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Characterization of Ferric Reducing Activity in the Rhizosphere of Chickpea Plants (*Cicer arietinum* L)

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Abstract. Chickpea seedlings (cv. ILC-195) were precultured in an iron free nutrient solution for 7 days. Thereafter, iron was supplied to one set of plants (Treatment A) and the second set of plants (Treatment B) continued to grow in a -Fe nutrient solution. Measurements were carried out to investigate the activity of the two reduction systems (extra cellular reductants and reductase) and the accumulation of organic acids.

Fe-stress appeared to enhance slightly the release of reductants capable of reducing Fe^{+3}. The quantity of Fe^{+3} reduced by reductants was however extremely low compared to the capacity of the intact root system to reduce iron. The rate of Fe reduction by the basal roots was significantly greater than that by root tips. The use of agar plate technique provided another evidence that iron reduction was all over the root system and was restricted to the apical zones and so was the acidification of the rhizosphere.

Regardless of the iron nutritional status, the concentrations of organic acids were much higher in the shoots than roots. The distribution of organic acids between tops and basal shoots was in favour of basal shoots. There was little difference between the different zones of the roots. The accumulated organic acids were mostly citric and malic in the shoots whereas, malonic and oxalic in the roots.

Introduction

The discovery that plant roots can reduce Fe^{3+} to Fe^{2+} [1] was important not just because reduction of Fe^{+3} increases the solubility of iron, but because, Fe^{2+} concentration in the external medium appears to determine iron availability for the plant [2-4]. As iron is present in the soil solution mostly as Fe^{3+}, the obligatory step of iron reduction at the root surface [4] is crucial in determining its uptake by the plant.

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Several contradictory models have been proposed to explain the reduction mechanism. At first, the reduction of Fe^{3+} was attributed to the release of "reductants" iinto the nutrient solution of iron-deficient plants [5,6]. Iron-deficient plant roots have also been shown to accumulate phenolics with a high reducing capacity [6,7,8,9], indicating possible Fe^{+3} reduction in the free space of the root system. Both the release of reductants and their capacity to reduce Fe are strongly pH dependent [9,10,11]. A pH lower than 4.5 is required for substantial Fe^{3+} reduction [6,7]. Such pH values are unlikely in the rhizosphere of plants grown in highly calcareous and probably other soils.

The alternative hypothesis of enzymatic reduction of Fe^{3+} at the plasma membrane of rhizodermal cells was proposed by Chaney *et al.* [4]. Römheld and Marschner [12] claim that the enzymatic reduction of Fe^{3+} occurs only at the apical root zone. These cells respond to Fe deficiency by enhanced Fe^{3+} reduction and the release of Fe^{4+} into the external medium [13]. The local coincidence of these two distinct response mechanisms may indicate an efficient cooperation since the membrane-bound reductase has optimum activity at pH 4-5, and is strongly inhibited at pH higher than 7.5 [9].

Römheld and Marschner [12] suggest that under field conditions, a combination of release of "reductants" and reduction by a membrane-bound reductase is responsible for the enhanced mobilization and uptake of iron by dicots in response to iron stress. This proposal was based on the observed additional ability of "reductants" (*e.g.* phenolics) to chelate iron [4].

The sites of iron reduction and proton efflux differ between plant species. In sunflower, enhanced capacity to reduce iron appeared to be restricted to "swollen" root tips around which there is also a decrease in pH [15]. These authors also carried out measurements of the capacity of various zones of the roots of intact chlorotic sunflower plants to reduce Fe^{3+} and showed that root tips [0-1.5 cm) had a higher efficiency than basal zones (1.5-5.5 cm). In other plant species (*e.g.* cucumber and peanuts) iron stress leads to enhanced reduction of Fe and increased proton efflux over the entire root system [15].

The known Fe-efficiency of chickpea has so far been attributed to its ability to acidify the rhizosphere and to release organic acids [16]. In this investigation, the ability of the roots to reduce Fe was investigated by a variety of techniques. Measurements were carried out on plants grown with and without iron supply to investigate the activity of the two reduction systems (extracellular reductants and reductase). The activity of different zones of the roots was also investigated using an *in vivo* assay.

Materials and Methods

Plant cultivation

Chickpea seeds (cv. ILC-195) were surface sterilized in 2% sodium hypochlorite with trace of teepol (detergent) for 2 min. and germinated on moist filter paper at room temperature. After 7 days, seedlings were transferred to two 50 litre tanks, each supporting 24 plants. Plants were allowed an establishment period of 7 days during which iron free one-tenth strength Long Ashton nutrient solution was supplied. Thereafter, one of two different regimes of iron supply was imposed. In treatment (A) plants received iron (Fe-EDTA) at 5.6 $mgL^{-\varnothing}$; and in treatment (B) plants continued to receive no iron.

Treatment	Establishment period (-7 to 0 days)	Experimental period (0 to 20 days)
A	0 mg Fe L^{-1}	5.6 mg Fe L^{-1}
B	0 mg Fe L^{-1}	0 mg Fe L^{-1}

During the experimental period, plants received half-strength Long Ashton nutrient solution. The experiment was carried out in a glasshouse with supplementary lighting (day temperature, 25°C; night, 20°C; photoperiod, 16 h.). The nutrient solutions were completely renewed every 7 days.

Collection and measurement of extracellular "reductants"

Three plants from each treatment (A,B) were placed with their roots in flasks containing 100 ml of freshly prepared half-strength nutrient solution, but with micronutrients supplied at one-tenth the rates of that recommended in the Long Ashton formula. Iron was supplied at 0 and 5.6 mg/liter as before. Blank flasks containing fresh nutrient solutions ± Fe (3 replicates each) were also prepared in the same way as the samples. The pH of all nutrient solutions (samples and blanks) was adjusted to 6.0 using NaOH (0.1 M). Air was bubbled through the flasks continuously. Plants were left in the flasks for 24 hours for collection of "reductants" released by the root system. Thereafter, plants were removed and fresh weights of shoots and roots were separately recorded. The volumes of the nutrient solution remaining in the flasks were also measured. Thereafter, to each of those flasks containing no Fe (samples and blanks), iron was added to give final Fe^{3+} concentrations of 5.6 mgL^{-1}.

3 × 20 ml aliquots of the remaining nutrient solutions were taken from each replicate and transferred to 50 ml flasks. The following were added with mixing: 1 ml of 2 M HCl, 4 ml of 2.5 mM BPDS (bathophenanthroline disulphonic acid), 1 ml of 4 M sodium acetate and 1 ml of distilled water.

A range of standards containing Fe^{2+} (0- 20 ppm in 2 M HCl) were prepared and treated exactly as for the plant samples. All samples, blanks and standards were incubated in the dark at room temperature and their absorbances were measured at 535 nm after 24 hours. The mean absorbance values of the relevant blanks were subtracted from those of samples to allow calculation of the Fe^{3+} reduced during incubation. The method used for collection and measurement of reductants was modified after Chaney *et al.* [4].

Measurement of Fe^{+3} reduction by the root tissues

Preparation of the test media: Nutrient solutions were prepared as above. Iron was added as Fe-EDTA (5.6 mg Fe/L). BPDS was added to all nutrient solutions to give a final concentration of 0.4 mM of BPDS. Thereafter, the pH of the nutrient solutions was adjusted to 6.0 using 0.1 M of NaOH or HCl.

Serial measurement of Fe^{+3} reduction by intact plant root system: Three plants from each treatment (A and B) were placed in separate flasks containing 100 ml of nutrient solution + BPDS, bubbled with air. Flasks were covered with aluminium foil to ensure that the roots were in darkness. the absorbance of the Fe^{2+}-BPDS complex which accumulated was measured on samples (5 ml) withdrawn every 10 min up to 60 min. Thereafter, samples were withdrawn every hour until hour 5, and then again at hour 24. Plants were then removed and fresh weights of shoots and roots were measured. The volume of solution remaining in each flask was also measured and recorded. Control solutions (without plants) were also ampled at the same intervals. The absorbance of the samples withdrawn from the flasks was detected at 535 nm and compared to those of the standards (1-20 mg L^{-1} Fe^{2+}) containing the same quantities of BPDS.

Measurement of Fe^{3+} reduction by different root zones: Root tissue samples (0.8-1g) consisting of tips (0-2 cm) or basal (> 2 cm) were taken from three replicate plants from each of treatments A and B. Both tips and basal root samples were incubated in 25 ml of nutrient solution + BPDS + Fe-EDTA for 24 hours in the dark at room temperature. Thereafter, the accumulated Fe-BPDS complex was measured as described above. The lengths of roots in the samples were estimated using the line intercept method [17]. Control samples, having no roots, received the sample treatments previously mentioned.

Organic acid determination in the plant tissue

Three plants from each treatment (A&B) were harvested for the determination of organic acid contents of different plant tissues. Each plant was divided into shoots and roots. From the shoots, 1-1.5 g samples of fresh material were taken from tops and basal shoots. From the roots, samples (0.8-1.5 g fresh material) were taken from the root tips (0-2 cm) and from basal roots (segments taken at random from 0-2 cm behind tip zone).

Samples of the different plant parts were ground in a mortar and their saps were extracted with 25 ml of hot water (70°C). The resulting extracts were centrifuged for 15 min at 8000 rpm. The purification of supernatants by ion-exchange chromatography, organic acid derivatization, gas-liquid chromatography setting and analysis of peaks and calculations were carried out as mentioned by Stumpf and Burris [18].

100 mg portion of each of the following organic acids: malonic, oxalic, fumaric, succinic, malic, citric, tartaric and acontic were dissolved in 100 ml of distilled water. The organic acid mixture (2 × 10 ml portions) were passed through chromatography colums, treated and analyzed for organic acids in exactly the same way as the samples. The percentage recovery for each of the organic acids was calculated in conformity with the known standard mixture.

Results

Release of reductants: The release of reductants from the roots was accompanied by proton efflux which caused the pH of the nutrient solution to fall from 6 to 4.8. In Fe-sufficient plants, the pH increased during 24 h. from 6 to 6.4. The activity of the Fe^{3+} -reducing compounds ("reductants") released by the Fe-deficient chickpea plants was significantly greater than that by the control plants (Table 1).

Table 1. The activity of "reductants" released by chickpea plants as affected by iron supply. Treatments are A and B

Treatment	Fe^{3+} reduced µmol/100ml/24h	Total fresh weight (g)	pH of nutrient solution	"Reductant" activity after 24 h. incubation expressed as µmol of Fe^{3+} reduced	
				µmol/g root fwt.	µmol/plant
+Fe(A)	0.088	9.19[a]	6.4	0.0126[b]	0.048[b]
+Fe blank	0.040				
−Fe(B)	0.129	8.4[b]	4.8	0.0270[a]	0.088[a]
−Fe blank	0.041				

−numbers followed with the same letter within each column are not significantly different.

Ferric reducing activity of the roots: The total of Fe^{3+} reduced by the roots over short and long time periods are shown in (Fig. 1 a,b). Over short times, reduction of ferric ions by the roots of Fe-stressed chickpea plants *e.g.* fresh root tissues increased quite rapidly, and was higher than that by the control roots (Fig. 1a). In the long term observation, Fe^{3+} reduction by the roots of Fe-stressed plants continued to increase rapidly up to 5 hours, whereas in the control plants the reduction of Fe^{3+} tended to slow after 4 hours (Fig. 1b). However, after 24 hours incubation, Fe^{3+} reduction by Fe-stressed plant roots was about two times greater than that by the control (Fe-stressed plants, 0.0270; control plants, 0.0126 μmol/g root fwt).

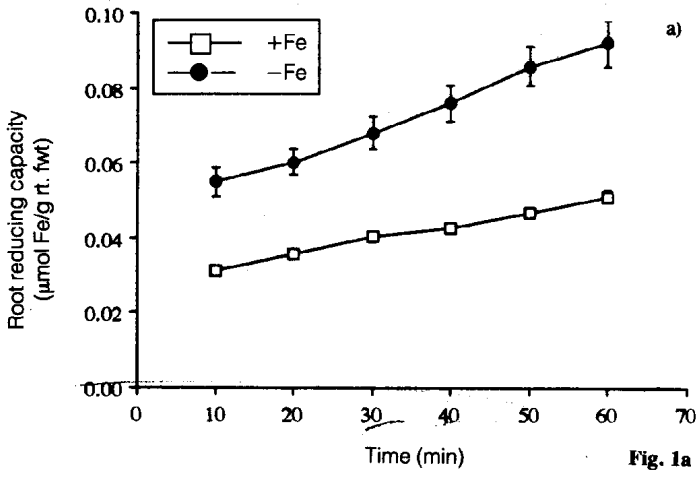

Fig. 1a

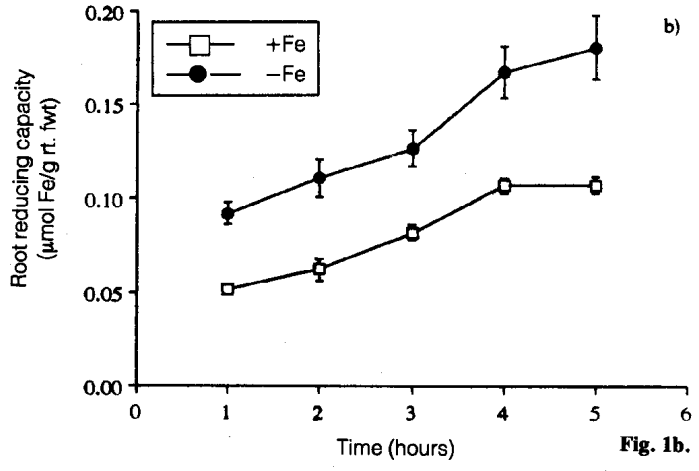

Fig. 1b.

Fig. 1. The time course of cumulative reduction of Fe^{3+} expressed as μmol of Fe^{2+} reduced per gram of root fresh weight.

Data given in Table 2 showed that iron stress reduced root fresh weight by about 60%. The production of swollen root tips and inhibited growth of the lateral roots (Fig. 2) was responsible for the smaller root lengths per gram of each root zone compared to the control plants (Table 2). The Fe^{3+} -reducing capacity of both tips and basal roots, calculated on a weight basis, were significantly higher under iron stress as compared to the control. Also, under both regimes of iron supply, the activity in the root tips was higher than in the basal roots.

Table 2. The effect of iron stress on root growth, root length and ferric reducing capacity of tips and basal roots

Treatment	Total root fwt. (g/plant)	root length (m/g)	Fe^{3+} reduction after 24 h. incubation	
			µmol/g root fwt.	µmol/m root length
+Fe basal	7.92[a]	0.44[c]	0.098[c]	0.226[c]
tip		0.82[a]	0.213[b]	0.258[c]
−Fe basal	3.05[b]	0.32[d]	0.233[b]	0.884[a]
tip		0.75[b]	0.366[a]	0.503[b]

−numbers followed with the same letter within each column are not significantly different.

However, a different picture was obtained when Fe^{3+} reduction was expressed per meter of root length. In the Fe-stressed plants, the reducing activity of the basal roots was found to be significantly higher than that of the tips, whereas in the control plants, the activity was similar in both tips and basal roots. Fe^{3+} reduction by both tips and basal roots under iron stress conditions was higher than the control, but the difference was greater for basal roots (4 times increase) than tips (2 times increase).

Organic acid: 10 organic acids were run as standards on the GLC. Seven of these acids were tentatively identified and measured in the samples. Organic acid contents per gram of fresh weight of plant tissues varied according to iron nutritional status and also to plant part. A summary of the analysis of variance is given in (Table 3).

The effect of iron stress on organic acid accumulation in different parts of chickpea plants, all expressed in µeq per gram of fresh weight, is given in (Table 4).

Regardless of the iron nutritional status, the concentrations of organic acids were much higher in shoots than roots. The biggest differences in the concentrations of organic acids between shoots and roots occurred in the case of citric and malic acids.

Fig. 2. [A] general view of the experiment. Fe-stressed plants have chlorotic tops (−Fe). Control plants remained green (+Fe).
[B] The root system of control plants. Roots were white with long laterals.
[C] The root system of Fe-stressed plants. Roots were slightly brown and had short and thick laterals with swollen tips (arrows).

Table 3. Summary of the analysis of variance of organic acid concentration in different plant parts in relation to iron supply

Acid	Iron supply		Plant part			
	+Fe	−Fe	shoot top	shoot basal	root tip	root basal
Malonic	***	***	***	ns	**	ns
Oxalic	***	ns	ns	ns	ns	ns
Succinic	ns	*	nd	nd	nd	nd
Malic	***	***	ns	**	ns	ns
Citric	***	**	**	**	nd	nd
Aconitic	ns	*	ns	ns	nd	nd

* 5% level of significance; ** 1% level of significance; *** 0.1% level of significance; ns non-significant; nd not detected.

There was a tendency for organic acid concentrations to be higher in basal shoots than top shoots which was most pronounced in the case of malic and citric acids, but, there was little difference between basal roots and tip zones.

The concentrations of malonic, malic and citric acids were significantly greater in the shoot tops of Fe-stressed plants compared to control plants. In the basal shoots, there was a significant difference only in the case of citric acid. In Fe-stressed plants, malonic and malic acids concentrations were significantly higher in the root tips compared to those of the control. There was no significant difference in organic acid concentrations in basal roots between Fe-stressed and unstressed plants. However, the total organic acid concentrations per gram of fresh weight were significantly greater in both tops and basal shoots of Fe-stressed plant compared to those in the control plants. In the roots, total organic acid concentrations were greater in Fe-stressed plants, but significantly so only in the root tips.

Discussion

Chickpea plants grown under iron stress showed morphological changes (stunted root system, etc.) typical to those reported for other plant species [15]. The growth rate of the roots was decreased considerably by iron stress leading to reduction in root weight and also in root length (Table 2).

Table 4. The effect of iron supply on organic acid concentrations in different parts of the chickpea plants expressed as μeq/g fresh weight

Part	Malonic		Oxalic		Succinic		Fumaric		Malic		Citric		Aconitic		Total	
	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe	+Fe	−Fe
Shoots																
Tops	18b	30a	62a	55a	tr.	16	5.5a	6a	36b	112a	8b	138a	13a	16a	142.5b	373a
Basal	23a	30a	80a	72a	tr.	10	6a	4.5a	109a	132a	75b	169a	13a	13a	306b	430.5
Roots																
Tips	6b	17a	22a	19a	tr.	7	tr.	2.2	3b	5a	tr.	3	tr.	2	31b	86.2a
Basal	6a	13a	25a	14a	tr.	2	tr.	0.9	2a	4a	tr.	2	tr.	2	33a	70.9a
LSD*	5.1	8.2	27.8	22	tr.	8.5	7.8	1.7	13.3	17.9	40	58	5.2	8.6	69	94

* LSD at 5% level of significant; − numbers followed with the same letter horizontally for each organic acid are not significantly different.

Although used by a number of other researchers [4,5,9], the method used here for measurement of reductants released by the roots and root reducing capacity must be subject to criticism. The method for measuring "reductants"; 1) does not distinguish between the Fe^{2+} reduced by microbial activity from that by reductants during collection and incubation periods, 2) does not account for the activity of that fraction of reductants that could be quickly oxidized after being released from the roots or used as substrate by microorganisms and 3) does not distinguish the fraction of Fe^{2+} produced by reduction in the roots and subsequent release from that by the "reductants". The high Fe^{3+} reduction observed for ± Fe blanks give concern, since the quantities of Fe^{2+} reduced in these blanks were up to 50% of those reduced by "reductants" released by the control plants (Table 1).

In the measurements of root reducing capacity, it is not certain whether the BPDS added to the nutrient solution does or does not affect the root system and its properties. It would have been wiser to measure the Fe^{3+} reducing capacity of a root system growing in a system which allowed continuous sampling and replacement of the nutrient solution.

The results presented do however provide a general picture of the ferric reducing activity of the roots of chickpea. Fe-stress appears to enhance slightly the release of substances capable of reducing Fe^{3+}. However, this was probably trivial compared to the capacity of the intact root system to reduce iron (Table 5). It is therefore, unlikely that released reductants can make important contribution to the reduction of iron by Fe-stressed chickpea plants. This is in agreement with the finding of Römheld and Marschner [9] for sunflower plants, and support the proposal that Fe^{3+} is mainly reduced enzymatically in the roots [4,19].

Table 5. Capacity of the root system of intact chickpea plants to reduce Fe^{3+} chelates at the root surface compared to that reduced by the released reductants after 24 hours of incubation period

Treatment	Roots reducing capacity μmol Fe^{2+}/g rt.fwt	Reductants reducing activity μmol Fe^{2+}/g rt. fwt
+Fe	0.384 ± 0.017	0.0126 ± 0.0019
−Fe	0.821 ± 0.090	0.0270 ± 0.0017

"Reductants" may be more important as chelators of iron [9,14]. Römheld and Marschner [12] were probably correct in speculating that in soil-grown plants a combination of both mechanisms, the release of "reductants" which may also act as chelators and reduction by membrane-bound reductase, are responsible for the mobilization and uptake of iron in response to iron deficiency.

Iron stress increased the capacity of both tips and basal roots to reduce Fe^{3+} as compared to the control plants. In the Fe-stressed plants, the rate of Fe reduction by the basal roots was significantly greater than that by root tips if values are expressed per meter of root length (Table 2). This is not in agreement with Römheld and Marschner [15] who suggested that root tips reduced Fe at a greater rate. As reduction of Fe^{3+} by root rissue is apparently a process involving a membrane-bound enzyme. The capacity of the roots to reduce iron may be related to the surface area of plasmalemma. The use of the agar plate technique [13] provided evidence that Fe^{3+} was reduced by the Fe-stressed plants more or less uniformly along the whole root system (Figs. 3,4) and there was no indication of specifically enhanced Fe^{3+} reduction in the apical zone. The reductase is therefore probably not restricted to the apical zones but is more or less uniformly distributed along the whole length of the root system.

Proton extrusion induced by cation/anion imbalance during uptake is a well known phenomenon, especially when NH_4_N is supplied. Proton extrusion was uniformly distributed along the whole root system in NH_4_ fed plants [20]. Proton extrusion in response to iron stress also occurred along the whole root system (Fig. 5). Enhanced Fe^{3+} reduction and increased proton extrusion along the whole length of the chickpea root system were similar to those reported for cucumber and peanut [13].

The formation of the so called "transfer cells" in chickpea plants [21] may constitute another response mechanism to iron stress, but it is impossible from the available evidence to reach a firm conclusion concerning their specific role or importance.

In conclusion, Fe-stressed chickpea plants showed physiological changes in their roots similar to those reported for other plant species. These were: increased rate of Fe^{3+} reduction, enhanced release of reducing substances ("reductants"), proton efflux, organic aicd accumulation and the formation of so called "transfer cells". The chickpea cultivar tested can therefore be classified as a "Fe-efficient".

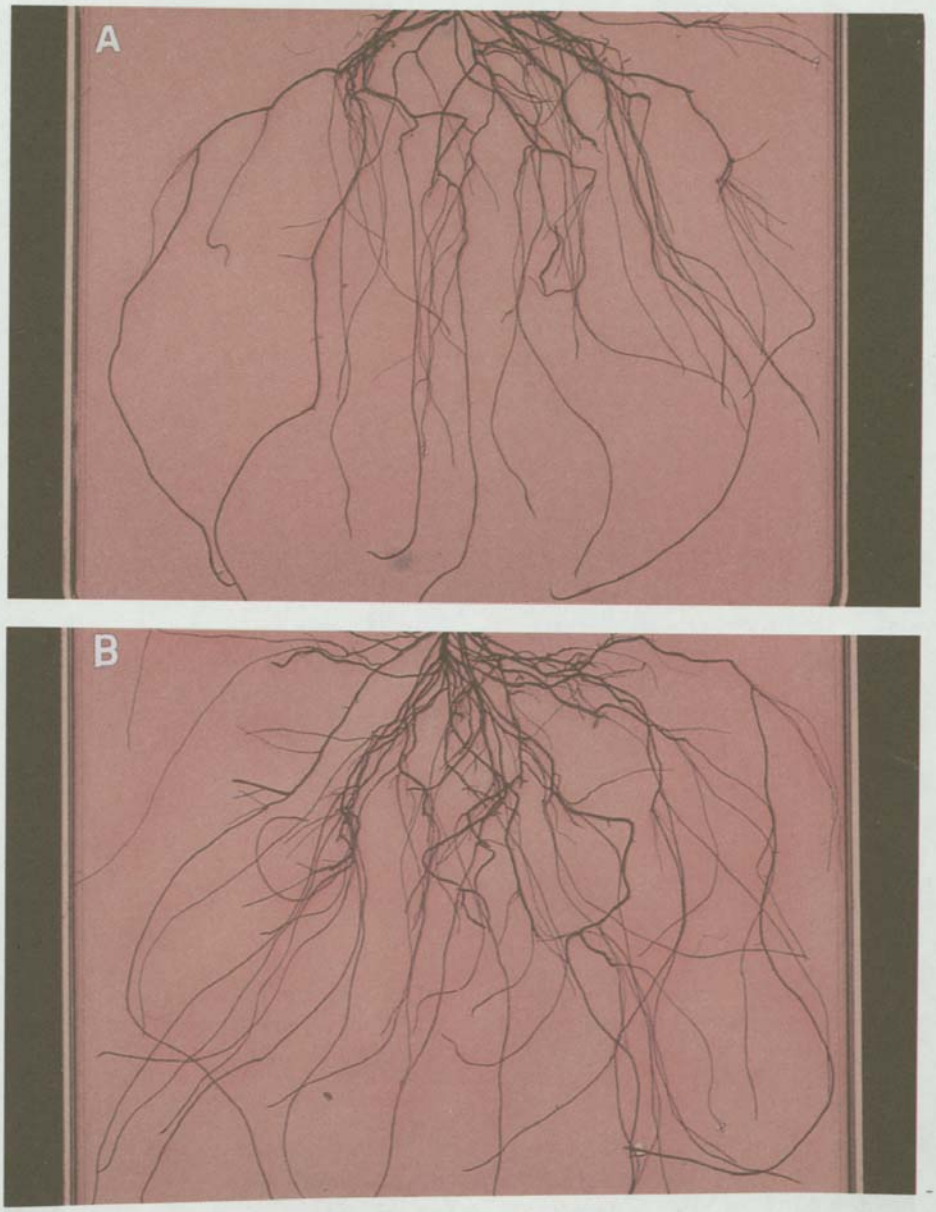

Fig. 3. The effect of iron supply on the Fe^{3+} reduction by the root system of L-SSO after 16 hours in agar containing Fe-EDTA and BPDS.
[A] Control plant showed little ability to reduce Fe^{3+}-EDTA.
[B] Fe-stressed plants reduced Fe^{3+} along the whole length of the root system, indicated by the pink zone around the roots.

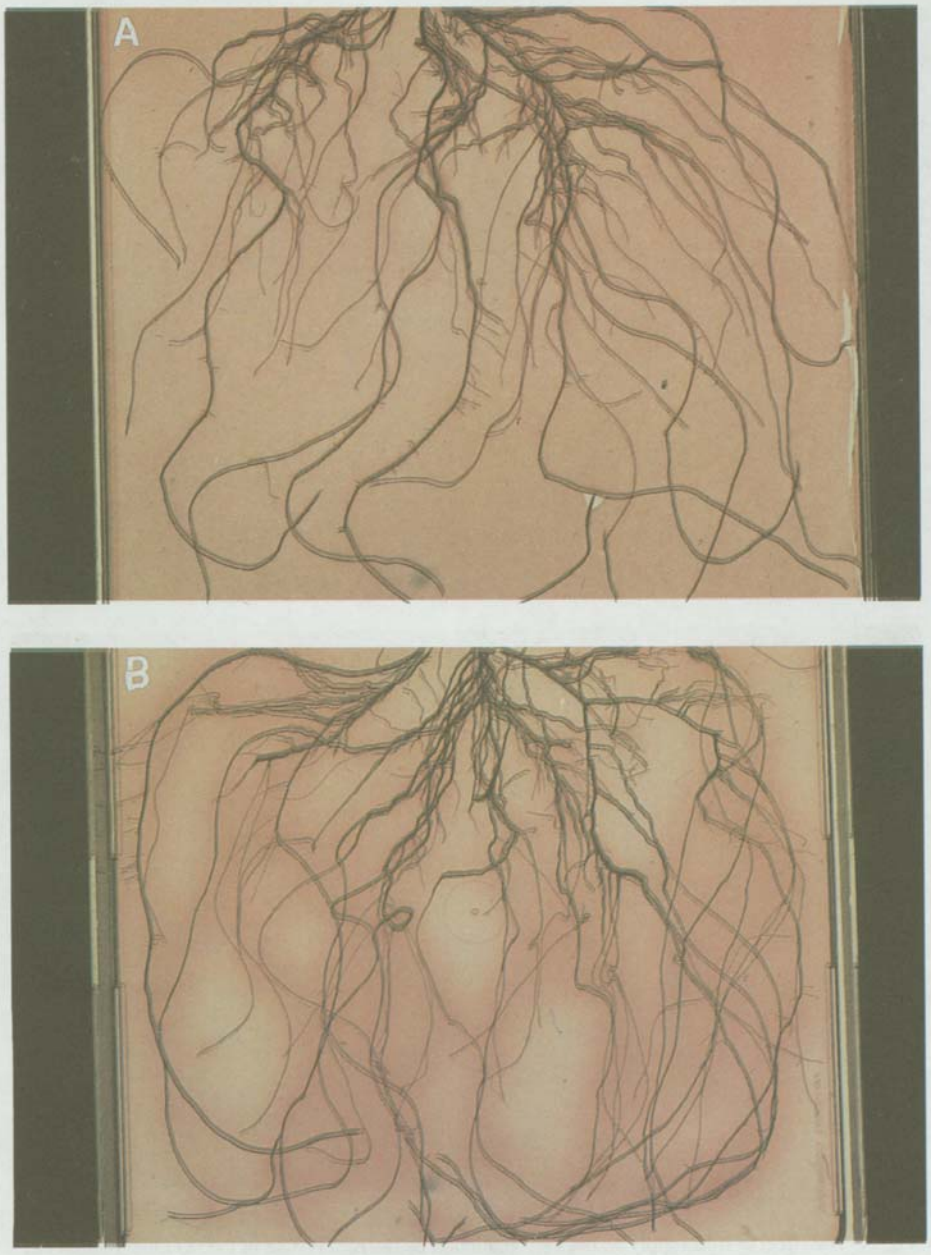

Fig. 4. The effect of iron supply on the Fe^{3+} reduction by the root system of K-850 after 16 hours.
[A] Contrtol plant showed no ability to reduce Fe^{3+}-EDTA.
[B] Fe-stressed plants reduced Fe^{3+} along the whole length of the root system.

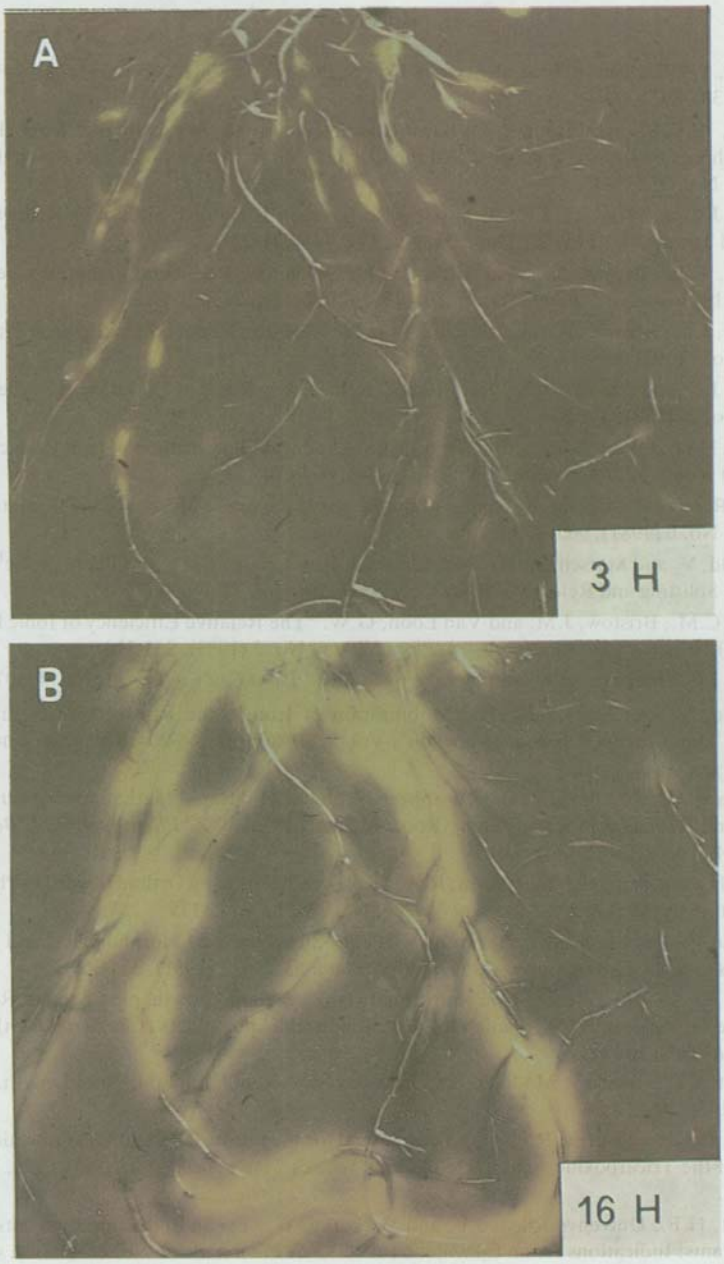

Fig. 5. pH after 3 hours [A] and after 16 hours [B] around the roots of Fe-stressed ILC-195 embedded in agar adjusted to pH 6 (red). The yellow colour indicates acidification to pH 4. This occurred along the whole length of the root system.

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خواص النشاط الاختزالي للحديد في رايزوسفير نباتات الحمص *(Cicer arietinum L.)*

غياث علوش وفرانسس ساندرز

قسم العلوم البيولوجية التطبيقية، جامعة ليدز، بريطانيا

(ورد البحث في ١٤١٦/٦/١٦هـ؛ وقُبل للنشر في ١٤١٦/٦/٢٢هـ)

ملخص البحث. نميت بادرات الحمص (صنف ILC-195) في محلول خال من الحديد لمدة سبعة أيام ومن ثم نقلت مجموعة من البادرات إلى محلول يحتوي على الحديد (معاملة A) ومجموعة أخرى استمرت في النمو في محلول خال من الحديد (معاملة B). جدد المحلول الغذائي تمامًا وباستمرار حتى نهاية التجربة حيث أجريت مجموعة من القياسات تتقصى النظام الاختزالي للحديد (الخارجي بواسطة المواد المختزلة وبواسطة النشاط الأنزيمي)، وكذلك تتراكم الحموض العضوية ووجود الخلايا الناقلة في المجموع الجذري.

تبين أن نقص الحديد قد زاد قليلاً من إفراز الجذور لمركبات قادرة على اختزال الحديد. لقد كانت كمية الحديد المختزلة بواسطة المواد المختزلة قليلة جدًا إذا ما قورنت بمقدرة المجموع الجذري الكامل على اختزال الحديد. كما تبين أن معدل اختزال الحديد بواسطة الأجزاء القاعدية أكبر بكثير من مقدرة المناطق القمية من الجذر. إن استخدام تقنية أطباق الآجار قد قدم برهانًا أكيدًا على أن اختزال الحديد يتم على طول المجموع الجذري كله وكذلك الحال بالنسبة لانخفاض pH الرايزوسفير.

بغض النظر عن حالة التغذية بعنصر الحديد، فقد كانت تراكيز الحموض العضوية أعلى في الأوراق منها في الجذور. لقد كان توزع الحموض العضوية في الأفرع الخضرية القاعدية أكثر من الأفرع القمية. لم يكن هناك فروقات معنوية لتراكيز الحموض العضوية بين مناطق الجذور المختلفة. لقد شكلت حموض Citric & Malic معظم الحموض العضوية الموجودة في المجموع الخضري بينما شكل كل من Molonic & Oxalic معظم ما قد وجد في الجذور.