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Snap bean production from seeds treated with *Bacillus subtilis*[1]

Helio Fernandes Ibanhes Neto[2], Gustavo Henrique Freiria[3], Ananda Covre da Silva[2], Raissa Marrafon Ponce[2], Lúcia Sadayo Assari Takahashi[2]

ABSTRACT

In order to ensure an adequate final stand and good yields in agricultural crops, a fast healthy seedling establishment in the field is essential and can be improved using seed treatment techniques. This study aimed to assess the effect of treating snap bean seeds with *Bacillus subtilis* doses on the development and yield components of the crop. Seeds of a determinate variety were used. A 4 x 4 + 1 factorial scheme was applied, being four seed treatments (film coating with and without drying, pre-imbibition and biopriming), four doses of *B. subtilis*-based commercial product (c.p.) (0, 7, 14 and 28 mL c.p. kg^{-1} of seed) and an absolute control. The analyzed variables were seed moisture content, seedling emergence, leaf length, width and area, plant height and height to first pod at harvest, number of trifoliate leaves and pods, pod length and number of grains per pod, and pod fresh and dry weight. The pre-imbibition and biopriming treatments were equal or superior to the absolute control. Associating these treatments with doses of 7-14 mL c.p. kg^{-1} for pre-imbibition and 0-28 mL c.p. kg^{-1} for biopriming favors both the traits assessed during the reproductive stage and the yield components of the snap bean.

KEYWORDS: *Phaseolus vulgaris* L., biopriming, rhizobacteria.

RESUMO

Produção de feijão vagem oriunda de sementes tratadas com *Bacillus subtilis*

A velocidade e a sanidade no estabelecimento de plântulas a campo são imprescindíveis para a obtenção de um estande final adequado e para a produtividade em um cultivo agrícola, os quais podem ser melhorados por técnicas baseadas no tratamento de sementes. Objetivou-se verificar a influência do tratamento de sementes de feijão vagem com doses de *Bacillus subtilis* sobre o desenvolvimento e componentes de produção da cultura. Foram utilizadas sementes de tipo determinado. Utilizou-se esquema fatorial 4 x 4 + 1, sendo quatro tratamentos de sementes (peliculização com e sem secagem, pré-embebição e biopriming), quatro doses de produto comercial (p. c.) à base de *B. subtilis* (0; 7; 14; e 28 mL p.c. kg^{-1} de semente) e uma testemunha absoluta. As variáveis analisadas foram teor de água de sementes, emergência de plântulas, comprimento, largura e área foliar, altura de plantas e de primeira vagem na colheita, número de trifólios e de vagens, comprimento e número de grãos por vagem, massa de matéria fresca e seca de vagens. Os tratamentos de pré-embebição e biopriming foram iguais ou superiores à testemunha absoluta. A associação desses tratamentos com doses de 7-14 mL p.c. kg^{-1} para pré-embebição e de 0-28 mL p.c. kg^{-1} para biopriming favorece tanto as características avaliadas no período reprodutivo, como os componentes de rendimento de feijão vagem.

PALAVRAS-CHAVE: *Phaseolus vulgaris* L., biopriming, rizobactérias.

INTRODUCTION

The ideal traits in snap bean cultivation include plant vigor, yield, and pest and disease resistance, with light green commercially-sized and shaped pods, while the most appealing traits for consumers are a pleasant flavor and minimally fibrous pods (Moreira et al. 2009). Yield is estimated based on yield components by analyzing the stages and structures that determine their yield potential during plant development (Silva et al. 2019).

For short-cycle crops whose seeds are used for multiplication, the number of plants per unit area is the main factor in optimizing yield. Once established in the field, snap bean yield per unit area also depends on the number of plants per unit area and per pod, as well as seed weight. These yield components vary between individual plants according to spacing and

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[2] Universidade Estadual de Londrina, Centro de Ciências Agrárias, Departamento de Agronomia, Londrina, PR, Brazil. *E-mail/ORCID*: helioibanhes@hotmail.com/0000-0003-2153-9649; ananda_covre@hotmail.com/0000-0002-5933-8861; raissamp@hotmail.com/0000-0002-1139-7600; luciasadayo@gmail.com/0000-0001-6513-7591.
[3] Universidade do Estado de Minas Gerais, Departamento de Ciências Agrárias e Naturais, Ituiutaba, MG, Brazil. *E-mail/ORCID*: gustavo.freiria@uemg.br/0000-0003-2786-3810.

competition for assimilates (Ramos Júnior et al. 2005, Liu et al. 2010, Dalchiavon & Carvalho 2012).

Fast seedling development and establishment in the field under adverse conditions are associated with seed vigor (Nakagawa 1999). Thus, seeds with high vigor are crucial to the number of plants per area. Field establishment can be safeguarded or improved via pre-germination seed treatments (Ohse et al. 2014) such as film coating and pre-imbibition. Studying and enhancing these techniques enable an efficient application in agricultural crops.

Although seed treatment has been combined with chemical products to protect and stimulate seedlings, the need to mitigate environmental and operational risks has prompted new research on biological products with the same effect (Brito et al. 2013). In addition to sustainability, the low cost of inoculants and their variety of benefits, including stimulating secondary metabolism, solubilizing nutrients and biocontrol, are relevant. Fungi of the *Trichoderma* spp. genus and bacteria of the *Bacillus* spp. genus have been used successfully in soybean, corn, rice and cowpea crops (Chagas et al. 2017).

Bacillus subtilis is considered a plant growth-promoting bacteria (PGPB) due to its interaction with plant roots. Once present, the species has the potential to act in germination and seedling emergence, phosphate solubilization, hormone synthesis, root and shoot growth, biocontrol of plant pathogens and stimulating secondary metabolism (Buchelt et al. 2019).

Oliveira et al. (2016) studied *B. subtilis* inoculation in common bean seed lots with low and high vigor and observed an increase in seedling dry weight. Maciel et al. (2017) reported growth in *Pinus* spp. seedlings from seeds microbiolized with *B. subtilis*. Sá et al. (2019) analyzed cowpea (*Vigna unguiculata* L. Walp.) seeds treated with *B. subtilis* strains and observed an increased germination, lower fungi incidence and greater seedling dry weight.

Thus, given the lack of results regarding the performance of snap bean inoculated with *B. subtilis*, this study aimed to assess the performance (based on plant measurements) and yield of snap bean plants obtained from seeds treated with different processes and *B. subtilis* doses.

MATERIAL AND METHODS

The experiment was conducted in a greenhouse (23º20'28''S, 51º12'34''W and altitude of 548 m) at the Universidade Estadual de Londrina, using commercially determinate seeds of the Macarrão Baixo snap bean cultivar (Isla Sementes®).

Initially, the moisture content of the seed lot was obtained via the oven method at 105 ºC. Two 4.5 g subsamples were weighed on a 2 decimal place scale and dried for 24 hours. The final weighing was performed when the sample weight had stabilized, and the result on a wet basis (w.b.) expressed in percentage (Brasil 2009).

In order to determine the pre-imbibition treatments, the imbibition curve was obtained using four replications of twenty seeds with known weight and moisture content suitable for storage (11.32 %). The seeds were placed among three sheets of Germitest® paper soaked in distilled water at 2.5 times their dry weight and placed on trays in a Mangelsdorf germination chamber at 20 ºC. Weighing was performed every hour, for 14 hours, and then every two hours until the end of the germination phase II, identified by root protrusion and the onset of phase III. The analysis ended when 50 % of the seeds displayed a radicle. The results (w.b.) were expressed as percentage of seed moisture content.

A completely randomized two-factor factorial design was used, with five replications and the factor A consisting of types of seed treatment (film coating with and without drying, pre-imbibition and biopriming) and the factor B of *Bacillus subtilis* doses [0, 7, 14 and 28 mL of commercial product (c.p.) kg^{-1} of seed], and an absolute control that received no treatment.

Four seed treatments were used: film coating (FC) and coating and drying (CAD): the seed samples were placed in a plastic bag containing 100 mL of solution consisting of deionized water combined with the product doses for each treatment and then agitated for 3 min. Next, the seeds were submitted to testing immediately after the immersion treatment (FC), or after being left to dry on Germitest® paper for 96 hours under laboratory conditions, until reaching storage conditions at a moisture content of 11 % (CAD); pre-imbibition (PI) and biopriming (BP): the samples were placed among three sheets of Germitest® paper soaked in solution at 2.5 times their dry weight, then placed on trays in a Mangelsdorf germination chamber at 20 ºC for 48 hours, indicated by the imbibition curve as the end of phase II and the limit for desiccation tolerance. For the PI samples, testing was carried out immediately after soaking,

and for their BP counterparts, following drying on Germitest® paper under ambient conditions for 96 hours, until reaching a storage-suitable moisture content (around 11 %).

The solutions used in all the treatments consisted of 0.0, 7.0, 14.0 and 28.0 mL of Bayer Cropscience's® commercial product (c.p.) Serenade [*B. subtilis* strain QST 713 (minimum of 1 x 10^9 CFU g^{-1} of the active ingredient)] per kilogram of seed, with 100 mL of distilled water per kg of seed as a carrier. Samples in the treatments with 0.0 mL c.p. kg^{-1} of seed were immersed in distilled water, while the absolute control involved no treatments or contact with the solution.

Seeds from each treatment were planted in 9 L ceramic pots (22.3 cm high × 22.4 cm top diameter × 19.0 cm bottom diameter), with each pot corresponding to an experimental unit. The substrate consisted of Latossolo Vermelho Eutroférrico (Santos et al. 2006) or Ferralsol (FAO 2006) quantified by weighing to standardize the soil volume for all treatments.

The soil chemical analysis indicated 5.4 of pH ($CaCl_2$); 30.2 mg dm^{-3} of P^{3+}; 3.68 $cmol_c$ dm^{-3} of $H + Al^{3+}$; 0.0 $cmol_c$ dm^{-3} of Al^{3+}; 2.66 $cmol_c$ dm^{-3} of Ca^{2+}; 1.37 $cmol_c$ dm^{-3} of Mg^{2+}; 0.26 $cmol_c$ dm^{-3} of K^+; 3.9 g kg^{-1} of C; and 6.7 g kg^{-1} of organic matter. For the pH correction, the equivalent of 1.5 metric tons of lime was applied per hectare, and, for fertilization, 20 metric tons of organic compost containing 1.7 % of N; 2.46 % of P^{3+}; 6.4 % of Ca^{2+}; 1.1 % of K^+; 0.73 % of Mg^{2+}; 0.21 % of S; 28.72 % of organic C; and pH of 8.09.

The ambient temperature in the greenhouse was kept between 20 and 30 ºC, using a cold air intake cooling system. Irrigation was managed according to the crop requirements (Filgueira 2003). There was no need for fungicides, and pests were controlled with a solution of 1.5 L c.p. ha^{-1} of neem oil (*Azadirachta indica* A. Juss.) and 5 mL L^{-1} of neutral detergent diluted in 200 L ha^{-1} of water.

The seedling emergence was evaluated using 5 seeds pot^{-1}, with each pot representing one replication and each treatment replicated five times. The emerged seedlings were counted at 19 days after planting (DAP) and the result was the mean of the replication, expressed as percentage.

The height of one plant per replication was assessed at 10, 20, 30, 40, 50 and 60 DAP and expressed in centimeters, measured from the base of the plant to the tip of the youngest central trifoliate leaf at the top of the plant. The length (mm), width (mm) and area of the central leaflet on the youngest true trifoliate leaf (mm^2) of one marked plant per pot were measured at the same assessment times, using a millimeter ruler, and the results applied in the formula Leaf Area = 0.1026 x $Width^{1.6871}$ (Queiroga et al. 2003).

At the onset of the reproductive stage (R7 - pod formation; Oliveira et al. 2018), the number of true trifoliate leaves and height to first pod were calculated and expressed in centimeters. In the R8 stage (pod filling before fiber accumulation; CIAT 1983, Brandão 2001), the number of commercial fruits (larger than 10 cm), length and number of grains per pod and pod fresh and dry weight were determined and expressed in grams. Two harvests were performed, at 60 and 66 DAP, considering 42,000 plants ha^{-1}, with data expressed in kg ha^{-1}.

The data were standardized according to the following equation: $\hat{Y}_i = (Y_i - \bar{Y})/s$, where: Y_i is the mean of the treatment i; $\bar{Y}$ the mean of the trait, considering all the treatments; and s the standard deviation for the trait, considering all the treatments.

Then, the data were submitted to multivariate analysis of variance (Manova) and, when significant, to the principal component analysis (PCA). The unweighted pair-group method with arithmetic mean (UPGMA), based on the standardized Euclidean distance, was used to divide the treatments into clusters on the two-dimensional perceptual map. Analyses for the vegetative and reproductive traits were processed individually. Additionally, the Pearson's correlation was applied. The analyses were conducted in the R software (r-project.org), using the Candisc, Faraway, Qgraph and FactoMineR packages (R Development Core Team 2012) and Excel®.

RESULTS AND DISCUSSION

The hydration rate, represented by the soaking time as a function of seed moisture content, identified three germination phases (Figure 1).

The average absorption rate in the phase I was 1.83 % between 0 and 18 hours, and, in the phase II, the moisture content increased by 0.49 % per hour, with a total duration of 30 hours. In the phase III, 50 % of radicle protrusion were achieved at a moisture content of 58 %. The imbibition curve made it possible to control the pre-imbibition (PI) and

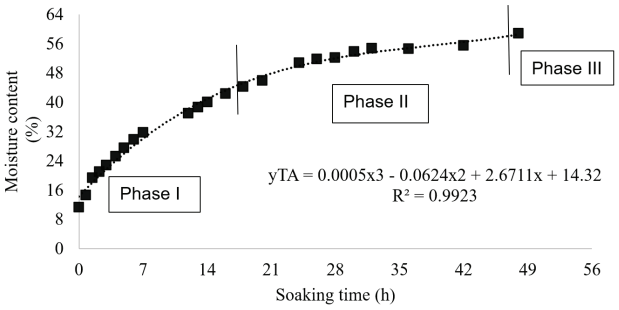

Figure 1. Imbibition curve characterizing the germination phases of the Macarrão Baixo cultivar snap bean seed lot. Source: Ibanhes Neto et al. 2021.

biopriming (BP) treatments, with samples treated for 48 hours until the onset of the phase III.

For the multiple interaction, two 2D perceptual maps were constructed, depicting the behavior of the vegetative traits assessed and the seed treatments and doses applied. The first principal component (PC1-Dim1) explained 51.16 % of the multiple interactions and the principal component 2 (PC2-Dim2) 18.23 %. The first two components explained 69.39 % of the interactions. The behavior of the absolute control in the assessments confirmed the high physiological quality of the original seed lot. The treatments applied and compared with the reference treatment should maintain or improve the traits (Figure 2).

The trait distribution places seedling emergence, plant height (PH) (PH10, PH20, PH30, PH40, PH50 and PH60), central trifoliate leaf length (LL) (LL10, LL20, LL30, LL40, LL50 and LL60), width (LW) (LW10, LW20, LW30, LW40, LW50 and LW60) and area (LA) (LA10, LA20, LA30, LA40, LA50 and LA60) to the right of the PC1, along with the absolute control, FCD0, CAD7, PI7, PI14, BP0, BP7, BP14 and BP28 (Figure 2).

In relation to the PC2, most of the initial assessments up to 30 DAP were in the upper right quadrant (CAD7, PI7, BP0 and BP14), while the final assessments, mostly from 40 DAP onwards, were concentrated in the lower right quadrant, along with the absolute control, FC0, PI14, BP7 and BP28 (Figure 2).

Film coating (FC) and coating and drying (CAD) largely showed an inferior performance, lying to the left of the principal components of reference for both the growth stages (vegetative and reproductive), what may be linked to seed vigor after the treatment. Lopes et al. (2018) studied drum priming in common bean and found that vigor is the main trait affected by soaking-induced damage and that extreme cases may affect seed viability.

Among the tested treatments, FC and CAD resulted in a fast seed imbibition, whereas the priming techniques (PI and BP) provided controlled imbibition. Ogawa et al. (2016) investigated black bean seed submersion and early plant development and observed seedling damage for soaking times from four hours onwards. The authors suggested that the abrupt entry of water into the seed causes hypoxia or anoxia, inverting the respiratory pathway from

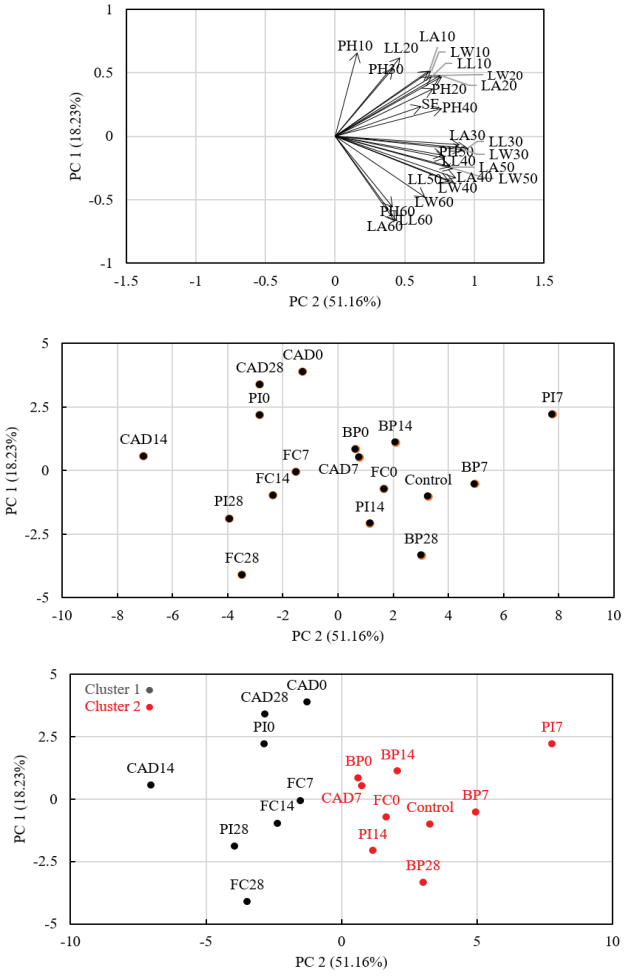

Figure 2. Two-dimensional perceptual map with and without clusters for the traits seedling emergence (SE), plant height (PH), leaf length (LL), width (LW) and area (LA) of the snap bean during the vegetative stage (10, 20, 30, 40, 50 and 60 days after planting), for the treatments absolute control, film coating (FC) and coating and drying (CAD), pre-imbibition (PI) and biopriming (BP), and *Bacillus subtilis* doses (0, 7, 14 and 28 mL c.p. kg^{-1} of seed).

aerobic to anaerobic, what directly affects seed vigor and initial development.

The treatments that provided controlled soaking, using the scenario proposed by the imbibition curve (Figure 1), performed better in the vegetative and reproductive assessments, particularly PI7, PI14, BP0, BP7 and BP28, which remained to the right of the PC1.

According to Silva et al. (2014), bean seeds absorb water at specific points on the seed coat, even when their contact with water occurs uniformly. Moreover, the meristematic growth zones, radicle and plumule are more sensitive to deterioration, for example, since they are considered active centers of vigor. This may explain the inferior development results in the present study, such as those obtained for plant height and leaf length, width and area.

Forti et al. (2009) reported that the emergence of the primary root and seedling growth require energy and reserve material to produce new tissues. This physiological process is initially triggered by a minimal amount of energy and then by the presence of water for hydration and catalysis of metabolic processes by enzymes in the cotyledons, peaking at ideal water availability, what explains the superior performance of the controlled hydration treatments.

Figure 2 also shows the segregation of the treatments into two groups (clusters), with the cluster consisting of FC7, FC14, FC28, FCS0, FCS14, FCS28, PI0 and PI28, and the cluster 2 the absolute control, FC0, FCS7, PI7, PI14, BP0, BP7, BP14 and BP28, the latter cluster exhibiting a positive correlation with the assessed traits. The BP treatment at all tested doses and the absolute control were associated with higher emergence rates after 10 days and greater plant height, length, width and area of the central leaflet (Figure 2).

Figure 3 depicts the 2D perceptual map with the traits evaluated in the reproductive stage. The PC1 explained 41.46 % of the data and the PC2 33.66 %, for a total of 75.12 % of explained variance, using both main components as a reference for interpreting the results, in order to obtain four quadrants, lower and upper left, lower and upper right.

Among the assessed traits, the yield components analyzed at the first harvest, namely number of trifoliate leaves, commercial pods and grains per pod, pod fresh and dry weight, and harvest yield were in the lower right quadrant in relation to the PC1. Those evaluated at the second harvest were in the upper right, except for the number of trifoliate leaves, while the lower left quadrant contained only the pod height (Figure 3).

For the treatment behavior analysis, in relation to the PC1, the treatments PI7, PI14, BP0, BP7 and BP28 were in the lower right quadrant and associated with high yield traits at the first harvest, whereas the second harvest and total yield were correlated with the absolute control, FC28 and PI28, and FC0, FC7, FCS0, FCS28 and BP14 with pod height (Figure 3).

Figure 3 shows four distinct clusters, the first comprising the PC14, which was not associated

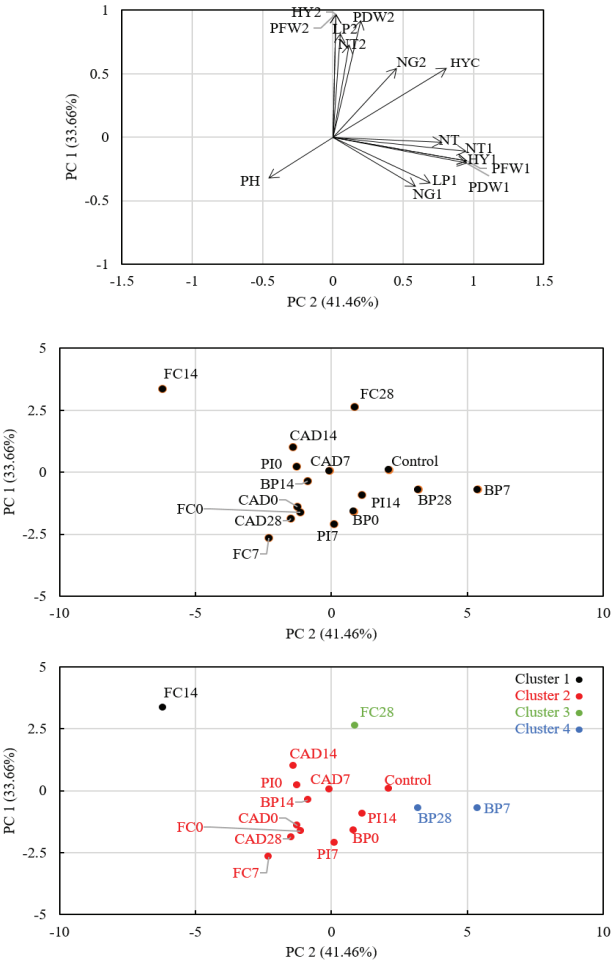

Figure 3. Two-dimensional perceptual map with and without clusters for the traits pod height (PH), number of trifoliate leaves (NT), number of grains per pod (NG), pod fresh (PFW) and dry weight (PDW) and harvest yield (HY) of the snap bean during the reproductive stage with two harvests (1 and 2), submitted to the treatments absolute control, film coating (FC), film coating and drying (CAD), pre-imbibition (PI) and biopriming (BP), and *Bacillus subtilis* doses (0, 7, 14 and 28 mL^{-1} c.p. kg of seed).

with the yield traits analyzed at the first harvest, and the second the absolute control, FC0, FC7, FC28, all the CAD treatments, PI0, PI7, PI14, BP0 and BP14.

The cluster 3 contained the PI28 and the cluster 4 the BP7 and BP28. The treatments BP 7 and BP28 performed better and were correlated with the principal components (vectors) at the first harvest (60 DAP), whereas the PI 28 showed a strong correlation with the second harvest (66 DAP).

Nunes et al. (2019) studied the pre-hydration of cowpea seeds and observed a better membrane organization in seeds submitted to this technique, which reduced the leachate content in the medium, when compared with seeds that did not undergo pre-imbibition. This corroborates the superior performance of PI and BP in relation to FC and CAD.

Aragão et al. (2002) analyzed the effect of soaking and drying cycles on bean seeds and observed positive effects for those soaked and dried over 6, 12 and 24-hour cycles, with an increase in the first germination count. Guimarães et al. (2013) reported that the 12-hour pre-imbibition of watermelon seeds favored seedling emergence, whereas soaking for 48 and 72 hours produced larger and heavier plants.

Kumari et al. (2018) also reported favorable results for *Vigna radiata* (L.) R. Wilczek plants grown from seeds bioprimed with *Pseudomonas aeruginosa* BHU B13-398 and *B. subtilis* BHU M., with respective increases of 32.26 and 13.38 % for shoot length and 84.60 and 61.94 % for root length. The authors attributed these findings to previous positive tests for the solubilizing action of siderophores, phosphate, ammonia and hydrogen cyanide.

Negi et al. (2019) tested the use of biopriming in *P. vulgaris* seeds with native isolates of the plant growth promoting rhizobacteria (PGPR) *Rhizobium* spp. and *Trichoderma* spp. and observed an increase in germination, plant growth, pod yield and seed quality when specific strains from each genus were applied individually or together.

Monalisa et al. (2017) applied *Trichoderma* spp. and *Pseudomonas fluorescence* via biopriming to common bean seeds and found that the latter increased germination, shoot and radicle length, and seedling dry weight. According to the authors, in addition to the beneficial effect of microorganisms, knowledge and use of the predetermined imbibition curve for the species help to prevent soaking-related damage and maximize the use of resources.

Mitra et al. (2021) highlighted this trend of biopriming with rhizobacteria in the modernization of agriculture, reinforcing the gains in the aforementioned traits, in addition to biocontrol of plant pathogens and better crop sustainability.

Figure 4 depicts a Pearson's correlation network graph, where the traits assessed up to day 20 showed a positive strong correlation, except for seedling emergence, despite its proximity.

However, after 30 DAP, the assessments in the vegetative stage are concentrated in the center of the graph and strongly correlated, but with clear segregation between the two harvest times. Better performance results at 50 and 60 days remained close to the first harvest, making it possible to infer that plants developed better, with greater PH, NT and LA, resulting in a superior performance at the first harvest (Figure 4).

The assessments at the second harvest maintained a negative weak correlation with vegetative traits measured up to 30 DAP (Figure 4). Some treatments were superior in only one period, such as FC0 and FCS7 in the vegetative and FC28 and PI28 in the reproductive stage. However, when compared to the best treatments to the right of the

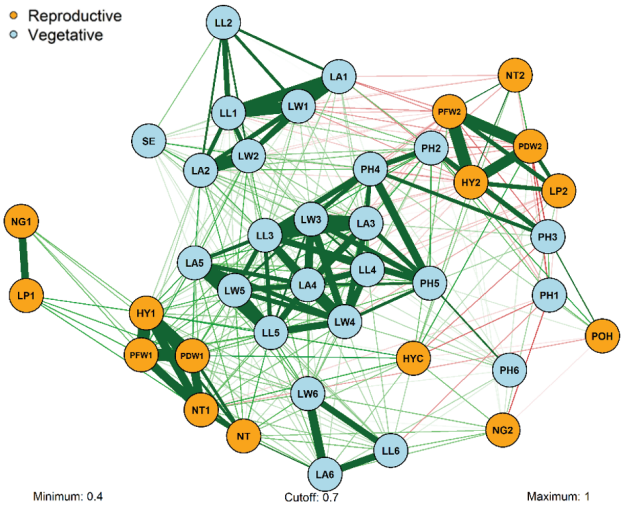

Figure 4. Pearson's correlation network (green line positive and red negative) consisting of the Macarrão Baixo cultivar snap bean traits evaluated in the vegetative stage [seedling emergence (SE), plant height (PH), leaf length (LL), width (LW) and area (LA)], at 10, 20, 30, 40, 50 and 60 days after planting, and during the reproductive stage [pod height (POH), number of trifoliate leaves (NT), number of pods (NP), number of grains per pod (NG), pod fresh (PFW) and dry weight (PDW) and total (TY) and harvest yield (HY)].

PC1 (Figures 2 and 3), the first two treatments were weakly correlated with vegetative trait vectors. The superior performance only in the reproductive stage was influenced by the highest dose of the *B. subtilis*-based commercial product, making it important to conduct further research on treatments with higher doses.

The dose used in the treatments is selected based on the cost effectiveness of the application, which varied among the applied treatments. The best dose responses were obtained between 7 and 14 mL c.p. kg^{-1} of seed when applied before soaking, whereas biopriming provided benefits at all the tested doses. As previously mentioned, doses can be adjusted in cases of plant pathogens that respond to *B. subtilis* application.

Lazzaretti & Bettiol (1997) tested the effect of a product consisting of wettable powder combined with *B. subtilis* cells and metabolites on variables such as biocontrol, nodulation and initial emergence in rice, wheat bean and soybean. A dose of 2.57 x 10^8 CFU g^{-1} did not affect the emergence crop, enabled the combined application with inoculants without compromising nodulation, and provided a biocontrol similar to that of the recommended fungicides for *Sclerotinia sclerotiorum*, *Rhizoctonia solani* and *Aspergillus* sp.

Costa et al. (2019) studied doses of 0-8 mL kg^{-1} of seed of a *B. subtilis*-based commercial product in two soybean cultivars and reported an increase in the SPAD index in both the plant materials, as well as a higher shoot fresh weight at 30 DAP and root fresh weight at 45 DAP for the TMG132 cultivar.

Tu et al. (2016) tested the longevity of microencapsulated *B. subtilis* seed coating agents and their effects on cotton seedling growth and found an increase of 2.70 % in seedling weight, 25.13 % in root length, 46.47 % in fresh weight and 33.21 % in dry weight, at a dose of 10 %.

Ibanhes Neto et al. (2021) investigated different treatments associated with *B. subtilis* doses between 0 and 28 mL c.p. kg^{-1} of seed and their effect on the physiological potential of snap bean seeds in a laboratory and obtained better vigor using a PI with 16-20 mL c.p. kg^{-1} of seed, whereas the BP improved the seedling dry weight at 18-22 mL c.p. kg^{-1} of seed. This may be associated with the superior response observed for the yield components and traits evaluated in the vegetative stage in the present study.

CONCLUSION

The pre-imbibition treatments combined with 7 and 14 mL of commercial product per kg of seed and biopriming at doses of 0, 7 and 28 mL of commercial product per kg of seed favored the snap bean growth traits and development during the vegetative stage. The same treatments improved yield components in the snap bean plants, which reached the harvest point faster, when compared to film coating with and without drying.

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