Organic acids alleviate iron chlorosis in chickpea grown on two p-fertilized soils

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Abstract

The objectives of this paper are to investigate the response of chickpea growing on two iron (Fe) chlorosis-inducing calcareous soils, with different Fe availability, generously supplied with phosphate (P), and to evaluate the effectiveness of a mixture of organic acids (citric, oxalic and malic) to alleviate Fe chlorosis symptoms. Application of P (+P treatment) aggravated Fe deficiency irrespective of the soil. Phosphorus-induced Fe chlorosis was alleviated when organic acids were also applied to the soil (+P+OA treatment), which was reflected in increased leaf chlorophyll concentration and decreased root ferric reductase activity in the plants growing on both soils; the effect was stronger for plants grown on the soil with the lowest Fe-available content. However, Fe chlorosis was still more severe than in the control (–P treatment) plants. The need of –P treatment plants to explore soil in order to take up nutrients is reflected in their root architecture, reduced root diameter and increased length, surface and volume relative to +P and +P+OA roots. Organic acids alleviate Fe chlorosis in function of non crystalline Fe oxides content but inhibit the root response mechanisms as a consequence of increasing Fe concentration in the soil solution.

Keywords: Calcareous soils, iron oxides, citrate, malate, oxalate, phosphate fertilization

1. Introduction

Calcareous soils cover about the 30 % of world's total land area, with their main properties being their intrinsically high calcium carbonate content and alkalinity (pH 7.5–8.5). Typically, the bioavailability of micronutrients (e.g. Fe, Mn, Cu, Zn) is low in these soils due to their poor solubility at high pH. Consequently, micronutrient deficiency in crop production is a worldwide problem affecting many crops (citrus, olive, grapevine, cereals and berries, among others) grown in calcareous soils.

The main visual symptom of Fe deficiency is leaf chlorosis (i.e. interveinal yellowing of the youngest leaves), which ultimately leads to leaf necrosis, reduced growth and poor yields. The high content and reactivity of carbonate combined with the low content of poorly crystalline Fe oxides in soil play a key role in Fe deficiency (Díaz *et al.*, 2009). Therefore strategies are needed to enhance Fe dissolution and diffusion in the rhizosphere to enhance plant growth.

The interactive effect of phosphorus (P) on plant Fe deficiency remains poorly understood. The high affinity of phosphate for the surface of Fe oxides and oxyhydroxides (Bigham *et al.*, 2002) reduces Fe dissolution and bioavailability (Cornell and Schwertmann, 2003). Furthermore, P can interfere with the reduction of Fe (III) complexed with different organic ligands (Brown and Olsen, 1980), such as organic acids (e.g. citrate) or phytosiderophores (e.g. mugineic acid).

Since the observation of de Kock and Hall (1955) that chlorotic leaves had a higher P/Fe ratio than non-chlorotic leaves in some plant species (e.g. *Bougainvillea glabra, Sambucusnigra, Ilex aquifolium, Pelargonium zonale and Spiraea japonica*), different experiments have suggested that high P fertilization exerts a negative effect on Fe nutrition. Examples of this phenomenon exists for a wide range of crop plants including, field-grown soybean and grapevine rootstocks (Marschner and Schropp, 1977) and hydroponically grown corn, oat, soybeans, barley, peanut and tomato (Brown and Olsen, 1980; Ladouceur *et al*., 2006). This had led some authors to suggest that P competes physiologically with Fe in plant tissues.

Sánchez-Rodríguez *et al*. (2013) showed that high levels of P aggravated Fe chlorosis in chickpea, peanut, lupin and sorghum grown on artificial substrates prepared with mixtures of Fe oxide (ferrihydrite)-coated, calcium carbonate and quartz sand. The effect was more evident at low concentrations of Fe oxide in the substrate when the phosphate ion was strongly adsorbed on its surface (Cornel and Schwertmann, 2003) decreasing the solubility of soil Fe.

By contrast, there are also studies rejecting the hypothesis that P can aggravate Fe chlorosis: Kovanci *et al*. (1978) in field-grown mandarin, Müllner (1979) and Mengel *et al*. (1979) in field-grown grapevine, Mengel *et al*. (1984) in pot-grown grapevine, Romera *et al*. (1991) in nutrient solution-grown peach rootstocks, Samar *et al*. (2007) in pot-grown 'Delicious' apple, and Balal *et al*. (2011) in two pot-grown Prunus rootstocks.

These authors support the idea put forward by Mengel *et al*. (1984) that the high P content found in chlorotic leaves was actually the result and not the cause for Fe chlorosis.

When tissue Fe levels fall below a critical threshold, plants can exploit a range of strategies to enhance the mobilization of Fe in the rhizosphere including: (1) acidification of the rhizosphere by the excretion of H^+, (2) stimulation of the Fe-reducing capacity through a ferric reductase system, (3) exudation of phytosiderophores by Strategy II plants, and (4) root exudation of organic acids and phenolic compounds. Furthermore, morphological changes occur in plants (except in grasses) in response to Fe deficiency, including inhibition of root elongation, an increase in the diameter of the apical root zones and abundant root hair formation.

Root exudation of organic acid anions (e.g. citrate, malate, oxalate) is thought to represent one of the main strategies used by plants growing on calcareous soils to enhance nutrient mobilization and acquisition under P and micronutrient limiting conditions (Jones, 1998). After release into the rhizosphere the organic acids can undergo a number of fates including complexation of metals which induces mineral dissolution and enhances the solubility and diffusion of micronutrients towards the root (Ryan *et al*., 2001). This response is typically enhanced by co-acidification of the soil via the roots H^+-ATPase or via release of organic acid anions in a protonated form (Jones, 1998). However, the behaviour of organic acids in soil is complex and a range of processes can occur which can reduce the magnitude of the nutrient mobilization response including: (1) consumption of the organic acids by the soil microbial community, (2) immobilization on anion exchange sites, (3) precipitation (e.g. Ca-oxalate), (4) abiotic mineralization, and (5) leaching down the soil profile (Jones, 1998). While organic acids such as citrate and malate have been shown to induce the dissolution of insoluble ferric oxyhydroxides in soil in the absence of plants, their ability to mobilize Fe in a complex rhizosphere environment remains largely unknown.

The aims of the present study were therefore, (1) to investigate the response of chickpea growing on two Fe chlorosis-inducing calcareous soils, with a different Fe bioavailability, when the soil was generously supplied with phosphate, and (2) to evaluate the effectiveness of a mixture of organic acids (citric, oxalic and malic) to alleviate Fe chlorosis symptoms under high-P conditions.

2. Materials and Methods

2.1. Soils

The experiments used two calcareous soils from southern Spain. The first called "soil poor in acid oxalate extractable Fe (Fe_{ox})" was collected from a vineyard in Montilla, Province of Córdoba (37º34'26"N, 4º38'25"W) whilst the second called "soil rich in Fe_{ox}" was collected from an olive orchard in the Province of Jaén (37º59'41"N, 3º13'37"W). Crops growing on both soils exhibited strong signs of Fe chlorosis.

Organic carbon content (rapid dichromate oxidation) was 15 and 16 g kg^{-1} for soil poor in Fe_{ox} and soil rich in Fe_{ox}, respectively, and clay content (pipette method) was 210 and 300 g kg^{-1} for soil poor in Fe_{ox} and soil rich in Fe_{ox}, respectively. Both soils were highly calcareous: calcium carbonate equivalent (CCE) (determined by weight loss upon treatment with 6 M HCl; van Wesemael, 1955) was 490 g kg^{-1} for soil poor in Fe_{ox} and 380 g kg^{-1} for soil rich in Fe_{ox}, and active calcium carbonate equivalent (ACCE) (Drouineau, 1942) was 70 g kg^{-1} for soil poor in Fe_{ox} and 150 g kg^{-1} for soil rich in Fe_{ox}. The pH (potentiometric measurement in a relation of 1:2.5 soil in water suspension) was typical of calcareous soils (8.6 for soil poor in Fe_{ox} and 8.4 for soil rich in Fe_{ox}), and cation exchange capacity (extraction with 1 M NH_4OAc buffered at pH 7) was 8.3 $cmol_c$ kg^{-1} for soil poor in Fe_{ox} and 19.5$cmol_c$ kg^{-1} for soil rich in Fe_{ox}, calcium being the dominant exchangeable cation due to the high carbonate content

in both soils. Electrical conductivity of the 1:5 (w/v) soil:water extract was 0.15 dS m^{-1} in soil poor in Fe_{ox} and 0.08 dS m^{-1} in soil rich in Fe_{ox}. Olsen P (extraction with 0.5 M $NaHCO_3$ buffered at pH 8.5; Olsen *et al*., 1954) was low in both soils (4.1 mg kg^{-1} in soil poor in Fe_{ox} and 2.4 mg kg^{-1} in soil rich in Fe_{ox}). Citrate/bicarbonate/dithionite-extractable Fe (Fed; Mehra and Jackson, 1960, with extraction carried out at 25 º C for 16 h) was 1.80 g kg^{-1} in soil poor in Fe_{ox} and 4.40 g kg^{-1} in soil rich in Fe_{ox}. Citrate/ascorbate –extractable Fe (Feca; Reyes and Torrent 1997) was 0.64 g kg^{-1} in soil poor in Fe_{ox} and 1.21 g kg^{-1} in soil rich in Fe_{ox}. Acid oxalate extractable Fe (Fe_{ox}; Schwertmann, 1964) was 0.23 g kg^{-1} in soil poor in Fe_{ox} and 0.71 g kg^{-1} in soil rich in Fe_{ox}, being the main difference between the two soils. Both Fe_{ox} and Feca are considered to provide a good estimate of the Fe present in the form of poorly crystalline Fe oxides (Reyes and Torrent, 1997); thus, the availability of Fe is higher in the soil rich in Fe_{ox} than in soil poor in Fe_{ox}.

2.2. Plant growth experiments

The experimental design included three treatments: a control (no P added to the soil; "–P" treatment) and two treatments where phosphate was applied by spraying the soil homogeneously with a Ca $(H_2PO_4)_2$ solution at a rate of 100 mg P kg^{-1} before transplantation without ("+P" treatment) or with organic acids ("+P+OA" treatment, the same P spraying than +P). In the latter treatment, plants were watered with 10 mL of a solution containing citric, oxalic and malic acids (10 mM) every day from the fifteenth day after transplantation (DAT), when roots were well established in the soil, until harvest. The concentration was chosen to reflect high rates of organic acid exudation (Jones, 1998).

Chickpea (*Cicer arietinum* L., cv. ICC 11224) was grown in cylindrical plastic pots 7.5 cm in diameter and 10 cm high provided with drainage holes at the bottom and filled with 250 g of soil. Seeds were germinated for 72 h at 20 ºC and three seedlings were transplanted to each pot, which was placed in a greenhouse with a photoperiod of 16 h d^{-1}, a temperature of 20

ºC and a relative humidity of 45−55% for 27 d. A micronutrient-rich, Fe-free, P-poor modified Hoagland nutrient solution was applied according to the needs of each crop. The composition of the nutrient solution was Ca $(NO_3)_2 \cdot 4H_2O$ (5 mM), KNO_3 (5 mM), $MgSO_4$ (2 mM), KCl (0.1 µM), H_2KPO_4 (0.25 mM), H_3BO_3 (50 µM), $MnSO_4 \cdot H_2O$ (4 µM), $ZnSO_4 \cdot 7H_2O$ (4 µM), $CuSO_4 \cdot 5H_2O$ (0.1 µM), Na_2MoO_4 (6 µM). The pots were weighed daily and the soil kept at near field capacity with deionized water. Five replicates were used in all treatments and soils.

After the appearance of the first symptoms of Fe chlorosis at 21 DAT, the leaf chlorophyll concentration was periodically monitored using a SPAD meter (SPAD 502 Portable Chlorophyll Meter, Minolta Camera Co., Osaka, Japan). To validate the SPAD readings, 30 leaves of the younger and completely expanded leaves exhibiting a variable degree of Fe chlorosis were collected from various plants. After measuring the leaflet fresh weight and SPAD, chlorophyll was extracted with 96 wt.% ethanol. Because SPAD and leaf chlorophyll concentration per unit surface were highly correlated ($r = 0.81$, $p < 0.001$) the SPAD value was adopted as a reliable proxy for chlorophyll concentration.

During the growth period, plant height and number of leaves were measured periodically. Soil solution was extracted from each pot 20 DAT and the day before harvest using Rhizon® samplers (Rhizosphere Research Products, Wageningen, Netherlands) and analysed for Fe and P. At harvest, the plants were removed from the moist soil carefully and the soil adhering to the roots (about 30 g per pot) was collected and stored at 4 ºC. The plant was then taken to the laboratory and its roots were washed and soaked for 30 min in plastic vessels with 50 mL of a nutrient solution analogous to the one described previously except that it contained no micronutrients and adjusted to pH 5.0 with KOH (10 mM). To determine their ferric reductase activity, the plant roots were submerged for 1 h in 40 mL of a reduction assay solution without micronutrients that contained 100 µM Fe (III)-EDTA,

300 µM Ferrozine and 5 mM Mes buffer adjusted to pH 5.0 with KOH. The amount of Fe (II)-ferrozine was determined spectrophotometrically at 562 nm using an extinction coefficient of 29800 M^{-1} cm^{-1}. Subsequently, the roots were washed with deionized water, scanned and their length determined using WinRHIZO® (Regent Instruments Inc., Canada). N_2-fixing nodules were then separated from the roots by hand. Root, shoot and nodule dry weight were determined after oven drying (80 ºC, 72 h) and mineral element concentrations in aerial parts and roots were determined after dry-ashing and digestion with hydrochloric acid (Adrian, 1973). Magnesium, Fe and Mn in solution were determined using a Varian 720 ES ICP-OES, K and Ca by flame emission and P by the molybdenum blue method of Murphy and Riley (1962). The N and C concentration was determined by direct combustion of the plant material using a TruSpec® analyser (Leco Corp., St Joseph, MI, USA).

2.3. ^{14}C organic acid mineralization

To determinate the rate of microbial organic acid degradation, ^{14}C-labelled citrate, oxalate and malate, were added to soil and the rate of $^{14}CO_2$ evolution determined over a 72 h period. Briefly, 200 µL of an organic acid solution (10 mM) containing citrate, oxalate and malate (of which only one was ^{14}C-labeled) was placed in 60 mL polypropylene tubes containing 5.0 g of field-moist rhizosphere soil obtained from the harvested pots. The specific activity of citrate was 0.20 kBq mL^{-1}, 0.44 kBq mL^{-1} for oxalate and 0.45 kBq mL^{-1} for malate. After organic acid addition, a 1 M NaOH trap (1 mL) was placed on top of the soil and the tubes sealed and incubated in the dark at 20 ºC. The NaOH traps were removed at 1, 2, 4, 8, 10, 24, 32, 48 and 72 h after organic acid addition and their $^{14}CO_2$ content determined by liquid scintillation counting. Five replicates of each treatment (the same number of pots) were performed.

2.4. Statistical analyses

Statistical analyses were performed using STATISTIX 9.0 software (Analytical Software, Tallahassee, FL, USA). The analysis of variance (ANOVA) was

based on a completely randomized design with three treatments (−P, +P and +P+OA) in each soil. Means were separated using the Least Significant Difference (LSD) test at a probability level of 0.05. Unless otherwise indicated the term "significant" refers to this probability level.
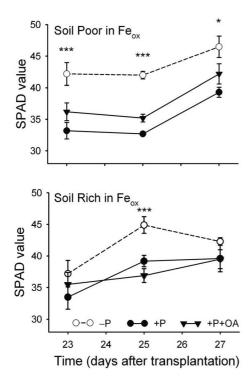
3. Results and Discussion

3.1. SPAD values, chlorophyll content and ferric reductase activity

Figure 1 shows the time course of the SPAD value for the youngest leaves of chickpea as a function of soil and treatment. Iron deficiency symptoms (leaf chlorosis) appeared at the end of the third week after transplantation. Generally, both P fertilization and organic acid addition to the soil (+P and +P+OA treatments) resulted in reduced SPAD readings in the plants grown on both soils. The SPAD value of the control plants (−P) was significantly higher than the rest of the treatments at all measurement times for the soil poor in Fe_{ox} ($p < 0.001$ at 23 and 25 DAT, and $p < 0.05$ at 27 DAT) and at 25 DAT ($p < 0.001$) for the soil rich in Fe_{ox}. Relative to the +P treatment, the +P+OA treatment resulted in higher SPAD readings for the plants grown on the soil poor in Fe_{ox}; this effect, however, was not observed for the soil with a higher content in Fe_{ox}. Similar results were obtained for direct measurements of chlorophyll content in the last leaflet and two last leaflets (Table 1). The key role played by the poorly crystalline Fe oxides in supplying Fe to the plant (Díaz *et al.*, 2009) was probably responsible for the higher chlorophyll content in plants grown on the soil rich in Fe_{ox} (Table 1 and Figure 1), however, no significant differences were found in SPAD values between soils.

The ferric reductase activity of plant roots was significantly higher in plants grown on soils supplied with P (+P and +P+OA treatments) than in the control treatment (–P) (Table 1). Furthermore, the ferric reductase activity of plants treated with P and organic acids (+P+OA treatment) was lower than those treated only with P (+P treatments) (Table 1). The chlorophyll content was negatively correlated with the ferric reductase activity ($r = -0.65$, p = 0.016, for plants grown on the soil poor in Fe_{ox}; and $r = -0.60$, $p = 0.027$, for plants grown on the soil rich in Fe_{ox}), as a result of the Fe-stress reaction. This correlation was reported previously for other crops, (Romera *et al.*, 1991; Ellsworth *et al.*, 1997).

Figure 1. Influence of soil P (+P) and P + organic acid (+P+OA) addition on the chlorophyll content (SPAD value) of chickpea leaves grown in two calcareous soils (soil poor in Fe_{ox} and soil rich in Fe_{ox}). Values represent mean ± standard error (n = 5). Fe_{ox}: Acid oxalate extractable Fe. * indicates *p*<0.05 and *** indicates *p*<0.001.

Table 1. Chlorophyll concentration in the last leaflet (1) and the two last leaflets (1-2), root ferric reductase activity, dry weight (DW), last leaflet weight and root nodule weight in chickpea in response to the addition of P (+P) and P plus organic acids (+P+OA) to soil poor in Fe_{ox} and soil rich in Fe_{ox}. Values represent mean ± standard error ($n = 5$). Letters *a* and *b* denote significant differences between treatments at the $p < 0.05$ level. Fe_{ox}: Acid oxalate extractable Fe.

Treatment	Chlorophyll 1	Chorophyll 1-2	Ferric reductase	DW	Leaflet weight	Nodules
	mg g^{-1}		nmol g^{-1} h^{-1}	g plant^{-1}	mg plant^{-1}	
	Soil Poor in Feox					
−P	4.9 ± 0.4 a	4.5 ± 0.2 a	263 ± 25 b	0.29 ± 0.02 a	3.9 ± 0.1	3.4 ± 0.5 b
+P	3.5 ± 0.2 b	3.4 ± 0.2 b	430 ± 36 a	0.31 ± 0.01 a	4.9 ± 0.5	17.1 ± 2.3 a
+P +OA	4.3 ± 0.3 ab	4.1 ± 0.2 a	378 ± 46 ab	0.23 ± 0.02 b	3.7 ± 0.3	13.7 ± 2.5 a
P-value	0.019	0.007	0.035	0.003	0.051	0.001
	Soil Rich in Feox					
−P	6.4 ± 0.2	7.1 ± 0.6	301 ± 62 b	0.25 ± 0.01	2.8 ± 0.2	6.0 ± 0.7 b
+P	5.6 ± 0.4	6.4 ± 0.7	1023 ± 227 a	0.29 ± 0.01	3.3 ± 0.4	14.8 ± 2.2 a
+P +OA	6.2 ± 0.1	5.8 ± 0.1	784 ± 91 a	0.24 ± 0.03	2.5 ± 0.1	18.5 ± 2.4 a
P-value	0.656	0.324	0.019	0.093	0.150	0.002

3.2. Plant growth

Vigorous plant growth was observed on both soils. Plants grew slightly taller on soil poor in Fe_{ox} than on soil rich in Fe_{ox} (Figure 2), which was not unexpected because of the significantly different physical and chemical properties of the two soils. Significant differences in plant height were found at harvest, with plants grown under the +P treatment being significantly taller that the other two treatments. Dry weight (DW) and leaflet weight at harvest were lower in the +P+OA treatment than in the −P and +P plants, but differences were significant only for the soil poor in Fe_{ox} (Table 1). Nodule weight was significantly greater in the +P and +P+OA treatments than in the −P plants (Table 1), as expected from the known favorable effect of P on nodulation (Cassman *et al.,* 1980).

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Properties of root architecture as a function of soil type and amendment are shown in Table 2. Root diameter increased in the order −P < +P < +P+OA plants but differences were significant only between −P and +P+OA plants grown on the soil rich in Fe_{ox}. Root length, surface area and volume, and number of tips decreased in the order −P > +P > +P+OA plants, most of the significant differences being found in the root of plants grown on the soil poor in Fe_{ox} (Table 2).
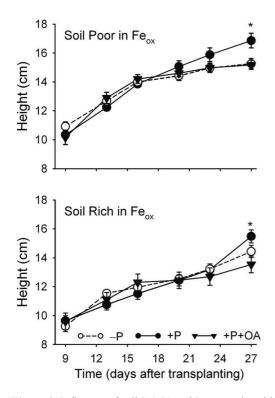
Figure 2. Influence of soil P (+P) and P + organic acid (+P+OA) addition on the height of chickpea plants grown in two calcareous soils (soil poor in Fe_{ox} and soil rich in Fe_{ox}). Values represent mean ± standard error (n = 5). Fe_{ox}: Acid oxalate extractable Fe. * indicates $p<0.05$.

As expected, the concentration of P in the soil solution was higher for the +P and +P+OA treatments than for the –P treatment (Table 3). Both the concentration of Fe and the pH of the soil solution were significantly higher for the +P+OA than for the other two treatments.

Root and root hair production are typically stimulated by low P tissue concentrations. No stimulatory effect on root hair production by Fe deficiency was observed in comparison with other experiments possibly because the effect of a macronutrient like P in soil solution is more important from a regulatory perspective.

Amendment of the soil with organic acids (+P+OA) increased the concentration of Fe in soil solution (Table 3). This would increase the availability of Fe and may be responsible for reducing Fe chlorosis symptoms in comparison with the P treated plants (+P) (see chlorophyll content in the last leaflet, Table 1).

3.3. Organic acid mineralization

Figure 3 shows the time-dependent mineralization of the mixture of citrate, oxalate and malate for each soil and labeled organic acid. All the $^{14}CO_2$ evolution data fitted well to sigmoidal curves ($0.95 < r^2< 0.99$, $p < 0.001$) Overall, mineralization was faster for citrate than for the other two anions which exhibited similar rates of mineralization. Except for oxalate in the soil poor in Fe_{ox}, the mineralization rate was higher or similar to in the soils supplied with P than in the control soils, consistent with the idea that P promotes soil microbial activity. The low mineralization rate of oxalate in soil poor in Fe_{ox} not previously supplied with organic acids (treatments –P and +P) agree with previous data on the resistance of oxalate to microbial degradation (Ström *et al.*, 2002) and may have relationship with the rapid precipitation of Ca oxalate in a calcareous soil not previously supplied with this anion. However, the mineralization patterns of oxalate in soil rich in Fe_{ox} differ markedly from those of the soil poor in Fe_{ox}. The results also suggest that the microbial community may have upregulated its ability to use oxalate in the soils which had received organic acids on a regular basis.

3.4. Mineral element concentration in plant

The mineral nutrient concentrations in the chickpea aerial biomass and root are shown in Table 4. Although nutrient concentrations were above established critical levels (Benton Jones *et al.*, 1991), some data deserve attention. Carbon content in the aerial part was inversely related to root nodulation (Tables 1 and 4), because bacteria in nodules need carbohydrates (and other nutrients) from the host plant.

Table 2. Root architecture characteristics for chickpea plants grown in two calcareous soils (soil poor in Fe_{ox} and soil rich in Fe_{ox}) amended without (−P) or with P (+P) and P + organic acids (+P+OA). Values represent mean ± standard error (n = 5). Letters *a, b* and *c* denote significant differences between treatments at the $p < 0.05$ level. Fe_{ox}: Acid oxalate extractable Fe.

Treatment	Diameter	Total Length	Surface area	Root volume	Tips
	mm	m plant^{-1}	cm^2 plant^{-1}	cm^3 plant^{-1}	No. plant^{-1}
	Soil Poor in Fe_{ox}				
−P	0.83 ± 0.02	3.48 ± 0.16 a	91 ± 5 a	1.91 ± 0.01 a	166 ± 8 a
+P	0.85 ± 0.04	2.72 ± 0.18 b	72 ± 4 b	1.53 ± 0.01 b	136 ± 24 ab
+P+OA	0.91 ± 0.02	1.84 ± 0.19 c	53 ± 5 c	1.20 ± 0.01 b	85 ± 8 b
P-value	0.146	<0.001	<0.001	0.005	0.012
	Soil Rich in Fe_{ox}				
−P	0.80 ± 0.02 a	2.47 ± 0.18 a	68 ± 3	1.36 ± 0.07	234 ± 46
+P	0.84 ± 0.03 ab	2.37 ± 0.15 ab	62 ± 3	1.31 ± 0.05	207 ± 63
+P+OA	0.89 ± 0.02 b	1.91 ± 0.18 b	53 ± 5	1.19 ± 0.11	166 ± 22
P-value	0.040	0.015	0.051	0.344	0.594

Table 3. Composition of soil solution obtained from the rhizosphere of chickpea plants grown in two calcareous soils (soil poor in Fe_{ox} and soil rich in Fe_{ox}) amended without (−P) or with P (+P) and P + organic acids (+P+OA). Values represent mean ± standard error (n = 5). Letters *a, b* and *c* denote significant differences between treatments at the $p < 0.05$ level. Fe_{ox}: Acid oxalate extractable Fe.

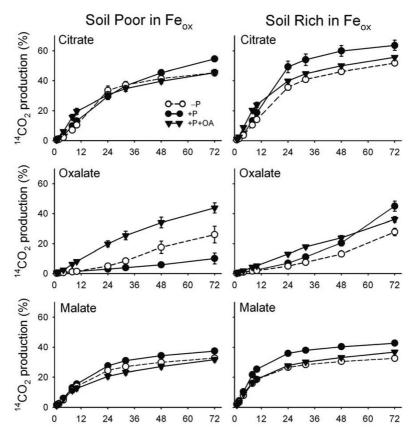| Treatment	P	Fe	pH
	mg L^{-1}		
	Soil Poor in Fe_{ox}		
−P	0.7 ± 0.1 b	12.2 ± 12.2 b	7.65 ± 0.02 b
+P	24.9 ±1.2 a	42.8 ± 18.3 b	7.62 ± 0.02 b
+P +OA	23.6 ± 2.8 a	295.6 ± 112.1 a	7.97 ± 0.03 a
P-value	< 0.001	0.004	< 0.001
	Soil Rich in Fe_{ox}		
−P	0.3 ± 0.1 b	79.5 ± 37.0 b	7.75 ± 0.01 b
+P	21.7 ± 0.3 a	30.6 ± 30.6 b	7.70 ± 0.01 b
+P +OA	22.2 ± 1.9 a	193.7 ± 36.8 a	8.00 ± 0.04 a
P-value	< 0.001	0.016	< 0.001

Figure 3. Time course of microbial $^{14}CO_2$ production (% of total organic acid applied) after the addition of ^{14}C-labeled citric, oxalic or malic acid to two calcareous soils (soil poor in Fe_{ox} and soil rich in Fe_{ox}) previously treated without P (−P), with P (+P) or with P + organic acids (+P+OA). Values represent mean ± standard error (n = 5). Fe_{ox}: Acid oxalate extractable Fe.

Control plants (−P) followed by those treated with phosphorus (+P) and then by those treated with organic acids (+P+OA) had the highest biomass N concentration despite having less nodulation (Table 1 and 4). Wang *et al*. (2009) found that organic acids (oxalic, citric and malic) had an inhibitory effect on root nitrogen accumulation, nodulation and nitrogen fixation of soybean grown in sand culture under phosphorus deficient conditions. The conditions in our experiment in which plants were watered with modified Hoagland solution rich in N would, however, be expected to have a greater regulatory effect on N fixation.

Plants grown on soil poor in Fe_{ox} treated with organic acid (+P+OA) increased their aerial P concentration (Table 4). The same effect was observed for roots of plants grown on treated P soils (+P) and treated with organic acids (+P+OA) for both soils (data not shown).

Table 4. Nutrient concentrations in aerial part of chickpea grown in two calcareous soils (soil poor in Fe$_{ox}$ and soil rich in Fe$_{ox}$) amended without (−P) or with P (+P) and P + organic acids (+P+OA). Values represent mean ± standard error (n = 5). Letters *a, b* and *c* denote significant differences between treatments at the $p < 0.05$ level. Fe$_{ox}$: Acid oxalate extractable Fe.

Treatment	C g kg^{-1}	N	P	K	Ca	Mg	Fe mg kg^{-1}	Mn	Cu
	Soil Poor in Fe$_{ox}$								
−P	443.5 ± 1.7 a	45.8 ± 1.3 a	1.52 ± 0.05 b	16.4 ± 3.0 b	17.8 ± 0.8 b	1.2 ± 0.1 b	67 ± 11b	20 ± 3 b	11 ± 0 a
+P	417.1 ± 1.9 b	35.1 ± 0.9 b	1.62 ± 0.05 b	30.6 ± 2.4 a	20.4 ± 0.4 a	1.6 ± 0.1 a	64 ± 7 b	41 ± 3 a	9 ± 0 b
+P+OA	421.2 ± 2.3 c	27.3 ± 0.9 c	2.07 ± 0.17 a	28.0 ± 6.6 ab	15.6 ± 0.4 c	0.9 ± 0.0 c	142 ± 32 a	25 ± 2 b	11 ± 1 a
P-value	<0.001	<0.001	0.004	0.039	<0.001	<0.001	0.018	<0.001	0.015
	Soil Rich in Fe$_{ox}$								
−P	440.9 ± 0.8 a	46.4 ± 1.3 a	1.57 ± 0.10	21.4 ± 1.6	16.5 ± 0.3 b	1.7 ± 0.0 b	76 ± 4	26 ± 1 b	11 ± 0 a
+P	424.1 ± 1.5 b	40.5 ± 1.0 b	1.43 ± 0.09	25.3 ± 1.6	19.2 ± 0.3 a	2.2 ± 0.0 a	90 ± 7	41 ± 5 a	9 ± 0 b
+P+OA	423.9 ± 1.4 b	32.6 ± 1.4 c	1.88 ± 0.27	28.8 ± 8.6	16.5 ± 0.2 b	1.3 ± 0.1 c	72 ± 16	47 ± 7 a	11 ± 1 a
P-value	<0.001	<0.001	0.226	0.601	<0.001	<0.001	0.432	0.011	0.032

An increasing effect in K concentration was observed in plants grown on both soils (especially in root, not shown) when organic acids were added (+P+OA) treatment). It could be because an excessive positive charge is produced by H$^+$ from organic acids out of the root cells, decreasing the H$^+$ excretion by the roots that uptake K$^+$ in order to reduce the positive charge, increasing slightly and significantly the pH of the rhizosphere in both soils (Table 3).

Calcium concentration decreased for plants treated with organic acid (+P+OA) possibly due to the formation of Ca-oxalate precipitates reducing the bioavailability of Ca.

4 . Conclusions

Non-crystalline Fe oxides have an important role in the availability of Fe for plants. Therefore, leaf chlorophyll content was higher in chickpea grown on the soil rich than on the soil poor in Fe$_{ox}$. The negative effect of high P fertilization on Fe chlorosis in chickpea was partially alleviated (increased SPAD value and leaf chlorophyll content, and reduced root ferric reductase activity) with the addition of organic acids to the soil, especially for the soil with the lower

Fe$_{ox}$ content. However, the Fe chlorosis symptoms were more severe in chickpea growing on soils treated with organic acids than in control plants which received no P. The increase in Fe availability (Fe in soil solution) as a result of organic acids addition could indirectly inhibit the root response mechanisms (i.e. increase of root diameter in comparison with control plants), reducing the production of fine roots needed to explore the soil for nutrient acquisition.

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