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Formulation of *Trichoderma asperellum* TV190 for biological control of *Rhizoctonia solani* on corn seedlings

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Abstract

Environmental conditions affect biocontrol agents in a field, being appropriate formulations an alternative to overcome this problem. Formulations based on *Trichoderma asperellum* TV190 were prepared by emulsified mineral or vegetable oils, which protected spores from ultraviolet radiation, showing greater viability of 37–43% (mineral) and 56–63% (vegetable) than the control (8–12%). These formulations improved an antagonism of *T. asperellum* on *Rhizoctonia solani* under greenhouse conditions, reducing infected corn seedlings by 72% (mineral) and 59% (vegetable). Necrotic spot size was reduced by 90.04% (mineral) and 87.29% (vegetable). A granular formulation, prepared with degreased corn germ and *T. asperellum* spores, protected the corn seedlings from *R. solani* under greenhouse conditions, with 73% reduction of infected plants and 93% reduction of necrotic spot size. Both granular and liquid formulations were able to improve *T. asperellum* antagonism, suggesting that these formulations could be included in agricultural pest control strategies.

Keywords: *Zea mays*, *Trichoderma asperellum*, Antagonistic fungi, Granular formulation, Liquid formulation, Ultraviolet UV protection

Background

Corn (*Zea mays* L.) is one of the most important cereals in the world. The production of this crop is affected by biotic and abiotic factors, where phytopathogenic fungi play an important role by decreasing production levels and generating global economic losses. The phytopathogenic fungus *Rhizoctonia solani* (Kühn) is a major pest in corn attacking all belowground plant parts, including seeds, hypocotyls, and roots (Da Silva et al. 2017). In maize, *R. solani* is the causal agent of banded leaf and sheath blight, albeit other symptoms like stalk lesions (rind spotting), stalk breakage, clumping and caking of styles (silk fibers), horseshoe-shaped lesions with banding on caryopses, and sclerotial formation on styles, glumes, cupules, and caryopses have been reported

(Chander and Payak 1982). Consequently, it has required the implementation of control measures, especially the chemicals, which have environmental and health implications (Kim et al. 2017).

Application of living organisms for pest control is an alternative to the use of agrochemicals. Biological control of *R. solani* using *Trichoderma* spp. is a real alternative to the use of agrochemicals. In in vitro tests, antagonism was registered from *Trichoderma* spp. against this phytopathogen (Wang and Zhuang 2019). Field applications of *Trichoderma* spp. have demonstrated a positive effect on biocontrol of *R. solani* (Barnett et al. 2019). However, field environmental conditions are still one of the main limitations for the use of biocontrol agents (Bashan et al. 2014). Parameters such as ultraviolet radiation (UVR) (Costa et al. 2016), relative humidity (Swaminathan et al. 2016), temperature (Domingues et al. 2016), and storage conditions (Locatelli et al. 2018) could have a negative influence on inoculum viability. Using appropriate formulations is a way to overcome this problem, which create

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microclimates that protect spores from adverse environmental conditions (Doni et al. 2014).

Formulations of biological control agents can be classified in (a) dry powders, (b) granules, and/or (c) wettable powders. Additionally, the use of adherents such as oil, gelatin, and gum as well as humectants such as propylene glycol and polyethylene glycol has been used to reduce evaporation (Zhang et al. 2016).

Application of biocontrol agents using a suitable formulation that protects the inoculum against UV radiation is the main objective of this work, proposing liquid and granular formulations based on *Trichoderma asperellum* spores to control *R. solani* under in vitro and greenhouse conditions.

Materials and methods

Fungal strains and seeds

Trichoderma asperellum (TV190) isolated from maize fields of Monagas State, Venezuela (Pavone and Dorta 2015), was obtained from the Centro de Biotecnología Aplicada (CBA), Universidad de Carabobo, Venezuela. The strain was maintained by alternate subculture on sterile soil and potato dextrose agar (PDA) plates. *R. solani* strain P2AB2 (AG1-IA) was donated by Dr. Alex González (Fundación DANAC) San Felipe, Venezuela, and maintained by subculture on PDA plates. White corn seeds H2020 were donated by "Semillas Híbridas de Venezuela C.A." (SEHIVECA).

Granulated formulation

Degreased corn germ (DCG), donated by "Refinadora de Maíz Venezolana" (REMAVENCA) Aragua State, Venezuela, was sterilized at 120 °C for 30 min in an autoclave and inoculated with the necessary volume of a *T. asperellum* spore suspension in water (10^6 spores/g) to reach a final water content of 50% (w/w). Preparation was mixed and processed through a meat grinder with a 4-mm screen. Finally, the preparation was air-dried to a moisture content of 8% (w/w) and stored at 8 °C for 24 h until use.

Liquid formulation

Formulations were prepared using 20 ml of vegetal oil (VO) Vatel®, 10 ml of Surfatron®, and 970 ml of water. Alternatively, emulsified mineral oil (MO) Aceite Blanco® was diluted in water according to the manufacturer's instructions (20 ml/l). Lignosulfonate (1% w/v) was also added as UVR filter. Concentration of *T. asperellum* was adjusted to 10^6 spores/ml. A *T. asperellum* spore suspension in pure water was used as the negative control. Liquid formulations were used immediately after preparation.

Viability of *T. asperellum* spores

Water agar plates (1.7% w/v) were inoculated with 0.1 ml spore suspensions (formulated or not). Plates were incubated at 25 ± 2 °C for 20 h in the dark. Germinated

spores were counted, using a stereoscopic microscope ($\times 400$), in 3 individual plates (replicates), 100 spores in each one.

Effect of ultraviolet radiation (UVR) on *T. asperellum* spores

In plates prepared as in viability assays, spores were exposed to UVR in uncovered plates. Exposition to UVR was performed using a lamp Model UVLMS-38, UVP® (Ultra-Violet Products), with wavelengths of 254 nm (UV-C) and 302 nm (UV-B) applied separately. Intensities used for UV-B and UV-C were $1900 \mu\text{W}/\text{cm}^2$ and $250 \mu\text{W}/\text{cm}^2$ for 1, 2, and 5 min, respectively. UVR measurements were performed by a UVP® Model UVX radiometer, equipped with 3 sensors (Models UVX-25, UVX-36, and UVX-31). After irradiation, plates were incubated for 18–24 h and viability was evaluated.

Effect of liquid and granular formulations on *R. solani*

White corn seeds were planted in bags (15 cm diameter \times 20 cm tall) in soil, and irrigated daily. After 10 days, a *R. solani* sclerotia, obtained from PDA plates grown for 8 days, was placed on a plant bud. Immediately, 1.5 ml of the liquid formulations (emulsions with 10^6 *T. asperellum* spores/ml) or a granule of the solid formulation (approximately 0.1 g) was added to the infected bud. Water was sprayed daily to maintain high relative humidity. Plants with *R. solani* sclerotia without *Trichoderma* and emulsions or granules without *T. asperellum* spores were used as controls. Treatments consisted of 30 plants. After 3 to 8 days in open greenhouse conditions (average temperature 30 °C), the number of infected plants and the necrotic spot size produced by *R. solani* on leaf were evaluated. The reduction in the number of infected plants $[(1 - \frac{\text{number of infected plants in treatments}}{\text{number of infected plants in control}}) \times 100]$ and the decrease in necrotic spot size (NSS) caused by *T. asperellum* $[(1 - \frac{\text{NSS in treatments}}{\text{NSS in control}}) \times 100]$ were also calculated.

Despite *R. solani* is a soil-borne pathogen, sclerotia was inoculated on the plant bud because it demonstrated a high infection rate on corn plants than the experiments inoculating *R. solani* on soil, with very clear and evident symptoms. Additionally, using *R. solani* as leaf pathogen was more suitable to evaluate the effect of UVR. This method is very convenient albeit it is clear to be only an approach previous to field validation on natural conditions.

Statistical analysis

Mean and standard error were calculated for each treatment. Variance analysis (ANOVA) was performed in order to detect significant differences between treatments. In case of not fulfilling the assumptions for the ANOVA, non-parametric tests were performed (Kruskal-Wallis). A

comparison of means (Tukey) was also performed. The statistical package used was Past 3.1 (Hammer et al. 2001). All experiments were repeated three times.

Results and discussion

Oil formulations

One of the first steps for implementing the formulations with biocontrol fungi was to determine its compatibility with the microorganisms involved in the study. The percentage of *T. asperellum* TV190 spore germination in oil formulations without irradiation were 95.32, 96, and 97.66%, for the mineral oil (MO), vegetable oil (VO), and control, respectively (Figs. 1 and 2). It was observed that *T. asperellum* and emulsions seemed to be compatible, with non-significant differences ($F = 0.018$; $df = 4$; $P = 0.99$) between spore germination in formulations with mineral or vegetable oil. For the treatments with spores, exposed to UVR, a negative effect was evident on spore viability. Statistical differences were found in treatments with MO under UVB ($F = 109$; $df = 32$; $P < 0.05$) and UVC ($F = 132.82$; $df = 32$; $P < 0.05$) and VO under UVB ($F = 176.3$; $df = 32$; $P < 0.05$) and UVC ($F = 132.39$; $df = 32$; $P < 0.05$). In general, UVR decreased spore viability to 8–12% levels after 5 min of treatment on unformulated *T. asperellum* spores (NF), the effect being dependent on time exposure (Figs. 1 and 2).

However, in systems treated with oil formulations, partial protective effects were detected. After 5 min UVR exposure, viability decreased to 43–56% in systems with MO and to 56–63% in VO systems (Figs. 1 and 2). Once again, the negative effect was dependent on the exposure time. In treatments with lignosulphonate, non-significant differences were detected in the combination with VO under the same time of exposure to UVR ($F = 0.16$; $df = 5$; $P = 0.71$) (Fig. 2), while in the treatments with MO, differences were detected only when spores were exposed to UVB ($F = 24.89$; $df = 5$; $P < 0.05$) or UVC ($F = 4.84$; $df = 5$; $P < 0.05$) for 5 min (Fig. 1).

Results strongly suggested a protective effect of oil formulations and lignosulphonate to *T. asperellum* spores, when irradiated with UVR under laboratory conditions. Exposure time was a crucial parameter because spore viability was drastically reduced, when exposure increases from 1 to 5 min. A greater negative effect of UV-C radiation was also evident in spore viability. The UVR effect on fungal metabolism was related to DNA degradation in conidia and mycelium of *Aspergillus nidulans* (Braga et al. 2015). In addition, Seyedmousavi et al. (2014) reported that UVR affected proteolytic activity, cell growth, and carbohydrate synthesis in *Candida albicans*. Besides, it had also been reported that UV-B inhibited various fungal processes such as spore germination and hyphae elongation (Suthaparan et al. 2016) and affects negatively several fungi such as

Botrytis cinerea (Janisiewicz et al. 2016). Mutagenesis has been induced using UVR to obtain modifications in the genetic structure of two *Trichoderma* biocontrol agents, *T. virens* and *T. asperellum* (Alfiky 2019). If exposure time was longer, DNA damage will be great, producing a higher rate of mutations and reduced spore germination (Begum et al. 2009). UV-C radiation reduced the spore germination by more than 80% (Bell and Wheeler 1986). A decrease in spore viability by UVR depends on spore coloration, the medium in which it was evaluated, and time of exposure to radiation, the darker the spore, the greater its resistance to UV radiation, probably due to melanin that protects it from this radiation (Carzaniga et al. 2002). The photoprotective properties of melanin were considered to be important for the survival and longevity of spores (Bell and Wheeler 1986).

A protective effect of oil formulations against UVR has also been reported in other studies (Fernandes et al. 2015), observing mineral and vegetable oil protection on entomopathogenic fungi spores against UVR. Several oil-based formulations with *T. asperellum* have been developed to control cacao black pod disease caused by *Phytophthora megakarya*, in which the half-life of the conidia reached 22.5 and 5 weeks in aqueous and oil suspension, respectively (Mbarga et al. 2014). Oil and aqueous formulations have been proven to control frosty pod rot caused by *Moniliophthora roreri* on cocoa (Crozier et al. 2015), finding that an inverted corn oil formulation significantly enhanced cocoa yield, providing a promising model for optimizing *Trichoderma*-based biocontrol strategies. Finally, some vegetable oils are able to absorb UV radiation (Montenegro and Santagati 2019) suggesting the possibility to use them as UV blockers.

Greenhouse assays

The ability of *T. asperellum* formulations (granular and liquid) to control *R. solani* was evaluated by determining the number of infected plants and necrotic spot size (NSS) on corn leaves. Results showed statistical differences in treatments with *T. asperellum* ($F = 1875.892$; $df = 29$; $P < 0.05$) producing a decrease in the number of infected plants after being treated with *T. asperellum* (Fig. 3). The number of infected plants was similar (70%) in treatments with oil formulations (MO and VO) and with granules (G), both without *T. asperellum*, and in the treatment with *R. solani* alone (R) ($F = 13.63$; $df = 8$; $P = 0.2$). After applying *T. asperellum* spores to oil formulations (MOT and VOT) or to granules (GTR), the percentage of infected plants decreased to 20, 29, and 19%, respectively. The number of infected plants was reduced by 72% (MOT), 59% (VOT), and 73% (GTR).

Necrotic spot size (NSS) produced by *R. solani* on corn seedlings was another evaluated parameter (Fig. 4). Treatments without *T. asperellum* resulted in spot sizes

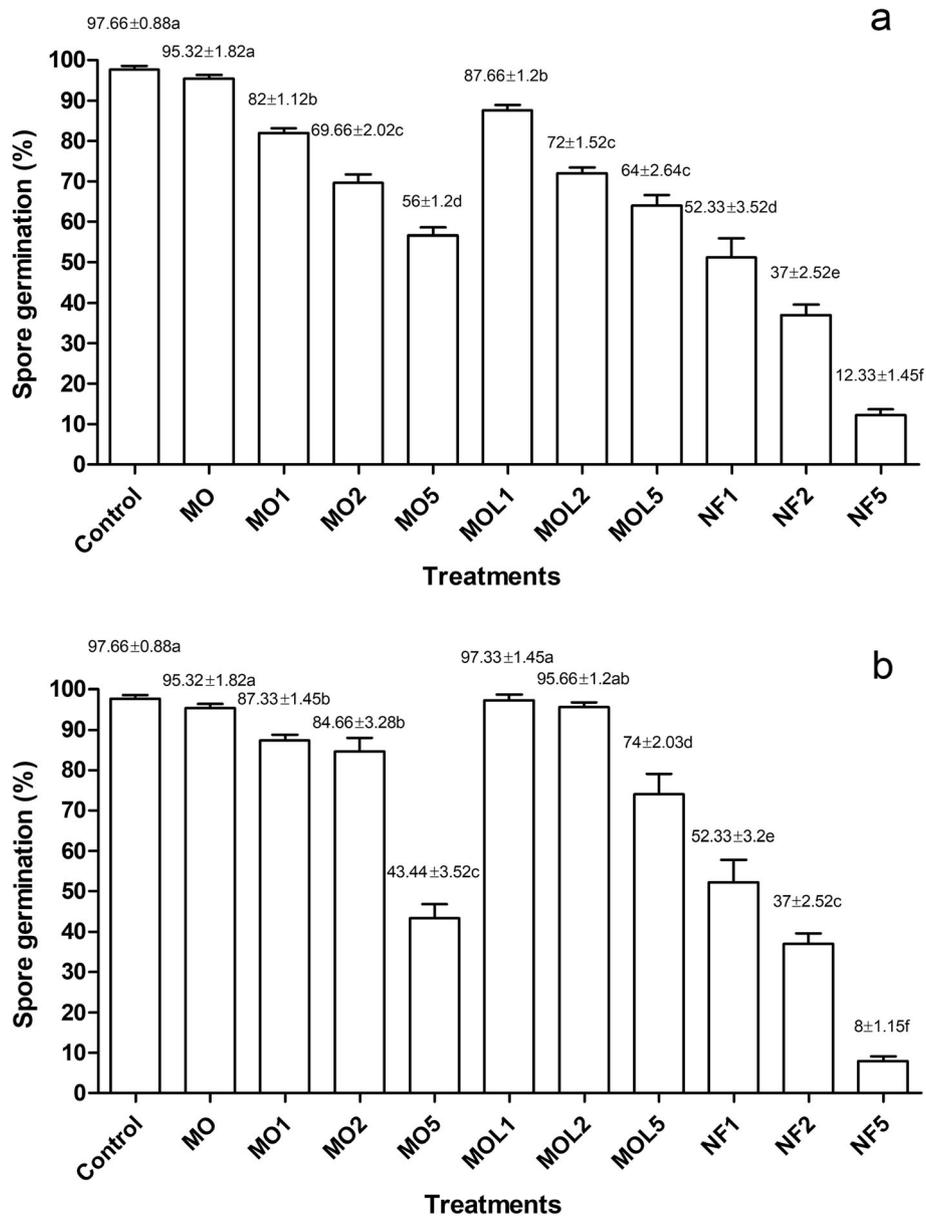


Fig. 1 Viability of *Trichoderma asperellum* spores in formulations with mineral oil (MO) and exposed to UVR. **a** Spores in MO + UV-C. **b** Spores in MO + UV-B. MO, spores + MO without UVR; MOL, MO + lignosulfonate without UVR; NF, no formulation. Numbers in each treatment indicate exposure time to UVR (minutes). Letters indicate statistical differences ($P < 0.05$)

larger than 20 mm (MO = 26.8 mm, VO = 28.31 mm, GR = 20.33 mm, R = 27.53 mm). In contrast, by using *T. asperellum* without formulation, NSS was 12.23 mm. When *T. asperellum* was included in oil formulations, spot size decreased to 2.67 mm (MOT) and 3.6 mm (VOT), with significant differences in relation to other treatments ($H = 113.972$; $df = 9$; $P < 0.05$), but not with each other ($F = 0.222$; $df = 1$; $P = 0.64$). The decrease in spot size caused by *T. asperellum* was 55.58%, when applied alone, compared to 90.04 and 87.29%, when

applied with MO or VO, respectively. In GTR formulation, spot size decreased to 1.36 mm (93.32%).

In greenhouse assays, *R. solani* incidence was evaluated when liquid and granular formulations were applied. However, it is not possible to determine if the effect observed is due to the protection they exert against the UVR, since it was not possible to determine how much radiation these spores received. Obtained results were similar to those of Battan (2004) who used an oil formulation of *T. harzianum* to evaluate its

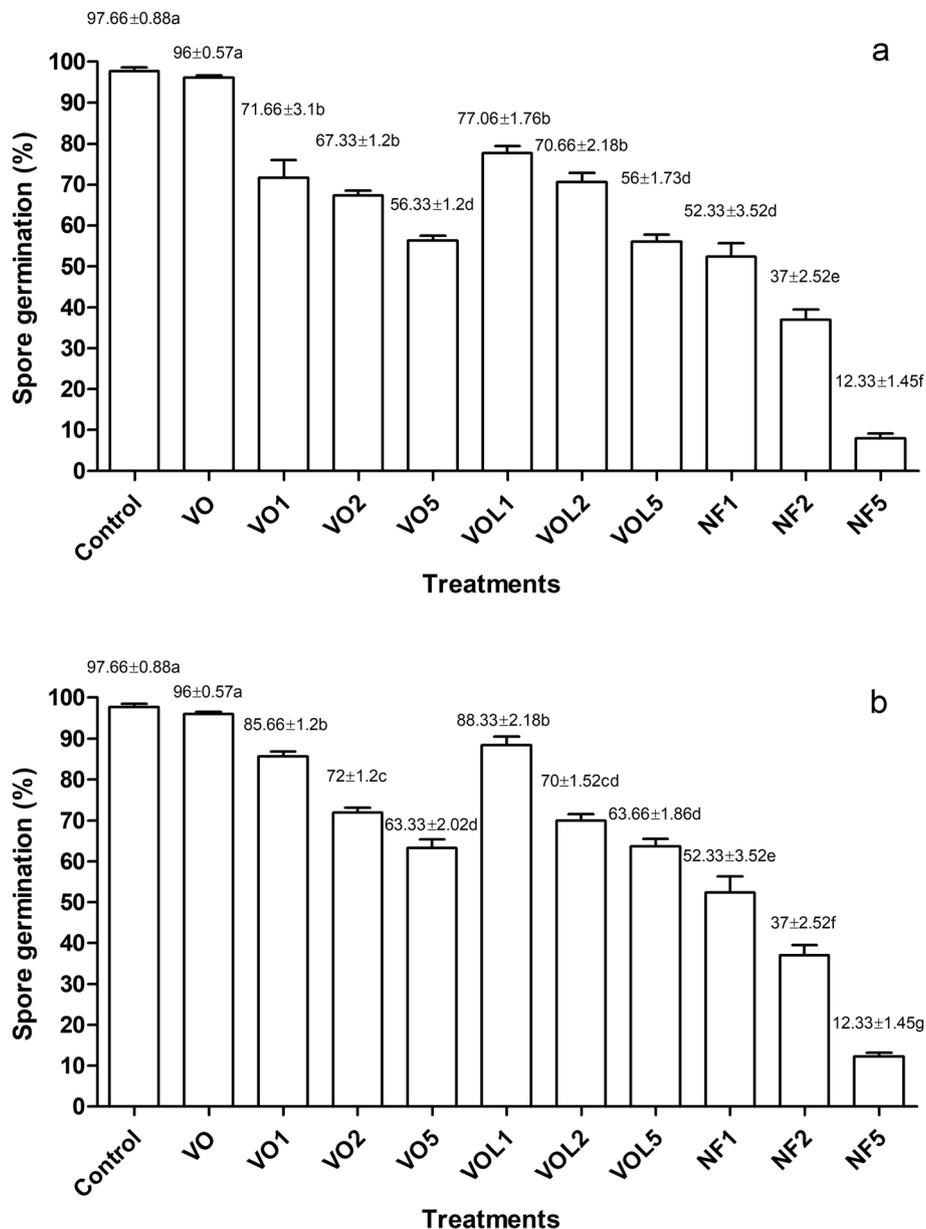


Fig. 2 Viability of *Trichoderma asperellum* spores in oil formulations with vegetable oil (VO) and exposed to UVR. **a** Spores in VO + UV-C. **b** Spores in VO + UV-B. VO, VO without UVR; VOL, VO + lignosulfonate without UVR; NF, no formulation. Numbers in each treatment indicate exposure time to UVR (minutes). Letters indicate statistical differences ($P < 0.05$)

biocontrol effect on *Rhizopus stolonifer*, *Botrytis cinerea*, and *Penicillium expansum*, fungi that affected apple, peach, pear, and strawberry. Although it was not studied in this work, one of the objectives of granular formulation was to serve as a substrate for fungal sporulation to increase spore number. The substrate used for granular formulation (DCG) has been characterized by the manufacturer containing 65.06% carbohydrates, 13.82% protein, 10.97% water content, 5.37% crude fiber, 4.19% of ash, and 0.59% of fats. It was also rich in minerals such as phosphorus, magnesium, iron, and zinc. DCG could be an

excellent substrate for sporulation of several filamentous fungi. In this sense, a granulated formulation with DCG using the entomopathogenic fungus *Nomurea rileyi* spores was used to increase inoculum over 600 times and to protect spores from UVR (Pavone et al. 2009), probably because spores were immersed within a granule matrix, where UVR cannot reach them. Protection exerted by granule against UVR could be important for maintaining inoculum viability in the field.

Extruded granular formulations, containing rice flour, gluten, and biomass of *Gliocladium virens* and *Trichoderma*

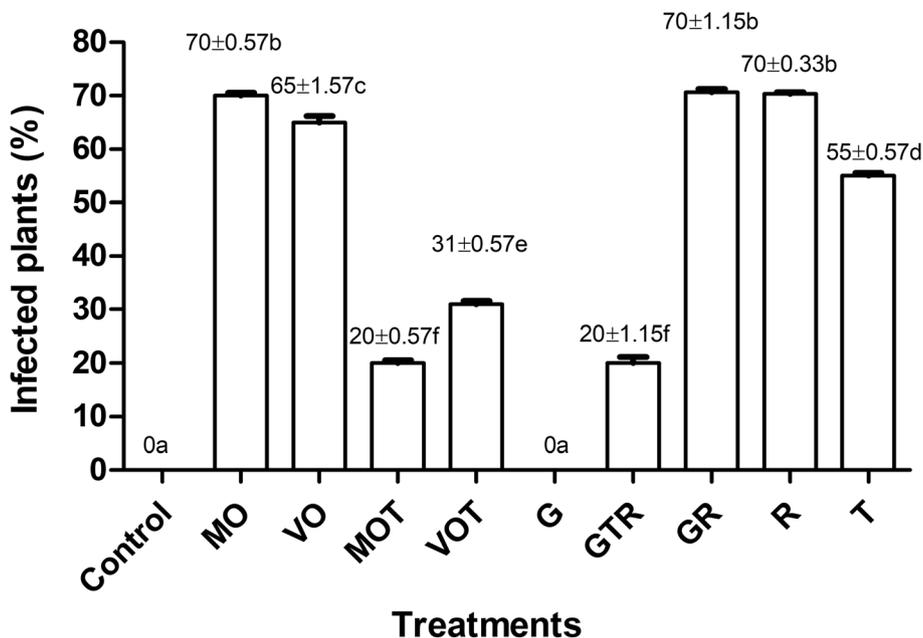


Fig. 3 Corn seedlings infected with *R. solani*. MO, mineral oil without *Trichoderma asperellum* or *Rhizoctonia solani*; VO, vegetable oil without *T. asperellum* or *R. solani*; MOT, mineral oil with *T. asperellum*; VOT, vegetable oil with *T. asperellum*; G, granular formulation without *T. asperellum* or *R. solani*; GTR, granular formulation with *T. asperellum*; GR, granular formulation without *T. asperellum*; R, *R. solani* without treatment; T *T. asperellum* without formulation. Letters indicate statistical differences

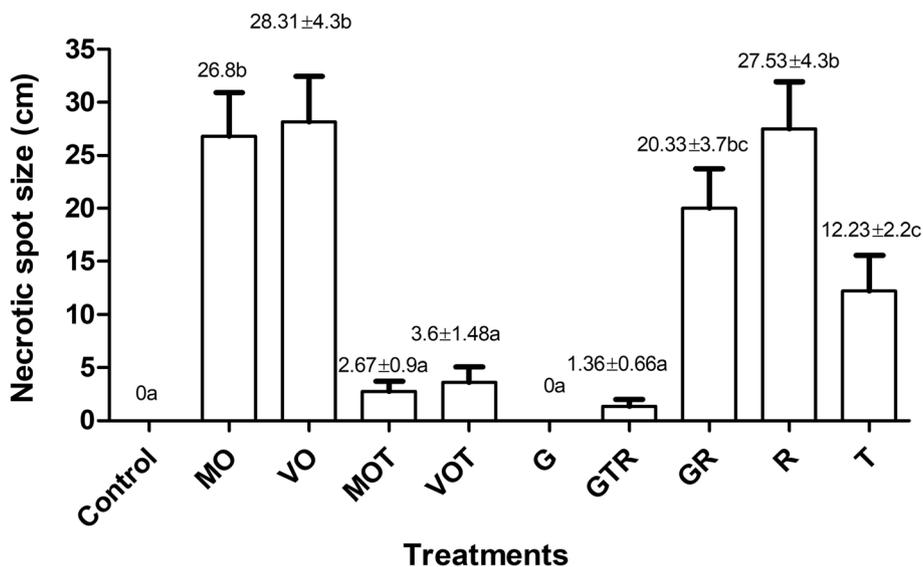


Fig. 4 Necrotic spot size produced by *Rhizoctonia solani* in corn seedlings. MO, mineral oil without *Trichoderma asperellum* or *R. solani*; VO, vegetable oil without *T. asperellum* or *R. solani*; MOT, mineral oil with *T. asperellum*; VOT, vegetable oil with *T. asperellum*; G, granular formulation without *T. asperellum* or *R. solani*; GTR, granular formulation with *T. asperellum*; GR, granular formulation without *T. asperellum*; R, *R. solani* without treatment; T, *T. asperellum* without formulation. Letters indicate statistical differences

spp. among other components reduced eggplant damping-off caused by *Rhizoctonia solani* (Lewis and Larkin 1997). Formulations prepared with several components like talc and lignite were produced for seed treatment and control of tomato damping-off caused by *Pythium aphanidermatum*, in which active colonization of *T. harzianum* in the rhizosphere was observed (Jayaraj et al. 2006). Microencapsulation has been proposed to prolong shelf life and enhance application efficiency of *Trichoderma* (Cumagun 2014) focusing on seed treatment using solid matrix priming, liquid coating, and double coating.

It will be important to test these formulations under field conditions to obtain conclusive results that will allow their use as commercial products. Formulation compatibility with other control measures such as insecticides and herbicides commonly used in field should also be evaluated. Due to its mode of action and good performance under in vitro and greenhouse conditions, protecting spore against UVR and plants from *R. solani*, oil and granular formulation seems to have great potential to be incorporated in Integrated Pest Management Programs.

Conclusions

Obtained results evidenced a great potential to use *T. asperellum* in liquid and granular preparations. Oil formulations efficiently protected *T. asperellum* spores from UVR (UV-B and UV-C) in vitro. Lignosulphonate enhanced spore protection against UVR, only when applied with MO. It was also verified that oil and granulated formulations improve *T. asperellum* performance in protecting corn seedlings from *R. solani* attack under greenhouse conditions. Fungal formulations should become a standard in biocontrol applications in order to increase efficacy.

Abbreviations

DCG: Degrease corn germ; G: Granular formulation without *T. asperellum* or *R. solani*; GR: Granular formulation without *T. asperellum* with *R. solani*; GTR: Granular formulation with *T. asperellum* and *R. solani*; MO: Mineral oil; MOL: Mineral oil with lignosulfonate; MOT: Mineral oil with *Trichoderma*; NF: No formulation; NSS: Necrotic spot size; PDA: Potato dextrose agar; R: *R. solani* without treatment; T: *T. asperellum* without formulation with *R. solani*; UVR: Ultraviolet radiation; VO: Vegetable oil; VOL: Vegetable oil with lignosulfonate; VOT: Vegetable oil with *Trichoderma*

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Authors' contributions

WH: Collection and/or assembly of data; data analysis and interpretation; writing the article; critical revision of the article. OV: Writing the article; critical revision of the article. DP: Research concept and design; data analysis and interpretation; writing the article; critical revision of the article. The authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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