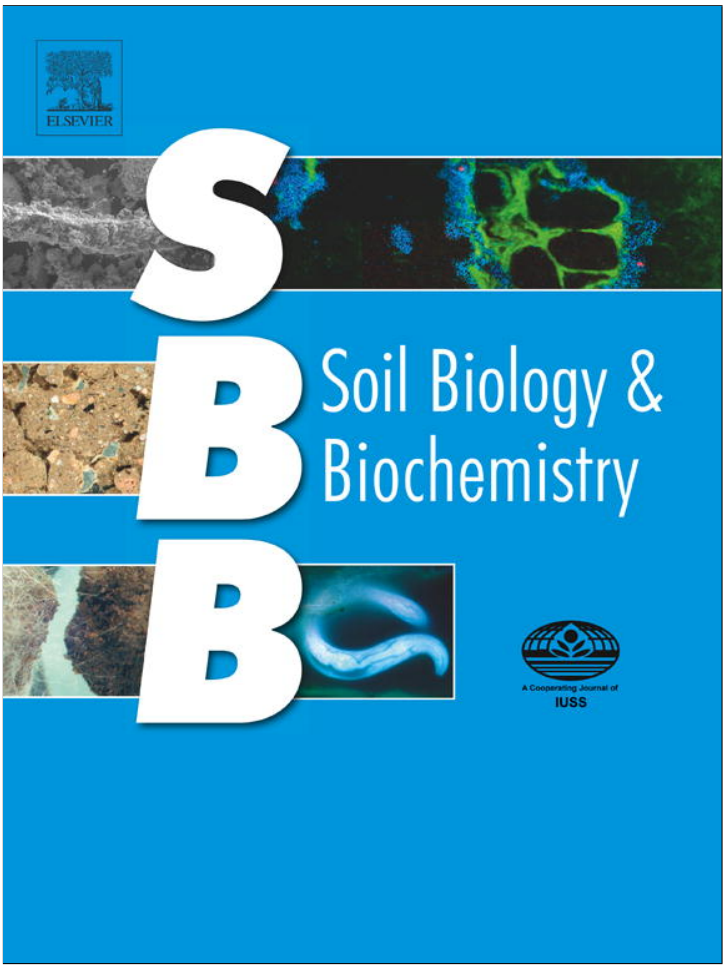

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Effect of *Trichoderma asperellum* strain T34 and glucose addition on iron nutrition in cucumber grown on calcareous soils

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ABSTRACT

The fungus *Trichoderma asperellum* T34 is a biological control agent which has been shown to enhance Fe uptake by plants. The objective of this research was to study the contribution of T34 to Fe availability to cucumber plants in soils and how this potential supply can be affected by soil properties and in particular, by soil microbial activity. To this end, a completely randomised experiment was performed twice involving three factors: soil (LB9, LB11, and LB14), plant inoculation with T34 (inoculated and non-inoculated with T34), and glucose supply to achieve a priming effect (supply and no supply). Inoculation with T34 was effective in increasing plant growth and total accumulation of Fe and Cu in aerial parts. This increase was not related to an increased accumulation or concentration of Fe or Cu in roots. Glucose addition was only effective in increasing Fe accumulation in the aerial parts of plants grown in the soil with the lowest organic C content and native β-glucosidase activity (LB9 soil). The effect of T34 on Fe concentration in the aerial parts of plants differed depending on the soil and the glucose addition. In the LB9 soil, the simultaneous application of T34 and glucose increased the development of the fungus and resulted in an increased Fe concentration in plant shoots when compared to the control without T34 and glucose. In the soil where β-glucosidase activity was increased by glucose addition (LB14 soil), this addition resulted in an increased Fe concentration in aerial parts of plants, thus revealing the effect of increased soil microbial activity on improving Fe uptake by plants; T34 alone produced a similar effect on Fe concentration to that achieved with glucose in this soil. Glucose or T34 did not affect Fe concentration in aerial parts of plants grown in the soil with the highest Fe availability index (LB11), thus showing that the effect of T34 or microbial activity can be less evident when the availability of nutrients is not so restricted.

1. Introduction

Microbial communities can alter nutrient cycling in the rhizosphere, thus affecting nutrient availability to plants (Marschner et al., 2004; Violante et al., 2005; Robin et al., 2008; Browne et al., 2009). Many rhizobacteria and fungi release iron chelators (siderophores) which can contribute to increased Fe availability to plants (Chen et al., 1998; Sharma and Johri, 2003; Khan et al., 2006; Jin et al., 2006; Lemanceau et al., 2009). Excretion of chelators also explains increased mobilisation of other metals (Altomare et al., 1999; Renshaw et al., 2002). Additionally, microbial activity in soil can be involved in acidification and reduction processes, which can also contribute to the mobilisation and uptake of Fe and other metals by plants (Marschner et al., 2003; Stemmler and Berthelin, 2003; Valencia-Cantero et al., 2007; Rakshit et al., 2009; Sánchez-Alcalá et al., 2011).

The contribution of rhizosphere microbial communities to improved plant nutrition is of particular interest in environments with restricted nutrient availability, and supports the strategy of manipulation of microbial activity in the rhizosphere in order to overcome nutritional deficiencies (Aseri et al., 2008; Zuo and Zhang, 2011). This can be relevant in the case of Fe deficiency chlorosis, an extended nutritional disorder which constraints agricultural production in many areas of the world where alkaline soils predominate (Sharma et al., 2003; de Santiago and Delgado, 2007; Naeve and Rehm, 2006). Other deficiencies, such as of Mn or Zn, are also frequent in this type of soil, and also give rise to significant agronomic problems (Rashid and Ryan, 2004; Husted et al., 2005; Sayyari-Zahan et al., 2009).

The capacity of soil microbes to mobilise and uptake nutrients is usually part of a competition strategy involving antagonisms,

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which explains their use in the control of plant pathogens (Mercado-Blanco and Bakker, 2007; Chaiharn et al., 2009; Lemanceau et al., 2009; Hartmann et al., 2009). In fact, many known biological control agents are effective in mobilising nutrients in the rhizosphere, supporting their use in integrated management of plant diseases and nutritional deficiencies (Vassilev et al., 2006; de Santiago et al., 2011). The soil fungus *Trichoderma* spp. is an efficient and widely used biological control agent (Howell, 2003; Segarra et al., 2007; Schuster and Schmoll, 2010) which has been shown to enhance nutrient uptake by plants (Rudresh et al., 2005; Yadav et al., 2009). The release of siderophores by this fungus (Haas et al., 2008) comprises one of the competition mechanisms between this fungus and pathogenic fungi (Verma et al., 2007; Segarra et al., 2010; Fontanelle et al., 2011) and explains why plants inoculated with it show increased Fe uptake (Hoyos-Carvajal et al., 2009). In addition, *Trichoderma* can increase the mobilisation and absorption of Cu and Mn by plants (Altomare et al., 1999; Harman et al., 2004). Recently, *Trichoderma asperellum* T34 (hereafter referred to as T34) was shown to enhance the uptake of Fe from calcareous growing media by lupins and wheat: the effect on uptake of other micronutrients differed depending on the plant and the Fe content of the growing medium (de Santiago et al., 2009; de Santiago et al., 2011).

To elucidate the contribution of T34 to Fe availability to plants in soils and how this potential supply can be affected by soil properties, and in particular by soil microbial activity, here we inoculated cucumber (*Cucumis sativus* L) with T34 and studied the effects on Fe uptake. Plants were grown in three calcareous soils differing in $CaCO_3$, nutrients and organic C content, key factors affecting the incidence of Fe deficiency chlorosis. In previous research, the effect of native soil microbial activity on plant nutrition has been studied by assessing the consequences of autoclave sterilisation on nutrient uptake (Masalha et al., 2000; Rroço et al., 2003). This treatment can alter chemical soil properties, including availability of nutrients (Wolf and Skipper, 1994; Urbanek et al., 2010), and thus potential changes in nutrient uptake cannot necessarily be ascribed to decreased microbial activity. Instead of sterilisation, we applied an easily available substrate for microorganisms (glucose) in order to promote increased microbial activity in soil.

2. Material and methods

2.1. Experimental design

An experiment was performed twice at different times – two trials – under the same growing conditions in a growing chamber. The design was exactly the same for both trials, completely randomised with five replications and involving three factors: soil (three), plant inoculation with T34 (inoculated and non-inoculated with T34) and glucose supply to achieve a priming effect (supply and no supply). For statistical analysis, each trial was considered a separate block in order to exclude the variation associated with repetition of the experiment. Each replication in each trial corresponded to a pot with a plant.

Three representative calcareous soils from the Guadalquivir Valley (SW Spain) were used in this study. Samples were collected from the Ap horizon (0–30 cm). Prior to analysis, soils were air-dried and ground to pass through a 2 mm sieve. Particle size analyses were carried out using the densimeter method (Gee and Bauder, 1986). Organic C (OC) was determined by dichromate oxidation (Walkley and Black, 1934), and the cation exchange capacity (CEC) by using 1 M NH_4OAc buffered at pH 7 (Sumner and Miller, 1996). The total $CaCO_3$ equivalent (CCE) was determined by the calcimeter method and the "active" $CaCO_3$ equivalent (ACCE) according to Drouineau (1942). Electrical conductivity (EC) was measured in the saturation extract, and pH was measured in water and $CaCl_2$ at a soil:extractant ratio of 1:2.5. DTPA extraction of micronutrients was performed according to Lindsay and Norvell (1978) to assess their availability in soils. The three soils differed in CCE, ACCE, OC, and DTPA extractable nutrients (Table 1).

Addition of glucose to soil was carried out at a rate of 200 mg kg^{-1} soil as a solution before transplanting according to Chander and Joergensen (2007). To this end, 20 mL of a solution with 2.75 g L^{-1} of glucose was carefully applied to 275 g of soil in trays. The applied volume humidified the soil but did not promote any drainage. After that, soils were carefully mixed and disposed in pots. Since the amount of C applied was lower than the expected C biomass in soil (García-Orenes et al., 2010), increased microbial activity without significant alteration of community structure could be expected (Blagodatskaya and Kuzyakov, 2008).

Inoculation with T34 was carried out by immersing roots in a suspension in water containing 10^3 conidia m L^{-1} before transplanting (de Santiago et al., 2009), and by adding 10^4 conidia g^{-1} soil after transplanting; this addition was carried out by applying 20 mL of a conidia suspension in water ($1.375 \bullet 10^5$ conidia m L^{-1}) with a micropipette on the soil surface in five points around the plants; conidia were prepared following Segarra et al. (2007).

2.2. Plant material and growth conditions

Cucumber (*C. sativus* L cv Serena) was used for the experiments since it is a strategy I plant of economic interest. Although it can be consider a Fe-efficient plant, symptoms of Fe deficiency chlorosis can be observed when it is grown in strongly calcareous growing media (Bacaicoa and García-Mina, 2009). Seeds were germinated in peat and fifteen days after germination, at the two true-leaf stage, individual plants were transplanted into pots (350 mL, 5.5 cm diameter, 15 cm-high polystyrene cylinder) containing 275 g of soil and grown for 33 additional days. The experiments were conducted in a growing chamber under the same controlled environmental

Table 1
Properties of soils.

Soil	Taxonomy[a]	Sand (g kg^{-1})	Silt (g kg^{-1})	Clay (g kg^{-1})	OC (g kg^{-1})	CCE (g kg^{-1})	ACCE (g kg^{-1})	pH ($CaCl_2$)	pH (H_2O)	EC (dS m^{-1})	CEC ($cmol_c$ kg^{-1})	DTPA extractable nutrients			
												Fe (mg kg^{-1})	Cu (mg kg^{-1})	Mn (mg kg^{-1})	Zn (mg kg^{-1})
LB11	Typic Calcixerolls	180	409	184	15	365	98	7.86	8.59	0.81	24	17.3	1.3	11.2	1.3
LB14	Aquic Haploxeralfs	570	184	246	8	89	19	7.69	8.19	1.87	9	6.3	1.3	5.4	0.3
LB9	Haplic Xerarents	120	450	400	3	857	109	7.86	8.79	0.42	6	2.3	1.6	5.2	0.1

OC, organic carbon, CCE, calcium carbonate equivalent; ACCE. Active calcium carbonate equivalent; EC, electrical conductivity in the saturation extract; CEC, cation exchange capacity.

[a] According to Soil Taxonomy (Soil Survey Staff, 2010).

conditions: photoperiod of 14 h, a 25/20 °C day/night temperature, 65% RH, and 22 w m^{-2} light intensity. Plants were irrigated daily with a Fe-free Hoagland-type nutrient solution. Two different nutrient solutions were used: twice a week, the composition of the solution applied (concentrated one) was (all concentrations in mmol L^{-1}): $MgSO_4$ (2), $Ca(NO_3)_2$ (5), KNO_3 (5), KH_2PO_4 (1), KCl (0.05), H_3BO_3 (0.009), $MnCl_2$ (0.0023), $CuSO_4$ (0.0005), $ZnSO_4$ (0.002), and H_2MoO_4 (0.0005); the other days of the week, a nutrient solution (diluted one) with one tenth of the concentration of nutrients indicated above was used. At the end of the experiment, a total of 225 mL of the concentrated nutrient solution and 425 mL of the diluted one were applied per pot. The pH of the nutrient solution ranged between 5.5 and 6. This type of nutrient was used in order to study the soil as Fe source for plants. The application of other micronutrients with nutrient solution was carried out in order to avoid the other micronutrient deficiencies which can be frequent in calcareous soils.

2.3. Soil analysis

β-glucosidase activity, a measure of microbial activity in soil, was determined in the rhizospheric soil at the end of both experiments as the amount of PNP (*p*–nitrophenol) formed from PGN (*p*–nitrophenyl-β-D-Glucoside), according to Eizavi and Tabatabai (1988). This enzyme activity has been deemed an enzymatic index which is highly sensitive to changes in soil properties, including organic matter supply (de Santiago et al., 2008) and is considered a basic soil quality indicator related to soil microbial activity involved in the C cycle (Stott et al., 2010).

The densities of T34 in the rhizospheric soil after each of both experiments were determined by dilution plating using the *Trichoderma* semi-selective medium of Chung and Hoitink (1990), as described by Borrero et al. (2012). To this end, soil (0.5–1 g) was suspended in 10 mL water agar (2 g L^{-1}). The suspension was shaken and a 10-fold dilution series (from 10^{-1} to 10^{-4}) was prepared with water agar (2 g L^{-1}). Suspensions were pipetted onto three plates per dilution. Colony forming units (CFU) were counted 4 days after plating and expressed as CFU g^{-1} of soil sampled. No CFU were detected in treatments without T34 inoculation.

2.4. Plant analysis

Chlorophyll was measured in triplicate in the last completely expanded leaf at the end of each experiment using a Minolta SPAD-502 (Minolta Camera Co, Ltd., Osaka, Japan). Accurate correlation between SPAD units and leaf chlorophyll content was previously checked ($Chlorophyll = 0.3 \ln(\text{SPAD}) - 0.48$; $R^2 = 0.85$; $P < 0.001$, $n = 18$).

The shoots and roots of each plant were separated and their dry weight was determined after drying in a forced-air oven at 65 °C until constant weight. Dried plant material was ground to pass through a 1-mm sieve prior to mineralisation. An aliquot of 0.25 g was then mineralised in porcelain crucibles in a furnace at 550 °C for 8 h. After that, 10 mL of 1 M HCl was used to dissolve ashes, heated at 100 °C for 15 min, and Fe, Cu, Mn, and Zn were determined by atomic absorption spectrometry in the digest. Certified plant material was also analysed to assess complete recovery of nutrients by this procedure.

2.5. Statistical analysis

An analysis of variance was performed to identify the effects of the three studied factors on SPAD readings, dry matter (DM) production (shoots and roots), nutrient content in the aerial parts and roots, and β-glucosidase activity. Only two factors (soil and glucose addition) were considered in the analysis of variance of T34 CFU in soil because non CFU were detected in non-inoculated soil. To this end, the General Linear Model procedure in Statgraphics Plus 5.1 (StatPoint, 2000) was used. Means were compared via Tukey's test, except when the interaction between factors was significant: in this case, the main effects could not be evaluated in a combined analysis and it was not possible to conduct a mean comparison between treatments of each factor. In this case, and only when the soil factor soil was involved in a significant interaction, a mean comparison for the effect of glucose addition, T34 inoculation, or both were performed separately for each soil.

3. Results

The soil significantly affected DM in aerial parts, SPAD meter readings in plants, the total amount of Cu and Zn in aerial parts, and the concentration and total amount of Cu and Mn in roots of cucumber plants (Table 2). Inoculation with T34 was significantly effective in increasing DM yield in shoots and their total Fe and Cu content (Table 2, Table 3); in roots, however, it resulted in a decreased concentration of Cu (Table 3).

The effect of T34 on total Mn accumulation in shoots and Zn concentration and accumulation in plant roots differed across soils, as revealed by the significant interaction between the two factors (Table 2). Thus, T34 significantly increased Mn accumulation in the aerial parts of plants grown in LB14 and resulted in decreased Zn concentration and accumulation in the roots of plants grown in LB11 when compared with non-inoculated plants (Table 4).

Glucose addition significantly increased DM yield and Zn accumulation in the aerial parts of cucumber plants (Table 3), but its effect on increasing total Fe accumulation and Zn concentration in aerial parts and the population of T34 in soil at the end of the experiment was different depending on the soil, as shown by the significant interaction observed between the two factors (Table 2). Glucose significantly increased total Fe accumulation and the population of T34 (measured as CFU after the experiment) in LB9, and increased Zn concentration in the aerial parts of plants grown in LB14 (Table 4).

Significant interactions between the three factors were observed for the other variables studied (Table 2). In the case of Fe concentration in aerial parts, it only increased significantly as a result of the simultaneous application of T34 and glucose in plants grown in the LB9 soil when compared to the control without T34 and glucose ($P < 0.1$): meanwhile, in the LB14 soil, T34 and glucose were equally effective in increasing Fe concentration in plant shoots, increasing it two-fold when compared to the control without treatments, (Table 5). In this latter soil, however, the combination of both treatments produced non-significantly higher Fe concentrations than those found in non-treated plants (Table 5). In LB14, the concentration of Mn was increased by the application of T34 alone, whereas the effect of the different combination of treatments on the concentration of Fe, Cu, and Mn in aerial parts was non-significant in LB11 (Table 5).

In LB9, inoculation with T34 significantly increased the concentration and total accumulation of Mn in roots (Table 5). β-glucosidase activity in LB14 was only increased significantly by glucose addition or its combination with T34 when compared to the control without treatments ($P < 0.1$), whilst in LB11, T34 inoculation without glucose resulted in decreased activity when compared to the other treatments (Table 5).

4. Discussion

In general terms, the best development of cucumber plants was achieved in the LB14 soil (Table 3). This was the soil with the lowest

Table 2
Significance levels (*P* values) in the analysis of variance of various properties of cucumber (*Cucumis sativus* L.) plants and microbial properties in soil.

Source of variation	Aerial part								Roots							Soil	
	DM	SPAD	Concentration of nutrients				Total amount in aerial part			DM	Concentration of nutrients				Total amount in roots				β-glucosidase	CFU T34[a]
			Fe	Cu	Mn	Zn	Fe	Cu	Mn	Zn		Fe	Cu	Mn	Zn	Fe	Cu	Mn	Zn		
Factors																					
A:Soil	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0184	0.0000	0.0000	0.0000	0.1015	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.5913
B:T34	0.0107	0.6575	0.1579	0.7092	0.0593	0.2012	0.0061	0.0230	0.0024	0.1145	0.7023	0.5468	0.0006	0.6094	0.0046	0.8577	0.1052	0.5864	0.2956	0.0321	
C:Glucose	0.0471	0.3004	0.1864	0.7452	0.8442	0.4234	0.0064	0.1206	0.2128	0.0194	0.8376	0.1505	0.2348	0.1508	0.4173	0.3563	0.2822	0.2466	0.7088	0.0861	0.0410
Interactions																					
AB	0.7231	0.1219	0.2249	0.5363	0.0064	0.2749	0.0871	0.5369	0.0096	0.6703	0.5092	0.0591	0.8338	0.2904	0.0216	0.0780	0.2944	0.1089	0.0371	0.3922	
AC	0.0758	0.6666	0.5739	0.9379	0.8605	0.0175	0.0259	0.0576	0.1262	0.1444	0.6896	0.7264	0.2728	0.9214	0.0762	0.8667	0.2441	0.5583	0.2616	0.2129	0.0046
BC	0.1967	0.2062	0.7540	0.7001	0.3409	0.1433	0.2261	0.2704	0.0867	0.9168	0.2815	0.0635	0.2993	0.0036	0.1884	0.0886	0.3310	0.0277	0.0859	0.0336	
ABC	0.4449	0.9548	0.0048	0.0108	0.0403	0.6155	0.1351	0.0949	0.0623	0.1949	0.0382	0.2033	0.6601	0.0382	0.5469	0.0354	0.2301	0.0410	0.1234	0.0039	

DM, dry matter; CFU, colony forming units.
P values lower than 0.05 indicate a significant effect of the source.
[a] CFU was 0 in soil without T34 inoculation. Thus, the analysis of variance was performed considering only two factors, soil and glucose addition.

content of CCE and ACCE, but not that with the highest content of available nutrients as estimated by DTPA extraction (Table 1), thus revealing the key role of carbonate content in contributing to reduced plant development and nutrition in calcareous soils. According to the observed concentrations of Zn in aerial parts (Table 3), this nutrient probably accounts for the highest restriction in micronutrient supply in the soils studied. The highest Zn accumulation in aerial parts and roots was observed in LB14 (3–4 times higher than in the other two soils; Tables 3 and 4), and this soil was the only one providing Zn concentrations in aerial parts close to a sufficiency level (Huett et al., 1997). This probably accounts for increased plant development in this soil, thus indicating, in agreement with Rashid and Ryan (2004), that Zn availability frequently restricts productivity in calcareous soils.

Inoculation with T34 was effective in increasing Fe accumulation in the aerial parts of cucumber plants. This increase was not associated with an increased accumulation or concentration of this nutrient in roots. This may indicate that enhanced Fe uptake by plants due to T34 was not only the consequence of increased mobilisation from sources in soil (e.g. poorly crystalline oxides as a source of Fe, Mikutta and Kretzschmar, 2008) but also of enhanced transport to the aerial parts previously described as an effect of siderophore-producing microorganisms (Carrillo-Castañeda et al., 2005; De Maria et al., 2011). The effect of T34 on increased Fe uptake by plants has previously been described by de Santiago et al. in lupins (2009) and wheat (2011), and has been ascribed at least in part to the effect of siderophores. The effect on cucumber is thus not surprising, since it is known that this plant can use siderophore bound Fe (Wang et al., 1993; Hördt et al., 2000).

Glucose addition was only effective in increasing Fe accumulation in the aerial parts of plants grown in LB9 (Table 4), which was not associated with increased β-glucosidase activity in the soil. This would appear to suggest that changes in microbial communities due to glucose addition rather than to increased microbial biomass in soil can contribute to enhanced Fe accumulation in plants. In this low organic C soil, where native microbial activity is low and oligotrophs are necessarily dominant, the addition of glucose seems to promote a shift to copiotroph communities (Langer and Rinklebe, 2011). Leita et al. (2011) observed increased populations of metal reducing microorganisms in glucose-amended soils. This effect of glucose addition to LB9 reveals the influence of soil microorganisms on Fe uptake by plants and supports the well-known effect of organic amendments on improving Fe uptake by plants grown in low organic C soils (Bar-Ness and Chen, 1991; Ye et al., 2008).

Inoculation with T34 affected Fe concentration in the aerial parts of plants, depending on the soil and glucose addition. In the LB9 soil, only the simultaneous application of T34 and glucose increased Fe concentration when compared to the control without T34 and glucose ($P < 0.1$). With this addition, Fe concentrations within the sufficiency range were achieved in plant shoots, in comparison to deficient levels observed in control plants (Huett et al., 1997). On the other hand, T34 or glucose addition in LB14 promoted a similar increment in Fe concentration when applied separately (Table 5). In LB11, neither treatment produced a significant effect on Fe concentration in aerial parts (Table 5). These results probably indicate that soil conditions affecting the availability of Fe (mainly extractable amount and pH-buffering capacity; de Santiago and Delgado, 2006), native microbial activity and successful development of T34 may affect Fe concentration in cucumber. LB9 is a poor soil with a very low organic content which accounts for the lowest β-glucosidase activity observed in the soil at the end of the experiment (Table 5), and a probably reduced availability of hydrocarbon sources for T34, restricting the effective development of this fungus. T34 inoculation with glucose increased

Table 3
Significant effects of main factors on different properties of cucumber (*Cucumis sativus* L.) plants.

Factor	Aerial part					Roots
	DM (g plant^{-1})	SPAD (Arbitrary units)	Total amount			Concentration
			Fe (μg plant^{-1})	Cu (μg plant^{-1})	Zn (μg plant^{-1})	Cu (mg kg^{-1})
Soil						
LB11	3.59 ± 0.11 b	30.3 ± 0.62 b		15 ± 0.6 b	28 ± 0.9 b	13 ± 0.3 c
LB14	4.02 ± 0.11 a	34.4 ± 0.62 a		16 ± 0.8 b	80 ± 2.8 a	15 ± 0.9 b
LB9	2.65 ± 0.11 c	33.1 ± 0.45 a		18 ± 0.5 a	21 ± 0.9 c	32 ± 1.4 a
T34						
+	3.54 ± 0.12 a		262 ± 28 a	17 ± 0.6 a		19 ± 1.2 b
−	3.31 ± 0.11 b		238 ± 32 b	15 ± 0.6 b		22 ± 1.5 a
Glucose						
+	3.51 ± 0.11 a				45 ± 3.8 a	
−	3.33 ± 0.12 b				42 ± 3.6 b	

DM, dry matter.
Only significant effects of factors have been considered when the factor was not involved in a significant interaction.
Mean ± standard error, $n = 40$ for soil, and 60 for T34 and glucose.
Means followed by different letters within a column are significantly different according to the Tukey test ($P < 0.05$) for each factor.

the development of T34, as shown by the increased CFU of T34 at the end of the experiment in the soil (Table 4), and resulted in an increased Fe concentration in plant shoots (Table 5). However, this increased development of T34 was not reflected in an increased β-glucosidase activity (Table 5).

In contrast to LB9, LB14 presented a much higher native β-glucosidase activity, which was increased by glucose addition. This increased microbial activity probably accounted for the increased Fe concentration observed in the aerial parts of plants when glucose was applied to the soil (Table 5). This observation is consistent with previous studies demonstrating the key contribution of soil microbial activity to the Fe nutrition of plants (Masalha et al., 2000; Rroço et al., 2003). In LB14, T34 also increased Fe concentration in the aerial parts of plants, but this was not associated with a significant increase of β-glucosidase activity (Table 5), suggesting that this microorganism has a positive effect on the Fe nutrition of cucumber in this soil. However, the combined application of T34 and glucose resulted in worse results than the application of either treatment separately. This probably reflects the effect of competition between native microorganisms in the soil and T34, which does not result in a significantly decreased CFU in soil after the experiment (Table 4). This competition could explain the decreased effectiveness of the combined application of T34 and glucose in improving Fe uptake by plants when compared with the application of T34 alone in LB14. On the other hand, this probable competition also restricts the effect of increased native microbial activity in increasing Fe concentration in plants due to the application of T34 in LB14. One indication of the potential competition between T34 and other microorganisms is the effect observed in the LB11 soil (Table 5), where β-glucosidase activity was decreased by T34 without glucose addition when compared with the control or treatments with glucose addition. This suggests that T34 can negatively affect other saprophytic microorganism populations in this soil through competition for C sources (Harman et al., 2004). Glucose or T34 did not affect Fe concentration in aerial parts of plants grown in LB11; this can be explained by the DTPA-extractable Fe content of this soil, which was the highest of the three soils studied (Table 1), thus showing that Fe availability was not so restricted in this soil. The clearer effect of glucose addition or T34 inoculation on Fe concentration in plants was observed in a soil with restricted Fe availability but without limitation in Zn supply to plants (LB14, Table 5). This may reveal that a low Zn availability in soil can contribute to restrict the effect of soil microbial activity or T34 on Fe nutrition of plants.

In general terms, an increased accumulation or concentration of Fe in the aerial parts of plants was not associated with an increased concentration or accumulation in roots. As stated above, this may reflect not only increased mobilisation from soil but also enhanced transport to the aerial parts. The positive relationship observed between the total amount or concentration of Fe in aerial parts of plants and β-glucosidase activity in the soil at the end of the experiment (Fig. 1) is not only the consequence of the effect of soil microbial activity on Fe nutrition but also of the soil factors contributing to high microbial activity (e.g. organic C content or pH), which are also involved in a good Fe supply to plants (de Santiago and Delgado, 2006).

Nutrients other than Fe were applied with fertigation, and their accumulation and concentration was also affected by treatments

Table 4
Double interactions of factors on different properties of cucumber (*Cucumis sativus* L.) plants and microbial properties of soil.

Soil	G	Aerial part		Soil	Soil	T34	Aerial part	Roots	
		Total amount	Concentration	T34 CFU g^{-1}			Total amount	Concentration	Total amount
		Fe (μg plant^{-1})	Zn (mg kg^{-1})				Mn (μg plant^{-1})	Zn (mg kg^{-1})	Zn (μg plant^{-1})
LB11	+	262 ± 53	8 ± 0.3	2030 ± 500	LB11	+	210 ± 14	18 ± 0.4 b	11 ± 0.6 b
	−	280 ± 68	8 ± 0.5	1370 ± 510		−	212 ± 16	21 ± 1.1 a	14 ± 0.7 a
LB14	+	389 ± 51	21 ± 0.4 a	1730 ± 250	LB14	+	134 ± 12 a	26 ± 1.3	25 ± 2.1
	−	350 ± 53	19 ± 0.5 b	2590 ± 1050		−	90 ± 12 b	27 ± 1.1	24 ± 1.6
LB9	+	131 ± 14 a	8 ± 0.3	3650 ± 1260 a	LB9	+	470 ± 18	21 ± 0.8	14 ± 1.2
	−	89 ± 7 b	8 ± 0.4	700 ± 300 b		−	443 ± 30	22 ± 1.5	14 ± 1.4

Mean ± standard error, $n = 20$.
G, glucose CFU, colony forming units (expressed per g of dry soil).
Data in table are included only if there is a significant interaction between soil and one of the other two factors (glucose amendment or inoculation with T34).
Means followed by different letters in a column were significantly different according to Tukey test at a probability level of 0.05 for each factor (T34 or G) within the same soil.

Table 5
Triple interaction of factors on different properties of cucumber (*Cucumis sativus* L.) plants and microbial properties of soil.

Soil	T34	Glucose	Aerial part			Roots				Soil
			Concentration			DM (g plant^{-1})	Concentration	Total amount		β-glucosidase[a] (μg PNP g^{-1} h^{-1})
			Fe (mg kg^{-1})	Cu (mg kg^{-1})	Mn (mg kg^{-1})		Mn (mg kg^{-1})	Fe (μg plant^{-1})	Mn (μg plant^{-1})	
LB11	+	+	91 ± 37.9	4.4 ± 0.4	62 ± 8.1	0.65 ± 0.04	64 ± 6.3	1560 ± 177	42 ± 4.7	114 ± 10 a
	+	−	61 ± 11.3	4.2 ± 0.2	58 ± 6.8	0.63 ± 0.07	72 ± 6.5	1692 ± 320	47 ± 8.5	47 ± 12 b
	−	+	64 ± 11.2	4.1 ± 0.2	60 ± 7.4	0.64 ± 0.05	74 ± 5.1	1783 ± 268	49 ± 7.1	82 ± 16 a
	−	−	105 ± 38.1	4.3 ± 0.3	69 ± 9.8	0.65 ± 0.03	75 ± 4.9	1794 ± 138	49 ± 4.5	98 ± 14 a
LB14	+	+	84 ± 10.6 ab	3.7 ± 0.3	28 ± 2.2 ab	0.94 ± 0.10	62 ± 16.6	2576 ± 528	52 ± 9.6	136 ± 9
	+	−	121 ± 25.7 a	4.4 ± 0.3	37 ± 4.5 a	1.04 ± 0.10	73 ± 11.0	3039 ± 429	82 ± 18.7	117 ± 8
	−	+	125 ± 29.6 a	4.1 ± 0.5	27 ± 5.0 ab	0.89 ± 0.08	60 ± 10.8	2644 ± 336	55 ± 11.8	136 ± 12
	−	−	58 ± 5.6 b	3.6 ± 0.5	21 ± 3.1 b	0.90 ± 0.08	61 ± 9.9	2540 ± 287	51 ± 6.7	106 ± 8
LB9	+	+	61 ± 11.4	7.0 ± 0.2	177 ± 10.4	0.60 ± 0.06	193 ± 18.5 b	1522 ± 149	123 ± 22.5 b	26 ± 4
	+	−	42 ± 8.4	6.5 ± 0.4	177 ± 10.8	0.71 ± 0.06	330 ± 45.3 a	2561 ± 450	240 ± 41.2 a	24 ± 3
	−	+	36 ± 2.9	6.6 ± 0.3	178 ± 13.3	0.71 ± 0.07	271 ± 27.3 ab	2690 ± 607	206 ± 36.1 ab	31 ± 4
	−	−	38 ± 3.0	7.2 ± 0.4	171 ± 11.8	0.54 ± 0.04	208 ± 28.6 b	1656 ± 315	114 ± 22.1 b	32 ± 5

Mean ± standard error, $n = 10$.
DM, dry matter.
Data in table are included only if there is a significant interaction of the three factors.
Means followed by different letters in a column were significantly different according to Tukey test at a probability level of 0.05 for the four treatments (combination of two factors) consider within the same soil.
[a] According to Eizavi and Tabatabai (1988), and measured as the amount of PNP (*p*–nitrophenol) formed from PGN (*p*–nitrophenyl-β-D-Glucoside).

(Table 3–5). The total accumulation of Cu in aerial parts was increased by T34 (Table 3), in agreement with previous findings for lupins in calcareous growing media (de Santiago et al., 2009). Glucose addition enhanced Zn accumulation in plant shoots (Table 3), providing an interesting indication for overcoming a significant nutritional disorder in calcareous soils. As stated for Fe, these increments were not associated with increased accumulation in roots; in fact, T34 decreased the concentration of Cu in roots. This could be explained by an enhanced transport from roots to aerial parts, probably due to the effect of the chelating compounds produced by T34. In the case of Zn, the increased accumulation in aerial parts was not associated with increased β-glucosidase activity in soil (only observed in LB14), suggesting that changes in microbial communities due to glucose addition rather than increased microbial activity in soil may contribute to enhanced Zn uptake by plants.

In LB9, T34 resulted in an increased concentration and total accumulation of Mn in roots when compared to the control without T34 and glucose addition, which was not reflected in changes in the aerial parts (Table 5). This was probably due to enhanced mobilisation from soil without a significant increase in the transport of this nutrient from roots to shoots. Enhanced development of T34 due to glucose addition is probably unnecessary to increase Mn mobilisation from soil and absorption by roots. The effect of T34 on plants grown in LB14, increasing Mn concentration in aerial parts but not associated with increased Mn accumulation in roots (Table 5), is of particular interest and can probably be ascribed at least in part to the production of chelating compounds (Altomare et al., 1999; Harman et al., 2004). The probable reason why this effect was only observed in LB14 is that this was the soil where the best development of the fungus was observed (Table 4). The only negative effect of T34 was observed in the concentration and total amount of Zn in roots of plants grown in LB11; however, this was not associated with any significant effect on the aerial parts of plants (Table 5). Negative effects of T34 on the accumulation of Zn in plants have previously been described by de Santiago et al. (2011) in wheat grown in Fe-rich calcareous growing media, and have been ascribed at least in part to conditions which decrease Zn availability due to antagonism with Fe (Heitholt et al., 2003; Assimakopoulou, 2006) or adsorption on Fe oxides (Montilla et al., 2003).

The effects of T34 and glucose on increasing DM in aerial parts could be partially ascribed to the improved nutrition of plants;

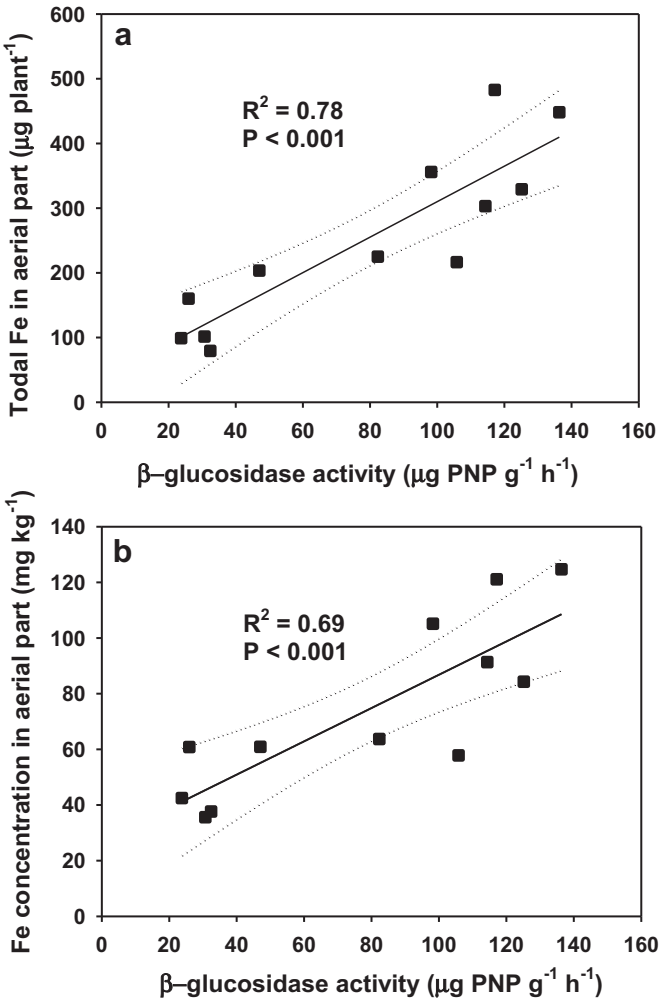

Fig. 1. Relationship between total Fe in the aerial parts of cucumber plants and β-glucosidase activity in soil at the end of the experiments (a), and between Fe concentration in the aerial parts of cucumber plants and β-glucosidase activity (b) in soil at the end of the experiments. Each data point corresponds to the mean of all the replications performed for each combination of treatments ($n = 10$). Dotted lines indicate the regression certainty interval at a probability level of 0.05.

other effects such as phytohormone production by rhizosphere microorganisms inducing a direct promotion of growth cannot be ruled out (Hoyos-Carvajal et al., 2009; Schuster and Schmoll, 2010).

5. Conclusions

T. asperellum T34 contributed to increased Fe and Cu accumulation in the aerial parts of cucumber plants, whereas its effect on Mn and Zn depended on the soil. Glucose addition was only effective in increasing Fe accumulation in the aerial parts of plants grown in the soil with the lowest organic C content. This increase can probably be ascribed to changes in microbial communities. T34 increased Fe concentration in shoots of plants grown in a soil with restricted Fe availability and relatively high microbial activity, estimated as β-glucosidase activity. In this soil, the increased microbial activity obtained with glucose addition also contributed to increased Fe concentration in aerial parts. In the soil with the lowest organic C content, it was necessary to apply T34 with a C source to obtain positive effects on Fe concentration in plant shoots. In the soil with the highest Fe availability index, non-significant results of T34 or glucose on Fe concentration in aerial parts were observed. These results are relevant with a view of improving Fe nutrition of plants and to achieve a sustainable control of Fe deficiency in calcareous soils.

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