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## Techniques microbiolization seed forage radish with *Trichoderma* spp. and *Bacillus subtilis*

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### ABSTRACT

The objective of this study was the effect of leverage microbiolization forage radish seed with *Trichoderma* spp. and *Bacillus subtilis* by physiological conditioning techniques, suspension of biological structures and film coating. The microbiolization suspension of biological structures was carried out with commercial products Rhizoliptus® and Agrotich plus®. Fluid restriction was held in PDA medium + mannitol (- 0.7 MPa), when was the first seed radicle protrusion in the other were removed and dried in a laboratory environment for 48 h. The film coating was performed with the addition of the polymer Color Seed® (300 mL kg<sup>-1</sup>) treatment of the syrup containing *Trichoderma* spp. or product Rhizoliptus®. We used a treatment covering the seeds primed with organisms, or their association. The microbiolization with *Trichoderma* spp. by physiological conditioning controls pathogens infecting the seeds. *Trichoderma* spp. and *Bacillus subtilis* promote the growth and development of seedlings of forage radish, and the coating of the seeds primed in the presence of organisms which provides greater shoot growth of seedlings in the field.

**Key words:** conditioning; germination; polymer; health

## Técnicas de microbiolização de sementes de nabo forrageiro com *Trichoderma* spp. e *Bacillus subtilis*

### RESUMO

O objetivo deste trabalho foi potencializar o efeito da microbiolização de sementes de nabo forrageiro com *Trichoderma* spp. e *Bacillus subtilis* pelas técnicas de condicionamento fisiológico, suspensão de estruturas biológicas e peliculização. A microbiolização com suspensão de estruturas biológicas foi realizada com os produtos comerciais Agrotich plus® e Rhizoliptus®. A restrição hídrica foi realizada em meio BDA + Manitol (- 0,7 MPa), ao ocorrer protrusão radicular na primeira semente, as demais foram retiradas e secas em ambiente de laboratório por 48 h. A peliculização foi realizada com a adição do polímero Color Seed® (300 mL kg<sup>-1</sup>) à calda de tratamento contendo *Trichoderma* spp. ou ao produto Rhizoliptus. Foi utilizado um tratamento recobrimo as sementes condicionadas com os organismos isolados ou em associação. A microbiolização com *Trichoderma* spp. pelo condicionamento fisiológico controla os patógenos infectantes das sementes. *Trichoderma* spp. e *Bacillus subtilis* promovem o crescimento e desenvolvimento de plântulas de nabo forrageiro, sendo o recobrimento das sementes condicionadas na presença dos organismos o que proporciona maior crescimento de parte aérea de plântulas no campo.

**Palavras-chave:** condicionamento; germinação; polímero; sanidade

## Introduction

Microbiolization consists in the use of live organisms for the treatment of seeds with the objective of controlling diseases and/or promoting plant growth, favoring seed germination, seedling emergence and development, and grain and fruit production (Harman, 2000). This technique represents an alternative to the chemical treatment of seeds, which can cause damages such as poisoning and contamination, selection of resistant strains, imbalance in the soil microbial population, reduction of biodiversity and elimination of beneficial organisms responsible for the cycling of nutrients.

Among the organisms that stand out in microbiolization are fungi of the genus *Trichoderma* and bacteria of the genus *Bacillus*. *Trichoderma* spp. works as biocontrol agent in several pathologic systems (Carvalho et al., 2011; Figueirêdo et al., 2010; Vinale et al., 2008). Microbiolization enhances plant growth and development, nutrient availability, agricultural production, and induces disease resistance (Jegathambigai et al., 2010). In addition, *B. subtilis* also plays a role in the control of pathogens (Martins et al., 2013), and in the promotion of plant growth (Araújo, 2008; Araújo & Guerreiro, 2010; Junges et al., 2013).

Many microorganisms with biocontrol ability and growth promoters have been studied, as well as the technique used in their incorporation with the seeds (Harman, 2000). Araújo (2008) demonstrates that the formulation has a significant role in determining the final efficacy of the product based on rhizobacteria promoting plant growth.

In order to potentiate the effect of the microbiolization in seeds and the synergism of the benefits, the association of techniques such as physiological conditioning and seed filming is used. The physiological conditioning is based on the control of the speed of water imbibition by seeds, using osmotic solutions adjusted to water potentials that allow the onset of physiological processes before reaching the humidity necessary for cellular elongation and consequent radicle protrusion to occur (Bonome et al., 2006). Some researchers have used microbes in seeds. For example, Junges et al. (2013) saw that the integration of matrix conditioning with a biological treatment (*Bacillus*) is effective to increase the vigor of maize seeds. Furthermore, the use of polymer for filming provides good appearance, color and distribution of products on the seed surface, as demonstrated for soybean seeds in a study by Avelar et al. (2015).

This work aims to potentiate the effect of the microbiolization in forage turnip seeds with *Trichoderma* spp. and *Bacillus subtilis* through the techniques of physiological conditioning, suspension of biological structures and filming.

## Material and Methods

A batch of forage turnip seeds (*Raphanus sativus* L.) supplied by a rural producer from the municipality of Cruz Alta - RS, Brazil, was used. Before the determination of the adequate water potential and the application of the treatments, the seeds were disinfested in 1% sodium hypochlorite solution

for 1 minute, followed by 70% alcohol for 1 minute and three baths in distilled and sterilized water.

### Determination of adequate water potential for forage turnip seeds

Physiological conditioning was performed in petri dishes containing 50 mL of the BDA medium added of the mannitol solute in different water potentials (0.0 -0.6, -0.7, -0.8 and -0.9 MPa) (Coutinho et al., 2001). One hundred seeds were conditioned on the plates poured with the culture medium in the different potentials. Plates were incubated in growth chambers (12h light at 25°C) until root protrusion in a first seed. The seeds whose radicle was not visible were removed and dried in laboratory conditions for 48 hours, and after this, the germination test in paper roll was installed. Two hundred seeds were used per each potential tested, separated in eight replicates of 25 seeds. The paper rolls were kept in a germinator under 12h light at 25°C temperature for four days, when the first germination count was carried out and. At the tenth day, the percentage of normal and abnormal seedlings and dead seeds were determined.

### Treatment application

Different methods of microbiolization (water restriction, filming and suspension) on forage turnip seeds were evaluated, and two commercial biological products were tested, one based on the bacterial agent (*Bacillus subtilis*) (Rhizoliptus®) and another based on the fungic agent (*Trichoderma* spp.) (Agrotrich®).

Application of suspension of biological structures on seeds: Spores suspension of *Trichoderma* spp. was obtained from 10g of Agrotrich®plus placed in 100 ml of distilled and sterilized water, and 0.5 ml of the suspension was applied for each group of 100 seeds. For *Bacillus subtilis*, 0.5 ml of the commercial product was applied for each group of 100 seeds. After the microbiolization, the seeds were dried on filter paper under laboratory conditions for 48 h.

Physiological conditioning: PDA culture medium with mannitol at -0.7MPa, on which the organisms were harvested, was used for physiological conditioning. *Trichoderma* spp. was isolated from the Agrotrich® commercial product, and the plates were incubated for 10 days so that the fungus sporulated abundantly. *Bacillus subtilis* was harvested with 0.5 mL of the Rhizoliptus® commercial product on each plate, incubated for 48h for growth and bacterium development on the medium. In the joint microbiolization of the two organisms, 0.5 ml was applied to each 100 seeds of the product based on *Bacillus subtilis* and these were conditioned on plates containing *Trichoderma* spp. In each plate with the different organisms, 100 pre-disinfested turnip seeds were incubated in a germinator with 12-h photophase at 25°C, until root protrusion in a first seed. The remaining seeds were removed from the medium and dried on filter paper under laboratory conditions for 48 h.

Filming: The Collor Seed® He Red polymer was used for filming, following the manufacturer's recommendation, i.e. 300 mL of the product for each 100 kg of seeds. For each 100 seeds, a solution was prepared for treatment by adding the amount of polymer corresponding to the weight of the sample

in 0.5 mL of *Trichoderma* spp. or of the Rhizoliptus® product. After that, the seeds were dried on filter paper under laboratory conditions for 48 h.

Untreated seeds: seeds were disinfested in 1% sodium hypochlorite solution for 1 minute, followed by 70% alcohol for 1 minute and three baths in distilled and sterilized water. Soon after, the seeds were dried in laboratory conditions for 48 h.

Chemical treatment: The fungicide Captan SC (120 i.a.g/100 kg seeds) diluted in 0.5 mL treatment solution for 100 seed was used.

Nine microbiolization treatments were used: T1: Physiological conditioning + *Trichoderma* spp.; T2: Filming + *Trichoderma* spp.; T3: Spore suspension of *Trichoderma* spp.; T4: Physiological conditioning + *Trichoderma* spp. followed by filming; T5: Physiological conditioning + *Bacillus subtilis*; T6: Filming + *Bacillus subtilis*; T7: Suspension of *Bacillus subtilis* bacterial cells; T8: Physiological conditioning + *Bacillus subtilis* followed by filming; T9: Physiological conditioning + *Trichoderma* spp. and *Bacillus subtilis* followed by filming; T10: Chemical treatment (positive control); and T11: untreated seeds (negative control).

### Treatment evaluation

In order to evaluate the performance of seeds submitted to the different treatments, sanity, germination, emergence speed index, growth and seedling development were measured in greenhouse and in the field.

For the sanity, 200 seeds were used per treatment, divided into eight replicates of 25 seeds. These seeds were placed in gerbox boxes previously disinfested with 70% alcohol and 1% sodium hypochlorite containing two sheets of sterile filter paper moistened with herbicide 2,4-D at 0.5% to inhibit germination. Seeds were incubated at under a 12-h photophase at 25°C for five days and analyzed under stereoscopic and optical microscopes to observe the morphological structures of the fungi, which were identified at the genus level with the aid of specialized bibliography (Barnet & Hunter, 1972), determining the percentage of incidence of each fungal genus.

For the germination tests, 200 seeds divided into eight replicates of 25 seeds sown in roll of filter paper moistened with distilled water in the proportion of 2.5 times the dry weight of the paper were used per treatment. The rolls containing the seeds were kept in a growth chamber with 12-h photophase at 25°C. Two counts were performed at four and ten days according to the Rules for Seed Analysis (Brasil, 2009), and the normal seedlings of each replicate were evaluated at the first count.

Ten seedlings were randomly selected from the germination test and had the length of the shoot and of the root measured. Due to the low volume, the total average dry mass was determined in an oven at 60°C for 48 h. At the 10th day of incubation, seedlings were classified as strong, weak or abnormal, or dead seeds, and the percentage of germination was obtained in each treatment according to the Rules for Seed Analysis (Brasil, 2009).

An experiment was carried out to evaluate the seedlings in a greenhouse under daily irrigation. Four replicates of five seeds were seeded in plastic cups containing 60 g of commercial substrate (Carolina Soil®). A completely randomized design was used and the evaluation was performed 10 days after sowing. The width of the first leaf, diameter of the shoot, length of the root system and of the shoot and the dry mass of the root system and of the shoot were determined.

The determination of the emergence speed index (ESI) was performed in 200-cell alveolar trays, one seed per cell, using commercial substrate (Carolina Soil®). Emergence counts were performed in daily basis until a constant number was obtained and, thus, the emergence speed index could be determined. The occurrence of emergence corresponded to the rise of seedling cotyledons from the substrate level. The ESI was calculated for each repetition, summing the number of plants emerged each day and dividing this value by the number of days elapsed from the sowing, according to Maguire (1962). After emergence became constant, the percentage of emerged seedlings was determined.

The evaluation of seedling performance in the field was conducted in a completely randomized block design with four replications. For each plot, four cultivation lines 20 cm apart from each other were sown, and evaluations were performed in the two central lines. Untreated seeds were sown at the margins, and the spacing between plants was 5 cm, and sowing was done manually, placing two seeds per pit.

At the 18th day after sowing, the percentage of emergence followed by thinning was performed, leaving only one plant per pit. At the 28th day after sowing, seedling height was determined using a millimeter ruler, and the number of leaves per seedlings was counted. When the plants presented 50% of flowering (75 days after sowing), one of the central lines of each block was collected. The plants were sectioned at the shoot/root junction and the average dry mass of the plants was determined after oven dried at 60 °C until reaching constant weight.

Means of all variables were calculated and the analysis of variance was applied using F test with error probability of 5%, and the differences between the means were compared using the Scott-Knott test at the error probability of 5%, using the statistical application SASM - Agri (Canteri et al., 2001).

## Results and Discussion

### Determination of the appropriate water potential for forage turnip seeds

Root protrusion of the first seed occurred 18h after incubation in PDA medium at -0.6 MPa, 22h after incubation at -0.7 MPa, and 29h after incubation under -0.8 and -0.9 MPa. Water restriction from -0.8 MPa on was harmful to germination and to first germination count and increased the percentage of abnormal seedlings (Table 1). Although the lower potentials did not differ from one another and from the use of the culture medium without restrictor, the potential of -0.7 MPa presented higher absolute values than the others in all analyzed variables. Therefore, this was the potential used for conditioning the seeds in culture medium. Other researchers have observed that



**Table 1.** Means (%) of normal seedlings at the fourth day (NS 4d), normal seedlings at the tenth day (NS 10d), dead seeds (DS) and abnormal seedlings (AS) of forage turnip seeds conditioned in PDA culture medium with mannitol in different water potentials.

Treatments (MPa)	NS 4d	NS 10d	DS	AS
0.0	86 a*	88 a	0 a	8 b
-0.6	85 a	85 a	3 a	11 b
-0.7	87 a	92 a	1 a	7 b
-0.8	58 b	63 b	10 a	26 a
-0.9	57 b	62 b	6 a	30 a
CV (%)	8	9	2	46

\* Means with the same letter in the column do not differ from each other according to the Scott-Knott test at error probability of 5%.

more negative osmotic potentials reduce germination of seeds in corn and beans (Junges et al., 2013; 2014; 2015).

### Treatment evaluation

All microbiolization methods had similar efficiency of colonization by *Trichoderma* spp. The use of water restriction potentiated the effect of *Trichoderma* spp. in the control of *Fusarium* sp. and *Alternaria* sp., and incidence of pathogens did not occur when this form of microbiolization was performed (Table 2). However, the use of other technique than water restriction for *Trichoderma* spp. either favored or did not affect the incidence of *Fusarium* sp. Carvalho et al. (2011) used spore suspension as a form of microbiolization with *Trichoderma* spp. and obtained a reduction of up to 51% in the incidence of *Fusarium oxysporum* in bean seeds. Ethur et al. (2006) used a powdered product based on *Trichoderma* spp. and obtained 100% control of all fungi associated with forage turnip seeds and demonstrated that the organism used in seed treatment was able to colonize the soil of the seedling rhizosphere. Moreover, *T. harzianum* acts as an inducer of resistance, extending its effect throughout the crop cycle (Pedro et al., 2012; Vinale et al., 2014).

Regardless of the technique used in the microbiolization with *B. subtilis*, there was no control of *Fusarium* sp.; treatments either favored or did not interfere on its incidence, and promotion of the incidence of *Alternaria* sp. was also observed (Table 2). Although the use of *B. subtilis* in the treatment of seeds was not efficient to control the associated pathogens, the benefits may be observed in later moments of the cycle of the culture, since several authors have reported the action of *B. subtilis* as inducer of responses of resistance to diseases (Lahlali et al., 2013; Sbalcheiro et al., 2009).

**Table 2.** Incidence (%) of *Trichoderma* spp. (TRI), *Fusarium* sp. (FUS), *Alternaria* sp. (ALT) in forage turnip seeds submitted to different treatments.

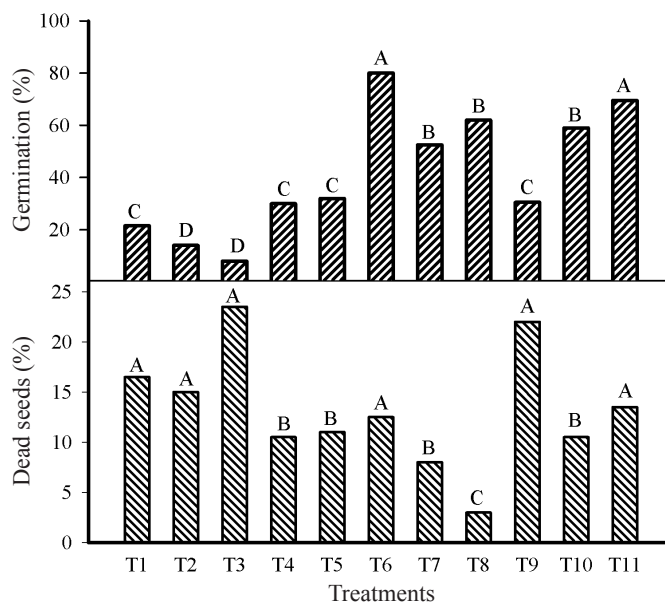
Treatments	TRI	FUS	ALT
<i>Trichoderma</i> spp. + water restriction	100 a*	0 c	0 c
<i>Trichoderma</i> spp. + filming	100 a	12 a	0 c
Spore suspension of <i>Trichoderma</i> spp.	98 a	15 a	1 c
<i>Trichoderma</i> spp. + water restriction + filming	100 a	0 c	0 c
<i>B. subtilis</i> + water restriction	1 c	9 a	23 a
<i>B. subtilis</i> + filming	0 c	7 a	12 b
Spore suspension of <i>B. subtilis</i>	0 c	2 b	9 b
<i>B. subtilis</i> + water restriction + filming	0 c	10 a	21 a
<i>Trichoderma</i> spp. + <i>B. subtilis</i> + water restriction + filming	100 a	0 c	0 c
Chemical treatment	0 c	2 b	1 c
No treatment	19 b	5 b	1 c
CV (%)	7	56	60

\* Means with the same letter in the column do not differ from each other according to the Scott-Knott test at error probability of 5%.

In the evaluation of germination, microbiolization with *B. subtilis* had better results than with *Trichoderma* spp., which was favored by the use of filming (Figure 1). The other treatments applied to forage turnip seeds harmed the germination; *Trichoderma* spp. was the most harmful. The use of spore suspension did not reach 10% of germination. This method associated with filming of the conditioned seeds in the presence of the two organisms caused mortality of more than 20% of the seeds. The conditions to which the germination rolls are exposed, i.e. high humidity and temperature, induce the saprophytic action of *Trichoderma* spp., which ultimately compromises germination. Filming of conditioned seeds in the presence of *Trichoderma* spp. and *B. subtilis* separately reduced seed mortality. Diniz et al. (2006) reported that the transmission of *Trichoderma viride* through the technique of filming promoted an increase in emergence and in the emergence speed index of lettuce seedlings.

Microbiolization with *B. subtilis* with the use of filming stimulated the vigor of the seedlings, resulting in higher values in the first count and higher percentage of strong seedlings, as well as promoted low interference on seedling abnormalities (Table 3). However, the other forms of microbiolization caused abnormalities in the seedlings. *Trichoderma* spp. impaired the vigor of the seedlings in all the methodologies used. Similarly, damages to the vigor of seedlings were observed with the use of water restriction associated with *B. subtilis*.

Microbiolization with *B. subtilis* associated to polymer promoted greater shoot and root growth as well as greater accumulation of dry mass (Figure 2). Beneficial effects on plant growth of *Bacillus subtilis* has been reported for corn plants (Araújo, 2008). Under the conditions of the paper roll test, the isolated use of the microbiolization techniques with *Trichoderma*



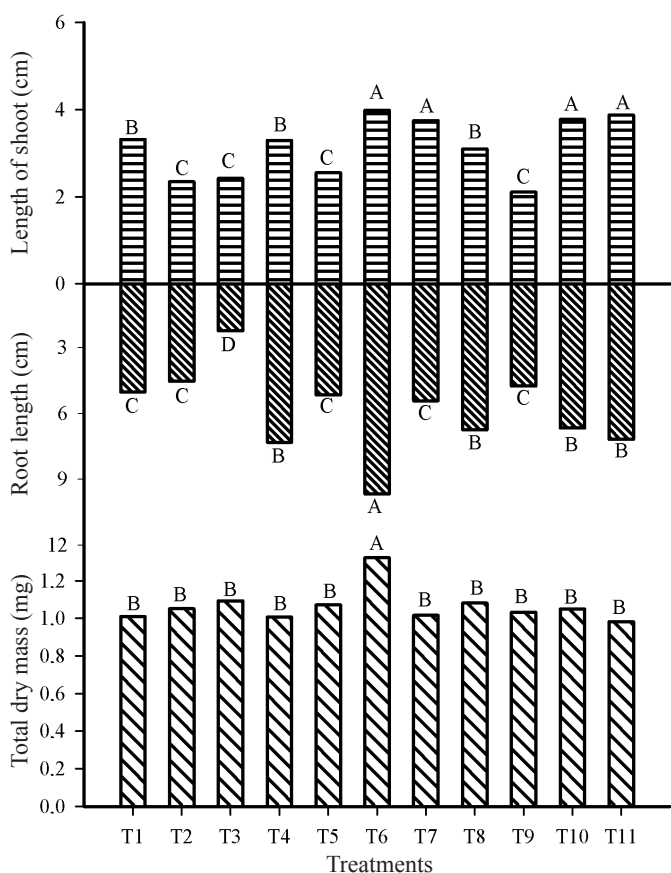
Treatments: T1: *Trichoderma* spp. + water restriction; T2: *Trichoderma* spp. + Filming; T3: Spore suspension of *Trichoderma* spp.; T4: *Trichoderma* spp. + water restriction + filming; T5: *Bacillus subtilis* + water restriction; T6: *Bacillus subtilis* + filming; T7: Suspension of *Bacillus subtilis* bacterial cells; T8: *Bacillus subtilis* + water restriction + filming; T9: *Trichoderma* spp. + *Bacillus subtilis* + water restriction + filming; T10: Chemical treatment; T11: No treatment. VC (%) germination: 28.7; Dead seeds: 37.6. Means with the same letter do not differ from each other according to the Scott-Knott test at error probability of 5%.

**Figure 1.** Percentage of dead seeds and germination in the germination test forage turnip seeds submitted to different treatments.

**Table 3.** Means (%) of the first count (FCO), normal seedlings (NOS), weak seedlings (WS) and abnormal seedlings (AS) of forage turnip seed submitted to different treatments.

Treatments	FCO	NOS	WS	AS
<i>Trichoderma</i> spp. + water restriction	20 c*	20 c	1 c	62 a
<i>Trichoderma</i> spp. + filming	14 d	14 d	0 c	71 a
Spore suspension of <i>Trichoderma</i> spp.	8 d	8 d	0 c	68 a
<i>Trichoderma</i> spp. + water restriction + filming	23 c	24 c	6 a	59 b
<i>B. subtilis</i> + water restriction	29 c	29 c	3 b	57 b
<i>B. subtilis</i> + filming	75 a	77 a	2 b	7 d
Spore suspension of <i>B. subtilis</i>	49 b	49 b	3 b	39 c
<i>B. subtilis</i> + water restriction + filming	55 b	57 b	5 b	35 c
<i>Trichoderma</i> spp. + <i>B. subtilis</i> + water restriction + filming	28 c	28 c	2 b	47 b
Chemical treatment	42 b	50 b	9 a	30 c
No treatment	55 b	59 b	10 a	17 d
CV (%)	28	28	80	28

\* Means with the same letter in the column do not differ from each other according to the Scott-Knott test at error probability of 5%.



VC (%) root length: 22.1; shoot length: 14.6; total dry mass: 18.1.

**Figure 2.** Root length, aerial part and total dry mass of seedlings of the germination test of radish seeds submitted to different treatments.

spp. affected the growth of the aerial part as well as of the root system of the forage turnip seedlings. However, the filming of the conditioned seeds has been observed to reduce the damage to the plant root system in other crops such as corn. The association of water restriction with filming for microbiolization with *Trichoderma* spp. led to control of pathogens associated with the seeds and maintained the seedling vigor (Junges et al., 2014).

The deleterious behavior of the microbiolization observed in the variables measured in the germination test in paper roll was not reproduced in the cultivation in substrate under greenhouse conditions (Table 4). Under these conditions, microbiolization

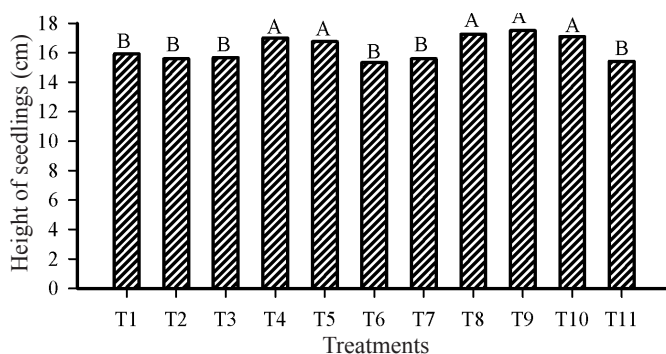
promoted greater length of the root system and width of the first leaf (Figure 3), giving evidence of the persistent benefits even regardless of seed reserves. Some treatments reduced the emergence speed index, but there was no reflection on the percentage of emerged seedlings. No effect of treatments on leaf number, shoot diameter, shoot length and accumulation of dry mass was observed. Ethur et al. (2006) observed that *Trichoderma* spp. did not influence the percentage of emerged seedlings and the speed of emergence of turnip seedlings. However, the seedlings produced from seeds treated with the fungus, associated or not to the chemical treatment, presented greater height.

The use of seed microbiolization techniques with *Trichoderma* spp. and *B. subtilis* confirmed the action of these organisms as growth promoters of forage turnip seedlings, producing seedlings with greater shoot length (Figure 3). The rapid initial growth of turnip seedlings favors the closure of the canopy, a characteristic of vital importance because this is a plant with a soil cover purpose. The best performances were observed with filming conditioned seeds in the presence of the organisms either separately or associated, opposing the results of Hölbjg et al. (2011) who observed that the use of films in filming tests with hydroponic onion seeds impaired the vigor of seedlings. Similarly, a beneficial effect of physiological conditioning was also observed in the isolated microbiolization of *B. subtilis*, leading to a similar performance to that of chemical treatment. However, no difference between treatments was observed in the evaluation of emergence, number of leaves and dry mass of plants.

**Table 4.** Mean of root length (MRL), total length (TL), width of the first true leaf (WTL), emergence speed index (ESI) of seedlings from forage turnip seed submitted to different treatments in greenhouse.

Treatments	MRL	TL	WTL	ESI
<i>Trichoderma</i> spp. + water restriction	34 a*	46 a	3 b	6 a
<i>Trichoderma</i> spp. + filming	33 a	44 a	3 b	5 b
Spore suspension of <i>Trichoderma</i> spp.	38 a	48 a	3 b	5 b
<i>Trichoderma</i> spp. + water restriction + filming	28 b	40 b	3 b	5 a
<i>B. subtilis</i> + water restriction	32 a	44 a	4 b	6 a
<i>B. subtilis</i> + filming	34 a	45 a	4 b	5 a
Spore suspension of <i>B. subtilis</i>	28 b	39 b	3 b	5 b
<i>B. subtilis</i> + water restriction + filming	32 a	43 a	4 b	6 a
<i>Trichoderma</i> spp. + <i>B. subtilis</i> + water restriction + filming	25 b	36 b	5 a	6 a
Chemical treatment	29 b	41 b	3 b	6 b
No treatment	22 b	33 b	3 b	6 a
CV (%)	17	13	15	7

\* Means with the same letter in the column do not differ from each other according to the Scott-Knott test at error probability of 5%.



VC (%) mean height of seedlings: 7.6.

**Figure 3.** Mean height of forage turnip seedlings from seeds submitted to different treatments conducted in the field.

## Conclusions

*Trichoderma* spp. microbiolized by water restriction controls *Fusarium* sp. and *Alternaria* sp. associated with forage turnip seeds.

*Trichoderma* spp. and *Bacillus subtilis* promote the growth and development of turnip seedlings.

Microbiolization of forage turnip seeds with suspension of biological structures does not express the potential of organisms.

The use of filming in the microbiolization of *B. subtilis* produces improvements in the growth and accumulation of dry mass of seedlings.

The filming of conditioned seeds in the presence of *Bacillus subtilis* and *Trichoderma* spp. stimulates the growth of shoots of seedlings in the field.

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