



Efficiency of application of *Trichoderma* on the physiological quality and health of cowpea seeds

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Abstract

One of the main causes of low productivity of cowpea crops in Brazil is the use of low quality seeds. Low quality seeds have a high degree of infestation by phytopathogens, mainly fungi. Seed treatment with biological control agents, such as fungi of the genus *Trichoderma*, is a useful alternative to reduce or inhibit the transmission of diseases via seeds. In view of the above, this study aims to evaluate the physiological and sanitary quality of cowpea seeds treated with conidia of *Trichoderma* sp. (1.2 , 2.4 , 4.8 and 9.6×10^8 CFU g⁻¹). We evaluated the germination and the sanity of seeds, length of seedling radicles, and production of indole acetic acid (IAA) by fungi. The experimental design was completely randomized in all experiments. In general, there was a predominance of *Fusarium* spp. and *Rhizoctonia solani*. The treatment of seeds with the conidia of *Trichoderma* sp. was positive for the parameters related to germination and radicle length of cowpea beans up to the concentration 4.8×10^8 CFU g⁻¹. We conclude that the application of *Trichoderma* sp. was efficient in reducing the incidence of *Fusarium* spp., and promoted the germination and elongation of the radicle in cowpea.

Keywords: *Vigna unguiculata*, germination, fungus, indole acetic acid.

Introduction

In the state of Tocantins, cowpea has been cultivated mainly in tropical lowland areas. The planted area is approximately 3.5 thousand hectares, the annual production is 4.5 thousand tons, and there is a low productivity (SEAGRO, 2012). One of the main causes of such a low productivity of cowpea crops in Brazil is the use of low quality seeds (Rey et al., 2009). The increase in investments in technologies aiming to increase the productivity of cowpea in the region has led to an increase in the acceptance of cowpea by traders, agroindustries, distributors and consumers (Freire Filho et al., 2009; Dutra et al., 2012).

The best quality cowpea seeds are the

consequence of a positive interaction of genetic, environmental, physiological and, especially, health factors (Teixeira et al., 2012; Dutra et al., 2012). Low quality seeds have a high degree of infestation by phytopathogens, mainly fungi. They reduce germination and can disseminate, establishing primary foci of infection (Barbosa et al., 2011; Kobayashi & Pires, 2011; Dutra et al., 2012). In this context, seed health analysis is an important tool for providing information on the incidence of phytopathogens, and serves as the basis for the choice of control methods (Rey et al., 2009; Dutra et al. 2012).

Seed treatment with biological control agents, such as fungi of the genus *Trichoderma*,

is a useful alternative to reduce or inhibit the transmission of diseases via seeds (Corrêa et al., 2008; Harman, 2011; Oliveira et al., 2012). The fungi of the genus *Trichoderma* easily propagate. They are able to survive in unfavorable environments and act efficiently as biological control agents (Brotman et al., 2010; Mastouri et al., 2010; Machado et al., 2011). In addition, they are antagonists of several phytopathogen species, in addition to promoting improvements in plant growth and defense against pathogen populations in different soil types without causing a negative impact on the environment (Vinale et al., 2008; Silva et al., 2011; Zhang et al., 2012).

In view of the above, this study aims to evaluate the physiological and sanitary quality of cowpea seeds inoculated with different concentrations of conidia of *Trichoderma* sp.

Material and Methods

We used cowpea seeds (*Vigna unguiculata* (L.) Walp.) of the 'Vinagre' cultivar. Prior to the beginning of the tests, the seeds were treated with conidia of *Trichoderma* sp. at concentrations of 1.2, 2.4, 4.8 and 9.6×10^8 CFU g^{-1} . Adjustment of concentrations of *Trichoderma* sp. conidia was performed using a Neubauer chamber. The conidia of *Trichoderma* sp. were obtained from a mixture of isolates of different species of *Trichoderma* and *T. harzianum*. The control consisted of cowpea seeds not inoculated with conidia of *Trichoderma* sp.

For the germination and sanity test, the seeds were evenly distributed in Petri dishes containing sterile filter paper sheets moistened with sterile water. The plates were then randomly placed in an incubation chamber at 20.0 ± 2.0 °C and a 12-h photoperiod for seven days. The germination test was performed by counting the number of seeds that emitted a radicle after a period of 24 h, and the percentage of seeds germinated per treatment was calculated. For the evaluation of sanity, the fungi present in seeds were identified with a magnifying glass and an optical microscope. Then, the percentage of infected seeds with each fungal genus identified was determined. In addition, seedlings considered normal were evaluated as for radicle length using a digital pachymeter.

To determine the rate of germination and the rate of seed-seedling fungi transmission in each treatment, the cowpea seeds were seeded in plastic trays containing washed and sterilized sand (121 °C, 101 kPa; 1 h). The trays were kept in greenhouse conditions throughout the evaluation period. The germination speed index (GSI) was calculated according to Maguire (1962). The first count rate was determined based on the number of seedlings germinated in each treatment after two days of sowing, and the data were expressed as percentage.

The evaluation of transmission was performed through daily observations of symptomatic emerged seedlings. Seedlings with lesions were collected, and samples of infected plant tissue were transferred to Petri dishes containing PDA (potato-dextrose-agar) culture medium. The plates were incubated for seven days at 20.0 ± 2.0 °C with a 12-h photoperiod for observation of fungal structures as described by Sá et al. (2011). The identification of fungi was carried out using a stereoscopic and optical microscope and specialized literature. The fungi identified in the samples were inoculated into healthy cowpea seedlings for evaluation of Koch's postulates.

The production of indole acetic acid (IAA) was evaluated in the fungal isolates found in the sanity test. For this, the isolates were transferred to Petri dishes containing TSA (Tryptone Soy Agar) culture medium, covered with a nitrocellulose membrane, and incubated at 28.0 °C for 24 hours. After, the nitrocellulose membrane was withdrawn, transferred to another plate, and then moistened with Salkowski' solution (2% solution of 0.5 M ferric chloride in 35% perchloric acid; v/v). The presence of red halos in the nitrocellulose membrane, in an interval of 30 min to two hours, indicates the production of IAA by the isolates tested.

For the germination and sanity test, the experimental design was completely randomized with 10 replications, each replication consisting of a Petri dish containing 20 seeds. A completely randomized experimental design with four replicates was used to determine the germination speed index and the rate of seed-seedling fungi transmission. Each replicate comprised a plastic

tray containing 25 seeds. The percentage data were previously transformed into sine-arc $\sqrt{x} + 100$, and then submitted to statistical analysis. Regression analysis was used to evaluate the effects of different concentrations of conidia of *Trichoderma* sp. on the seeds. Data were submitted to analysis of variance (ANOVA) and Tukey test at 5% of probability using the statistical software ASSISTAT.

Results and Discussion

The sanitary analysis of cowpea beans treated with different concentrations of conidia of *Trichoderma* sp. did not show efficient results in relation to the control of fungi in the seeds (Table 1). The *Trichoderma* are able to inhibit phytopathogen growth mainly due to their fast growth and, consequently, a high ability to compete for space and nutrients and to produce inhibitory compounds (Siameto et al., 2010). In view of this, it is probable that, in this study, there was an inability of the fungus *Trichoderma* sp. to

compete with fungi recorded in the seeds and/or produce antifungal substances. The incidence of pathogenic fungi was recorded in all treatments, however the incidence of *Fusarium* spp. in the seeds treated with *Trichoderma* decreased when compared to the control (Table 1).

The genus *Fusarium* encompasses a range of widely distributed microorganisms that causes fusariosis in cowpea plants and can cause approximately 90% reductions in seed production in susceptible cultivars (Rodrigues et al., 2006; Silva et al., 2011; Teixeira et al., 2012). The use of resistant cultivars is recommended for the control of fusariosis in cowpea, but the difficulties in obtaining such cultivars encourage the search for viable alternatives to control the disease (Rodrigues et al., 2006; Teixeira et al., 2012). The use of antagonistic fungi for biological control, such as *Trichoderma* sp., may be a viable alternative for reducing the incidence of fungi of the genus *Fusarium* on cowpea.

Table 1. Sanitary analysis of cowpea seeds cv. 'Vinegar' treated with different concentrations of conidia of *Trichoderma* sp.

Mycoflora	Control ^a	Concentrations of conidia of <i>Trichoderma</i> sp.			
		1.2	2.4	4.8	9.6
		(x 10 ⁸ CFU g ⁻¹)			
<i>Fusarium</i> spp.	79.1% a	59.4% b	56.8% b	60.4% b	51.0% c
<i>Rhizoctonia solani</i>	19.4% c	24.9% b	25.8% b	17.9% c	32.4% a
<i>Aspergillus</i> spp.	0.5% c	14.9% b	16.7% a	16.8% a	14.3% b
<i>Rhizopus</i> spp.	1.0% b	0.4% c	0.4% c	1.5% a	-
<i>Penicillium</i> spp.	-	-	-	3.4%	2.3%
<i>Curvularia</i> spp.	-	0.4%	0.4%	-	-

^aSeeds not inoculated with conidia of *Trichoderma* sp. Data represent the mean of 10 replications and, when followed by the same lowercase letter, do not differ statistically from each other by Tukey test at 5% probability.

In our study, there was no inhibition of infestation of *Rhizoctonia solani* in the treatment of cowpea seeds with *Trichoderma* sp. in relation to the control (Table 1). The fungus *R. solani* presents numerous resistance structures or sclerotia that ensures it long periods of survival (Anees et al., 2010; Nechet & Halfeld-Vieira, 2011). In addition, a high saprophytic capacity and a wide range of host plants, cultivable or not, make this pathogen difficult to control (Nechet & Halfeld-Vieira, 2011). In addition to not controlling infestation by *R. solani*, there was a significant increase in the percentage of infestation by *Aspergillus* spp. in the seeds of cowpea independently from the concentration of conidia of *Trichoderma* applied (Table 1).

The fungi of the genus *Aspergillus*, *Penicillium* and *Rhizopus* cause diseases in stored seeds, and their occurrence increases in the post-harvest period and may cause seed rot (Lazarotto et al., 2010; Siameto et al., 2010; Danelli et al., 2011). Fungi of the genera *Trichoderma* and *Aspergillus* may act synergistically and effectively in suppressing the growth of *Fusarium oxysporum* (Usha et al., 2012). Thus, it is probable that the reduction in the percentage of infestation in seeds by *Fusarium* spp. (Table 1) was the result of an effective interaction between isolates of *Trichoderma* sp. and *Aspergillus* spp.

Seed treatment with conidia of *Trichoderma* sp. did not inhibit the transmission of the fungi *Fusarium* spp. and *Rhizoctonia solani*

from cowpea seeds to seedlings (Table 2). This result indicates that the treatment of seeds with the fungus *Trichoderma* sp. was inefficient in the control of these phytopathogens, allowing their occurrence in cowpea seedlings. According to Kobayasti & Pires (2011), the pathogens carried

by seeds may associate with them superficially or colonize their internal tissues, and then contaminate them. It is probable that the plant pathogens identified in cowpea seedlings were already in the inner tissues of the seeds and, after germination, infected the seedlings.

Table 2. Seed-seedling transmission and indole acetic acid (IAA) production in fungi isolated from cowpea cv. 'Vinagre' seeds inoculated with conidia of *Trichoderma* sp.

Mycoflora	Transmission test*	Production of IAA*
Positive control[§]	Not tested	+
<i>Fusarium</i> spp.	Yes	-
<i>Rhizoctonia solani</i>	Yes	-
<i>Aspergillus</i> spp.	No	-
<i>Rhizopus</i> spp.	No	-
<i>Penicillium</i> spp.	No	-
<i>Curvularia</i> spp.	No	-
<i>Trichoderma</i> sp.	Not tested	+

[§]*Aspergillus niger*. * Data represent the mean of four replications.

The fungi *Aspergillus* spp., *Rhizopus* spp., *Penicillium* spp. and *Curvularia* spp., found on the surface of cowpea seeds, were not transmitted from the seed to the seedling (Table 2). Although the fungal inoculum, in the form of mycelium or spores, is susceptible to transportation by the seed, the transmission of the pathogen depends mainly on the amount and location of the inoculum in the seed and its ability to infect the plant (Akhtar et al. 2007; Kobayasti & Pires, 2011; Sá et al., 2011).

In our study, the fungi found by the sanity test and the isolates of *Trichoderma* sp. were tested as for indole acetic acid (IAA) production. Among all fungi tested, only the isolates of *Trichoderma* sp. produced IAA (Table 2). IAA is a plant hormone that promotes differential cell elongation and, consequently, plant growth. *Trichoderma* sp. isolates are efficient producers of this plant hormone (Gravel et al., 2007; Carvajal et al., 2009; Oliveira et al., 2012). According to Carvajal et al. (2009), several species of the genus *Trichoderma* produce both IAA and metabolites with an activity analogous to plant hormones. Once produced in excess by *Trichoderma* sp., the IAA plant hormone may cause a negative or inhibitory effect on germination and on seedling development (Taiz & Zeiger, 2009).

The germination of cowpea seeds presented a positive correlation with the application of conidial concentrations of *Trichoderma* sp. (Figure 1A). The results indicate

that *Trichoderma* sp. was able to stimulate the early germination of cowpea seeds. Species of *Trichoderma* may improve plant growth and development by producing metabolites whose activities are analogous to plant hormones (Gravel et al., 2007; Carvajal et al., 2009). In our study, the isolates of *Trichoderma* used were IAA producers, as shown in Table 2. IAA is considered a root initiation hormone (Brotman et al., 2010; Inácio et al., 2011).

The increase in the concentration of conidia of *Trichoderma* sp. stimulated more root growth (Figure 1B) and initial germination (Figure 2A) in relation to the control. Moreover, the stimulation caused by the treatment of seeds with *Trichoderma* sp. increased up to the concentration 4.8×10^8 CFU g⁻¹ for the parameters related to germination and the length of the bean radicle (Figure 1 and 2). The application of 9.6×10^8 CFU g⁻¹ conidia of *Trichoderma* sp. did not cause greater stimuli. It was even responsible for the decrease in the germination speed index (Figure 2B).

Although it is reported that the fungus *Trichoderma* sp. has several beneficial effects on plants, which include the activation of seed germination (Shoresh et al., 2010), high concentrations of this fungus imply high concentrations of auxins. Auxins and cytokinins are the main hormones related to the regulation and maintenance of cell division (Inácio et al., 2011). However, levels of auxin over the

optimal may induce ethylene synthesis in plant tissues (Brotman et al., 2010). Ethylene is a plant

hormone known to inhibit cell stretching (Taiz & Zeiger, 2009).

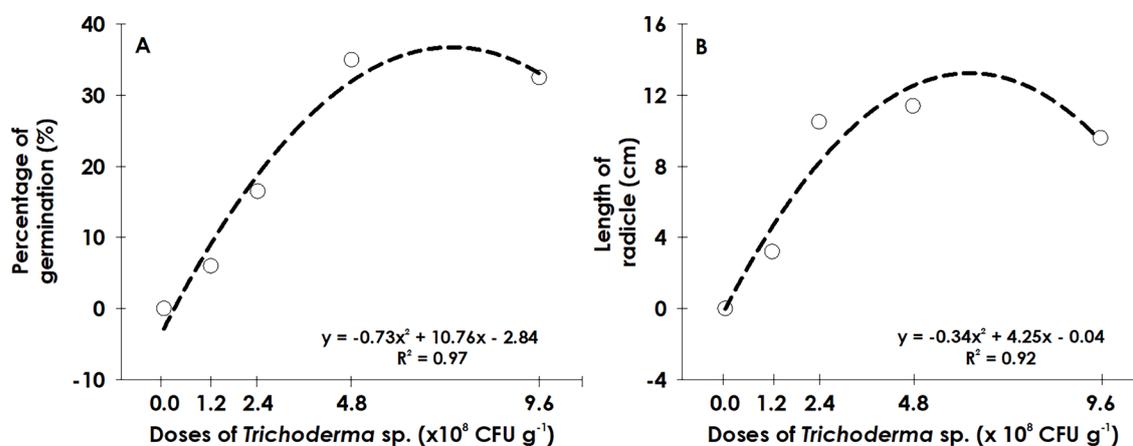


Figure 1. Percentage of germination (A) and length of radicle (B) of cowpea seeds cv. 'Vinegar' inoculated with conidia of *Trichoderma* sp. at the concentrations 1.2, 2.4, 4.8 and 9.6×10^8 CFU g^{-1}

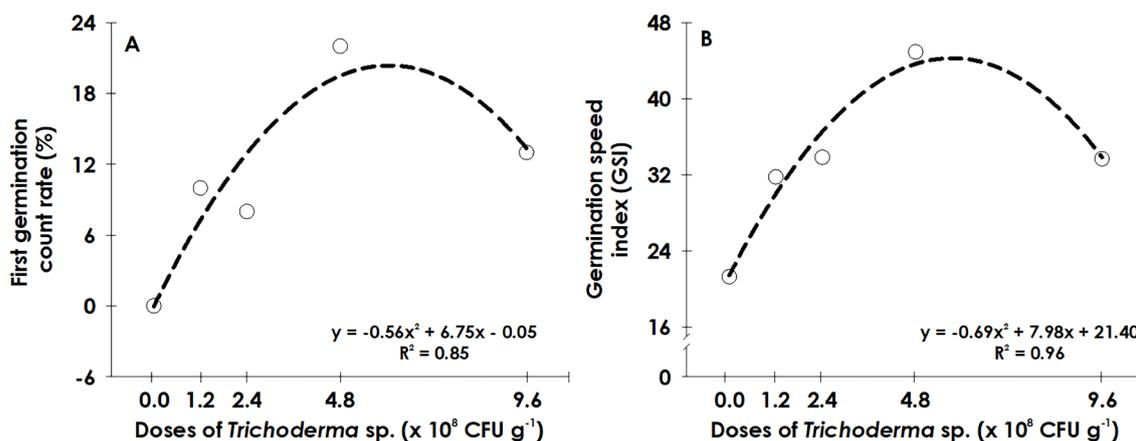


Figure 2. First germination count rate (A) and germination speed index (B) of cowpea cv. 'Vinegar' inoculated with conidia of *Trichoderma* sp. at the concentrations 1.2, 2.4, 4.8 and 9.6×10^8 CFU g^{-1}

The IAA produced by isolates of *Trichoderma* acts as a promoter of root growth of plant species of agricultural importance (Carvajal et al., 2009; Oliveira et al., 2012). Thus, these microorganisms may be suggested as plant growth promoters. However, it is necessary to take care with the doses applied so that there are no negative effects on plant growth, as observed with the application of 9.6×10^8 CFU g^{-1} conidia of *Trichoderma* sp. on the seeds evaluated. The IAA plant hormone produced by the microorganisms at low concentrations acts to stimulate growth, whereas high concentrations of this plant hormone may impair root development (Gravel et al., 2007; Inácio et al., 2011).

The treatment of seeds with antagonistic microorganisms may result in improved plant

development and significant effects on germination and seedling emergence and development (Akhtar et al., 2007; Harman, 2011; Silveira et al., 2011; Oliveira et al., 2012). The fungi of the genus *Trichoderma* are able to act as stimulants of root growth and development, of nutrient assimilation and, consequently, of resistance to biotic stresses (Anees et al., 2010; Mastouri et al., 2010; Shores et al., 2010). Studies on the application of *T. harzianum* to common bean seeds did not show any symptom of toxicity or damage to seed germination. There was indeed an improvement in seed germination rates, always higher than 80%, and in the percentage of normal seedlings (Carvajal et al., 2009; Mastouri et al., 2010; Carvalho et al., 2011), confirming the significant effects of the use of

Trichoderma sp. inoculants on germination and development of cowpea seedlings.

Conclusions

The application of *Trichoderma* sp. was efficient in reducing the incidence of *Fusarium* spp., and promoted the germination and elongation of the radicle in cowpea beans.

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