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Inoculation with entomopathogenic fungi reduces seed contamination, improves seed germination and growth of chilli seedlings

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The use of entomopathogenic fungi is a common practice for integrated pest management. It has recently been observed that they also play a role as growth promoters and plant disease antagonism. In this study, the effect of inoculation with two strains of *Beauveria bassiana* [(Bals.-Criv.) Vuill. 1912] (strains BB42 and BB09) and two strains of *Metarhizium anisopliae* [(Metschn.) Sorokīn 1883] (strains MA25 and MA28) on the percentage of seed germination and development of chilli seedling (C*apsicum annuum* L.) was evaluated. In the *in vitro* test, we did not find significant differences between percentages of germination, but in the *in vivo* test, differences were significant, where sMA28 and BB09 strains obtained the highest germination percentage (85%). It was also found that seedlings inoculated with entomopathogenic fungi generate longer roots and produced more biomass in both tests, as well as lower percentages of contaminated seeds in *in vitro* and *in vivo* tests. All strains evaluated had inhibitory effects against two seed borne fungi isolated from contaminated seed, belong to genus *Alternaria sp*.

Key words: Germination, antagonims, entomopathogenic fungi.

INTRODUCTION

Capsicum annuum L. is one of most important vegetable worldwide, with a global consumption of 400,000 t approximately, and it account of 16% of the world's total spice trade, but yields are usually very low (Olatunji and Afolayan, 2018). Research efforts to ensure sanity and quality of chilli seeds are of the main importance (Matthews et al., 2012). In optimum conditions, seeds must be present with a high germination percentage and a good seedling development, however, seedlings are commonly affected by exposed biotic and abiotic factors (Penella and Calatayud, 2018). In chilli seeds, there have been isolated and identified several pathogens that can inhibit germination and generate infections, causing in some cases the death of the plant (Chigoziri and Ekefan,

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2013). Fungal diseases reduce yield losses of up to 50%, mainly seed and seedling rot, causing by fungi such as *Aspergillus niger* (Chauhan Rinkal et al., 2018).

Chilli production is also limited by low germination rate. In nature, seed borne microorganism have strongly associated with seed germination and seedling growth (Shearin et al., 2018). Different pre-germination treatments have been tried to increase the range of chilli seed germination, but it implies the use of chemical agents (Prado-Urbina et al., 2015). Microbial inoculation of seeds with fungi favours germination and emergency of the embryo (Lee et al., 2010). In *Opuntia*, artificial inoculation with native fungi of the soil rhizosphere (*Penicillium chrysogenum*, *Phoma* sp., and *Trichoderma* sp.) helped to break their dormancy, growth of pathogens was inhibited and germination percentage increased (Delgado-Sánchez et al., 2011, 2013). Another rhizosphere fungus *P. chrysogenum* improved seed germination, and reduced disease incidence and disease protection against plant pathogens (Murali et al., 2013).

Beauveria bassiana [(Bals.-Criv.) Vuill. 1912] and *Metarhizium anisopliae* [(Metschn.) Sorokīn 1883] are more abundant entomopathogenic fungi (EF) in subtropical environments and their use is a common practice for integrated pest management (IPM) (Pérez-González et al., 2014). These fungi have an excellent biocontrol capacity against insects such as whitefly (*Bemisia tabaci*) in eggplants (Islam et al., 2010), fall armyworm (*Spodoptera frugiperda*) in *Zea mays* (Ramirez-Rodriguez and Sánchez-Peña, 2016), *B. tabaci* Gennadius (Aleyrodidae), potato/tomato psyllid, *Bactericera cockerelli* Sulc. (Triozidae), and western flower thrips, *Frankliniella occidentalis* (Pergande) in tomato (*Solanum lycopersicum* L.), (Ríos-Velasco et al., 2014) among others. They also have been shown to be a plant symbiont which have plant-root-promoting properties (Sasan and Bidochka, 2012) and antagonistic activity against plants pathogens (Jaber and Alananbeh, 2018) and we proposed that these can significantly improve germination and facilitate establishment of seedlings.

This study presents the first report about the effect of *B. bassiana* and *M. anisopliae* on the inhibition of seed borne fungi in chilli seeds. The effect of these fungi on the promotion and seedlings growth of *C. annuum* L. is also evaluated and discussed.

MATERIALS AND METHODS

Fungi strains

Four strains of entomopathogenic fungi were evaluated; two of *B. bassiana* (BB09 and BB42 strains), and two of *M. anisopliae* (MA28 and MA25 strains). Native strains used were BB42 strain, isolated from a bug *Lygus* sp. in El Copal, Guanajuato, and strain MA25, isolated from a white grub that was found in Puruaga town, Guanajuato, both provided by the Laboratory of Beneficial Organisms Reproduction, which belongs to the State Committee of strains used were active ingredient in commercial products: BB09 strain (Bassianil Wp®) and MA28 strain (Metabich ®). All mono-spore cultures were generated and were sown in Sabouraud Dextrose Agar (SDA) and incubated at 25 ± 1°C (Villegas-Rodriguez et al., 2014).

C. annuum L. seeds

In both experiments, we evaluated seeds of native poblano chilli pepper, native from the state of San Luis Potosi, Mexico.

In vitro inoculation of *C. annuum* L. seeds with entomopathogenic fungi

Chilli seeds were superficially disinfected by soaking for 5 min in 20% sodium hypochlorite (NaOCl) of purity (6% of free chlorine) after washing in 70% ethanol for 5 min. In following step, seeds were rinsed four times in sterile distilled water. One hundred seeds were allocated in 5 petri dishes (20 per dish) with Water Agar Medium. Each seed treated with EF was inoculated with 2 µL of conidia solution (final concentration, 1 x 10^8 spores ml^{-1}) and in the case of control seeds, 2 µL of INEX-A® solution at 0.2% were added to each one (Lohse et al., 2015). Each treatment was repeated five times. The petri dishes were kept in a growth chamber with a photoperiod of 16:8 (L:D) h. Germination percentage, length of roots and shoots, fresh weight and biomass production of the seedlings were measured after 15 days (Elena et al., 2011).

Trials of antagonisms among seed borne fungi and entomopathogenic fungi

Three seed borne fungi were isolated to non-inoculated seeds used in the *in vitro* test. Infected seeds were marked and placing to new petri dish with SDA and incubated at 25 ± 1ºC. Active discs of each strain of EF mixed with SDA medium were placed in one edge of the petri dishes, and we placed each seed borne fungi evaluated in the opposite edge. The petri dishes were maintained in a growth chamber at 25 ± 1ºC and after fifteen days, antagonist effect of each strain of EF over the seed borne fungi was evaluated (Jaber and Alananbeh, 2018). The experiment was repeated three times.

Inoculation tests of *C. annuum* L. seeds with entomopathogenic fungi under greenhouse conditions

Chilli seeds were planted singly in pots of 100 ml containing sterile peat moss (Sunshine Grow Mix #3®, Sun Gro Horticulture Canada). 500 µL of solution containing 1 x 10^8 conidia ml^{-1} of each EF tested, and commercial surfactant solution INEX –A® at 0.2% in the case of control was applied directly to the substrate. When the seeds began their germination, after seven days, inoculations were carried out again (Farias et al., 2018). It was considered that the seed germinated when the cotyledons emerged on the surface of the soil. Dead plants that had presence of mycelium during the experiment were considered as contamination. Variables evaluated were length, biomass production and fresh weight in roots and shoots 45 days after germination (Moloinyane and Nchu, 2019).

Statistical analysis

One-way ANOVA analysis was conducted on the experimental data that are presented as the mean ± standard error. Tukey's test was used to compare the treatment with a significant F value in the

ANOVA ($p \leq 0.05$). The analyses were performed with the Statistica software (ver. 7.0, StatSoft Inc., Tulsa, OK, USA).

RESULTS

In vitro inoculation of *C. annuum L.* seeds with entomopathogenic fungi

Although the statistical analysis did not show significant differences among treatments in ANOVA test at the level of 5%, germination of seeds inoculated with BB09 and MA28 strains was almost 10% greater than non-inoculated control and MA25 treatment. Strain BB42 had a germination percent greater than 80% as described in Figure 1. In the *in vitro* test, seeds inoculated with the strain BB42 generated seedlings with longer roots and shoots (5.18 and 1.51 cm, respectively), compared with shortest roots from the seedlings inoculated with the strain MA25 (3.8 cm), while shortest shoots belonged to the control and to the seeds inoculated with the strain MA28(1.17 cm). Seedlings inoculated with *B. bassiana* had a better colour as well as a greater size compared with other ones as presented in Figure 2.

A higher root fresh weight was observed in the control seedlings compared to those inoculated with BB09, with means of 13.58 and 6.6 mg, respectively as described in Figure 3A. Seedlings inoculated with the strains BB09, MA28, BB42 and the control ones showed the lowest root dry weight with values oscillating among 1.36 and 1.42 mg, without finding statistically significant differences between their weights ($p < 0.05$) as described in Figure 3B.

Seedlings inoculated with BB42 and MA28 strains generated seedlings with the highest weight, with 30.18 and 28.37 mg, respectively followed by seedlings inoculated with BB09, which obtained a fresh weight of 25.61 mg, forming the same group with the control (26.85 mg). With respect to dry weight or shoots, all the seedlings inoculated with EF showed higher values than the control (3.22 mg). Seedlings inoculated with BB42 had the highest biomass production with 3.93 mg (Figure 3B).

The control treatment had 18% of contaminated seeds while the seeds inoculated with EF showed a lower contamination percentage than the control: 5% in the case of seeds inoculated with the strain BB42, 2% of seeds inoculated with MA25 and there was no contamination in seeds inoculated with the strains BB09 and MA28 as presented in Figure 1.

Antagonisms among seed borne fungi and entomopathogenic fungi in *C. annuum* L. seeds

Based on the results of microscopy, it was determined that these colonies belong to genus *Alternaria sp.* and *Cladosporium sp.* All EF strains had inhibitory effects on seed borne fungi isolates one and three belong to genus *Alternaria* sp. (light brown and gray colonies, respectively) and MA28 and BB09 strain formed a characteristic halo zone surrounding the colony as shown in Figure 4. None of the EF isolates tested could inhibit the growth of colony 2, belonging to genus *Cladosporium sp*.

In vivo inoculation of *C. annuum* L. seeds with entomopathogenic fungi

Significant differences between treatments were found in the germination percentage of chilli seeds under greenhouse conditions in Tukey test. Seeds inoculated with strain MA25 showed the highest percentage of germination with 85%, and the lowest percentage obtained by seed inoculated with BB42 strain was 70% as shown in Figure 5. The highest percentage of contamination (50%) was observed on the control seedlings, unlike of EF inoculated seeds where percentage of contamination oscillated among 5 and 15% as presented in Figure 5.

With respect to the seedling development, seedlings inoculated with MA28 strain have the largest number of leaves (4.8), roots with lengths up to three times longer than the control (12.94 cm) and the higher height of the seedlings (4.03 cm). Control treatment obtained the lowest values for the number of leaves (1.6 cm), the length of the root (4.09 cm) and the height of the seedling (1.75 cm) as described in Figures 6 and 7.

Seedlings inoculated with the strain MA28 also showed the highest fresh weight (269.17 mg) and biomass production of roots and shoots, while seedlings inoculated with BB42, MA25 and BB09 strains got shoots with fresh weights that oscillated within 142 and 172 mg as presented in Figure 7A. Comparatively, seedlings inoculated with the strain BB09 and the control treatment produced the seedlings with the lowest fresh weights of roots (18.39 and 19.16 mg, respectively).

Regarding the dry weight of shoots, the control treatment produced the lowest value (4.91 mg), followed by the seedlings inoculated with the strains MA25, BB09 and MA25, with values at least twice as large as described in Figure 7B. Seedlings inoculated with the MA28 strain produced 23.22 mg of biomass, a value almost five times heavier than the control as shown in Figure 7B. In the case of root dry weight, control and treatments inoculated with BB09 produced similar values (3.34 and 4 mg), followed by the seedlings inoculated with MA25 and BB42 (6.33 and 8.36 mg). The heaviest root dry weight was produced by the seedlings inoculated with the MA28 strain with roots of 13.88 mg as shown in Figure 7B.

DISCUSSION

EF have been employed mainly to combat insect pests of several crops with economic importance (Villegas et al.,

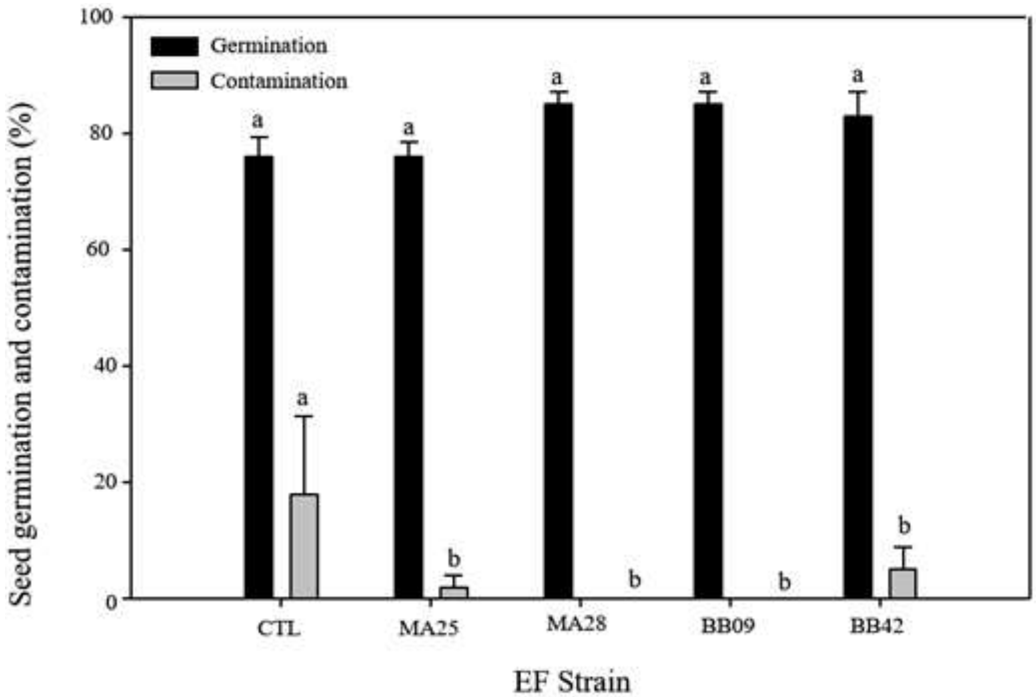

Figure 1. *In vitro* effect on the germination and contamination of chilli seeds inoculated with different isolates of entomopathogenic fungi (EF). CTL: Control; bb09: *Beauveria bassiana* strain 09; bb4: *Beauveria bassiana* strain 28. Different letters indicate significant difference (Tukey test; $p \leq 0.05$).

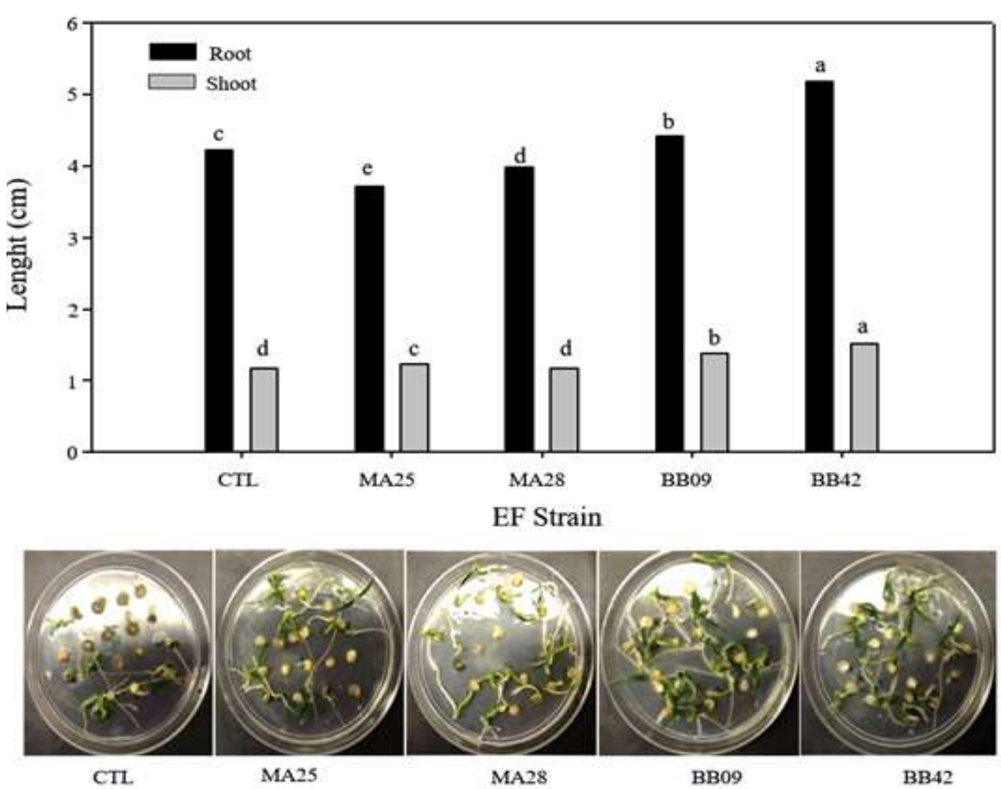

Figure 2. *In vitro* effect on the development of chilli seedlings inoculated with entomopathogenic fungi (EF). CTL: Control; BB09: *Beauveria bassiana* strain 09; BB42: *Beauveria bassiana* strain 42; MA25: *Metarhizium anisopliae* strain 25; MA28: *Metarhizium anisopliae* strain 28. Different letters indicate significant differences (Tukey test; $p \leq 0.05$).

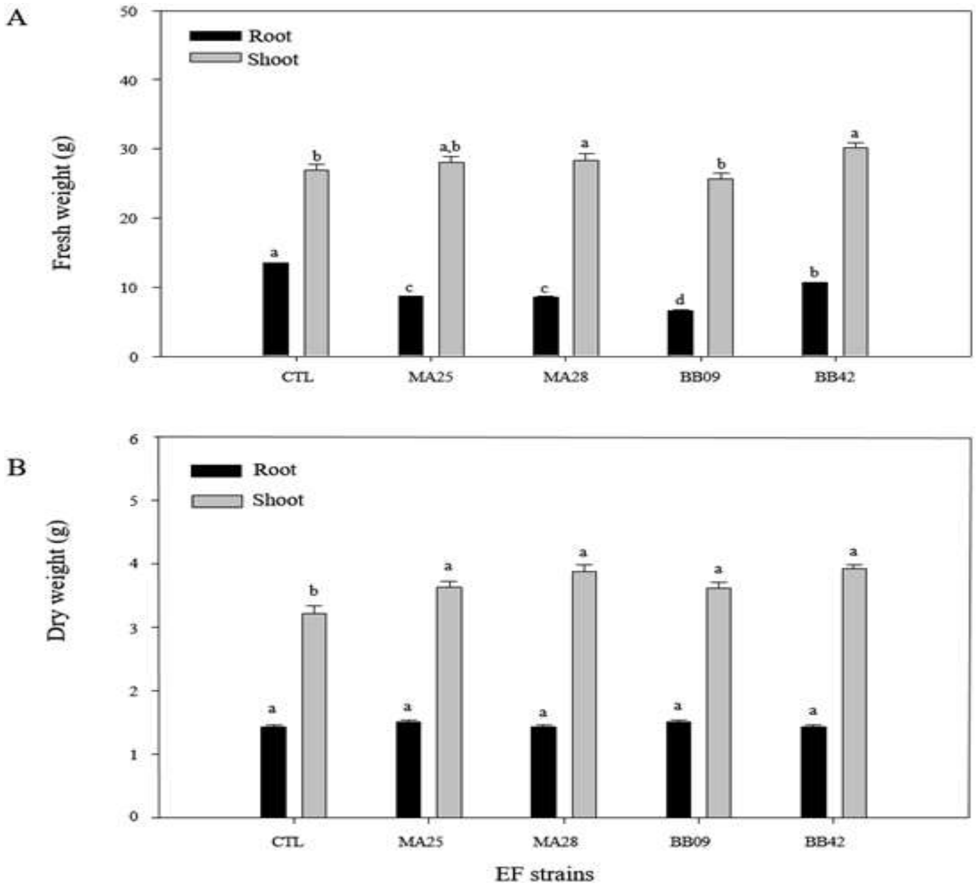

Figure 3. *In vitro* effect in the biomass production on chilli seedlings inoculated with different entomopathegenic fungi (EF). CTL: Control; BB09: *Beauveria bassiana* strain 09; BB42: *Beauveria bassiana* strain 42; MA25: *Metarhizium anisopliae* strain 25; MA28: *Metarhizium anisopliae* strain 28. Different letters indicate significant differences (Tukey test; $p \leq 0.05$).

Figure 4. In vitro effect of entomopathogenic fungi strains on seed borne fungies isolated from seeds (1,2,3). *Mettarhizium anisopliae* strain MA28, dark green mycelium; *Beauveria bassiana* strain BB09, white mycelium.

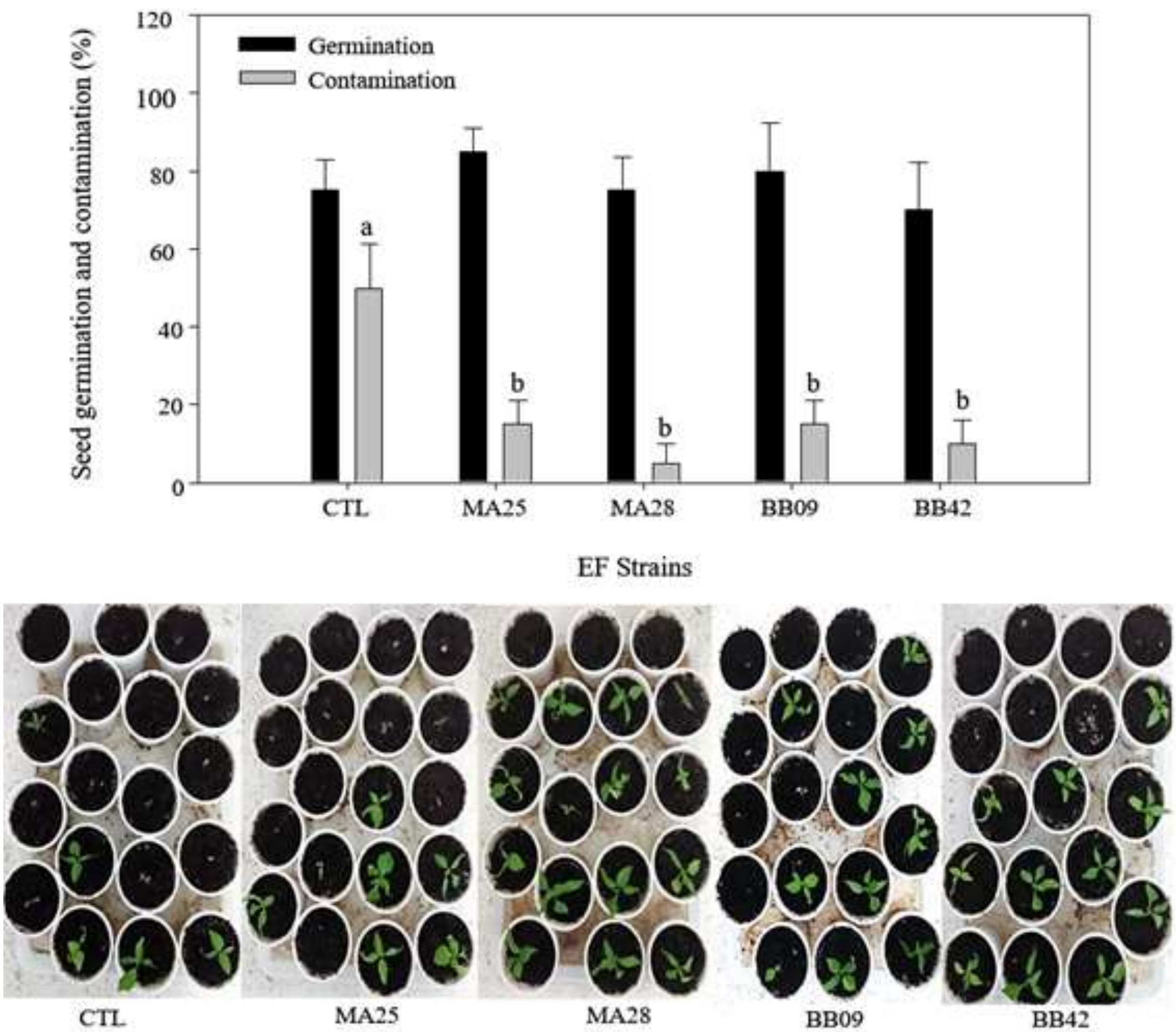

Figure 5. Effect of inoculation with entomopathegenic fungi (EF) on chilli seedlings (*Capsicum annumm* L.) germination and reduction of fungal seed contamination in substrate. CTL: Control; BB09: *Beauveria bassiana* strain 09; BB42: *Beauveria bassiana* strain 42; MA25: *Metarhizium anisopliae* strain 25; MA28: *Metarhizium anisopliae* strain 28. Different letters within each column indicate significant differences (Tukey test; p ≤ 0.05).

2014; Maniani and Ekesi, 2013), in addition to be insect pests and plant pathogens antagonist, and their ability to promote plant growth (Rai et al., 2014). Our results showed that inoculation with EF produced seedlings with greater height and weight. Results from this study is in agree with others, where EF was able to promote plant growth parameters (Bamisile et al., 2018; Jaber and Enkerli, 2016, Tall and Meyling, 2018), but in other cases, differences in growth parameters were not significant (Tefera and Vidal, 2009).

Even though the effect of EF on the germination of *C. annuum* L. was not significant in this study, a trend of germination increment was observed. At the end of greenhouse experiment, low germination and high contamination percentages was observed in control seedlings. From a practical perspective, this situation would be a limiting factor because having enough healthy plants at the beginning of the production cycle is crucial. In our case, the chilli seedlings inoculated with EF showed a higher survival percentage.

With respect to the length of shoot and roots, no significant differences were found in *in vitro* test, but potted chilli seedlings inoculated with MA28 strain was higher compared to other treatments. Sasan and Bidochka (2012) reported that *Metarhizium robertsii* colonized plant roots and stimulates the growth of lateral roots *in vitro* and *in vivo* test, using an appropriate fungal dose on soil is vital to ensure the plant growth proprieties of EF (Raya-Diaz et al., 2017). Substrate where the inoculated seeds are sown is another source of variation, and *Metarhizium* is not always successful in invading plant tissue and competing with seed borne fungus or

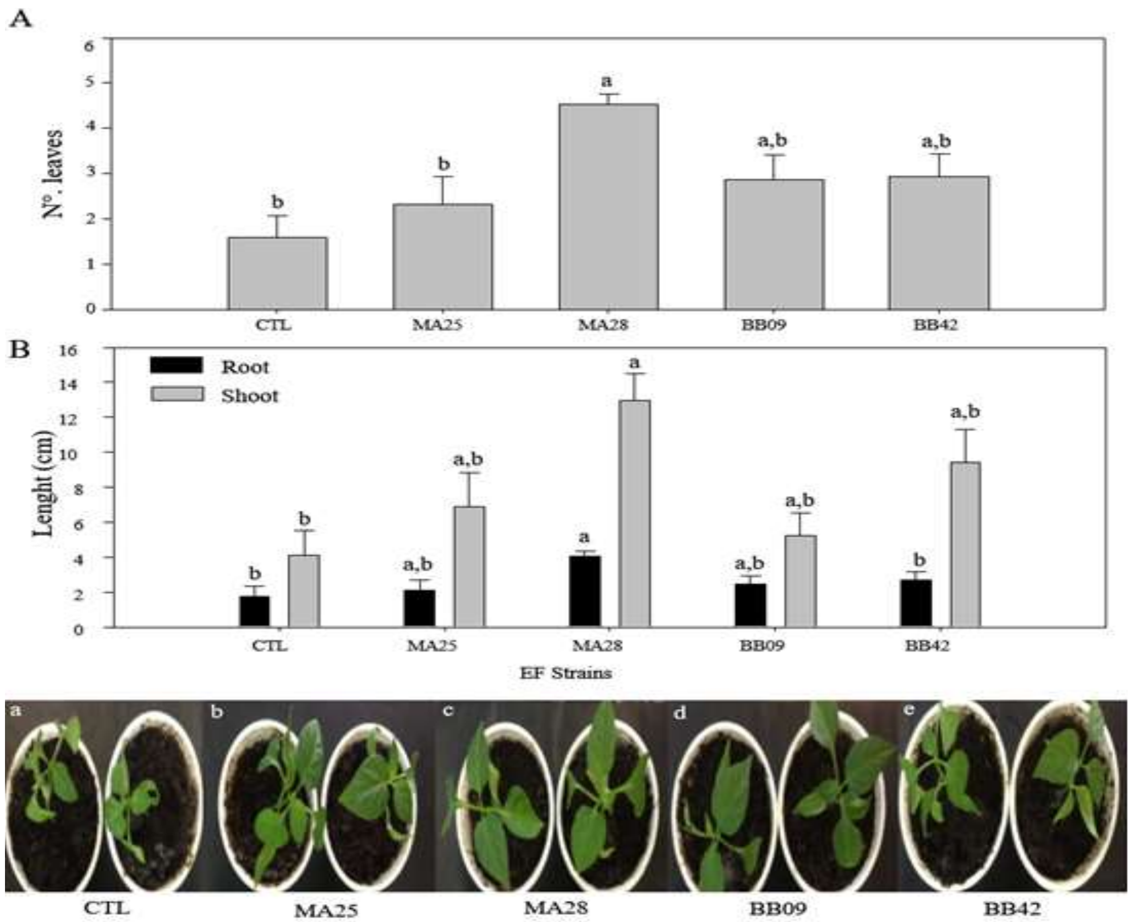

Figure 6. Effect on the development of potted chilli seedlings inoculated with entomopathogenic fungi (EF). a) Number of leaves and b) length of seedlings. CTL: Control; BB09: *Beauveria bassiana* strain 09; BB42: *Beauveria bassiana* strain 42; MA25: *Metarhizium anisopliae* strain 25; MA28: *Metarhizium anisopliae* strain 28. Different letters within each column indicate significant differences (Tukey test; $p \leq 0.05$).

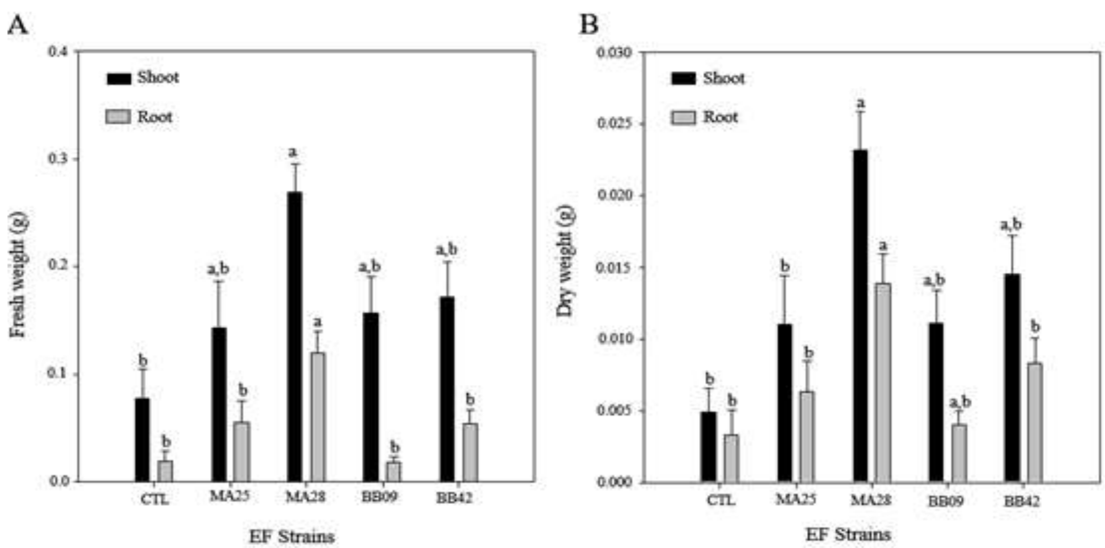

Figure 7. Effect on biomas production in potted chilli seedlings inoculated with entomopathogenic fungi. CTL: Control; BB09: *Beauveria bassiana* strain 09; BB42: *Beauveria bassiana* strain 42; MA25: *Metarhizium anisopliae* strain 25; MA28: *Metarhizium anisopliae* strain 28. Different letters indicate differences (Tukey test; $p \leq 0.05$).

other microorganism in the soil (Parsa et al., 2016).

Biological control of plant pathogens could complement chemical control, such as *P. chrysogenum* which can alter rhizosphere soil and becoming available soil nutrients and suppressing diseases in grasses (Murali et al., 2013). In this model fungal endophytes, that live asymptomatically within plant tissues without causing symptoms of disease (Khan et al., 2016), modulate plant defensive hormones, repressed jasmonic acid and salicylic acid pathways and produce alkaloids which are related with plant defence (Bastias et al., 2017). In *Capcisum annuum* L., actinomycetes isolated by medicinal plants improved a growth parameter and there is evidence that these fungi produced indole-3-acetic acid, chitinase, can solubilise inorganic phosphorous and produce HCN (Passari et al., 2015).

EF fulfil very different functions (defence against pathogens, nutrient acquisition, symbiotic interactions) so they must produce many secondary metabolites (Macheleidt et al., 2016). Plants colonized by EF caused significantly differential accumulation of metabolites and may also influence how plants respond to plant pathogen (Dastogeer et al., 2017), and some of them can act against diverse soil pathogens such as *Pythium spp*., *Rhizoctonia spp.* and *Fusarium spp*. affecting the canopy (Ownley and Gwinn, 2010). Isolates of *Metarhizium brunneum* and *B. bassiana* showed strong inhibition of the mycelial growth of olive pathogens *Verticillium dahlia* and *Phytophthora megasperma* (Lozano-Tovar et al., 2017). More important mechanism elicited by EF is induced systemic resistance (ISR), which includes reduction of disease symptoms in parts of the plant distant from the site where the inducing agent is active (Pieterse et al., 2014). It has been reported that *B. bassiana* induces systemic resistance against *Xanthomonas axonopodis* pv. *malvacearum* (bacterial blight) when inoculated on cotton and tomato seeds, and previously inoculated on tomato seeds, *B. bassiana* can induce resistance against soil pathogens such as *Rhizoctonia solani* and *Pythium myriotylum* (Ownley et al., 2008). Field research suggests that *B. bassiana* and *M. anisopliae* applicated as endophyte in maize showed suppression of maize stem borer damage caused by *Chilo partellus* Swinhoe reported reduction in steam tunnelling in maize plant, mainly due to systematic activity of this EF isolates (Ramanujam et al., 2017).

In the present study, seeds inoculated with EF prevented contamination by inhibiting the growth of seed borne fungi; therefore it can be employed as symbiotic insecticides that may offer protection against plant pathogens as well as others EF (Jaber and Ownley 2017).

Conclusion

EF used in this work could ensure optimal, safe germination and seedlings production, even if the seeds used were contaminated. In general, seeds inoculated with EF showed the biggest sizes and weights, and seedlings inoculated with BB42 and MA28 showed the seedlings with the biggest size and weight as shown in Figures 2 and 3). MA28 and BB42 strains would be an attractive alternative to be included in integrated pest management, but more research will be needed to determine other effects on the plant development and plant defence response.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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