

SANITARY AND PHYSIOLOGICAL QUALITY OF BEAN SEEDS TREATED WITH *Trichoderma* spp.

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ABSTRACT - The presence of pathogens associated in the bean seeds can represent great risks for the development of the crop. Thus, the seed treatment is essential, and the use of antagonistic microorganisms is an effective tool for the control of various pathogens. Therefore, the objective of the study was to evaluate the sanitary and physiological quality of bean seeds treated with spore suspension and culture filtrates of *Trichoderma* spp. and the effect of the antagonist on the health quality of bean seeds inoculated with *Fusarium oxysporum* f. sp. *phaseoli* and *Macrophomina phaseolina*. For this, five *Trichoderma* spp. isolates were used in the form of culture filtrate and spore suspension. Sanity tests were carried out, sanity with inoculation of *F. oxysporum* f. sp. *phaseoli* and *M. phaseolina* and germination test by the paper roll incubation method. The spore suspension of *Trichoderma* spp. obtained the highest percentage of reduction in the incidence of the pathogen in relation to the culture filtrates, in the seed health tests. Culture filtrates of isolates Eco, TR4 and SC were the most promising ones in relation to the analysis of growth and dry mass of seedlings.

Keywords: biological control, *Fusarium oxysporum*, *Macrophomina phaseolina*, *Phaseolus vulgaris*.

QUALIDADE SANITÁRIA E FISIOLÓGICA DE SEMENTES DE FEIJÃO TRATADAS COM *Trichoderma* spp.

RESUMO - A presença de patógenos associados às sementes de feijão pode representar grandes riscos para o desenvolvimento da cultura. Dessa forma, o tratamento de sementes é indispensável, e a utilização de micro-organismos antagonistas uma ferramenta efetiva para o controle de diversos patógenos. Portanto, o objetivo do trabalho foi avaliar a qualidade sanitária e fisiológica de sementes de feijão tratadas com suspensão de esporos e filtrados de cultura de *Trichoderma* spp. e o efeito do antagonista sobre a qualidade sanitária quando inoculadas com *Fusarium oxysporum* f. sp. *phaseoli* e *Macrophomina phaseolina*. Para isso, foram utilizados cinco isolados de *Trichoderma* spp., sob a forma de filtrado de cultura e suspensão de esporos. Foram realizados os testes de sanidade, sanidade com inoculação de *F. oxysporum* f. sp. *phaseoli* e *M. phaseolina* e teste de germinação pelo método de incubação em rolo de papel. A suspensão de esporos de *Trichoderma* spp. obteve maior percentual de redução da incidência do patógeno em relação aos filtrados de cultura, nos testes de sanidade de sementes. Filtrados de cultura dos isolados Eco, TR4 e SC foram os mais promissores em relação às análises de crescimento e massa seca das plântulas.

Palavras-chave: controle biológico, *Fusarium oxysporum*, *Macrophomina phaseolina*, *Phaseolus vulgaris*.

INTRODUCTION

The sanitary quality of the seeds is a key point for the installation of the culture in the field, since pathogens associated with the seeds can interfere in their development and in the production process. Bean seeds can serve as an inoculum for the survival and spread of various pathogens. According to a study developed by Mambrin et al. (2015), *Fusarium* sp. and *Macrophomina phaseolina* were the predominant pathogens when the health of bean seeds was evaluated. Both are soil-dwelling pathogens and are characterized by difficulty in controlling them, especially as they produce survival structures, which keep them viable under adverse conditions.

The use of seed treatment becomes an indispensable practice for the phytosanitary management of

the crop, since the presence of pathogens in the seeds can act in the dispersion of more aggressive races, not yet existing in the area, or else the introduction of pathogens already in the early stages of plant development (OOTANI et al., 2016). The use of fungicides or biocontrol agents in seed treatment helps eliminate pathogens, protecting the crop against pre- and post-emergence diseases and ensuring an effective establishment (CARVALHO et al., 2011).

The genus *Trichoderma* is recognized for its ability to parasitize phytopathogens, in addition to inducing defense mechanisms in plants, so a wide range of species is used as commercial biofungicides (MUKHERJEE et al., 2013). There are also reports of the antagonist promoting growth in different cultures (CARVALHO et al., 2011; CHAGAS et al., 2017). Several studies have reported the

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efficiency of using *Trichoderma* spp. in the control of soil pathogens in the common bean crop (CARVALHO et al., 2014; ARAUJO et al., 2019). However, microorganisms can also be cultured in a liquid medium through fermentation processes to induce them to produce substances and enzymes that can be used to control phytopathogens and induce resistance in plants (CHEN et al., 2019).

Thus, the objective of this work was to evaluate the sanitary and physiological quality of bean seeds treated with spore suspension and culture filtrates of *Trichoderma* spp. and the effect of the antagonist on the sanitary quality of

bean seeds inoculated with *Fusarium oxysporum* f. sp. *phaseoli* and *Macrophomina phaseolina*.

MATERIAL AND METHODS

The works were carried out at the Laboratory of Phytopathology Elocy Minussi, Department of Plant Protection, Federal University of Santa Maria (UFSM). The phytopathogenic and antagonistic isolates used in the experiments are described in terms of species, code, *GenBank* registration and origin in Table 1. Bean seeds of the cultivar Fepagro Triunfo, provided by Fepagro de Maquiné/RS state, were used for all tests.

TABLE 1 - Description of isolates from *Macrophomina phaseolina*, *Fusarium oxysporum* and *Trichoderma* spp. regarding code, *GenBank* registration and origin.

Isolated	Code	<i>GenBank</i> record	Source
<i>Macrophomina phaseolina</i>	PEL	MK450343	Federal University of Pelotas BRM 28134- Collection of Multifunctional and Phytopathogenic Microorganisms from Embrapa – Rice and beans
<i>Fusarium oxysporum</i> f. sp. <i>phaseoli</i>	Fop 101	-	Bean rhizospheric soil- Roque Gonzales/RS state
<i>Trichoderma virens</i>	TF1	MK450344	Stimu Control commercial product
<i>Trichoderma harzianum</i>	SC	-	Ecotrich commercial product
<i>Trichoderma harzianum</i>	ECO	-	Pecan rhizospheric soil
<i>Trichoderma asperellum</i>	TR1	MK982653	Pecan rhizospheric soil
<i>Trichoderma asperellum</i>	TR4	MN082152	Pecan rhizospheric soil

Culture filtrates of *Trichoderma* spp.

For the production of culture filtrates, submerged fermentations were carried out separately for each isolate of *Trichoderma* spp. It was carried out a study of the isolates of *Trichoderma* spp. in 250 mL Erlenmeyers containing liquid culture medium, composed of 100 mL of distilled water, 7.5 g L⁻¹ of yeast extract, 20 g L⁻¹ of corn steep liquor, 100 g L⁻¹ of sucrose and pH adjusted to 5.0. Then, the media were autoclaved at 120°C for 20 min. and after reaching room temperature, 1 mL of the spore suspension of each isolate was added at a concentration of 10⁷ spores mL⁻¹. Subsequently, the Erlenmeyers were incubated in a Shaker (Tecnal® shakermodel TE-4200), at 28°C, for 96 h, under constant agitation at 180 rpm. After the incubation period, the culture media were first filtered through an 11 µ millipore® membrane, with the aid of a vacuum pump, removing the microbial biomass. Next, a second filtration of the media was made in a millipore® membrane of 0.22 µ, to remove the fungal cells.

Seed health test

The seed health test was performed using 11 conditions, namely: untreated seeds, seeds treated with viable spore suspension (S) of five isolates of *Trichoderma* spp. (10⁷ spores mL⁻¹) and seeds treated with culture filtrate (F) of five isolates of *Trichoderma* spp. For every 200 seeds, was used 1 mL of the spore suspension or culture filtrate of *Trichoderma* spp.

To evaluate the health of the seeds, the filter paper method (“blotter test”) was used. In order to do this, 100 seeds were used, which were distributed in four plastic boxes (11 x 11 x 3.5 cm) previously disinfected with 70%

alcohol. Three sheets of sterilized filter paper were placed in each box, moistened with a 10% agar-water solution in the proportion of three times the dry mass of the paper. Twenty five seeds were deposited on the paper, then incubated in an environment with a temperature of 25°C and a photoperiod of 12 h. After seven days of incubation, the seeds were individually examined under a stereoscopic microscope and, when necessary, slides were made for the visualization and identification under an optical microscope. The result was expressed as a percentage of incidence of each fungus detected for each condition.

Sanity test of seeds inoculated with *Fusarium oxysporum*, f. sp. *phaseoli* and *Macrophomina phaseolina*

The treatments used were the same as in the previous sanity test, plus the inoculation of the seeds with the pathogens, and the control treatment received only the inoculation of the pathogen. Each pathogenic isolate was transferred to Petri dishes, in potato-dextrose-agar (PDA) culture medium osmotically modified, by adding mannitol to the medium, in order to reach an osmotic potential equal to -0.7 MPa, and incubated for 11 days.

The amount of mannitol used in g L⁻¹ of PDA medium was based on the study by Coutinho et al. (2001). Before inoculation of the pathogens, the seeds were disinfected in a bath of 70% alcohol, 1% sodium hypochlorite and two baths of distilled water and sterilized. Both for *M. phaseolina* and for *F. oxysporum* f. sp. *phaseoli*, inoculation was performed via direct contact of the seed with the pathogen for 48 h.

After the period of contact between seed and pathogen, the seeds were treated, separately, with the

suspension of spores or culture filtrates of the isolates of *Trichoderma* spp. and then the test was installed. The methodology used for seed installation, incubation and evaluation was the same as in the previous sanity test.

Germination test by paper roll incubation method

For the seed germination test, 11 conditions were used, namely: non-disinfested seeds, seeds treated with viable spore suspension of five isolates of *Trichoderma* spp. (10^7 spores mL^{-1}) and seeds treated with culture filtrate of 5 *Trichoderma* spp. For every 200 seeds, 1 mL of the spore suspension or culture filtrate of *Trichoderma* spp. For each treatment, 200 seeds were used, divided into four replications of 50 seeds.

Filter paper was used as substrate, with two sheets placed on the base and one on the cover, previously moistened with distilled and sterilized water in the proportion of 2.5 times the dry biomass of the paper. The seeds were sown on the two leaves at the base and then covered with a leaf, rolls were made and placed in plastic bags, which were later incubated in BOD at 25°C and 12 h photoperiod. On the fifth day, the percentage of normal seedlings in the first count and final germination on the 9th day was counted (BRASIL, 2009). In the first germination

count, ten normal seedlings were randomly chosen from the upper third of the sheet of paper of each repetition and the length of the shoot and root was measured by separating the parts with a cut in the hypocotyl/radicle axis. Then, each part was placed separately in paper bags and taken to a drying oven at 65°C for 48 h.

The design used was completely randomized, with four replications per treatment. The data were submitted to analysis of variance and the means compared by the Scott-Knot test ($p \leq 0.05$) using the statistical program Sisvar (FERREIRA, 2011).

RESULTS AND DISCUSSION

Seed health test

In the sanity test, it was possible to observe that the treatments using the spore suspension of the *Trichoderma* spp. were those with the lowest incidence of fungal genera (Figure 1). The fast growth of the antagonist on the pathogens is often favored by the stimulus of the host itself (HURMANN et al., 2020). A study reports that bean seeds treated with *Trichoderma harzianum* spore suspension inhibited the incidence of *Aspergillus*, *Cladosporium* and *Sclerotinia sclerotiorum* (CARVALHO et al., 2011).

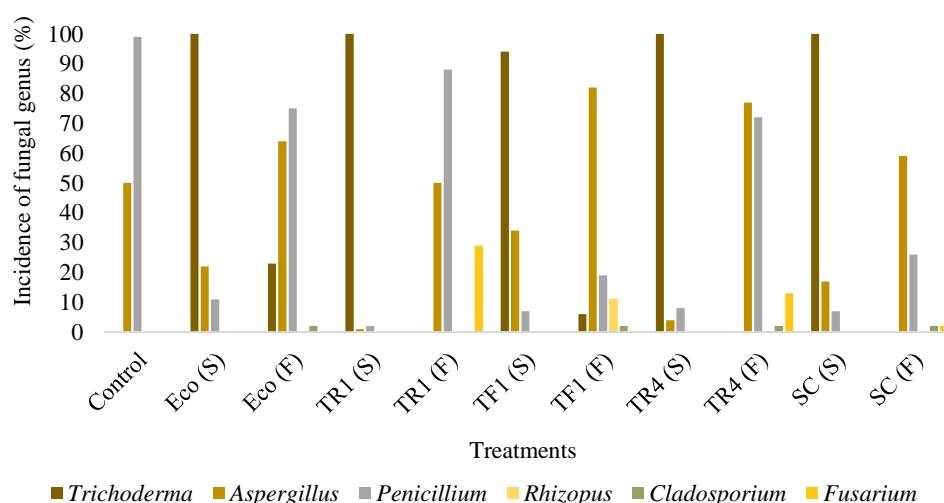


FIGURE 1 - Incidence of fungal genera (%) in bean seeds cultivar Fepagro Triunfo, submitted to the sanity test. S = spore suspension, F = culture filtrate.

For the other treatments, the genera *Penicillium* and *Aspergillus* presented higher incidence, being these characterized as storage fungi. *Penicillium* and *Aspergillus* are genera commonly found in bean seed health tests, with potential to produce mycotoxins (AMARAL et al., 2013). These fungal genera were also identified in a health test of caupi bean (*Vigna unguiculata*) seeds, and when microbiolized with a suspension of *Trichoderma* sp. showed a reduction in the percentage of incidence of these fungi (DE SÁ et al., 2019). The presence of fungi of the genus *Aspergillus* sp. and *Penicillium* sp. in addition to depreciating the quality of the seeds by reducing the germination power, affecting the viability of the commercial and nutritional value of the seeds, they can also

produce mycotoxins, substances toxic to both humans and animals (NASCIMENTO et al., 2012).

Sanity test of seeds inoculated with *Fusarium oxysporum* f. sp. *phaseoli* and *Macrophomina phaseolina*

In the sanity test in which the bean seeds were inoculated with *F. oxysporum* f. sp. *phaseoli*, treatments with spore suspension of *Trichoderma* spp. reduced, on average, 7% the incidence of the pathogen, however, treatments Eco (S) and TR4 (S) showed the greatest reductions in incidence, 17 and 16%, respectively (Figure 2). The percentage of reduction in the incidence of the pathogen found at work, especially in SC and Eco treatments, derived from commercial formulations, may be

associated with the lower concentration used at work when compared to the commercial package insert. In the work developed by Carvalho et al. (2014), the spore suspension of *T. harzianum* isolates (2.5×10^8 spores mL⁻¹) significantly reduced (40 and 31%) the incidence of *F. oxysporum* from bean seeds in relation to the control, highlighting the other isolates tested. Regarding the cultivar used, there is no information on resistance to soil pathogens, such as *F. oxysporum* and *M. phaseolina*, therefore, intrinsic

characteristics of the cultivar may be acting on resistance or susceptibility to the pathogen and its incidence.

In treatments with *Trichoderma* spp. culture filtrates, incidence of the pathogen was observed in all seeds, however, with lower growth intensity on them (Figure 3). However, the study developed by Guimarães et al. (2014) observed that both bean seed treatment with conidia suspension and *T. harzianum* autoclaved filtrate were effective in reducing the population of *Cladosporium herbarum*.

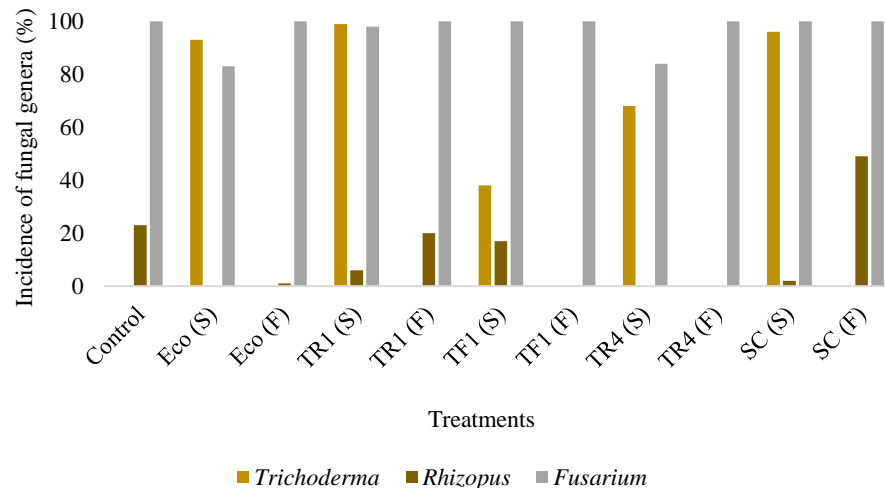


FIGURE 2 - Incidence of fungal genera (%) in beans, cultivar Fepagro Triunfo, inoculated with *Fusarium oxysporum* f. sp. *phaseoli* and submitted to the sanity test. S = spore suspension, F = culture filtrate.

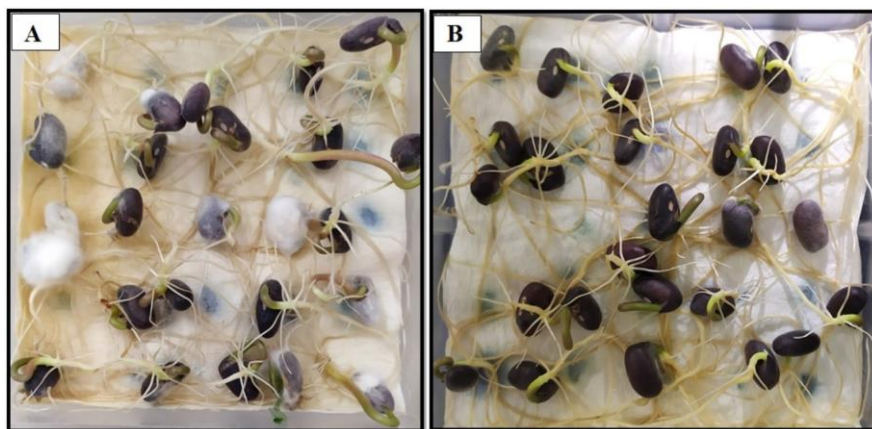


FIGURE 3 - Health test of common bean seeds, cultivar Fepagro Triunfo, inoculated with *Fusarium oxysporum* f. sp. *phaseoli*, control treatment (A), treatment with *Trichoderma virens* culture filtrate (TF1) (B).

For the sanity test of seeds in which *Macrophomina phaseolina* was inoculated, it was observed that treatments with the suspension of spores of *Trichoderma* spp. were the ones that mostly reduced the incidence of the pathogen in the seeds, with emphasis on treatments Eco (S) and TR4 (S), with 93 and 92% reduction in incidence (Figure 4). This fact may have occurred due to the rapid growth of *Trichoderma* spp. isolates, as their mycelia completely covered the seed in some treatments (Figure 5).

In the same way as for the test with *Fusarium oxysporum* f. sp. *phaseoli*, although in the treatments with culture filtrate the incidence of the pathogen was similar to that of the control, with an average reduction of 0.6% of the incidence, it was observed less intensity of growth of the pathogen on the seed. The fact that *Trichoderma*, in the form of a living organism, rapidly colonizes the seed, occupying spaces that would belong to the pathogen, and the production of volatile metabolites is more related to the biocontrol of pathogens in seeds, justifies the use of non-volatile metabolites (represented by the culture filtrates) to

obtain less satisfactory results (CARVALHO et al., 2014). According to the authors, there are no reports of non-

volatile metabolites as a key mechanism in the biological control of seed-borne pathogens.

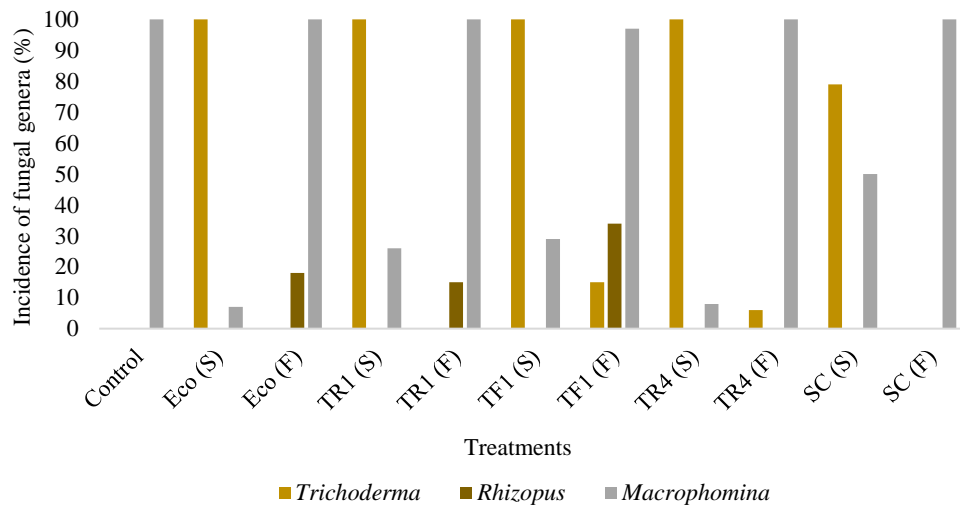


FIGURE 4 - Incidence of fungal genera (%) in beans, cultivar Fepagro Triunfo, inoculated with *Macrophomina phaseolina* and submitted to the sanity test. S = spore suspension, F = culture filtrate.

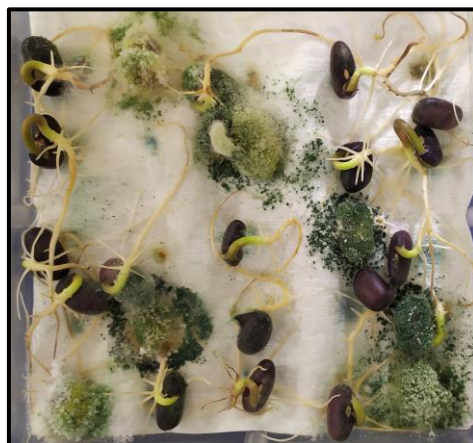


FIGURE 5 - Health test of common bean seeds, cultivar Fepagro Triunfo, treated with spore suspension of *Trichoderma asperellum* (TR4) and inoculated with *Macrophomina phaseolina*.

Thus, it is necessary to study measures that favor the action of antagonists, whether by suspension of spores or by culture filtrate, to be more effective in the control of pathogens transmitted by seeds, so that their physiological quality can act in a different way, according to its full capacity, and pathogens do not become a hindrance to this. In addition, an effective seed treatment ensures a more homogeneous plant stand in the field, ensuring better competitiveness with weeds, in addition to keeping the plants healthy for a longer period of the cycle, allowing greater productive gain in the crop.

Germination test

The increment of germinated seeds between the evaluation of the first germination count and the germination was not verified, therefore, only the values of the first count were displayed. Regarding the germination data of bean seeds, it was observed that the Eco (S) treatment had a negative effect, significantly different from

the control and the other *Trichoderma* isolates (Table 2). In a study developed by Marques et al. (2014), observed that isolates of *Trichoderma* spp. helped in the germination of bean seeds, interfering in the speed of germination.

For the shoot length, only treatments Eco (S), Eco (F), TR1 (S), TR4 (F) and SC (F) showed a positive effect, different from the control. In terms of root length, only treatments Eco (S), TR1 (S), TR1 (F), TR4 (S) and TF1 (S) differed from the control, showing a negative effect. A study carried out by De Sá et al. (2019) identified that microbiolization with *Trichoderma* sp. in caupi bean (*Vigna unguiculata*) seeds also promoted increases in radicle length. According to Zhao et al. (2020) the siderophores produced by *Trichoderma asperellum* are able to increase the indole acetic acid (IAA) content of *Arabidopsis* roots, contributing to the conversion of poorly soluble iron and promoting plant growth. Auxin in association with cytokinin are phytohormones responsible for regulating plant growth and development (SCHALLER et al., 2015).

For shoot dry biomass, only treatments Eco (F), TR4 (F) and SC (F) had a positive effect on the control. In the radicle dry mass, Eco (F), TR1 (F) and TR4 (F) were superior to the control. The use of conidia suspension or

autoclaved filtrate of *T. harzianum* were also effective in the accumulation of biomass in common bean seedlings (GUIMARÃES et al., 2014).

TABLE 2 - First germination count (FG), shoot length (SL), radicle length (RL), shoot dry biomass (SDB) and radicle dry biomass (RDB) of common bean seedlings submitted to treatment of seeds with *Trichoderma* spp.

Treatments	FG (%)	SL (cm)	RL (cm)	SDB (g)	RDB (g)
Control	97 a*	5.50 b	13.02 a	0.29 b	0.17 b
Eco (S)	79 c	5.70 a	9.76 c	0.29 b	0.14 c
Eco (F)	95 b	5.83 a	14.57 a	0.34 a	0.19 a
TR1 (S)	95 b	5.95 a	11.82 b	0.30 b	0.17 b
TR1 (F)	98 a	4.93 c	11.91 b	0.30 b	0.18 a
TR4 (S)	98 a	4.46 c	11.42 b	0.20 d	0.12 c
TR4 (F)	99 a	5.88 a	14.82 a	0.32 a	0.20 a
SC (S)	97 a	5.35 b	14.38 a	0.28 b	0.16 b
SC (F)	98 a	6.02 a	14.54 a	0.36 a	0.19 a
TF1 (S)	91 b	5.42 b	12.66 b	0.25 c	0.16 b
TF1 (F)	98 a	4.93 c	14.19 a	0.29 b	0.20 a
CV(%)	7.24 ¹	6.23	8.27	8.24	8.41

*Means followed by the same letter in the column do not differ from each other, by the Scott-Knott test, at 5% error probability.

¹Data transformed by Box-Cox ($\lambda = 2.5$). CV = coefficient of variation, S = spore solution, F = culture filtrate.

In general, each *Trichoderma* isolate and its form, spore suspension or culture filtrate, acted in different ways on each of the analyses. However, it is worth mentioning that the treatments using the culture filtrates of the isolates Eco, TR4 and SC were the most promising in the development of the seedlings, under the conditions and concentrations used in the study. While the spore suspension of *Trichoderma* spp. guaranteed greater effect on the inhibition of the pathogen in the sanity tests. However, it is necessary to carry out studies using different concentrations of mL⁻¹ husband and growth conditions of the antagonist, in order to observe its effects in relation to the control of the incidence of pathogens in the seeds and in the development of the seedlings.

CONCLUSIONS

The spore suspension of *Trichoderma* spp. obtained the highest percentage of reduction in the incidence of the pathogen in relation to the culture filtrates, in the seed health tests.

Culture filtrates of isolates Eco, TR4 and SC were the most promising in relation to the analysis of seedling growth and dry biomass.

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