Redefining the dose of the entomopathogenic fungus *Metarhizium brunneum* (Ascomycota, Hypocreales) to increase Fe bioavailability and promote plant growth in calcareous and sandy soils

Silvia RAYA–DÍAZ[1], Enrique QUESADA–MORAGA[1], Vidal BARRÓN[2], María Carmen DEL CAMPILLO[2], Antonio Rafael SÁNCHEZ–RODRÍGUEZ[1,2*]

[1]Departamento de Ciencias y Recursos Agrícolas y Forestales, Universidad de Córdoba, ETSIAM, 14071 Córdoba, Spain

[2]Departamento de Agronomía, Universidad de Córdoba, ETSIAM, 14071 Córdoba, Spain

***Corresponding Author**: e–mail: l02saroa@uco.es (Antonio Rafael Sánchez–Rodríguez)

Abstract

Background and aims Entomopathogenic mitosporic ascomycetes *Beauveria, Metarhizium* and *Isaria* sp. are commonly used for pest control but can also serve other, lesser known functions such as increasing nutrient bioavailability or promote plant growth. The objective of this work was to identify the doses of entomopathogenic fungi (EF) to be applied to soil in order to modify iron (Fe) uptake by plants and promote their growth.

Methods We used an *in vitro* assay to assess the ability of *Beauveria bassiana, Metarhizium brunneum* and *Isaria farinosa* to mobilize Fe from nine Fe oxides differing in composition, particle size and crystallinity, including ferrihydrite, hematite, goethite and magnetite. We also conducted an *in vivo* assay by applying five different doses (viz., 0, 5×10^2, 5×10^4, 5×10^6 and 5×10^8 conidia ml^{-1}) of a conidial suspension of *M. brunneum* to the surface of a calcareous soil, which induced Fe chlorosis and a non–calcareous soil which did not induce chlorosis to explore the ability of the fungus on improving Fe nutrition and plant growth of sorghum and sunflower plants.

Results In the *in vitro* assay, all three EF increased Fe availability differently depending on particle size and crystallinity, and *I. farinosa* and *B. bassiana* increased the pH of the culture medium, whereas *M. brunneum* did not produce a great effect. In the *in vivo* assay, the highest dose (5×10^8 conidia ml^{-1}) of *M. brunneum* alleviated Fe chlorosis symptoms of sorghum plants grown in the calcareous soil, and the two highest doses (5×10^6 and 5×10^8 conidia ml^{-1}) increased plant height and inflorescence production of sunflower grown in both soils.

Conclusions The observed benefits of EF on plant growth and nutrition provide support for more sustainable and cost–effective use of these biocontrol agents.

Keywords *Isaria farinosa, Beauveria bassiana*, Plant growth promoter, Calcareous soil, Non–calcareous soil, Sandy soil, Iron nutrition, Iron chlorosis.

Introduction

Entomopathogenic fungi (EF) such as *Beauveria*, *Metarhizium*, *Lecanicillium* and *Isaria* (Hypocreales, Ascomycota) are commonly found in agricultural and uncultivated soils (Quesada–Moraga et al. 2007). Apart from being natural enemies of insect pests frequently occurring in agricultural ecosystems in temperate regions (Vega et al. 2012), entomopathogenic hypocreales have been used to develop commercial mycoinsecticides for innundative use in integrated pest management programs because they fulfil the principles of sustainable agriculture and hence those of the Common Agricultural Policy of the European Union (EU), embodied in Directive 91/414/EEC. However, recent studies have identified new ecological roles of entomopathogenic ascomycetes as rhizosphere colonizers (Sasan and Bidochka 2012), plant endophytes (Vega et al. 2009), systemic resistance inducers (Bayat et al. 2009), plant growth promoters (Liao et al. 2014, Sánchez–Rodríguez et al. 2017), sources of active secondary metabolites used by the pharmaceutical and agricultural industries (Schulz et al. 2002), and enhancement of plant nutrition (Behie and Bidochka 2014).

The results obtained in two previous works developed by our research group using entomopathogenic hypocreales showed an improvement in the bioavailability of certain nutrients such as iron (Fe). In the first work, *Beauveria bassiana* was applied to tomato and wheat seeds before growing them in artificial calcareous substrates with variable Fe content (Sánchez–Rodríguez et al. 2015). In the second work, *Metarhizium brunneum* was applied to calcareous soils where sorghum, wheat and sunflower plants were grown (Sánchez–Rodríguez et al. 2016). The chlorosis arising from Fe deficiency, is typical of sensitive plants (fruit trees, olive trees, citrus, cereals, berries) grown on calcareous soils. The chlorosis is due to the inhibition of chlorophyll synthesis and to a limited ability to redistribute Fe in the plant phloem. The main symptoms of Fe deficiency in plants are internerval yellowing of young leaves, causing a reduction of plant growth and quality (Díaz et al. 2009).

Calcareous soils are usually found in regions with an arid or semi–arid climate and span almost 30 % of the worldwide area of arable land; they have pH range of 7.5 — 8.5, conditions which Fe and other nutrients are poorly soluble for effective plant nutrition (Diaz et al. 2009). One other factor influencing Fe bioavailability in calcareous soils is the dominance of crystalline Fe oxides (goethite, hematite). Unlike poorly crystalline Fe oxides such as ferrihydrite, which is an effective source of Fe for plants, crystalline Fe oxides are very sparsely soluble (Vempati and Loeppert 1988).

Using an appropriate fungal dose on soil is vital to ensure its persistence in the environment over a desirable period of time. The dose must be high enough to guarantee the presence of the fungus while performing its regulatory function within the agroecosystem but not as high as to cause a detrimental effect to health or the environment (Directive 2005/25/EC, point 2.7.7). The effects of the entomopathogenic hypocreales doses applied to soil and the types of Fe oxides present in it on the ability of the fungi in mobilizing Fe and improving its bioavailability to crops remain unknown. The objective of this manuscript is to determine the minimum dose of EF that produces an improvement in growth and Fe nutrition of plants. For this, we developed two different assays. The first one consisted of an *in vitro* assay using three strains of EF to evaluate its ability to mobilize Fe from nine Fe oxides that differ in crystallinity and surface area. The second one consisted of a pot assay in which five doses of *M. brunneum*, which was selected based on the results obtained from the *in vitro* assay, were applied to two different soils, one a non–calcareous soil and the other a calcareous soil able to induce Fe chlorosis, where sorghum and sunflower plants were grown.

Materials and methods

In vitro assay

Synthetic iron oxides

Nine Fe oxides differing in specific surface areas were prepared as follows:

(a) Two ferrihydrite samples (Fh350 and FhP350) and one magnetite sample (Mag100) according to Schwertmann and Cornell (2000).

(b) Three hematite samples (Hm109, Hm40 and Hm15) according to Colombo et al. (1994).

(c) Three goethite samples (Gt115, Gt65 and Gt25) according to Torrent et al. (1990).

The specific surface area ($m^2\ g^{-1}$), which is the number following the initials for each Fe oxide is related to their reactivity, was determined by using the BET method (Brunauer et al. 1938) to obtain N_2 adsorption measurements. A suspended portion of each Fe oxide was lyophilized and ground in a mortar, the resulting powder being analyzed on a Siemens D5000 X–ray diffractometer using Co Kα radiation and a JEOL JEM 2010 transmission electron microscope. Fig. 1 shows electron micrographs including the average particle size and X–ray diffraction patterns in addition to the characteristic peaks for each Fe oxide. Particle size was inversely proportional to the surface area of each oxide (Fig. 1).

Fungal strains, experimental design and analysis

Three different entomopathogenic fungal strains deposited at the EF Collection of the Entomology Unit of the C.R.A.F. Department of the University of Córdoba (Spain) were used, namely:

(a) *Beauveria bassiana* EABb 04/01–Tip isolated from an *Iraella luteipes* larva collected in the field in the town of Carmona (Sevilla, Spain). This strain was previously found to exhibit an endophytic behavior on opium–inoculated plants (Quesada–Moraga et al. 2006). This strain was deposited with accession number CECT 20744 following the Budapest Treaty in the Spanish Collection of Culture Types (CECT), located at the University of Valencia (Spain).

(b) *Metarhizium brunneum* EAMa 01/58–Su isolated from soil in the town of Hinojosa del Duque (Córdoba, Spain). This strain was deposited with accession number CECT 20764 following the Budapest Treaty in the Spanish collection of culture types (CECT), located at the University of Valencia (Spain).

(c) *Isaria farinosa* 10/01–Msp isolated from a *Monochamus* insect of an unknown species.

The three entomopathogenic fungi were grown on Petri dishes containing Sabouraud Dextrose Agar supplemented with 0.5 g L^{-1} chloramphenicol (SDAC; Biolife, Italy) at 25 °C in the dark for 15 days to enable sporulation. A razor blade was used to scrape the sporulated mycelia off the surface of the plates and suspended in 50 ml of water containing Tween 80 (0.1 % v/v). Three suspensions of each entomopathogenic fungus were prepared, sonicated for 2 min, filtered to remove mycelia and adjusted to a concentration of 5×10^8 conidia ml^{-1} using a hemocytometer (Malassez chamber). Finally, 0.1 ml aliquots of each fungal suspension were homogeneously spread onto the surface of Petri dishes containing SDAC medium. All three entomopathogenic fungi were incubated at 25 °C in the dark for 4 (*M. brunneum*) or 6 days (*B. bassiana* and *I. farinosa*) —*M. brunneum* was incubated for a shorter time owing to its faster growth rate—.

Then, circular agar plugs (10.9 mm diameter) of actively growing fungi were cut out and carefully placed face down in the center of new Petri dishes of 90 mm diameter containing 20 ml of Czapek–dox solid medium (3 g $NaNO_3$, 1 g KH_2PO_4, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7\ H_2O$, 30 g glucose and 15 g agar per liter) supplemented with three doses (0, 50 or 250 mg Fe L^{-1}) of each one of the synthetic Fe oxides mentioned above. The control plates had SDAC plugs without fungus growing on them. The volume of 20 ml of Czapek–dox provided a thin layer allowing the fungi to successfully use the nutrients and grow.

In summary, nine Fe oxides (two ferrihydrite samples, one magnetite sample, three hematite samples and three goethite samples) at three different Fe doses (0, 50 and 250 mgFe L^{-1}) were set up in a

125 complete randomized design for each fungal strain (*M. brunneum, B. bassiana, I. farinosa* and control
126 without fungus) with four replications per combination of the two factors (Fe oxide and Fe dose) . The
127 experimental unit was one Petri dish and, in total, we used 480 Petri dishes.

128 Fungal colony diameter was measured daily over the period until each entomopathogenic fungus
129 reached a growth diameter of 30 mm, namely: 7 days for *M. brunneum*, 8 for *B. bassiana* and 18 for *I.*
130 *farinosa.* To do this, two perpendicular measurements with a gauge were taken from the center of the SDAC
131 piece to the outer edge of colony concentric growth of the fungus. The assay was ended when each fungus
132 fully covered the surface of the Petri dishes (viz., after 11 days for *B. bassiana*, 15 for *M. brunneum* and 42
133 for *I. farinosa*). Fungal mycelia masses were carefully scrapped from the surface of the dishes and culture
134 media (clean of fungus) was cut into small pieces with scissors. pH of the growth medium was measured
135 with a pH–meter in a 1 M KCl solution (1:2.5 w/v).The extractable Fe was determined after extraction with
136 0.005 M diethylenetriaminepentaacetic acid (Fe_{DTPA}) using atomic absorption spectroscopy (1:2 w/w
137 medium:DTPA) after shaking at 120 rpm for 2 h and centrifuging at 5000 rpm. Fe_{DTPA} is typically used as
138 Fe availability index (Lindsay and Norvell 1978).
139
140 ***In vivo* assay**
141 **Soil properties**
142 Soils were collected at a depth of 0 — 30 cm from two different locations. One was a Fe chlorosis–inducing
143 calcareous soil from Las Tablas, Jerez de la Frontera (Spain) (36° 41' 42" N, 6° 13' 10" W) with an alkaline
144 pH and low Fe availability (Fe_{DTPA} below 5 mg kg^{-1}). The other was a non–calcareous soil from the
145 Rabanales University Campus in Córdoba (37° 56' 04" N, 4° 43' 05" W); this was a sandy soil with a higher
146 Fe availability (above the critical level, 4.5 mg kg^{-1}, proposed by Lindsay and Norvell 1978). Both soils
147 were air–dried for 1 week and passed through a 1 cm sieve before use. Then, 100 g of each soil was further
148 to 2 mm and analysed for their physico–chemical properties. Table 1 shows the main properties of the both
149 soils.
150
151 **Plant material, fungal treatment and experimental design**
152 Seeds of *Sorghum bicolor* L. Moench cv. 03CS900/899 and *Helianthus annuus* L. were washed with 5 %
153 sodium hypochlorite solution for 2 min and then rinsed with abundant deionized water. *Sorghum bicolor* is
154 very sensitive to Fe chlorosis but *H. annuus* is not. Seeds were pre–germinated at 25 °C under moist

conditions for 72 h in the dark. Then, germinated seeds of each species were planted into free–draining cylindrical PVC pots (15 cm high and 5 cm in diameter, four seeds per pot) filled with either 260 g of calcareous soil or 280 g of non–calcareous soil. One week after planting three seedlings were removed from each pot to leave one seedling per pot.. The assay was conducted in a growth chamber maintained at 20 °C and 75 % relative humidity under 350 µmol m^{-2} s^{-1} photosynthetically active radiation and a photoperiod of 15 h light / 9 h dark. The length of the assay was 75 days for sorghum and 90 days for sunflower.

Four suspensions containing *M. brunneum* doses at 5×10^2, 5×10^4, 5×10^6 or 5×10^8 conidia ml^{-1} were prepared as described above. Fungal suspensions (5 ml per concentration and pot) were applied to the soil surface of the pots after sowing. Control pots received 5 ml of sterile deionized water (free of fungus) containing Tween 80 (0.1 % v/v) instead.

For each plant species, a completely randomized design was performed with five fungal doses (0, 5×10^2, 5×10^4, 5×10^6 or 5×10^8 conidia ml^{-1}) for each soil (calcareous and non–calcareous) with five replications (six in the non–calcareous soil); i.e., 100 pots for the calcareous soil and 120 pots for the non–calcareous soil, each pot as an experimental unit.

The pots were watered daily to keep soil moisture near 80 % field capacity and avoid conidial losses through draining. Also, 10 ml of a solution containing 5 mM $Ca(NO_3)_2$ was added to each pot on a weekly basis in order to supply the crops with nitrogen. Previous tests identified the fertilizers needed for proper plant growth of sorghum and sunflower in the two soils (Sánchez–Rodriguez et al., 2016).

Soil and plant analyses

The Colony Forming Units (CFU, conidia $g^{-1}$of soil) of *M. brunneum* were determined 5, 39 and 75 days after sowing (DAS) from in three pots randomly chosen from each combined treatments of crop, soil and fungal dose. One gram of soil was collected at a depth of 0 — 3 cm, suspended in 10 ml of sterile deionized water containing Tween 80 (0.1 % v/v) and the mixture shaken with rotary stirrer (Orbit, J.P. Selecta 3000445) at 12 rpm, for 90 min. Dilutions were made and 0.1 ml aliquots of these dilutions spread on Petri dishes containing SDAC to determine CFU after 2–3 days (Goettel and Inglis 1997).

Plant height and SPAD values (as a proxy of the chlorophyll concentration in leaf) were measured on a weekly basis after appearance of the earliest Fe chlorosis symptoms in sorghum (26 DAS). No interveinal yellowing was observed in the sunflower plants, however. SPAD was measured with a portable chlorophyll meter (SPAD 502 Minolta Camera Co., Osaka, Japan) on the two youngest leaves and

measurements validated by determining the amount of chlorophyll extracted by 99.5 % methanol (Wintermans and de Mots 1965) at the end of culture (75 DAS for sorghum and 90 DAS for sunflower) (R = 0.89, $P < 0.001$ in sorghum; R = 0.74, $P < 0.001$ in sunflower; 50 leaves per crop).

Above–ground plant weights of sorghum and sunflower were determined at harvest at 75 DAS and 90 DAS, respectively. Plants were dried at 70 °C for 72 h before grinding. Mineral element concentrations were determined after digestion with a nitric–perchloric acid mixture (Zasoski and Burau 1977). Ca, Mg, Fe, Mn, Zn, Cu were determined by atomic absorption spectrophotometry, K by flame emission and P with the molybdenum blue method (Murphy et al. 1986). C and N were determined by direct combustion on a Eurovector Analyser EA3000 (Eurovector SpA, Milan, Italy). Finally, Fe, Mn, Zn and Cu extracted by DTPA as described above for Fe_{DTPA} and K extracted by 1 N $CH_3CO_2NH_4$ at pH 7 and 25 °C after shaking for 30 minutes were determined in the soil at the end of the experiment.

Statistical analyses

Statistical analyses were performed with the software STATISTIX 9.0 (Analytical Software, Tallahassee, FL, USA). Previously, the data were checked for normal distribution by using the Kolmogorov–Smirnov test and homoscedasticity by using the Levene's test. When the requirements to perform parametric analyses were not met, logarithmic transformations were done. An analysis of variance (ANOVA) was performed to identify the effects of the studied factors (Fe oxide and Fe dose) on slope of fungal growth, pH and Fe_{DTPA} for each fungal strain in the *in vitro* assay. An additional factorial ANOVA was done for slope of fungal growth, Fe_{DTPA} and pH for each combination of fungal strain, Fe oxide and dose of Fe for the *in vitro* assay. Means were separated via the Least Significant Difference (LSD) test ($P < 0.05$) and additional correlations were performed. For data obtained from the *in vivo* assay, means and standard errors were used to describe the time course of CFU for the two soils and crops. A factorial ANOVA (soil and fungal doses) was developed for plant height, SPAD, plant dry weight, mineral nutrient concentration in plant and in soil for each plant species used in the *in vivo* experiment. Then, LSD was used to identify differences between the two soils, and orthogonal contrasts to identify differences between different fungal doses (control and the lowest doses vs highest doses) for plant height, SPAD, dry weight, mineral nutrient concentration in plant and in soil were established. When an interaction soil x fungal dose occurred, orthogonal contrasts were done for each soil separately to study the effect of the different fungal doses.

214 Where possible, these variables were fitted to a curve by using the software SigmaPlot 10.0 (Systat Software
215 Inc., Chicago, Illinois, USA). Additional Pearson's correlations were done between variables.
216
217 **Results**
218 ***In vitro* assay**
219 *Metarhizium brunneum* was the fastest–growing fungus (4.13 ± 0.05 mm day^{-1}), followed by *B. bassiana*
220 (3.68 ± 0.06 mm day^{-1}) and *I. farinosa* (1.25 ± 0.03 mm day^{-1}) according to mean values (data not shown).
221 Table 2 shows the slope of fungal diameter growth (growth rate) as a function of the Fe source and Fe dose
222 for each fungal strain. There were significant differences between the interaction of Fe oxide and Fe dose
223 for *M. brunneum* ($P < 0.001$) and *B. bassiana* ($P < 0.005$) but significant differences did not occurred for *I.*
224 *farinose* ($P > 0.05$, for each factor and the interaction). In order to clarify how each fungal strain behaved
225 for the different Fe oxides and Fe doses Fig. 2 is shown. The lowest slope was determined for *Isaria farinosa*
226 in all combinations of Fe oxide x Fe dose. *Metarhizium brunneum* and *B. bassiana* were the fungi that had
227 a faster growth without significant differences between them except when the Fe source was FhP350 and
228 Fh350 at the highest Fe doses (Fig. 2a), Hm109 at the lowest Fe dose, Hm40 (Fig. 2b), Gt115 at the lowest
229 Fe dose, and Gt25 (Fig. 2c). Increasing Fe doses reduced growth in *B. bassiana* ($P < 0.001$, $R^2 = 0.81$) and
230 *M. brunneum* ($P < 0.001$, $R^2 = 0.99$), but increased it slightly in *I. farinosa* ($P < 0.001$, $R^2 = 0.29$).
231 From the mean values (data not shown), the three strains increased Fe availability (Fe_{DTPA}, mg L^{-1})
232 at the end of the assay in the following decreasing sequence (mean ± standard error) relative to the control
233 without fungus (1.3 ± 0.2), 13.2 ± 2.3 (*B. bassiana*), 12.5 ± 3.4 (*M. brunneum*) and 8.0 ± 1.9 (*I. farinosa*).
234 *Beauveria bassiana* and *I. farinosa* considerably altered pH (to 8.3 ± 0.0 and 6.1 ± 0.1, respectively) in
235 relation to the control treatment (5.6 ± 0.0), whereas *M. brunneum* hardly changed it (5.6 ± 0.0).
236 Significant interactions between Fe oxide and Fe dose occurred for Fe_{DTPA} and pH in the medium
237 after culturing each one of the three fungal strains (Table 3). The two ferrihydrite samples and the magnetite
238 sample resulted in the highest Fe_{DTPA} concentrations in the medium for the three fungal strains, with Fh350
239 > FhP350 > Mag100 (Table 3 and Fig. 3). The presence of P in the structure of ferrihydrite (FhP350)
240 decreased Fe_{DTPA} mean values (Table 3; the effect was less evident for *B. bassiana*). As can be seen from
241 Table 3 and Fig. 3, the crystalline Fe oxides (hematite and goethite) resulted in lower Fe_{DTPA} values than
242 the poorly crystalline oxides (ferrihydrite and magnetite). As expected, Fe_{DTPA} increased with increasing
243 Fe dose in the majority of cases (Fig. 3 a–c). The increase in Fe_{DTPA} due to the effect of the fungi was more

evident when the Fe source was a poorly crystalline Fe oxide and especially at the highest doses (Fig. 3 a–c). The lowest increases occurred or even an increase was not observed with the crystalline oxides that had the lowest specific surface areas.

While *B. bassiana* increased the pH in a similar way with the different Fe sources, *I. farinosa* increased the pH especially in the presence of Fh350, FhP350, Mag100 and also, to a lesser extent, the three hematite samples, and to an even lesser degree the three goethite samples —with slight differences in any case (Fig. 3 a–c)— in comparison with the control without fungus. On the other hand, *M. brunneum* had a lower (negligible in the majority of cases) effect on pH.

***In vivo* assay**

Colony Forming Units (CFU)

Metarhizium brunneum was detected in none of the control samples. The number of CFU was greater in the first week (5 DAS) than in the following samplings (39 and 75 DAS). Overall, CFU increased with increasing fungal dose ($5\times10^8 > 5\times10^6 > 5\times10^4 > 5\times10^2$ conidia ml^{-1}); by exception, the differences between 5×10^2 and 5×10^4 conidia ml^{-1} were no significant (Fig. 4).

SPAD chlorophyll measurements

The SPAD values results differed between crops and soil types (Table 4 and Fig. 5). The mean SPAD values were significantly lower in the plants grown on the calcareous soil than in those on the non–calcareous soil, throughout the cropping period in sorghum ($P < 0.001$ for 26 DAS and 37–75 DAS) but only at the beginning in sunflower ($P < 0.001$, 26–34 DAS). An interaction between the kind of soil and the fungal dose occurred for SPAD of sorghum plants in the period 34–75 DAS ($P = 0.019$, Table 4 and Fig. 5b). The SPAD values 26 DAS in sorghum grown on the calcareous soils were negatively fitted to a logarithmic curve according to the fungal dose ($P = 0.016$, $R^2 = 0.89$; Fig. 5a). This trend changed 34 DAS to one that remained through the end of culture (75 DAS), when increased SPAD values were found in the sorghum plants grown on the calcareous soil treated with the highest fungal dose only (5×10^8 conidia ml^{-1}) relative to the others ($P = 0.009$; Fig. 5b). No significant differences between doses were detected (Table 4) in sorghum grown on the non–calcareous soil (Fig. 5a and 5b).

Plant growth

Plant height was higher in sorghum grown on the sandy soil than in these plants grown on the calcareous soil 14 DAS ($P < 0.001$) and 75 DAS ($P < 0.001$, Table 5). The same occurred in sunflower plants 21 DAS ($P < 0.001$) but the opposite 90 DAS ($P < 0.001$). Only the dry weight of sorghum was significantly higher for these plants grown on the sandy soil at the end of the experiment ($P < 0.001$, Table 5). The effect of fungal dose on plant height differed between crops (Table 5). At the beginning (14 DAS), the two highest fungal doses had a negative effect on height in sorghum ($P = 0.009$). However, these two doses produced a positive effect in sunflower plants 21 DAS ($P = 0.056$). At the end of the experiment (75 DAS in sorghum and 90 DAS in sunflower), the fungal dose did not affect sorghum plant height but an interaction (soil x fungal dose, $P = 0.012$) occurred for sunflower plants: a positive effect was observed in sunflower with the two highest fungal doses (46.1 ± 1.9 for control plants, 47.9 ± 1.4 for 5×10^2, 48.2 ± 3.2 for 5×10^4, 60.5 ± 3.0 for 5×10^6 and 57.3 ± 4.0 for 5×10^8 conidia ml^{-1}) in the plants grown on the calcareous soil ($P = 0.003$), and with the three highest fungal doses (42.0 ± 2.2 for control plants, 45.7± 0.5 for 5×10^2, 51.9 ± 2.5 for 5×10^4, 47.0 ± 1.0 for 5×10^6 and 48.6 ± 1.7 for 5×10^8 conidia ml^{-1}) in those on the non–calcareous soil ($P = 0.010$, data not shown). Higher dry weights were found in sorghum plants grown on the calcareous soil at the end of the experiment but not in sunflower plants (Table 5). Plant height and dry weight were correlated in sorghum ($P < 0.001$, $R = 0.79$) but not in sunflower.

The sunflower plants inoculated with the fungus flowered earlier than the control plants. The plants grown on the non–calcareous soil started flowering earlier (34 DAS) than those on the calcareous soil (46 DAS) (results not shown). A higher diameter ($P < 0.001$) and dry weight ($P < 0.001$) of the inflorescence of sunflower were obtained for plants grown on the sandy soil and for plants grown on the soils treated with the two highest doses of *M. brunneum* (Fig.6 and Table S1; $P = 0.001$ for diameter and $P = 0.001$ for dry weight). In addition, these variables were fitted to a logarithmic curve ($P = 0.024$, $R^2 = 0.86$ for diameter in calcareous soil, and $P = 0.010$, $R^2 = 0.92$ for diameter in sandy soil, Fig. 6a; and $P = 0.026$, $R^2 = 0.85$ for dry weight in calcareous soil, Fig. 6b).

Total mineral nutrient contents in above–ground plant biomass

No deficiency symptoms other than those of Fe chlorosis in the youngest leaves of sorghum grown on the calcareous soil were observed. There was significant differences in total nutrient in the above–ground biomass of sorghum and sunflower, and in the inflorescence of sunflower between soils and the different

fungal doses (Table 6). Nevertheless, the effect of the soil was more evident than these of the fungal dose. Higher amounts of C, N, K, P and Mg were found in sorghum plants, and of P, Mn, and Zn in sunflower plants grown on the sandy soil (Table 6). This effect was also found in inflorescence of sunflower for C, N, K, P, Mg, Fe, Mn, and Cu (Table 6). On the other hand, a lower amount of Ca in sorghum and sunflower plants, and Cu in sunflower plants grown on the sandy soil was obtained (above–ground biomass). The highest fungal doses reduced the total amount of K in sorghum and the total amount of K and P in above–ground biomass of sunflower (Table 6) but increased the total amount of N in the inflorescence of sunflower (Table 6). There were two interactions in Zn (P = 0.039) and Cu (P = 0.034) in above–ground sorghum plants because the total content of these mineral nutrients was reduced in plants grown on calcareous soils, where the two highest doses were applied (Table 6). This was not observed in plants grown on the non–calcareous soil. The effect of fungal doses in Fe content in inflorescences of sunflower was not clear (Table 6). The same occurred for the interaction soil x *M. brunneum* dose for Ca (P = 0.037) and Zn (P = 0.011, Table 6).

Total mineral nutrient content in rizhospheric soil

The calcareous soil had lower Fe_{DTPA} ($P < 0.001$) and Mn_{DTPA} ($P < 0.001$) contents than the non–calcareous soil —but also, as expected—, and higher Zn_{DTPA} contents with both crops (Table S2). The application of *M. brunneum* reduced Mn_{DTPA} contents in the soil where sunflower was pot–grown (P = 0.044) and altered Zn_{DTPA} contents in a different way in each crop. An interaction soil x fungal dose occurred for Cu_{DTPA} (P = 0.005 and P = 0.007) and K_{aa} ($P < 0.001$ and P = 0.023, for sorghum and sunflower, respectively). These interactions did not have a clear explanation because the effect of the fungal dose caused inconsistent differences in each soil, except for K_{aa} in the soil of sorghum. In this case, the fungal dose increased the amount of K_{aa} (mg kg^{-1}) in the sandy soil (89.3 ± 3.1 for control plants, 94.1± 4.5 for 5×10^2, 92.0 ± 0.9 for 5×10^4, 94.9 ± 2.2 for 5×10^6 and 97.2 ± 2.8 for 5×10^8 conidia ml^{-1}; logarithmic curve, P = 0.045, R^2 = 0.79) but reduced these values in the calcareous soils (134.8 ± 1.1 for control plants, 127.5 ± 2.5 for 5×10^2, 117.3 ± 1.1 for 5×10^4, 110.4 ± 2.9 for 5×10^6 and 113.8 ± 4.9 for 5×10^8 conidia ml^{-1}; logarithmic curve, P = 0.021, R^2 = 0.87).

Discussion

Based on the results of the *in vitro* assay, the three EF strains studied increased Fe availability in the culture medium. *Metarhizium brunneum* and *B. bassiana* were more efficient than *I. farinosa* in improving Fe availability; they especially effective in the presence of amorphous Fe oxides such as ferrihydrite (FhP350, Fh350) and poorly crystalline Fe oxides with a small particle size such as magnetite (Mag100). The increased bioavailability of Fe can be ascribed to the high specific surface area of ferrihydrite and magnetite relative to hematite and goethite, which are less reactive by effect of their being more crystalline and having a larger particle size (Vempati and Loeppert 1986). The changes in Fe_{DTPA} and pH observed in this first assay can be explained in two different ways. Thus, EF are known to alter the concentrations of elements such as Fe, Cu and Ag in their environment by producing organic acids (Joseph et al. 2012) to lower pH and increase the solubility of nutrients —Fe in this case— as a result. Based on the results of the *in vitro* assay, the increased availability of Fe was not due to the ability of the fungi to lower the pH of the culture medium (except in two occasions, one for *I. farinose* and another for *M. brunneum*). Most fungi —entomopathogenic hypocreales included— produce and release substances of low molecular weight called "siderophores" which act as chelators for mineral elements such as Fe, Mn, Zn and Cu without altering the pH of the medium (Jirakkakul et al. 2015). Siderophores have a high affinity and selectivity for Fe and facilitate its uptake by microorganisms. In spite of increased pH, fungi *B. bassiana* (especially) and *I. farinosa* (somewhat less effectively) increased Fe_{DTPA}, this can be likely ascribed to mobilized large amounts of Fe, so they must be very effective in secreting siderophores, at least when the medium is not calcareous (*in vitro* assay).

Our choice of *M. brunneum* for the *in vivo* assay was based on the results of the previous *in vitro* assay, its being a rhizospheric competent fungus (St. Leger 2008) and its persistence over long periods of time in soil (Bidochka et al. 2001). This fungus successfully colonized the rhizosphere in both soils and remained in the soil column throughout the culture period, especially at the highest doses (5×10^6 and 5×10^8 conidia ml^{-1}). The vertical movement of fungal propagules in soil appears to depend largely on texture, organic matter content and pH (Quesada–Moraga et al. 2007). A gradual decrease in fungal propagules (CFU) over time was observed during crop development in both soils; however, CFU remained above the natural background levels throughout the assay (Bruck 2005, Scheepmaker and Butt 2010). Our results are consistent with those of Garrido–Jurado et al. (2011), whose conidia retention experiments on soil–filled columns with different textures, pH and organic matter revealed a lower retention of the *Metarhizium*

conidia in the clay particles due to its larger size (compared to other fungi such as *Beauveria*) and its hydrophobic nature. Also, Salazar et al. (2007) suggested that the movement of *Metarhizium* in the soil is favored by the larger macropores and less tortuousity of sandy soils, compared to the clayey soils, favoring the vertical movement. These expected reductions in CFU with time may have resulted from leaching and translocation of fungal propagules to deeper levels in the soil.

The benefits of *M. brunneum* to promote plant growth and increase root growth, and ultimately increase crop yield, have been previously reported by several authors (Kabaluk and Ericsson 2007; Vega et al. 2009; Sasan and Bidochka 2012). In this trial we can also include the positive effect of this EF against the Fe chlorosis in a sensitive plant such as sorghum and on the flowering of the sunflower. As can be seen from our results, the highest fungal doses (5×10^6 and 5×10^8 conidia ml^{-1}) were crucial with a view to ensuring a positive effect in plant growth promotion. These fungal doses having such effects are similar to those recommended for biological control (Scheepmaker and Butt 2010). Nevertheless, Gurulingappa et al. (2010) in previous studies remarks, that the fungal doses effect on plant height was dependent on the particular crop. Thus, the sorghum plants inoculated with the highest doses (5×10^6 and 5×10^8 conidia ml^{-1}) grew less at the beginning (14 DAS), probably because of initial competition in the fungus–plant association having an energy cost for the host plant (Partida–Martínez and Heil 2011). At the end (75 DAS), however, fungal dose had no effect on growth in sorghum. In sunflower, the plants grown on both soils treated with the highest fungal doses were higher, flowered earlier and produced larger inflorescences. These increase of sunflower height was reflected in the diameter and dry weight of the inflorescences that were considerably raised (higher production).

There was no effect on the sorghum plants grown on the non–calcareous soil, nor on the sunflower —which are less sensitive to Fe chlorosis— plants grown on both soils in relation to Fe chlorosis. In general, SPAD values were lower in the plants grown on the calcareous soil owing to the less Fe bioavailability in this soil. At the beginning (26 DAS), the sorghum plants grown on the calcareous soil —which contained less Fe_{DTPA}— exhibited symptoms of interveinal yellowing, the observed decrease in SPAD values in the sorghum plants with increasing fungal dose probably being the result of initial competition for nutrients between the fungus and the plants. However, a general beneficial effect of *M. brunneum* on sorghum was observed throughout the experiment in the plants grown on the calcareous soil treated with the highest fungal dose only. According to Mikami et al. (2011), sorghum is one of the most inefficient monocots producing phytosiderophores (viz., Fe chelators mobilizing Fe from the soil under low Fe availability

conditions); therefore, the Fe chlorosis symptoms were to be expected in this crop. The highest dose of *M. brunneum* (5×10^8 conidia ml^{-1}), which increased Fe bioavailability at some growth stages in sorghum can be defined as the lowest dose having a positive effect in alleviating Fe chlorosis of sorghum plants in this experiment, which agrees with previous experiments (Sánchez–Rodríguez et al. 2016). As shown in this experiment, this fungal dose depends on the crop and is different to the fungal dose that could produce a positive effect on plant growth.

The increase of SPAD in plants grown on the calcareous soil treated with the highest dose of *M. brunneum* was not related to an increase in the concentration of Fe in the above–ground plant biomass. Although Fe participates in the production of chlorophyll, which is responsible of the green color of the plants, its concentration is not normally correlated with chlorophyll content in plants under Fe deficiency, known as "Fe chlorosis paradox" (Römheld 2000). Other fungi used in biological control such as *Trichoderma* are able to increase the activity of Fe–containing enzymes but at the same time reduce the chlorophyll concentration in young leaves (de Santiago et al. 2009). The highest SPAD values measured in sorghum plants grown on the calcareous soil treated with the 5×10^8 conidia ml^{-1} of *M. brunneum* indicates that the fungus increased the availability of Fe to be used for chlorophyll synthesis.

Although some alterations (increase and reduction) in nutrient content in plants and soils were found due to the application of *M brunneum* in this work, especially the highest doses, they resulted in a positive effect for alleviating Fe chlorosis symptoms in sorghum and in an increase in plant growth and production (inflorescence) in sunflower plants. Special mention requires the decrease in K in sorghum and sunflower plants grown on the soils treated with the highest fungal doses. Under low Fe availability, dicot plants (sunflower) increase the production of H^+ and organic acids to acidify the rhizosphere facilitating the absorption of Fe (and other micronutrients) while monocot plants (sorghum) produce phytosiderophores to chelate Fe without reducing the pH of the rhizosphere (Marschner and Römheld 1994). Potassium plays an important role in the mechanisms that regulate Fe nutrition in both monocot and dicot plants (Marschner and Römheld 1994; Neuman and Römheld 2001). An increase in the uptake of K is expected in plants exhibiting more Fe chlorosis symptoms to compensate the emission of H^+ (higher in these plants) in order to regulate the membrane potential (Jolley and Brown 1995). The different effect that the highest fungal doses caused in micronutrient and K_{aa} contents in soil could be related to the different strategies to uptake Fe of these two plants grown on the two different soils.

There is evidence that the host association of the plant rather than association with insect hosts plays a central role in the evolutionary divergence of different species in the genus *Metarhizium* (Wyrebek and Bidochka 2013). Liao et al. (2014) found the beneficial effect of *Metarhizium* on plant growth and productivity to be related to the success of the fungus–plant association, their results suggesting that physical root colonization is a prerequisite for most of the beneficial effects of this fungus. Our results show that the fungus had no adverse effect on the crops; rather, the highest fungal doses had a greater effect on growth and production in the sunflower plants and only the highest one was able to alleviate Fe chlorosis symptoms in sorghum plants grown on a calcareous soil; the success of the fungus–plant association could be achieved only for these doses in these plants. The presence of a high density of fungal propagules in the soil samples treated with these doses —which exceeded the natural concentrations in the field— makes the fungus highly competitive with other microorganisms. The doses used are typical of most laboratory tests and consistent with field sampled doses following application of EF–based commercial products.

Conclusions

These results demonstrate the ability of the three fungal strains of EF to improve the bioavailability of Fe under different conditions (Fe availability and Fe concentrations). Furthermore, we conclude that the positive effects of *M. brunneum* on Fe nutrition and plant growth depend on the particular crop, soil type and fungal dose. In this work, we found that *M. brunneum* is able to increase Fe availability for sorghum grown on a calcareous soil (only the highest fungal dose applied to soil), and to boost plant growth and inflorescence production in sunflower in both a calcareous and a non–calcareous soil. These results are useful to encourage the use of EF in new sustainable strategies in agriculture to provide the plant with other advantages besides the well–known ability to kill insects as microbial control agents.

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567

568 **FIGURE CAPTIONS**

569 **Fig. 1** *In vitro* assay. Electron micrographs showing the average particle size, the X–ray diffraction patterns
570 and the characteristic peaks for each Fe oxide. Fh= Ferrihydrite, Hm= Hematite, Mag= Magnetite, Gt=
571 Goethite. The number in each Fe oxide means the specific surface area (m^2 g^{-1}).

572 **Fig. 2** *In vitro* assay. Diameter growth (slope in mm day^{-1}) as a function of the Fe source (a), Fe dose (b)
573 for each fungal strain and Fe source × Fe dose for the three fungal strains.

574 **Fig. 3** *In vitro* assay. Fe_{DTPA} and pH for each fungal strain as a function of the Fe source and Fe dose. Fh=
575 Ferrihydrite, Hm= Hematite, Mag= Magnetite, Gt= Goethite. The number in each Fe oxide means the
576 specific surface area (m^2 g^{-1}).

577 **Fig. 4** *In vivo* assay. Colony Forming Units (CFU) of *M. brunneum* in the soil as a function of the plant
578 species (sorghum and sunflower), kind of soil (calcareous soil and non–calcareous soil), days after sowing
579 (DAS) and fungal dose applied to the surface of each pot at the beginning of the experiment. Mean ±
580 standard error for $n = 5$ (calcareous soil) and $n = 6$ (non–calcareous soil).

581 **Fig. 5** *In vivo* assay. SPAD chlorophyll measurements (SPAD value) in sorghum and sunflower plants as a
582 function of the kind of soil (calcareous soil and non–calcareous soil), days after sowing (DAS) and fungal
583 dose applied to the surface of each pot at the beginning of the experiment. Mean ± standard error for $n = 5$
584 (calcareous soil) and $n = 6$ (non–calcareous soil).

585 **Fig. 6** *In vivo* assay. Flower diameter and dry weight in sunflower as a function of the kind of soil
586 (calcareous soil and non–calcareous soil), days after sowing (DAS) and fungal dose applied to the surface
587 of each pot at the beginning of the experiment. Mean ± standard error for $n = 5$ (calcareous soil) and $n = 6$
588 (non–calcareous soil).

Table 1 *In vivo* assay. Selected properties of the soils (mean value, n = 2).

Soil	Latitude	Longitude	Crop	Clay	CCE	$pH_{1:2.5}$	K_{aa}	P_{Olsen}	Fe_d	Fe_{ox}	Fe_{ca}	Fe_{DTPA}	Mn_{DTPA}	Zn_{DTPA}	Cu_{DTPA}
				g kg^{-1}	g kg^{-1}		mg kg^{-1}	mg kg^{-1}	g kg^{-1}			mg kg^{-1}			
Calcareous	36° 41' 42" N	6° 13' 10" W	Vineyard	335	630	8.4	210	37.5	1.20	0.40	0.80	3.3	10.2	2.8	1.2
Non-calcareous	37° 56' 04" N	4° 43' 05" W	Olive	98	≈ 0	6.5	84	14.0	13.00	1.12	1.20	56.6	58.9	1.7	20.4

Clay: Pipette method following dispersion with sodium hexametaphosphate after shaking for 2 h (Bouyoucos & McCool 1915).
CCE: Weight loss upon treatment with 6 M HCl after shaking for 30 min (Van Wesemael 1955).
$pH_{1:2.5}$: Potentiometric measurement in a 1:2.5 soil:water suspension after shaking for 30 min.
K_{aa}: Ammonium acetate-extractable potassium, extracted with 1 N $CH_3CO_2NH_4$ at pH 7 at 25 °C after shaking for 30 min.
Fe_d: Dithionite/Citrate/bicarbonate-extractable Fe at 25 °C after shaking for 16 h (Mehra & Jackson 1960).
Fe_{ox}: Ammonium oxalate-extractable Fe at pH 3 at 25 °C after shaking for 2 h (Schwertmann 1973).
Fe_{ca}: Citrate/ascorbate-extractable Fe at 25 °C after shaking for 16 h (Reyes & Torrent 1997).
Fe_{DTPA}, Mn_{DTPA}, Zn_{DTPA} and Cu_{DTPA}: Diethylenetriaminepentaacetic acid-extractable Fe, Mn, Zn and Cu, respectively, at 25 °C after shaking for 2 h (Lindsay & Norvell 1978).
P_{Olsen}: P extracted with 0.5 M $NaHCO_3$ buffered at pH 8.5 at 25 °C after shaking for 30 min (Olsen et al., 1954).

Table 2 *In vitro* assay. Factorial ANOVA of the slope of the growth (mm day^{-1}) for each fungus as a function of the Fe oxide and Fe dose (mean ± standard error, n = 4 Petri dishes per combination of fungal strain, Fe oxide and Fe dose). P_{ANOVA} means the *P* value for each factor and interaction is the *P* value of Fe oxide x Fe dose.

Factor		Slope (mm day^{-1})		
		M. brunneum	***B. bassiana***	***I. farinosa***
Fe oxide	Control	4.27 ± 0.15	4.20 ± 0.21	1.17 ± 0.17
	FhP350	3.68 ± 0.21	2.92 ± 0.26	1.22 ± 0.18
	Fh350	3.73 ± 0.20	2.88 ± 0.13	1.31 ± 0.14
	Mag100	3.75 ± 0.09	3.65 ± 0.07	1.29 ± 0.05
	Hm109	4.28 ± 0.12	3.83 ± 0.04	1.33 ± 0.11
	Hm40	4.57 ± 0.10	3.70 ± 0.10	1.30 ± 0.12
	Hm15	4.56 ± 0.11	3.61 ± 0.09	1.44 ± 0.02
	Gt115	3.90 ± 0.13	3.41 ± 0.22	1.18 ± 0.14
	Gt65	3.75 ± 0.20	3.32 ± 0.22	1.17 ± 0.16
	Gt25	4.37 ± 0.13	3.42 ± 0.11	1.35 ± 0.10
P_{ANOVA}		**< 0.001**	**< 0.001**	0.947
Fe dose (mgFe L^{-1})	[a]0	4.27± 0.04	4.20 ± 0.06	1.17 ± 0.05
	50	4.18 ± 0.10	3.61 ± 0.07	1.30 ± 0.05
	250	3.95 ± 0.07	3.22 ± 0.09	1.28 ± 0.07
P_{ANOVA}		**< 0.001**	**< 0.001**	0.250
Interaction		**< 0.001**	**0.005**	0.816

[a]Control, without Fe. Fh= Ferrihydrite, Hm= Hematite, Mag= Magnetite, Gt= Goethite. The number in each Fe oxide means the specific surface area (m^2 g^{-1}).

Table 3 *In vitro* assay. Factorial ANOVA of Fe_{DTPA} and pH (mean ± standard error, n = 4 Petri dishes per combination of fungal strain, Fe oxide and Fe dose) in the medium after culturing the three different strains for 11 (*M. brunneum*), 15 (*B. bassiana*) and 42 (*I. farinosa*) days (when each fungal strain fully covered the surface of the Petri dishes) at 25 °C, as a function of the Fe oxide and Fe dose. P_{ANOVA} means the P value for each factor and interaction is the P value of Fe oxide x Fe dose.

Factor		***Metarhizium brunneum***		***Beauveria bassiana***		***Isaria farinosa***		***Without fungus***	
		Fe_{DTPA} (mg L^{-1})	pH_{KCl}	Fe_{DTPA} (mg L^{-1})	pH_{KCl}	Fe_{DTPA} (mg L^{-1})	pH_{KCl}	Fe_{DTPA} (mg L^{-1})	pH_{KCl}
Fe oxide	Control	0.5 ± 0.0	5.4 ± 0.0	0.4 ± 0.0	8.3 ± 0.1	0.5 ± 0.0	5.8 ± 0.3	0.7 ± 0.0	5.5 ± 0.1
	FhP350	45.0 ± 15.2	5.8 ± 0.3	55.4 ± 8.3	8.5 ± 0.1	29.5 ± 11.9	7.5 ± 0.2	4.1 ± 1.2	5.5 ± 0.1
	Fh350	94.6 ± 28.7	5.2 ± 0.2	57.4 ± 9.6	8.4 ± 0.0	45.7 ± 14.9	7.4 ± 0.2	4.6 ± 1.2	5.5 ± 0.1
	Mag100	18.9 ± 7.1	5.6 ± 0.1	42.9 ± 8.8	8.4 ± 0.0	30.1 ± 10.1	7.2 ± 0.2	1.2 ± 0.2	5.6 ± 0.1
	Hm109	0.9 ± 0.1	5.8 ± 0.1	7.5 ± 2.5	8.3 ± 0.1	4.1 ± 1.0	6.4 ± 0.1	0.6 ± 0.1	5.7 ± 0.1
	Hm40	2.6 ± 0.7	5.6 ± 0.0	2.7 ± 0.6	8.2 ± 0.0	0.8 ± 0.2	6.1 ± 0.2	1.1 ± 0.3	5.9 ± 0.1
	Hm15	0.8 ± 0.1	5.6 ± 0.1	1.1 ± 0.2	8.3 ± 0.0	0.5 ± 0.0	6.0 ± 0.3	0.5 ± 0.0	5.6 ± 0.1
	Gt115	2.0 ± 0.4	5.7 ± 0.0	5.2 ± 0.9	8.2 ± 0.1	2.8 ± 0.5	5.7 ± 0.2	0.6 ± 0.1	5.7 ± 0.1
	Gt65	0.7 ± 0.0	5.7 ± 0.1	2.2 ± 0.3	7.9 ± 0.1	2.0 ± 0.3	4.8 ± 0.2	0.8 ± 0.1	5.8 ± 0.1
	Gt25	0.7 ± 0.1	5.6 ± 0.1	1.6 ± 0.2	8.1 ± 0.0	0.6 ± 0.0	5.8 ± 0.3	1.2 ± 0.3	5.6 ± 0.1
P_{ANOVA}		**< 0.001**	**0.045**	**0.002**	**< 0.001**	**0.018**	**< 0.001**	**< 0.001**	0.224
Fe dose (mg L^{-1})	[a]0	0.5 ± 0.0	5.4 ± 0.0	0.4 ± 0.0	8.3 ± 0.0	0.5 ± 0.0	5.8 ± 0.1	0.7 ± 0.0	5.5 ± 0.0
	50	5.0 ± 1.4	5.7 ± 0.0	10.9 ± 2.4	8.3 ± 0.0	2.0 ± 0.3	6.0 ± 0.2	1.0 ± 0.2	5.6 ± 0.1
	250	31.9 ± 9.5	5.6 ± 0.0	28.2 ± 5.6	8.2 ± 0.1	21.6 ± 5.0	6.5 ± 0.2	2.3 ± 0.5	5.7 ± 0.0
P_{ANOVA}		**< 0.001**	**0.001**	**< 0.001**	0.095	**< 0.001**	**< 0.001**	**< 0.001**	0.129
Interaction		**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.001**	**< 0.001**	**< 0.001**	0.541

[a]Control, without Fe. Fh= Ferrihydrite, Hm= Hematite, Mag= Magnetite, Gt= Goethite. The number in each Fe oxide means the specific surface area (m^2 g^{-1}). Different letters indicate significant differences based on LSD test ($P < 0.05$).

Table 4 *In vivo* assay. Factorial ANOVA for SPAD at the beginning and at the end of the experiment (mean ± standard error; calcareous soil: $n = 5$, non-calcareous soil: $n = 6$).

	Sorghum		**Sunflower**	
	SPAD 26	SPAD 34 – 75	SPAD 26 – 34	SPAD 40 – 90
Soil				
Calcareous soil	27.7±0.4b	16.0±0.9	36.6±0.5b	32.1±0.5
Sandy soil	39.4±0.3a	27.7±0.2	40.9±0.4a	32.3±0.5
P_{ANOVA}	**< 0.001**	**< 0.001**	**< 0.001**	0.708
***M. brunneum* dose (conidia ml^{-1})**				
Control	34.6±1.9	21.1±2.3	38.8±1.4	32.7±0.8
5×10^{2}	34.2±1.9	22.4±2.0	38.9±0.9	30.8±0.6
5×10^{4}	33.8±2.1	22.4±2.1	39.4±1.1	34.2±0.5
5×10^{6}	34.5±2.2	22.0±2.5	38.4±0.8	32.0±1.1
5×10^{8}	34.1±2.1	24.9±1.3	39.1±1.1	31.4±0.6
P_{ANOVA} / $P_{orthogonal}$	0.227	**0.027**	0.798	**0.027 / 0.155**
Interaction	0.456	**0.019**	0.123	0.778

P_{ANOVA} is the P value of the factorial ANOVA / $P_{orthogonal}$ is the P value of the orthogonal contrast between doses of *M. brunneum*. Interaction is the P value of Soil x *M. brunneum* dose of the factorial ANOVA.

The lowercase letters shows the difference of means between the different soils with the LSD test. These letters were not included when an interaction Soil x *M. brunneum* dose occurred.

Table 5 *In vivo* assay. Factorial ANOVA for plant height (at the beginning and at the end of the experiment) and above-ground plant dry weight (without inflorescence for sunflower; mean ± standard error; calcareous soil: n = 5, non-calcareous soil: n = 6).

	Sorghum			**Sunflower**		
	Height 14 DAS (cm)	Height 75 DAS (cm)	Dry weight (g)	Height 21 DAS (cm)	Height 90 DAS (cm)	Dry weight (g)
Soil						
Calcareous soil	11.8±0.6b	52.9±0.9b	0.9±0.0b	7.4±0.3b	51.6±1.7	1.7±0.1
Sandy soil	19.0±0.5a	59.4±0.6a	1.6±0.0a	10.0±0.3a	47.0±0.9	1.5±0.1
P_{ANOVA}	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**0.004**	0.123
***M. brunneum* dose (conidia ml^{-1})**						
Control	16.0±1.5A	56.1±1.5	1.3±0.1	8.1±0.5B	43.8±1.6	1.7±0.2
5×10^2	17.6±1.1A	57.4±1.3	1.3±0.1	8.7±0.7B	46.7±0.7	1.7±0.2
5×10^4	16.1±1.7A	56.6±1.2	1.2±0.2	8.3±0.6B	50.1±2.0	1.8±0.2
5×10^6	15.0±1.6B	56.4±2.2	1.3±0.2	9.3±0.6A	53.0±2.7	1.3±0.1
5×10^8	14.4±1.2B	56.2±1.4	1.3±0.1	9.9±0.8A	52.4±2.4	1.5±0.1
P_{ANOVA} / $P_{orthogonal}$	**0.009** / **0.001**	0.796	0.377	0.056 / **0.005**	**0.001**	0.521
Interaction	0.771	0.222	0.147	0.329	**0.012**	0.700

P_{ANOVA} is the P value of the factorial ANOVA / $P_{orthogonal}$ is the P value of the orthogonal contrast between doses of *M. brunneum.* Interaction is the P value of Soil x *M. brunneum* dose of the factorial ANOVA.
The lowercase letters shows the difference of means between the different soils with the LSD test.
The capital letters shows the difference of means between the different doses with the orthogonal analysis.
These letters were not included when an interaction Soil x *M. brunneum* dose occurred.

Table 6 *In vivo* assay. Factorial ANOVA for total nutrient content in the above-ground plant biomass of sorghum and sunflower plants, and in sunflower´s inflorescence, grown on the two different soils (mean ± standard error with $n = 5$ for the calcareous soil and $n = 6$ for the non-calcareous soil).

Factor	C (mg)	N (mg)	K (mg)	P (mg)	Ca (mg)	Mg (mg)	Fe (µg)	Mn (µg)	Zn (µg)	Cu (µg)
Soil	**Sorghum**									
Calcareous soil	376.8±22.2b	10.5±0.2b	29.0±1.0b	1.4±0.0b	6.4±0.3	1.9±0.1b	152.4±10.0	130.1±6.4	30.7±1.6	11.3±0.9
Sandy soil	656.5±14.3a	17.8±0.3a	38.3±1.1a	2.0±0.0a	6.4±0.2	2.6±0.1a	158.7±13.7	137.6±5.2	13.9±0.7	11.9±0.4
*P*value	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.817	**< 0.001**	0.966	0.698	**< 0.001**	0.479
***M. brunneum* dose (conidia ml^{-1})**										
Control	535.6±59.5	14.3±1.3	37.5±2.6A	1.7±0.1	7.2±0.4	2.5±0.2	160.6±12.5	144.5±9.2	25.3±4.5	12.8±1.8
5×10^2	545.7±44.3	14.5±1.3	36.3±2.0A	1.6±0.1	6.5±0.2	2.3±0.1	162.9±10.1	142.4±6.9	21.4±2.9	12.5±0.5
5×10^4	486.9±58.4	14.3±1.3	31.7±2.1B	1.6±0.1	6.2±0.5	2.4±0.3	124.8±14.8	125.0±11.6	19.5±2.4	11.1±0.7
5×10^6	529.7±69.6	15.1±1.2	32.8±2.4B	1.8±0.1	6.0±0.4	2.4±0.2	138.4±23.2	130.4±9.0	21.0±2.5	11.3±0.7
5×10^8	563.2±33.4	14.8±1.3	32.3±1.6B	1.8±0.2	6.0±0.3	2.0±0.2	189.3±29.1	127.5±8.7	19.2±3.4	11.2±0.5
P_{ANOVA} / $P_{orthogonal}$	0.274	0.963	**0.024/0.001**	0.362	0.193	0.177	0.192	0.442	0.172	0.613
Interaction	0.112	0.941	0.575	0.264	0.291	0.522	0.689	0.641	**0.039**	**0.034**
Soil	**Sunflower**									
Calcareous soil	659.6±42.1	24.6±1.5	29.1±1.8	0.6±0.1b	52.4±3.3a	6.3±0.4	84.3±5.0	56.9±4.3b	38.9±2.4b	5.5±0.5a
Sandy soil	576.8±41.7	22.6±1.5	26.4±1.3	1.1±0.1a	33.3±1.2b	6.8±0.4	96.2±13.5	323.3±19.2a	45.6±3.2a	3.6±0.4b
P_{ANOVA}	0.265	0.365	0.273	**< 0.001**	**< 0.001**	0.223	0.183	**< 0.001**	**< 0.001**	**< 0.001**
***M. brunneum* dose (conidia ml^{-1})**										
Control	646.1±74.5	24.1±2.7	28.8±2.3A	1.0±0.1A	40.1±3.9	6.9±0.7	75.8±8.8	245.6±63.2	46.7±4.3A	4.8±0.9A
5×10^2	641.0±79.2	23.4±2.9	28.5±2.9A	1.1±0.2A	46.7±5.6	7.1±0.6	78.7±6.9	202.66±47.3	46.1±5.0A	6.1±0.9A
5×10^4	680.8±65.9	26.3±2.3	32.2±2.0A	1.1±0.1A	43.6±5.4	6.4±0.4	78.0±6.6	180.1±41.8	51.4±3.5A	5.1±0.8A
5×10^6	520.0±50.5	21.8±1.5	25.7±1.8B	0.6±0.1B	39.9±4.8	6.4±0.5	120.5±31.5	157.8±35.2	32.2±2.7B	2.8±0.4B
5×10^8	572.54±54.2	21.5±2.6	22.1±1.3B	0.7±0.1B	38.6±3.5	6.2±0.7	104.6±17.6	221.8±61.2	34.6±4.7B	3.1±0.4B
P_{ANOVA} / $P_{orthogonal}$	0.562	0.709	**0.043 / 0.008**	**0.012 / 0.001**	0.479	0.832	0.355	0.222	**0.010/0.001**	**0.006/0.001**
Interaction	0.708	0.134	0.833	0.778	0.510	0.270	0.137	0.180	0.967	0.700
Soil	**Sunflower´s inflorescence**									
Calcareous soil	269.8±25.6b	9.4±0.7b	14.1±1.1b	1.0±0.1b	7.5±0.8	1.2±0.1b	32.9±4.8b	5.3±0.6b	20.1±2.3	4.5±0.4b
Sandy soil	483.3±24.9a	16.8±0.8a	26.0±1.1a	3.1±0.2a	7.7±0.4	2.5±0.1a	45.2±3.9a	63.1±3.8a	18.3±1.2	9.1±0.5a
P_{ANOVA}	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	0.896	**< 0.001**	**0.017**	**< 0.001**	0.449	**< 0.001**
***M. brunneum* dose (conidia ml^{-1})**										
Control	342.7±51.9	11.3±1.3B	17.5±2.2	2.0±0.4	6.8±0.7	1.6±0.2	38.3±9.9AB	38.0±11.4	17.2±2.2	5.4±0.8
5×10^2	349.8±46.4	12.5±1.6B	21.2±3.0	2.0±0.3	6.9±0.8	1.9±0.3	28.8±4.9B	30.4±8.1	17.1±2.2	7.5±1.1
5×10^4	352.0±49.6	12.3±1.5B	20.2±2.6	2.4±0.4	6.7±1.0	1.7±0.2	42.3±7.9A	32.5±9.8	16.7±2.1	6.4±1.0
5×10^6	485.11±43.8	16.3±1.2A	22.8±1.7	2.1±0.3	10.1±0.9	2.2±0.2	53.7±4.2A	42.4±12.0	26.9±4.1	8.3±1.0
5×10^8	409.3±66.9	14.9±2.3A	20.9±3.2	2.4±0.6	7.7±0.8	2.0±0.4	36.9±5.7AB	41.4±13.2	18.4±2.3	7.1±1.2
P_{ANOVA} / $P_{orthogonal}$	0.086	**0.024/0.002**	0.317	0.568	**0.010**	0.191	**0.029/0.022**	0.421	**0.010**	0.132
Interaction	0.344	0.217	0.517	0.181	**0.037**	0.157	0.051	0.573	**0.011**	0.945

P_{ANOVA} is the *P* value of the factorial ANOVA / $P_{orthogonal}$ is the *P* value of the orthogonal contrast between doses of *M. brunneum*. Interaction is the *P* value of Soil x *M. brunneum* dose of the factorial ANOVA. The lowercase letters shows the difference of means between the different soils with the LSD test. The capital letters shows the difference of means between the different doses with the orthogonal analysis.

Figure 1

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Fig. 1

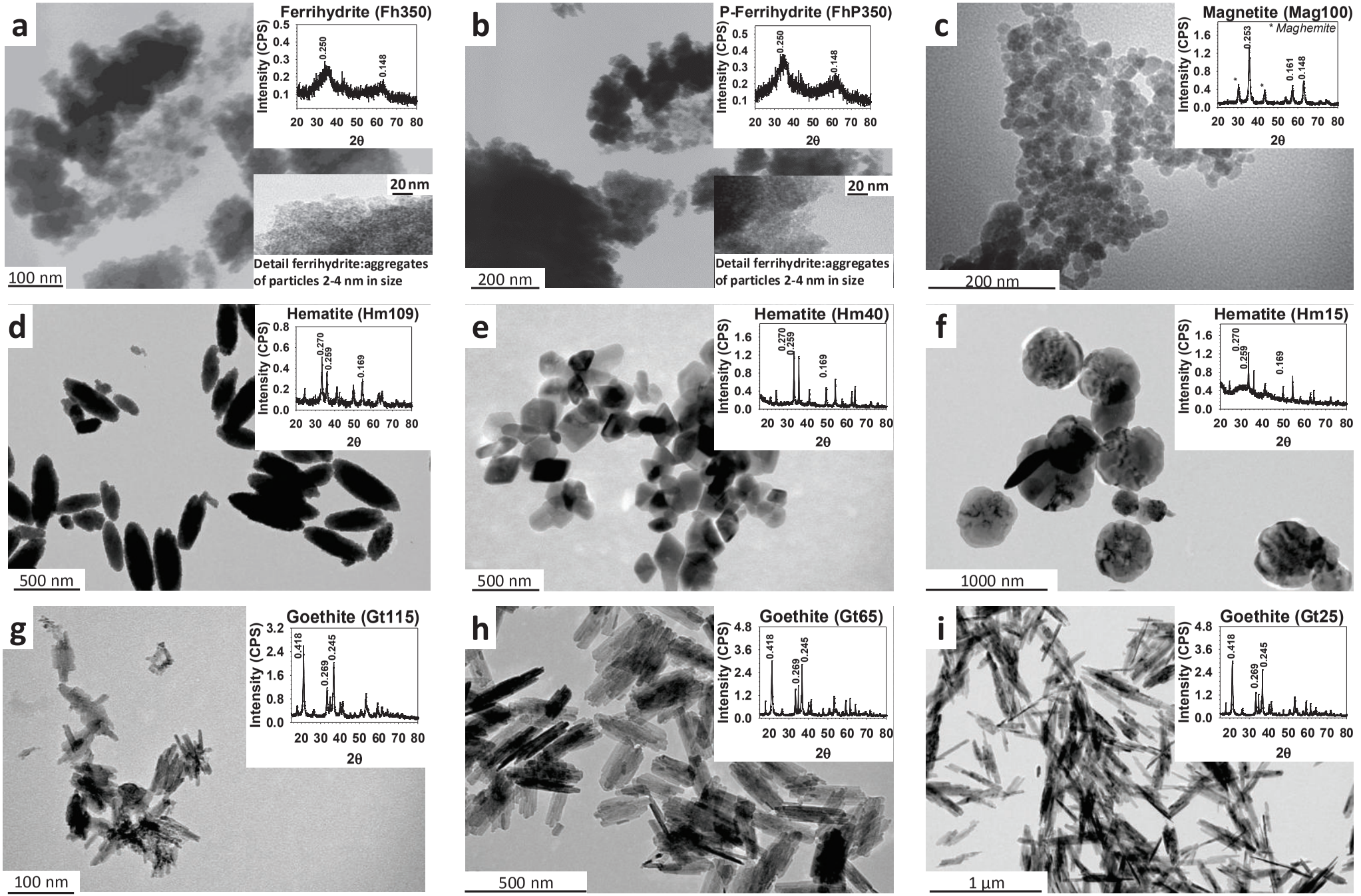

Figure 4

Fig. 4

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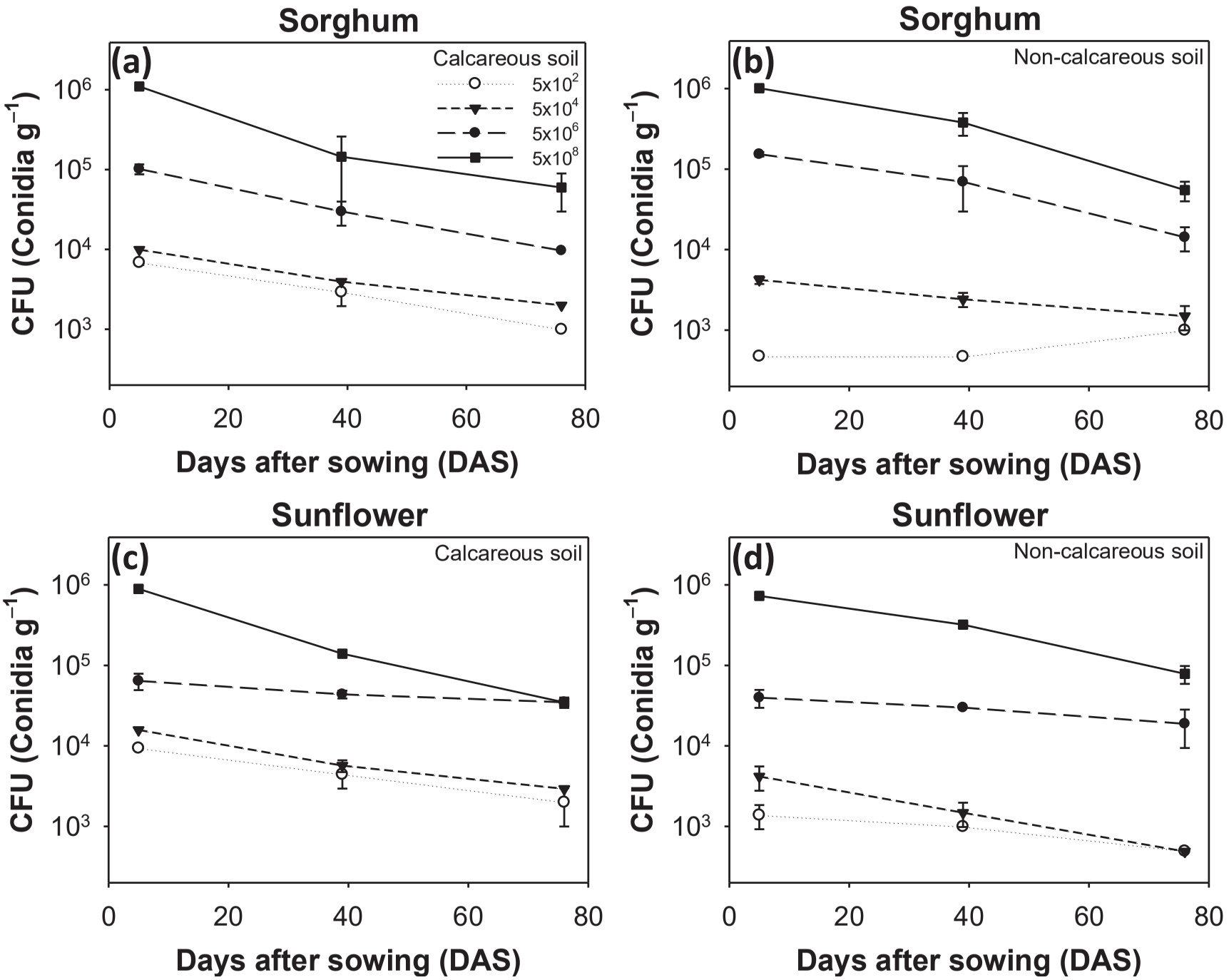

Figure 5
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Fig. 5

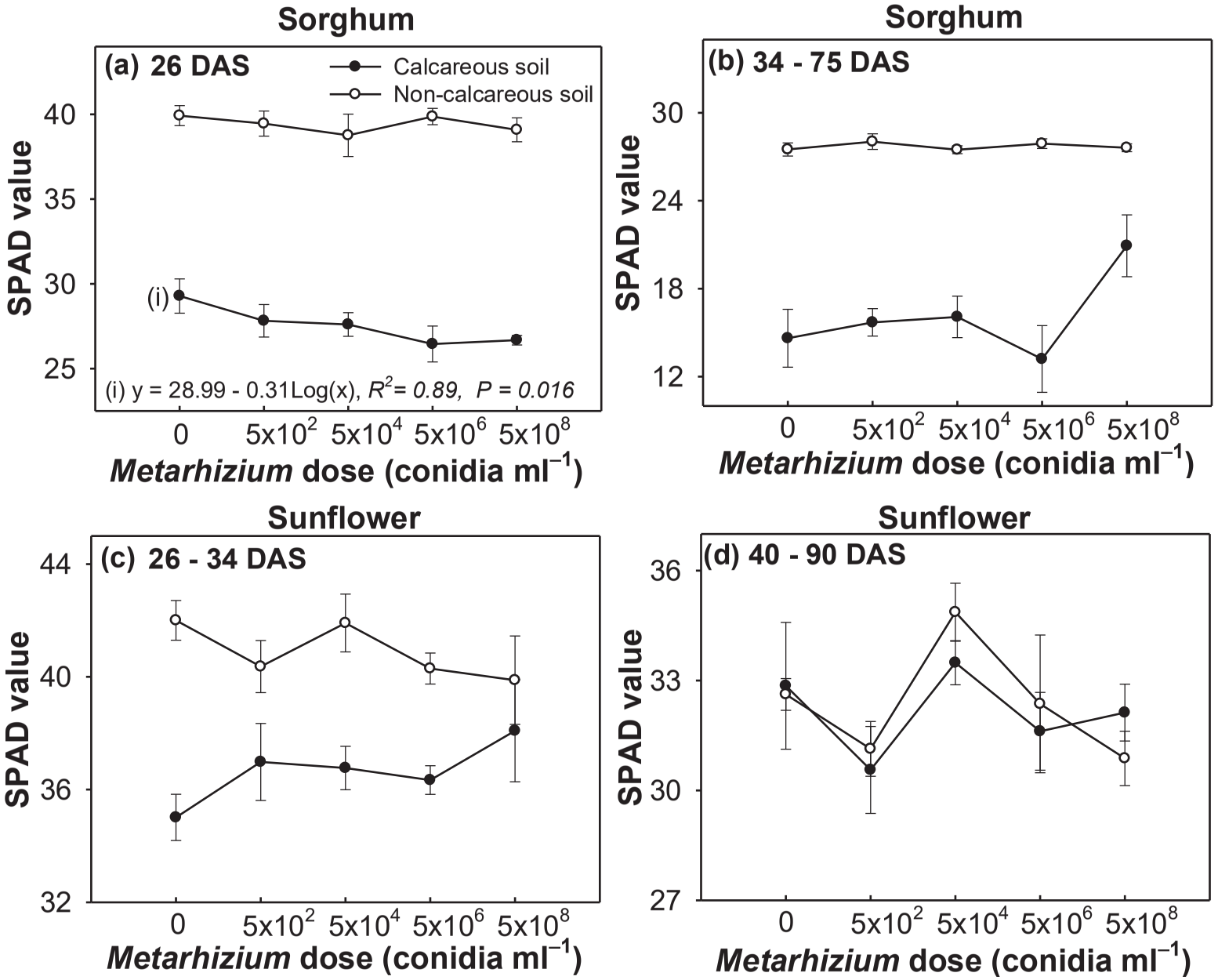

Figure 6

Fig. 6

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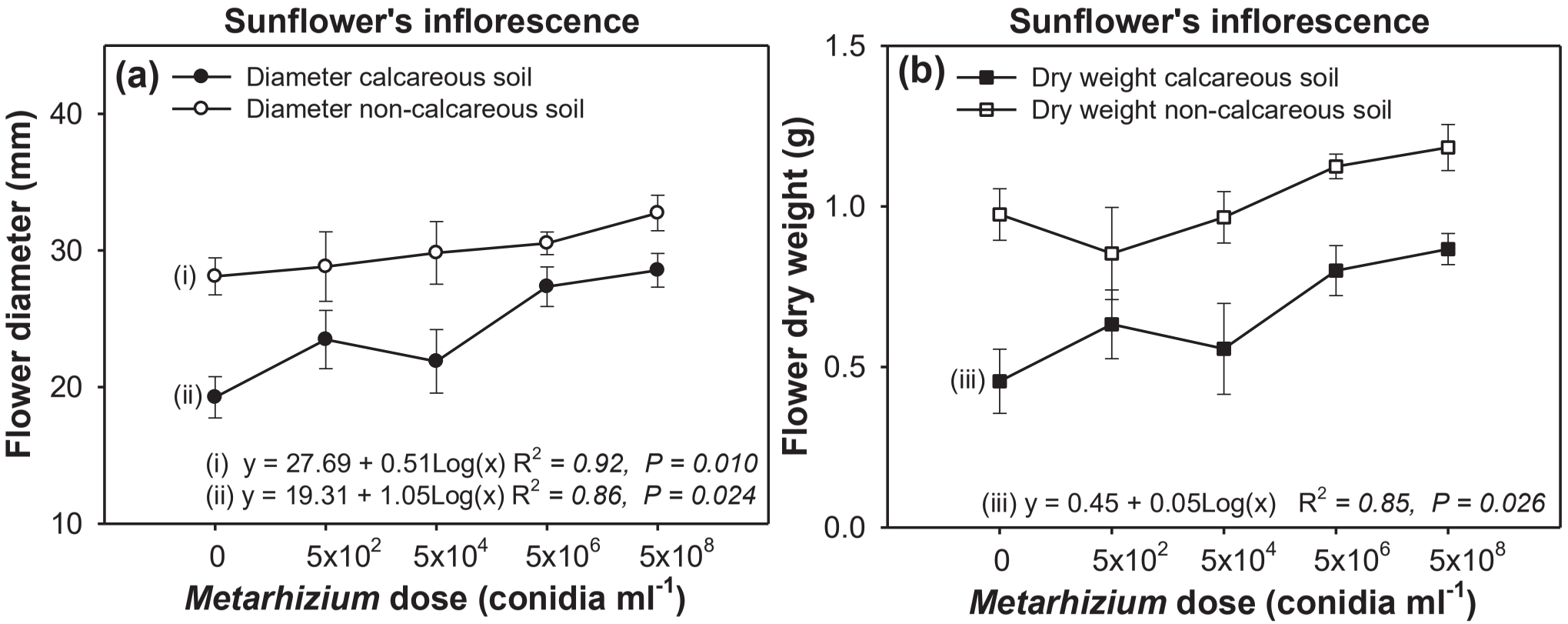

Figure 2
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Fig. 2

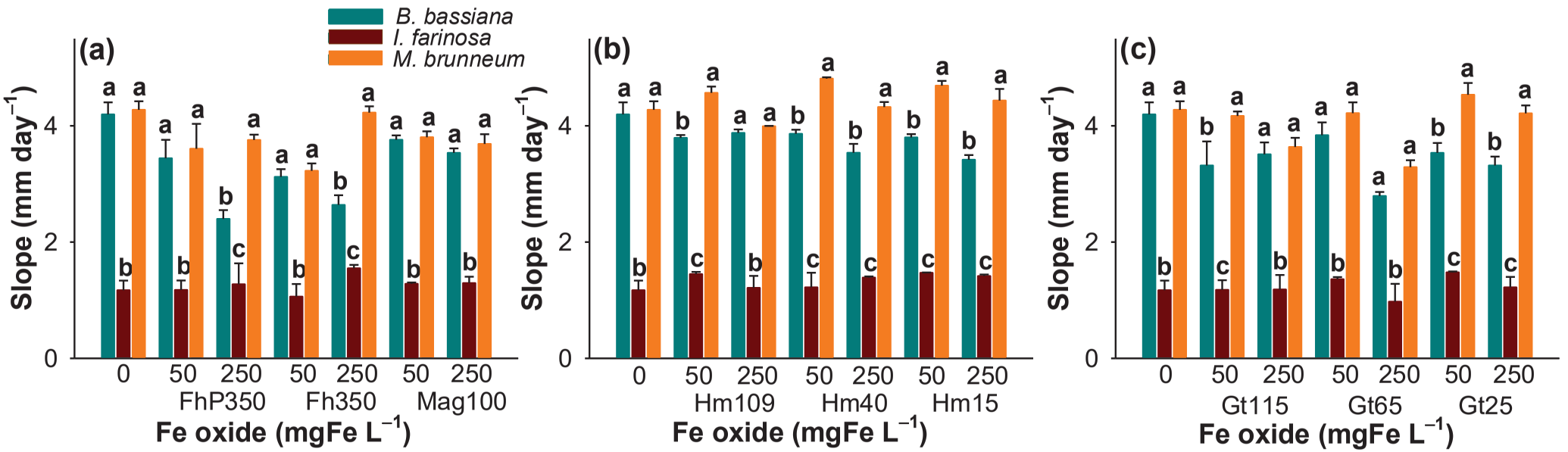

Figure 3
Fig. 3

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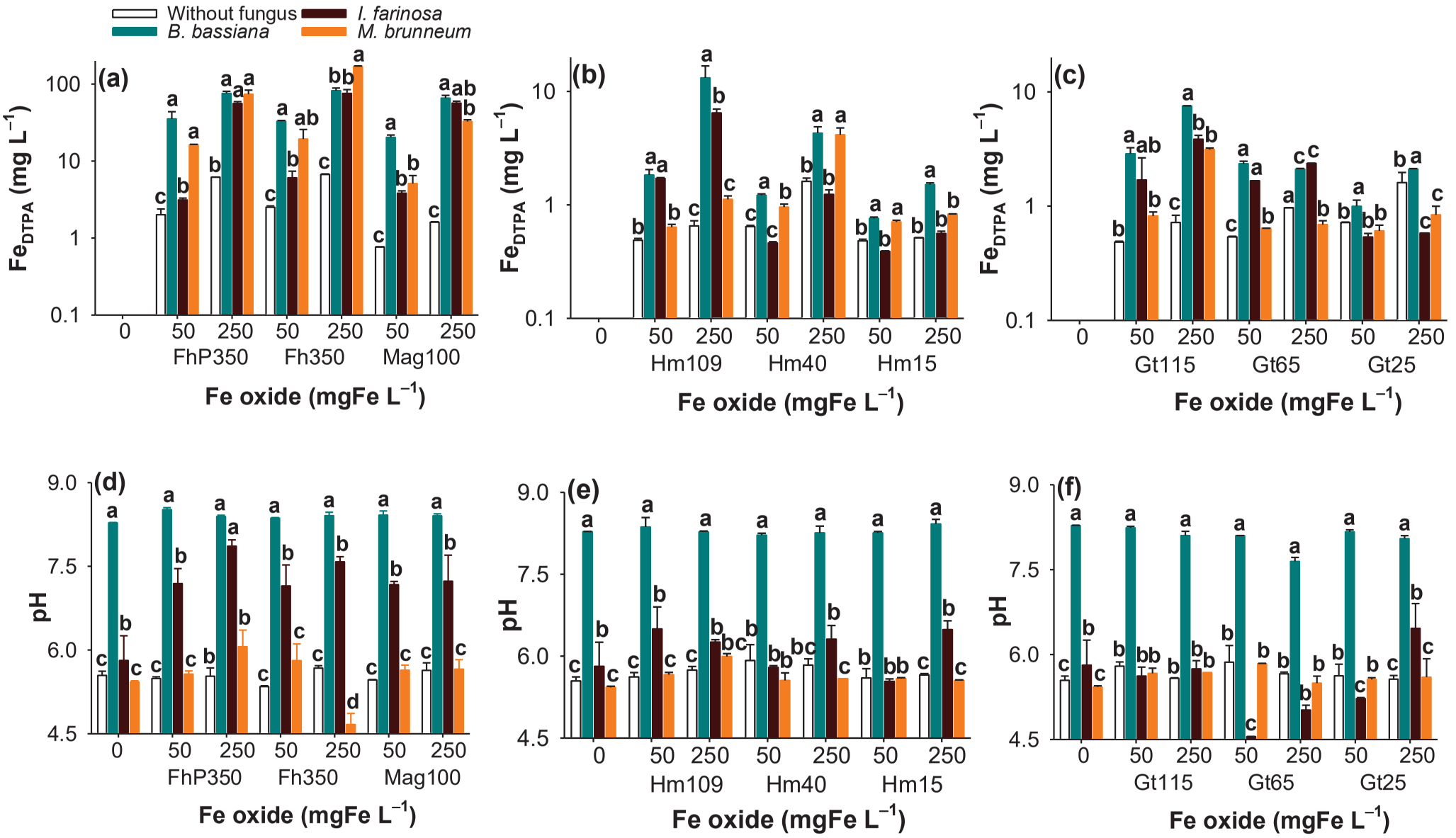

Table S1 *In vivo* assay. Factorial ANOVA for diameter and dry weight of sunflower inflorescence (mean ± standard error; calcareous soil: $n = 5$, non-calcareous soil: $n = 6$).

	Inflorescence of sunflower	
	Diameter (cm)	Dry weight (g)
Soil		
Calcareous soil	24.0±1.1b	0.7±0.1b
Sandy soil	29.9±0.8a	1.0±0.0a
P_{ANOVA}	**< 0.001**	**< 0.001**
***M. brunneum* dose (conidia ml^{-1})**		
Control	24.1± 1.8B	0.7±0.1B
5×10^2	26.4± 1.8B	0.8±0.1B
5×10^4	25.8±2.0B	0.8±0.1B
5×10^6	29.1±0.9A	1.0±0.1A
5×10^8	30.9±1.1A	1.0±0.1A
P_{ANOVA} / $P_{orthogonal}$	**0.007 / 0.001**	**0.011 / 0.001**
Interaction	0.454	0.624

P_{ANOVA} is the P value of the factorial ANOVA / $P_{orthogonal}$ is the P value of the orthogonal contrast between doses of *M. brunneum.* Interaction is the P value of Soil x *M. brunneum* dose of the factorial ANOVA.

The lowercase letters shows the difference of means between the different soils with the LSD test.

The capital letters shows the difference of means between the different doses with the orthogonal analysis.

Table S2 *In vivo* assay. Factorial ANOVA for nutrient concentrations in rhizospheric soil at the end of the experiment (mean ± standard error with $n = 5$ for the calcareous soil and $n = 6$ for the non-calcareous soil).

	Fe_{DTPA} (mg kg^{-1})	Mn_{DTPA} (mg kg^{-1})	Zn_{DTPA} (mg kg^{-1})	Cu_{DTPA} (mg kg^{-1})	K_{aa} (mg kg^{-1})
Soil	**Sorghum**				
Calcareous soil	2.5±0.0b	3.1±0.1b	2.9±0.0a	19.8±0.1	121.7±2.3
Sandy soil	50.5±0.5a	48.4±0.9a	1.3±0.0b	4.0±0.0	93.7±1.4
P_{ANOVA}	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
***M. brunneum* dose (conidia ml^{-1})**					
Control	25.7±7.7	24.6±7.2	2.2±0.3A	11.9±2.6	112.0±7.7
5×10^2	29.2±7.7	28.6±7.4	2.1±0.3A	11.5±2.6	109.3±5.9
5×10^4	28.4±8.2	26.6±7.6	1.9±0.3B	10.8±2.7	103.2±4.5
5×10^6	32.3±8.1	31.4±7.8	1.9±0.3B	10.1±2.5	101.1±3.0
5×10^8	31.2±7.8	31.0±7.6	1.9±0.3B	10.5±2.6	103.8±3.6
P_{ANOVA} / $P_{orthogonal}$	0.320	0.496	**0.039/0.003**	**< 0.001**	**0.007**
Interaction	0.261	0.383	0.361	**0.005**	**< 0.001**
Soil	**Sunflower**				
Calcareous soil	2.5±0.0b	4.3±0.2b	2.8±0.0a	19.8±0.3	137.4±2.6
Sandy soil	53.9±0.7a	52.4±2.1a	1.3±0.0b	3.9±0.1	99.1±2.1
P_{ANOVA}	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**	**< 0.001**
***M. brunneum* dose (conidia ml^{-1})**					
Control	32.4±9.5	37.2±10.5A	2.0±0.3B	11.0±2.8	123.0±7.5
5×10^2	30.5±8.1	32.0±7.9B	2.0±0.2B	10.6±2.3	113.8±7.7
5×10^4	26.7±8.1	24.2±7.1B	2.0±0.3B	11.8±2.6	112.9±4.8
5×10^6	32.2±9.4	31.2±9.4B	2.0±0.3B	10.9±2.8	111.3±6.5
5×10^8	30.4±8.8	27.6±7.4B	2.1±0.3A	11.6±3.1	123.5±9.5
P_{ANOVA} / $P_{orthogonal}$	0.124	**0.044 / 0.008**	**0.005 / 0.005**	**0.014**	**0.003**
Interaction	0.109	0.185	0.165	**0.007**	**0.023**

P_{ANOVA} is the P value of the factorial ANOVA / $P_{orthogonal}$ is the P value of the orthogonal contrast between doses of *M. brunneum.* Interaction is the P value of Soil x *M. brunneum* dose of the factorial ANOVA.

The lowercase letters shows the difference of means between the different soils with the LSD test.The capital letters shows the difference of means between the different doses with the orthogonal analysis. Fe_{DTPA}, Mn_{DTPA}, Zn_{DTPA} and Cu_{DTPA}: Diethylenetriaminepentaacetic acid-extractable Fe, Mn, Zn and Cu, respectively; K_{aa}: Ammonium acetate-extractable potassium.