Treatments for *Sclerotinia sclerotiorum* on Inoculated Bean Seeds and Effects on Health and Physiological Quality

Brenda Tortelli

Plant Pathology Laboratory, Federal University of Fronteira Sul - Campus Erechim

ERS 135 - Km 72, Nº 200, PO Box 764, Zip Code: 99700-970, Erechim - RS, Brazil

E-mail: bre.tortelli@hotmail.com

Suelen Cappellaro

Plant Pathology Laboratory, Federal University of Fronteira - Campus Erechim

ERS 135 - Km 72, Nº 200, PO Box 764, Zip Code: 99700-970, Erechim - RS, Brazil

PRO-ICT/UFFS Scholarship

E-mail: suelen02cappellaro@hotmail.com

Júlia Andrade

Plant Pathology Laboratory, Federal University of Fronteira Sul - Campus Erechim

ERS 135 - Km 72, Nº 200, PO Box 764, Zip Code: 99700-970, Erechim - RS, Brazil

UFFS - Academic Monitoring Scholarship

E-mail: julia_andrade09@hotmail.com

Márcio Paulo Mezzomo

Plant Pathology Laboratory, Federal University of Fronteira Sul - Campus Erechim

ERS 135 - Km 72, Nº 200, PO Box 764, Zip Code: 99700-970, Erechim - RS, Brazil

E-mail: marciopaulo.mezomo@gmail.com

Péricles Roberto Steffen

Plant Pathology Laboratory, Federal University of Fronteira Sul - Campus Erechim

ERS 135 - Km 72, Nº 200, PO Box 764, Zip Code: 99700-970, Erechim - RS, Brazil

E-mail: periclessteffenagro2015@gmail.com

Vanessa Neumann Silva

Seed and Grain Laboratory, Federal University of Fronteira Sul - Campus Chapecó

Rodovia SC 484 - Km 02, Fronteira Sul, Zip Code: 89815-899, Chapecó - SC, Brazil

E-mail: vanessa.neumann@uffs.edu.br

Paola Mendes Milanesi (Corresponding author)

Plant Pathology Laboratory, Federal University of Fronteira Sul - Campus Erechim

ERS 135 - Km 72, Nº 200, PO Box 764, Zip Code: 99700-970, Erechim - RS, Brazil

Tel: 55-54-3321-7336   E-mail: paola.milanesi@uffs.edu.br

Abstract

Considering the importance of bean cultivation, the objective was to prove the effectiveness of the seed microbiolization method in the control of *Sclerotinia sclerotiorum* (*Ss*) on black bean seeds (cv. IPR Tuiuiú), inoculated by the water restriction method. The treatments were fungicide methyl thiophanate + fluazinam (350.0 g L^{-1} + 52.5 g L^{-1}); *Trichoderma asperellum* BV-10 (1.0 x 10^{10} conidia mL^{-1}); *T. harzianum* strain CCT 7589 (1 x 10^9 CFUs L^{-1}); *Bacillus subtilis* BV-02 (minimum 3.0 x 10^9 CFU mL^{-1}); *B. amyloliquefaciens* isolated BV03 (minimum 3.0 x 10^9 CFU L^{-1}); Positive control (seeds exposed to *Ss*); fungicide; *T. asperellum* BV-10; *T. harzianum* strain CCT 7589; *B. subtilis* BV-02; *B. amyloliquefaciens* isolate BV03; and Negative control (PDA medium plus restricting). The effects of these treatments on germination, vigor, and the health of bean seeds, and seedling emergence in the field, were evaluated. Microbiolization with *T. asperellum* and *T. harzianum*, provides improvement in germination, vigor, and health, but is not superior to fungicide treatment. *Bacillus subtilis* and *B. amyloliquefaciens*, in seeds not inoculated with the pathogen, produce the best results for fresh and dry mass (g). These may be used in the integrated management of white bean mold.

Keywords: white mold, *Phaseolus vulgaris* L., water restriction, microbiolization

1. Introduction

The bean (*Phaseolus vulgaris* L.) is a legume of great economic and social importance in Brazil as it is a source of income for many family farmers, who depend on bean production and also because it is part of the population's diet (Binotti et al., 2009). In 2019, the first crop season of beans reached a production of 973.7 thousand tons and, by the end of the third crop, it is estimated to reach 3.1 million tons (Conab, 2019).

However, several factors can affect bean production capacity; one of these is disease incidence. Diseases affecting bean crops include white mold, caused by *Sclerotinia sclerotiorum* (Ascomycota; *Sclerotiniaceae* family) (Mycobank, 2019), which is considered one of the main and most destructive bean diseases (Wendland et al., 2016). White mold can cause 100% yield losses in crops with high severity of the disease, that is spread from infected seeds, where the pathogen survives for long periods, increasing the source of inoculum at each seeding with the same host (Wu and Subbarao, 2008).

Thus, the treatment of seeds with fungicides is an important procedure in bean production, since they are efficient in controlling the pathogens associated with seeds and also with soil (Machado, 2000). When performed effectively and in quality seeds, the treatment ensures an adequate initial stand (Tonello et al., 2019). The integration of white mold management measures provides, in addition to crop rotation, the application of fungicides with different active ingredients, including multisites, preventing or delaying the emergence of fungicide-resistant *S. sclerotiorum* populations (Lehner et al., 2017).

The search for disease control methods that are not considered only in chemical control has intensified in recent years. In this context, the use of biological control agents is one of the alternatives for seed treatment, aiming at greater sustainability in agriculture (Xu et al., 2011). The fungi *Trichoderma harzianum* (Zhang et al., 2016) and *T. asperellum* (Kipngeno et al., 2015), as well as the bacteria *Bacillus subtilis* (Wang et al., 2018) and *B. amyloliquefasciens* (El-Gremi et al., 2017), have been successfully used in the microbiolization of seeds of various agricultural crops. This is a safe method that does not cause environmental imbalance, allowing subsequent sowing with minor disease-related problems. These microorganisms can also act as biostimulants, promoting plant growth (Harman, 2006; Moura et al., 2018).

In order to enable seed inoculation with a pathogen and subsequent treatment (chemical and biological), there are methods that guarantee the reproduction and expression of symptoms and signs typical of a particular seed-associated plant pathology. Therefore, it is essential that such methods enable seed infection but without compromising germination potential and seedling emergence (Rocha et al., 2014), to allow future evaluation. The most used seed inoculation methods involve immersion of the seeds in a spore suspension (Pedroso et al., 2010); direct contact between the seeds and the fungal culture developed in culture medium (Migliorini et al., 2018); and osmotic conditioning, with the use of water restriction (Venturoso et al., 2015).

In this context, the objective of this study was to evaluate the effect of chemical and biological treatment on germination, vigor and the health of bean seeds (cv. IPR Tuiuiú) inoculated with *Sclerotinia sclerotiorum* by water restriction.

2. Material and Methods

The experiment was carried out in Erechim - Rio Grande do Sul (RS), Brazil (27° 37' 50'' S, 52° 14' 11'' W, altitude: 753 m), in a completely randomized design with four replications. Bean seeds of cultivar IPR Tuiuiú (black group, medium cycle: 88 days), 2019 crop, classification C1, were used. The seeds had the following characteristics: *i)* humidity: 15%, this is above the recommended value of 13% (Zucareli et al., 2015); *ii)* electrical conductivity: 54, 35 µS cm^{-1} g^{-1}, considered of high vigor (Smaniotto et al., 2013); *iii)* germination: 96%, above the minimum standard required in Brazil for commercialization, which is 80% (Brasil, 2013); and *iv)* health, storage fungi only: *Rhizopus* spp. (14%), *Aspergillus* spp. (5%), and *Penicillium* spp. (17%).

The inoculum of *Sclerotinia sclerotiorum* was obtained from pathogen sclerotia collected from soybean plants with symptoms of white mold in a field located in Erechim (RS). The sclerotia were submitted to asepsis with 1% sodium hypochlorite for 1 min and sterile distilled water (three rinses of 1 min each). In Petri dishes containing potato-dextrose-agar (PDA) culture medium, the sclerotia were distributed, and then the plates were incubated at 23 °C with a 12-hour photoperiod (Pereira et al., 2009). After 7 days of incubation, the pathogen structures were subcultured onto plates containing PDA culture medium to obtain the pure culture.

Through the water restriction technique (Coutinho et al., 2001; Cruciol and Costa, 2017), the pathogen was cultivated on PDA culture medium supplemented with the osmotic restrictor mannitol ($C_6H_{14}O_6$) in order to guarantee a water potential of -0.6 MPa (44.88 g of mannitol L^{-1} PDA) (Coutinho et al., 2001). Plates were incubated at 23 °C with a 12-hour photoperiod for 7 days when mycelial growth of *S. sclerotiorum* reached the edges of the petri dish. The bean seeds were distributed on the PDA + mannitol medium in a single layer and then incubated at 23 ºC with a photoperiod of 12 hours, remaining on the medium in direct contact with the pathogen with different exposure times (2, 6, 12, 18, 24, 36, and 48 hours).

After this initial test, the previous procedure was repeated with 12 hours of exposure to the pathogen (23 ºC and a photoperiod of 12 hours), due to the infection and maintenance of germination and seed vigor. These were removed from the culture medium and kept at room temperature, properly covered with sterile paper towels, to dry for 24 hours (Cruciol and Costa 2017).

Inoculated bean seeds were submitted to the following treatments:

T1 - fungicide methyl thiophanate + fluazinam (350.0 g L^{-1} + 52.5 g L^{-1}; 180 mL/100 kg of seeds);

T2 - *Trichoderma asperellum* BV-10 (1.0 x 10^{10} viable conidia mL^{-1}; 200 g L^{-1}; 1 mL/kg);

T3 - *Trichoderma harzianum* strain CCT 7589 (1 x 10^9 CFUs/L; 5 g L^{-1}; 2 mL/kg);

T4 - *Bacillus subtilis* BV-02 (minimum 3.0 x 10^9 CFU/mL; 42 g L^{-1}; 2 mL/kg);

T5 - *Bacillus amyloliquefaciens* isolate BV03 (minimum 3.0 x 10^9 CFU/L; 42 g L^{-1}; 2 mL/kg);
T6 - Positive control (seeds exposed to the pathogen only);

T7 - Fungicide methyl thiophanate + fluazinam;

T8 - *Trichoderma asperellum* BV-10;

T9 - *Trichoderma harzianum* strain CCT 7589;

T10 - *Bacillus subtilis* BV-02;

T11 - B*acillus amyloliquefaciens* isolate BV03; and

T12 - Negative Control (PDA medium supplemented with mannitol only).

The seeds remained at room temperature for 24 hours for complete drying.

In order to verify the efficiency of treatments on the physiological quality of bean seeds, after exposure to *Sclerotinia sclerotiorum*, the following tests were performed:

Sanitation: from eight repetitions of 25 seeds, according to the methodology from the Manual for Sanitary Analysis of Seeds (Brasil 2009a). Using the freeze-free blotter test method, the seeds were distributed among gerbox boxes (11.0 x 11.0 x 3.0 cm) containing two sheets of blotter paper. In order to suppress seed germination, the paper was moistened with 2,4-D salt solution (5 ppm). The seeds were incubated at 20 ± 2 ºC with a photoperiod of 12 hours for 7 days and analyzed with the aid of stereoscopic and optical microscopes. The percentage (%) of fungal incidence, identified according to specialized literature (Barnett and Hunter, 1999; Brasil, 2009a), was determined.

Germination: the method of Seed Analysis Rules (Brasil, 2009b) was used, with four replications of 50 seeds. The seeds were placed on germitest paper moistened with distilled water at 2.5 to 3 times their dry weight. After sowing, rolls were made and placed in a germination chamber at 25 ± 2 ºC, with a photoperiod of 12 hours. The evaluations occurred by counting at five and nine days after sowing (DAS). In the first count, all germinated seeds that originated normal seedlings were counted. At the second count, the seedlings were classified as normal, abnormal, and non-germinated seeds (hard and dead).

Germination speed index (GSI): performed from eight repetitions of 25 seeds each, distributed on sterile moistened germitest paper. The samples were incubated at 25 ± 2 °C with a photoperiod of 12 hours. Germinated seeds were evaluated daily by counting normal seedlings until the fifth day, together with the first twinning count (Brasil, 2009b). After obtaining daily data on the number of normal plants, the GSI was calculated (Maguire, 1962).

Cold without soil: conducted in a similar way to the germination test, the seed-containing rolls remained in an incubator at 10 ± 2 °C, with no photoperiod, for three days (Miguel and Cicero, 1999; Guiscem et al., 2010). Thereafter, the rolls were placed in an incubator at 25 ± 2 °C with a 12-hour photoperiod for four days, and the usual seedling counts were performed (Brasil, 2009b).

Accelerated aging: four samples of 50 seeds for each treatment were distributed on aluminum screens, suspended in gerbox, containing 40 mL of distilled water, and kept in an incubator for 72 hours at 42 ± 2 °C. Soon after, the germination test was assembled, performing the first and

second count of germinated seeds, as previously described in the bean germination test (Marcos Filho, 1999).

Field emergence: four replications with 50 seeds were used, and emergence evaluation was performed at 30 days after sowing (DAS), accounting for the number of emerged plants, that is, plants that had fully expanded primary leaflets (Migliorini et al., 2017). At 30 DAS the plants present in each plot were collected to quantify plant fresh and dry mass (g). For this, 25 plants were removed from each repetition and placed in kraft paper bags (capacity 3 kg). The samples were placed in a drying oven with forced air circulation at 65 ºC until they reached a constant weight, and the values were expressed in g seedling^{-1} (Dutra et al., 2012).

Statistical analysis: the data obtained were submitted to analysis of variance by the F test ($p \leq 0.05$) and, when significant, the means were compared by the Tukey test ($p \leq 0.05$). The analyses were performed using the statistical software SISVAR version 5.6 (Ferreira, 2011).

3. Results and Discussion

The inoculation of *Sclerotinia sclerotiorum* in bean seeds by the water restriction method was efficient and reached up to 100% colonization when the seeds were left in direct contact with the pathogen for 48 hours (Figure 1). In this study, we opted for 12 hours of exposure to the pathogen, which resulted in a colonization rate of 50% of the seeds, allowing the evaluation of germination and vigor. This behavior corroborates what has already been observed for *Colletotrichum lindemuthianum vs.* beans (Migliorini et al., 2017) and also for *S. sclerotiorum vs.* beans, in which there was a reduction of over 60% in seedling emergence in infected seeds (Venturoso et al., 2015).

Seeds that were not inoculated with *Sclerotinia sclerotiorum* showed the best performance in the analyzed variables and no incidence of the fungus (Table 1). These results demonstrate the importance of the use of quality seeds to obtain an adequate crop establishment in the field (Araujo et al., 2011). However, the higher the initial inoculum level of a given pathogen, the higher the percentage of dead seeds (Rey et al., 2009).

Of the treatments that were inoculated, only fungicide-treated seeds did not differ statistically from those that were not inoculated (Table 1), indicating the efficiency of the product. The active ingredient fluazinam is also used to control white mold on crops, and according to Sumida et al. (2015), this fungicide is effective at inhibiting mycelial growth and carpogenic germination of *S. sclerotiorum* sclerotia, ensuring high efficiency in soybean disease control under field conditions.
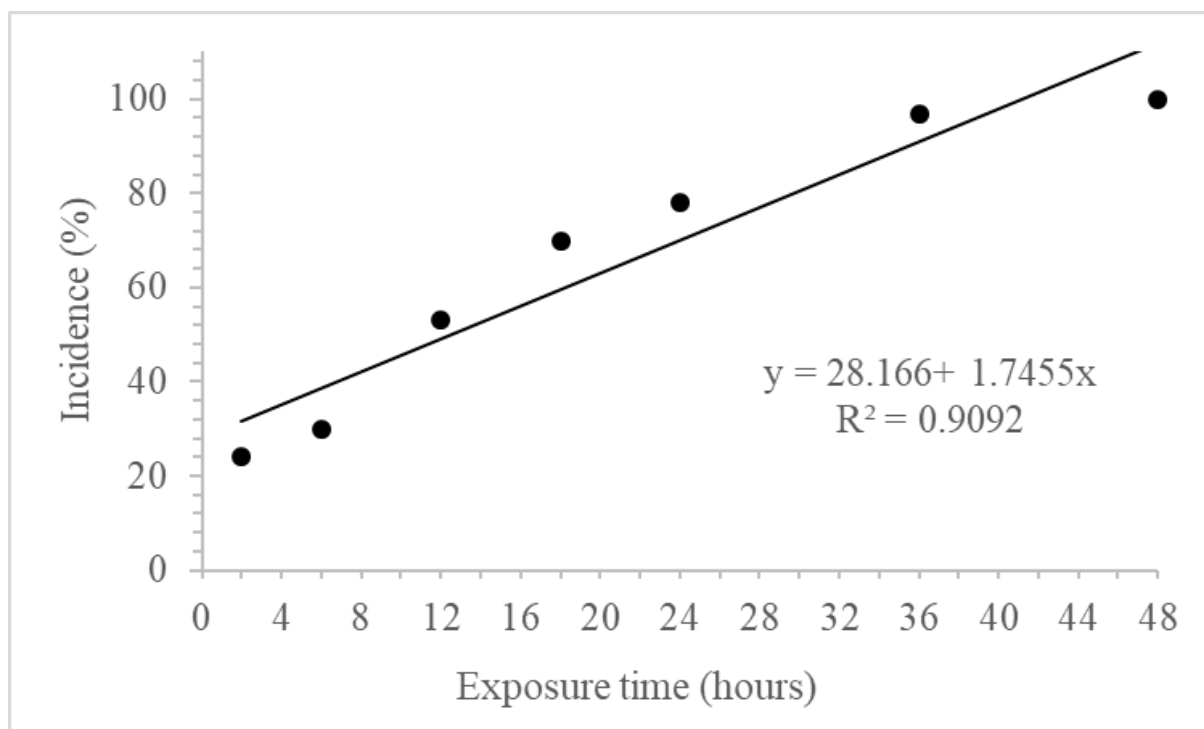
$$y = 28.166 + 1.7455x$$
$$R^2 = 0.9092$$

Figure 1. Incidence of *Sclerotinia sclerotiorum* in bean seeds, cv. IPR Tuiuiú, inoculated or not inoculated with the pathogen by the water restriction method with mannitol (-0.6 MPa), as a function of exposure time (hours)

In the T6 (Positive control) treatment, inoculation with *Sclerotinia sclerotiorum* caused damage to the bean seeds, as the lowest averages in all examined variables were obtained for this treatment (Table 1). Because it is the Control, where the seeds have not received any treatment, the pathogen can cause the seeds to die before they even begin the germination process, which may be related to the aggressiveness of *Sclerotinia sclerotiorum*, as well as conditions favorable to its establishment and rapid colonization of plant tissues by the pathogen (Venturoso et al., 2015).

Treatment with the thiophanate-methyl + fluazinam fungicide ensured that even in seeds inoculated with the pathogen, the analyzed variables achieved similar results to those of non-inoculated seeds. Figueirêdo et al. (2010) also verified efficient control of *Sclerotinia sclerotiorum* in the common bean after application of a fungicide with thiophanate methyl as the active ingredient.

The treatments performed with *Trichoderma harzianum* (T2) and *T. asperellum* (T3) produced superior results to the others in the inoculated seeds (T1-T6) (Table 1). Similarly, Carvalho et al. (2011) found that three *T. harzianum* isolates provided higher germination percentages in common bean seeds, cv. 'Jalo Precoce', which were not treated. The fact that *Trichoderma* species have the potential for white mold control can be attributed to the action of metabolites and parasitism exerted on *S. sclerotiorum* hyphae (Harman, 2006). Moreover, in plants colonized by *T. harzianum,* there was a greater ability to combat pathogenic fungal attacks

(Zhang et al., 2017), showing that this antagonist causes a significant increase in the activation and accumulation of plant-related biomolecules (Singh et al., 2019).

Table 1. Incidence (Inc.,%) of *Sclerotinia sclerotiorum* (Ss), germination (G,%), germination speed index (GSI), accelerated aging (A.A.,%) and cold test (C.T.,%) in bean seeds, cv. IPR Tuiuiú, inoculated or not inoculated with the pathogen by the water restriction method with mannitol (-0.6 MPa) and subsequently treated

Treatments	Inc.	G	GSI	A.A.	C.T.
	%			%	
T1) Fungicide (methyl thiophanate + fluazinam) + Ss	2 ab[*]	97 a	8.8 abc	86.0 a	98.5 ab
T2) *Trichoderma asperellum* BV-10 + Ss	26 de	92 ab	7.8 bc	40.5 bc	93.5 ab
T3) T*richoderma harzianum* CCT 7589 + Ss	30 ef	83 ab	7.4 c	48.0 b	93.5 ab
T4) *Bacillus subtilis* BV-02 + Ss	20 cd	73 c	5.6 d	48.0 b	94.5 ab
T5) *Bacillus amyloliquefaciens* isolate BV03 + Ss	11 bc	82 bc	5.0 d	30.0 de	82.0 c
T6) Positive Control	40 f	52 d	4.2 d	26.5 e	68.0 d
T7) Fungicide methyl thiophanate + fluazinam	0 a	98 a	8.2 abc	89.0 a	100.0 a
T8) *Trichoderma asperellum* BV-10	0 a	98 a	9.2 ab	46.5 b	98.5 ab
T9) *Trichoderma harzianum* CCT 7589	0 a	98 a	8.8 abc	38.0 cde	92.5 ab
T10) *Bacillus subtilis* BV-02	0 a	99 a	9.5 a	41.5 bc	89.0 bc
T11) *Bacillus amyloliquefaciens* isolate BV03	0 a	97 a	8.2 abc	37.5 cde	93.5 ab
T12) Negative Control	0 a	97 a	7.4 c	31.0 de	90.0 bc
C.V. (%)[**]	24.2	5.9	7.6	9.9	4.2

[*]Means followed by the same lower-case letter in the column do not differ statistically by Tukey's test (p ≤ 0.05). [**]Coefficient of variation.

Regarding the GSI, the treatments that produced a lower germination rate were T4 (*B. subtilis*), T5 (*B. amyloliquefasciens*), and the positive control (T6) (Table 1). Fungicide (T1) and *Trichoderma* (T2 and T3) treatments favored rapid germination, which is important because it reduces the time required for bean establishment, providing competitive ability against weeds, which cause competition and reduced yield between 35% and 80% (Galon et al., 2016).

In the accelerated aging test, only fungicide treatments were superior, in seeds with and without exposure to the pathogen (Table 1). The incubation temperature of various fungal isolates, such as *Fusarium* sp., *Penicillium* sp., *Graphium* sp., and *Trichoderma,* was 30 °C, and it should be noted that none of the *Trichoderma* species grows when the temperature is above 40 °C (Singh et al., 2014)**.** Thus, considering that the seeds were exposed to 42 °C for 72 hours during the accelerated aging period, it can be understood why treatments with *Trichoderma* and *Bacillus* did not benefit seed vigor.

However, in the cold test, the results were similar to those of germination (Table 1). On soybean, cv. BRS 133, according to seed lot, percentages higher than or equal to those obtained in the germination test were also evidenced in the cold test (Vieira et al., 2010). This reinforces the indication that the seeds used in this work had high vigor.

In the field emergence analysis, the treatments in which the seeds were not inoculated and those treated with fungicide showed the best results at 30 days after sowing (Table 2). In soybeans, *Sclerotinia sclerotiorum* is a very aggressive pathogen and therefore reduces seedling emergence, in which case biological control with *T. harzianum* is not efficient (Silva et al., 2017).

Table 2. Emergence (%) of bean plants, cv. IPR Tuiuiú, fresh and dry mass (g), up to 30 days after sowing (DAS) of plants whose seeds were inoculated or not inoculated with *Sclerotinia sclerotiorum* (Ss) by the water restriction method with mannitol (-0.6 MPa) and subsequently treated

Treatments	Emergence	Fresh mass	Dry mass
	(%)	(g)	
T1) Fungicide (methyl thiophanate + fluazinam) + Ss	79 abc	3.8 abcd[*]	0.6 abc
T2) *Trichoderma asperellum* BV-10 + Ss	67 cd	2.8 bcd	0.4 c
T3) T*richoderma harzianum* CCT 7589 + Ss	74 abc	4.1 abc	0.5 abc
T4) *Bacillus subtilis* BV-02 + Ss	68 cd	3.5 bcd	0.5 bc
T5) *Bacillus amyloliquefaciens* isolate BV03 + Ss	72 bcd	4.0 abcd	0.5 abc
T6) Positive Control	57 d	2.6 cd	0.4 c
T7) Fungicide methyl thiophanate + fluazinam	88 a	3.2 bcd	0.5 bc
T8) *Trichoderma asperellum* BV-10	79 abc	3.7 abcd	0.5 abc
T9) *Trichoderma harzianum* CCT 7589	84 ab	3.4 bcd	0.5 bc
T10) *Bacillus subtilis* BV-02	77 abc	5.1 a	0.7 ab
T11) *Bacillus amyloliquefaciens* isolate BV03	75 abc	5.1 a	0.7 a
T12) Negative Control	72 bc	2.4 d	0.4 c
C.V. (%)[**]	4.9	26.8	28.8

*Means followed by the same lower-case letter in the column do not differ statistically by Tukey's test ($p \leq 0.05$). **Coefficient of variation.

The treatment of seeds with systemic and/or contact fungicides acts on several metabolic processes of phytopathogenic fungi (Cameron et al., 2017), inhibiting the growth of these pathogens with environmental conditions conducive to the development of a certain disease, ensuring that the seeds germinate without the interference of pathogens (Urrea et al., 2013).

Also, in this test, it was found that treatments with *Bacillus*, without inoculation of the pathogen in bean seeds, presented the best results for fresh and dry mass (g). The other

treatments did not differ statistically for these variables; however, the Control, both positive and negative, presented the worst results (Table 2). According to Araujo et al. (2005) this would be related to the fact that *Bacillus* spp. is a soil bacterium that produces hormones such as indole acetic acid (IAA) and indole butyric acid (IBA), secreting important enzymes for plant nutrition, mainly by facilitating nodulation.

Chowdappa et al. (2013) showed that IAA levels increased by 45% in roots of tomato seedlings inoculated with *Trichoderma* when compared with uninoculated plants; similarly, Zhang et al. (2017) found that soybean lateral roots increased up to 34.3% in relation to plants that were not inoculated with *Trichoderma*, indicating that there is induction in the production of auxins by *T. harzianum,* and this phenomenon is related to the plant growth stimulus (Medina et al., 2010). Canbolat et al. (2006) reported increased nutrient availability to plants provided by increased nodulation in corn and cotton plants when inoculated with *Bacillus subtilis.*

The application of biological control agents can also be increased by combination with other compounds of organic origin, increasing the population of antagonists and making the soil suppressive to phytopathogens. In this case, the use of sewage sludge biochar combined with *T. harzianum* maximizes the antagonistic effect, improving seed germination, as well as increasing the fresh and dry mass of bean plants inoculated with *Macrophomina phaseolina* (Araujo et al., 2019). In this context, it is noted that plants with more abundant root systems exploit a larger soil volume, improving nutritional conditions and thus providing greater tolerance to adverse conditions that can be found in the field, becoming less sensitive to possible soil and shoots pathogens.

With this study, we confirmed that the technique of water restriction provides efficient infection of bean seeds, cultivar IPR Tuiuiú, with the pathogen *S. sclerotiorum*, even when using shorter inoculation times. Furthermore, the chemical treatment with fungicide ensures the maintenance of the physiological quality of the seeds, which reflects on the germination, vigor and health of seeds inoculated with *S. sclerotiorum* in relation to the evaluated antagonists.

It is clear that the most efficient method for controlling *Sclerotinia sclerotiorum* in bean seeds is still the adoption of chemical treatment. However, the use of *Trichoderma* and *Bacillus* were also efficient when compared with the positive control. Therefore, in some aspects, the biological control agents evaluated in this work improved the potential establishment of seedlings and provided them with protection.

4. Conclusion

The integration of management measures for white mold in common beans must been consider not only chemical control, but also biological control. The use of methodologies, such as microbiolization of seeds, allows the infection by the pathogen, ensuring the manifestation of symptoms, making the observation of the effects of better or worse control for a given treatment more assertive.

Therefore, the microbiolization with both *Trichoderma asperellum* and *Trichoderma harzianum* improved germination, vigor, and health of bean seeds but is not yet superior to fungicide. *Trichoderma harzianum*, *T. asperellum*, *Bacillus subtilis,* and *B. amyloliquefaciens*

in seeds not inoculated with *Sclerotinia sclerotiorum*, improvement the seed germination, vigor, and health, as well as the fresh and dry mass (g) of plants, wherein *Bacillus subtilis* and *B. amyloliquefaciens* stand out for the best results.

In view of an agricultural perspective less dependent on pesticides, the biological treatments evaluated in this study can be successfully used to control white mold in common beans, in the context of the integrated management of this disease.

Acknowledgments

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