Microbiolization of cowpea seeds with commercial strains of *Trichoderma asperellum* and *T. harzianum*[1]

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ABSTRACT

Seed microbiolization has been increasingly contributed to researches due to its beneficial action in the initial growth of seedlings and control of phytopathogens. Thus, the aim of this study was to evaluate the initial growth and control of fungi associated with cowpea seeds by the seed microbiolization with commercial strains of *Trichoderma* spp. Seeds of cowpea cultivar 'BRS Gurguéia' were analyzed in a completely randomized design, with four replications, using five commercial strains based on *Trichoderma asperellum* and *T. harzianum* (Trichodermax®, Quality®, Trichodermil®, Agroguard® and Ecotrich®) and two control treatments, represented by untreated seeds (negative control) and the fungicide carbendazim (positive control). Sanity, germination, emergence and electrical conductivity tests of seeds were carried out to confirm the hypotheses. Seed microbiolization with *Trichoderma asperellum* and *Trichoderma harzianum* are effective in reducing the incidence of fungi and have antimicrobial activity similar to synthetic fungicide. The strains *T. asperellum* T-211 (Trichodemax®), *T. asperellum* URM-5911 (Quality®) e *T. harzianum* ESALQ-1306 (Trichodermil®) promote increases for seed vigor. The percentage of seed emergence is maximized with the application of strains *T. asperellum* (Quality® and Trichodemax®). The commercial strains of *Trichoderma* tested increase the initial growth of cowpea seedlings cv. 'BRS Gurguéia'.

Keywords: biological control; *Vigna unguiculata*; biological products; plant pathogens.

INTRODUCTION

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the most cultivated and consumed grain legumes in Brazil, with emphasis on the North and Northeast regions, where it is an important source of income and subsistence for small producers who live on family farming (Lima *et al.*, 2014).

According to data from the Brazilian National Supply Company (Conab), cowpea production was estimated at 637.7 thousand metric tons of grains in 2018/2019 (Conab, 2019). Although it is a very important crop in Brazil, its average productivity is still low, as the result of low technological level used in cultivation (Oliveira *et al.*, 2015).

As a limiting factor for cowpea cultivation, it is possible to verify the difficulty in obtaining seeds with good physiological and sanitary quality, since these are considered one of the main inputs of agricultural production, acting as a preponderant factor to obtain uniform and vigorous plant stands, interfering directly with productivity (Nogueira *et al.*, 2014).

By the way, the control of microorganisms associated with seeds is an essential measure to guarantee high productivity, which seed microbiolization has become an important study for researchers (Cruz *et al.*, 2020; De Sá *et al.*, 2019; Farias *et al.*, 2019; Pereira *et al.*, 2019), once it has reached out prominence in relation to synthetic products to be an alternative in reducing environmental risks caused by the excessive use of pesticides, thus aiming the use of microorganisms that are able to promote growth and direct action in the control of phytopathogens associated with seeds.

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In the biological control of phytopathogens, *Trichoderma* spp. stands out due to its bioprotective action against the attack of pathogens during germination, promoting initial seedling growth, as well as the solubilization of insoluble micronutrients in the soil, increasing the vigor and yield of plants (Missio *et al.*, 2016; Gomes *et al.*, 2019).

For this reason, the aim of this work was to evaluate the initial growth and control of fungi associated with seeds of cowpea by the seed microbiolization with commercial strains of *Trichoderma* spp.

MATERIAL AND METHODS

The experiment was carried out in a completely randomized design, at the Phytopathology Laboratory (LAFIT) of the Department of Phytotechnics and Environmental Sciences at the Federal University of Paraíba (UFPB), with seeds of cowpea cultivar 'BRS Gurguéia' given by the Brazilian Agricultural Research Corporation (Embrapa) – Mid-North.

The commercial strains we used were *T. asperellum* T-211 (Trichodermax® EC, Novozymes BioAg Produtos para Agricultura Ltda), *T. asperellum* (Quality® WG URM-5911, Laboratory of BioControle Farroupilha Ltda), *T. harzianum* ESALQ -1306 (Trichodermil® SC, Koppert do Brasil Holding Ltda.), *T. harzianum* DSM 14944 (Agroguard® WG, Live Systems Technology S.A.) and *T. harzianum* IBLF 006 (Ecotrich® WP, Ballagro Agro Tecnologia Ltda).

The inoculation of seeds with strains of *Trichoderma* spp. was carried out following the methodology proposed by Carvalho *et al.* (2014), in suspension of 2 mL $100g^{-1}$ of seeds (2.5 x 10^8 conidia mL^{-1}). The efficacy of the isolates, untreated seeds and treated seeds with fungicide carbendazim (100 mL 100 kg^{-1} of seeds), was compared by using negative and positive controls, respectively.

The effect of seed microbiolization on the incidence of fungi was appraised by sanitary test (Blotter test) (Brasil, 2009), using 200 seeds, divided into ten repetitions of twenty seeds, placed on Petri dishes (Ø 15 cm), under aseptic conditions, containing a double-layer of filter paper which was previously sterilized and moistened with sterile distilled water. The plates were incubated in a *Biochemical Oxygen Demand* (B.O.D) growth chamber for eight days, at constant temperature of 25 ± 2 °C with 12-hour photoperiod. We used optical microscope and specialized literature (Seifert *et al.*, 2011) to identify and assess the incidence of fungi associated with seeds, and results were expressed as percentage of the occurrence of fungi.

The initial seedling growth was evaluated by the germination and emergence tests, using four replications of 50 seeds. The emergency test was carried out in a greenhouse and seeds were sown in plastic trays of dimensions 66 x 33 x 6 cm, containing previously sterilized washed sand. For the germination test according to Brasil (2009), seeds were distributed on previously sterilized Germitest® paper substrate and moistened with equivalent to 2.5 times the dry paper weight. After sowing, papers were wrapped in roll forms, placed inside transparent plastic bags and then taken to B.O.D., regulated at 25 ± 2 °C with 12-hour photoperiod.

The counting of germinated and emerged seeds, for the germination and emergence tests respectively, were performed daily from the fifth to the eight days after application of commercial strains (Brasil, 2009). The first germination and emergence counts were done together with the tests, which allowed to calculate the germination and emergence speed indices (GSI and ESI, respectively), according to the formula proposed by Maguire (1962).

The seedling length was measured by shoot lengths and the longest root lengths, with a digital caliper presenting an accuracy of 0.001 mm, and results were expressed in centimeters. Afterwards, we determined shoot and root dry matter of by a forced air circulation oven at 65 °C for 48 hours and then samples were weighed on an analytical balance with an accuracy of 0.001 g, and results were expressed in grams.

The electrical conductivity test was carried out thirty days after the application of treatments, using four replicates of 100 seeds, which were weighed and placed inside plastic glasses (200 mL), adding deionized water in the amount of 100 mL and subsequently kept in B.O.D. at temperature of 25 ± 2 °C. The electrical conductivity was determined after 12 and 24 hours and results were expressed in $\mu S\ cm^{-1}\ g^{-1}$.

The statistical analysis was performed using the statistical software R (R Development Core Team, 2020), results were submitted to normality test for residuals by Shapiro-Wilk and homogeneity of variances by Bartlett's test. The results that presented normal distribution and homoscedasticity were submitted to analysis of variance (ANOVA), and the means of the treatments were compared using the Tukey test ($p \leq 0.05$). The values of the incidence of fungi were previously transformed into $\sqrt{y+1}$.

RESULTS AND DISCUSSION

The effect of *Trichoderma* spp. commercial strains on the incidence of fungi is reported in Figure 1. Genera we identified were *Aspergillus* spp. (57.0%), *Penicillium* sp. (26.0%), *Fusarium* sp. (34.0%), *Colletotrichum* sp. (28.0%), *Macrophomina* sp. (27.0%).

We verified that the application of *Trichoderma* spp. strains reduced the incidence of these fungi earlier mentioned. Results showed that no differences were verified between the action of antagonists when compared to the application of the fungicide carbendazim, with the exception of *Trichoderma harzianum* IBLF 006 (Ecotrich® WP) in the control of *Penicillium* sp., which means these strains were as efficient as the chemical treatment to control these microorganisms.

According to Singh *et al.* (2018) modes of action of *Trichoderma* spp. that inhibit the growth of fungi may be several, such as mycoparasitism, which involves direct activity on phytopathogenic fungi, the antibiosis, antagonistic interaction involving low molecular weight diffusible secondary metabolites or antibiotics harmful to fungal phytopathogens; and the competition for substrate, which is the most important factor for the establishment of fungi in the search for space and nutrients.

These phytopathogenic genera we verified in seeds are keystone species responsible to cause diseases in cowpea (Amorim *et al.*, 2016), and they can be transmitted directly to seedlings, besides affecting directly seed quality, causing the loss of vigor, reduction of germination and signs of decay (Figure 2).

The variables of first germination (FGC) and emergency count (FEC), and root dry matter in the germination (RDMG) and emergence (RDME) did not show significant differences between the treatments applied.

In the germination speed index (GSI), *T. harzianum* ESALQ-1306 (Trichodermil®) and the fungicide were the highest, with values of 9.69 and 9.64 respectively, besides showing statistical differences in relation to the negative control. In the emergency speed index (ESI), *T. asperellum* T-211 (Trichodermax®) stood out, presenting 9.51, and it was the only treatment with significant difference in relation to the control treatment (Figure 3A).

Regarding the germination percentage (GP), both strains of *T. harzianum* DSM 14944 (Agroguard®) and *T. asperellum* T-211 (Trichodermax®) differed from the control treatment and achieved 97.5% and 97.0% respectively (Figure 3B). For the emergency percentage (EP), only *T. asperellum* T-211 (Trichodermax®) showed difference when compared to the control treatment, with 94.5%.

This increase in germination potential associated with *Trichoderma harzianum* in beans has already been described by Carvalho *et al.* (2011). The results are associated with the release mechanism of plant growth regulator but also the solubilization of nutrients (Hajieghrari, 2010; Saber *et al.*, 2017) that activate the production of enzymes and phytohormones involved in seed germination, thus increasing the germination speed and its vigor (Singh *et al.*, 2018).

Bezuidenhout *et al.* (2012) reported the similarity of gliotoxin, secondary metabolite produced by *T. harzianum*, in relation to the gibberellic acid, plant growth hormone involved in the seed germination process, indicating a possible perception of gliotoxin as gibberellic acid, influencing the germination process positively.

We observed that the strain *T. harzianum* ESALQ-1306 (Trichodermil®) for the germination test had the shortest (5.15 cm) shoot length (SL) as well as the control treatment (4.33 cm). In the emergence, the fungicide and all the commercial strains of *Trichoderma* spp. showed the highest SL, differing from the control treatment (8.32 cm).

For the root length (RL) in the germination and emergence, *T. harzianum* IBLF 006 (Ecotrich®) was responsible for the highest root development of seedlings with 7.06 cm in germination and 14.18 cm in emergence, differing statistically from the control treatment, which presented the lowest RL value in the germination (5.70 cm) and emergence (11.33 cm) (Figure 4B). The chemical treatment and *T. asperellum* T-211 (Trichodermax®) also differed from the control treatment for RL in the germination.

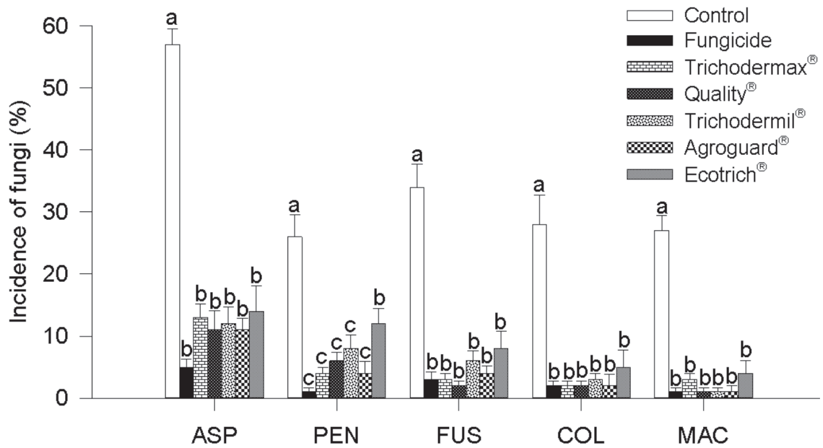

Figure 1: Incidence of fungi associated with seeds of cowpea cultivar 'BRS Gurguéia' treated with commercial strains of *Trichoderma* spp. ASP – *Aspergillus* spp., PEN – *Penicillium* sp., FUS – *Fusarium* sp., COL – *Colletotrichum* sp., MAC – *Macrophomina* sp.

These results corroborate those reported by Pereira *et al.* (2019) that used strains similar to our research and found an increase for shoot and root growth in lettuce, thus evidencing the efficiency of the strains to promote the growth in cowpea.

Evaluating the shoot dry matter (SDM) in the germination, the strain *T. harzianum* IBLF 006 (Ecotrich®) achieve values of 0.048g and the fungicide of 0.045g, both differing significantly from the control treatment (0.028g) (Figure 5A). We also verified this difference in the emergency for the strains *T. harzianum* IBLF 006 (Ecotrich®), *T. harzianum* ESALQ-1306 (Trichodermil®) and *T. asperellum* URM-5911 (Quality®), with values of 0.100g, 0.098 and 0.095g respectively, and 0.060g for the control treatment, with lowest production of SDM.

The promotion of the initial plant growth is probably related to the mechanisms of indirect pathway, with growth inhibition of phytopathogens (Gava & Menezes, 2012) and direct pathway, through the production of hormones or analogues (Saber *et al.*, 2017) and phosphate solubilization (Oliveira *et al.*, 2012).

Trichoderma spp. is directly related to the balance of gibberellic acid, ethylene and mainly auxin by the production of indole-3-acetic acid (IAA), a hormone responsible for plant vegetative development, promoting

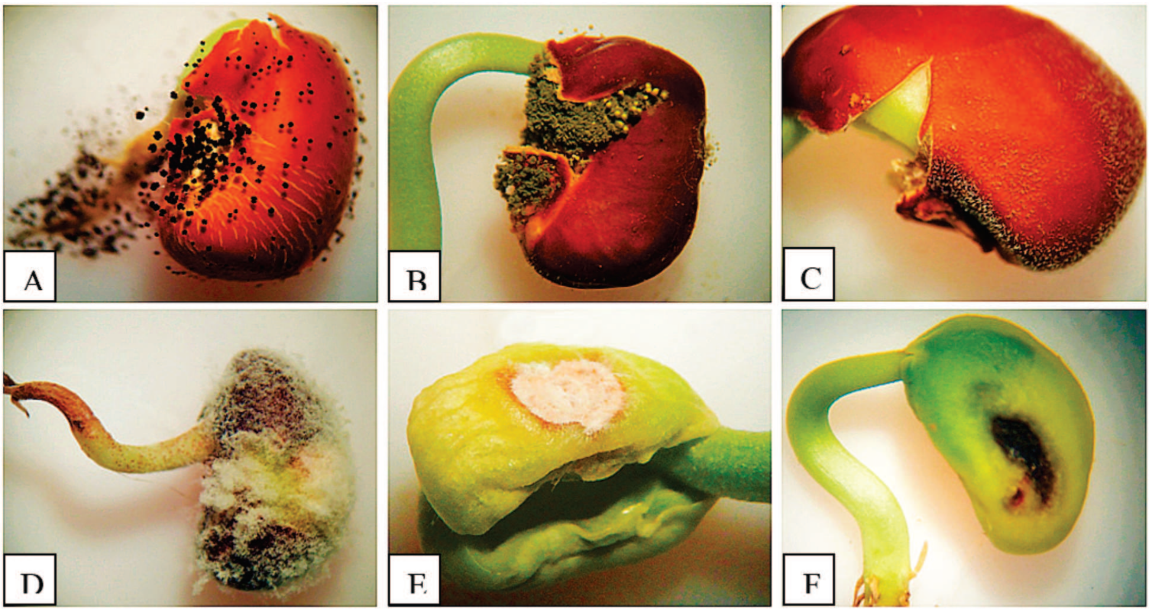

Figure 2: Seedlings of cowpea cultivar 'BRS Gurguéia' from the negative control infected by *Aspergillus* spp. (A) and (B), *Penicillium* sp. (C), *Fusarium* sp. (D), *Colletotrichum* sp. (E), *Macrophomina* sp. (F).

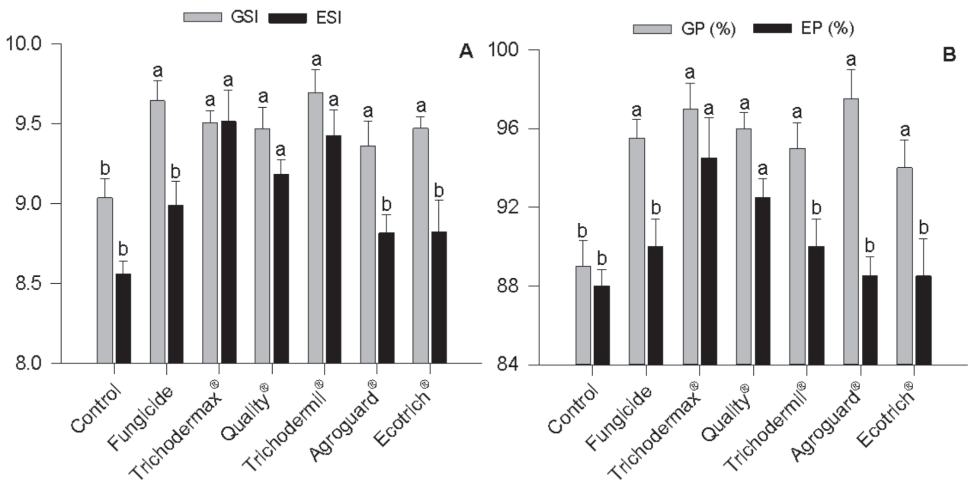

Figure 3: The germination speed index (GSI), emergence speed index (ESI), germination percentage (GP) and emergence percentage (EP) from seeds of cowpea cultivar 'BRS Gurguéia' treated with commercial strains of *Trichoderma* spp. Columns with the same color and followed with similar letters do not differ statistically by Tukey test ($p \leq 0.05$).

cellular elongation, formation of secondary roots, production of apical and lateral buds (Oliveira *et al.*, 2012; Stewart & Hill, 2014).

Regarding electrical conductivity (EC), there is a positive activity with the application of *Trichoderma* spp. on seed vigor (Figure 5B). In the first 12 hours, the lowest values were verified for *T. asperellum* T-211 (Trichodermax®) and *T. harzianum* ESALQ-1306 (Trichodermil®), with 19.72 µS $cm^{-1}g^{-1}$ and 20.10 µS cm^{-1} g^{-1} respectively, both with statistical difference in relation to the control. In the 24-hour period, only *T. harzianum* IBLF 006 (Ecotrich®) and *T. harzianum* DSM 14944 (Agroguard®) did not differ statistically from the control treatment and the lowest values were 40.25 µS cm^{-1} g^{-1} and 40.92 µS cm^{-1} g^{-1}, as well as in the first 12 hours, with strains *T. harzianum* ESALQ-1306 (Trichodermil®) and *T. asperellum* T-211 (Trichodermax®), respectively. The control showed the highest values than other treatments according to the periods of 12 (28.35 µS cm^{-1} g^{-1}) and 24 hours (56.53 µS cm^{-1} g^{-1}).

This change in electrical conductivity may be indirectly linked to the control of fungi associated with seeds, providing seed a greater protection in the integrity of cell membranes. Once seed quality is indirectly evaluated by the amount of leachate in the seed immersion solution in which, the least vigorous release greater amounts of solutes to the external environment (Machado *et al.*, 2011; Marcos-Filho, 2015).

To sum up, electrical conductivity is able to detect seed deterioration in its initial growth, due to the integrity of cell membranes (Prado *et al.*, 2019). This quantification is essential, once seeds with lower vigor in the field have less vigorous seedlings and much difference in emergence when compared to seeds presenting higher vigor, because of the differences in seedling initial growth rates (Munizzi *et al.*, 2010).

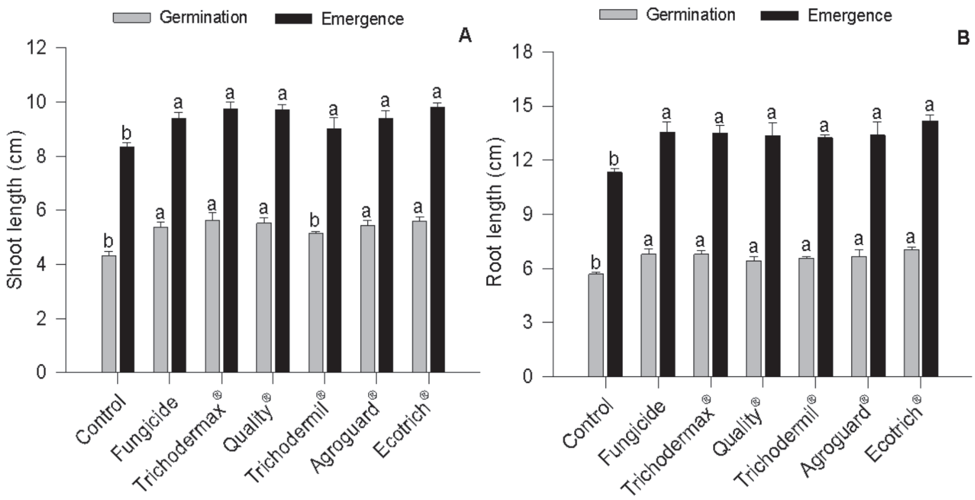

Figure 4: Shoot length (SL) and root length (RL) from seeds of cowpea cultivar 'BRS Gurguéia' treated with commercial strains of *Trichoderma* spp. Columns with the same color and followed with similar letters do not differ statistically by Tukey test ($p \leq 0.05$).

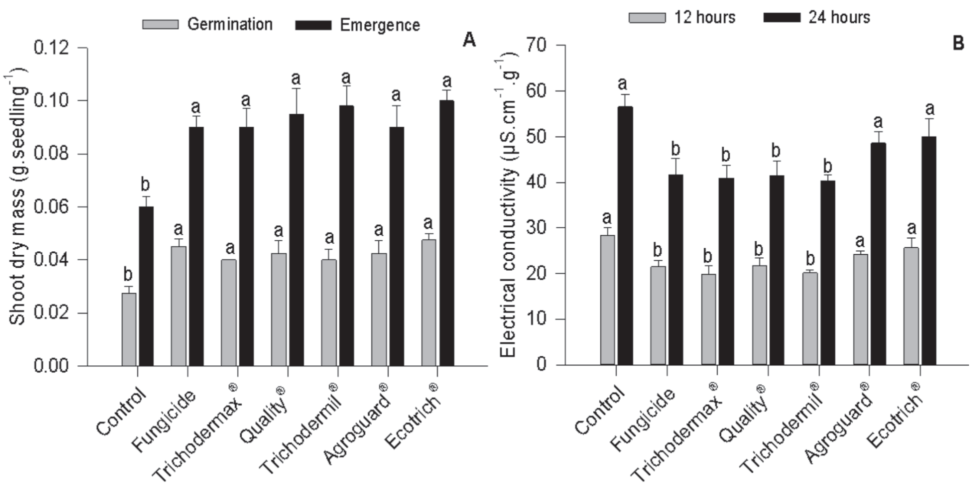

Figure 5: Shoot dry matter (SDM) and electrical conductivity (EC) from seeds of cowpea cultivar 'BRS Gurguéia' treated with commercial strains of *Trichoderma* spp. Columns with the same color and followed with similar letters do not differ statistically by Tukey test ($p \leq 0.05$).

CONCLUSIONS

Seed microbiolization with *Trichoderma asperellum* and *Trichoderma harzianum* are effective in reducing the incidence of fungi and have antimicrobial activity similar to synthetic fungicide.

The strains *T. asperellum* T-211 (Trichodemax®), *T. asperellum* URM-5911 (Quality®) e *T. harzianum* ESALQ-1306 (Trichodermil®) promote increases for seed vigor.

The percentage of seed emergence is maximized with the application of strains *T. asperellum* (Quality® and Trichodemax®).

The commercial strains of *Trichoderma* tested increase the initial growth of cowpea seedlings cv. 'BRS Gurguéia'.

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