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Alleviation of Fe-induced chlorosis of soybean plants grown in calcareous soil by a freeze-dried iron fertilizer containing siderophores produced by *Rhizobium radiobacter*

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ABSTRACT

The concerns about the unsustainability of traditional synthetic Fe chelates lead to the search for new environmentally friendly alternatives, such as siderophores-based fertilizers. In this work, the focus was on the evaluation of a bio-based Fe-chelate fertilizer obtained from the culture of the siderophore-producer bacterium *Rhizobium radiobacter*. The suitability of a freeze-dried fertilizer formulated from a *R. radiobacter* culture labeled with ^{57}Fe to alleviate Fe chlorosis in soybean plants cultivated in calcareous soil was analyzed and the new potential Fe fertilizer was evaluated in comparison to the traditional synthetic chelate *o,o*EDDHA/$^{57}Fe^{3+}$. This natural chelate was able to maintain chlorophyll content stable during all the pot trials and presented greater Fe concentration in the remaining soil fractions serving as an Fe pool for a long time whereas *o,o*EDDHA/$^{57}Fe^{3+}$ could supply Fe quickly. The new bio-based Fe siderophore fertilizer, derived from *R. radiobacter* culture, could be a green substitute to conventional synthetic chelates to address Fe chlorosis in calcareous soil conditions.

1. Introduction

Iron (Fe) is one of the essential micronutrients required for plant nutrition. Its role is crucial in various essential plant processes, such as chlorophyll biosynthesis [1,2]. The lack of Fe leads to a reduction in chlorophyll levels in plant leaves, showing characteristic visual symptoms through interveinal leaf yellowing [3]. This chlorosis is a major agricultural problem leading to a yield reduction or crop failure [4], especially in calcareous soil. This type of soil constitutes a third of the extension of harvested soil [5,6] characterized by a high pH ranging between 7.5 and 8 [7], maintained by the buffer effects calcium and bicarbonate ions in the soil solution. Only a small fraction of Fe in the soil is available to plants due to the poor Fe solubility in high pH values [8]. Fruit crops are among the most susceptible plants affected by Fe chlorosis such as pear, peach [9] or citrus [10]; however, other crops such as soybean are highly sensitive to Fe deficiency [11,12], being an economically important legume [13].

To alleviate Fe chlorosis, a common and effective strategy is the use of Fe synthetic chelates treatments. Most of them are derived from aminopolycarboxylic acid (APCAs) compounds, being the most frequently used: ethylenediamine tetraacetic acid (EDTA) and ethylenediamine-N,N′-bis(o-hydroxyphenylacetic) acid (*o,o*EDDHA) [14]. In general, Fe chelates maintain Fe in the soil solution in a wide pH range. Once the chelate has supplied Fe to the plant, a fraction of the ligand is capable of mobilizing Fe from natural sources, maintaining the Fe nutrition for a longer period (named “shuttle effect”) [8]. The Fe chelate *o,o*EDDHA/Fe^{3+} is a well-known effective Fe fertilizer since it is stable at different pH values and soils. However, due to its high cost, this fertilizer is mainly used in high-value crops [15]. Conversely, in the last years, conventional Fe chelates are under scrutiny. Some concerns about them include their persistence in soil and the mobilization of heavy metals

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Abbreviations

AAS-FA	atomic absorption spectroscopy flame atomization
APCAs	aminopolycarboxylic acids
CC	capacity of complexation
DAT	days after treatment
DW	dry weight
DTPA	diethylenetriaminepentaacetic acid
EDDHA	ethylenediamine-N,N′-bis(o-hydroxyphenylacetic)
EDTA	ethylenediamine tetraacetic acid
Fe_{Fer}	fertilizer-derived Fe
Fe_{Nat}	naturally-derived Fe
ICP-OES	Inductively Coupled Plasma Optical-Emission Spectrometry
ICP-MS	Inductively Coupled Plasma-Mass spectrometry
ICC	Iron Complexation Capacity
NS	nutrient solution
RR	*Rhizobium radiobacter* secretion
SPAD	Soil and Plant Analyzer Development
WHC	water-holding capacity

[16].

Therefore, many studies are currently focused on assessing more sustainable options to replace traditional Fe chelates to correct the Fe deficiency. These alternatives should achieve similar effectiveness to amend Fe deficiency, cause low environmental impact, and be feasible in the long-term [17]. Among different sustainable alternatives, siderophores have been studied as Fe sources and potential substitutes for synthetic APCAs in plants grown in hydroponic [18] and soil [17]. Siderophores are biomolecules with low molecular-weight capable of complex Fe since they present common functional groups, such as catechol and hydroxamate which provide them high affinity to bind Fe^{3+} [19]. Some plants and microorganisms, mainly by bacteria, can generate siderophores in response to Fe scarcity, as was recently reviewed [20]. They can bind Fe, giving rise to strong Fe complexes that are potentially biodegradable [21]. *Rhizobium radiobacter* is a non-pathogenic plant growth-promoting bacteria, classified as a biological risk group 1, considered unlikely to cause human disease safety. This bacterium produces a siderophore, named agrobactin, based on tri-catecholate, [21,22]; thus, it looks to be suited for the complexation of Fe under alkaline conditions.

The use of ^{57}Fe-fertilizer as a tracer has been demonstrated advantageous in studying the efficiency of Fe treatments in soil [23,24]. The use of stable ^{57}Fe-labeled was applied in different previous studies to evaluate the Fe uptake in different plant organs [10,25,26] and to assess the Fe distribution from natural sources (Fe_{Nat}) or provided by the application of Fe fertilizer under study (Fe_{Fert}) [11,24]. This approach provides information about the behavior of the Fe fertilizer monitoring the Fe uptake, translocation, and distribution among the plant organs [15] with a stable isotope.

This work aimed to evaluate the application of a freeze-dried formulation prepared from a *R. radiobacter* culture, chelated with the stable isotope ^{57}Fe (named RR/$^{57}Fe^{3+}$), to amend Fe deficiency of soybean plants cultivated in calcareous soil. The effectiveness of the Fe fertilizer was studied in both a single and repeated dosage, in comparison with the conventional chelate *o,o*EDDHA/$^{57}Fe^{3+}$.

2. Materials and methods

2.1. Preparation of the freeze-dried product

Rhizobium radiobacter DSM 30205 was obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), Germany. For siderophore production, cultures were prepared as previously described [27]. Briefly, the bacteria, in the exponential phase of growth, were inoculated in 5 L Erlenmeyer flasks containing 3 L of Fe-deficient minimal medium (MM): 10.0 g L^{-1} glucose, 1.47 g L^{-1} glutamic acid, 3.0 g L^{-1} K_2HPO_4, 1.0 g L^{-1} KH_2PO_4, 0.5 g L^{-1} NH_4Cl, 0.1 g L^{-1} NH_4NO_3, 0.1 g L^{-1} Na_2SO_4, 10 mg L^{-1} $MgSO_4{\cdot}7H_2O$, 1 mg L^{-1} $MnSO_4.4H_2O$, and 0.5 mg L^{-1} $CaCl_2$; the pH was set to 7.0 ± 0.1. Cells were incubated on an orbital shaker at 150 rpm, at 30 °C. After 72 h of growth, bacterial cells were removed by centrifugation (2500×*g*, 10 min, 4 °C), and the supernatant was filtered through a 0.45-μm pore size membrane.

Before freeze-drying the culture, the estimation of the siderophore concentration and its qualification was accomplished (see Section 2.2). Moreover, the ability of the siderophores, present in the resultant filtrate to complex Fe was evaluated (see Section 2.3). The filtrate obtained was mixed with 10 g L^{-1} corn starch, serving as an anticaking agent. Subsequently, the mixture was freeze-dried (Labconco FreeZone 2.5 L coupled with a VacuuBrand RC 6 pump, USA), and homogenized, obtaining the freeze-dried fertilizer objective of the study. The product was stored at 4 °C.

2.2. Estimation of siderophore concentration and respective qualification

After the 72 h growth period, the siderophore concentration in the filtered supernatants was estimated by UV–Vis spectroscopy, recording the maximum absorbance at 310–320 nm and using the molar extinction coefficient (ε) of 9600 M^{-1} cm^{-1} [22]. The presence of siderophore in the filtrate with a catecholate group was confirmed using Arnow's method [28].

2.3. Assessment of siderophore (Fe) complexation capacity

The iron complexation capacity (ICC) of the siderophore in solution was determined using a procedure adapted from Villén et al. [29]. For this purpose, to a fixed volume of culture filtrate, increasing amounts of $FeCl_3$ were added and the pH was adjusted to 9.0 ± 0.1 and let to rest for 30 min. Then, pH was corrected again and let to settle for 3 h. Subsequently, the solution was centrifuged (3000×*g*, 10 min) and filtered by a 0.45 μm pore size membrane and the amount of Fe in solution was quantifed by atomic absorption spectroscopy with flame atomization. The ICC was determined graphically after plotting the [Fe]complex versus [Fe]added, where [Fe]complex is the concentration of Fe in the filtrate and [Fe]added is the total Fe concentration added.

2.4. Fe chelated solutions

Fertilizer containing siderophores obtained from *R. radiobacter* culture and the *o,o*EDDHA ligands were chelated with ^{57}Fe solutions. For that, ^{57}Fe (metal, 96.66 %; Isoflex, Moscow, Russian Federation) already dissolved in HNO_3 Suprapur (Merck, Germany), was gradually mixed with the corresponding ligand solution.

For the *o,o*EDDHA/$^{57}Fe^{3+}$ treatment, an excess of 2 % of ligand *o,o*EDDHA (97.94 %; LGC Standards, UK) over the molar amount of Fe (molar ratio ligand:Fe 1:1), was firstly dissolved in NaOH (1:3 M ratio). During the addition of the Fe solution for the chelation process, the pH was maintained within the range of 6 and 8 and then fixed to 7.0. The chelate solution, after being kept overnight in darkness to prevent potential photodecomposition, was filtered through 0.45 μm Millipore filter. After filtration, solution was made up to volume with ultrapure water (Millipore, Milford, USA), with a final concentration of 570 mg $^{57}Fe{\cdot}L^{-1}$.

For the preparation of the filtrate (from *R. radiobacter* culture) labeled with ^{57}Fe (RR/$^{57}Fe^{3+}$), the freeze-dried powder, obtained previously, was similarly prepared by dissolving in ultrapure water and added to be 2 % in excess taken into account the Fe complexation capacity determined in the filtrate as mentioned in Section 2.3 After

allowing the solution to settle for 3 h, it was filtered using a 0.45-μm pore size filter, resulting in a solution containing 52.2 mg $^{57}Fe{\cdot}L^{-1}$.

2.5. Plant experiments

Germination of soybean *(Glycine* max L, cv. RGT Speeda) seeds was done using a standard procedure on sterile trays with water-moistened perlite (1–5 mm grain; Projar, Spain) in the absence of light in a climate chamber fixed at 30 °C and 60 % relative humidity for four days. Seedlings were then placed on a perforated plate floating in a new tray, situated above the nutrient solution (NS) 1/5 diluted containing 5 μmol L^{-1} EDTA/Fe^{3+} for 4 days. Then, to induce Fe deficiency, plants were grown with a full-strength NS containing a low Fe dose of 2 μmol L^{-1} EDTA/Fe^{3+} for three more days. The composition of NS was: macronutrients (mmolL^{-1}): 1.0 $Ca(NO_3)_2$, 0.9 KNO_3, 0.3 $MgSO_4$, and 0.1 KH_2PO_4; 115.5 μmol L^{-1} Na_2EDTA buffered micronutrients (μmolL^{-1}) 2.5 $MnSO_4$, 1.0 $CuSO_4$, 10 $ZnSO_4$, 1.0 $NiCl_2$, 1.0 $CoSO_4$, and other micronutrients (μmolL^{-1}): 35 NaCl, 10 H_3BO_3, and 0.05 Na_2MoO_4. The pH was fixed to 7.5 with 1.0 mol L^{-1} KOH to replicate calcareous soil conditions.

After the hydroponic pre-growth period, seedlings of the same size and appearance were moved to pots. Three plants per pot were transferred to methacrylate cylinders measuring 9 cm in diameter and 14 cm in height. Each pot contained 0.95 kg of a soil:sand mixture (70:30, w:w). The soil used was an agricultural soil from Picassent (Valencia, Spain) whose main characteristics were previously described in López-Rayo et al. [30] (Table S1). The soil was sieved at 4 mm and mixed with a standard calcareous sand (975 g kg^{-1} $CaCO_3$, Table S2). Two days before transplantation, the pots were watered until they reached 80 % of their water-holding capacity (WHC). Throughout the experiment, pots were irrigated four days a week to maintain 80 % WHC monitored by weight. The irrigation water contained 0.1 g L^{-1} $CaCO_3$ and 0.1 g L^{-1} $NaHCO_3$ (pH between 8.0 and 8.5) to simulate typical conditions of the Mediterranean agronomic areas. To manage the possible leaching, pots were placed over a Petri dish.

At this point, the Fe treatments were applied to the pots. They included the RR/$^{57}Fe^{3+}$, RR/$^{57}Fe^{3+}$(2), *o,o*EDDHA/$^{57}Fe^{3+}$ as the positive control (C+), and no Fe application (C-) as the negative control. The C+ was applied as 2.8 mL of ^{57}Fe *o,o*EDDHA/$^{57}Fe^{3+}$ concentrated solution described above and the RR/$^{57}Fe^{3+}$ as 30 mL of the mentioned prepared RR/$^{57}Fe^{3+}$ solution. They were applied on the top of each pot to achieve a dose of 2.4 mg of ^{57}Fe per kg of soil, being similar to those assayed in similar studies with other Fe sources [17,24]. Also, a macronutrient solution was added to the pots corresponding to the positive and negative controls to balance other nutritional elements different than Fe presented in the *R. radiobacter* culture and previously analyzed. A second application was done in pots amended with RR/$^{57}Fe^{3+}$ (named RR/$^{57}Fe^{3+}$ (2)), thus, also assaying a repeated application at 16 DAT (days after first treatment) of the same quantity was done. Five replicate pots with three plants were assayed per treatment.

2.6. Plant analysis

The chlorophyll index was determined using a chlorophyll meter SPAD (Soil and Plant Analyzer Development, Minolta). SPAD values indirectly assess chlorophyll concentration in leaves by measuring the transmittance of two wavelengths (650 and 940 nm) [13]. The SPAD measurement was taken at different leaf levels in triplicate throughout the experiment ranging from the cotyledons to the topmost part of the plant. The SPAD measurements were performed for 3rd and 5th expanded leaves in all the plants. The harvest was carried out at 14 and 32 days after treatment (DAT). In the first sampling, the leaves and stems of two plants were collected. In the second sampling, the remaining plant and the three roots were harvested.

Plant samples were washed with a solution containing 0.1 % (w/v) HCl and 0.01 % (w/v) non-ionic detergent (Tween 80, Probus, Barcelona, Spain), and twice with MilliQ water [31]. The dry weight (DW) was determined after 6 days at 65 °C in a forced air oven. Subsequently, plant samples were milled with a titanium mill (Retsch ZM200, Retsch Gmbh, Haan, Germany). To analyze the mineral concentration, plant parts were calcined at 500 °C for 4h and then, the ashes were digested with HNO_3 Suprapur (Merck).

The total Fe, Mn, and Zn concentrations in the filtrate samples were measured by Atomic Absorption Spectroscopy Flame atomization (AAS-FA) (PerkinElmer AAnalyst 400 spectrometer (Norwalk, CT, USA), and Cu concentration was determined by Inductively Coupled Plasma Optical-Emission Spectrometry (ICP-OES) (Thermo iCAP 7400 series, Bremen, Germany). The concentration of Fe isotopes was assessed using an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) (NexION 300XX, PerkinElmer, Waltham, MA, USA). The total Fe concentration was mathematically divided into naturally-derived Fe (Fe_{nat}) and fertilizer-derived Fe (Fe_{fer}) through isotope pattern deconvolution analysis [32].

2.7. Soil and root analysis

At 32 DAT, the roots were carefully extracted from the soil and washed with the same procedure described earlier for aerial plant samples. To quantify the Fe in the soluble and available fractions of the soil, the entire pot content was immersed in 0.6 L of distilled water and shaken for 10 min [33]. Subsequently, 40 mL of the suspension was centrifuged (6000 cycles min^{-1}) for 5 min. The resulting supernatant was filtered through a 0.45 μm membrane, followed by the addition of HNO_3 Suprapur (1 %) to obtain the Fe soluble fraction. Afterward, to obtain the available fraction, the soil remaining in the centrifuge tube was extracted with 25 mL of diethylenetriaminepentaacetic acid (DTPA) solution [34] and then filtered [35]. Both the soluble and available soil fractions were analyzed for Fe isotope concentrations by ICP-MS.

2.8. Statistical analysis

The results obtained for all the plant and soil parameters after the experiment were submitted to a statistical analysis through the SPSS software (version 26.0; SPSS Inc., Chicago, IL, USA). To discern significant differences among the treatments, a one-way analysis of variance (ANOVA) was conducted, and subsequently, a Duncan's post hoc test ($p < 0.05$ or $p < 0.01$) was performed.

3. Results and discussion

3.1. Bacterial siderophore production: quantification and iron complexation in the bacterial culture filtrate

In this work, after culturing *R. radiobacter* in a mineral iron-deficient medium, and subsequent harvest and filtration of the culture supernatant at 72 h after inoculum, siderophore production was detected by UV–Vis spectroscopy (Fig. 1), at 310–320 nm, a wavelength typically associated with catecholate-based molecules [22]. The Arnow's test [28] (detection of catecholates) further confirmed the presence of catecholates in the culture supernatant (data not shown). The concentrations of siderophore were estimated by UV–Vis in distinct culture times (Table 1). The similarity between the spectra presented in this work and those described in the literature [22] suggests that agrobactin should be the main, if not the only, siderophore produced by the bacteria. Together, these results demonstrate that no siderophores were present in the initial culture medium, but they were produced throughout the 72 h of the bacteria growing. Moreover, the ability of the Fe complexation in the bacterial culture filtrate was evaluated (Fig. 2), at pH 9.0, with increasing concentrations of Fe added, following the adaptation of a method validated according to the European regulations on fertilizers [36]. Plot in Fig. 2 evidences a slope close to 1 as a result of the high

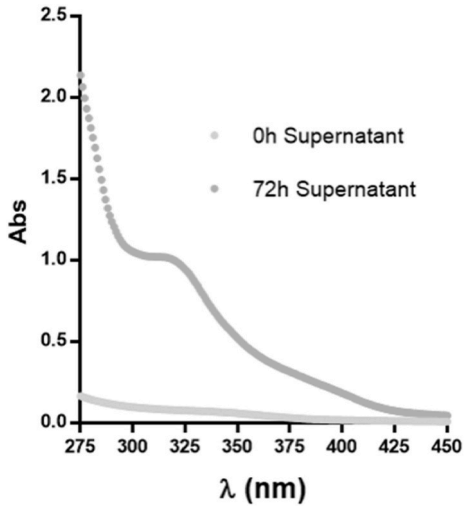

Fig. 1. UV–Vis absorption spectra of *R. radiobacter* culture supernatants collected at 0 and 72 h.

Table 1
Evolution of siderophore production during *R. radiobacter* culture.

Time (h)	[siderophore] (μmol L^{-1})
0	12 ± 5
24	35 ± 2
48	72 ± 2
72	103 ± 4

Data are means ± standard error ($n = 8$).

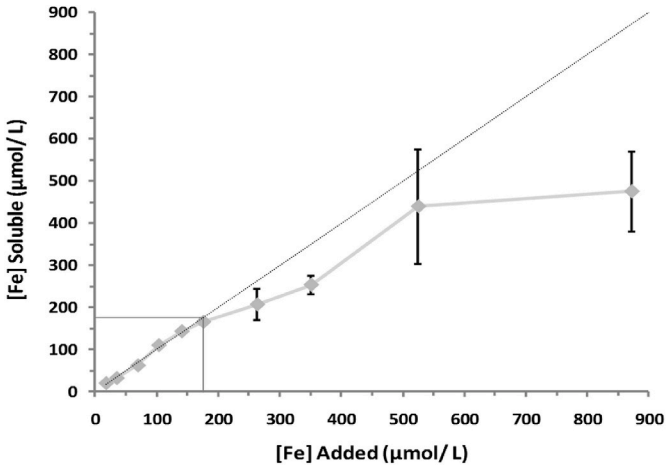

Fig. 2. Depiction of the amount of iron complexed at pH 9.0 in function of the quantity of Fe added to supernatant samples. Each value represents the mean and standard deviation of at least three determinations ($n \geq 3$). The black traced line represents a line with a slope = 1. The black vertical line defines the Iron Complexion Capacity (ICC).

stability of the chelate formed between iron and the siderophore(s) present and a consequent effectiveness of complexation. The capacity of complexation (CC) of the supernatant samples for iron corresponds to 175 μmol L^{-1} however, increasing the iron addition it is possible complex Fe till near 450 μmol L^{-1}, corresponding to the formation of weaker complexes. The modification of the *R. radiobacter* supernatant color with the increasing addition of Fe (Fig. S1), suggests the formation of a wine-red colored complex between iron and siderophore in a 1:1 ratio. Probably this siderophore is agrobactin, since catecholates form colored complexes with Fe (III), matching the color observed [37]. Furthermore, the color intensity does not change after 180 μmol L^{-1}, a value coherent with the CC (Fig. 2).

3.2. SPAD index and growth parameters

During the experiments, the "regreening" of the plants was assessed by the SPAD chlorophyll index for the 3rd (the youngest leaf at the moment of treatment application, Fig. 3) and 5th leaf levels (the youngest fully developed leaf at the end of the experiment, Fig. 4), being the most relevant to observe the effects of the treatments. The results presented in both figures correspond to expanded leaves. Additionally, the visual aspect of plants 14 DAT is presented in the Supplementary Material Fig. S2. With a single application of RR/$^{57}Fe^{3+}$, uniform SPAD levels were achieved throughout the experiment and reached similar values to C+ (Fig. 3). Fig. 3 shows that SPAD values at the 3rd leaf stage were higher than those of the untreated control (C-), with differences remaining consistent throughout the experiment. Although these differences were not statistically significant at the 5th leaf level, a similar trend was observed.

Soybean plants without Fe application (C-) showed the lowest SPAD values at 3rd stage during the whole experiment compared to the rest of the treatments, indicating the Fe-chlorosis status of these plants. In contrast, positive control (C+) plants showed the highest levels of chlorophyll content until 16 DAT but towards the end of the experiment, they finally decreased. The SPAD index for C+ decreased more than that of the RR/$^{57}Fe^{3+}$ treatment at the 3rd stage (Fig. 3), with no significant differences between these two treatments by the end of the experiment at either the 3rd or 5th stages (Fig. 5). This result aligns with findings from Ferreira et al. [38] and Nadal et al. [24], which suggest that Fe from *o,o*EDDHA/Fe^{3+} is rapidly taken up by plants but after a short period, plant uptake mechanisms are inhibited or repressed, leading to Fe dilution in plant and reduced chlorophyll content during later stages. This fact will be also discussed based on the Fe_{Fert} concentrations (section 3.3.).

Repeated application of the fertilizer containing siderophores [RR/$^{57}Fe^{3+}$(2)] at 16 DAT produced an increase in chlorophyll index, which was maintained throughout the experiment (32 DAT) (Fig. 3). Similar SPAD results were obtained by Martins et al. [18] when Fe-based fertilizer with synthetic siderophores (azotochelin) were applied to cucumber plants grown in hydroponics, and by Ferreira et al. [17] when using an Fe-based fertilizer containing natural siderophore from *Azotobacter vinelandii* in soybean in calcareous soil conditions. Thus, positive results were obtained when applying a double dose of a Fe fertilizer containing the secretion of siderophore-producing bacteria.

This was expected since the Fe mobility in the plant is scarce thus, an additional Fe supplementation by the RR/$^{57}Fe^{3+}$(2) could improve the Fe chlorophyll concentration. Although chlorosis typically appears more evident in younger leaves (5th stage) than older leaves, Fig. 4 shows that, by 32 DAT, Fe chlorosis was most pronounced at the 3rd leaf stage, while SPAD indexes measured for the 5th levels did not show differences between the treatments. This is in consonance to the low mobility of Fe in plants. While soybean plants primarily uptook Fe from the Fe treatments, by the end of the experiment, Fe from the native soil, despite being Fe-deficient, could have served as Fe source, even in the untreated control (C-). This affected the chlorophyll index and reduced the differences between treatments.

Table 2 shows the dry weight (DW) results at 14 and 32 DAT for each plant organ. At 14 DAT, soybean plants supplied by *o,o*EDDHA/Fe^{3+} (C+) showed the highest DW in both leaves and stems. Plants treated with fertilizer containing siderophore (RR/$^{57}Fe^{3+}$) presented an intermediate value of the DW, i.e., between positive and negative control, being negative control plants those with the less leaf yield. In the second sampling time (32 DAT), RR/$^{57}Fe^{3+}$(2) showed less DW in leaves while RR/$^{57}Fe^{3+}$ and C- were similar. Again, the positive control showed higher DW in leaves. However, there were no differences in stems DW.

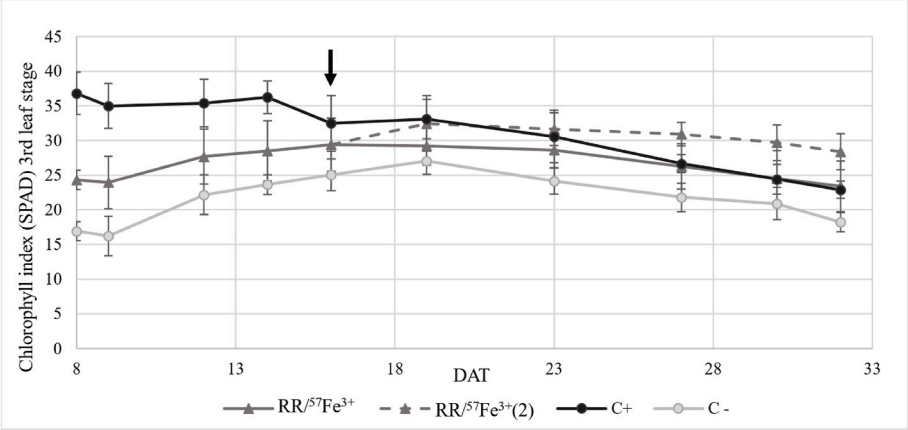

Fig. 3. Chlorophyll SPAD index of 3rd leaf stage during the pot experiment. Treatment of plants: **RR/^{57}Fe^{3+}**- treatment with ^{57}Fe chelated with siderophores produced by *R. radiobacter*; **RR/^{57}Fe^{3+} (2) –** second treatment with *R. radiobacter* siderophores, at 16th day after treatment (DAT) (arrow); positive control **(C+)** - treatment with Fe chelated with *o,o*EDDHA); negative control **(C—) –** no treatment. Error bars indicate standard error ($n = 5$). The average values and statistical analysis using one-way ANOVA and Duncan's test ($p < 0.05$) for each day are presented in Table S3 of the Supplementary material.

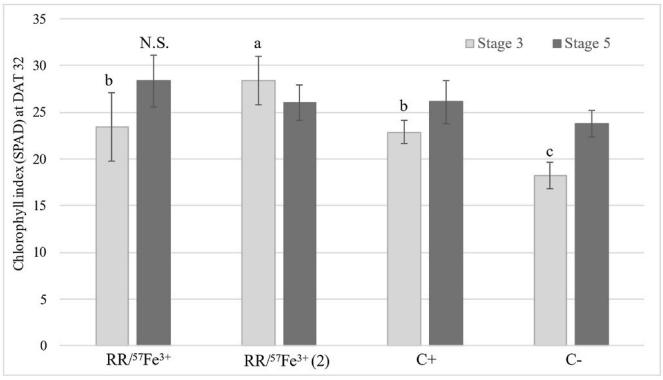

Fig. 4. Chlorophyll SPAD index of 3rd and 5th leaf stage at 32 DAT. Treatment of plants: please see the legend of Fig. 1. Different letters denote significant differences among the treatments according to Duncan's test $p < 0.05$), N.S.: no significant differences. Capital letters correspond to 5th leaf stage.

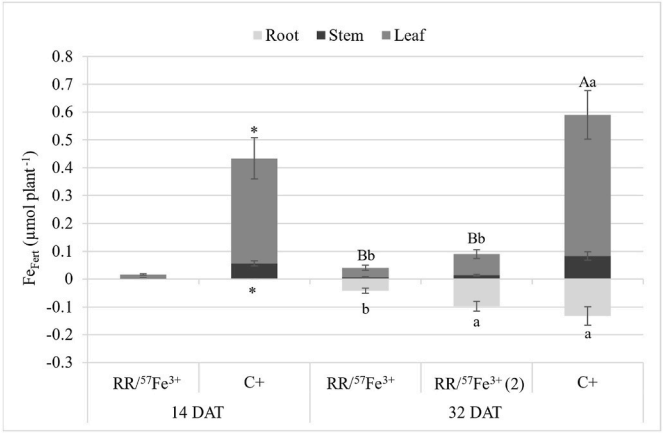

Fig. 5. Distribution of labeled ^{57}Fe (Fe_{Fert}) between plant organs for both samplings (14 and 32 DAT). Treatment of plants: please see the legend of Fig. 1. Mean ± standard. Different superscript letters denote significant differences among the treatments according to Duncan's test $p < 0.05$), ns: no significant differences. Capital letters correspond to the leaves. *denotes significant differences based on the Student'test ($p < 0.05$).

Table 2
Dry weight (DW, g·plant^{-1}) at the first and second sampling in plant materials.

Treatment	Leaves		Stems		Root
	14 DAT	32 DAT	14 DAT	32 DAT	32 DAT
RR/^{57}Fe^{3+}	0.34 ± 0.11 ab	1.60 ± 0.19 ab	0.26 ± 0.08 ab	1.29 ± 0.15 n.s.	0.28 ± 0.05 ab
RR/^{57}Fe^{3+}(2)	–	1.26 ± 0.42 b	–	1.00 ± 0.31	0.23 ± 0.07 ab
C+	0.46 ± 0.06 a	1.67 ± 0.22 a	0.32 ± 0.03 a	1.30 ± 0.26	0.30 ± 0.06 a
C-	0.27 ± 0.12 b	1.39 ± 0.12 ab	0.22 ± 0.06 b	1.10 ± 0.19	0.22 ± 0.04 b

Treatment of plants (RR/^{57}Fe^{3+}, RR/^{57}Fe^{3+}(2), C+, and C-): please, see the legend of Fig. 1. Data are means ± standard error ($n = 5$). Different letters in the same column denote significant differences according to the Duncan test ($p < 0.05$) between treatments for each DAT and plant organ; n.s.: no significant differences.

Roots from positive control (C+) showed the highest DW while negative control was the lowest one. The application of one dosage of RR/^{57}Fe^{3+} on soybean plants produced a slight increase compared to the negative control, being RR/^{57}Fe^{3+} closer to the positive control without significant differences.

3.3. *Isotopically Fe partitioning in plant and the remaining soil*

The results of the allocation of labeled ^{57}Fe (Fe_{Fert}) among various plant organs for both samplings are shown in Fig. 5. At 14 DAT, the Fe content naturally-derived showed no difference among the treatments for the aerial part (data not shown). Regarding Fe_{Fert} at 14 DAT (Fig. 5), the Fe chelate *o,o*EDDHA/^{57}Fe^{3+} (C+) provided higher Fe to soybean stem and leaves than the RR/^{57}Fe^{3+} treatment, which also correlates with the evolution of the SPAD (Fig. 3) in both treatments. The *o,o*EDDHA/^{57}Fe^{3+} chelate provided Fe faster than the other treatments, in good agreement with the findings, already reported [39,38].

Similarly, as in the first sampling time, at 32 DAT, C+ showed a greater response to supply Fe to the plants (Fig. 5). Nevertheless, the effects of the second dosage of RR/^{57}Fe^{3+} can be qualitatively appreciated since a relatively high increase of Fe_{Fert} in RR/^{57}Fe^{3+}(2) was obtained in the aerial part, although no significant differences were obtained between the two treatments in either the stem or leaves (Fig. 5). When comparing the two sampling times, Fe_{Fert} (µmol·plant^{-1}) increased by approximately 140 % in RR/^{57}Fe^{3+}, while the application of the second dose of RR/^{57}Fe^{3+} [RR/^{57}Fe^{3+}(2)] resulted in a

remarkable increase in the ^{57}Fe content in the aerial part, from approximately 0.02 to 0.09 μmol·plant^{-1}, representing an increase of around 430 %. Conversely, the C+ exhibited only a slight increase (36 %) at 32 DAT.

The calculation of the ratio between the Fe content in leaves and stems was performed to assess the Fe translocation from the stems to the leaves (Table 3). Greater values of the translocation ratio are associated with faster translocation, i.e., Fe from the stem moves faster to the upper organ (leaves). Conversely, a lower ratio indicates slower translocation or slower Fe uptake by the plant [17,40]. Regarding Fe supplied directly by the fertilizer (Fe_{Fert}), higher ratios were found at both sampling times. Positive control showed a faster Fe supplied on 14 DAT than RR/$^{57}Fe^{3+}$ treatment, although soybean plants were able to move fast with the Fe obtained from both fertilizers. However, at 32 DAT results were somewhat different. Iron translocation in C+ was reduced by approximately 22 % as compared to 14 DAT. On the other hand, there was an increase in the translocation ratio in RR/$^{57}Fe^{3+}$ treatments of which the second application of RR/$^{57}Fe^{3+}$ [RR/$^{57}Fe^{3+}$(2)] was higher. It was probably related to the increase in Fe dosage and, was comparable to positive control. Iron translocation from natural sources (Fe_{Nat}) was lower than from the fertilizer (Fe_{Fert}), because of the low Fe pool existing in this soil. However, in this case, there were two different situations between the sampling times. At 14 DAT, negative control showed the lowest value of Fe ratio while at 32 DAT, all the plants moved Fe from stems to leaves in the same way since no significant differences were found among all of them. Soybean plants grown without Fe supply (C-) absorbed low amounts of Fe since the beginning of the experiment, and in response to Fe deficiency, they translocated it from the stem to the leaves rapidly, so that the translocation ratio improved in the second sampling time.

Regarding the Fe_{Fert} in soybean roots (Fig. 5), it seemed a relatively low ^{57}Fe mobility from root to aerial part. Normally, Fe in roots explains the Fe effectively taken up by the plant, but also by the Fe precipitated or chelate retained in the apoplast. In RR/$^{57}Fe^{3+}$ treatment, ^{57}Fe was mainly accumulated in the root in comparison to that in the aerial part, this retention may occur but a low mobility of ^{57}Fe from the root to the aerial part is also possible [35]. Conversely, translocation from roots in plants supplied by *o,o*EDDHA/Fe^{3+} seemed to be faster since ^{57}Fe was mainly accumulated in the aerial part. This result agreed with Martín-Fernández et al. [35] and Cieschi et al. [11] in soybean in calcareous soil.

Table 3 suggests an important translocation from stem to leaves. When Fe reached the stem, it was rapidly translocated to the leaves, so Fe could further move to the flowers or fruits. Taking all into account, the results suggested that in both treatments with fertilizer containing siderophore [RR/$^{57}Fe^{3+}$ and RR/$^{57}Fe^{3+}$(2)], Fe could be mobilized from roots to leaves. Similar results have been already reported for other Fe-siderophores [17]. In addition, ^{57}Fe in the roots may be a future long-term source of Fe but longer assay times would be needed to prove this hypothesis.

However, would a single dose of RR/$^{57}Fe^{3+}$ be sufficient? SPAD data showed that RR/$^{57}Fe^{3+}$ kept chlorophyll indices stable throughout the experiment, closer to positive control. With the second application of the treatment [RR/$^{57}Fe^{3+}$(2)], an increase in the SPAD index was observed until the end of the experiment. Otherwise, a slight positive effect caused by the second dosage of the Fe chelated siderophore [RR/$^{57}Fe^{3+}$(2)] was observed in Fe content in the aerial part compared to the single application of RR/$^{57}Fe^{3+}$. However, the amount of Fe in roots increased in plants supplied with RR/$^{57}Fe^{3+}$(2) and the increase observed in the translocation ratio could be an improvement related to the increase of the SPAD index of soybean plants. In any case, a single dosage of the chelate originating from *R. radiobacter* was capable of alleviating Fe chlorosis symptoms at the time the experiment lasted.

Table 4 displays the data on soluble and plant available Fe fractions in the remaining calcareous soil at 32 DAT. Following the methodology proposed by Rodríguez-Castrillón et al. [32], the differences between the Fe amount supplied from the fertilizer under study (Fe_{Fert}) and from natural sources (Fe_{Nat}) were calculated. In general, the amount of Fe_{Fert} obtained in the soluble fraction of the soil was low, being higher in the available fraction for both *o,o*EDDHA/Fe^{3+} and remarkably relevant in the RR/Fe^{3+} treatments. The results obtained for the siderophore-based treatment indicate that it presented a strong complexing capacity, being able to solubilize native Fe not-directly available. In fact, this Fe solubilization was not found in the C+ treatment. An explanation could be that the RR can complex more Fe than that used for the preparation of the Fe fertilizer. The maximum complexing capacity was determined considering the value until a slope of 1 but an additional complexation can be observed (Fig. 2), and thus, the possibility to complex more Fe is possible. What was unexpected, in any case, was the fact that the solubilization of Fe occurs from a very-low available Fe pool presented in this soil.

The results found in available Fe_{Fert} remaining in soil agree with those reported by Ferreira et al. [39] for Fe chelates from other bacterial siderophores. Both treatments with fertilizer produced from *R. radiobacter* cultures showed higher ^{57}Fe compared with both controls, where RR/$^{57}Fe^{3+}$(2) treatment reached the maximum value. Iron from the siderophore could be influenced by interaction with soil components [41] but the results suggested the interaction would not be large enough to prevent an increase in the bioavailability of Fe. In the case of soluble Fe_{Nat}, both controls (positive and negative) were significantly lower than the RR/$^{57}Fe^{3+}$ treatments whereas Fe content obtained through DPTA extraction was greater in plants supplied with RR/$^{57}Fe^{3+}$(2), due to the application of second Fe dosage. This result along with the fact that ^{57}Fe was mainly found in this soil fraction strongly supports the hypothesis that RR/$^{57}Fe^{3+}$ could be used as a reserve for Fe long-term

Table 3
Average ratio of Fe content in leaves to stem among treatments for both Fe sources at 14 and 32 DAT.

Treatments	Fe content$_{leaves}$/Fe content$_{stem}$			
	14 DAT		32 DAT	
	Fe_{Fert}	Fe_{Nat}	Fe_{Fert}	Fe_{Nat}
RR/$^{57}Fe^{3+}$	3.74 ± 0.51 *	1.05 ± 0.19 a	4.42 ± 0.25 b	0.90 ± 0.17 n. s.
RR/$^{57}Fe^{3+}$(2)	–	–	5.22 ± 0.17 a	1.17 ± 0.23
C+	6.54 ± 0.48	1.42 ± 0.20 a	5.12 ± 0.20 a	1.14 ± 0.25
C-	–	0.31 ± 0.05 b	–	1.80 ± 0.42

Fe_{Fert} represents Fe from fertilizer and Fe_{Nat} from natural sources. Treatment of plants (RR/$^{57}Fe^{3+}$, RR/$^{57}Fe^{3+}$(2), C+ and C-): please, see the legend of Fig. 1. Different letters in the same column denote significant differences between treatments for each DAT and plant organ, according to the Duncan test ($p < 0.05$). *denotes significant differences based on the Student'test ($p < 0.05$); n.s.: no significant differences.

Table 4
Fe content (μmol·pot^{-1}) in the remining soil at 32 DAT.

	RR/$^{57}Fe^{3+}$	RR/$^{57}Fe^{3+}$ (2)	C+	C-
Fe_{Fert}				
Soluble	0.018 ± 0.005 b	0.07 ± 0.03 ab	0.11 ± 0.08 a	–
Available	15.6 ± 2.8 b	30.3 ± 1.8 a	4.1 ± 0.7 c	–
Fe_{Nat}				
Soluble*	19.8 ± 5.5 a	22.6 ± 9.9 a	3.0 ± 0.9 b	4.1 ± 1.0 b
Available	209 ± 38 ab	225 ± 69 a	151 ± 48 b	170 ± 43 ab
Fe_{Total}				
Soluble*	19.8 ± 5.4 a	22.7 ± 9.9 a	3.1 ± 0.9 b	4.1 ± 1.0 b
Available	224 ± 36 ab	255 ± 70 a	155 ± 48 b	170 ± 43 b

Treatment of plants (RR/$^{57}Fe^{3+}$, RR/$^{57}Fe^{3+}$(2), C+, and C-): please, see the legend of Fig. 1. Mean ± standard error (n = 5). Different letter in the same row denotes significant differences among the treatments according to Duncan's test ($p < 0.05$, *$p < 0.01$).

plant nutrition.

3.4. Total Fe and other micronutrient concentrations in plants

The soil selected in our study was chosen due to slight Fe availability, but a non-sensible crop variety could be enough to supply Fe to the plants [23]. No clear differences in total Fe concentration in the aerial part were obtained among the treatments (Table 5) despite the visual (Fig. S2) and SPAD values (Fig. 3) differences observed. However, significant differences were obtained in roots. The RR/$^{57}Fe^{3+}$ fertilizer promotes natural Fe accumulation in this organ, mainly apoplast retention, which could not be completely removed by washing [33]. However, a part of Fe accumulated in the root was related to Fe found in the remaining soil (Table 4) which would support the hypothesis that Fe supplied chelated by siderophores produced by *R. radiobacter* improved the Fe bioavailability for later nutrition. It should be noticed that negative control plants were able to reach a similar Fe concentration to the rest of the treatments in the aerial part. This fact highlights that the Fe chlorosis in the soybean cultivar used in this study has more relevance on plant growth (Table 2) and chlorophyll synthesis (Fig. 3) than on Fe concentration in aerial part.

Table 5
Total Fe, Mn, Cu, and Zn concentrations in leaves, stem, and root (mg kg^{-1} DW) and Fe/Mn ratio 14 DAT and 32 DAT.

	14 DAT			
	RR/$^{57}Fe^{3+}$	RR/$^{57}Fe^{3+}$ (2)	C+	C-
Fe (mg·kg^{-1})				
Leaves	53.8 ± 6.5 b	–	85.3 ± 15.3 a	54.9 ± 3.4 b
Stem	82.3 ± 50.1 b	–	53.9 ± 15.7 b	169.1 ± 53.4 a
Mn (mg·kg^{-1})				
Leaves	92.9 ± 21.7 b	–	19.3 ± 6.2 c	113.8 ± 31.3 a
Stem	30.1 ± 4.5 a	–	7.3 ± 2.7 b	30.4 ± 9.8 a
Fe/Mn				
Leaves*	0.64 ± 0.24 b	–	4.7 ± 1.6 a	0.44 ± 0.14 b
Cu (mg·kg^{-1})				
Leaves	6.0 ± 0.8 a	–	4.9 ± 0.9 b	5.5 ± 0.2 ab
Stem	5.0 ± 1.6 n.s	–	6.7 ± 2.8	6.7 ± 2.4
Zn (mg·kg^{-1})				
Leaves	26.6 ± 5.1 ab	–	19.5 ± 5.2 b	27.1 ± 5.1 a
Stem	17.0 ± 4.5 n.s.	–	15.1 ± 3.1	22.4 ± 4.3
	32 DAT			
	RR/$^{57}Fe^{3+}$	**RR/$^{57}Fe^{3+}$ (2)**	**C+**	**C-**
Fe (mg·kg^{-1})				
Leaves	38.8 ± 4.9 n.s.	44.1 ± 5.2	40.0 ± 8.4	47.0 ± 10.3
Stem	63.3 ± 23.9 n.s.	69.2 ± 57.2	39.1 ± 13.8	32.5 ± 12.7
Root	2509 ± 506 a	2521 ± 601 a	1638 ± 31 b	1618 ± 271 b
Mn (mg·kg^{-1})				
Leaves	107.6 ± 18.6 b	90.3 ± 14.7 bc	72.8 ± 15.1 c	149.0 ± 15.4 a
Stem	15.4 ± 1.5 bc	17.6 ± 1.7 b	14.0 ± 0.9 c	21.4 ± 2.9 a
Fe/Mn				
Leaves	0.36 ± 0.04 b	0.50 ± 0.08 a	0.50 ± 0.09 a	0.32 ± 0.07 b
Cu (mg·kg^{-1})				
Leaves	5.1 ± 1.1 b	6.0 ± 0.6 ab	4.0 ± 0.4 c	6.3 ± 0.7 a
Stem	4.6 ± 1.5 n.s.	3.9 ± 0.4	7.0 ± 4.0	6.2 ± 3.6
Zn (mg·kg^{-1})				
Leaves	18.2 ± 13.0 n.s.	11.5 ± 3.5	9.1 ± 1.4	13.1 ± 5.0
Stem	4.6 ± 1.5 n.s.	3.9 ± 0.4	7.0 ± 4.0	6.2 ± 3.6

Treatment of plants (RR/$^{57}Fe^{3+}$, RR/$^{57}Fe^{3+}$(2), C+, and C-): please, see the legend of Fig. 1. Mean ± standard error ($n = 5$). Different letters in the same row denote significant differences among the treatments according to Duncan's test ($p < 0.05$, $^{*}p < 0.01$), n.s.: no significant differences.

The impact of the treatments on the concentrations of other metal micronutrients was also evaluated (Table 5). Soybean plants supplied with RR/$^{57}Fe^{3+}$ showed 14 DAT an intermediate Mn value between both controls being negative control plants those accumulating more Mn in leaves [42]. At 32 DAT, C- still showed a higher accumulation of Mn in leaves and C+ presented the lowest values. Both treatments with fertilizer containing siderophore [RR/$^{57}Fe^{3+}$ and RR/$^{57}Fe^{3+}$(2)] presented less Mn concentration than the negative control. The Fe and Mn antagonism has been already described in the bibliography as being especially relevant in plants treated with the Fe synthetic chelate commonly used *oo*EDDHA/Fe. An increase in Fe uptake will infer a lower rate of Mn uptake [43,44]; so, an increase of Fe/Mn ratio would imply an alleviation of chlorosis. Numerous authors have used the Fe/Mn molar ratio in leaf as a useful index to diagnose balanced nutrition between the two micronutrients and as a tool to assess recovery from Fe chlorosis [45]. In similar pot experiments with soybean grown in soil, the optimal values reported were in the range of 0.5–1.2 [24,33,42,46]. In this study 14 DAT, an adequate Fe/Mn ratio was obtained for the RR/$^{57}Fe^{3+}$. Remarkably, a very high Fe/Mn ratio was obtained in plants treated with *oo*EDDHA/$^{57}Fe^{3+}$, coincident with a high Fe but notably low Mn concentrations (Table 5). At 32 DAT, a more equilibrated concentration was found, noticing that plants supplied with RR/$^{57}Fe^{3+}$ and non-treated plants C- presented lower Fe/Mn ratios because of their higher Mn concentration.

For the Cu concentration in leaves 14 DAT, higher values were found in the RR/$^{57}Fe^{3+}$ treatment than the positive control, but they were similar to the negative control. The chelating agent *o,o*EDDHA has a high affinity for Cu^{2+} [47], which may prevent its uptake by the plant by competition between the chelate and the roots. Regarding Zn, significant differences were only observed in leaves 14 DAT (Table 5), presenting the treatment C+, the lowest value, due to the well-known Fe/Zn antagonism. In the case of repeated application of the Fe-chelated RR, similar concentrations of Cu and Zn were obtained and similar to negative control; therefore, the effect of the second addition dose did not hinder the uptake of these micronutrients.

4. Conclusions

A new freeze-dried Fe fertilizer produced from the culture of *R. radiobacter* has been tested to alleviate Fe chlorosis of soybean plants cultivated in calcareous soil. The use of the ^{57}Fe isotopic labeling technique helped to assess the Fe translocation from the soil to the leaves in soybean plants.

Despite dry weight being only different in the first sampling time, the application of one dosage of RR/$^{57}Fe^{3+}$ was effective in amending Fe chlorosis in soybean plants and comparable to the positive control according to the SPAD index whereas the second application could also contribute to prolonging its effect. The Fe levels in plants treated with the RR/$^{57}Fe^{3+}$ were lower than those for the *o,o*EDDHA/$^{57}Fe^{3+}$ but a remarkably higher Fe concentration, especially in the soluble fraction, remains in the soil, indicating a high Fe available pool. Moreover, when a double dosage of RR/$^{57}Fe^{3+}$ [RR/$^{57}Fe^{3+}$(2)] was applied better results were found in Fe_{Fert} in the available soil fraction. Presumably, in longer periods more than that evaluated in this experiment, plants could utilize Fe from this available fraction, ensuring not just plant initiation but also a sufficient supply of Fe from products with RR.

Finally, these results pointed out that the bio-based Fe fertilizer produced from *R. radiobacter* cultures could be an eco-friendly option instead of traditional synthetic Fe chelates to alleviate Fe chlorosis in calcareous soil for the medium term.

CRediT authorship contribution statement

Alejandra Arcas: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation. **S. Maryam Sadeghi:** Investigation, Formal analysis, Data curation. **Juan J. Lucena:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Joao M. Vindeirinho:** Investigation, Visualization. **Eduardo V. Soares:** Writing – review & editing, Visualization, Funding acquisition, Conceptualization, Supervision, Project administration. **Helena M.V.M. Soares:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization, Project administration. **Sandra López-Rayo:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jafr.2024.101504.

Data availability

Data will be made available on request.

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