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Effects of Microbial Inoculants and Organic Amendments on Wheat Nutrition and Development in a Variety of Soils

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Abstract

Manipulation of soil microbial communities through inoculants or amendments can improve crop nutrition. However, to what extent these benefits vary depending on soil properties is not yet understood. Thus, here we studied the effects of microbial inoculants and the application of labile organic C on the yield and uptake of micronutrients and P in wheat (*Triticum durum*) in different soils. The application of *Bacillus subtilis* QST713, *Trichoderma asperellum* strain T34, and cellulose was tested in ten soils varying greatly in properties in a pot experiment. Microbial inoculants and cellulose increased dry matter (between 5 and 10%) and grain yield (between 15 and 20%), regardless of the soil. Some treatments triggered nutrient mobilization mechanisms such as phosphatase and oxalate production. However, total Zn and P in plants did not increase with treatments, and their effect on Fe and Mn varied depending on soils. The effect of *B. subtilis* and *T. asperellum* improving Fe uptake by plants decreased with increasing pH and also with decreased microbial activity in soil. Inoculants and cellulose increased the Zn harvest index and decreased the P-to-Zn ratio in grains independently of the soil. This was probably ascribable to changes in the distribution of phytohormones in plants. Microorganisms and cellulose improved wheat yield, the portion of absorbed Zn accumulated in grains, and grain quality. These effects did not depend on the soil. However, the effect on Fe and Mn nutrition was affected by soil pH and microbial activity.

Keywords Microbial activity · Microbial inoculant · Enzyme activity · Phosphorus · Biofortification

1 Introduction

Micronutrient deficiency is a relevant agronomic problem that restricts crop yields and quality in soils with basic pH (Alloway 2009; Mousavi 2011; Ryan et al. 2013; Rengel 2015; Moreno-Lora et al. 2020). Furthermore, low Fe and Zn concentration in cereals can cause nutritional problems for humans in regions with cereal-based diets (Cakmak et al. 2010; Borrill et al. 2014; Wang et al. 2014; Zhao et al. 2014; Cakmak and Kutman 2018). Overcoming micronutrient deficiency and biofortification of crops to increase their concentration in edible parts requires the application of fertilizers (McBeath and McLaughlin 2014; Moreno-Lora et al. 2019). However, these fertilizers are expensive and not always efficient due to their reactions in the soil (Alloway 2009; White and Broadley 2009; Zhang et al. 2012).

Phosphorus deficiency can also be frequent in soils prone to micronutrient deficiency. On the other hand, this nutrient can promote nutritional antagonisms with Fe and Zn, decreasing its availability to plants (Moreno-Lora et al. 2022; Recena et al. 2021). Therefore, more cost-effective and sustainable practices are required to supply micronutrients to crops (Moreno-Lora et al. 2019). Regarding this, some microbial inoculants have been shown to be effective in increasing the availability of micronutrients to plants (de Santiago et al. 2009; 2011; 2013; Khande et al. 2017).

The soil microbiota plays a key role in the soil nutrient cycle, solubilizing insoluble compounds that lead to an increase in exchangeable micronutrient concentration with increase in availability to plants (Ramesh et al. 2014). Soil microorganisms trigger mobilization mechanisms in response to nutrient deficiency (Marschner et al. 2011). This may enhance the availability of nutrients to plants (García-López et al. 2021). These mechanisms involve acidification, the release of low molecular weight organic acids, chelating agents (e.g., siderophores), and hydrolytic enzymes (Zhao et al. 2011; Rengel 2015). As a particular case, the efficiency

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in mobilizing Zn by the *Bacillus* genera has been mainly ascribed to the production of organic acids and decreased rhizosphere pH (Ramesh et al. 2014; Mumtaz et al. 2019). However, the microbial mechanisms that contribute to the improvement of micronutrient uptake by crops and the functioning of these mechanisms under different soil conditions are not fully elucidated.

Many soil microorganisms influence plant nutrition and development by producing phytohormones or affecting their transport and signaling pathways (Spaepen 2015; Kudoyarova et al. 2019). Indole-3-acetic acid (IAA) produced by rizhospheric microorganisms promotes plant growth and morphological root changes. This leads to an increased root surface that allows for an enhanced uptake of nutrients (Sukumar et al. 2013; Owen et al. 2015; Gouda et al. 2018). Gibberellins (GA3), cytokinins, and abscisic acid (ABA) produced by microorganisms such as *B. subtilis* may also affect root and plant development (Arkhipova et al. 2005; Singh et al. 2017). Therefore, the benefits of microorganisms are attributed not only to improved nutrient mobilization from the soil, but also to the effects on plant physiology that lead to an increased capacity to absorb nutrients (Qiu et al. 2019; de los Santos-Villalobos and Parra-Cota 2020). Consequently, it is necessary to take into account the possible effects on phytohormone homeostasis as a relevant mechanism that explains the effect of microorganisms on plant growth and nutrition.

The activity of rhizospheric microorganisms is strongly affected by soil properties (Shi et al. 2013; Owen et al. 2015; Ma et al. 2017; Nguyen et al. 2019a, b). In particular, the composition, size, and activity of microbial communities in the rhizosphere can be modulated by the quantity and quality of carbon (C) sources in soil (Hobbie and Hobbie 2013). Therefore, applying a labile C source to the soil can stimulate the activity of native soil microorganisms (Demoling et al. 2007). This may promote the mobilization of nutrients that induce benefits on plant nutrition (Kuzyakov 2002; de Santiago et al. 2013; Moreno-Lora et al. 2019).

In addition to organic carbon, other soil properties affect the establishment, activity, and potential benefits of microbial inoculants (García-López et al. 2018). Different authors have reported the effects of microbial inoculants on micronutrient nutrition and biofortification (Singh et al. 2017; Moreno-Lora et al. 2019; Nguyen et al. 2019a, b). However, most of these studies have been conducted with a reduced number of soils. Therefore, the influence of soil factors on the benefits of microbial inoculants has been poorly evaluated. This is a relevant aspect for fully understanding how microbial mechanisms may enhance the nutrition of plants.

The benefits of microbial inoculants and the supply of organic C in plant nutrition and biofortification must be confirmed in a wide range of soils to provide solid recommendations in their use. We hypothesized that the application of organic amendments or microbial inoculations will have three positive effects on crops: (i) improve the growth and yield, (ii) improve the uptake of micronutrients and P, and (iii) increase the portion of micronutrients accumulated in the edible part of the crop, that is, the harvest index of nutrients. This work aims to assess the effects of the application of a readily available source of C and two microbial inoculants, *Trichoderma asperellum* T34 and *Bacillus subtilis* QST713, on the nutrition of micronutrients and P and the growth of plants in different soils. Both microbial inoculants were selected because they have a capacity to mobilize nutrients (de Santiago et al. 2013; Garca-López et al. 2018). To explain possible effects, we will study the effect of these treatments on organic acids and hydrolytic enzymes release in the rhizosphere and on the concentration of phytohormones in plant roots. The study has been conducted in a set of soils with a wide range of properties to better understand the microbial mechanisms involved in the supply of nutrients to plants and to assess the consistency of the results in different edaphic environments.

2 Material and Methods

2.1 Experimental Design

A randomized block experiment with four replications was performed using durum wheat plants (*Triticum durum* cv Amilcar). It was carried out in pots under controlled environmental conditions in a growing chamber, involving two factors: (i) soil type and (ii) treatments that affect microbial activity in the rhizosphere. The treatments were two microbial inoculants (*Bacillus subtilis* QST713 and *Trichoderma asperellum* strain T34), source of labile C, ß-cellulose (CF11, Whatman), and a control without treatment.

2.2 Soils

Ten soils were selected for the study in different locations in Spain (Figure S1, Supplementary material). Soil samples were taken from 0 to 20 cm depth, defining an area of around 1000 m^2 in each sampling site from which at least 12 soil cores were taken. The sampling sites were selected to achieve a wide range of soil properties, such as clay, Fe oxides, and carbonate content, that can affect the availability of P and micronutrients to plants. This selection was made on the basis of color and a proxy estimate of textural classes in the upper soil horizon. The soil samples were air dried and sieved to < 4 mm for the pot experiment and to < 2 mm for soil characterization (Table 1).

For soil characterization, the following properties were analyzed: particle size distribution by pipette method (Gee and Bauder 1986), soil organic carbon (SOC) by the

Table 1 Soil taxonomy (soil orders according to Soil Survey Staff, 2014) and main properties and P and Zn availability indices of studied soils

Soil and taxonomy	Sand	Silt	Clay	SOC	ACCE	CCE	pH	EC	CEC	DTPA extractable				Zn_{pyr}	Olsen P
										Fe	Cu	Mn	Zn		
	——————————————gkg^{-1}——————————							µS cm^{-1}	cmocl$_c$ kg^{-1}	———————— mg kg^{-1} ————————					
BLGZ2 Inceptisol	450	220	108	4.9	72	231	8	397	14.4	3.6	3	5.3	0.41	1	26
BLGZ3 Inceptisol	475	253	116	5.1	56	153	8.3	132	14.8	3.1	1.2	4.4	0.27	1	7.5
LCVZ1 Alfisol	484	102	385	7.4	28	32	8.2	163	24.5	6.6	5.4	13.1	0.28	0.8	9.3
LCVZ4 Alfisol	426	135	387	7.4	16	44	8.1	178	24.6	6.6	2.4	11.5	0.40	1	16.7
OTRZ1 Inceptisol	264	184	383	3.2	71	174	8.2	116	20.0	5.9	0.3	7.8	0.1	0.4	4.7
OTRZ2 Inceptisol	311	191	355	10.8	54	150	8.2	197	18.1	6	0.5	12.7	0.24	1	7.5
RCNZ1 Inceptisol	767	43	162	5.5	0	0	7.4	55	12.1	13.7	12.8	10	0.31	1.1	9.4
RCNZ2 Inceptisol	794	41	170	5.5	0	0	7.2	132	13.9	10.9	15.7	14.7	0.25	1.5	21.7
VGTZ1 Inceptisol	787	89	127	5.8	0	0	8	102	8.0	10.7	0.7	15.4	0.38	1.1	22.8
VGTZ2 Inceptisol	810	100	92	5.8	0	0	8	119	7.7	10	0.6	15.9	0.49	0.5	23.1

SOC soil organic carbon, *ACCE* active calcium carbonate equivalent, *CCE* calcium carbonate equivalent, *EC* electrical conductivity in the saturated extract, *CEC* cation exchange capacity, Zn_{pyr} pyrophosphate-extractable Zn

Walkley and Black oxidation method (1934), total equivalent CaCO$_3$ (CCE) using a calcimeter, active Ca carbonate content (ACCE; Drouineau 1942), soil pH, and electrical conductivity (EC) in a 1:2.5 soil/water extract and cation exchange capacity (CEC) by the NH$_4$OAc method (Sumner and Miller 1996).

The availability of micronutrients and phosphorus in soils was assessed by DTPA extraction (Lindsay and Norvell 1978) and as Olsen P (Olsen et al. 1954), respectively. For DTPA extraction, soil samples were mixed with extractant (0.005 M diethylenetriaminepentaacetic acid, 0.1 M triethanolamine, and 0.01 M CaCl$_2$ at pH 7.3) at a rate of 1:4 (w:v). The suspension was shaken in a falcon tube at a frequency of 2.7 s^{-1} for 120 min. Subsequently, suspensions were centrifuged at 1260 g for 15 min, and micronutrient concentration in supernatants was determined by atomic absorption spectrometry in a Soolar M device (Thermo, Madrid, Spain). The Olsen P (Olsen et al. 1954) was determined by extraction with 0.5 M NaHCO$_3$ at pH 8.5 in a soil extractant ratio of 1:20 (w:v) shaking at 2.7 s^{-1} during 30 min. After extraction, suspensions were centrifuged at 1260 g for 10 min, and the concentration of P in the extract was determined colorimetrically by the molybdate blue method (Murphy and Riley 1962) in a Unicam UV2 spectrophotometer (Thermo, Madrid, Spain). Zinc bound to the organic matter fraction weakly associated to mineral matrix (Lopez-Sangil and Rovira 2013), which can be indicative of available Zn for plants (Moreno-Lora et al. 2020), was determined. This was done by extraction with sodium pyrophosphate (Zn$_{pyr}$) following Reed and Martens (1996). To do this, 0.25 g of soil were mixed in a ratio of 1:100 (w:v) with 0.1 M Na-pyrophosphate and shaken at 4 s^{-1} for 16 h. The suspension was then centrifuged (1260 g, 15 min), and the collected

supernatant was centrifuged again at 17,900 g for 15 min to decant colloids. The concentration of Zn in the extract was determined by atomic absorption spectrometry using the same device mentioned above.

2.3 Plant Growing Conditions

The seeds of durum wheat were disinfected by immersion in 5% NaClO according to de Santiago et al. (2011) and pre-germinated at 8 °C under darkness in Petri dishes before transplanting to a perlite seedbed. Later, at stage Z1.3 of Zadoks scale (Zadoks et al. 1974), individual plants were relocated into 350-ml polystyrene cylinders (15 cm high × 5.5 cm diameter) containing 300 g of soil. The cultivation was carried out in a growing chamber with a photoperiod of 16 h (300 µmol m^{-2} s^{-2} of light intensity), at 25/18 °C of temperature and 40/60% relative humidity day/night. The plants were daily irrigated using a modified Hoagland nutrient solution without P and Zn and with a limited supply of other micronutrients. This solution contained (mmol l^{-1}): Ca(NO$_3$)$_2$ (5), KNO$_3$ (5), MgSO$_4$ (2), KCl (0.05), H$_3$BO$_3$ (0.024), MnCl$_2$ (0.0023), Fe-EDDHA (0.02), CuSO$_4$ (0.0005), and H$_2$MoO$_4$ (0.0005). A total volume of 800 ml per pot was applied during cultivation that ended when the plants reached the Z9 stage of the Zadoks scale.

2.4 Application of Treatments

Commercial microbial inoculants were applied as described by García-López et al. (2016). *Bacillus subtilis* (Serenade Max, Bayer CrospScience, with an inoculum concentration of 5·10^{13} CFU kg^{-1}) was inoculated at a rate of 2·10^4 colony-forming units (CFU) per gram of soil. To this end, 20 ml of

an aqueous suspension contain $3 \cdot 10^5$ CFU ml^{-1} over the soil surface in each pot at four different points around the plants. The product was tested before inoculation in a nutrient agar medium following the procedure of Tuitert et al. (1998). *Trichoderma asperellum* (Biocontrol Technologies, Barcelona, Spain, with an inoculum density of 10^{12} CFU kg^{-1}) was inoculated at a rate of 10^4 conidia per gram of soil, as described by de Santiago et al. (2009). To do this, before transplantation, plants were immersed in a water suspension with 10^3 conidia ml^{-1}, and later 20 ml of an aqueous suspension ($1.5 \cdot 10^5$ conidia mL^{-1}) were applied over the soil surface in each pot at four different points around the plants. ß-cellulose treatment was applied at a rate of 200 mg C per kg of soil split in two applications: at the beginning (Z1.3) and in the middle (Z5.1) of the crop cycle, following the same procedure as in inoculation: applying a volume of 20 ml of water suspension (3.4 g of ß-cellulose per l) on the soil surface in each pot at four different points around the plants. The application of cellulose was split to avoid losses due to irrigation and to maintain a more constant effect throughout the crop cycle. The C content was 0.44 g per g of ß-cellulose. The pots without treatments (controls) were irrigated with 20 ml of water to balance the water content in all the pots. For both inoculants, the concentration of C, N, and P was in the range 7–8%, 1–1.1%, and 0.12–0.13%, respectively; in both cases, micronutrient concentration was not detectable. This implies that the supply of C or nutrients at the dilutions at which inoculants were used is negligible.

2.5 Harvest and Analysis of Plants

Immediately after harvest, roots, shoots, and grains were separated and dried (65 °C, 48 h) in a forced air oven to determine the dry matter (DM) in each organ. Roots were cleaned by immersion in distilled water and by ultrasonic treatment for 1 min. Subsequently, fresh weight was determined, and a portion of fresh material was kept for hormone determination. The other portion was dried as described above and weighed to determine DM. The harvest index (HI) was calculated as the ratio of grain DM to total in all aerial parts.

The portion of fresh roots for hormone analysis was immediately weighed and frozen in liquid nitrogen (-70 °C) after sampling. The samples were then ground (<1 mm) using liquid nitrogen and extracted overnight in the dark with 10 ml of 80% cold aqueous methanol (<0 °C). The extract was centrifuged (2470 g, 15 min at 4 °C) in a 15-ml falcon tube, and the supernatant was collected. The remnant was extracted following the same procedure 3 additional times. The total volume of the supernatant was dried in a rotary evaporator and dissolved in 2.5 ml of methanol. Indoleacetic acid (IAA), abscisic acid (ABA), and gibberellins 3 (GA3) in roots were determined by injection

of the extract into a reverse phase HPLC Varian ProStar 410 instrument furnished with a column C18 (Varian, 250 mm $\times$ 34.6 mm, 8-μm particle size) using as mobile phase methanol-0.6% ethanoic acid, with gradient elution, column temperature 35 °C, injection volume of 10 μl, and a flow rate of 1 ml min^{-1}. Detection was performed at 254 nm according to Tang et al. (2011).

For mineral nutrient analysis, 0.25 g of dried and milled (<1 mm) plant material was mineralized in a muffle furnace at 550 °C for 8 h. After calcination, the ashes were dissolved in 10 ml of 1 M HCl. This solution was heated (100 °C, 15 min) to ensure full recovery of nutrients. Micronutrient concentrations (Zn, Fe, Mn, and Cu) in the digest were determined by atomic absorption spectrometry and P concentration colorimetrically (Murphy and Riley 1962). The total P and micronutrients in the plants were calculated as the sum of product of the DM in each organ and its nutrient concentration. Nutrient harvest indices (HI) were calculated as the ratio of the total nutrient accumulated in the grain to that accumulated in all aerial parts of the plant. The P-to-Zn and P-to-Fe molar ratios were calculated as the quotients of P and Zn or Fe concentrations in grains. The phosphate in grains is present mainly as phytate, which inhibits the absorption of Zn and Fe during digestion, thus decreasing the grain value for the supply of Zn and Fe in diets. Therefore, these molar ratios are commonly used as an index of potential nutritional quality for humans (Miller et al. 2007; Gómez-Coronado et al. 2019).

2.6 Chemical, Microbiological, and Biochemical Analysis of the Soil

Soil samples for analysis were collected at harvest from rhizospheric soil by shaking it off from the roots in air as described by Wang et al. (2009). Thus, only the soil adherent to the roots was considered (Nazih et al. 2001). Immediately after sampling, the wet soil samples were sieved to <2 mm and homogenized for chemical, biochemical, and microbial analysis. A portion of them was air-dried and used to determine pH and EC (1:2.5 in water) and further chemical analyses.

The population density of *B. subtilis* and *T. asperellum* in rhizospheric soil was measured by dilution plating according to Tuitert et al. (1998). To this end, a suspension with 5 g of soil and 90 ml of 0.1% Na-pyrophosphate was shaken (2.5 s^{-1}, 30 min) to promote the disruption of soil aggregates. Then, five-fold dilution series were prepared from an original dilution of 1 ml of vortexed suspension in 9 ml of 0.1% water agar. From each dilution, 0.1 ml were pipetted into 3 replicate plates, containing a modified semiselective culture media for *T. asperellum* (Chung and Hoitink 1990) prepared according to Borrero et al. (2012). The colony-forming units (CFU) were counted 4 days after

plating and expressed as CFU per gram of soil. Similarly, *B. subtilis* was isolated in a semiselective culture medium (400 ml of filtered V-8 juice®, 40 g of NaCl, 1 g of dextrose, 20 g of agar, and 600 ml of Millipore water, adjusted to pH 5.2 before autoclaving) as described by Turner and Backman (1991). The suspensions were heated (80 °C, 10 min) in a water bath before preparing the dilution series (Tuitert et al. 1998). The CFU were counted twice, 48 h after plating to determine *Bacillus* spp. and a week after plating to determine and confirm the presence of *B. subtilis* based on colony morphogenesis as described by Aguilar et al. (2007).

The concentrations of low molecular weight organic anions in the rhizosphere were determined by high-performance liquid chromatography (HPLC) according to Gao et al. (2012). Soil extractions with 0.1 M NaOH were carried out in a 1:1 ratio (w:v) by shaking the mix (4 s^{-1}, 90 min) (Radersma and Grierson 2004) as described by García-López and Delgado (2016). The suspensions were centrifuged at 1260 g for 10 min, and the collected supernatants were centrifuged again at 17,900 g for 10 min. The extracts were then acidified to pH 2–3 with 0.1 M H$_2$SO$_4$ and filtered through a 0.20-µm membrane filter before being separated on an HPLC Varian ProStar 410 instrument. To this end, the HPLC was furnished with a column C18 (Varian, 250 mm × 34.6 mm, particle size of 8-µm particle size). The elution method was isocratic with 98% 5 mM H$_2$SO$_4$ at pH 2 plus 2% methanol at 0.8 ml min^{-1} as carrier solution and 20 µl injection volume. Detection of organic anions was performed at 215 nm, using a Varian 486 photodiode array detector. Individual standard solutions of acetic, oxalic, citric, malic, fumaric, and succinic acid, all from Sigma (Barcelona, Spain), were used for the identification of organic anions. Only oxalate was detected in the extracts.

The siderophores in the rhizosphere were determined by measuring the amount of Fe^{3+} complexed by them. The colorimetric method (Schwyn and Neilands 1987) uses the affinity of siderophores for Fe^{3+} and a ternary complex (chrome azurol S/iron (III)/hexadecyltrimethylammonium bromide –CAS–) as an indicator, with an extinction coefficient of 100,000 M^{-1} cm^{-1} at 630 nm. The siderophore moves Fe from the indicator which changes from blue to orange at pH 5.6. To extract siderophores from the rhizosphere, the same soil extraction procedure was used for organic anions, and 0.5 ml of this extract was mixed with 0.5 mL of CAS assay solution (Schwyn and Neilands 1987). After reaching equilibrium (6 h), the absorbance was measured at 630 nm and compared with a reference without soil extract.

Enzyme activities in the rhizospheric soil were determined at harvest. Dehydrogenase activity was determined after soil incubation (37 °C in darkness, 24 h) in falcon tubes containing 1 g of soil, 0.01 g of CaCO$_3$, 0.25 ml of 3% 2,3,5-triphenyl-tetrazolium chloride (TTC), and 0.875 ml of water. The triphenyl formazan (TPF) produced was sequentially extracted (three times) by adding ethanol (up to 15 ml of total volume) and centrifuging (1260 g, 10 min) to separate it from the soil. The concentration of TPF in the supernatant was determined colorimetrically at 485 nm as described by Casida et al. (1964), using a Lambda 35 spectrophotometer (Perkin Elmer, USA). The ß-glucosidase activity was determined according to Eivazi and Tabatabai (1988) by determining the *p*-nitrophenol produced from soil incubation (37 °C, 1 h) with 0.05 M 4-nitrophenyl-ß-D-glucopyranoside (PNG) as an enzymatic substrate buffered with the modified universal buffer (MUB) at pH 6. A similar procedure was used to determine alkaline phosphatase, using 0.05 M 4-nitrophenyl phosphate as substrate buffered with MUD at pH 11 and measuring the amount of *p*-nitrophenol (PNP) produced (Tabatabai and Bremner 1969; Eivazi and Tabatabai 1977).

2.7 Statistical Analysis

The effect of soil type and treatments affecting microbial activity in the rhizosphere was evaluated using a two-way analysis of variance. Previously, normality according to the Shapiro-Wilks test and homoscedasticity according to the Levene test were checked. Power transformations were performed if necessary to fully meet both criteria. Analysis of variance was performed with the General Linear Model procedure in Statgraphics Plus 5.1 (StatPoint 2000). The means for each factor level were compared using the LSD test (P < 0.05), except when the interaction between factors was significant. In the case of significant interactions, the effect of main factors cannot be assessed, and only the interaction can be discussed, since the effect of one factor depends on the level of the other. In this case, this means that the effect of treatments depends on soils, and it was not possible to conduct a mean comparison between treatments for each main factor (de Santiago et al. 2013). Then, two analyses were performed; first, the combined effect of both factors was evaluated with a one-way analysis of variance and mean comparison for the combination of both factors in order to see differences between particular soils; second, a general linear model (GLM) involving two factors, one categorical (treatment) and another quantitative (specific soil property), was performed in order to identify the soil property with particular interaction with treatments. This was done for the main physicochemical and biochemical properties of soils. Pearson's correlation coefficients and regressions were calculated using the same software mentioned above.

3 Results

3.1 Effect of Soil on Plant Development and Nutrition

The soils studied encompassed a wide range of properties, in particular, textural classes and nutrient availability indices (Table 1). The initial DTPA extractable Zn (Zn_{DTPA}) was below the threshold value for nutrient deficiency (0.5 mg kg^{-1}) in all the soils. The pyrophosphate extractable Zn (Zn_{pyr}) ranged from 0.4 to 1.5 mg kg^{-1} (Table 1). In the case of P, the soils showed a wide range of availability status, Olsen P ranging from 4.7 to 26 mg kg^{-1}. The pH of the soils varied between 7.2 and 8.3.

The yield and nutritional status of the crop differed widely between soils (Table S1, Supplementary material). Initial Olsen P explained 72% of the variation in total dry matter (DM) production (DM = 0.72 + 0.025 Olsen P; $P = 0.002$; $n = 10$). The total P in the plant was not related to the initial Olsen P in the soil (not shown). The total Zn in the plant or the Zn concentration in the grain was not related to Zn_{DTPA} or Zn_{pyr}. However, the ratio of Zn_{pyr} to Olsen P explained 54% of the variation in total Zn in the plant (Total Zn = 6.7 + 211 Zn_{pyr}/Olsen P; $P = 0.0152$; $n = 10$) and 80% of the variation in Zn concentration in grains ([Zn] in grain = 9.4 + 308 Zn_{pyr}/Olsen P; $P < 0.001$; $n = 10$). In general, the concentration of Zn in grains was low relative to the standards of good nutritional quality. In this sense, this concentration was below 32 mg kg^{-1} in 5 of the soils (Table S1; Supplementary material). The dry matter yield was not related to the extractable Fe or

Mn. These extractions did not relate to the total nutrient content of the plants. A wide range of enzyme activities were also observed in the rhizospheric soil after crop harvest (Table S2, Supplementary material). The phosphatase activity of the soil was not related to the initial Olsen P nor other soil properties (not shown). In general, soils with the highest DM yield of the crop were those that showed the lowest indolacetic acid (IAA) and abscisic acid (ABA) in roots (Table S1, Supplementary material).

3.2 Effect of Microbial Inoculants and Cellulose on Crop

After cropping, both inoculants were detected in rhizopheric soil, without significant differences between soils. Colony-forming units (CFU) were in the range 5.4·10^3–2.2·10^5 g^{-1} soil for *B. subtilis* and 1.5·10^4–3.9·10^4 g^{-1} soil for *T. asperellum*. The two microbial inoculants and the cellulose amendment (treatments) increased the total root DM and grain yield compared to the control (Table 2). When the aboveground biomass was considered, only cellulose improved it significantly relative to the control (Table 2). Dry matter in non-reproductive organs in above-ground biomass was not significantly affected by inoculants or cellulose (not shown). In general, *B. subtilis* and cellulose promoted lower levels of indolacetic acid (IAA), abscisic acid (ABA), and gibberellins 3 (GA3) than the control. No significant differences in IAA and ABA were observed between *B. subtilis* and *T. asperellum* (Table 2). The effect of treatments on GA3 varied depending on the soil, as revealed by the significant

Table 2 Effect of the different factors on the dry matter (DM) yield, harvest index (HI), and plant hormones

Source of variation	Dry matter yield				HI	Hormones		
	Total	Grain	Root	Shoot		IAA	ABA	GA3
Treatment	——————————— g pot^{-1} ———————————					——————————— µg kg^{-1}———————————		
B. subtilis	1.09 ± 0.28a	0.40 ± 0.11a	0.121 ± 0.08a	0.97 ± 0.23ab	0.41 ± 0.07	0.35 ± 0.15b	0.10 ± 0.04b	3.0 ± 1.6b
Cellulose	1.13 ± 0.23a	0.42 ± 0.11a	0.127 ± 0.05a	1.00 ± 0.2a	0.42 ± 0.07	0.31 ± 0.1b	0.09 ± 0.03b	3.2 ± 0.92b
T. asperellum	1.08 ± 0.25a	0.39 ± 0.12a	0.116 ± 0.08ab	0.96 ± 0.2ab	0.41 ± 0.09	0.40 ± 0.2b	0.12 ± 0.05b	4.5 ± 2.17a
Control	1.03 ± 0.25b	0.35 ± 0.15b	0.101 ± 0.05b	0.93 ± 0.22b	0.37 ± 0.11	0.42 ± 0.16a	0.13 ± 0.04a	3.9 ± 1.39a
ANOVA	*P value*							
Soil	0.0000	0.0000	0.0000	0.0000	0.0005	0.0000	0.0004	0.0008
Treatment	0.0030	0.0134	0.0031	0.0164	0.0949	0.0006	0.0021	0.0007
Soil*treatment	0.1630	0.8707	0.0514	0.0640	0.1043	0.0564	0.2000	0.0001

Mean ± standard deviation; means followed by the same letter are not significantly different according to the LSD test ($P < 0.05$)

HI harvest index, *IAA* indole-acetic acid, *ABA* abscisic acid, *GA3* gibberellic acid

Shoot DM includes grain DM

Hormones were not detected in soil OTRZ1 and OTRZ2

In the case of potential transformation to meet ANOVA requirements, back transformed results are shown

Mean comparison for soils and interaction effects shown in supplemental material

interaction between both factors (Table 2). Although *T. asperellum* increased GA3 content compared to other treatments (Table 2), this effect was more marked with increasing soil pH (Fig. 1).

The microbial inoculants and the cellulose had no effect on the total content of P and Zn in the plants, the concentration of P in the different organs, or the concentration of Zn in the grains compared to the control. However, a trend of decreasing Zn concentration with these treatments was observed in roots and above-ground biomass (Table 3). All treatments increased the Zn harvest index (Zn HI) and decreased the molar ratio of P to Zn in grains relative to the control (Table 3). The effect of treatments on total Fe and Mn in plants differed between soils, as revealed by the significant interaction between factors ($P = 0.04$ and $P = 0.001$, respectively; Table S3). In three of the soils, microbial inoculants or cellulose increased total Fe or Mn in plants relative to the control (Fig. 2; Table S3). In one, these concentrations were decreased (VGTZ2, Fig. 2). According to GLM, the effect of *B. subtilis* and *T. asperellum* increasing total Fe in plants decreased with increased soil pH (Fig. 3a). On the other hand, the effect of these microorganisms on the improvement of total Fe in plants was more marked with increased soil microbial activity estimated as the β-glucosidase activity in controls without treatment after harvest (Fig. 3b). The total Cu in the plants; the concentration of Fe, Cu, and Mn in the different organs; and their harvest indices were affected only by soil (not shown). The molar ratio of Fe to P in the grain was not affected by treatments, only by soils (not shown).
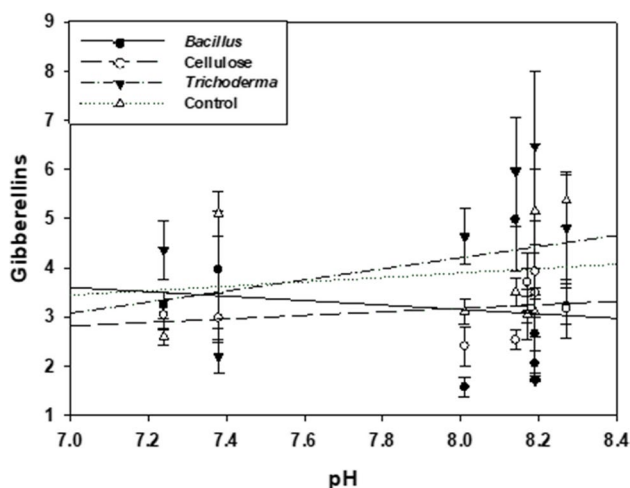
Fig. 1 Effect of the different treatments (microorganisms and organic amendment) on gibberellins concentration (μg kg.$^{-1}$) as affected by soil pH according to the general linear model (GLM); the lines represent the trend in the effect of each treatment as a function of soil pH; there are significant differences in the regression slopes between treatments at $P = 0.0011$. The effect of the interaction between soil and treatment was significant at $P = 0.0015$

3.3 Effect of Microbial Inoculants and Cellulose on Rhizospheric Soil

Bacillus subtilis tended to increase the concentration of oxalate relative to *T. asperellum*. In general terms, *T. asperellum* and cellulose increased phosphatase activity, and both inoculants and cellulose increased β-glucosidase activity (Table 4). Actually, the effect of inoculants or cellulose on enzyme activities in the rhizospheric soil varied depending on the soil, as revealed by the significant interactions in the ANOVA between both factors (treatments and soil, Table 4). Overall, *T. asperellum* was the most effective treatment increasing dehydrogenase and phosphatase activities (Table S3). This inoculant promoted higher dehydrogenase activity than control in BLGZ2, BLGZ3, and OTRZ2 soils and phosphatase activity in LCVZ4 and VTGZ1 (Table S3). *Bacillus subtilis* increased the activity of dehydrogenase compared to the control in LCVZ1 and the activity of phosphatase in RCNZ2 (Table S3). Cellulose was the most effective improving β-glucosidase activity, with a significant effect in three soils (LCVZ4, RCNZ1, and RCNZ2; Table S3). The concentration of siderophores was only increased by *T. asperellum* in the soil LCVZ4 (Table S3). Only in the soil RCNZ1, inoculants and cellulose promoted a significant decrease in pH (Table S3).

3.4 Relationships Between the Variables Studied

In the controls without treatments, the concentration of abscisic acid in the roots decreased with increased total Fe (Fig. 4a), total Mn (Fig. 4b), and total P in plants (ABA = 1/[0.003 + 0.009 Total P]; $R^2 = 0.19$; $P < 0.001$, $n = 30$). In the whole dataset, phosphatase and β-glucosidase were positively correlated between them ($r = 0.62$; $P < 0.001$; $n = 160$) and both activities with total plant DM ($r = 0.21$; $P < 0.01$ and $r = 0.41$; $P < 0.001$, respectively; $n = 160$) and total P in plants ($r = 0.44$ and 0.47, $P < 0.001$, respectively; $n = 160$). The concentration of siderophores in the rhizosphere was correlated with dehydrogenase activity ($r = 0.37$; $P < 0.001$; $n = 160$).

4 Discussion

The results validate our first hypothesis, that is, the two microbial inoculants and the cellulose amendment enhanced plant development and grain yield (Table 1). These effects did not depend on the soil, since the interaction between the treatments that affect microbial activity and the soil was not significant. These benefits cannot always be ascribed to

Table 3 Effect of the different factors on plant nutritional variables

Source of variation	Total nutrient in plants					P concentration			Zn concentration			Harvest index		Molar ratio
	P	Zn	Fe	Mn	Cu	Grain	Shoot	Root	Grain	Shoot	Root	P	Zn	P/Zn
	µg plant⁻¹					mg kg⁻¹								
Treatment														
B. subtilis	883±254	23±12	477±229	17±5.6	10±8.2	2050±911	454±398	628±283	33.1±14.3	8.5±5.78	22.8±8.16b	0.85±0.07	0.57±0.08a	72.6±39b
Cellulose	872±206	24±12	415±164	17±3.8	8±5.4	1960±832	364±227	596±176	33.5±16	7.9±5.26	23.8±8.51b	0.86±0.04	0.57±0.07a	71.3±41b
T. asperellum	860±244	23±12	470±330	17±5	8±5.2	1982±742	396±308	596±229	33.0±14.3	8.1±5.84	22.4±8b	0.85±0.07	0.57±0.09a	72.4±37b
Control	906±274	23±11	371±167	16±4.5	7±5.3	2523±1360	525±389	654±280	34.9±15.4	9.0±6.36	25.9±8.05a	0.82±0.09	0.52±0.11b	82.9±42a
ANOVA P value														
Soil	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Treatment	0.9950	0.6615	0.4445	0.1846	0.1794	0.0964	0.1581	0.6685	0.5259	0.0568	0.0232	0.0892	0.0263	0.0112
Soil*treatment	0.4273	0.8679	0.0289	0.0046	0.5141	0.8104	0.5794	0.0720	0.2574	0.3472	0.0041	0.3202	0.0624	0.6600

Mean±standard deviation; means followed by the same letter are not significantly different according to the LSD test ($P < 0.05$)

Zn HI and P HI, proportion of total nutrient in plants that is accumulated in grain

In the case of potential transformation to meet ANOVA requirements, back transformed results are shown

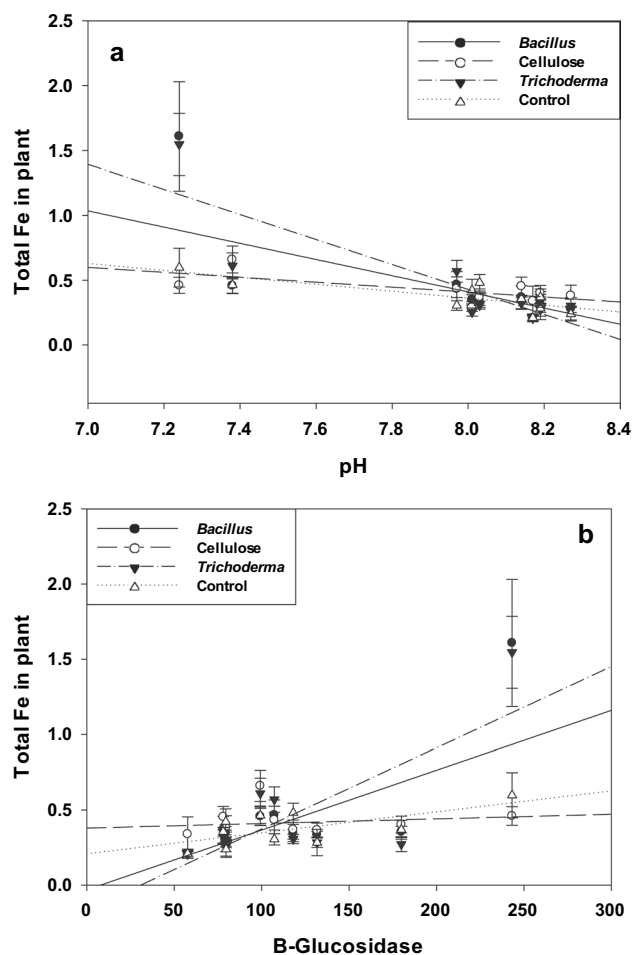Mean comparison for soils and interaction effects shown in supplemental material
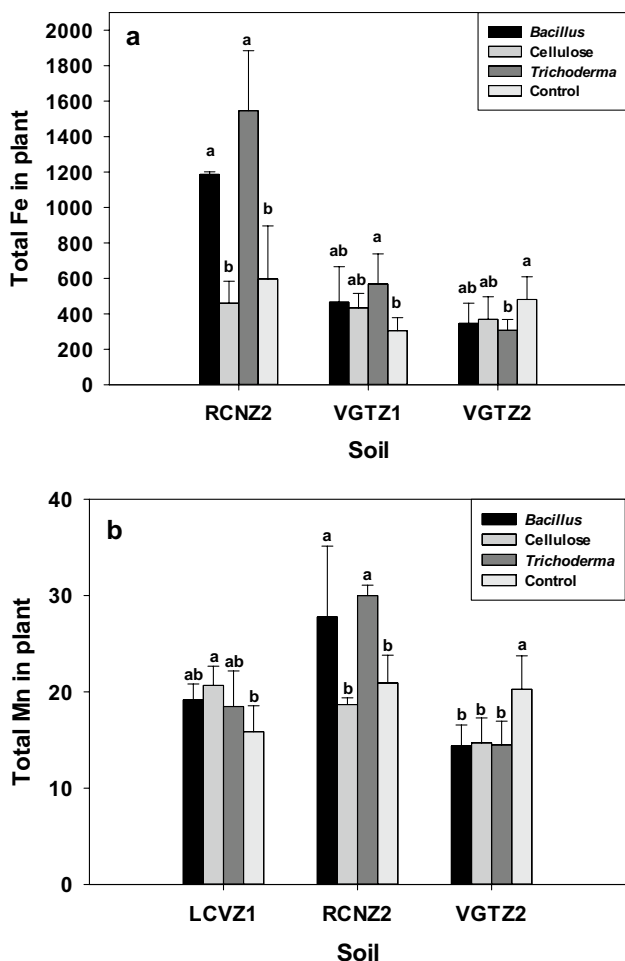
Fig. 2 a Effect of the different treatments (microorganisms and organic amendment) on total Fe in plants (μg plant^{-1}) in the soils where this effect was significant. (**b**) Effect of the different treatments (microorganisms and organic amendment) on total Mn in plants (μg plant.$^{-1}$) in the soils where this effect was significant. Differences significant according to LSD ($P < 0.05$)

Fig. 3 a Effect of the different treatments (microorganisms and organic amendment) on total Fe in plants (μg plant^{-1}) as affected by soil pH according to the general linear model (GLM); the lines represent the trend in the effect of each treatment as a function of the soil pH. There are significant differences in the slope of the regressions between treatments at $P = 0.0000$. The interaction between soil and treatment was significant at $P = 0.0026$. **b** Effect of the different treatments (microorganisms and organic amendment) on total Fe in plants (μg plant^{-1}) as related to β-glucosidase activity in control (without treatment) at the end of the experiment for each soil (in mg PNP kg^{-1} h.$^{-1}$) according to the general linear model (GLM); the lines represent the trend in the effect of each treatment as a function of β-glucosidase activity in control. There are significant differences in the slope of the regressions between treatments at $P = 0.0000$. The interaction between soil and treatment was significant at $P = 0.0135$

an improved plant nutrition. In this sense, increased uptake was observed only for Fe or Mn in three of the soils studied.

The accumulation of ABA in roots is known to be a response to abiotic stresses, particularly nutrient deficiency in durum wheat (Trapeznikov et al. 2003; Vysotskaya et al. 2008). Furthermore, ABA concentration has been proven to increase with Fe deficiency in plants (Lei et al. 2014). This agrees with our observation of a decreased ABA concentration in roots with an increase in total Fe, Mn, and P in plants (Fig. 4). Therefore, reduced bioavailability of these nutrients, attributed in part to soil properties such as carbonates, basic pH, or high adsorption capacity (Ryan et al. 2013), seems to promote accumulation of ABA in roots. High levels of this hormone in roots are one of the reasons for restricted plant development under nutrient deficiency conditions (Vysotskaya et al. 2008). It appears that

the promotion of plant growth by *B. subtilis*, *T. asperellum*, and cellulose was attributed to a reduction in the concentration of ABA in the roots. This decrease in ABA concentration by microorganisms may alter, and in some cases stimulate, plant growth (Belimov et al. 2014). This effect of rhizospheric microorganisms that reduce ABA concentration has previously been described in cereals (Dodd et al. 2010). However, increased Fe or Mn uptake by treatments can contribute to the improved plant growth and decreased

Table 4 Effect of the different factors on rhizospheric soil variables

Source of variation	Oxalate	Siderophores	Enzymatic activities			pH
			DHA	Phos	β-GLU	
Treatment	mmol kg^{-1}	mg Fe^{3+} kg^{-1}	——————— mg kg^{-1} h^{-1}———————			
B. subtilis	73±29	13.4±4.2a	2.3±2.3	187±59b	122±58a	7.78±0.38
Cellulose	65±22	11.8±4.9b	2±1.9	199±76a	125±55a	7.83±0.37
T. asperellum	63±27	13.4±4a	2.3±2.2	200±63a	128±52a	7.79±0.43
Control	69±26	13.4±4.2a	1.9±1.7	186±68b	117±55b	7.80±0.34
ANOVA	*P values*					
Soil	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Treatment	0.0660	0.0095	0.2263	0.0090	0.0000	0.0169
Soil*treatment	0.4165	0.0071	0.0000	0.0000	0.0002	0.0000

Mean ± standard deviation; means followed by the same letter are not significantly different according to the LSD test ($P < 0.05$)

DHA dehydrogenase activity measured as the amount of triphenyl-formazan produced, *β-GLU* β-glucosidase activity measured as the amount of p-nitrophenol released from M 4-nitrophenyl-ß-D-glucopyranoside, *Phosp* alkaline phosphatase activity measured as the amount of p-nitrophenol released from p-nitrophenol phosphate

In the case of potential transformation to meet ANOVA requirements, back transformed results are shown

Mean comparison for soils and interaction effects shown in supplemental material

ABA concentration in the roots of plants grown in some soils when stress signals are diminished (Sah et al. 2016). However, the effect of *T. asperellum* appears to be related to an increased concentration of GA3 in roots, which is known to stimulate plant growth (Hedden 2016). This augmented GA3 concentration with *T. asperellum* was more evident with increasing pH, which are conditions more restrictive for plant nutrition and consequently where plant development should be more restricted.

We found evidence of the triggering of nutrient mobilization mechanisms by treatments, such as enhanced phosphatase activity (Table 4), or the trend of *B. subtilis* to increase oxalate in the rhizosphere. Total P in plants was related to phosphatase activity in the rhizosphere. However, microbial inoculants and cellulose had no effect on total P, Zn, and Cu. This is explained because the properties of the soil also affect nutrient uptake, such as the ratio of Zn_{pyr} to Olsen P in the case of Zn (Recena et al. 2021). The effect of cellulose and microbial inoculants on total Fe and Mn in plants depended on the soil. The effect of *B. subtilis* and *T. asperellum* on the improvement of Fe uptake by plants was more evident at lower soil pH (Fig. 3). This probably reveals that these microbial inoculants are more effective at improving Fe nutrition in soil conditions that are not so restrictive for Fe availability to plants. Furthermore, these benefits were also more relevant with increasing microbial activity in soils (as estimated by the average β-glucosidase activity in nontreated soils). This reveals that more suitable conditions for microorganisms likely enhance inoculant development and consequently their effects.

Microbial inoculants and cellulose improved microbial activity in the rhizosphere as revealed by the increased β-glucosidase activity (Table 4). This enzyme is retained in the soil and reflects long-term fluctuations in microbial activity, likely along the crop cycle (Stott et al. 2010; Moreno et al. 2015). Phosphatase is ascribed in part to microbial activity as revealed by its correlation with β-glucosidase. The correlation between β-glucosidase and phosphatase activity and DM of plants may suggest that enhanced plant growth may promote improved colonization and microbial activity in the rhizosphere, perhaps through increased root exudation (Zhao et al. 2020; Vora et al. 2021). The production of siderophores was related to microbial activity at harvest, as revealed by the positive correlation with the dehydrogenase activity. Therefore, the activity of rhizospheric microorganisms may contribute to the potential for nutrient mobilization from the soil.

Zinc uptake by plants was not affected by microbial inoculation or cellulose amendment. However, these treatments increased the proportion of Zn accumulated in grains (Zn HI) independently of soil, in agreement with Moreno-Lora et al. (2019). This may be explained by the decrease in the Zn concentration in roots and shoots without a significant effect on grains promoted by inoculants and cellulose. Thus, this confirms our hypothesis of an alteration of the Zn partitioning in plants leading to a higher accumulation in grains relative to other organs. This may be attributed to the observed changes in auxin or ABA concentration, since these hormones can regulate transporters involved in metal homeostasis in plants (David-Assael et al. 2006; Barickman et al. 2019). Increased Zn HI with inoculants and cellulose compared to control led to a decreased molar P-to-Zn ratio in the grain with these treatments and consequently to an improved grain quality, as Zn digestibility is enhanced.
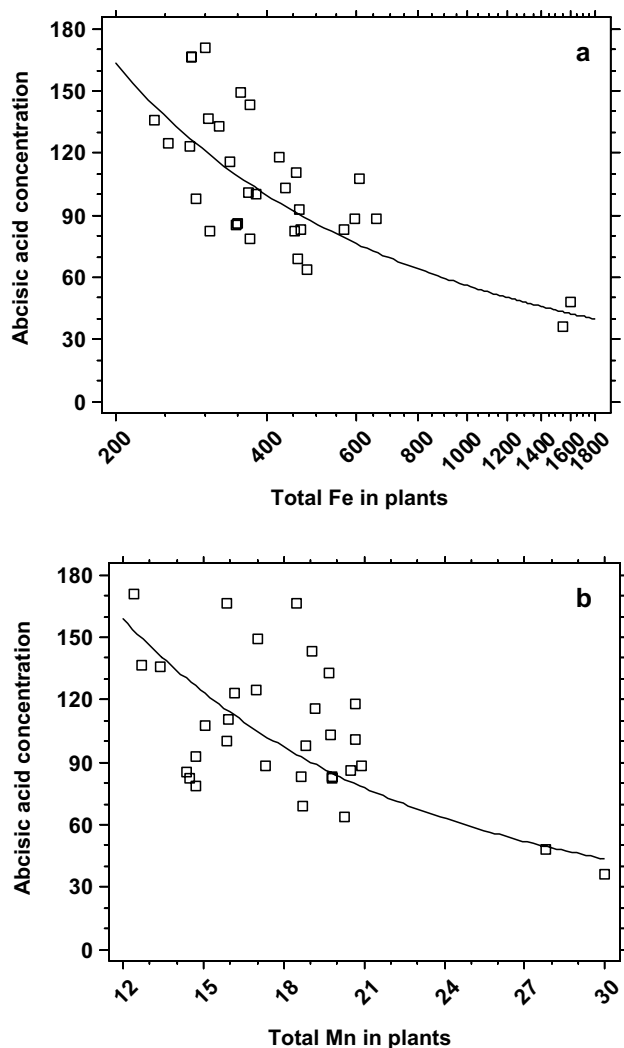
Fig. 4 **a** Relationship between the abscisic acid concentration in roots (in ng g^{-1}) and total Fe in plants (μg plant^{-1}) in the controls without treatments; $Y = 1/(0.009 + 6.2 \cdot 10-9\ X^2)$, $R^2 = 0.68$, $P < 0.0001$, $n = 30$. **b** Relationship between the abscisic acid concentration in roots (in ng g^{-1}) and total Mn in plants (μg plant^{-1}) in the control without treatments; $Y = 1/(0.003 + 2.2 \cdot 10^{-5}\ X^{1/2})$, $R.^2 = 0.61$; $P < 0.0001$, $n = 30$

5 Conclusions

Microorganisms and cellulose improved DM and grain yield in wheat, and this effect was independent of soil. In general, neither microorganisms nor cellulose increased total P and Zn in plants. However, the Zn HI and the P to Zn molar ratio were enhanced independently of the soil. Therefore, our results provide evidence of the benefits of using microorganisms and organic amendments as a tool to improve the yield, the portion of absorbed Zn accumulated in grains, and the grain quality in wheat grown in a set of soils that vary widely in their properties. The effect on Fe nutrition and gibberellins varied depending on the soil. These effects were affected by soil pH, while the microbial activity of the soils seemed relevant to explain the effect of the inoculants on total Fe in the plant.

Declarations

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