Soil Nutrients Effects on the Performance of Durum Wheat Inoculated with Entomopathogenic Fungi

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Abstract: Entomopathogenic fungi (EFs) are widely used as biological control agents. However, some strains of *Beauveria bassiana* and *Metarhizium brunneum* can also promote plant growth and increase nutrient uptake. We examined the effects of soil properties on the performance of *Triticum durum* inoculated by seed dressing with these EFs and grown on 12 agricultural soils. The plants were supplied with all nutrients except P and Zn (essential for yield and the grain quality of wheat). Fungal inoculation increased the grain yield and harvest index significantly with *B. bassiana* (17% and 14%, respectively) but not with *M. brunneum* (6% and 6%, respectively). The increase in grain yield was positively and moderately correlated with the soil available phosphorus (P_{Olsen}) in plants inoculated with *B. bassiana* and with the soil content in poorly crystalline Fe oxides with *M. brunneum*. In addition, the increase in aerial dry matter resulting from inoculation with *B. bassiana* was negatively correlated with soil available Zn. Furthermore, the observed increase in grain yields due to fungal inoculation resulted in P and Zn grain dilution (grain nutrient concentrations decrease). Inoculation with *B. bassiana* increased grain Zn uptake and the proportion of Zn in grain relative to that in aerial dry matter. Success in the mutualistic relationship between EF and wheat plants depends on the fungal strain and soil properties.

Keywords: grain yield; growth promotion; phosphorus; plant–soil–fungus interaction; zinc

1. Introduction

The rapid increase in human population in recent decades has required an increase in food production and nutritional quality in staple crops (maize, wheat and rice) without jeopardizing the environmental health of agroecosystems or their ability to provide ecosystem services. In this respect, using sustainable alternatives to inorganic fertilizers and pesticides has provided a powerful tool for reducing the adverse side effects of traditional agricultural production [1]. For instance, a number of studies have exposed the central role of rhizobacteria and mycorrhiza as plant growth promoters and nutritional enhancers for crops [2]. The use of microorganisms to combat crop pests, known as "biological control", is a widespread successful practice to minimize the environmental impact of synthetic products [3]. More specifically, entomopathogenic mitosporic ascomycetes (EMAs) such as *Beauveria bassiana* (Bals) Vuill. and *Metarhizium brunneum* (Petch) are effective biocontrol agents against pests [4] as well as disease antagonists [5]. These microorganisms can penetrate many plants and move through them as endophytes [6–8].

The question posed by Vega et al. (2009) [9] whether other positive synergistic or side effects might have been overlooked when using entomopathogenic fungi (EF) solely as biopesticides against insects, aroused the interest of researchers to explore new or unknown roles of these fungi. Accordingly, EFs have

been investigated as plant growth promoters [10–12] and nutritional enhancers [10,13–16]. Although *B. bassiana*, *M. brunneum* or both fulfill the expectations of most studies, these studies were largely conducted under conditions far from typical field settings. In fact, a number of studies were performed on sterile soils or artificial substrates; the effects of the fungi were only examined during early crop growth stages [10,11,13], so neither potential interactions with crops grown on natural soils nor their effects throughout the crop cycle were considered. In addition, although some physical soil properties are known to affect the availability, mobility and virulence of EF conidia in soil [17], there is a knowledge gap on the role that physical and chemical soil properties play in the crosstalk between EF and plants. This knowledge is key to achieving high crop performance. The soils of southern Spain, and generally those under Mediterranean climates, are usually deficient in P and Zn, both of which are crucial for adequate plant performance [18,19]. While P deficiency mainly decreases crop growth and yield [19,20], Zn deficiency reduces the Zn contents of most of the edible parts of crops; this is particularly common with grain crops [21] and is responsible for malnutrition in nearly 30% of the world's population [22]. Zinc deficiency is, therefore, especially important in countries with diets based on cereals grown in soils with low soil Zn availability [22] but also in countries such as Spain, where farmers seek to improve wheat quality. Although previous studies have shown *Beauveria bassiana* and *Metarhizium brunneum* to increase the bioavailability of micronutrients such as Fe [14,16], the effects of EF on biomass in the plants they colonize, P and Zn plant uptake and grain quality remain largely unexplored. Further, there is a well-known negative interaction between P and Zn in soil [23], and a lack of information about how EFs may affect such interactions in soils with limited bioavailability of these nutrients.

The main objective of this study was to elucidate the effects of soil properties on the relationship between EFs (*B. bassiana* and *M. brunneum*) and durum wheat plants, with an emphasis on plant growth and the uptake of nutrients (primarily P and Zn). In addition, we also evaluated how the differences in soil nutrient availability before and after the experiment were related to wheat performance parameters. We hypothesized that inoculation with EFs enhances plant performance and nutrient uptake, mainly when the plant is able to fulfil its own needs; meaning that success in the EF–plant relationship will depend on soil properties.

2. Materials and Methods

2.1. Experimental Design

Triticum durum L. (durum wheat), twelve soils and three treatments (Control, *B. bassiana* and *M. brunneum*) were assayed in a completely randomized factorial design with 6 replicates per soil × treatment combination. This resulted in 216 experimental units or pots, each containing one wheat plant.

2.2. Crop

Durum wheat (*Triticum durum* L. cv. Calero) was chosen because it is a staple crop cultivated worldwide with relevant importance in the Mediterranean belt, including southern Spain where the agricultural soils were collected. Furthermore, durum wheat is a good candidate for this study because its susceptibility to endophytic colonization by EFs was previously reported [10,12] to improve plant growth [12] and Fe uptake [13].

2.3. Soils and Soil Properties

The twelve soils used in this study were obtained from the collection of 47 topsoil (0–25 cm) samples characterized by Sacristán et al. (2019) [24] (see Table 1 and Table S1 in the Supplementary material). The selection of these soils was done based on several criteria: The soils were representative of the Mediterranean region, varied widely in their properties, covered a broad range of P content and were all Zn-poor soils, which resulted in an uneven P/Zn ratio. The wide variety of soil properties represented in these 12 soils also enabled us to evaluate the presence of key parameters in plant–EF crosstalk. Three of the 12 soils were non-calcareous and nine were calcareous (Table 1). Their content

in carbonate-free clay ranged from 62 (Soil 4) to 645 g kg^{-1} (Soil 8), and organic carbon (OC) ranged from 5 (Soils 2, 21 and 22) to 13 g kg^{-1} (Soil 4). The cation-exchange capacity (CEC) ranged from 11 to 43 cmol$_c$ kg^{-1} and was significantly correlated with clay content ($R = 0.83$). All soils had similar pH values (8.0–8.8, except for no. 4, which had a pH of 6.6). The soils were non-saline (the EC value of the 1:5 soil:water extract was below 284 μS cm^{-1}) and the calcium carbonate equivalent (CCE) of the calcareous soils ranged from 32 to 596 g kg^{-1}.

For the soil micronutrient availability, Zn$_{DTPA}$ was low (<0.52 mg kg^{-1}; (0.39 ± 0.09) mg kg^{-1}) and Cu$_{DTPA}$ and Mn$_{DTPA}$ varied widely (0.18–6.3 and 3.2–25 mg kg^{-1}, respectively). The amount of poorly crystalline, highly reactive, Fe oxides (Fe$_{ox}$) was low, except in Soils 8, 31 and 48, where it exceeded 1 g kg^{-1}. The available K differed widely among soils and was correlated with CEC ($R = 0.460$; $p = 0.009$) and hence with that type of clay and its content in the soil. Finally, the P$_{Olsen}$ ranged from 5.5 mg kg^{-1} to 37.9 mg kg^{-1}, and the mass P$_{Olsen}$/Zn$_{DTPA}$ ratio ranged from 13 to 111 in Soil 8 and Soil 21, respectively.

At harvest (102 days after sowing), half of the soil from the bottom part of the pots was reanalyzed (4 replicates per soil–fungal combination) to determine the variation of available P (P$_{Olsen}$), Fe (Fe$_{DTPA}$), Cu (Cu$_{DTPA}$), Mn (Mn$_{DTPA}$) and Zn (Zn$_{DTPA}$) caused by the inoculated and non-inoculated plants. For this process, the soil samples were air-dried and sieved through a 2 mm mesh (removing roots) and homogenized in the laboratory. Micronutrients (Fe, Cu, Mn and Zn) were extracted with diethylenetriaminepentaacetic acid (DTPA) (Fe$_{DTPA}$, Cu$_{DTPA}$, Mn$_{DTPA}$ and Zn$_{DTPA}$) by suspending 10 g of soil in 20 mL of extractant at 25 °C, shaking the suspension at 2 Hz and centrifuging it at ~10^4 m s^{-2} for 15 min [25] before measuring the samples by atomic absorption spectrophotometry. Available soil P was determined following Olsen et al. (1954) [26] and measured by the method of Murphy and Riley (1962) [27]. A more detailed description of this procedure can be found in Sacristán et al. (2019) and in the Supplementary material.

The naturally present *M. brunneum* and *B. bassiana* in the soils were discarded before the pot experiment by counting the corresponding colony forming units (CFUs). For this purpose, 1 g of each soil (four replicates) was mixed with 10 mL of sterile deionized water (SDW) and shaken for 60 min in a rotational shaker at 2.5 Hz. Then, two 100 μL aliquots from 1/100 and 1/1000 dilutions of soil solution samples were plated in Petri dishes containing a Sabouraud Dextrose Chloramphenicol Agar (SDCA) medium and kept at 25 °C in the dark. The CFUs were counted after 6–12 days of inoculation. Neither *B. bassiana* nor *M. brunneum* was detected in any of the soils.

Table 1. Selected properties [‡] of the soils.

Soil Code	Clay	OC	CCE	pH	EC	CEC	Fe$_{ox}$	Fe$_{DTPA}$	Cu$_{DTPA}$	Mn$_{DTPA}$	Zn$_{DTPA}$	K$_{available}$	P$_{Olsen}$	P$_{CaCl2}$	P$_{Olsen}$/Zn$_{DTPA}$
	g kg^{-1}				µS cm^{-1}	cmol$_c$ kg^{-1}	g kg^{-1}			mg kg^{-1}				mg L^{-1}	
Non-calcareous soils															
2	286	5	0	8.8	176	17	0.76	5.3	1.45	11.6	0.50	148	30.1	0.03	60
4	62	13	0	6.6	30	11	0.90	36.7	0.18	25.0	0.43	78	8.4	0.01	20
8	645	8	0	8.1	158	43	1.06	4.9	0.64	15.4	0.42	293	5.5	0.00	13
Calcareous soils															
19	200	11	596	8.3	213	22	0.25	4.5	6.11	14.1	0.51	382	12.0	0.00	24
21	108	5	231	8.0	397	14	0.18	3.7	3.03	5.9	0.39	101	37.9	0.04	96
22	116	5	153	8.4	132	15	0.18	3.6	1.69	3.2	0.32	86	11.1	0.00	34
26	371	8	346	8.5	156	34	0.69	15.4	2.07	3.6	0.28	585	10.5	0.01	37
27	362	6	330	8.3	284	34	0.68	9.8	1.80	12.1	0.38	663	17.0	0.00	45
28	163	8	587	8.6	170	23	0.31	4.1	6.30	8.4	0.46	254	5.9	0.01	13
31	385	7	32	8.0	163	25	1.06	5.6	5.13	6.5	0.29	546	8.3	0.00	29
37	185	10	61	8.1	245	17	0.27	5.3	0.40	8.2	0.21	164	22.9	0.01	111
48	120	12	43	8.3	173	29	1.55	8.6	0.95	6.5	0.51	585	16.9	0.07	33

[‡] Clay, carbonate-free clay; OC, organic carbon; CCE, calcium carbonate equivalent; EC, electrical conductivity of 1:5 soil:water extract; CEC, cation-exchange capacity; K$_{available}$, exchangeable K; Fe$_{ox}$, NH$_4$ oxalate-extractable Fe; Fe$_{DTPA}$, Cu$_{DTPA}$, Mn$_{DTPA}$ and Zn$_{DTPA}$, Fe, Cu, Mn and Zn extracted by diethylenetriaminepentaacetic acid (DTPA); P$_{CaCl2}$ and P$_{Olsen}$ are the most labile P extracted with CaCl$_2$ and the P availability extracted with Olsen.

2.4. Fungi

2.4.1. Fungal Isolates

Beauveria bassiana (Balsamo) Vuill., strain EABb 04/01-Tip, is registered with the identification number 20744 in the Spanish Collection of Culture Types (CECT), as is *Metarhizium brunneum* 01/58-Su, with the number 20784. Both came from the Entomopathogenic Fungi Collection of the University of Córdoba (Research group AGR 163 of the Department of Agronomy).

2.4.2. Fungal Preparation

For the experiment, both fungi were multiplied in SDCA, scraped with a razor and kept at $-20\,°C$ for 10 days before application to the wheat seeds. The concentrations obtained were 4.7×10^9 conidia g^{-1} for *B. bassiana* and 2.3×10^9 conidia g^{-1} for *M. brunneum*. Conidial viability was determined with a hemocytometer (Malassez chamber) 48 h before application and it exceeded 90% for both fungi.

2.4.3. Fungal Treatment: Seed Dressing

A total of 648 seeds (216 pots $\times$ 3 seeds pot^{-1}) were used for sowing. Twenty additional seeds were checked after surface sterilization to ensure the absence of *B. bassiana* and *M. brunneum* inside before fungal inoculation of the seeds. Disinfection involved immersing the seeds in 70% ethanol for 2 min, then in a 3.6% NaClO solution for 5 min and finally, in sterile deionized water twice for 2 min each time. Two 100 µL aliquots of water from the last wash were plated in Petri dishes containing an SDCA medium and kept at 25 °C in the dark to thoroughly ensure external disinfection.

In each fungal treatment (*B. bassiana* and *M. brunneum*), 216 seeds (12 soils $\times$ 6 replicates $\times$ 3 seeds pot^{-1}) were spread on a large disinfected plastic tray to obtain a monolayer of seeds and sprayed with 0.4 mL of a sterilized sugar solution (15% w/v) to make them moist and sticky. Then, 0.85 g of *B. bassiana* and 1.7 g of *M. brunneum* conidia were mixed with 0.2 g of peat—sterilized and sieved through 150 µm—, separately. Fungus, peat and seeds were rubbed by hand to spread the fungus and peat over the seeds homogeneously. No fungus was mixed with the peat in the Control treatment. The number of conidia per seed was estimated to be $\sim1.8 \times 10^7$ for *B. bassiana* and *M. brunneum*. All seeds were sown immediately after treatment.

2.5. Pot Experiment

Cylindrical PVC pots 5 cm in diameter and 15 cm tall with a 6 mm drainage hole at the bottom and fitted with a cellulose acetate filter were used. Different amounts of soil (Table S1) were used to fill the pots up to 1.5 cm below the pot rim (between 245 and 465 g per pot depending on the soil) and then immersed for 12 h (until saturation) in a tray filled with P- and Zn-free Hoagland solution [5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 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 $ZnSO_4 \cdot 7H_2O$, 10mM Fe (as EDDHA), 0.1 µM $CuSO_4 \cdot 5H_2O$ and 6 µM Na_2MoO_4] diluted four-fold, except for the N (undiluted) and Fe in calcareous soils (0.3 g Fe kg^{-1} soil) to ensure no iron deficiency in plants. The soil was allowed to drain freely for 24 h, and three treated seeds per pot were sown before transferring the pots to a growth chamber with a photoperiod of a 16 h day^{-1}, with light intensity around 325 µmol m^{-2} s^{-1}, a temperature of 24 °C in the day and 19 °C at night and 65% relative humidity. Plants were watered with de-ionized water or the nutrient solution mentioned above but without Fe (adding a total of ~60 mL of Hoagland solution per plant) at one- or two-day intervals to maintain the soil moisture at field capacity and supply the plants with the nutrients required to fulfill their needs. Fifteen days after sowing (DAS), the plants were thinned to leave one per pot (experimental unit).

2.6. Plant Growth, Gas Exchange, Water Use Efficiency (WUE) and Nutrients Analyses

Plant growth rate, as the increase in plant height over time, was measured at 13, 19, 26, 39, 48 and 73 DAS. The gas exchange parameters [viz., photosynthesis rate (A_n), conductance (g_s), intercellular CO_2 concentration (C_i) and transpiration rate] were measured in wheat plants grown on five randomly selected soils (No. 2, 19, 26, 31 and 48) by using a portable infrared CO_2 gas analyzer (LiCor Li6400XT, Inc.; Lincoln, NE, USA) at 62 DAS (stage 50 in the Zadoks growth scale). Plant water use efficiency (WUE) was calculated as the ratio of net photosynthesis to the transpiration rate. The experimental conditions for these measurements involved a CO_2 concentration of 400 ppm, a flux of 300 μm^3 s^{-1} and photosynthetic active radiation (PAR) of 500 μmol m^2 s^{-1} at temperatures from 23 to 26 °C. A total of 4 plants (replicates) per soil–fungal treatment combination were randomly chosen and used. Three measurements per plant were taken in the middle portion of the flag leaf at 15 s intervals from minute 2 to 2.5 to allow the leaves to adjust to the above-described conditions. Further, all measurements were made 2.5 to 7.5 h after dawn, when photosynthesis rates were expected to peak. The gas exchange variables were analyzed in the flag leaf (stage 50 in the Zadoks growth scale) because the flag leaf is the greatest source of assimilates during spike formation, so it may influence grain yield [28]. Soil, plant replicates and fungal treatments were randomly selected to minimize the effect of sampling at different times.

At harvest (102 DAS), the plants were cut 1 cm above the soil surface to avoid the presence of soil adhering to the stem base, dried at 60 °C for 72 h, split into grain and straw (stem, rachis plus glume, palea and lemma) and portions of 0.2–0.3 g of each division were digested with a mixture of concentrated nitric (3 mL) and perchloric acid (1.5 mL) [29]. Subsequently, the nutrients in the solution were measured using a Lambda 35 UV/VIS spectrometer (P), a Jenway PFP 7 flame photometer (K and Na) or a Perkin–Elmer AAnalyst 200 atomic absorption spectrometer (Ca, Mg, Fe, Mn and Zn). The nutrient uptake values were calculated by multiplying the concentration of each nutrient in each part of the plant (straw and grain) by its dry weight or yield. Finally, the proportion of grain Zn uptake was calculated as the ratio of grain Zn uptake to plant Zn uptake (grain + straw).

2.7. Statistical Analysis

A factorial analysis of variance (ANOVA) was performed on plant height at different times throughout the crop cycle, with soil (12 soils) and fungal treatment (Control and inoculation with *B. bassiana* or *M. brunneum*) as factors. Differences in biomass, number of tillers and nutrient uptake at harvest between the Control plants and those inoculated with each EF were assessed in separate paired *t*-tests that included the 12 soils. The soils were split into two groups to identify any alterations in plant nutrition associated with grain yield changes due to the fungal treatments. One included the group of soils where the grain yield increased by more than 15% (GSGYI > 15) in the inoculated plants relative to the non-inoculated plants. Such soils included no. 2, 4, 8, 21, 26, 37 and 48 with *B. bassiana* and no. 2, 4, 8, 26 and 48 with *M. brunneum*. The other group of soils contained soils where the grain yield increased by less than 15% (GSGYI < 15), namely, no. 19, 22, 27, 28 and 31 with *B. bassiana* and no. 19, 21, 22, 27, 28, 31 and 37 with *M. brunneum*. For each group of soils and EF, a factorial ANOVA with soil and fungal treatment as factors was performed for the ADM (straw + grain yield), grain yield, nutrient uptake and grain nutrient concentration. A second paired *t*-test analysis was performed to compare the variation in soil nutrient availability between treatments, both for the 12 soils and for the group of soils described above. Further, regression models for the differences in aerial dry matter (ADM) and grain yield between the inoculated and Control plants in relation to the soil properties were developed. Pearson correlations between the increase in ADM or grain yield and the increase in nutrient uptake with the fungal treatments relative to the Control treatment were calculated in order to identify any key nutrients influencing the growth promoting effects of the fungi. Pearson correlations between the variation in soil nutrient availability with ADM, grain yield and nutrient uptake of the crop, as well as with the relative grain yield and ADM variation between the fungi and Control, were also performed.

Gas exchange (net photosynthesis, stomatal conductance and intercellular CO_2 concentration) and WUE values (4 replicates) for each fungus and the Control were compared in 5 randomly selected soils (No. 2, 19, 26, 31 and 48) separately over time. The importance of the factor of time to these variables led us to conduct a repeated measures ANOVA.

When the assumptions for the parametric analysis were not fulfilled with transformations, a non-parametric Kruskal–Wallis test was used. Outliers (viz., values lower than *Q1 − 1.5(IQR)* or higher than *Q3 + 1.5(IQR)*) were detected and excluded. Unless otherwise stated, the term 'significant' is used here to denote significance at a $p < 0.05$ level. Statistical analyses were performed using the statistical package STATISTIX 10.0 (Analytical Software, Tallahassee, FL, USA) and IBM SPSS Statistics (Version 25).

3. Results

3.1. Plant Growth and Yield

Plant height was significantly affected by the factor of soil but not by the factor of fungal treatment (results not shown). However, a significant interaction between these two factors was found at 39 DAS in plants inoculated with *B. bassiana*; thus, inoculation with this fungus significantly decreased plant height in Soils 28 and 37 (Figure S1).

The ADM at harvest widely differed among the soils, from 1.16 g plant^{-1} in Soil 27 to 2.73 g plant^{-1} in Soil 48 (Table S2). Further, the grain yield ranged from 0.37 g plant^{-1} in Soil 31 to 1.05 g plant^{-1} in Soil 48, and the harvest index (grain yield/ADM ratio) ranged from 0.19 in Soil 31 to 0.51 in Soil 19 (Table S2). The ADM was positively correlated with the initial $CaCl_2$-P ($R = 0.754$; $p < 0.01$), as was grain yield ($R = 0.579$; $p < 0.05$).

Based on the results of the paired *t*-tests (Table 2), the grain yield and harvest index were significantly greater in plants inoculated with *B. bassiana* than in the Control plants; the number of grains per plant was significantly greater in plants inoculated with *B. bassiana* or *M. brunneum* than in the Control plants (14.5% and 6.3% greater, respectively—mean values). Figure 1 compares the grain yield of the plants inoculated with *B. bassiana* (Figure 1A) and *M. brunneum* (Figure 1B) with that of the Control plants in the twelve soils. In the group of soils where grain yield increased by more than 15% (GSGYI > 15) with *B. bassiana* relative to the non-inoculated (Control) plants (viz., Soils 2, 4, 8, 21, 26, 37 and 48, which fall slightly above the 1:1 line in Figure 1A), the mean increase in grain yield and harvest index was 37.1% and 31.3%, respectively. In five of these soils (No. 2, 4, 8, 26 and 48), *M. brunneum* increased the grain yield by more than 15% (GSGYI > 15). The mean increase in this parameter was 19.2%, and that for the harvest index was 17.6%. Fungal inoculation had no significant effect on grain yield in the group of plants grown on the GSGYI < 15 (Table S3).

Conversely, the number of tillers per plant was significantly greater in the Control plants (1.29 tillers plant^{-1}) than in the inoculated plants (1.19 with *B. bassiana* and 1.11 with *M. brunneum*; Table 2). No significant differences in straw weight between treatments were observed, however.

Table 2. Paired *t*-test for biomass variables (mean ± SE, *n* = 72) of inoculated versus non-inoculated plants at harvest (102 DAS). Significant *p*-values ($p < 0.05$) are in boldface.

Treatments	Grain Yield	Straw	Harvest Index ‡	Grains	Tillers
	g Plant^{-1}			**No. Plant^{-1}**	
Control	0.634 ± 0.034	1.20 ± 0.06	0.35 ± 0.02	17.3 ± 0.96	1.29 ± 0.16
B. bassiana	0.741 ± 0.034	1.14 ± 0.05	0.40 ± 0.01	19.8 ± 0.86	1.19 ± 0.15
p	**0.018**	0.314	**0.034**	**<0.001**	**0.011**
M. brunneum	0.670 ± 0.039	1.17 ± 0.06	0.37 ± 0.02	18.4 ± 1.03	1.11 ± 0.14
p	0.288	0.668	0.243	**<0.001**	**<0.001**

‡ Harvest index = Grain yield/aerial dry matter weight.
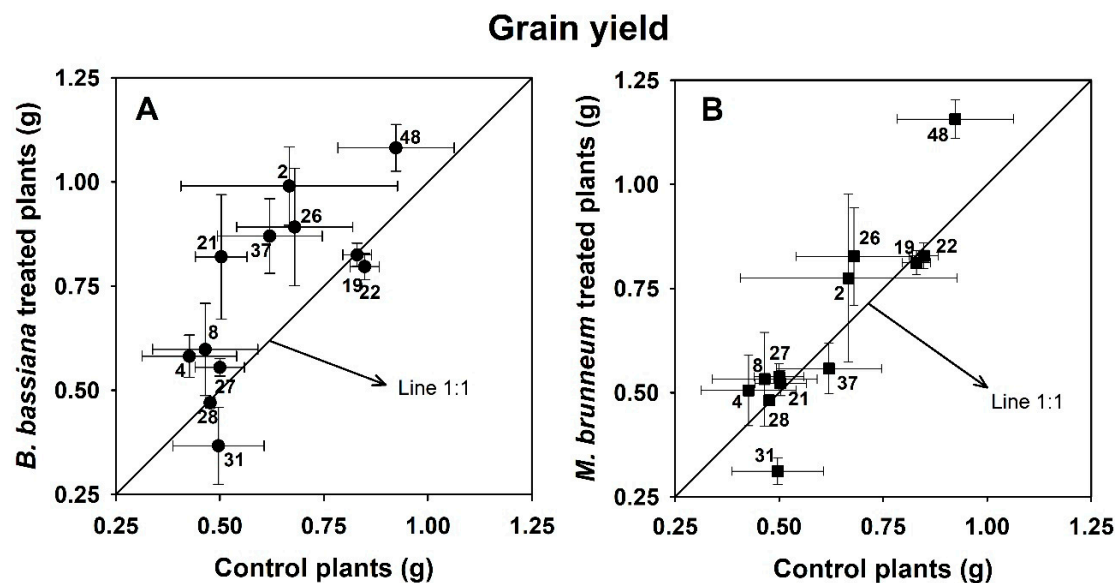
Figure 1. Grain yield (mean ± SE, *n* = 6) of durum wheat plants inoculated with *B. bassiana* (**A**) and *M. brunneum* (**B**) (*y*-axis) against grain yield of the Control plants (*x*-axis) on the different soils. The numbers by the symbols are the soil codes. The 1:1 line represents the mean value of the Control treatment for each soil, the horizontal error bars of the spots correspond to the standard errors for non-inoculated plants and the vertical bars to those for the fungus-treated plants.

3.2. Nutrient Uptake and Grain Nutrient Concentration

Table 3 shows results of the factorial ANOVA (soil × fungal treatment) for nutrient uptake and grain nutrient concentrations (P, K, Na and Zn) at harvest for the GSGYI > 15. Inoculation with *B. bassiana* significantly decreased P uptake and increased Na uptake; it decreased grain P and Zn concentrations. Both fungi significantly increased K uptake relative to the Control plants, but only *M. brunneum* increased grain K concentrations. A significant soil × fungal treatment interaction for K uptake in *B. bassiana* treated plants for the GSGYI > 15 was found, which reflected the significant positive influence of the fungus on this nutrient in plants grown on Soils 26 and 48 (Figure S2) and also on some soils included in the GSGYI < 15 (Figure S3).

Grain Zn uptake and the proportion of Zn in grain relative to that in ADM were significantly higher in the plants inoculated with *B. bassiana* than in the Control plants (p_{FT} = 0.009 and p_{FT} < 0.001, respectively). This is apparent in Figure 2A,C, where most of the data points lie above the 1:1 line. This pattern was also observed in the plants inoculated with *M. brunneum*, albeit with no significant differences (p_{FT} = 0.480 and p_{FT} = 0.191, respectively; Figure 2B,D). The effect of the factor of soil was significant in all cases (p_{soil} < 0.001) but the interactions between the two factors (soil × fungal treatment) were not significant in any cases ($p_{interaction}$ > 0.05).

Finally, Mg, Ca, Fe and Mn uptake in the GSGYI > 15 exhibited no statistically significant differences. However, a dilution effect—i.e., a decrease in grain nutrient concentration due to the observed grain yield increase [30,31]—of Mg and Ca with *B. bassiana* and *M. brunneum*, respectively, was observed (Table S4). The Supplementary material provides additional information about the effects of the fungal treatments on the yield and nutrient uptake variables in the GSGYI < 15 (Tables S3 and S5) and on nutrient uptake and grain nutrient concentration in all soils (Tables S6 and S7).

Table 3. Factorial ANOVAs for biomass, nutrient uptake and grain nutrient (P, K, Na, Zn) concentration (mean ± standard error, n = 6), with soil and fungal treatment as factors, for GSGYI > 15. [‡]

| Treatment | Aerial Dry Matter | | Nutrient Uptake | | | | Grain Nutrient Concentration | | | | Grain |
| | Yield | Straw | P | K | Na | Zn | P | K | Na | Zn | P/Zn Ratio |
	g plant^{-1}		g plant^{-1}		μg plant^{-1}		g kg^{-1}		mg kg^{-1}		
Control	0.63 ± 0.05	1.45 ± 0.08	3.89 ± 0.36	46.2 ± 2.5	939 ± 146	52.2 ± 2.9	3.98 ± 0.24	4.73 ± 0.12	50.6 ± 2.9	51.6 ± 4.5	100 ± 11
B. bassiana	0.83 ± 0.05	1.31 ± 0.07	3.42 ± 0.32	50.5 ± 2.7	1102 ± 163	49.6 ± 2.3	3.19 ± 0.23	5.07 ± 0.16	56.3 ±4.4	42.5 ± 3.5	91 ± 10
p_{FT} [§]	**0.002**	**0.04**	**0.022**	**0.011**	**0.042**	0.306	**0.000**	0.067	0.549	**0.027**	0.776
$p_{interaction}$	0.977	0.295	0.181	**0.049**	0.217	0.707	0.496	0.332	[†]	0.871	[†]
Control	0.66 ± 0.07	1.38 ± 0.09	3.60 ± 0.44	44.8 ± 3.1	1077 ± 201	51.2 ± 1.84	3.78 ± 0.29	4.64 ± 0.09	54.2 ± 3.9	51.2 ± 5.2	99 ± 15
M. brunneum	0.78 ± 0.07	1.30 ± 0.08	3.62 ± 0.42	51.5 ± 3.9	1206 ± 238	49.2 ± 1.4	3.54 ± 0.25	5.00 ± 0.13	54.6 ± 4.2	46.4 ± 5.1	101 ± 14
p_{FT} [§]	0.187	0.205	0.882	**0.023**	0.399	0.319	0.46	**0.041**	0.788	0.468	0.650
$p_{interaction}$	0.970	0.978	0.976	0.186	0.524	0.522	1.000	0.227	0.264	0.978	0.982

[‡] Group of soils in which the fungal treatment increased grain yield by more than 15% with respect to the Control treatment. [§] Only the probability values (p) for the fungal treatment (p_{FT}) and the soil × fungal treatment interaction ($p_{interaction}$) are shown because those for the factor soil (p_{soil}) were all significant ($p < 0.05$). [†] Absence of p for the interaction means that the variance fulfilled neither the homoscedasticity nor the normality criterion, so a Kruskal–Wallis test was performed instead.
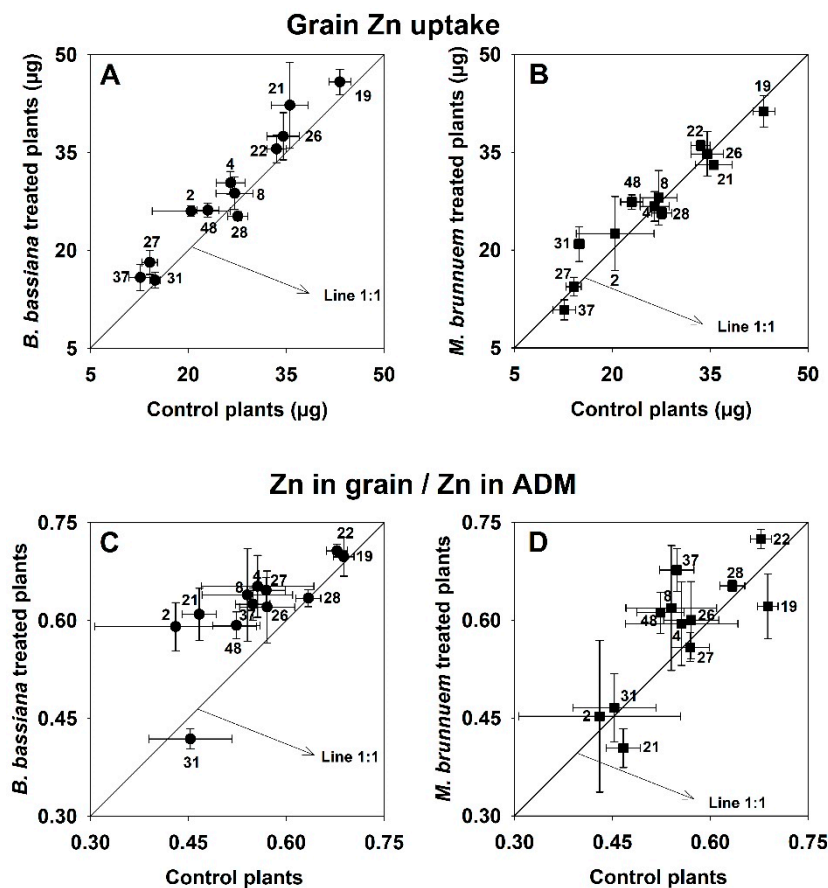
Figure 2. Grain Zn uptake (μg plant^{-1}) and proportion of Zn in grain relative to that in aerial dry matter (mean ± SE, *n* = 6) of durum wheat plants inoculated with *B. bassiana* (**A** and **C**, respectively) and *M. brunneum* (**B** and **D**, respectively) relative to the Control plants at harvest. The numbers by the symbols are the soil codes. The 1:1 line represents the mean value of the Control treatment for each soil, the horizontal error bars of the spots correspond to the standard errors for non-inoculated plants and the vertical bars to those for the fungus-treated plants.

3.3. Gas Exchange Variables and Water Use Efficiency

Figure 3 shows the gas exchange variables (net photosynthesis, stomatal conductance and intercellular CO_2 concentration), as well as water use efficiency (WUE) at 62 DAS for plants grown on five randomly selected soils. As can be seen, the effect of fungal inoculation differed between soils. Thus, there was no significant effect on plants grown on Soils 2 and 31 but there was a variable effect on those grown on Soils 19, 26 and 48. The net photosynthesis and stomatal conductance were significantly higher in plants inoculated with *B. bassiana* grown on Soil 48 and with *M. brunneum* on Soils 26 and 48 (Figure 3). The intercellular concentration of CO_2 was significantly higher in plants inoculated with *B. bassiana* grown on Soils 19 and 26 and with *M. brunneum* on Soil 26; however, it was significantly lower in plants treated with *M. brunneum* grown on Soil 48. Finally, the WUE of plants inoculated with *B. bassiana* or *M. brunneum* was significantly lower than that for non-inoculated plants grown on Soil 19—where the difference was only significant with *B. bassiana*—and soil 26; the opposite was observed for plants inoculated with *M. brunneum* grown on Soil 48 (Figure 3).
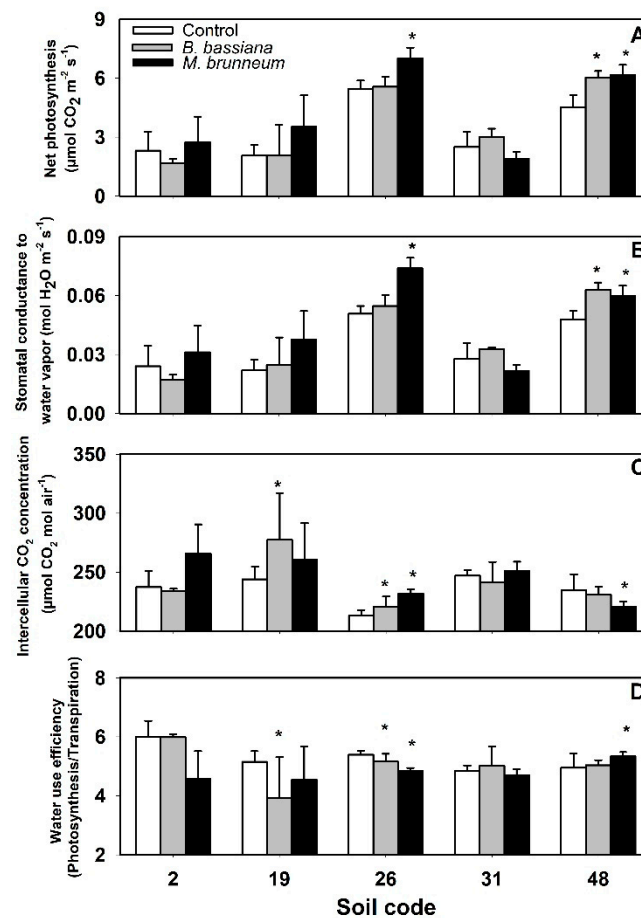
Figure 3. Repeated measures ANOVA (mean ± SE, *n* = 4) for net photosynthesis (**A**), stomatal conductance (**B**), intercellular CO_2 concentration (**C**) and water use efficiency (**D**, WUE) in durum wheat plants as a function of fungal treatment and soil (No. 2, 19, 26, 31 and 48) 62 DAS. Asterisks (*) denote significant differences (*p* < 0.05) between each fungal treatment and the Control plants.

3.4. Effect of Fungal Inoculation on Plant Performance in Relation to Soil Properties

Positive linear relationships were found between the difference in grain yield and P_{Olsen} in plants inoculated with *B. bassiana* (Figure 4A; adjusted $R^2_{linear\ regression}$ = 0.426, *p* = 0.013), as well as between the difference in grain yield and soil Fe_{ox} in plants inoculated with *M. brunneum* (Figure 4B; adjusted $R^2_{linear\ regression}$ = 0.698, *p* < 0.001), relative to the Control plants. In other words, the increase in grain yield in the inoculated plants relative to the non-inoculated (Control) plants was more marked by increased values of initial P_{Olsen} (*B. bassiana*) or Fe_{ox} (*M. brunneum*). On the other hand, an inverse relationship was found between the difference in ADM and soil Zn_{DTPA} for plants inoculated with *B. bassiana* (Figure 4C; adjusted $R^2_{inverse\ regression}$ = 0.565, *p* = 0.003), so the greatest beneficial effect of the fungus on ADM occurred under low soil Zn_{DTPA} values (<0.30 mg kg^{-1}).
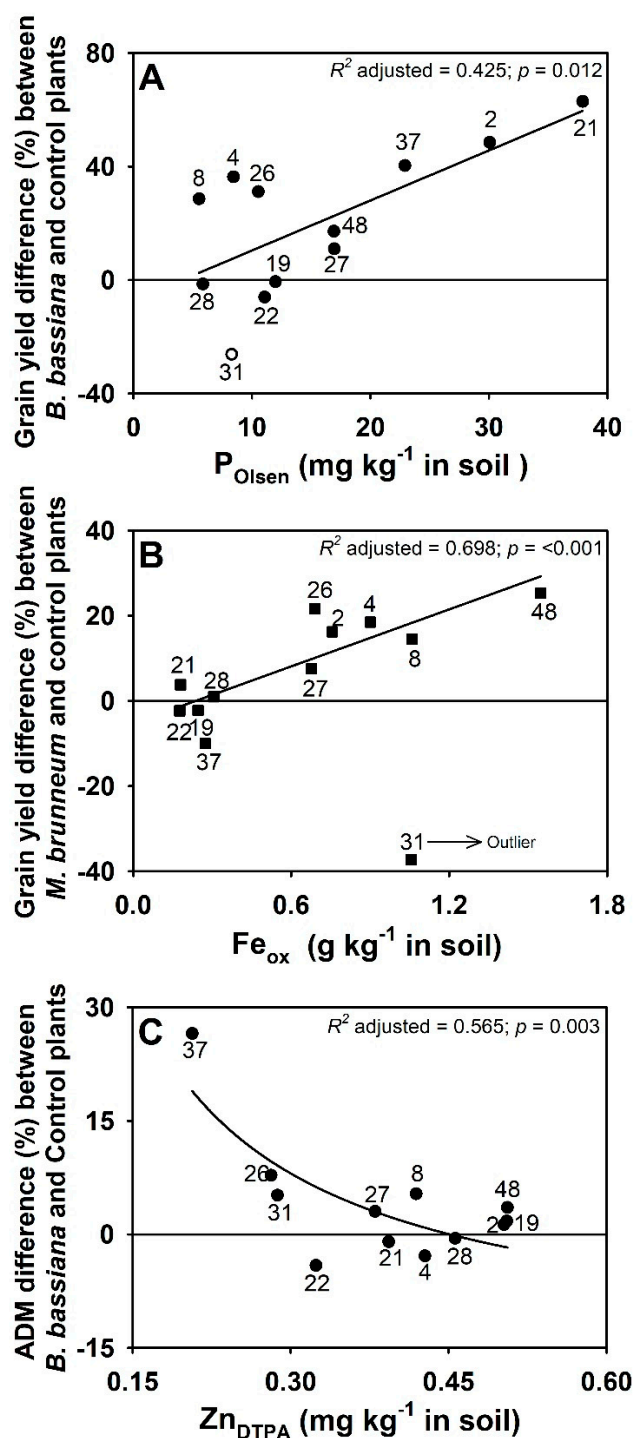
Figure 4. Regression models for the difference in grain yield between the Control plants and those inoculated with *B. bassiana* or *M. brunneum* as a function of the initial soil available P (P_{Olsen}, **A**) and Fe in poorly crystalline Fe oxides (Fe_{ox}, **B**), respectively; and differences in aerial dry matter (ADM) between the Control plants and those inoculated with *B. bassiana* as a function of soil available Zn (Zn_{DTPA}, **C**) at harvest. The numbers by the symbols are soil codes.

3.5. Changes in Nutrient Availability

Figure 5 and Figure S4 (Supplementary material) show the initial and post-harvest values of P_{Olsen}, Mn_{DTPA}, Zn_{DTPA}, Fe_{DTPA} and Cu_{DTPA} as a function of the soils and the fungal treatments. Cropping decreased nutrient availability except for some soils, in the case of Mn and Fe (which were in the

nutrient solution added to the pots at the beginning of the experiment). No statistical differences in post-harvest nutrient availability were seen between the Control and any fungal treatment for any nutrient except for available manganese (Mn_{DTPA}) with *M. brunneum* in the group of 12 soils studied ($p = 0.026$, Figure 5D). A similar pattern was observed for this element and Zn_{DTPA} with *B. bassiana* treatment relative to the Control ($p = 0.132$ and $p = 0.111$; Figure 5E,F, respectively). Nevertheless, focusing on the GSGYI > 15 highlights that the decrease in Fe availability (Fe_{DTPA}) was lower in soils with *B. bassiana* respect to Control treatment ($p = 0.059$; Figure S4).
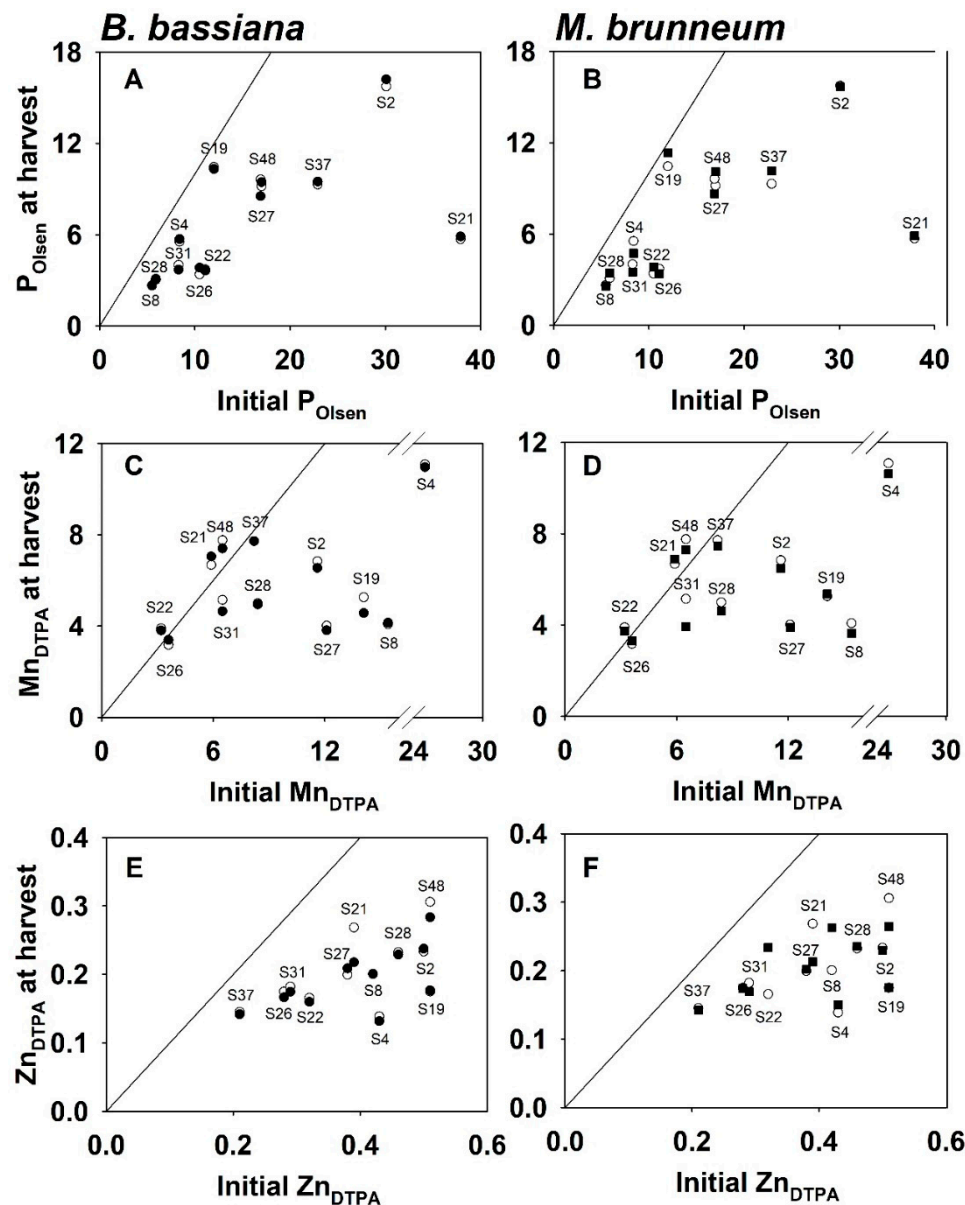
Figure 5. Nutrient availability of Control (white circles) against *B. bassiana* (black circles) treatment for P (**A**), Mn (**C**) and Zn (**E**) and against *M. brunneum* treatment (black squares) (**B**, **D** and **F**; respectively) in soils before (*x*-axis) and after (*y*-axis) cropping. The line 1:1 divides the soils with higher soil nutrient availability at harvest than at initial (over it) and vice versa (beneath it).

4. Discussion

4.1. Plant Growth in Relation with Soil Nutrients

The fact that ADM and grain yield were positively correlated with the initial $CaCl_2$-P and ADM with a decrease in soil P availability (ΔP_{Olsen}) ($R = 0.442$, $p = 0.007$) is consistent with the well-known and essential role of P in wheat development. Moreover, the ΔP_{Olsen} is positively correlated with P uptake ($R = 0.335$, $p = 0.046$) as expected. Negative correlations were observed between ΔP_{Olsen} and ΔCu_{DTPA}, ΔMn_{DTPA} and ΔZn_{DTPA} ($R = -0.396$, $p = 0.017$; $R = -0.676$, $p \leq 0.001$ and $R = -0.712$, $p \leq 0.001$). These strong correlations can be partly explained by the fact that most of the soil was rhizospheric at harvest due to the high root density in the pots used. This implies the release of micronutrients co-adsorbed to P as the latter was taken up by the plant, the acidification of the rhizosphere, and the higher excretion of organic acids and phytosiderophores, which all increase the availability of these micronutrients [32].

4.2. Effect of Fungal Inoculation on Plant Growth, Grain Yield, Photosynthesis Rate and Water Use Efficiency

In our experiment, plant growth and grain yield on the twelve soils were affected by inoculation with either EF (*B. bassiana* or *M. brunneum*). The increased number of grains, grain yield and harvest index observed here, especially in plants inoculated with *B. bassiana*, is suggestive of the stimulation of reproductive organs. Similar results were previously obtained with *Helianthus annuus* [10] and *Triticum aestivum* [12] grown on soils inoculated by the same EF strains used here. However, the former experiment was ended before the sunflowers reached maturity, and the latter was performed under different conditions (pre-germinated seeds, sterilized sandy soil and high fertilizer levels). In addition, our plants were inoculated using a different method (viz., seed dressing rather than application to soil). It is unclear why inoculation with *B. bassiana* and, to a lesser extent, with *M. brunneum*, increased the growth of the reproductive parts of plants (grain yield) and the harvest index of the inoculated plants relative to the non-inoculated (Control) plants. This phenomenon could be partially explained by the higher ability of *B. bassiana* compared to *M. brunneum* to penetrate, move through plant vessels, reach higher parts of the plant (e.g., the flag leaf or the spike) and establish themselves within their host plants as endophytes [8,12]. Indeed, this may increase the need for nutrients to satisfy them both (acting as a photoassimilate sink) [2,33]. This could explain the temporal reduction in plant height (at 39 DAS for *B. bassiana*; Figure S1) and the number of tillers (Table 2) relative to the Control plants in the first phenological stages but not at harvest (no significant reduction in ADM or grain yield). This negative initial effect or cost of fungal inoculation on plant growth was also observed by other authors [10,12,13] and could have been caused by the activation of the defensive mechanism of the host plants [34]. Consistent with this last claim, the increase in grain yield was related to Mn, a key nutrient in the plant immune system [35,36]. The increase in grain yield was significantly and positively correlated with a relative increase in Mn_{DTPA} at harvest with *B. bassiana* and *M. brunneum* treated plants compared to the Control ($R = 0.708$, $p = 0.009$ and $R = 0.639$, $p = 0.025$; respectively). However, Mn_{DTPA} was lower under both fungal treatments compared to the Control ($p = 0.132$ and $p = 0.026$; respectively). Plants recognizing the fungus as a pathogen/alien and stimulate their immune system, thereby enhancing Mn uptake as the stress level increases, which explains these correlations. The beneficial effect is dependent on the plant stress degree because the plants with higher grain yield compared to the Control resulted in a higher Mn_{DTPA} content in the soil at harvest. In this sense, *B. bassiana* stressed the plant less than *M. brunneum* and more strongly increased the grain yield, presenting higher values of Mn_{DTPA} at harvest. Likewise, Sánchez-Rodríguez et al. (2016) noted a decrease in soil Mn availability after growing sunflower and sorghum inoculated with the same strain of *M. brunneum* used here. From a lower Mn availability value in soil at harvest, one should expect higher Mn uptake values. However, such a correlation was not found in our study. This could be due to Mn accumulating in the roots, as a response of the plant to the presence of fungus within themselves rather than in other plant parts, likely because the fungus was applied by seed dressing in our experiment. This idea is

supported by the study of Sánchez-Rodríguez et al. (2018), who found that in bread wheat treated with the same strain of *B. bassiana*, the Mn concentration in the ADM decreased in the following order by application method: leaf spraying > soil application > seed dressing. This suggests that Mn is more readily translocated to the upper plant parts to activate the immune system when the fungus is sprayed on the leaves, rather than when it is applied to the soil or located on the surface of the seed Thus Mn is translocated to where the fungus is directly interacting with the plant.

This work demonstrates that soil fertility is a key factor in the crosstalk between fungi and plants and, therefore, in the ability of plants to overcome the initial fungal cost. Although the EF–host plant–soil interaction is far from being completely understood, the response of the plant to the fungal inoculation observed here seems to be related to the properties of the soil and appears to depend on the specific requirements of the endophytic fungus. We demonstrated this phenomenon for the first time in this study. Firstly, the increase in grain yield of the host plant due to inoculation with *B. bassiana* relative to Control was related to the soil availability of P (P_{Olsen}) (Figure 4). Thus, a medium or high soil P content helped the plants inoculated with *B. bassiana* overcome the initial cost of endophytism and led to increased yields relative to those that were non-inoculated (Control). This result is consistent with the contention of Crush (1975) [37] that "endophyte–host relationships vary between parasitism and mutualism depending on soil available P levels". Our results also support the idea that these EFs have the potential to increase ADM when available Zn levels in the soil are low (Figure 4C) and likely limited [38]. In particular, the plants inoculated with *B. bassiana* and grown on Soils 26, 31 and 37—where Zn_{DTPA} was limited to 0.28, 0.29 and 0.21 mg kg^{-1}, respectively—yielded 7.2%, 5.3% and 26.1% more ADM, respectively, than the Control plants (Figure 4C). On the other hand, inoculation with *M. brunneum* was only successful in this respect for plants grown in Soil 31 (ADM increase, 11.2%; results not shown). Secondly, the role of *M. brunneum* as a growth promoter seems to be related to the content of poorly crystalline Fe oxides in the soil (Figure 4B). This may be a result of *M. brunneum* increasing soil Fe availability [14,16] via the mean increase in Fe_{DTPA} found in the group of 12 soils (0.23 mg kg^{-1}), especially for the GSGYI > 15 (0.68 mg kg^{-1}) after harvest (Figure S4). Moreover, the positive and significant correlation found between the relative increase of Fe available between *M. brunneum* and the Control at harvest with the relative increase in ADM ($R = 0.585$, $p = 0.045$) supports this hypothesis, as the fungus was able to directly mobilize more Fe in the soil or stimulates the plant to do so, thereby triggering a growth promotion effect.

Although the results for net photosynthesis, stomatal conductance and WUE differed little between fungal treatments and soils, they afford some interesting observations. The increase in net photosynthesis and stomatal conductance in the flag leaf at 62 DAS in the plants inoculated with EFs (Figure 3) and grown on Soils 26 (*M. brunneum*) or 48 (both EF) is in line with the results of a previous study [10], where inoculation with the same *M. brunneum* strain increased leaf chlorophyll concentration in young leaves of sorghum plants in terms of their SPAD (as a proxy of chlorophyll content). This supports our comment on the initial fungal cost (a sink of photoassimilates) stimulating an increase in photosynthesis to supply more nutrients. Given the importance of gas exchange in the flag leaf to grain development [28], differences in these parameters could affect grain yield, as occurred in these two soils (26 and 48). Rubio et al. (2017) [39] found a similar pattern in tomato seeds coated with *Trichoderma harzianum* T34 under salinity stress in the absence of NPK fertilization. Unlike the net photosynthesis rate, the general decrease in WUE (Soil 48 excepted) can be ascribed to the greater carbon requirements of the host plant to sustain the EFs, thus leading to a stomatal opening greater than that of the Control plants.

4.3. Effect of Fungal Inoculation on Plant Nutrition and Grain Quality

Wheat breeding to increase grain yield has been historically associated with a decrease in nutrient concentration [31]. A dilution effect in nutrient content was observed in the plants grown on the GSGYI > 15 (Table 3; Table S4), which was especially apparent in plants inoculated with *B. bassiana*—those exhibiting the higher yields. Although weaker, this dilution effect was also found in plants grown

on the GSGYI < 15 (Table S5), which was already described by Bethlenfalvay (1983) [40] for plants inoculated with endophytes.

The dilution effect was obvious for P and some micronutrients, particularly Zn (Table 3), which is typically diluted as grain yield increases [22]. The fungal treatments resulted in no biofortification of grain with Fe, Mn, or Zn (Tables S4–S7). However, if not only grain Zn concentration but also grain yield is considered, then grain Zn uptake and the proportion of Zn in grain relative to that in ADM (Figure 2) were significantly increased in plants inoculated with *B. bassiana*. In particular, Zn uptake in Soils 26, 31 and 37 (presenting the lowest values of Zn_{DTPA}) increased by 0%, 7.1% and 10.5%. The increased efficiency of these plants in mobilizing Zn to grain can be ascribed to the ability of *B. bassiana* to act as an endophyte and colonize new plant tissues. The main reason for the reduced P uptake observed in plants inoculated with *B. bassiana* might be that wheat plants absorb about 60% of their total P content during their early phenological stages, when shoot biomass is about 20% of the final aerial biomass [41]. In our experiment, the EFs clearly created an initial cost for their host plants (reduced growth), which may have limited the amount of P initially absorbed (especially in soils with $P_{Olsen} \leq 10$ mg kg^{-1}).

The increase in net photosynthesis to satisfy fungal requirements here was associated to an increased stomatal conductance in Soils 26 and 48. The observed increase in K and Na uptake—the latter was not significant in *M. brunneum* treated plants (Figure S2)—may have resulted from the increase in net photosynthesis since these two nutrients are dissolved in the water that is absorbed and transpired by plants [42]. Specifically, the significant increase in K uptake under both fungal treatments, and the increase in K grain concentration with *M. brunneum* (Table 3), may have resulted from stimulated growth [43]. Former experiments [44] showed K uptake to be related to an increase in grain yield and harvest index. The percentage of increase in K uptake found here was significantly correlated with the increase in ADM in plants inoculated with *B. bassiana* (adjusted $R^2_{\text{linear regression}}$ = 0.322; p = 0.031) and *M. brunneum* (adjusted $R^2_{\text{linear regression}}$ = 0.274; p = 0.047, respectively), which is consistent with the stimulated growth hypothesis. Additionally, the increase in K uptake in treated plants relative to the Control plants was related to initial higher levels of available K (Table 1).

5. Conclusions

Our results support the idea that EFs, which are normally used as effective biocontrol agents, can also influence plant growth and nutrition. However, the EF–host plant relationship is complex; its success is dependent on the host plant and, as shown here, the endophyte species and soil properties where the host plant is grown. Grain yield was generally higher in inoculated plants, by up to a 63% with *B. bassiana* and by up 26% with *M. brunneum;* however, the yield increase was only significant with the former fungus. Specific soil properties were found to have a key role in this triple interaction: the increase in grain yield due to fungal inoculation was more marked in soils with high values of P_{Olsen} (*B. bassiana*) and Fe_{ox} (*M. brunneum*) and the greatest benefits in ADM production provided by the fungi were observed when soil Zn_{DTPA} was scant (<0.30 mg kg^{-1}). The increase in grain yield resulting from inoculation was associated with a dilution effect in nutrients (primarily P and Zn). However, the inoculated plants absorbed more Zn, and a greater proportion of Zn in ADM was present in grain. Therefore, these EFs are seemingly a promising choice for enhancing crop performance. Their success, however, depends on certain soil properties, among other factors.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/4/589/s1, Figure S1: Time course of plant height (mean ± standard error, n = 6) in plants inoculated with *B. bassiana* and non-inoculated (Control) plants grown on Soils 28 and 37. Significant (p < 0.05) differences are marked with an asterisk, Figure S2: Nature of soil × fungal treatment interaction in K uptake by plants on soils pertaining to the GSGYI > 15 (those where grain yield was increased by more than 15%) inoculated with *B. bassiana*. Significant (p < 0.05) differences are marked with an asterisk, Figure S3: Nature of soil × fungal treatment interaction in K and Na uptake by plants on soils pertaining to the GSGYI < 15 (those where grain yield was increased by less than 15%) inoculated with *B. bassiana* (A and B) or *M. brunneum* (C and D). Significant (p < 0.05) differences are marked with an asterisk, Figure S4: Nutrient availability of Control (white circles) against *B. bassiana* (black circles) treatment for Fe (A) and Cu (C) and against *M. brunneum* treatment (black squares) (B and D; respectively) in soils before (*x*-axis)

and after (*y*-axis) cropping, Table S1: Additional soil properties[‡] of the soils described in Sacristán et al. (2019) [24], Table S2: One-way ANOVA or Kruskal–Wallis test and comparison of means[‡] for wheat biomass, nutrient uptake and grain nutrient concentration (mean value, *n* = 18) on the different soils at harvest, Table S3: Factorial ANOVA (soil × fungal treatment) and Kruskall–Wallis test ([†], when data failed to fulfill the criteria for parametric analysis) for grain yield, straw and nutrient uptake of durum wheat plants grown on GSGYI < 15.[§] Significant differences (*p* < 0.05) are in boldface. Table S4: Factorial ANOVAs for nutrient uptake and grain nutrient concentration (mean ± standard error) with soil and fungal treatment as factors for the GSGYI > 15. Significant differences (*p* < 0.05) are in boldface, Table S5: Factorial ANOVA (soil × fungus) and Kruskall–Wallis test ([†], when data failed to fulfill the ANOVA criteria) for grain nutrient concentration in the GSGYI < 15.[§] Significant differences (*p* < 0.05) are in boldface, Table S6: Paired t-test for nutrient uptake (mean ± SE) of fungus-treated plants against non-inoculated plants (*n* = 72) at harvest (102 DAS). Significant *p* values (*p* < 0.05) are in boldface, Table S7: Paired t-test for grain nutrient concentration (mean ± SE) of fungus-treated plants against non-inoculated plants (*n* = 72) at harvest (102 DAS). Significant *p* values (*p* < 0.05) are in boldface.

Author Contributions: In this manuscript the division of tasks was as follow: Conceptualization, M.C.d.C. and J.T.; methodology, D.S. and A.G.-G.; software, A.G.-G.; validation, J.T., M.C.d.C., V.B. and A.R.S.-R.; formal analysis, A.G.-G.; investigation, A.G.-G.; resources, M.C.d.C. and V.B.; data curation, A.G.-G.; writing-original draft preparation, A.G.-G.; writing-review and editing, A.G.-G., M.C.d.C., J.T. and A.R.S.-R.; supervision, M.C.d.C., J.T. and A.R.S.-R.; project administration, M.C.d.C. and V.B.; funding acquisition, M.C.d.C. and V.B. All authors have read and agreed to the published version of the manuscript.

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