Iron availability thresholds for the inoculation of cucumber with *Trichoderma asperellum* T34

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Abstract

Inoculation with biocontrol agents can affect iron (Fe) uptake by plants. The objective of this research was to study the necessity of defining a Fe threshold in growth media for the inoculation with the biocontrol agent *Trichoderma asperellum* T34. A completely randomized experiment with cucumber (*Cucumis sativus* L.) was performed involving two factors: Fe rate in the growth medium in the form of ferrihydrite (0, 8, 16, 32.5, and 75 mg kg^{-1} of citrate-ascorbate-extractable Fe (CA-Fe), and plant inoculation with T34. Dry matter (DM) of aerial parts of cucumber was decreased by T34. This was related to a decreased accumulation of Fe in plants, more in aerial parts than in roots. However, at the highest Fe rate (75 mg kg^{-1}), differences in DM yield, plant height, and the content and concentration of Fe in shoots between inoculated and noninoculated plants were not significant. The threshold of CA-Fe in the medium for DM yield of cucumber was 37 mg kg^{-1} without T34. With T34, this threshold was 65 mg kg^{-1}, which implies that, below this limit, additional Fe supply is required for inoculation with T34.

Key words: biocontrol / *Cucumis sativus* / fertilization / ferrihydrite / micronutrients

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1 Introduction

Iron (Fe) is an essential element for microorganisms and plants. Although it is relatively abundant in soils, Fe availability is constrained by the low solubility of Fe^{III} species and the slow dissolution of Fe minerals, particularly under nonacidic conditions (*Kraemer* et al., 2006; *Lemanceau* et al., 2009). An accurate definition of Fe sufficiency thresholds, below which the deficiency of this nutrient can be expected, is necessary for a sustainable management of Fe nutrition depending on plant sensitivity to Fe deficiency (*de Santiago* and *Delgado*, 2006). Thresholds have been defined on the basis of readily reducible Fe (*e.g.,* citrate-ascorbate-extractable Fe), mainly related to poorly crystalline oxides, which are usually dominant sources of this nutrient for plants in calcareous soils (*de Santiago* et al., 2008a, b).

Plants and microorganisms have developed similar mechanisms to mobilize Fe from the solid phase and take up Fe, based on the release of Fe chelators (siderophores) (*Sharma* and *Johri*, 2003; *Khan* et al., 2006; *Jin* et al., 2006), acidification (*Lemanceau* et al., 2009), and reduction processes (*Valencia-Cantero* et al., 2007; *Rakshit* et al., 2009; *Marschner* et al., 2004; *Sánchez-Alcalá* et al., 2011). Alteration of the Fe availability by these mechanisms is more evident in the rhizosphere, where microbial density and activity is greater due to root exudation (*Marschner* et al., 2004; *Sánchez-Alcalá* et al., 2011; *Violante* et al., 2005; *Robin* et al., 2008).

The manipulation of the rhizosphere by inoculating with microorganisms may lead to the control of soil-borne diseases (biocontrol) and to an improved plant nutrition (*Vassilev* et al., 2006; *Zuo* and *Zhang*, 2011; *de Santiago* et al., 2011). Interaction between the Fe-acquisition mechanisms of the different organisms in the rhizosphere may lead to intense competition for this nutrient (*Winkelmann*, 2007), which is known to be one of the mechanisms explaining the biological control of many soil-borne plant diseases (*Mercado-Blanco* and *Bakker*, 2007; *Chaiharn* et al., 2009), and also can result in an improved (*Lemanceau* et al., 2009; *Violante* et al., 2005; *Renshaw* et al., 2002) or decreased Fe uptake (*Marschner* et al., 2011) by plants.

The soil fungus *Trichoderma* spp. is an efficient and widely used biological control agent (*Howell*, 2003; *Schuster* and *Schmoll*, 2010; *Fontanelle* et al., 2011) which increases Fe uptake by plants (*Haas* et al., 2008; *Hoyos-Carvajal* et al., 2009; *de Santiago* et al., 2009; *Segarra* et al., 2010). However, potential benefits from the inoculation with the biocontrol agent *Trichoderma asperellum* T34 on Fe nutrition of plants seem to be related to soil conditions, in particular Fe availability (*de Santiago* et al., 2011). Thus, it seems necessary to clarify under which conditions the potential use of biocontrol agents can promote negative or positive effects on plant nutrition, in particular, how Fe uptake by plants can be affected depending on the availability of this nutrient in soil. The particular case of the contributory effect of T34 on Fe uptake by cucumber (*Cucumis sativus* L.) plants in a calcareous growth medium differing in the concentration of poorly crystalline Fe oxides concentration was studied in this work

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with the main objectives of: (1) clarifying the potential effect of T34 on the sufficiency threshold of Fe in the growth media for plants, and (2) if it is necessary to define a threshold of Fe for the inoculation with T34. Both objectives are focused on the sustainable management not only of the biocontrol agent T34, but also of the Fe nutrition of cucumber plants.

2 Material and methods

2.1 Experimental design

A completely randomized experiment with five replications was performed involving two factors: Fe rate in the growth medium in the form of ferrihydrite (0, 8, 16, 32.5, and 75 mg kg^{-1} of citrate-ascorbate-extractable Fe), and plant inoculation with T34 (inoculated and noninoculated with T34). Each replication corresponded to a pot with one plant. The experiment was performed twice at different times under the same growth conditions in a growth chamber.

The growth medium was prepared by carefully mixing siliceous sand (> 99% quartz) with calcareous sand (> 99.5% $CaCO_3$). Both sands were sieved, and particles above 0.15 mm diameter were selected in order to obtain good aeration and hydraulic conductivity in the medium. 380 g of a mixture of calcareous (75%) and siliceous (25%) sand was placed in each pot. Siliceous sand was previously washed with a solution containing 0.27 M Na citrate + 0.11 M $NaHCO_3$ + 2% Na dithionite to reduce the concentration of Fe oxides (*de Santiago* and *Delgado*, 2006). The aim of this treatment was to achieve a citrate-ascorbate-extractable Fe concentration of less than 3 mg kg^{-1}. The Fe concentration of the calcareous sand was negligible. Iron enrichment of the medium was achieved by introducing a fraction of the siliceous sand coated with ferrihydrite, after washing with diluted Na_2CO_3 (*de Santiago* et al., 2009).

For inoculation with T34, a commercial inoculum provided by Biocontrol Technologies S.L. (Barcelona, Spain) was used; this product contained 11.3% of dry conidia and 88.7% of inert material. Inoculation was performed following the method of *Segarra* et al. (2007) for cucumber by immersing plant roots in a suspension in water containing 10^3 conidia mL^{-1}. After transplanting, the medium was inoculated with 10^4 conidia mL^{-1} growth medium; this inoculation was carried out by applying 20 mL of a conidia suspension in water (1.375×10^5 conidia mL^{-1}) with a micropipette on the growth-medium surface in five points around the plants (*de Santiago* et al., 2009).

2.2 Plant material and growth conditions

Cucumber (*Cucumis sativus* L. cv. Serena) was used for the experiments. Although it can be considered an Fe-efficient plant, symptoms of Fe-deficiency chlorosis can be observed when it is grown in strongly calcareous growth medium (*Bacaicoa* and *García-Mina*, 2009). It is an important horticultural crop in which the use of T34 is recommended to control soil-borne diseases (*Segarra* et al., 2007). Seeds were germinated in peat, and 15 d after germination individual plants were transplanted to pots (polystyrene cylinder: 350 mL, 5.5 cm diameter, 15 cm height). The experiments were conducted in a growth chamber over a period of 27 d under the same controlled environmental conditions: photoperiod of 14 h, a 25°C/23°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. The composition of the solution was (all concentrations in mM): $MgSO_4$ (2), $Ca(NO_3)_2$ (5), KNO_3 (5), KH_2PO_4 (1), KCl (0.05), H_3BO_3 (0.009), $Mn\,Cl_2$ (0.0023), $CuSO_4$ (0.0005), $ZnSO_4$ (0.002), and H_2MoO_4 (0.0005). 300 mL of the nutrient solution were applied per pot during the growth period. The pH of the nutrient solution ranged between 5.5 and 6.

2.3 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 concentration was previously checked (Chlorophyll (mg [kg fresh weight]$^{-1}$) = 0.3 ln (SPAD) – 0.48; R = 0,85; $P < 0.1\%$, 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 (approximately 48 h). Dried plant material was ground to pass a 1-mm sieve prior to dry-combustion mineralization. Aliquots of 0.25 g were then mineralized in porcelain crucibles in a furnace at 500°C for 8 h. After that, 10 mL of 1 M HCl were used to dissolve ashes, heated at 100°C for 15 min, and Fe, Cu, Mn, and Zn were determined using atomic-absorption spectrometry (Unicam Solaar M Ficher Scientific, Madrid, Spain) in the digest. Certified plant material was also analyzed to assess complete recovery of nutrients by this procedure.

2.4 Growing-media analysis

β-glucosidase activity, as a general measure of microbial activity involved in the carbon cycle in the growth medium (*de Santiago* et al., 2008b), was determined at the end of both experiments in the rhizospheric growth medium as the amount of PNP (*p*-nitrophenol) formed from PGN (*p*-nitrophenyl-β-D-glucoside) (*Eizavi* and *Tabatabai*, 1988).

At the end of the experiments, the densities of T34 in the rhizospheric medium were determined by dilution plating using the *Trichoderma* semiselective medium of *Chung* and *Hoitink* (1990), as described by *Borrero* et al. (2012). The growth medium (five replicates) was suspended in 90 mL of a pyrophosphate solution in water (1 g L^{-1}). The suspension was shaken and a tenfold dilution series (from 10^{-1} to 10^{-4}) was prepared with water agar (1 g L^{-1}). Suspensions were pipetted onto three plates per dilution. Colony-forming units (CFU) were counted 7 d after plating and expressed as CFU per mL of growth medium sampled. No CFU were detected in treatments without T34 inoculation.

2.5 Statistical analysis

An analysis of variance was performed to identify the effects of the two factors on SPAD readings, dry-matter (DM) production (shoots and roots), nutrient concentrations in the aerial part and roots, and β-glucosidase activity in the growth medium. Only Fe rate was considered in the analysis of variance of T34 CFU in the growth medium because no CFU were detected in noninoculated growth medium. In the analysis of variance, each replication of the experiment was considered a separate block in order to exclude the variation associated with the repetition of the experiment. The General Linear Model procedure in Statgraphics Plus 5.1 was used (*Statpoint*, 2000). Linear and quadratic responses (L and Q) to Fe rate were considered in the model. In a preliminary analysis, all terms (factors and interactions) were included. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from the final models (*Borrero* et al., 2012). Means were compared with 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 the mean comparison between treatments of each factor could not be performed. Regressions to fit total DM yield and total Fe accumulation in plants to Fe rate were performed with the same software. Data were fitted to an exponential rise to maximum equation with three parameters, $y = a + b \times (1 - \exp[-c \times x])$, and the maximum adjustable DM was considered $a + b$; sufficiency Fe-threshold values were considered those to achieve 90% of the maximum DM (*Black,* 1993).

3 Results

Overall, β-glucosidase activity increased with increasing Fe rates, but Fe in the growth medium did not affect T34 CFU at the end of the experiments (Tab. 1).

The development of cucumber plants, measured as shoot-DM yield, shoot height, and root-DM yield increased at higher Fe rates in the form of ferrihydrite in the growth medium. In addition, the chlorophyll concentration of leaves measured using SPAD was positively affected by Fe supply (Tab. 2). In general terms, the total content of studied nutrients in the aerial part of plants was higher at increased Fe rates (Tab. 3). However, Fe concentration in the aerial parts was not increased by Fe addition and the concentration of Cu and Mn decreased at increased Fe rates (Tab. 4). The decreasing response of Zn concentration in shoots to increasing Fe rates was different in T34-inoculated and noninoculated media, as revealed by the significant interaction observed between both factors (Tab. 4). In roots, increased Fe rates accounted for increased contents of Fe, Mn, and Zn, increased Fe concentrations, and decreased concentrations of Mn (Tabs. 5 and 6); for total Cu content and concentration the response to increasing Fe rates was significantly affected by T34, as revealed by the significant interaction between both factors.

Table 1: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the β-glucosidase activity and T34-colony-forming units (CFU) in the growth medium at the end of the experiments.

	β-glucosidase activity[a]		**CFU[c]**	
	– T34	**T34**	**+ T34**	
Fe rate / mg kg^{-1}	/ μg PNP g^{-1} h^{-1}			
0	10.88 ± 3.29[b]	13.72 ± 1.99	15795 ± 3322	
8	8.21 ± 1.79	9.88 ± 2.93	10130 ± 3526	
16	15.45 ± 3.59	13.60 ± 4.73	11126 ± 3547	
32.5	24.66 ± 8.55	19.62 ± 6.95	11605 ± 2669	
75	24.06 ± 4.33	16.57 ± 3.38	15846 ± 2574	
T34 inoculation	16.63 ± 2.27	14.82 ± 1.97	12900 ± 1386	
ANOVA results[d]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	11.05	0.13	0.7	41.04
Fe rate (Q)	NI		2.21	14.96
T34	1.93	16.82		
Fe rate (L) × T34	NI			
Fe rate (Q) × T34	NI			

[a] Measured as the amount of PNP (*p*–nitrophenol) formed from PGN (*p*–nitrophenyl-β-D-glucoside)
[b] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[c] CFU, colony-forming units per g of growing medium; only Fe rate was considered in the analysis of variance of T34 CFU in the growth medium because no CFU were detected in noninoculated growth medium.
[d] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For the two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

Table 2: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the DM yield and chlorophyll meter readings (SPAD) of cucumber grown on a calcareous growth medium.

	Shoots				**Roots**		**SPAD**	
	DM		**height**		**DM**			
	– T34	T34	– T34	T34	– T34	T34	– T34	T34
Fe rate / mg kg^{-1}	/ g plant^{-1}		/ cm		/ g plant^{-1}		/ arbitrary units	
0	0.94 ± 0.04[a]	1.09 ± 0.10	14.0 ± 1.43	16.4 ± 2.60	0.34 ± 0.03	0.37 ± 0.04	3.37 ± 0.92	4.28 ± 0.73
8	1.32 ± 0.10	1.05 ± 0.08	21.2 ± 2.15	15.2 ± 2.25	0.47 ± 0.04	0.46 ± 0.07	10.99 ± 1.04	11.89 ± 1.47
16	1.56 ± 0.13	1.18 ± 0.10	23.9 ± 2.98	18.3 ± 2.66	0.64 ± 0.10	0.47 ± 0.04	17.33 ± 1.11	14.92 ± 2.27
32.5	1.79 ± 0.10	1.48 ± 0.11	26.7 ± 2.57	23.3 ± 2.79	0.74 ± 0.10	0.65 ± 0.07	23.67 ± 1.33	20.60 ± 1.47
75	2.13 ± 0.15	1.99 ± 0.11	27.1 ± 2.54	24.3 ± 2.14	0.71 ± 0.08	0.76 ± 0.11	29.11 ± 0.83	28.23 ± 0.75
T34 inoculation	1.54 ± 0.08a	1.37 ± 0.07b	22.5 ± 1.23a	19.7 ± 1.20b	0.58 ± 0.04	0.55 ± 0.04	16.74 ± 1.42	16.34 ± 1.34
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	121.28	< 0.01	24.15	< 0.01	43.04	< 0.01	400	< 0.01
Fe rate (Q)	NI		8.25	0.51	12.43	0.07	57.35	< 0.01
T34	7.36	0.8	4.65	03.37	0.9	34.59	1.29	25.91
Fe rate (L) × T34	NI		NI		NI		NI	
Fe rate (Q) × T34	NI		NI		NI		NI	

[a] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant were removed from final models.

Table 3: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the total content of micronutrients in the shoots of cucumber grown on calcareous growth medium.

	Fe		**Cu**		**Mn**		**Zn**	
	– T34	**T34**	**– T34**	**T34**	**– T34**	**T34**	**– T34**	**T34**
Fe rate / mg kg^{-1}	/ μg plant^{-1}		/ μg plant^{-1}		/ μg plant^{-1}		/ μg plant^{-1}	
0	27.32 ± 3.45[a]	25.77 ± 4.24	4.05 ± 0.32	3.45 ± 0.25	27.42 ± 1.75	27.26 ± 3.26	11.05 ± 0.49	11.40 ± 1.18
8	50.34 ± 10.39	27.44 ± 5.20	5.89 ± 0.46	4.14 ± 0.49	31.92 ± 1.95	27.87 ± 3.22	14.25 ± 0.93	10.90 ± 0.94
16	47.64 ± 8.05	32.59 ± 6.51	6.05 ± 0.52	4.48 ± 0.55	34.23 ± 1.50	34.48 ± 3.05	14.74 ± 0.83	12.61 ± 1.00
32.5	49.44 ± 4.84	31.94 ± 4.70	6.42 ± 0.40	5.34 ± 0.52	36.32 ± 2.97	33.94 ± 3.29	15.17 ± 0.90	14.29 ± 1.20
75	61.88 ± 6.87	60.96 ± 6.55	7.05 ± 0.57	6.80 ± 0.51	40.69 ± 4.79	36.69 ± 2.31	16.67 ± 0.95	16.86 ± 0.94
T34 inoculation	46.96 ± 3.50a	36.20 ± 3.06b	5.88 ± 0.25a	4.89 ± 0.27b	34.07 ± 1.40	32.19 ± 1.41	14.35 ± 0.45	13.31 ± 0.56
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	57.58	< 0.01	44.21	< 0.01	23.29	< 0.01	39.37	< 0.01
Fe rate (Q)	NI		4.05	4.71	NI		NI	
T34	18.89	< 0.01	12.69	0.06	1.8	18.3	3.59	6.13
Fe rate (L) × T34	NI		NI		NI		NI	
Fe rate (Q) × T34	NI		NI		NI		NI	

[a] Means ± standard errors, $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

Table 4: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the concentration of micronutrients in the shoots of cucumber grown on calcareous growth medium.

	Fe		Cu		Mn		Zn	
	– T34	T34	– T34	T34	– T34	T34	– T34	T34
Fe rate / mg kg^{-1}	/ mg kg^{-1}		/ mg kg^{-1}		/ mg kg^{-1}		/ mg kg^{-1}	
0	28.72 ± 3.13[a]	24.17 ± 3.36	4.30 ± 0.29	3.32 ± 0.34	29.63 ± 2.41	25.32 ± 2.05	11.83 ± 0.50	10.41 ± 0.41
8	40.13 ± 9.60	26.14 ± 4.09	4.53 ± 0.27	4.10 ± 0.49	24.99 ± 1.74	26.29 ± 1.39	10.94 ± 0.45	10.49 ± 0.44
16	31.48 ± 4.98	26.30 ± 4.12	3.91 ± 0.18	3.74 ± 0.29	22.99 ± 1.88	29.52 ± 1.90	9.75 ± 0.52	10.76 ± 0.62
32.5	28.42 ± 3.40	21.04 ± 1.87	3.61 ± 0.16	3.59 ± 0.20	20.27 ± 1.25	23.31 ± 1.67	8.50 ± 0.27	9.68 ± 0.42
75	31.32 ± 5.27	31.86 ± 4.12	3.38 ± 0.24	3.47 ± 0.24	18.56 ± 0.99	18.91 ± 1.47	7.95 ± 0.34	8.64 ± 0.51
T34 inoculation	32.12 ± 2.61a	25.92 ± 1.62b	3.95 ± 0.12	3.64 ± 0.14	23.36 ± 0.94	24.52 ± 0.90	9.82 ± 0.28	9.96 ± 0.24
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	1.70	19.51	7.09	0.92	48.8	< 0.01	63.72	< 0.01
Fe rate (Q)	NI		NI		NI		4.82	03.09
T34	8.75	0.4	3.02	8.55	2.01	15.96	0.6	44.13
Fe rate (L) × T34	NI		NI		NI		8.14	0.54
Fe rate (Q) × T34	NI		NI		NI		4.57	3.54

[a] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

Table 5: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the total content of micronutrients in the roots of cucumber grown on calcareous growing medium.

	Fe		Cu		Mn		Zn	
	– T34	T34	– T34	T34	– T34	T34	– T34	T34
Fe rate / mg kg^{-1}	/ μg plant^{-1}		/ μg plant^{-1}		/ μg plant^{-1}		/ μg plant^{-1}	
0	53.51 ± 4.48[a]	61.35 ± 7.87	1.99 ± 0.18	2.03 ± 0.27	5.05 ± 0.48	4.74 ± 0.51	2.44 ± 0.19	2.92 ± 0.30
8	88.62 ± 10.94	85.97 ± 12.16	2.46 ± 0.24	2.69 ± 0.39	5.60 ± 0.47	5.74 ± 0.75	3.96 ± 0.42	4.24 ± 0.54
16	117.04 ± 12.62	82.75 ± 6.72	3.03 ± 0.39	2.64 ± 0.20	7.70 ± 1.21	5.56 ± 0.44	5.17 ± 0.82	4.50 ± 0.37
32.5	187.14 ± 26.40	129.52 ± 15.94	3.69 ± 0.43	3.17 ± 0.29	8.21 ± 1.00	7.13 ± 0.65	5.37 ± 0.54	5.21 ± 0.42
75	200.02 ± 15.81	198.11 ± 57.69	3.57 ± 0.48	4.75 ± 0.38	7.33 ± 0.79	7.90 ± 0.98	5.92 ± 0.45	6.43 ± 0.45
T34 inoculation	128.32 ± 10.57	120.10 ± 7.07	2.93 ± 0.18	3.09 ± 0.19	6.73 ± 0.39	6.27 ± 0.35	4.54 ± 0.28	4.71 ± 0.25
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	113	< 0.01	55.11	< 0.01	24.77	< 0.01	61.88	< 0.01
Fe rate (Q)	20.29	< 0.01	3.58	0.06.2	12	0.08	13.05	0.05
T34	2.98	8.70	0.41	52.26	2.24	13.84	0.12	72.6
Fe rate (L) * T34	NI		4.77	3.17	NI		NI	
Fe rate (Q) * T34	NI		4.23	4.26	NI		NI	

[a] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analysed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

Table 6: Effect of different Fe rates applied as ferrihydrite to the growth medium and T34 inoculation on the concentration of micronutrients in the roots of cucumber grown on calcareous growth medium.

	Fe		Cu		Mn		Zn	
	– T34	T34	– T34	T34	– T34	T34	– T34	T34
Fe rate / mg kg^{-1}	/ mg kg^{-1}		/ mg kg^{-1}		/ mg kg^{-1}		/ mg kg^{-1}	
0	159.86 ± 9.56[a]	168.75 ± 13.68	6.07 ± 0.57	5.85 ± 0.77	15.20 ± 1.28	13.33 ± 1.07	7.39 ± 0.52	8.13 ± 0.50
8	184.43 ± 13.21	183.95 ± 8.57	5.21 ± 0.29	5.89 ± 0.61	12.14 ± 1.09	12.82 ± 0.90	8.78 ± 1.25	9.47 ± 0.91
16	192.33 ± 10.86	177.42 ± 9.79	4.97 ± 0.34	5.66 ± 0.30	12.04 ± 0.86	12.02 ± 0.92	8.40 ± 0.72	9.68 ± 0.63
32.5	261.71 ± 18.35	197.65 ± 8.46	5.20 ± 0.36	4.97 ± 0.37	11.53 ± 0.92	11.28 ± 0.87	7.65 ± 0.45	8.36 ± 0.72
75	291.35 ± 14.84	295.63 ± 12.26	4.53 ± 0.34	7.32 ± 1.03	10.61 ± 0.81	10.78 ± 0.86	8.81 ± 0.72	10.57 ± 1.75
T34 inoculation	217.56 ± 9.38	204.53 ± 8.23	5.21 ± 0.19b	5.95 ± 0.31a	12.33 ± 0.49	12.00 ± 0.42	8.22 ± 0.35	9.25 ± 0.46
ANOVA results[b]	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**	**F ratio**	***P* value / %**
Fe rate (L)	158.18	< 0.01	0.25	61.56	15.79	0.01	2.99	08.72
Fe rate (Q)	NI		4.92	02.91	4.55	03.56	NI	68.13
T34	3.16	7.88	5.58	02.04	0.22	63.78	3.96	5.36
Fe rate (L) × T34	NI		8.35	0.49	NI		NI	
Fe rate (Q) × T34	NI		NI		NI		NI	

[a] Means ± standard errors; $n = 10$ (pooled data from the two experiments since the interaction "experiment × factor" was not significant)
[b] F ratios and significance levels of the ANOVA factors: T34 treatment, Fe ratio, and their significant interactions, $n = 10$. Fe ratio was analyzed using orthogonal polynomial contrasts. For every two sets of data, within a line, numbers followed by different letters are significantly different ($P < 5\%$, Tukey's test).
L = linear response; Q = quadratic response.
NI = not significant and not included in the final model. In preliminary analyses, all terms (factors and interactions) were included in the model. If Fe rate (Q) or interactions were found to be nonsignificant they were removed from final models.

The effect of T34 on β-glucosidase activity was not significant (Tab. 1). Inoculation with T34 decreased the development of the aerial part of cucumber plants (DM yield and height), but did not affect root DM and SPAD meter readings (Tab. 2). In addition, T34 decreased the total content of Fe and Cu in the aerial parts, whereas the effect on Mn and Zn contents was not significant (Tab. 3). Inoculation with T34 decreased Fe concentration in the aerial parts, while its effect on Zn concentrations varied depending on the Fe rate, as revealed by the significant interaction between both factors (Tab. 4). Inoculation with T34 did not affect the total content and concentration of nutrients in roots, with the exception of Cu; its effect on total Cu content and concentration in roots was different depending on the Fe rate, as evidenced by the significant interaction between both factors (Tabs. 5 and 6).

The maximum DM yield estimated from the regression fitting total DM production and Fe rate in the media without T34 inoculation (2.86 g plant^{-1}) was almost equal to that obtained with the highest Fe rate (mean 2.84 g plant^{-1}, Tab. 2). Sufficiency CA-Fe threshold for noninoculated plants was 37 mg kg^{-1}; for inoculated plants, it was necessary 65 mg kg^{-1} of CA-Fe in the growth media to achieve the same total dry matter (Fig. 1). Except for 0 and 75 mg CA-Fe kg^{-1}, the total DM yield and the total Fe uptake by plants was always lower with T34 inoculation than without inoculation (Fig. 1).

4 Discussion

The increased development and chlorophyll concentration of cucumber plants at increased Fe rates (Tab. 2; Fig. 1) demonstrates that this nutrient was a limiting factor for plant development and that ferrihydrite was an efficient Fe source for plants grown on a calcareous medium, in agreement with previous findings (*de Santiago* and *Delgado*, 2007). According to the DM-response curve to Fe rate in the growth medium (Fig. 1), the threshold below which additional Fe supply is necessary was 37 mg CA-Fe kg^{-1}. This reveals a greater Fe-acquisition efficiency than of Fe-chlorosis-sensitive plants grown in calcareous soils which can suffer this deficiency below 140 mg CA-Fe kg^{-1} (de *Santiago* and *Delgado*, 2006; *de Santiago* et al., 2008a).

As expected, increased Fe rates also resulted in increased total Fe content in the aerial parts and roots of plants (Tabs. 3 and 5), thus revealing increased Fe availability in the medium. However, the effect of Fe addition on Fe concentration in plant shoots was not significant (Tab. 4), in agreement with previous findings demonstrating that Fe concentration in aerial parts or leaves is not always related to Fe-deficiency symptoms (*de Santiago* and *Delgado*, 2006). In contrast to what was observed in aerial parts, Fe concentration in roots was increased at increased ferrihydrite concentration in the growth medium (Tab. 6). This can be explained in terms of an increased Fe mobilization from the growth medium and reveals that besides mobilization and accumulation in roots, Fe transport to and within aerial parts is a limiting factor.

The improved development of cucumber plants contributes to explain the increased total contents of other nutrients in aerial parts and roots (Tabs. 3 and 5). However, the concentrations of Cu, Mn, and Zn in shoots and roots decreased at increased Fe rates (Tabs. 4 and 6). This can be ascribed at

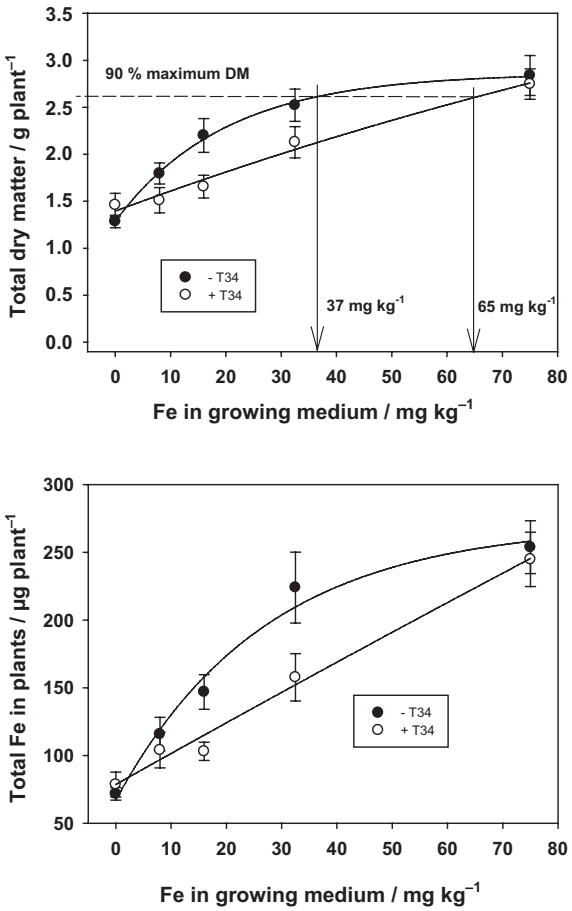

Figure 1: Total dry-matter (DM) yield and total iron accumulation in plants as a function of citrate-ascorbate-extractable Fe concentration in the growth medium. Error bars indicate twice the standard error (n = 10). Dry matter and Fe accumulation in plants was fitted to Fe concentration in the media using an exponential rise to maximum equation with three parameters, $y = a + b\ (1 - \exp[-c\ x])$, and the maximum adjustable maximum DM was considered $a + b$; R^2 was always > 0.99 and $P < 5\%$.

least in part to antagonism with Fe (*Heitholt* et al., 2003; *Assimakopoulou*, 2006) or, in the case of Zn, to adsorption on Fe oxides (*Montilla* et al., 2003).

Although the initial microbial activity in the growth medium was expected to be minimal due to the absence of hydrocarbon sources and to the heating of siliceous sand to adsorb ferrihydrite, some β-glucosidase activity was observed at the end of the experiment even in noninoculated pots (Tab. 1) indicating the development of microorganisms in the rhizosphere of plants. Microbial activity, measured as β-glucosidase activity, increased at increased Fe rates (Tab. 1). This can be related to an increased microbial activity with improved plant development because β-glucosidase activity was correlated with DM of shoots (r = 0.38, $P < 0.1\%$). This is in agreement with previous findings by *Valé* et al. (2005) with other herbaceous plants. Beside this, the positive effect of Fe on microbial activity can be also explained in terms of the reduced Fe availability which may be a limiting factor for microbial-biomass development. The number of CFU of T34 was not affected by Fe rate (Tab. 1), which contrasts with the effect on general microbial activity measured as β-glucosidase activity. Inoculation with T34 did not affect this enzymatic activity (Tab. 1) in agreement with *de Santiago* et al. (2013) indicating that β-glucosidase activity was affected by other rhizospheric microorganisms. The number of CFU of T34 was not correlated with DM of plants, thus revealing that T34 development was not affected by plant development contrasting with general microbial activity estimated as β-glucosidase activity. This contributes to explain the lack of effect of Fe rate on T34 CFUs. Also, this nonsignificant effect of Fe rate on T34 CFUs may indicate that T34 is more effective in the acquisition of Fe than other rhizospheric microorganisms.

Overall, inoculation with T34 reduced the development of the aerial parts of plants (DM yield and shoot height, Tab. 2). This can be related to a decreased Fe uptake by plants since inoculation with T34 reduced Fe concentration and the total content of Fe in shoots (Tabs. 3 and 4). However, differences in DM yield, height, and Fe content and concentration in shoots between inoculated and noninoculated plants were not significant when control without Fe or the highest Fe rate were only taken into account (Tab. 2, Fig. 1). At 0 Fe rate the extreme Fe deficiency accounted for a very serious limitation of plant development and masks other potential depressing factors. At other Fe rates, a negative effect of T34 on plant development can be ascribed to stress promoted by the fungus or other competition different from Fe. However, the absence of negative effect at the highest Fe rate in the medium may reveal a competition for Fe between T34 and plants in Fe-poor medium and the greater efficiency of T34 in the acquisition of this nutrient. The decreased total DM yield in T34 inoculated plants at Fe rates between 8 and 32.5 mg kg^{-1} was related to a decreased total Fe uptake by plants (Fig. 1) which contributes to support the hypothesis of a competition by Fe between plants and T34. The Fe-acquisition strategy of this fungus, mainly based on the release of siderophores (*Haas* et al., 2008), must be more efficient than the strategy of cucumber plants mainly based on the excretion of low-molecular-weight organic acids (*Kamilova* et al., 2006). Overall, it is accepted that microbial siderophores, such as those released by T34, show a much higher affinity towards Fe than chelators derived from nongraminaceous plants (*Marschner* et al., 2011).

The results contrast with previous studies reporting positive effects of T34 on Fe uptake by plants susceptible to Fe deficiency and grown in media with citrate-ascorbate-extractable Fe concentrations in the form of ferrihydrite above 150 mg kg^{-1} (*de Santiago* et al., 2009). These positive results were ascribed to the production of siderophores by T34. Fungal siderophores can be used as a source of Fe by cucumber plants at least in part through an increased reduction rate in roots (*Wang* et al., 1993; *Hördt* et al., 2000). However, the production of fungal siderophores can be increased under conditions of restricted Fe availability to take up Fe more efficiently (*Winkelmann*, 2007). A higher production of fungal siderophores in Fe-poor media, such as those with less than 75 mg kg^{-1} of CA-Fe for cucumber, in addition to their higher affinity towards Fe, could result in a decreased Fe uptake by plants with less effective acquisition mechanisms. This may also explain why T34 decreased Fe uptake by cucumber in a

growth medium with less than 75 mg Fe kg^{-1}, but not by lupins in a medium with 150 mg Fe kg^{-1} in the form of ferrihydrite (*de Santiago* et al., 2009).

The effect of T34 on plant development cannot be ascribed to other nutrients. Nutrient concentrations were high enough to cover plant and microbial biomass needs. Furthermore, T34 tended to decrease the antagonistic effect of Fe supply on Zn and Cu accumulation in plants: the decreasing response of Zn concentration in shoots to increasing Fe rates was more marked in noninoculated plants (Tab. 3), and T34 enhanced Cu concentration in roots (Tab. 5). Thus, it seems that T34 contributed to improve Cu and Zn nutrition in cucumber, which may be ascribed to the positive effect of siderophores produced by T34 on the uptake of other nutrients by plants (*Altomare* et al., 1999).

As stated above, sufficiency Fe-threshold for noninoculated cucumber below which Fe fertilizer should be applied was 37 mg kg^{-1} of CA-Fe. To achieve the same total DM production by inoculated cucumber plants, a minimum CA-Fe in the growth medium of 65 mg kg^{-1} is required (Fig. 1). Below this value, Fe fertilization must be considered in order to overcome a potential decrease of plant growth due to competition with T34. However, T34 increases the uptake of Fe by wheat (Strategy II) in a growth medium with an Fe concentration as low as 15 mg kg^{-1} of citrate-ascorbate-extractable Fe (*de Santiago* et al., 2011). Siderophores exuded by wheat have a much higher affinity towards Fe than the chelates derived from plants such as cucumber (*Kraemer* et al., 2006). Thus, the potential risk of competition between T34 and plants for Fe in growth media and hence the Fe threshold in the media for the inoculation with this microorganism seems to be related not only to the Fe concentration in the media, but also to the Fe-acquisition strategy of plants which determines, among other factors, plant susceptibility to Fe-deficiency chlorosis.

5 Conclusions

The results reveal the need to consider potential interactions between plants and microorganisms used in rhizosphere manipulation. Nutrient thresholds in growth media or soil above which inoculation does not promote a decreased uptake of the nutrient must be known, and fertilizer applied if competition between plants and microorganisms can be expected. Thresholds are only valid for a particular situation and they have to be newly adjusted for every plant–microorganism–substrate combination. It can be inferred that rhizosphere manipulation, including biocontrol strategies, must be integrated with nutrient-management strategies.

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