Title: An endophytic *Beauveria bassiana* strain increases spike production in bread and durum wheat plants and effectively controls *Spodoptera littoralis* larvae

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Abstract

Entomopathogenic fungi have traditionally been assumed to help regulate insect populations. However, some hypocrealean ascomycetes such as *Beauveria bassiana* play other, poorly understood ecological roles that might be useful with a view to developing novel strategies for increased crop production. The primary aims of this work were (*a*) to assess plant colonization in bread wheat and durum wheat plants inoculated with *B. bassiana* strain EABb 04/01-Tip; and (*b*) to examine the impact of various inoculation methods on growth, yield, phytohormone levels and nutrient uptake in the plants, and on mortality of *Spodoptera littoralis* larvae fed with leaves of inoculated plants. Three experiments involving different inoculation methods (viz., 'soil treatment', 'seed dressing' and 'leaf spraying'), and a fourth experiment intended to assess mortality in *S. littoralis* specimens fed with leaves of inoculated plants, were conducted according to a completely randomized design for this purpose. Based on the results, *B. bassiana* successfully established in, and colonized, bread wheat and durum wheat plants. The fungus was for the first time re-isolated from grains produced by plants inoculated with the 'seed dressing' or 'soil treatment'

method. The fungus boosted spike production in bread wheat inoculated by ‘seed dressing’ or ‘soil treatment’ methods, and also in durum wheat but only with ‘soil treatment’. ‘Seed dressing’ increased grain yield by about 40 %, and also root length, in bread wheat. Mortality in *S.* littoralis larvae fed with leaves of inoculated plants ranged from 30 % with ‘seed dressing’ to 57 % with ‘leaf spraying’. No fungal outgrow was detected in larval cadavers, however. The sustainability of crop production strategies based on *B. bassiana* therefore depends on the effectiveness of the inoculation method and on the particular host plant.

Highlights

Beauveria bassiana successfully establishes in, and colonizes, bread wheat and durum wheat throughout their life cycle.

Beauveria bassiana was re-isolated from grains of plants inoculated by ‘seed dressing’ or application to soil.

Beauveria bassiana boosted spike production with ‘seed dressing’ in bread wheat, and with ‘soil treatment’ in both bread wheat and durum wheat.

Spodoptera littoralis larvae fed with leaves of endophytically colonized bread wheat exhibited mortality rates of 30–57 % depending on the particular fungal inoculation method used.

Keywords: Entomopathogenic fungus, biological control, plant growth promotion, phytohormones.

1. Introduction

Developing sustainable strategies to increase crop production is a major agricultural challenge (Berg, 2009) and those based on microbial pest control are becoming increasingly important because they provide an environmentally sound alternative to chemical pesticides. Entomopathogenic fungi play an incompletely investigated ecological role in plant–microbial symbioses. There is growing evidence that they improve plant nutrient uptake, stimulate hormone production and increase tolerance to abiotic and biotic stresses, thereby boosting plant growth (Ownley et al., 2010; Sasan and Bidochka, 2012). Also, colonization by nonpathogenic fungi or bacteria can trigger a biocontrol mechanism of systemic resistance to disease in host plants (Benhamou, 2004; Vega et al., 2008; Ownley et al., 2010). These responses involve the production of phytohormones such as jasmonic acid, salicylic acid, abscisic acid (Anderson et al. 2004), gibberellins and indole acetic acid (Khan et al., 2012).

The entomopathogenic ascomycete *Beauveria bassiana* (Bals.) Vuill has been found in and isolated from a wide variety of insect orders (Vega et al., 2008), but also from soils and various plants (Quesada-Moraga et al., 2014; Garrido-Jurado et al., 2015). Although this fungus has received much attention by virtue of its high microbial control potential, recent studies have shown that it has endophytic lifestyle. According to some authors (Griffin, 2007; Ownley et al., 2008; Quesada-Moraga et al., 2009, 2014), the endophyte *B. bassiana* has the potential for protecting plants against various pests. However, other effects of colonization by this fungus on plant growth and responses to abiotic stresses have scarcely been studied. Recently, entomopathogenic fungi were found to increase nutrient availability and/or plant growth (Liao et al., 2014; Sánchez-Rodríguez et al., 2015, 2016; Greenfield et al., 2016).

The inoculation method used is seemingly the key to the reported positive effects of entomopathogenic fungi on their host plants. Fungi can be artificially inoculated by leaf spraying, stem injection, soil drenching and seed dressing, among other methods based on conidial suspensions. These methods allow plant tissues to be colonized with variable success (Bing and Lewis, 1992; Tefera and Vidal, 2009). The mechanisms behind endophytic penetration and colonization of plant tissues remain unclear, however. Quesada-Moraga et al. (2014) demonstrated for first the time vertical transmission of an entomopathogenic fungus upon application of *B. bassiana* strain 04/01-Tip to opium poppy seeds.

74 Interestingly, inoculation by leaf spraying with the same strain led to temporary, gradually decreasing
75 endophytic establishment in the treated area (Landa et al., 2013).

76 These endophytes can kill insects at an early growth stage due to the production of toxic
77 substances in plants against them (Schulz et al., 2002; Sree and Padmaja, 2008; Rohlfs and Churchill,
78 2011; War et al., 2012). Also, some endophytic species produce metabolites that inhibit insect feeding
79 (Amiri-Besheli et al., 2000; Daisy et al., 2002; Kim et al., 2002; Quesada-Moraga et al., 2006). Resquín-
80 Romero et al. (2016) found spray application of *B. bassiana* to alfalfa, tomato and melon plants to kill
81 *Spodoptera littoralis* larvae feeding on leaves of colonized plants. How inoculation methods other than
82 foliar application influence the pathogenicity of *B. bassiana* against larvae of chewing insects that are
83 pests for certain crops (e.g., *S. littoralis*) and feed on inoculated plant material is unknown.

84 The inoculation method is therefore a major factor in addition to the host plant species and fungal
85 strain in the persistence of entomopathogenic fungal endophytes and success of the plant–endophyte
86 association. In fact, the effectiveness of fungal applications against pests depends on the particular
87 inoculation method in an unclear manner.

88 The primary aims of this work were (*a*) to examine colonization by *B. bassiana* inoculated in
89 different ways to bread wheat (*Triticum aestivum* cv. Chinese spring) and durum wheat (*Triticum durum*
90 cv. Carpio) host plants; (*b*) to assess the effects of the inoculation methods on plant growth, nutrient
91 uptake and production; and (*c*) to elucidate the influence of the inoculation method on mortality in *S.*
92 *littoralis* larvae feeding on inoculated plants. To that end, four different experiments were conducted in
93 order to study the effect of *B. bassiana* on bread wheat growth (Experiment 1); bread wheat and durum
94 wheat yield (Experiment 2); bread wheat yield and root length (Experiment 3); and survival of *cotton*
95 *leafworm* larvae feeding on endophytically colonized bread wheat plants (Experiment 4). Experiment 3
96 used a higher fertilizer rate than Experiments 1 and 2 in order to check whether this variable had any
97 influence on the effectiveness of *B. bassiana*.
98

2. Materials and Methods

2.1. Experimental design

Table 1 shows the most salient features of the four experiments, including plant species, treatments, number of pots, plant per pots, plant and soil analyses, and statistics. Experiment 4 was the shortest (17 days), followed by Experiments 1 (31 days), 2 (120 days for bread wheat and 100 days for durum wheat) and 3 (140 days).

A completely randomized design and three inoculation methods ('soil treatment', 'seed dressing' and 'leaf spraying', in comparison with 'control' or noninoculated plants) were used in the four experiments, except in Experiment 3 in which only 'control' and 'seed dressing' were used. A total of 20 pots (replicates) per method were used in Experiment 1 and 25 in Experiments 2 and 3. Also, 30 larvae (per inoculation method) fed on bread wheat plants were used in Experiment 4 (Table 1). The experimental unit was a pot bearing two plants in Experiments 1 to 3, and a larva in Experiment 4 — where *S. littoralis* larvae fed on 15 plants per inoculation method (5 per pot) of bread wheat.

2.2. Plant material, soil properties and cropping

Seeds of bread wheat (*Triticum aestivum* L. cv. Chinese Spring) in the four experiments, and durum wheat (*Triticum durum* L. cv. Carpio) in Experiment 2 only, were immersed in a 6 % hydrogen peroxide solution for 15 min and washed with deionized water for 30 min, the water being replaced at 5 min intervals. Ten washed seeds per experiment were then plated onto Petri dishes containing sabouraud dextrose chloramphenicol agar (SDCA; Biocult, Madrid, Spain) in order to assess the efficacy of the method. All other seeds were germinated in sterile Petri dishes with autoclaved filter paper under high humidity conditions in a refrigerator at 4 ºC for 96 h and then at 25 ºC in the dark for 24 h. Four seedlings (5 in Experiment 4) were transplanted to each of several cylindrical PVC pots. The pots (3–25 depending on the experiment) were 15 cm in height × 5 cm in diameter (10 cm × 9 cm in Experiment 4) and furnished with a drainage hole at the bottom; each was filled with 300 g (500 g in Experiment 4) of a Haploxeral sandy soil. The soil was collected from the top sandy horizon (0–25 cm) in the Rabanales University Campus (Córdoba, southern Spain, 37° 56.03´N, 4° 43.13' W) and sterilized twice at 121 ºC for 45 min. The pH of the soil (1:2.5, weight:volume distilled water) was 6.5, its total C and N contents

were 9.7 and 1.13 g kg^{-1}, respectively (both as determined by direct combustion on an Euro Vector EA-3000 Elemental Analyzer from Euro Vector SpA, Milan, Italy), and its available P content was 14.0 g kg^{-1} (as determined by extraction with 0.5 *M* $NaHCO_3$ buffered at 8.5; Olsen et al., 1954). A detailed description of the soil properties can be found elsewhere (Sánchez-Rodríguez et al., 2016).

All pots were placed in a growth chamber (a 9 m^2 room furnished with a thermostat and a humidifier to keep the temperature and relative humidity at appropriate levels) with a photoperiod of 12 h day^{-1}, a light intensity of 250 µmol m^{-2} s^{-1}, a temperature of 21 ºC and a relative humidity of 65 %. Two plants per pot were removed 7 DAS (days after sowing) and the other two kept throughout the experiments —by exception, 5 plants per pot were kept throughout Experiment 4 (17 DAS).

After weighing, the pots were irrigated on a daily basis with deionized water to keep soil moisture near field capacity. Full Hoagland nutrient solution [5 m*M* $Ca(NO_3)_2 \cdot 4H_2O$, 5 m*M* KNO_3, 2 m*M* $MgSO_4$, 0.1 µ*M* KCl, 0.3 µ*M* $Ca(H_2PO_4)_2 \cdot H_2O$; 50 µ*M* H_3BO_3, 4 µ*M* $MnSO_4 \cdot H_2O$, 4 µ*M* $ZnSO_4 \cdot 7H_2O$, 0.1 µ*M* $CuSO_4 \cdot 5H_2O$ and 6 µ*M* Na_2MoO_4] was applied twice weekly until the end of cropping according to the specific needs of each plant and previous experience: 10 mL per pot per week in Experiments 1, 2 and 4; and 10 (first month), 15 (second month) and 30 mL (rest of the experiment) per pot per week in Experiment 3.

2.3. Fungus cultures and inoculation methods

Beauveria bassiana strain EABb 04/01-Tip was isolated from a dead *Iraella luteipes* larva collected from a field in Carmona (Seville, southern Spain). This strain, which had previously been found to behave as an endophyte in opium plants (Quesada-Moraga et al., 2009), is deposited in the CRAF Entomopathogenic Fungi Collection of the University of Córdoba. The fungus was routinely grown on slants of malt agar (MA; Biocult, Madrid, Spain) at 25 ºC in the dark. Fungal cultures were grown on malt agar containing 25 g malt agar and 7.5 g agar at 25 ºC in the dark for 2 weeks, and conidia then collected by scraping the surface of each culture with a sterile camel hairbrush into a 100 mL glass beaker containing 50 mL of sterile distilled water plus Tween 80 (0.1% v/v). The conidial suspension was stirred, filtered, adjusted to 1×10^8 conidia mL^{-1} and used to inoculate the plants and soil. A fresh culture was prepared for each experiment.

153 Three different methods were examined for their effects on the response of plants to inoculation
154 with *B. bassiana* in relation to noninoculated ('control') plants. The method labeled 'Soil treatment' was
155 started by applying 5 mL of conidial suspension per pot (1×10^8 conidia mL^{-1}) to the soil surface before
156 sowing (0 DAS). The other pots were watered with the same amount of sterile deionized water containing
157 Tween 80 (0.1% v/v). The 'seed dressing' (seed inoculation) method involved immersing 120 seeds in 30
158 mL of conidial suspension (1×10^8 conidia mL^{-1}) for 4 h under shaking at 180 rpm before sowing.
159 Inoculated seeds were then dried on sterile Petri dishes for 20 min and sown in pots (0 DAS). The process
160 was repeated for the other seeds except that the solution was conidia-free deionized water containing
161 Tween 80 (0.1 % v/v). The 'leaf spraying' (leaf inoculation) method involved using an airbrush to spray 1
162 mL of conidial suspension (1×10^8 conidia mL^{-1}) to the first two leaves at 8 DAS. Inoculation of the soil
163 during foliar application of the fungus was avoided by covering it with aluminum foil. The other plants
164 were sprayed with 1 mL of sterile deionized water containing Tween 80 (0.1 % v/v). A plastic bag was
165 used to cover each plant for 24 h in order to maintain a high humidity and facilitate foliar inoculation.

166 ***2.4. Soil and plant determinations***

167 Colony forming units (CFU) in the soil were determined on a periodic basis in samples from the 'control'
168 and 'soil treatment' groups (weekly in Experiment 1; monthly in Experiment 2; and 3, 9, 12 and 17 DAS
169 in Experiment 4). Each sample consisted of 1 g of soil collected at a depth of 0–3 cm and 9 mL of sterile
170 deionized water, the suspension being shaken for 90 min prior to plating of 100 µL diluted aliquots onto
171 Petri dishes containing malt agar medium. The CFU data were used to assess establishment of the fungus
172 in the rhizosphere.

173 Plant height was measured on a periodic basis (see Table 1). Five samplings per experiment were
174 conducted 8, 10, 13, 19 and 31 DAS in Experiment 1; one day after 'leaf spraying' at the tillering,
175 jointing, flowering and dough grain phenological stages in Experiments 2 and 3; and 12 DAS in
176 Experiment 4 . Each sampling operation involved removing and washing plants from 3 pots per crop for
177 each inoculation method, and assessing colonization of plant tissues by *B. bassiana*. Five plants per pot
178 per method were used in Experiment 4.

179 Colonization of plant tissues (leaves, roots, stems and grains whenever possible) was assessed by
180 re-isolating *B. bassiana* after removing the plants from the pots, and carefully washing their roots with tap
181 and deionized water. Eight small (0.5 cm^2) pieces from the first two leaves in each plant —irrespective of
182 inoculation method in the first and second samplings— and young leaves —third to last sampling—, five
183 0.5 cm long pieces from roots, 4 small (1 cm long) pieces from stems and 8 small pieces from grains —
184 stems and grains in Experiments 2 and 3— were washed with 2% sodium hypochlorite (4% for roots) for
185 2 min and sterile deionized water (twice) for another 2 min, and dried under sterile conditions for 5 min
186 before plating each tissue onto a different Petri dish containing sabouraud dextrose chloramphenicol Agar
187 (SDCA; Biocult, Madrid, Spain). The dishes were allowed to stand at 25 ºC for 2 weeks prior to
188 determining the presence or absence of *B. bassiana* in the tissues.
189 The efficacy of the surface sterilization method was checked by plating 50 µL aliquots of 10^{-1} to
190 10^{-3} dilutions from the water used to wash the plant material and incubating them at 25 ºC for 2 weeks to
191 determine CFU. As expected, no *B. bassiana* CFU were re-isolated from the water.
192 Additional determinations included freezing 0.50 g of aerial biomass per plant for each
193 inoculation method at –80 ºC to quantify the phytohormones abscisic acid (ABA), jasmonic acid and
194 salicylic acid (Bacaicoa et al., 2009, Sánchez-Romera et al., 2014) at the tillering, jointing, flowering and
195 dough grain stages in Experiment 2; and measure total plant root length (using the software WinRhizo)
196 throughout the life cycle (8, 22, 50 and 78 DAS) in Experiment 3.
197 The last sampling in Experiments 1 to 3 was used to determine biomass dry weight, spike dry
198 weight (not in Experiment 1) and grain production (not in Experiment 1). Mineral nutrients in aerial
199 biomass (only for Experiment 1) and grain (Experiments 2 and 3) were analyzed after digestion with a
200 nitric–perchloric acid mixture (Zazoski and Burau, 1977). Ca, Mg, Fe, Mn, Zn and Cu were determined
201 with an AAA Perkin Elmer AAnalyst 200 atomic absorption spectrophotometer; K with a Jenway PFP7
202 flame emission spectrometer; and P with the Molybdenum Blue method of Murphy and Riley (1962). The
203 total number of pots used in the last sampling was 5 in Experiment 1, and 10 in Experiments 2 and 3 —all
204 other pots were harvested in the previous samplings.
205

206 ***2.5. Spodoptera littoralis (Boisduval) (Noctuidae, Lepidoptera) culturing and feeding with inoculated***
207 ***plant leaves. Larval mortality***
208 *Spodoptera littoralis* (cotton leafworm) was bred under insectarium conditions (26 °C, 70 % RH and a
209 photoperiod of 16 h light/8 h darkness) (Poitout and Bues, 1974). The insects were originally collected
210 from different crops in the field and kept in the Department of Agricultural and Forestry Sciences of the
211 University of Córdoba (Spain).
212 Thirty second instar larvae were individually placed in Petri dishes (30 larvae × 3 inoculation
213 methods + 30 larvae × 3 inoculation 'control', 120 larvae). At 12 DAS (4 days after 'leaf spraying'), 5
214 plants per treatment were randomly selected for cutting and the larvae they contained fed with wheat discs
215 (0.5 cm^2). The larvae were fed with 5 fresh plants daily for 5 days and then on an artificial diet of alfalfa.
216 Larval deaths were recorded on a daily basis for 5 days to determine percent mortality, and also average
217 survival time, AST, as the number of days the larvae fed with inoculated leaves remained alive. Dead
218 larvae were immediately surface sterilized with 1 % NaOCl for 1 min, followed by sterile water twice,
219 drying under sterile conditions and placement on sterile wet filter paper in Petri dishes sealed with
220 Parafilm® for inspection of cadavers and fungal outgrowth on cuticle surfaces under a light microscope.
221 ***2.6. Statistical analysis***
222 Statistics (Table 1) were determined with the software SPSS v. 22.0. Re-isolation of *B. bassiana* was
223 measured as colonization frequency (viz., 100 × number of plant pieces containing the fungus in relation
224 to the total number of pieces). The results for plant height, root length (Experiment 3), *B. bassiana* re-
225 isolation from tissues —except in grain, where a Student's *t*-test was used— and phytohormone levels
226 (Experiment 2) were subjected to repeated measurement analysis of variance (MANOVA) to assess the
227 effects of time and the time × inoculation method interaction, and then to Bonferroni's multiple
228 comparison test at a probability level of 0.05. Plant biomass, nutrient concentrations in biomass and
229 spikes, grain production and nutrient concentrations in grains were subjected to analysis of variance
230 (ANOVA) in Experiments 1 and 2, and to Student's *t*-test in Experiment 3. Means were separated via the
231 Least Significant Difference (LSD) test at a probability level of 0.05 in Experiments 1 and 2. CFU data
232 were subjected to ANOVA —two-way ANOVA with crop and sampling time in Experiment 2— and

LSD. The data for both the 'control' group and the three inoculation methods were included in all MANOVA and ANOVA; by exception, the fungal re-isolation time course was only examined in relation to 'soil treatment', 'seed dressing' and 'leaf spraying'. In Experiment 4, ANOVA was applied with the software Statistix 9.0 to larval mortality and the LSD test was used to compare means. Average survival times were calculated by using the Kaplan–Meier method and compared via the log-rank test.

3. Results

Beauveria bassiana was re-isolated from no tissues in the 'control' plants in any experiment. Plants grew vigorously and exhibited no symptoms of mineral deficiency.

3.1. Effect of B. bassiana on bread wheat growth (Experiment 1)

The CFU results of Experiment 1 are not shown here because they were similar to those of Experiment 2 during the first 30 days (see Fig. 1 and Section 3.2). Fungal re-isolation from leaves and roots is shown in Fig. 2. As can be seen, a significantly increased proportion of *B. bassiana* (54 ± 8–100 ± 0 %) was re-isolated from leaves subjected 'leaf spraying' in relation to 'soil treatment' and 'seed dressing' in the first four samplings, namely: 8 ($P < 0.001$), 10 ($P = 0.001$), 13 ($P < 0.001$) and 19 DAS ($P = 0.006$). However, the differences were not significant ($P = 0.084$) and re-isolation from plants in the 'leaf spraying' group 31 DAS was much lower (8 ± 4 %, Fig. 2A). Re-isolation with 'soil treatment' and 'seed dressing' was lower than 5% in the five samplings. As regards roots (Fig. 2B), *B. bassiana* was found in no plants of the 'leaf spraying' group; also, re-isolation 10 DAS was increased by 'soil treatment' and 'seed dressing' ($P = 0.037$). The differences 13 DAS between the 'seed dressing' and 'leaf spraying' groups were significant ($P = 0.045$), and so were those 31 DAS between 'soil treatment' and the other two inoculation methods ($P < 0.001$).

Height in the 'control' plants was significantly greater than in the inoculated plants 9 DAS ($P < 0.001$ except with 'leaf spraying') and 12 DAS ($P < 0.001$). The trend changed in the period from 17 to 31 DAS, where the 'control' plants exhibited the lowest heights (42.1 ± 0.3 cm) at the end of the experiment relative to inoculated plants (viz., 48.0 ± 1.0, 46.1 ± 1.1 and 44.0 ± 1.0 cm with 'leaf spraying', 'soil treatment' and 'seed dressing', respectively; Fig. 3). Significant differences ($P < 0.001$) were found 26 and 31 DAS.

Table 2 shows the dry weight and nutrient concentrations of bread wheat aerial biomass. Biomass dry weight was not altered by the presence of *B. bassiana*. The P concentration was significantly higher (P = 0.026) with 'seed dressing' and 'leaf spraying' (2.6 ± 0.1 g kg^{-1} with both treatments) than in the 'control' plants (2.2 ± 0.0 g kg^{-1}); also, the Mn concentration was significantly increased (P = 0.005) by fungal inoculation (163 ± 8 mg kg^{-1} in the 'control' plants versus 252 ± 30, 453 ± 73 and 379 ± 47 mg kg^{-1} with 'seed dressing', 'leaf spraying' and with 'soil treatment', respectively).

In summary, the fungus succeeded in colonizing leaves and roots; also, it altered plant height and the concentrations of some nutrients (P, Mn) 31 DAS.

3.2. Effect of B. bassiana on bread and durum wheat yield (Experiment 2)

Figure 1 shows the time course of CFU in the soil in the Experiment 2. As can be seen, there were no significant differences in CFU counts (P = 0.485) between bread wheat and durum wheat. There were, however, significant differences (P < 0.001) among samplings. Thus, fungal counts peaked at ca. 1.55 × 10^6 CFU g^{-1} soil in the first sampling and then considerably decreased 31 DAS; however, they remained virtually constant from 31 to 90 DAS and decreased slightly in the last determination (from 0.85 × 10^6 to 0.65 × 10^6 CFU g^{-1} soil).

Colonization was uneven across treatments in both crops. Thus, *B. bassiana* was re-isolated by 45 % from all Petri dishes plated with inoculated plant material: 126 per crop —42 additional dishes containing material from the 'control' plants exhibited no signs of the fungus. Fig. 4 shows fungal re-isolation from leaves, roots, stems and grains in bread wheat and durum wheat. As can be seen, *B. bassiana* was re-isolated in higher proportions with 'leaf spraying' than with 'seed dressing' and 'soil treatment' at the two-leaf stage in bread wheat (P < 0.001, Fig. 4A), and at the tillering stage in durum wheat (P = 0.033, Fig. 4B). There were no significant differences among treatments at any other phenological stages. *Beauveria bassiana* was found in no leaves of plants in the 'soil treatment' group until the flowering stage in bread wheat (28.7 ± 8.0 %) and the tillering stage in durum wheat (5.0 ± 3.1 %). These values decreased in bread wheat (12.7 ± 5.7 %), and increased in durum wheat (10.0 ± 4.7 %), at the dough grain stage. Fungal colonization of leaves by effect of 'seed dressing' was detected at the tillering stage in bread wheat (25.6 ± 4.8 %) but decreased to negligible levels at the dough grain stage

(Fig. 4A). By contrast, durum wheat contained the fungus from the two-leaf stage (5.0 ± 3.1 %) through the last sampling (21.0 ± 9.1 %) (Fig. 4B). Finally, re-isolation in the 'leaf spraying' group was uneven in bread wheat (44.3 ± 2.1 % to 12.7 ± 6.7 %) and decreased from the tillering stage (23.3 ± 1.4 %) to the dough grain stage (0 ± 0 %) in durum wheat.

Differences in *B. bassiana* re-isolation from roots were only significant ($P = 0.051$) at the jointing stage in bread wheat, where 'seed dressing' led to a significantly higher value than 'leaf spraying' (22.7 ± 2.8 % versus 0.0 ± 0.0 %), and at the dough grain stage in durum wheat ($P = 0.018$), where 'soil treatment' resulted in much greater re-isolation than 'leaf spraying' (75.0 ± 0.0 % versus 27.2 ± 18.4 %). It should be noted that Figs 4C and 4D lack were constructed from incomplete data for 'seed dressing'. Overall, 'soil treatment' and 'seed dressing' led to a similar extent of re-isolation from roots in the bread wheat and durum wheat groups (<28%, two-leaf, tillering and jointing stages; Figs. 4C and 4D). No re-isolation from bread wheat roots was observed with 'leaf spraying'; on the other hand, re-isolation from durum wheat roots was 25.0 ± 11.7 % at the jointing stage and 27.3 ± 18.4 % at the dough grain stage. Other bacteria and fungi in proportions that increased with time (DAS) caused some data points to be missed —particularly as regards root re-isolation— at the flowering and dough grain stages.

Beauveria bassiana was re-isolated from stems of inoculated bread wheat and durum wheat plants already from the jointing stage except with 'soil treatment' in durum wheat. Re-isolation ranged from 4 % with 'leaf spraying' in bread wheat (Fig. 4E) to 33 % with 'soil treatment' in durum wheat (Fig. 4F). No significant differences with time among treatments were observed. Also, *B. bassiana* was re-isolated from grains at the dough grain stage with 'soil treatment' (10.0 ± 4.7 % in bread wheat and 13.3 ± 5.9 % in durum wheat) and 'seed dressing' (10.3 ± 4.2 % in bread wheat and 11.7 ± 4.9 % in durum wheat) (see Figs 4G and 4H).

Figure 5 shows the time course of plant height in Experiment 2. Durum wheat grew less tall than bread wheat (approximately 44 versus 70 cm). In general, the inoculation methods had no consistent effect on plant height. However, the 'control' plants were taller than the inoculated plants 31 DAS in both bread wheat ($P = 0.050$; 55.5 ± 0.6, 54.9 ± 0.6, 54.3 ± 0.4 and 53.1 ± 0.5 cm in the 'control', 'soil treatment', 'seed dressing' and 'leaf spraying' group, respectively; significant differences between

314 'control' and 'leaf spraying' only) and durum wheat ($P < 0.001$; 36.4 ± 0.4, 34.6 ± 0.4, 34.1 ± 0.3 and
315 33.8 ± 0.3 cm in the 'control', 'leaf spraying', 'soil' and 'seed dressing' group, respectively; significant
316 differences between 'control' plants and the three inoculation methods). No differences in plant height
317 were observed in bread wheat at the end of the cropping period. Durum wheat plants in the 'soil
318 treatment' group were taller than those in the other groups 17 DAS ($P < 0.001$; 30.0 ± 0.2, 28.9 ± 0.2,
319 28.8 ± 0.3 and 28.6 ± 0.3 cm in the 'soil treatment', 'control', 'seed dressing' and 'leaf spraying' group,
320 respectively; significant differences between 'soil treatment' and the other three groups). This was also
321 the case 38 DAS ($P = 0.046$), 44 DAS ($P = 0.004$), 53 DAS ($P = 0.003$) and 86 DAS ($P = 0.011$; 45.4 ±
322 0.2, 44.7 ± 0.2 , 44.7 ± 0.5 and 43.4 ± 0.4 cm in the 'soil treatment', 'control', 'leaf spraying' and 'seed
323 dressing' group, respectively; significant differences between 'soil treatment' and 'seed dressing' only in
324 the last four samplings; Fig. 5).

325 Table 3 (upper part) shows the leaf + stem, spike and plant dry weight, total grain weight, number
326 of grains per plant and grain weight for bread wheat and durum wheat at harvest. No significant
327 differences in leaf + stem dry weight, grains per plant or grain weight were found in bread wheat or
328 durum wheat. On the other hand, spike dry weight was significantly increased ($P = 0.002$) relative to the
329 'control' plants (1.81 ± 0.07 g) by 'soil treatment' (2.21 ± 0.18 g) and 'seed dressing' (2.43 ± 0.07 g) in
330 bread wheat, and by 'soil treatment' in durum wheat ($P < 0.001$; 2.50 ± 0.05 g as compared to 2.34 ±
331 0.04 g in the 'control' plants) (Table 3). Similar results were obtained for plant dry weight (leaf + stem +
332 spike) in bread wheat ($P = 0.027$) and durum wheat ($P = 0.007$); however, the latter crop exhibited no
333 significant differences in dry weight between 'seed dressing' and the other inoculation methods. As can
334 be seen from Table 3, there were no significant differences in spike or plant dry weight between 'control'
335 and 'leaf spraying' in bread wheat, nor among 'control', 'seed dressing' and 'leaf spraying' in durum
336 wheat.

337 Mean grain weight in bread wheat was increased by 41 % by 'seed dressing' and 14 % by 'soil
338 treatment' relative to the 'control' plants (Table 3); on the other hand, it was slightly reduced by 'leaf
339 spraying' ($P = 0.147$, not significant owing to the high standard deviations of the plant groups). In durum

wheat (P = 0.022), mean grain weight was increased by 3 % by 'soil treatment', and decreased by 3 and 5 % by 'seed dressing' and 'leaf spraying', respectively.

Table 3 (lower part) shows the mineral nutrient concentrations in grain at harvest. Although differences in plant nutrient uptake were generally small in bread wheat, some results are worth noting. For example, the P concentration in durum wheat grains was significantly increased (P = 0.014) by all inoculation methods (3.3 ± 0.1, 2.8 ± 0.1 and 3.2 ± 0.1 g kg^{-1} with 'soil treatment', 'seed dressing' and 'leaf spraying', respectively, relative to 2.8 ± 0.1 kg^{-1} in the 'control' plants; significant differences except between the 'control' and 'seed dressing' groups). Overall, the fungus increased the potassium (P = 0.006), Mg (P = 0.012) and Fe (P = 0.021) concentrations, and reduced the calcium concentration (P = 0.005), in durum wheat grains (see Table 3). The fungus also reduced the magnesium concentration, but only with 'leaf spraying' and 'seed dressing' (P = 0.033).

Table 4 shows the phytohormone levels in bread wheat and durum wheat. A reduced ABA content was found in all inoculated plants of bread wheat at the tillering stage (P = 0.004; 38 ± 9, 39 ± 3 and 28 ± 1 ρmol g^{-1} with 'soil treatment', 'seed dressing' and 'leaf spraying', respectively, vs 67 ± 3 ρmol g^{-1} in the 'control' plants). Although no similar effect was observed in durum wheat at the tillering stage, this species exhibited some changes worth noting. Thus, 'soil treatment' (800 ± 78 ρmol g^{-1}) and 'seed dressing' (510 ± 106 ρmol g^{-1}) led to significantly higher ABA concentrations (P < 0.001) than those of the 'leaf spraying' (173 ± 108 ρmol g^{-1}) and the 'control' group (95 ± 28 ρmol g^{-1}) at the jointing stage; however, the 'control' and 'soil treatment' groups exhibited the highest AB concentrations (>2000 versus < 300 ρmol g^{-1} with 'seed dressing' and 'leaf spraying') in the last determination. The salicylic acid concentration in bread wheat was only affected by 'soil' treatment (Table 4), with significantly increased levels relative to the other inoculation methods (P = 0.006) at the dough grain stage. On the other hand, the jasmonic acid concentration was significantly reduced by 'seed dressing' and 'leaf spraying' in bread wheat at the tillering stage (P = 0.001), and also in durum wheat at the dough grain stage (P = 0.004). No significant differences were observed in the other determinations.

Beauveria bassiana established in the rhizosphere of plants in the 'soil treatment' group after more than 100 days; also, it acted as an endophyte and colonized new tissues —leaves and stems with the

three inoculation methods, and grains only with 'soil treatment' and 'seed dressing'— throughout the life cycle of the plants. 'Soil treatment' and 'seed dressing' increased spike dry weight in bread wheat, and so did 'soil treatment' in durum wheat; also, 'soil treatment' altered grain production in durum wheat, and nutrient concentrations in grain and phytohormone levels in aerial biomass in both crops.

3.3. Effect of B. bassiana on bread wheat yield and root length (Experiment 3)

In Experiment 3, *B. bassiana* was re-isolated from plants in the 'seed dressing' group to an extent similar to that of bread wheat plants subjected to the same inoculation treatment in Experiment 2 (see Figs 4 and 6). The main difference was increased re-isolation from leaves (25.0 ± 6.2 % vs 0.0 ± 0.0 %, Figs. 6A and 4A) and stems (20–30 % vs 9–13 %, Figs. 6C and 4E) at the dough grain stage in Experiment 3. The fungus was also re-isolated from grain (13.5 ± 5.4 %, Fig. 6D).

A negative effect of fungal inoculation on plant growth was observed 7 DAS in the form of decreased plant height (P = 0.026, two-leaf stage); however, no significant differences between the 'control' and 'seed dressing' groups were found at the end of the experiment (P = 0.122, Fig. 7A). Although an initial negative effect was also detected in total root length 8 DAS (P = 0.028), no similar differences were observed at the tillering (22 DAS) or jointing stage (50 DAS); also, the opposite effect was observed at the flowering stage (78 DAS), where 'seed dressing' increased total root length relative to the 'control' group (P = 0.047). Although Experiments 2 and 3 were conducted under similar conditions, the bread wheat plants in the 'control' and 'seed dressing' groups had longer roots than those used for evaluation at different phonological stages (80.0 ± 1.01 vs 71.5 ± 0.6 cm, means and standard errors for the 'control' + 'seed dressing' combination).

As can be seen from Table 5, neither leaf + stem nor plant dry weight were altered by fungal inoculation (P = 0.337 and P = 0.102, respectively). On the other hand, spike dry weight, grain total weight and number of grains per plant were significantly increased [by 29.0, 43.5 and 63.1 %, respectively, in the 'seed dressing' plants (P = 0.015, P = 0.014 and P < 0.001, respectively]; also, grain weight was decreased by 13.3 % (P = 0.050, Table 5). Finally, *B. bassiana* altered no nutrient concentrations in grain except those of Mn.

In summary, *B. bassiana* applied by 'seed dressing' acted as an endophyte and colonized new plant tissues in bread wheat; also, it boosted spike production, altered nutrient concentrations in grain, and increased total root length and grain production in this species.

3.4. Survival of cotton leafworm larvae fed with endophytically colonized bread wheat (Experiment 4)

No CFU values for this experiment are shown here because they were similar to those obtained until 16 DAS in Experiment 1. *Beauveria bassiana* re-isolation from leaves peaked with 'leaf spraying' (86 ± 5%), followed by 'seed dressing' (16.0 ± 11.2 %) and 'soil treatment' (11.3 ± 5.6 %) ($P < 0.001$). On the other hand, re-isolation from roots peaked with 'seed dressing' (44.6 ± 18.8 %), followed by 'soil treatment' (33.8 ± 8.1 %) and 'leaf spraying' (0.0 ± 0.0 %) ($P = 0.048$). No colonies of the fungus were detected in 'control' plants.

Mortality in leafworm larvae feeding on leaves of plants subjected to the three inoculation methods was significantly higher than in 'control' larvae ($P < 0.001$); in fact, larval mortality amounted to 30.0 % with 'seed dressing', 53.3 % with 'soil treatment' and 56.7 % with 'leaf spraying' (see Table 6). On the other hand, no mortality was observed in larvae fed with 'control' plants. There were also significant differences in AST among larvae feeding on leaves of *B. bassiana* inoculated plants; thus, AST was 3.2 days with 'leaf spraying' and 'soil treatment', and 4.0 days with 'leaf spraying'. None of the dead larvae fed with colonized discs exhibited fungal growth.

410 **4. Discussion**

411 As can be seen from Fig. 1, *B. bassiana* succeeded in colonizing the rhizosphere upon application to the
412 soil surface; also, it persisted in soil throughout the experiments. Previous studies showed the persistence
413 of entomopathogenic fungi in soil and the migration of conidia from the surface to deeper layers to
414 depend on some soil properties (Kessler et al., 2003; Garrido-Jurado et al., 2011) and on the presence of
415 antagonistic organisms (Rumbos et al., 2008). The decline in CFU found in this work was also observed
416 in other studies using entomopathogenic fungi (Rumbos et al., 2008), where successful rhizosphere
417 colonization was found to rest on specific fungus–soil–plant interactions (Bruck, 2010). Our sterilizing
418 the soil before the three experiments may have helped *B. bassiana* colonize the rhizosphere.

419 As can be seen from Figs 2, 4 and 6, *B. bassiana* successfully colonized, established in and
420 moved within tissues of bread and durum wheat plants throughout their life cycle. The fungus was re-
421 isolated to a similar extent as in previous, shorter studies lasting less than one month (Gurulingappa et al.,
422 2010). In this work, *B. bassiana* was for the first time re-isolated from bread wheat and durum wheat
423 grains at the dough grain stage. Quesada-Moraga et al. (2014) demonstrated vertical transmission of *B.*
424 *bassiana* in opium poppies upon seed dressing; neither us nor these authors could identify the particular
425 inner tissues colonized by the fungus, however. To the best of our knowledge, no such information about
426 endophytic entomopathogens has to date been reported. We hypothesize that the fungus might reach the
427 ripening fruit/seed though the vascular system. This hypothesis is supported by the finding of Quesada-
428 Moraga et al. (2014) that *B. bassiana* was present mainly in the basal portion of the plant but
429 detected inside leaves at plant tips at the fruit/seed formation stage. These findings suggest that *B.*
430 *bassiana* adapts its growth strategy in order to reach reproductive tissues and ensure transmission to
431 progeny from endophytically colonized maternal plants.

432 'Soil treatment' and 'seed dressing' were the most effective inoculation methods as regards
433 colonization of plants by *B. bassiana*; unlike 'leaf spraying', both methods led to the fungus being re-
434 isolated from grain. Also, as can be seen from Fig. 4, 'leaf spraying' rarely led to re-isolation from roots
435 —and only in durum wheat. The success of *B. bassiana* in colonizing plants may rely on its ability to
436 reach the xylem (Wagner and Lewis, 2000) in order to spread throughout the plant. This assumption is

437 consistent with the finding that ‘soil treatment’ and ‘seed dressing’ resulted in systemic colonization of
438 the plants —*B. bassiana* was re-isolated from parts not directly in contact with the fungus—, and with the
439 results of previous studies (Ownley et al., 2008; Quesada-Moraga et al., 2009). On the other hand, Landa
440 et al. (2013) found *B. bassiana* applied by ‘leaf spraying’ to colonize aerial tissues of opium poppy plants
441 through intercellular spaces without reaching the cell lumen; also, fungal colonization was only
442 temporary. These results are consistent with our finding that no *B. bassiana* was re-isolated from grains at
443 the dough grain stage. In other studies, the applied fungus was re-isolated only from plant tissues in direct
444 contact with it (Tefera and Vidal, 2009; Greenfield et al., 2016). Therefore, the inoculation efficiency is
445 probably influenced by a number of variables including the particular host plant, fungal strain,
446 environmental conditions, substrate and soil.

447 The decreased plant growth initially observed in inoculated plants (9 and 12 DAS in Experiment
448 1; 31 DAS in Experiment 2; and 7 DAS for plant height and 8 DAS for total root length in Experiment 3)
449 may have resulted from the cost of the endophyte to the host plant. According to Partida-Martínez and
450 Heil (2001), plant–microbe interactions —those involving an endophyte included— can be explained in
451 terms of a cost–benefit balance. The presence of *B. bassiana* had both a cost (nourishing the
452 entomopathogen) and a benefit (biological control and, possibly, increased growth) for the host plant
453 (Vega et al., 2009). We hypothesize that the cost of inoculating the plant was temporarily lower growth
454 probably resulting from the amount of endophyte applied (Partida-Martínez and Heil, 2009) being
455 considerable for young plants but gradually decreasing in significance as the plants grew. After this initial
456 adverse effect, *B. bassiana* promoted plant growth and development. Thus, it increased plant height in
457 Experiment 1 (Fig. 3); spike production with ‘soil treatment’ and ‘seed dressing’ in bread wheat, and with
458 ‘soil treatment’ in durum wheat, in Experiment 2; and spike weight, grain yield and total root length with
459 ‘seed dressing’ in bread wheat in Experiment 3. Unlike ‘soil treatment’ and ‘seed dressing’, ‘leaf
460 spraying’ failed to promote plant growth except as regards to plant height in Experiment 1; in fact, it had
461 little or no effect on plant growth relative to the ‘control’ plants —it even caused a slight decrease in grain
462 weight in durum wheat.

463 Spike dry weight and grain production were more markedly increased in wheat bread than in
464 durum wheat, and also in Experiment 3 than in Experiment 2 —probably because the former used a
465 greater amount of fertilizer and the fungus may have been more effective as a result. The increased
466 number of grains in inoculated plants in Experiment 3 is specially worth noting because it testifies to the
467 ability of *B. bassiana* as a plant growth promoter. Our results underscore the importance of the fungal
468 inoculation method and plant–fungus interactions to the net effects on plant growth and grain production.
469 Contrary to previous studies (e.g., sorghum inoculated with *B. bassiana*; Tefera and Vidal, 2009), plant
470 growth was affected by the inoculation method used. In fact, our results with 'leaf spraying' contradict
471 those obtained with other microorganisms, where ecological costs led to yield losses (Brown, 2007) or no
472 growth differences between plants inoculated with *B. bassiana* and untreated plants were found (Lewis et
473 al., 2001). On the other hand, our results are consistent with those of Gualandi et al. (2014), who found
474 increased dry weight in *Echinacea purpurea* inoculated with *B. bassiana* via 'seed dressing', and those of
475 Waller et al. (2005), who reported increased yields in *Hordeum vulgare* grown on soil inoculated with the
476 endophytic fungus *Piriformis indica* —the increase, however, was much smaller than ours for bread
477 wheat with 'seed dressing' (11 % versus more than 40%).
478 The positive effects of *B. bassiana* on plant growth (viz., increasing spike production and total
479 root length) can be explained in various ways. Liao et al. (2014) found strains of another
480 entomopathogenic fungus, *Metarhizium spp.*, to increase spike biomass in inoculated corn seeds by
481 36−61 % and root colonization to be a prerequisite for the beneficial effects of this fungus. In this work,
482 'soil treatment' and 'seed dressing', which were the two inoculation methods leading to the greatest
483 extent of plant colonization —roots in all phenological stages and grain at the dough stage—, were also
484 the most effective in boosting spike production. Entomopathogenic fungi are known to produce
485 siderophores and organic acids (Joseph et al., 2012; Jirakkarul et al., 2015) that can alter the
486 bioavailability or certain nutrients. Sánchez-Rodríguez et al. (2015) recently found *B. bassiana* to
487 alleviate Fe chlorosis symptoms at an early stage in seed-inoculated tomato and wheat plants grown on
488 artificial calcareous substrates. Sánchez-Rodríguez et al. (2016) also found increased Fe bioavailability

489 and plant growth in sorghum, wheat and sunflower plants grown on (nonsterile) calcareous soils to which
490 the entomopathogenic fungus *Metarhizium brunneum* was applied at the beginning of the experiment.

491 Our mineral nutrient concentrations exceeded critical levels (Reuter et al., 1997). Application of
492 the fungus did not reduce such concentrations in aerial biomass (Experiment 1) and grain (Experiments 2
493 and 3). The most marked change was that in Mn for aerial biomass in bread wheat (Experiment 1).
494 Manganese is known to play a key role in plant growth and disease resistance (Huber et al., 1988). In
495 addition, some metalloproteins involved in biocontrol mechanisms of *B. bassiana* are Mn-cored (Xie et
496 al., 2012). Therefore, the increased amounts of Mn found in the inoculated plants could suggest a
497 response to fungal colonization as a way of protecting themselves from abiotic stress.

498 As regards phytohormones, high ABA contents have been associated to a response of plants to
499 abiotic stress (Ueguchi-Tanaka et al., 2007). Although *B. bassiana* inoculation altered phytohormone
500 levels at some phenological stages —in a different manner depending on the particular inoculation
501 method—, no clear-cut trend was observed. However, the changes suggest that the mechanisms by which
502 endophytic fungi colonize plants play a role here; thus, plant responses to stresses might depend on the
503 particular inoculation method.

504 The fungal strain used in this work proved a good endophyte; thus, mortality in larvae feeding on
505 leaf discs from inoculated bread wheat plants ranged from 30.0 to 56.7 %. These values are slightly
506 higher than those reported by Resquín-Romero et al. (2016) for the same insect feeding on *B. bassiana*
507 colonized melon, alfalfa and tomato leaves. Gurulingappa et al. (2010) found no insect dead but reduced
508 fitness, whereas Batta (2013) reported high mortality rates. Our results are consistent with those of
509 previous studies where no fungal outgrowth was detected in cadavers of insects fed with inoculated
510 plants. Resquín-Romero et al. (2016) detected dextrusin A, a mycotoxin secreted by *Metarhizium*
511 *brunneum*, in plants; this finding could partially account for larval mortality in the absence of fungal
512 outgrowth. It remains to be elucidated whether EABb 04/01-Tip strain secretes any metabolites
513 potentially able to kill larvae (e.g., beauvericin, oosporein, tenellin) *in planta*.

5. Conclusions

Beauveria bassiana successfully established as an endophytic fungus in roots, leaves, stems and —as found for the first time here— grains of bread wheat and durum wheat with some inoculation methods. The fungus increased yields (spike production) in *T. aestivum* ('soil treatment' and 'seed dressing') and *T. durum* ('soil treatment' only); this was especially so with 'seed dressing' in the former crop, where grain yield was 40 % higher than in the 'control' plants. The experiments conducted in this work demonstrate that *B. bassiana*, which is well-known for its microbial control potential, can be used to increase plant dry weight and spike production in wheat bread and durum wheat by inoculation with certain methods which also allow the fungus to efficiently kill *S. littoralis* larvae.

The differential effects on the target variables in bread wheat and durum wheat suggest that more specific knowledge about the plant–endophyte relationship is needed. In fact, studies on different plant species under variable field conditions will be required to confirm our results, and the ability of *B. bassiana* to promote plant growth and increase yields, and also to ascertain whether its establishment can have any adverse effects on insects in the long term.

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669 **Figure captions**

670

671 **Fig. 1.** Time course of Colony Forming Units (CFU) in soil treated with *B. bassiana* and used to grow
672 wheat and durum wheat in Experiment 2. Three replicates per treatment were used in each sampling. No
673 *B. bassiana* CFU were detected in the 'control' samples. CFU data were used to assess establishment of
674 the fungus in the rhizosphere.

675

676 **Fig. 2.** Mean ± standard error of *B. bassiana* re-isolation (%) from bread wheat leaves and roots as
677 influenced by inoculation method at different times after sowing (DAS) in Experiment 1. Two plants from
678 each of three pots per pot per treatment were collected in each sampling. No *B. bassiana* CFU were
679 detected in the 'control' plants. Different letters indicate significant differences at different DAS as per
680 Bonferroni's multiple comparison test.

681

682 **Fig. 3.** Mean ± standard error of plant height (mean value ± standard) for bread wheat in Experiment 1 as
683 a function of inoculation method at different times after sowing (DAS). The values shown are the means
684 for 17, 14, 11, 8, 8 and 5 pots bearing two plants each per treatment for each determination throughout the
685 experiment. Different letters indicate significant differences at different DAS as per Bonferroni's multiple
686 comparison test.

687

688 **Fig. 4.** Mean ± standard error of *B bassiana* re-isolation (%) from bread wheat and durum wheat leaves,
689 roots, stems and grains at different phenological stages as influenced by inoculation method in
690 Experiment 2. Two plants from each of three pots per treatment were collected in each sampling. No *B.*
691 *bassiana* CFU were detected in the 'control' plants. Different letters indicate significant differences at
692 different phenological stages as per Bonferroni's multiple comparison test.

693

694 **Fig. 5.** Time course of plant height (mean value ± standard) for bread wheat and durum wheat at different
695 times (DAS) as a function of inoculation method in Experiment 2. The values shown are the means of 22

(10, 14, 17, 21 and 24 DAS), 19 (28 and 31 DAS), 16 (38 and 44 DAS), 13 (56 and 71 DAS) and 10 (86 DAS) pots bearing two plants each per treatment for each crop. Different letters indicate significant differences at different DAS as per Bonferroni's multiple comparison test.

Fig. 6. Mean ± standard error of *B. bassiana* re-isolation (%) from bread wheat leaves, roots, stems and grains at different phenological stages as influenced by 'seed dressing' in Experiment 3. Three pots per treatment were used in each sampling. No *B. bassiana* CFU were detected in the 'control' plants.

Fig. 7. Time course of plant height (mean value ± standard) for bread wheat at different phenological stages as a function of inoculation method in Experiment 3. The values shown are the means of 22 (8, 10, 14, 18 and 21 DAS), 19 (24, 28, and 35 DAS), 16 (42 and 52 DAS), 13 (64, 73, 84 and 94 DAS) and 10 (108 DAS) pots per treatment. Different letters indicate significant differences as per Bonferroni's multiple comparison test.

Table 1

Table 1
Description of the experiments.

Experiment	Plant species (duration)	Treatments	Pots	Plants per pot	Analysis[a] (Assessments/replicates per treatment)	Statistical analysis
Effect of *B. bassiana* on bread wheat growth	Bread wheat (31 days)	Control	20	2	CFU[b] (4/3)	ANOVA+LSD
		Soil	20	2	Plant height (7/variable)	MANOVA+Bonferroni's
		Seed dressing	20	2	Fungal colonization[c] (5/3)	MANOVA+Bonferroni's
		Leaf spraying	20	2	Biomass dry weight (1/5)	ANOVA+LSD
					Biomass nutrient content (1/5)	ANOVA+LSD
Effect of *B. bassiana* on bread and durum wheat yield	Bread wheat (120 days) Durum wheat (100 days)	Control	25	2	CFU[b] (4/3)	ANOVA+LSD
		Soil	25	2	Plant height (11/variable)	MANOVA+Bonferroni's
		Seed dressing	25	2	Fungal colonization[c] (5/3)	MANOVA+Bonferroni's
		Leaf spraying	25	2	Phytohormones (5/3)	MANOVA+Bonferroni's
					Biomass and yield (1/10)	ANOVA+LSD
					Grain nutrient content (1/10)	ANOVA+LSD
Effect of *B. bassiana* on bread wheat yield and root length	Bread wheat (140 days)	Control	25	2	Plant height (15/variable)	MANOVA+Bonferroni's
		Seed dressing	25	2	Root length (4/3)	MANOVA+Bonferroni's
					Fungal colonization[c] (5/3)	
					Biomass and yield (1/10)	T-test
					Grain nutrient content (1/10)	T-test
Survival of cotton leafworm larvae fed with endophytically colonized bread wheat	Bread wheat (17 days)	Control	3	5	CFU (4/3)	ANOVA+LSD
		Soil	3	5	Fungal colonization[c] (1/5)	ANOVA+LSD
		Seed dressing	3	5	Average survival time of larvae (5/30[d])	Kaplan-Meier + Log-rank
		Leaf spraying	3	5		

[a]Plants from three pots were removed and used for fungal colonization 8, 10, 13, 19 and 31 DAS for the first experiment, at two-leaves, tillering, jointing, flowering and dough grain for the second and third experiments and 3, 9, 12 and 17 DAS for the fourth experiment. The rest of assessments did not involve a plant removal, except at the end of each experiment for biomass dry weight and biomass nutrient content, 5 pots were used in the first experiment and 10 pots in the second and third experiments. This assessment was not done for the fourth experiment.
[b]CFU: colony forming units were assessed weekly in the first experiment and monthly in the second and third experiments and 12 DAS in the fourth experiment.
[c]Fungal colonization. MANOVA+Bonferroni's were applied to the percentage of *B. bassiana* re-isolation from different vegetal tissues except for the fourth experiment (ANOVA, only one determination) and for grain in the second and third experiments (T-test, only one determination).
[d]Thirty larvae of *Spodoptera littoralis* were fed with inoculated plants (12 DAS) for 5 days, per inoculation method.

Table 2

Mean ± standard error of plant dry weight (aerial biomass) and nutrient concentration in aerial biomass of bread wheat as affected by inoculation method of *B. bassiana* 31 DAS in Experiment 1. Five replicates per treatment. Values followed by the same letter are not significantly different using least significant difference (LSD), $P < 0.05$.

Treatment	Plant g	P g kg^{-1}	K g kg^{-1}	Ca g kg^{-1}	Mg g kg^{-1}	Fe mg kg^{-1}	Mn mg kg^{-1}	Zn mg kg^{-1}	Cu mg kg^{-1}
Control	0.93 ± 0.01	2.2 ± 0.0 b	25.4 ± 2.1	4.7 ± 0.3	2.4 ± 0.1	101 ± 5	163 ± 8 c	67 ± 2 b	52 ± 3
Soil treatment	0.90 ± 0.01	2.3 ± 0.1 ab	26.6 ± 1.0	5.0 ± 0.1	2.3 ± 0.0	105 ± 4	379 ± 47 ab	62 ± 2 b	51 ± 1
Seed dressing	0.90 ± 0.01	2.6 ± 0.1 a	27.3 ± 1.9	5.3 ± 0.4	2.4 ± 0.1	120 ± 7	252 ± 30 bc	77 ± 3 a	53 ± 2
Leaf spraying	0.93 ± 0.01	2.6 ± 0.1 a	24.1 ± 3.0	5.1 ± 0.6	2.3 ± 0.2	108 ± 6	453 ± 73 a	67 ± 3 b	56 ± 2
P	0.160	0.026	0.784	0.782	0.942	0.179	0.005	0.010	0.416

Table 3

Mean ± standard error of plant dry weight of leaf+stem, spike, plant (leaf+stem+spike), total weight of grains, number of grains per plant and weight per grain (upper part), and mineral nutrient concentration in grain of bread wheat and durum wheat (bottom part) as affected by inoculation method of *B. bassiana* at the end of the crops in Experiment 2. Ten replicates per treatment. Values followed by the same letter are not significantly different using least significant difference (LSD), $P < 0.05$.

Treatment	Dry weight			Grain				
	Leaf + Stem	Spike	Plant	Total weight	Grains plant^{-1}	Weight grain^{-1}		
	g	g	g	g		mg		
	Bread wheat							
Control	2.83 ± 0.29	1.89 ± 0.12 b	4.68 ± 0.26 b	1.09 ± 0.18	39.5 ± 5.4	26.8 ± 1.8		
Soil treatment	3.14 ± 0.31	2.21 ± 0.18 a	5.35 ± 0.33 a	1.24 ± 0.22	46.1 ± 7.7	26.5 ± 0.9		
Seed dressing	2.91 ± 0.26	2.43 ± 0.07 a	5.33 ± 0.29 a	1.54 ± 0.11	53.0 ± 4.3	29.4 ± 0.7		
Leaf spraying	2.48 ± 0.20	1.81 ± 0.07 b	4.29 ± 0.18 b	1.05 ± 0.10	39.2 ± 2.7	26.6 ± 1.0		
P	0.407	0.002	0.027	0.147	0.220	0.361		
	Durum wheat							
Control	1.34 ± 0.09	2.34 ± 0.04 b	3.68 ± 0.10 b	1.75 ± 0.04 ab	38.8 ± 1.3	45.5 ± 1.3		
Soil treatment	1.62 ± 0.07	2.50 ± 0.05 a	4.13 ± 0.11 a	1.80 ± 0.02 a	36.6 ± 0.8	49.3 ± 0.9		
Seed dressing	1.54 ± 0.08	2.29 ± 0.03 b	3.84 ± 0.11 ab	1.70 ± 0.03 bc	35.7 ± 1.0	47.9 ± 1.0		
Leaf spraying	1.39 ± 0.08	2.24 ± 0.03 b	3.63 ± 0.09 b	1.66 ± 0.03 c	36.7 ± 1.1	45.7 ± 1.4		
P	0.074	< 0.001	0.007	0.012	0.212	0.081		
	P	K	Ca	Mg	Fe	Mn	Zn	Cu
	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
	Bread wheat							
Control	4.4 ± 0.2	3.8 ± 0.1	0.19 ± 0.01	1.5 ± 0.0 a	47 ± 2	136 ± 6	72 ± 4	7 ± 0
Soil treatment	4.5 ± 0.2	3.4 ± 0.3	0.18 ± 0.01	1.5 ± 0.1 ab	50 ± 4	156 ± 8	83 ± 8	8 ± 1
Seed dressing	3.9 ± 0.1	3.2 ± 0.1	0.18 ± 0.01	1.3 ± 0.0 c	43 ± 2	147 ± 3	66 ± 3	7 ± 0
Leaf spraying	4.0 ± 0.1	3.3 ± 0.1	0.19 ± 0.01	1.4 ± 0.0 bc	41 ± 2	143 ± 4	67 ± 1	7 ± 0
P	0.063	0.142	0.920	0.033	0.107	0.132	0.110	0.223
	Durum wheat							
Control	2.8 ± 0.1 b	2.8 ± 0.2 b	0.26 ± 0.01 a	0.9 ± 0.0 b	36 ± 1 b	91 ± 2	57 ± 1	7 ± 0
Soil treatment	3.3 ± 0.1 a	3.4 ± 0.2 ab	0.21 ± 0.01 b	1.1 ± 0.0 a	43 ± 2 a	95 ± 3	62 ± 2	7 ± 0
Seed dressing	3.0 ± 0.1 ab	3.2 ± 0.2 b	0.23 ± 0.00 b	1.0 ± 0.0 ab	41 ± 2 ab	91 ± 3	59 ± 1	7 ± 0
Leaf spraying	3.2 ± 0.1 a	4.0 ± 0.2 a	0.22 ± 0.01 b	1.1 ± 0.0 a	42 ± 1 a	98 ± 2	63 ± 2	7 ± 0
P	0.014	0.006	0.005	0.012	0.021	0.205	0.067	0.608

Table 4
Mean ± standard error of phytohormones time course in aerial biomass of bread wheat and durum wheat as affected by inoculation method of *B. bassiana* in Experiment 2. Three replicates per treatment and sampling. Values followed by the same letter are not significantly different using Bonferroni's multiple comparison test, $P < 0.05$.

Treatment	Bread wheat				Durum wheat			
	Tillering	Jointing	Flowering	Dough grain	Tillering	Jointing	Flowering	Dough grain
	ABA (ρmol g^{-1})							
Control	67 ± 3 a	250 ± 118	65 ± 14	433 ± 224	45 ± 3	95 ± 28 b	1131 ± 186	2122 ± 154 a
Soil treatment	38 ± 9 b	89 ± 5	63 ± 5	553 ± 171	45 ± 2	800 ± 78 a	1248 ± 125	2085 ± 453 a
Seed dressing	39 ± 3 b	271 ± 186	79 ± 14	193 ± 21	44 ± 2	510 ± 106 ab	1558 ± 314	282 ± 82 b
Leaf spraying	28 ± 1 b	344 ± 269	67 ± 4	247 ± 67	38 ± 3	173 ± 108 b	1167 ± 110	268 ± 62 b
P	0.004	0.770	0.713	0.335	0.252	< 0.001	0.465	< 0.001
	Salicylic acid (ρmol g^{-1})							
Control	330 ± 50 a	827 ± 419	288 ± 30	622 ± 120 b	1039 ± 585	552 ± 112	1198 ± 184	1839 ± 64
Soil treatment	1548 ± 1128 a	462 ± 122	241 ± 19	1306 ± 148 a	785 ± 133	955 ± 109	1310 ± 166	1395 ± 167
Seed dressing	502 ± 94 a	363 ± 48	180 ± 34	617 ± 107 b	1335 ± 722	672 ± 109	1416 ± 331	2325 ± 953
Leaf spraying	188 ± 33 a	463 ± 97	239 ± 28	546 ± 100 b	486 ± 47	736 ± 178	1020 ± 74	1513 ± 187
P	0.375	0.516	0.138	0.006	0.637	0.247	0.604	0.573
	Jasmonic acid (ρmol g^{-1})							
Control	357 ± 8 ab	217 ± 49	313 ± 30	393 ± 116	753 ± 62	858 ± 133	654 ± 162	1193 ± 215 a
Soil treatment	443 ± 33 a	149 ± 4	202 ± 22	428 ± 143	551 ± 92	1021 ± 147	529 ± 183	1047 ± 133 ab
Seed dressing	251 ± 44 bc	228 ± 46	449 ± 111	358 ± 59	475 ± 98	1156 ± 112	702 ± 82	369 ± 51 b
Leaf spraying	156 ± 20 c	233 ± 27	356 ± 92	296 ± 20	656 ± 134	1035 ± 201	555 ± 76	369 ± 114 b
P	0.001	0.387	0.210	0.800	0.293	0.605	0.777	0.004

Table 5

Mean ± standard error of dry weight of leaf+stem, spike, plant (leaf+stem+spike), total weight of grains, number of grains per plant and weight per grain and mineral nutrient concentration in grain of bread wheat as affected by inoculation method of *B. bassiana* at the end of the crop in Experiment 3. Ten replicates per treatment.

Treatment	Dry weight			Grain				
	Leaf + Stem	Spike	Plant	Total weight	Grains plant^{-1}	Weight grain^{-1}		
	g	g	g	g		mg		
Control	2.63 ± 0.22	2.55 ± 0.15	5.18 ± 0.21	1.84 ± 0.16	69.2 ± 6.4	26.9 ± 1.1		
Seed dressing	2.33 ± 0.21	3.28 ± 0.23	5.61 ± 0.12	2.64 ± 0.26	112.9 ± 8.8	23.3 ± 1.2		
P	0.337	0.015	0.102	0.014	< 0.001	0.050		
	P	K	Ca	Mg	Fe	Mn	Zn	Cu
	g kg^{-1}	g kg^{-1}	g kg^{-1}	g kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}	mg kg^{-1}
Control	4.6 ± 0.1	2.3 ± 0.1	0.47 ± 0.02	1.8 ± 0.1	65 ± 2	123 ± 6	52 ± 3	8 ± 0
Seed dressing	4.5 ± 0.3	2.5 ± 0.2	0.51 ± 0.02	1.8 ± 0.1	63 ± 2	96 ± 4	43 ± 4	8 ± 0
P	0.730	0.461	0.165	0.810	0.677	0.004	0.064	0.203

Table 6
Mean ± standard error of mortality (%) and average survival time (AST) of second instar *S. littoralis* larvae after 5 days of feeding with disc leaves from inoculated plants (three inoculation methods used). Thirty larvae (replicates) were used per treatment. Values followed by the same letter are not significantly different using LSD multiple comparison test (mortality) and log-rank test (AST), P < 0.05.

		Kaplan-Meier survival analysis	
Treatment	Mortality[a] %	AST[b] days	Confidence interval (95%)
Control	0.0 ± 0.0 c	5.0 ± 0.0 a	-
Seed dressing	30.0 ± 5.8 b	4.0 ± 0.3 b	3.4−4.5
Soil treatment	53.3 ± 3.3 a	3.2 ± 0.3 c	2.6−3.9
Leaf spraying	56.7 ± 3.3 a	3.2 ± 0.2 c	2.8−3.6
P	< 0.001		

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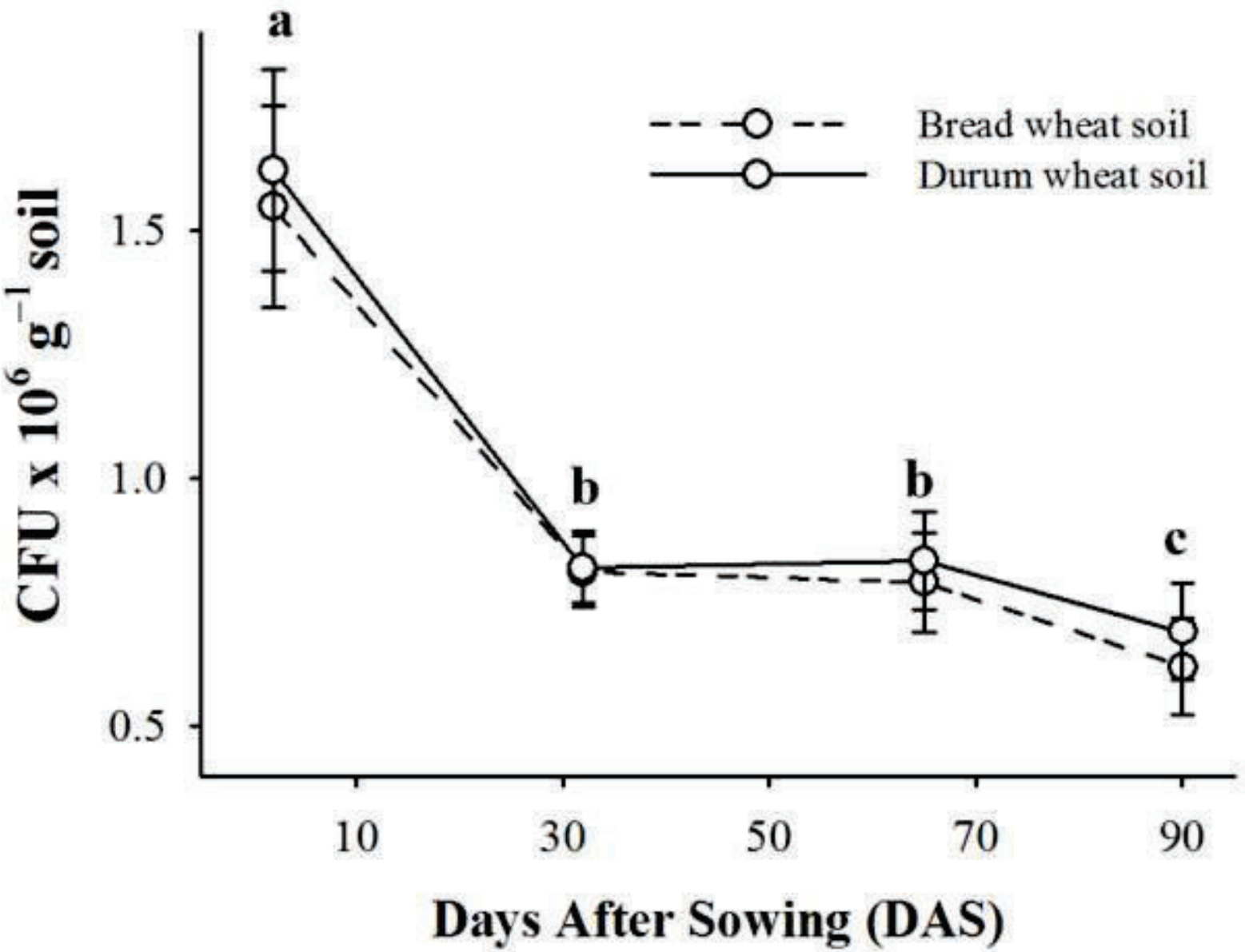

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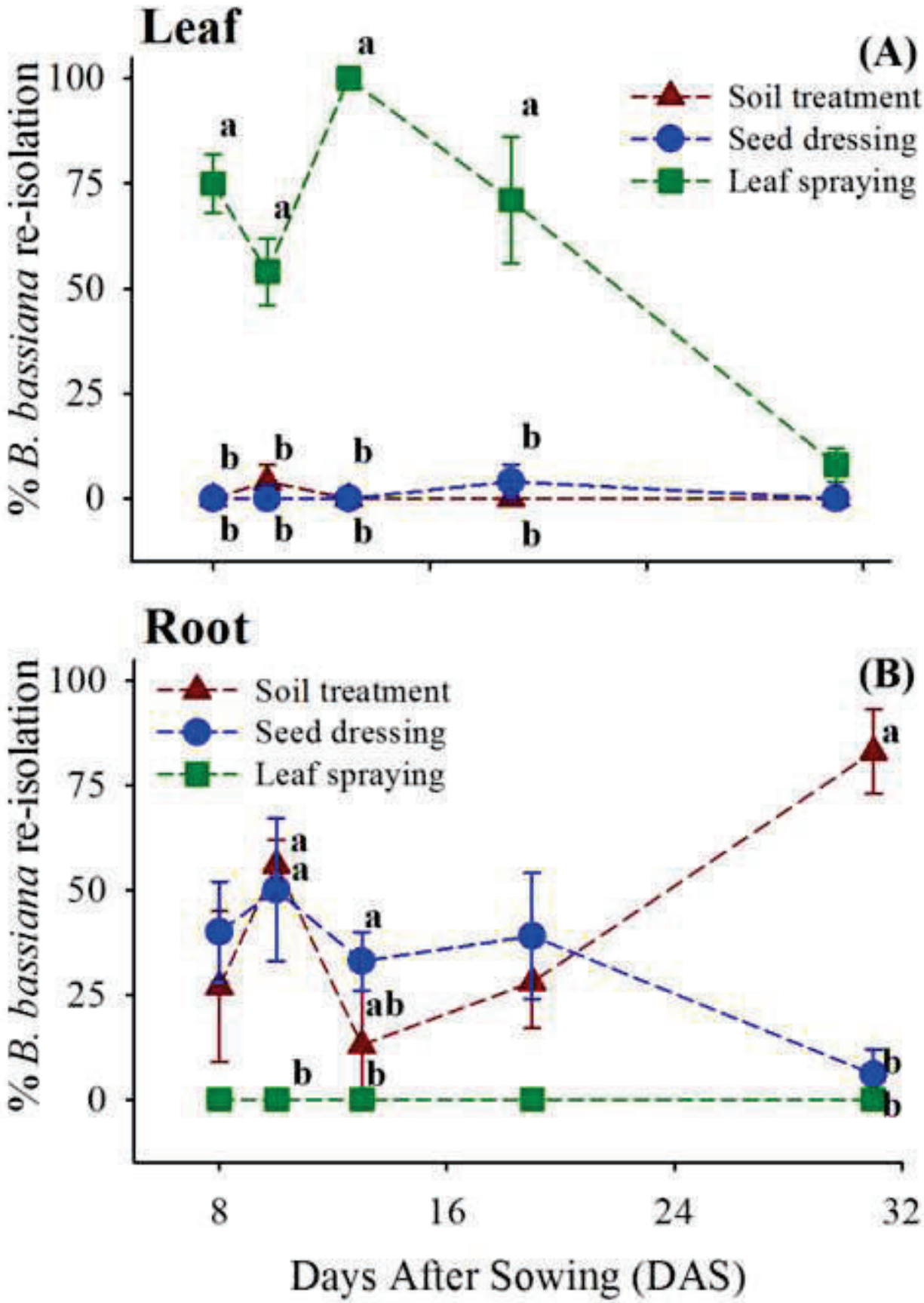

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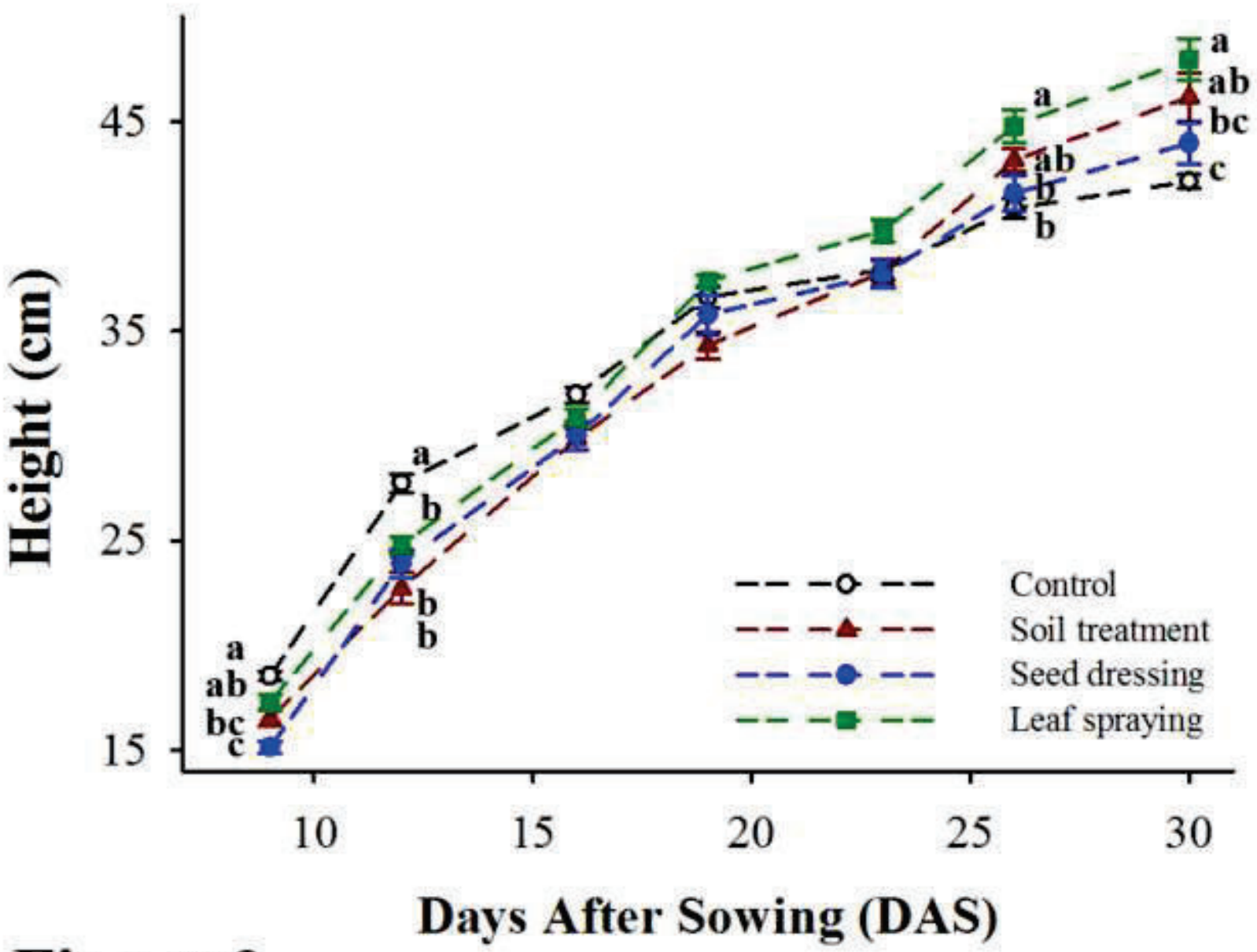

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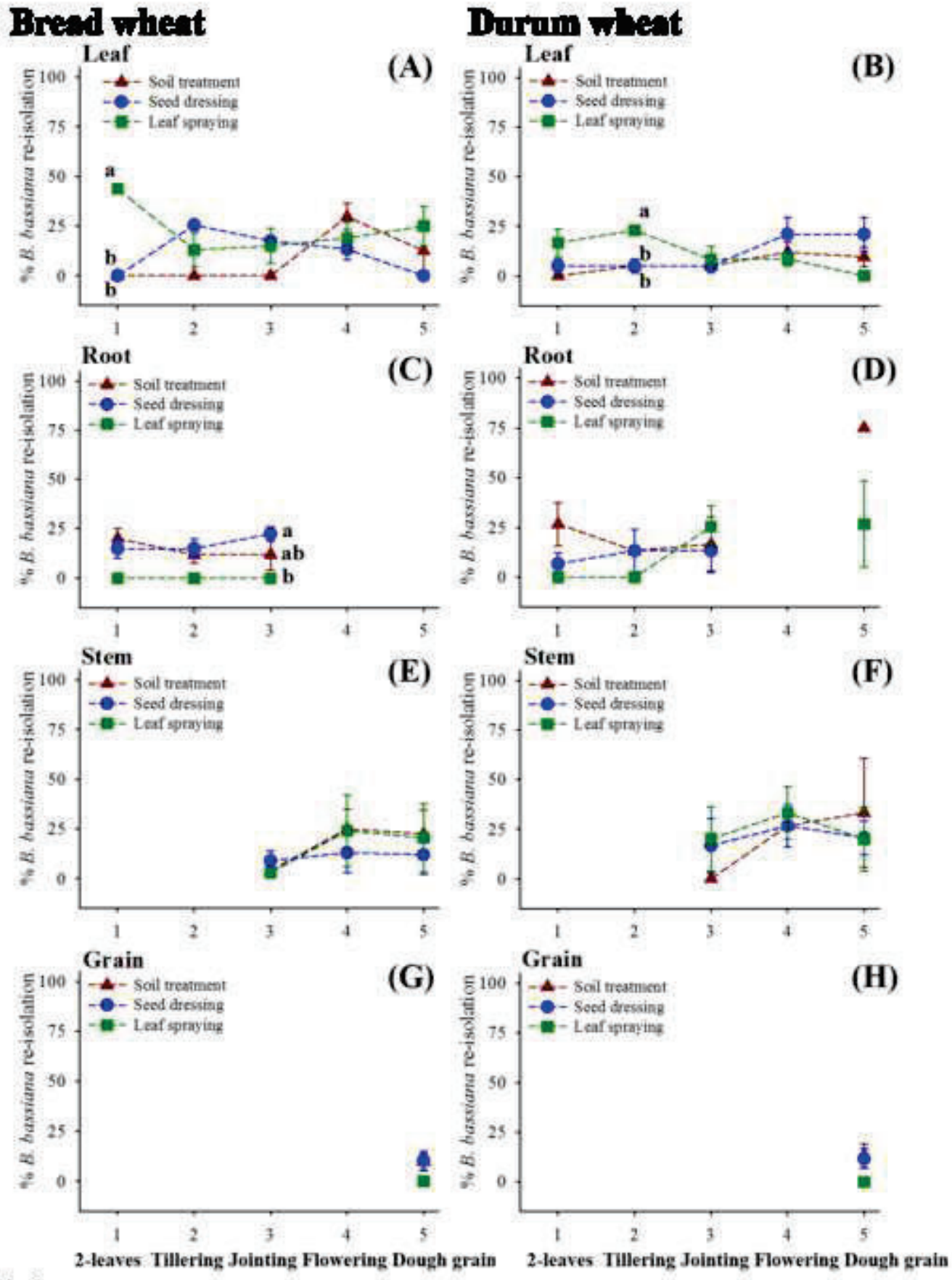

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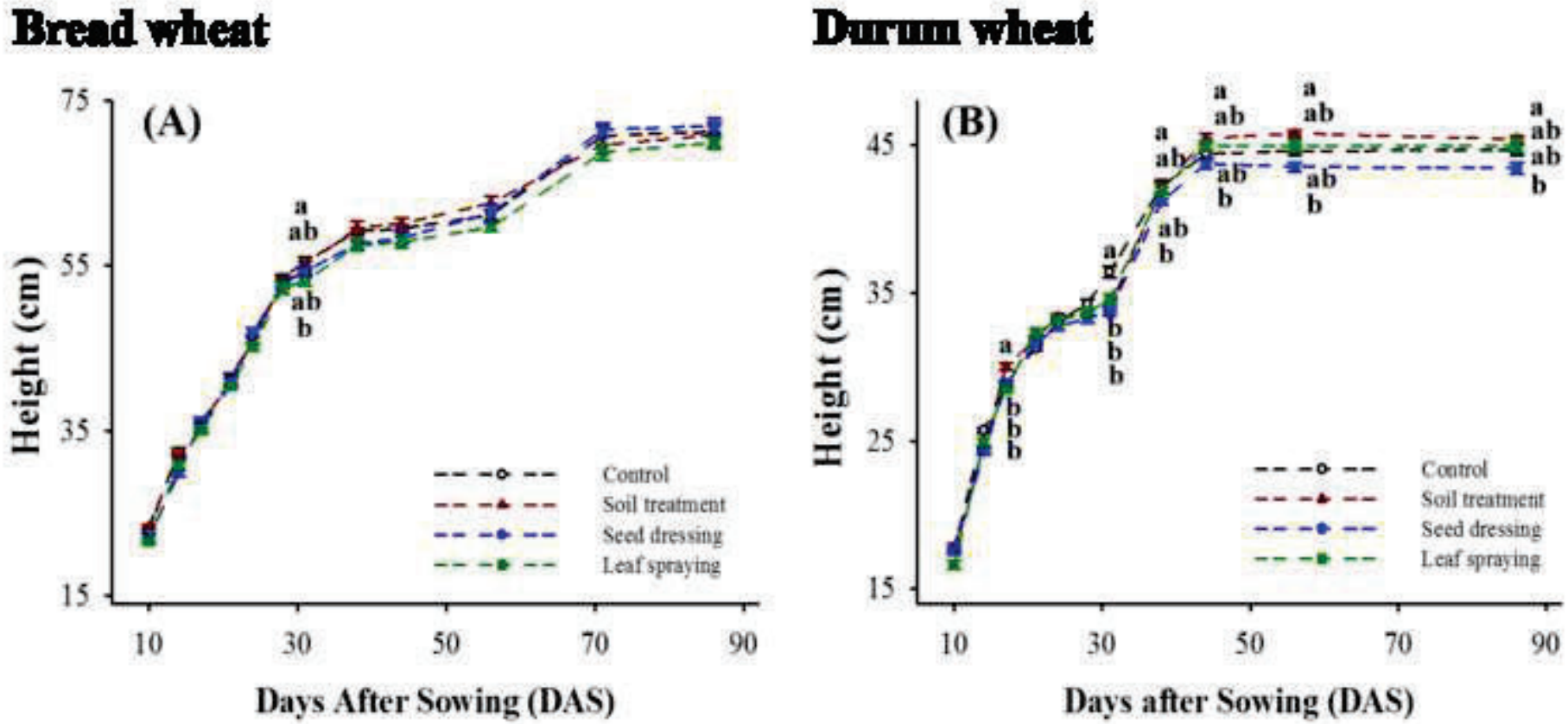

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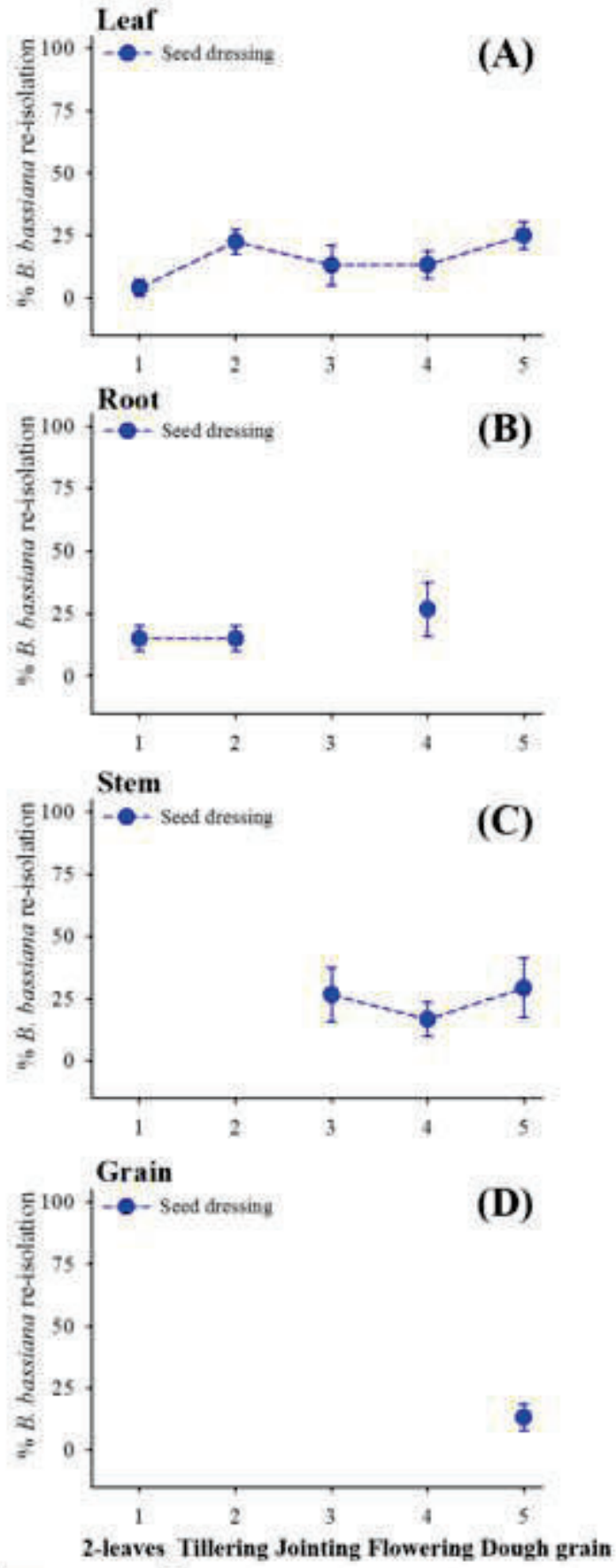

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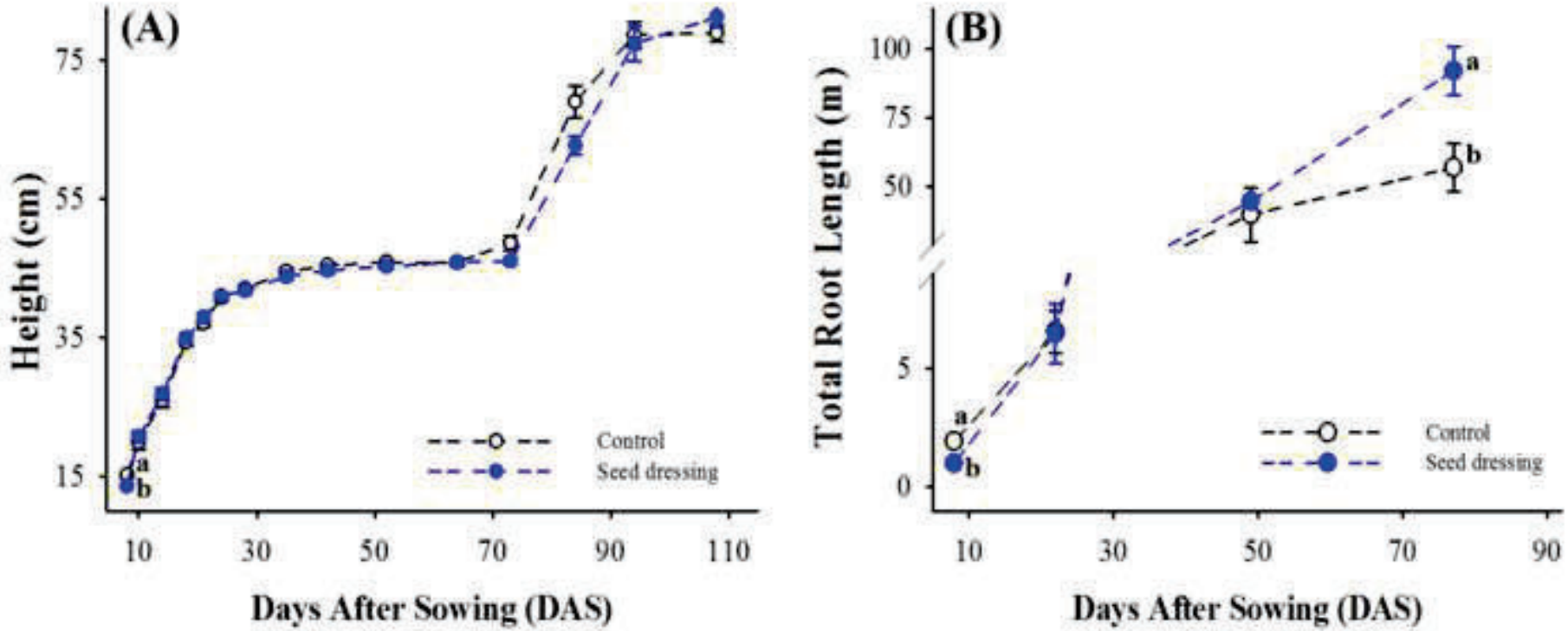

Figure 7