.; Casals, C.; Viñas, I. 2006. Improving low water activity and desiccation tolerance of the biocontrol agent *Pantoea agglomerans* CPA-2 by osmotic treatments. Journal of Applied Microbiology. 101: 927-937.
23. Teixidó, N.; Usall, J.; Torres, R. 2022. Insight into a Successful development of biocontrol agents: production, formulation, packaging and shelf life as key aspects. Horticulturae. 8: 305.
24. Vandemeer, J. 1995. The ecological basis of alternative agriculture. Annual review of Ecology and Systematics. 26: 201-224.
25. Vermelho, A.; Moreira, J.; Junior, A.; da Silva, C.; Cardoso, V.; Akamine, I. 2024. Microbial preservation and contamination control in the baking industry. Fermentation. 10: 231. doi.org/10.3390/fermentation10050231
26. Yánez-Mendizábal, V.; Viñas, I.; Usall, J.; Torres, R.; Solsona, C.; Teixidó, N. 2012. Production of the postharvest biocontrol agent *Bacillus subtilis* CPA-8 using low-cost commercial products and by-products. Biological control. 60: 280-289.
27. Younis, M.; Hezayen, F.; Nour-Eldein, M.; Shabeb, M. 2010. Optimization of cultivation medium and growth conditions for Bacillus subtilis KO strain isolated from sugar cane molasses. American Eurasian Journal of Agricultural Environmental Science. 7: 31-37.

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