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RESEARCH ARTICLE

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Optimizing wheat seed treatment with entomopathogenic fungi for improving plant growth at early development stages

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Abstract

Aim of study: Entomopathogenic fungi (EPF) are biocontrol agents, plant growth promoters, and increase tolerance to biotic-abiotic stresses. In this study we investigated the factors associated to the application method, which are crucial for the interaction between the fungus and the host plant at initial crop growth stages.

Area of study: The study was performed in Cordoba (Spain)

Material and methods: Three experiments were performed to investigate: (i) the effect of different concentrations of the surfactant Tween® 80 (0, 0.5, 1, 5, and 10%) on wheat seed coating with conidia of *Metarhizium brunneum* and seed and conidia viability; (ii) the performance of wheat seedlings at first growth stages after their inoculation with *Beauveria bassiana* or *M. brunneum* via seed coating or soil drenching; and (iii) the role of soil sterilization and seed disinfection on leaf concentration of chlorophyll (SPAD) and *B. bassiana* or *M. brunneum* colonization.

Main results: Tween® 80 concentration linearly improved seed coating (up to 127%) without altering wheat seeds and fungal conidia germination. Seedling length of inoculated plants was significantly increased with *B. bassiana* and *M. brunneum* (67% and 46%, respectively) via seed coating. Seed disinfection was key to achieve an enhancement in wheat SPAD (10-18%) with *B. bassiana* or *M. brunneum* concerning Control, that combined with sterilization of soil showed the highest endophyte colonization rates (up to 83.3% with both fungi).

Research highlights: The surfactant concentration, application method, seed disinfection, and soil sterilization are key parameters to improve the potential benefits on the EPF-plant relationship.

Additional key words: *Beauveria bassiana; Metarhizium brunneum*; seed inoculation; soil drenching; SPAD; cotyledon.

Abbreviations used: CECT (Spanish collection of culture types); D (disinfected seed); DAS (days after sowing); EPF (entomopathogenic fungi); LSD (least significant differences); ND (non-disinfected seed); NS (non-sterilized soil); ORI (Origuero farm soil); S (sterilized soil); SDCA (Sabouraud dextrose chloramphenicol agar); SDCA+D (SDCA + dodine); SDW (sterile deionized water); SPAD (single photon avalanche diode).

Authors' contributions: Conception and design of the experiment; data collection: AGG and MYY. Analysis of data AGG and ARSR. All authors interpreted the results and wrote, prepared, read and approved the final manuscript.

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Introduction

The use of microorganisms as a green alternative to pesticides or fertilizers is increasing in the last 20 years. Some of them act as biological control agents, plant growth promoters, biofortifiers, or even as a tool to alleviate drought stress in staple crops (maize, wheat, rice, etc) (Berg, 2009; Behie & Bidochka, 2013; Panpatte *et al.*, 2017; Quesada-Moraga *et al.*, 2020). Entomopathogenic fungi (EPF), a group of microorganisms used as biological control agents, have demonstrated that they play other roles beyond pest control providing the plant with additional benefits for better response to biotic and abiotic stresses (Quesada-Moraga *et al.*, 2019, 2020). Regarding the role of endophytic EPF in protecting the plant against abiotic stresses, they can help the plant under water (Dara *et al.*, 2017; Dara, 2019) and salt stresses (Khan *et al.*, 2012), enhance plant growth (Maniania *et al.*, 2003; Krell *et al.*, 2018a; Sánchez-Rodríguez *et al.*, 2018), plant nutrient uptake (Behie & Bidochka, 2014; Sánchez-Rodríguez *et al.*, 2015, 2016; Krell *et al.*, 2018b) or stimulate plant root length (Raya-Díaz *et al.*, 2017a).

Beauveria bassiana (Bals.) Vuill (Ascomycota; Hypocreales) and *Metarhizium brunneum* (Petch) (Ascomycota; Hypocreales) are two of the most studied EPF due to their potential and efficacy to control a wide range of insect pests (Quesada-Moraga *et al.*, 2020). Both EPF are described as endophytes, which means that they can inhabit the internal tissues of a plant without affecting it negatively (Hallmann *et al.*, 1997; Lugtenberg *et al.*, 2016). However, to optimise the success of the colonization, several factors should be considered such as the species –or even strain– of the microorganism and host plant, type of soil, weather conditions, fungal dose, and method of application, among others (González-Guzmán *et al.*, 2020a,b). Whilst the EPF must be applied following pest management decisions, it is key to elucidate under which environmental conditions and the extent to which the relationship plant-entomopathogenic fungus is facilitated to better exploit the full potential of the endophytes for plant nutrition and yield.

One of the multiple factors to be considered is the inoculation method of the host plant with the EPF endophyte, with soil inoculation, spraying to the aerial biomass of the host plant, and seed coating (seed dressing, film coating, and pelleting) as the three main explored application methods (Quesada-Moraga *et al.*, 2019, 2020; Rivas-Franco *et al.*, 2019).

Noteworthy, seed coating is known as an insecticide application technology with many advantages as higher economic viability, accurateness, and sustainability (Deaker *et al.*, 2004; Adholeya *et al.*, 2005; Vosátka *et al.*, 2012; Rocha *et al.*, 2019). Indeed, considering that smallholder families live on farms, which in many countries are significantly small (≤2 hectares; FAO, 2015), the methodology of applying the microorganisms inoculum must be as easy as possible and at the same time affordable. Different formulations have been investigated to enhance their viability, durability, and pathogenicity (Burges, 1998); however, few studies testing the different methodology to increase the conidial adherence to the seed have been performed. This should be addressed to enhance plant-fungus interaction because an increase in conidia adhesion may favour the percent of inoculated plants and the effectiveness of the fungal treatment.

In addition, most of the experiments in which EPF are applied to a crop have assessed fungal behaviour, plant colonization, plant growth, and plant nutrition in sterilized soils, artificial substrates, with seeds externally disinfested or supplying enough nutrients to avoid any nutrient deficiency (Garrido-Jurado *et al.*, 2011; Raya-Díaz *et al.*, 2017a; Sánchez-Rodríguez *et al.*, 2018). On the other hand, few studies include a comparison between different combinations of the conditions mentioned above (Kessler *et al.*, 2003; Partida-Martínez & Heil, 2011).

Wheat –a staple food worldwide– has shown a positive response in terms of growth promotion and nutritional enhancement when inoculated with EPF (Raya-Díaz *et al.*, 2017a; Bamisile *et al.*, 2018; Sánchez-Rodríguez *et al.*, 2018). However, many knowledge gaps, among which are surfactant and inoculum concentration, application methods, and plant species (Rocha *et al.*, 2019) must be elucidated.

Therefore, the objectives of this study were to: (1) assess the effect of the surfactant (Tween® 80) concentration on the efficiency of coating wheat seeds with EPF, and on the germination of both seed and conidia; (2) evaluate different inoculation methods (seed coating and soil drenching) on the initial crop growth stage; and (3) assess the efficiency of colonization rate and wheat performance under different combinations of environmental conditions (sterilization of soil and seed disinfection) with *B. bassiana* EABb04/01-Tip and *M. brunneum* EAMa01/58-Su strains by seed coating at early growth stages. We hypothesized that high doses of surfactant would increase conidia adhered to the seed but potentially could suppress seed germination, while different application methods –that add different amounts of conidia to the system– would produce a different wheat response, being wheat performance and fungal colonization rate increased in sterilized soils when seeds were initially disinfected.

Material and methods

Fungal origin and preparation

The *B. bassiana* and *M. brunneum* strains (EABb 04/01-Tip and EAMa 01/58-Su) used in these experiments belong to the culture collection of the Agricultural Entomology unit (AGR-163, Dept. of Agronomy),

University of Cordoba, Spain. They were originally isolated from a soil where wheat was grown and from a dead *Iraella luteipes* larva in Cordoba and Seville (Spain), respectively. Both strains were deposited in the Spanish collection of culture types (CECT) with accession numbers CECT 20744 and CECT 20764 following the Budapest Treaty (Quesada-Moraga *et al.*, 2009; Yousef *et al.*, 2013). Both strains had demonstrated endophytic behaviour (Quesada-Moraga *et al.*, 2009; Garrido-Jurado *et al.*, 2016). To obtain conidia, slant cultures of the isolates were subcultured on Sabouraud dextrose chloramphenicol agar (SDCA; Biolife) supplemented with 15 g L^{-1} of Roko agar for 15 days at 25 °C in darkness. After that, conidia were scraped and transferred to a sterile aqueous solution of 0.5% Tween® 80. This suspension was stirred vigorously for 5 min to homogenize it, and then, the concentrations of conidia were calculated using a Malassez chamber.

Experiment 1: Effect of Tween® 80 concentration on the efficiency of seed dressing method and germination of wheat seeds

Five suspensions with different concentrations of Tween® 80 [0, 0.5, 1, 5, and 10% (v/v)] and a concentration of 1×10^8 conidia mL^{-1} of *M. brunneum* strain EAMa 01/58-Su were prepared to evaluate the effect of Tween® 80 concentration within the sterile aqueous solution on the conidia adhesion. Four replicates were used, each one composed of 5 seeds of *Triticum aestivum* var. 'Chinese Spring', to which 1 mL of each fungal suspension was added in a 1.5 mL Eppendorf tube. The tubes were stirred for 1.5 h at 1.5 Hz in a rotational shaker (Rotabit, J.P. SELECTA). The seeds were then removed from the tubes and dried under a sterile airflow. To count the conidia adhered to its surface, the seeds from each Tween® 80 concentration were transferred to different 1.5-mL Eppendorf tubes containing 1 mL of sterile deionized water (SDW), which were vigorously stirred in a Vortex for 20 sec, sonicated 5 min, and stirred again for 1 min in a Vortex. Then, a 100 µL aliquot was charged into the Malassez chamber to count the number of conidia mL^{-1} to calculate conidia adhered per seed. To assess seed germination, 3 out of the 5 seeds per treatment (different Tween® 80 concentrations) were plated in Petri dishes with SDCA. Additionally, a seed germination test was done to evaluate the different concentrations of Tween® 80 (n = 12 seeds per concentration dose) with no fungal application, following the same protocol but using SDCA with methyl violet to easily observe radicle growth. The viability of conidia was observed from an aliquot of the same suspension where conidia were released from coated seeds, after keeping these Eppendorf tubes at ~25 °C temperature for 48 h. The aliquot was charged in a Malassez chamber and the number of conidia producing germ tubes was counted in the microscope.

Experiment 2: Effect of different EPF application methods in wheat growth at first stages of the crop

The same two strains of *B. bassiana* and *M. brunneum* used in Exp. 1 were applied to wheat before analysing the germination of wheat seeds and the growth of the seedlings in the first growth stages under sterile conditions. Three fungal treatments were used, soil drenching, seed coating, and no fungal application (Control). Before the application of these treatments, a total of 75 seeds were externally disinfected with 5% NaClO for 5 min and then immersed in SDW twice. For seed coating, a group of 15 seeds was stirred in a suspension with a concentration of 1×10^8 conidia mL^{-1} of *B. bassiana* plus 0.1 % Tween® 80 for 1 h. This was repeated for a second group of 15 seeds with *M. brunneum*. After that, seeds were plated in independent 15-cm diameter Petri dishes filled with 20 g of sterilized perlite and then covered by a sterilized filter paper. For soil drenching, the perlite was inoculated by adding 10 mL of fungal suspension (*B. bassiana* or *M. brunneum*) in the same above-mentioned Petri dishes. Then, a group of 15 seeds was placed in the Petri dishes, doing the same with both fungi, separately. Finally, a group of 15 seeds was placed in a new Petri dish and 10 mL of water plus Tween 0.1% was added to use as a Control. The perlite of the five Petri dishes used in this experiment was moistened with 10 mL of SDW before seed placement. The number of seeds that germinated was counted and the length of the cotyledon measured 11 days after sowing (DAS).

Experiment 3: Seed disinfection and soil sterilization effects on leaf chlorophyll content of wheat treated with *B. bassiana* and *M. brunneum*

Three factors were assessed in this experiment: the inoculation with different fungi (Control-no fungus, *B. bassiana*, and *M. brunneum*), the soil conditions in which the plants are grown (sterilized and non-sterilized), and seed sterilization (disinfected and non-disinfected) resulting in a total of 12 combinations (Fig. 1) with 6 pots per combination and two plants per pot (experimental unit).

Soil, pots, and seeds

The soil used in Exp. 3 was collected from the topsoil (0-20 cm) in the Origuero farm, in the province of Córdoba, southern Spain (37°50'40.6" N, 4°45'27.0" W). This soil is representative of a large area of Vertisols in the

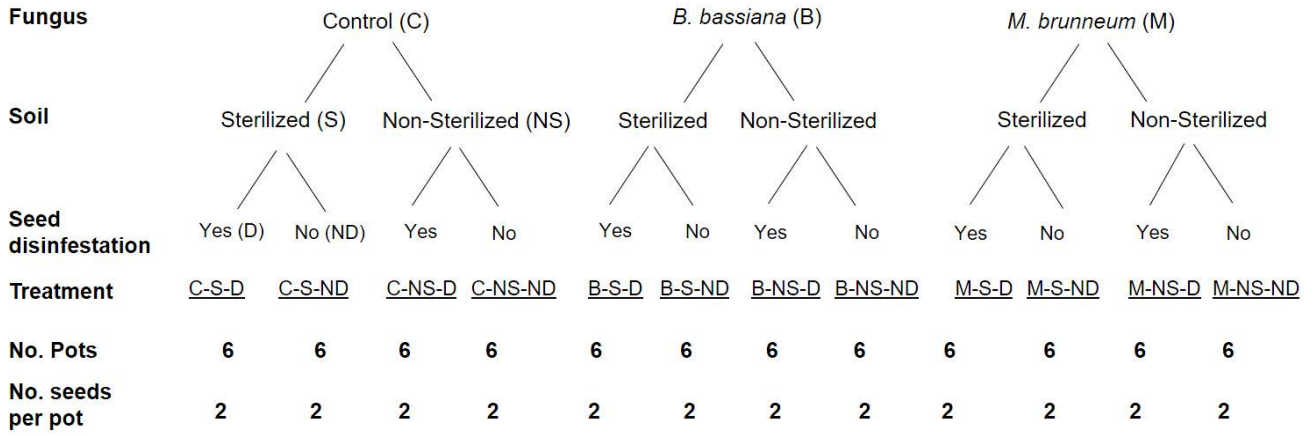

Figure 1. Experimental design scheme of Experiment 3. The 12 combinations of 6 replicates (pots) with 2 plants in each pot (pseudo-replicates) are shown. Abbreviations: C, Control (non-treated); B, *B. bassiana*; M, *M. brunneum*; S, sterilized soil; NS, non-sterilized soil; D, disinfected seed; ND, non-disinfected seed.

Guadalquivir River Valley and it is typically used for the wheat-sunflower rotation. An 8 kg composite soil sample was collected and homogenized, air-dried for one week, and sieved through a 0.5-cm pore size mesh. A subsample of 1 kg was grounded to pass through a 2-mm pore size mesh and used to analyse the main physical and chemical properties (see description in the Annex [suppl]). Falcon® 50 mL conical tubes with a 3-mm drainage hole at the bottom and a cotton wick through it (used to water the soil contained in the tube) were used as pots. A total of 72 pots were filled with 46 g of soil per pot and half of them were autoclaved twice for 20 min at 121 ºC with an interval of 24 h between each (S = sterilized soil; NS = non-sterilized soil). The pots were placed in racks (18 pots per rack), which were immersed in trays containing Hoagland solution [5 mM $(NO_3)_2 \cdot 4H_2O$, 5 mM KNO_3, 2 mM $MgSO_4$, 0.1 µM KCl, 0.3 µM $Ca(H_2PO_4)_2 \cdot H_2O$; 50 µM H_3BO_3, 4 µM $MnSO_4 \cdot H_2O$, 4 µM $ZnSO_4 \cdot 7H_2O$, 10 mM Fe (as EDDHA), 0.1 µM $CuSO_4 \cdot 5H_2O$ and 6 µM Na_2MoO_4] until the upper layer of the soil was wet. Then, the racks were removed from the trays and the soils were let drain for 24 h. Seeds of *T. aestivum* var. 'Chinese Spring' were used in this experiment, half untreated (ND) and the other half externally disinfested (D) with 70° ethanol and 3.7% bleach (NaClO) for 5 min each, then washed with SDW twice and let them dry out under sterile conditions. Finally, two seeds were sown in each of the 72 pots, previously inoculated with the same strains of *B. bassiana* or *M. brunneum* used in the other experiments (Fig. 1).

Analysis of natural presence of target fungi in soil and seed before the experiment

The natural presence of *B. bassiana* and *M. brunneum* was analysed in the soil after collection. A homogenized subsample of 1 g (3 replicates) was mixed with 10 mL of SDW containing 0.1% of Tween® 80 and the resulting suspension was stirred for 45 min at 2 Hz in a rotational shaker, and 100× and 1000× dilutions were prepared from it. Then, six 100 µL aliquots (three from each dilution) were plated in Petri dishes containing SDCA supplemented with 0.8 g L^{-1} of dodine (SDCA+D). Additionally, the absence of the target EPF inside the wheat seeds before fungal inoculation was ensured by using 35 seeds that were externally disinfested, as indicated in Exp. 2, and cut longitudinally and transversely before plating them on SDCA. Either soil or seeds were free of the target fungi as expected.

Fungal inoculation

Once the lack of *B. bassiana* and *M. brunneum* was ensured in soil and seeds, two conidia suspensions were prepared –obtained as described in Exp. 2– in SDW with Tween® 80 (0.1%) and the conidia concentration adjusted to 1×10^8 conidia mL^{-1} using the Malassez chamber. Two groups of 72 wheat seeds were prepared. The seeds of the first one were externally disinfected (D) while the seeds of the second group were non-disinfected (ND). The seeds in each group (24) were immersed in one of the two fungal suspensions (*B. bassiana* or *M. brunneum*) and the same number of seeds in water with 0.1% Tween® 80 for the Control treatment (C, without fungal inoculation). Then, the seeds were stirred horizontally for 1.5 h at 2 Hz in 150 mL sterile bottles, separately, and finally, removed from the suspension to let them dry out (12 h) in a flow chamber under sterile conditions. Five extra seeds were added to the 24 seeds of each fungus × seed disinfection combination to ensure there were enough seeds in case of a germination failure occurs. Finally, 12 of the disinfected and 12 of the ND seeds from each fungal treatment were sown in the previously sterilized soils and the non-sterilized ones (Fig. 1).

Growth conditions, monitoring and samplings

The pots were placed in a growth chamber where the plants grew with a photoperiod of 16 h day, a temperature of 25 ± 1 °C, a relative humidity of 65 ± 10 % and a light intensity of 334 ± 30 µmol m^{-2} s^{-1}. The pots were daily watered with Hoagland solution the first 15 DAS and with SDW the next 15 days, keeping the soil at field capacity.

Leaf chlorophyll content (estimated through SPAD readings by using a SPAD-502 Plus Konica Minolta chlorophyll meter) was measured in five different points along the second fully expanded leave of each plant 15 days after sowing (DAS) and in the third fully expanded leaf 30 DAS in 6 plants each time for the 12 treatments [combinations of fungus, seed (D, ND) and soil (S, NS)]. The percent of the variation between the two leaf chlorophyll estimations (ΔSPAD) was calculated to check the resilience of treated and untreated plants and the possible damping role of the assessed fungi in SPAD after removing the Hoagland solution (from 15 to 30 DAS).

The presence of the applied fungus within inoculated and non-inoculated plants was determined 15 DAS in one of the two plants initially grown in each pot (6 replicates per combination of factors) and 30 DAS with the remaining one; following the scheme shown in Fig. 2. Four pieces (1 cm long) of each tissue (root, stem, and leaf) were externally disinfected, plating each one in one of the 4 Petri dishes containing SDCA+D (replicates) for each combination of factors (treatments). Thus, a total of 144 Petri dishes (3 tissues × 4 replicates × 12 treatment combinations) were checked 7 to 12 days after sampling time. This protocol was based on Parsa *et al.* (2013).

Statistical analysis

In Exp. 1, linear regression and an one-way analysis of variance (ANOVA) followed by the Fisher's Least Significant Differences (LSD) post-hoc test were used to evaluate the effect of the concentration of Tween® 80 on the number of *M. brunneum* conidia adhered to the seed surface (4 experimental units for each concentration of Tween® 80).

In Exp. 2, a one-way ANOVA was performed to study the effect of each application method (seed coating and soil drenching) on the length of cotyledon for each fungus separately against Control. No statistical analysis was performed for comparing wheat seed germination since all of them germinated.

In Exp. 3, the number of plants colonized by EPF (*B. bassiana* or *M. brunneum*) depending on factor combinations [soil S/NS × seed D/ND] were analysed by Chi-square test (independent group), while the differences between sampling dates were performed through Cochran tests (dependent group). It should be noted that Control plants were not included in the analysis because

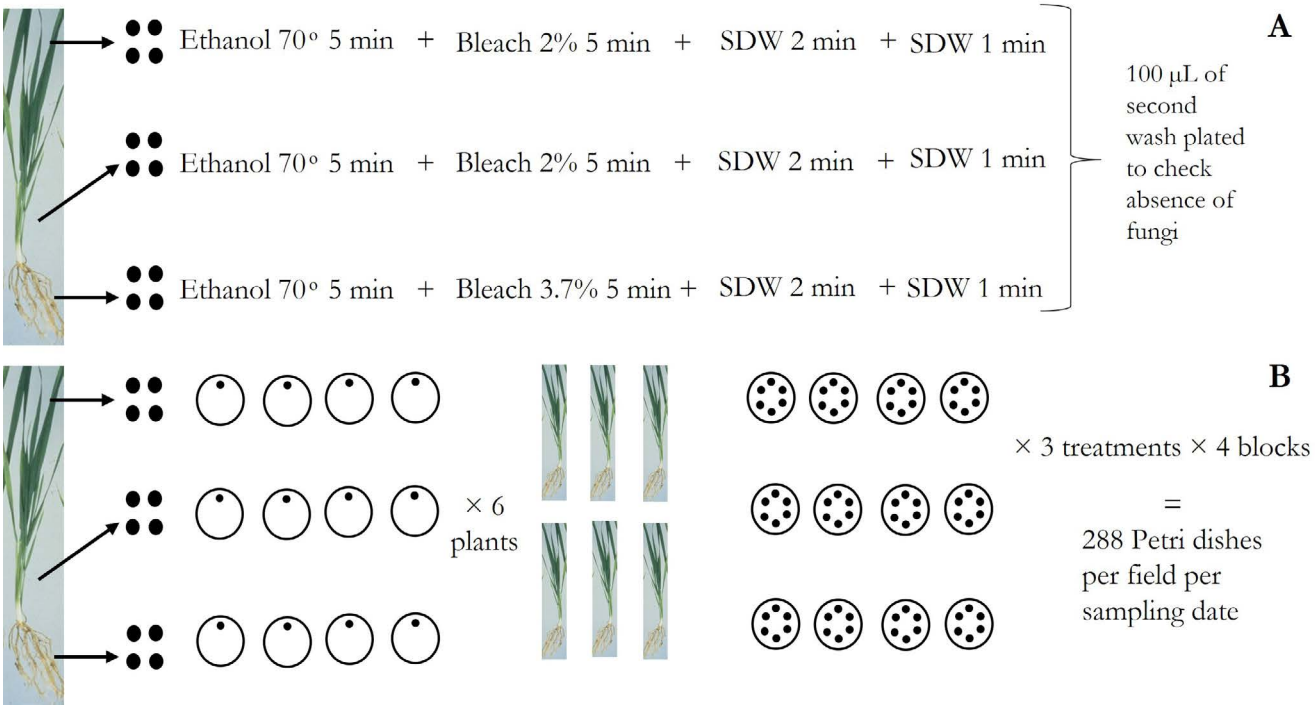

Figure 2. Scheme of the protocol followed for plant tissues disinfection and plating of vegetal tissue pieces in Petri dishes. In the upper-part of the figure it is shown the disinfection protocol while in the lower-part it is summarized the plating of the pieces from each vegetal tissue (black dots) in the Petri dishes (circles). Each Petri dish contains one piece of each tissue for each of the 6 replicate plants. Bleach is NaClO.

fungal colonization was always zero, as expected. Finally, the leaf chlorophyll content (SPAD values) was analysed by a factorial ANOVA with *fungus, sterilization,* and *disinfection* as factors. The *fungus* × *disinfection* interaction was significant in the first factorial and let us split the data into plants whose seeds were previously disinfected and non-disinfected and perform a second factorial ANOVA with *fungus* and *sterilization* as factors. The comparison was made between Control against each fungus, separately.

Results

Experiment 1

Seed germination was not affected by the concentration of Tween® 80 in the presence or absence of fungus (Fig. 3). A notable conidial growth on the surface and surrounding the 3 seeds even after the application of the highest Tween® 80 concentrations [10% (v/v)] was observed, and the viability of conidia was >75% (data not shown). Linear regression between the number of conidia adhered to each seed and the concentration of Tween® 80 was significant (R^2 = 0.895, p = 0.009; y = 87452 x + 704474, where y is the number of conidia per seed and x is the concentration of Tween® 80), indicating an increase in conidia adhesion of *M. brunneum* to the seeds with increasing the concentration of Tween® 80. In addition, differences were significant between the highest (10%) and the 3 lowest concentrations [0, 0.5, and 1% (v/v)] used in our study according to the LSD post hoc test (Fig. 4), with an increase up to 127% conidia adhered with the highest Tween® 80 concentration regarding Control.

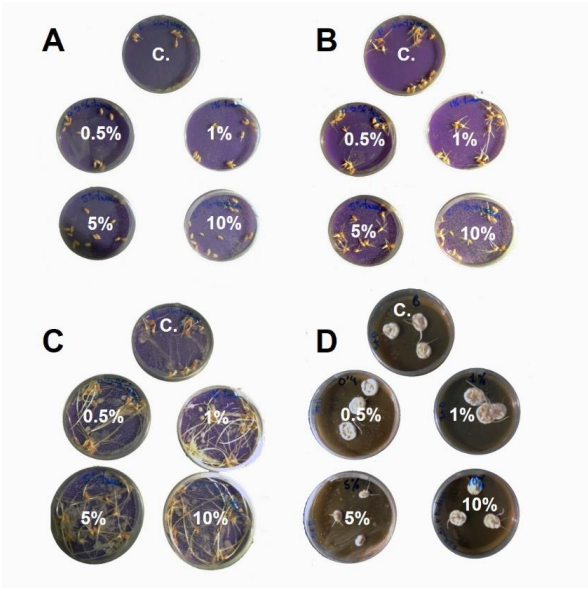

Figure 3. Seed germination (n = 15) through different concentrations with Tween® 80 (0, 0.5, 1, 5 and 10% v/v) 2 (A), 3 (B) and 6 (C) days after sowing (DAS). (D): outgrowth of *M. brunneum* conidia from the seeds treated with different concentrations of Tween® 80 described above plus 1 × 10^8 conidia mL^{-1} 3 DAS.

Experiment 2

Independently of the application method, all the seeds plated in the Petri dishes germinated. The seedlings showed differences in the length of the cotyledon. This variable was significantly increased ($p < 0.05$) in the seedlings previously treated by seed coating compared to the Control and soil drenching application methods, either with *B. bassiana* (67%) or *M. brunneum* (46%) (Fig. 5). However, root length was not measured due to they were strongly adhered to the filter paper and to each other.

Experiment 3

Fungal plant colonization

Beauveria bassiana or *M. brunneum* were not re-isolated from any wheat plant belonging to the control treatment. The number of plants colonized 30 DAS (up to 83% with both fungi) was significantly higher than those 15 DAS for *B. bassiana* (42% *vs* 9%, respectively; p = 0.020) but not for *M. brunneum* (37% *vs* 21%, respectively; p = 0.059) (Fig. 6). Root was the tissue where the highest percent of fungal colonization was detected, followed by stem and leaves (re-isolation did not occur in the last one) (Table 1). However, wheat plants inoculated with *B. bassiana* grown on sterilized soils with non-disinfected seeds (B-S-ND, 15 and 30 DAS) and on non-sterilized soils

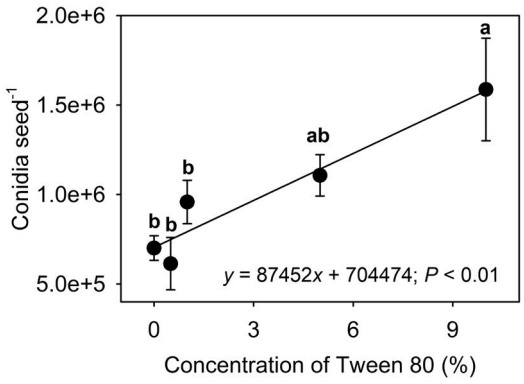

Figure 4. Linear regression showing the concentration of conidia adhered per seed (y axis) as a function of the concentration of Tween® 80 (x axis; 0, 0.5, 1, 5, 10%). Different letters indicate significant differences between the means of each different concentration in Tween® 80 according to the LSD post hoc test. Four replicates containing 5 seeds for each concentration were used. The adjusted R^2 of the linear regression = 0.895 and p = 0.009; being the equation y = 87452 x + 704474.

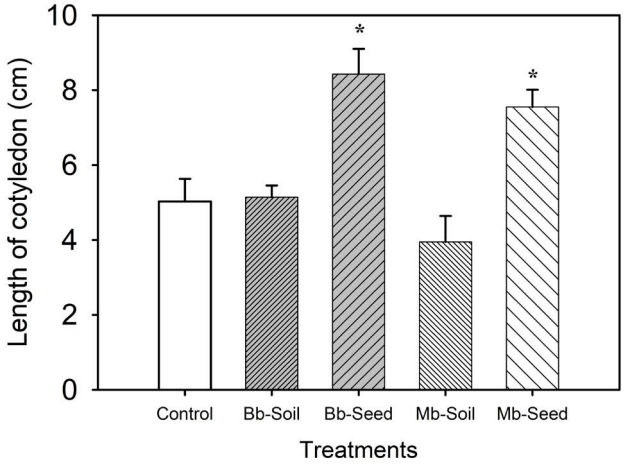

Figure 5. Length of wheat seedling cotyledon (*y* axis) 11 DAS under *B. bassiana* (Bb) or *M. brunneum* (Mb) treatment separately, applied as soil drenching or by seed relative to Control (untreated).

with disinfected seeds (B-NS-D, 30 DAS) showed higher re-isolation from stem than roots. No differences between both fungi for the same combination of treatments were found in any of the sampling dates (Table 1).

Significant differences between plants treated with *M. brunneum* S-D and NS were found 15 DAS (Fig. 6). Moreover, plants colonized by *B. bassiana* S-D and *M. brunneum* S-D were significantly higher than *B. bassiana* NS-ND ($p = 0.014$) and *M. brunneum* NS-D plants ($p < 0.010$), respectively, 30 DAS (Table 1).

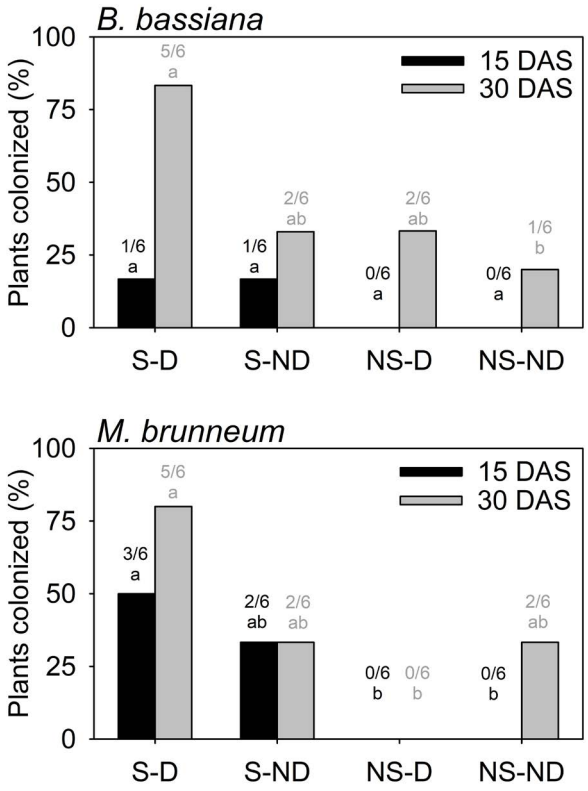

Figure 6. Percent of wheat plants colonized ($n = 6$) 15 and 30 DAS under the different combination of treatments (S = sterilized soil; D = disinfected seed; NS = non-sterilized soil and ND = non-disinfected seed). Different letters of the same DAS mean significant differences at $p = 0.05$ within each sampling date.

Leaf chlorophyll concentration

The significant interaction between *fungus* and *disinfection* factors found for leaf chlorophyll concentration when *M. brunneum* was used to inoculate seeds (Table S1 [suppl]) indicates that the inoculated plants behaved differently than the Control ones with D and ND seeds. *Beauveria bassiana* treated plants showed a similar pattern but no-significant differences were observed ($p = 0.103$, Table S1). Plants grown on S soils always had significantly higher SPAD readings than plants grown on NS soils 15 DAS (Table S2 [suppl]); whilst it was only observed within the group of plants with ND seeds 30 DAS (Table S2), specifically when *B. bassiana* was compared against Control. Fungal treatments were favoured by seed disinfection showing significantly higher SPAD readings than Control except for *B. bassiana* 30 DAS ($p = 0.072$) (Fig. 7A). However, when seeds were ND, SPAD values were similar between fungal treatments in both sampling times (Fig. 7B). The SPAD values were drastically decreased when Hoagland nutrient solution was restricted (ΔSPAD, Fig. 7, Table S2), with significant differences for plants grown in S soils related to NS ones only in the non-disinfected seeds group (Table S2). Finally, ΔSPAD was not affected by fungal treatments (Fig. 7).

Discussion

The implementation of microorganisms in agriculture is an appropriate tool to deal with one of the most important XXI century agricultural challenges, to achieve more sustainable agriculture. In this sense, EPF are considered among the most promising biopesticides to control a wide range of agricultural pests, while providing additional benefits to the plant as endophytes and rhizosphere competent microorganisms (Vega, 2018; Quesada-Moraga *et al.*, 2020). Of note, the past decade has witnessed increasing interest in the role of endophytic EPF as plant growth promoters and nutritional enhancers in soil drenching and seed coating treatments (Behie *et al.*, 2012; Jaber & Enkerli, 2016; Raya-Díaz *et al.*, 2017b). In the latter case, the surfactant concentration, application method, seed and soil disinfection, and sterilization respectively are of importance to improve the efficacy of these EPF in their facet as biofertilizers, while protecting the plant against insect pests.

Table 1. Rate of plants colonized (%, mean, $n = 6$) in each of the different vegetal tissues for each treatment combination 15 and 30 days after sowing (DAS) by seed dressing application method (1×10^8 conidia mL^{-1} fungal suspension).

Treatment combinations	15 DAS			30 DAS		
	Root	Stem	Leaf	Root	Stem	Leaf
B-S-D	16.7	0.0	0.0	83.3	33.3	0.0
B-S-ND	0.0	16.7	0.0	0.0	33.3	0.0
B-NS-D	0.0	0.0	0.0	16.7	33.3	0.0
B-NS-ND	0.0	0.0	0.0	16.7	0.0	0.0
M-S-D	50.0	0.0	0.0	83.3	33.3	0.0
M-S-ND	33.3	0.0	0.0	33.3	0.0	0.0
M-NS-D	0.0	0.0	0.0	0.0	0.0	0.0
M-NS-ND	0.0	0.0	0.0	33.3	0.0	0.0

B: *Beauveria bassiana*; M: *Metarhizium brunneum*; S: sterilized soil; NS: non-sterilized soil; D: disinfected seed; ND: non-disinfected seed.

In our study, no side effects in seed and conidia germination were found in the short-time due to the concentration of Tween® 80. In contrast, Gálvez *et al.* (2018) reported a negative impact of high concentrations of the surfactant Tween® 80 on lettuce and onion seed germination (0% and 50%, respectively; with 32.5 g L^{-1}; ~3.3% v/v). This is ascribed to the capability of the surfactant to bind membrane proteins, inhibit plant enzymes (Doige *et al.*, 1993), or alter the plant cell ultrastructure (de Bruin *et al.*, 2017). These differences could be due to the time that the seeds (and the radicle) were in contact with the surfactant; this was 3 days for lettuce and 10 days for onion (Gálvez *et al.*, 2018), and 6 days for *T. durum* (Rinallo *et al.*, 1988; Chang *et al.*, 2015), while this time was only 1.5 h in our experiments with seeds of *T. aestivum.* Furthermore, the surface holding the seeds was free of surfactant in our experiment. Gálvez *et al.* (2018) reported that Tween® 80 (and Brij35) were the harmless surfactants of the products they used, producing the highest fresh and dry biomass, root length, and germination rate in lettuce and onion seedlings using 0.6% concentration. This means that the time of contact between the surfactant with seeds and radicles is relevant. On the other hand, Rivas-Franco *et al.* (2019) determine that the efficiency of the coating was relatively higher with lower doses of conidia, which supports the importance of surfactant optimization. Finally, the increase in the concentration of the surfactant Tween® 80 may be a proper alternative to the seed coating application method to increase the number of EPF conidia adhered to the seed, when the cost of fungal production does not exceed that of the surfactant. Nonetheless, it is urgently required to unravel whether a higher number of conidia per seed leads to better wheat colonisation or whether the conidia per seed threshold could exist from which increasing the conidial load per seed does not result in higher colonisation rates.

The use of a more localized application of *B. bassiana* and *M. brunneum*, as is the case of seed coating application, increased the initial length of wheat seedlings cotyledon in Exp. 2. This implied an initial growth stimulation of aerial plant organs compared to control or the soil drenching methods. Thus, although the amount or concentration of fungi applied to the system is higher with soil drenching, the benefit (plant growth promotion) was lower. This could mean that for pest control decision-making, seed coating is a more cost-effective method and less environmentally disruptive than soil drenching because of the lower amount of inoculum applied to the system and the highest benefits for the host plant. It seems that the high presence of the conidia in the substrate leads to increases in the number of contacts between the fungus and the radicles, which can cause continuum stress, instead of a punctual one as in the seed coating method. As a consequence, a higher cost for the seedlings is caused by soil drenching than with seed coating at initial growth stages (Morgan *et al.*, 2005; Hermosa *et al.*, 2013), when seedlings are fed by seeds reserves. Therefore, the application method –and consequently the amount of inoculum applied to the system– is fundamental to keep the balance between fungus and plant at initial growth stages, requiring seed coating application method lower *B. bassiana* or *M. brunneum* conidia production, which makes it more environmentally friendly.

Although plant colonization by the EPF applied was observed at the first stages of the crop (Barelli *et al.*, 2018), a higher detection of fungi occurred 30 DAS relative to 15 DAS. This may be explained because fungal mass growth inside the plants was not enough to be detected by the method used in this experiment 15 DAS. Additionally, plant nutrient solution restriction during the second fortnight (in Exp. 3) may have triggered the association between plant-fungus, either due to the requirements of the former

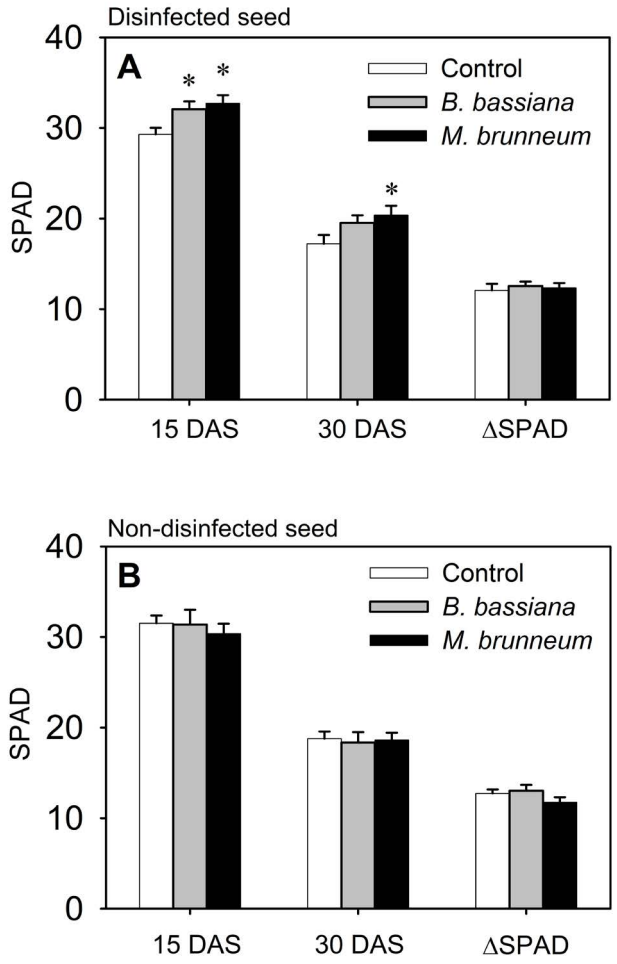

Figure 7. Readings of SPAD (n = 12, mean ± SE) in the group of plants, whose seeds were previously disinfected (A) or non-disinfected (B), showing the differences by fungal treatments (*B. bassiana* and *M. brunneum*) 15 and 30 DAS and SPAD variation between sampling dates respect to Control. Note: Here are represented all plants independently of the soil sterilization factor due to the lack of significant interaction with other factors in the factorial ANOVA statistical analysis.

to grow become higher (Blanke *et al.*, 2005; Balzergue *et al.*, 2013; Kobae *et al.*, 2016) or simply as its immune system is depressed (Pieterse *et al.*, 2012; Zamioudis & Pieterse, 2012) and penetration is facilitated under these circumstances.

The importance of seed disinfection and sterilization of soil to increase the probability of *B. bassiana* and *M. brunneum* colonizing the plant is highlighted here (Tefera & Vidal, 2009), even though the number of exogenous materials (more binders or fillers) to extend microbial survival to face to the soil antagonists was minimized with seed coating method (Rocha *et al.*, 2019). These two strategies minimize the competence with soil antagonistic microorganisms and facilitate the penetration (St. Leger, 2008), mainly providing an initial advantage due to the soil does not stand sterile anymore when is exposed to the air at the growth chamber. In addition, both fungi demonstrated to be rhizosphere competent since they colonized 16.7% (*B. bassiana*) and 33% (*M. brunneum*) of plants with seeds previously non-disinfected grown on non-sterilized soils, respectively; this results highlights the better rhizosphere competence of *Metarhizium* compared with Beauveria (Vänninen *et al.*, 2000; Hu & St. Leger, 2002; Bruck, 2010; Greenfield *et al.*, 2016). This ability is more developed in habitat-adapted fungal strains as is the case of both fungi used here (Rodriguez *et al.*, 2008). The higher colonization of *B. bassiana* relative to *M. brunneum* supports the better ability of the former to colonize and translocate into the plant (Vänninen *et al.*, 2000). The observed higher detection of both fungi in roots than in higher parts of the plant is due to the application method, which placed the conidia nearer this tissue; the colonization is produced among the first 72 h and the first contact between conidia and plant is the radicle. The absence of fungal detection in leaves may be due to a lack of time to hyphae translocation within plant tissues (Barelli *et al.*, 2018). Thus, time and inoculation method seem to be key to ensure a proper colonization rate (Akello *et al.*, 2009; González-Guzmán *et al.*, 2020a; Jaber & Enkerli, 2016) and, consequently, how these fungi influence the performance of the host plant. Besides those, nutritional solution affected leaf chlorophyll content (SPAD values) and consequently plant performance, being the decrease similar for all fungal treatments. Therefore, soil sterilization, which could improve soil nutrient availability, will also influence plant performance – presenting higher SPAD readings in plants grown in autoclaved soils (Williams-Linera & Ewel, 1984; Serrasolses *et al.*, 2008).

Seed disinfection (which is difficult to implement under field conditions so far) causes a seed priming effect, and this may favour the penetration of the fungus. However, this treatment may also facilitate the colonization of seedlings by other microorganisms in NS soils –other microorganisms that grown faster than EPF– occupying the ecological niche inside the plant as that could have happened with *M. brunneum* in NS soils, which can partially block the entrance of the target fungus. Therefore, in sterilized soils, the increasing colonization of the target fungi may trigger a higher demand of photoassimilates and consequently an increase in photosynthesis and concentration of chlorophyll –as in this study– to overcome this biotic carbon sink (Kaschuk *et al.*, 2009), though other authors did not find this trend (Greenfield *et al.*, 2016). This effect could be responsible for the slight differences between control treatments in S and NS soils, making control plants more vulnerable to the penetration of original soil microorganisms. Here, we only observed a significant ΔSPAD increase in S soils relative to NS in the group of plants whose seeds were not previously disinfected, which means that the disinfection – favouring fungal colonization – seems to buffer the negative effect of the Hoagland nutrient solution restriction.

Even though the number of exogenous materials (fillers) added in the seed coating method to extend microbial survival was minimized, seed disinfection was more efficient to increase plant colonization and improve plant performance than soil sterilization, at least, at the early crop-growth stages. This may be due to the colonization process by seed coating is developed at very early times (within 48 h), which minimizes the competence with other microorganisms in NS soils. In addition, when *B. bassiana* was applied in NS soils, higher colonized plants were found due to the better-known ability of this fungus compared to *M. brunneum* to colonize the host plant (Resquín-Romero *et al.*, 2016).

Finally, seed coating is gaining attention as an efficient, environmental, and economically sustainable delivery system for entomopathogenic endophytic fungi. Consequently, seed disinfection and soil sterilization should be considered in future research to improve the seed coating with EPF to facilitate the interaction between both, *B. bassiana* and *M. brunneum*, with wheat in a synergistic way and obtain a better benefit from the fungus-plant relationship.

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