Step-by-step morpho-physiological responses of *Arachis hypogaea* L. cv. NC 2 to iron deficiency

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ABSTRACT

Well-aerated and alkaline soils are proven to lack plant-available iron. Fe-efficient plants, however, induce morpho-physiological and biochemical mechanisms of adaptation. These changes in morphology and physiology of the shoot-root systems of peanut (*Arachis hypogaea* L. cv. NC 2) plants were studied by cultivating them hydroponically in the nutrient solution containing different levels of Fe^{3+}EDDHA (0.00, 0.125, 0.25, 0.50, 1 and 2 ppm). Three types of chlorosis and regreening (I, II, III) on the shoot system appeared simultaneously with three types of rooting (I, II, III) in different stages of plant growth under Fe-free media. The difference in the regreening processes of plants grown in Fe-free and Fe-treated media indicated that their signaling pathways for Fe localization might be different. The morphological responses were found to correlate to three types of regular and rhythmic pH changes in the nutrient solutions. The sites of pH responses and Fe^{3+} reducing activities in the roots showed dependence on the type of root and shoot morphology.

Keywords: Fe-deficiency; peanut; chlorosis; Fe uptake; Fe-efficiency

Iron is an essential element for all higher plants. Although it is abundant in most well-aerated and alkaline soils, it is often unavailable to the plant because it forms insoluble ferric hydroxide complexes in the presence of oxygen at neutral or basic pH (Guerinot and Yi 1994). In contrast, in anaerobic conditions in acidic soils cellular iron overloading causes serious damage to plants because bivalent free iron catalyzes the formation of reactive oxygen species (Briat and Lebrun 1999).

Utilization of iron in alkaline soil by plants is genetically controlled; a cultivar that can utilize it is called Fe-efficient, whereas a cultivar that develops Fe chlorosis is called Fe-inefficient (Brown and Jones 1976). To make iron more available, Fe-efficient plants have evolved adaptive or inducible mechanisms (Marschner 1986). They develop various morphological, physiological and biochemical changes in the shoot and root systems under iron-deficiency. Fe-efficient plants can be classified into two groups:

(a) Plants with strategy I, which include dicotyledonous and non-Graminaceae monocotyledonous species, often respond by increasing the capacity of the root system to reduce Ferric chelates (Bienfait et al. 1982, Schmidt et al. 1996, Robinson et al. 1999). This Fe-efficiency is achieved by transporting the resulting ferrous iron across the root (Robinson et al. 1999, Vert et al. 2002, Green and Rogers 2004), by acidification of rhizosphere (Romheld et al. 1984, Palmgren 2001), by excretion of phenolic and flavin compounds (Susín et al. 1994), and by accumulation of organic acids in iron-deficient leaves (Landsberg 1981) and roots (Alhendawi et al. 1997). In addition, strategy I plants develop different morphological changes in their root system to increase the surface available for iron uptake (Landsberg 1982, Bienfait 1988).

(b) Plants with strategy II, which include Graminaceae species, respond by increased synthesis and secretion of non-proteinogenic amino acids

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(phytosiderophores, specific Fe(III)-chelating compounds) to the rhizosphere (Marschner and Romheld 1986, 1994).

The cultivated peanut, *A. hypogaea* L., is one of the most important crops in the world. Iron-deficiency is an important limiting factor for this plant. Hence, peanut appears to be one of the most susceptible crops to lime-induced iron deficiency (Chen and Barak 1982). It has already been reported that Fe-deficiency is genetically controlled in this plant. *A. hypogaea* L. cv A124B is known as one of the iron-chlorosis resistant cultivars and can be cultivated in calcareous soils (Romheld and Marschner 1983), while *A. hypogaea* L. cv Tainan is susceptible (Tang et al. 1991). It seems that studies on genetically Fe-efficient peanut plants will be more important than the improvement of soils or other cultivation methods.

This paper presents and discusses some morphological and physiological responses in the shoot and root systems of a common peanut cultivar (*Arachis hypogaea* L. cv. NC 2) in Fe-free and Fe-treated media.

MATERIAL AND METHODS

Plant materials and culture conditions

Seeds of peanut cultivar (*Arachies hypogaea* L. cv. NC 2) were placed in an aerated solution of $CaSO_4 \cdot 2\ H_2O$ to remove the superficial mucilages for 3 days, surface sterilized with 1% NaOCl for 15 min and rinsed three times in distilled water. Sterilized seeds were germinated and grown until the seedling stage in vermiculite moistened with 0.5mM $CaSO_4 \cdot 2\ H_2O$ solution. The 14-day old seedlings were then transferred for five weeks to continuously aerated modified Hoagland solution (one liter, pH 5.75) after sterilization with 0.5% NaOCl for 10 min. The nutrient solution contained 1mM KH_2PO_4, 5mM KNO_3, 5mM $Ca(NO_3)_2$, 2mM $MgSO_4$, 11μM $MnSO_4$, 0.7μM $ZnSO_4$, 0.3μM $CuSO_4$, 0.16μM $(NH_4)_6Mo_7O_{24}$, and 46μM H_3BO_3. Six Fe treatments (0.00, 0.125, 0.25, 0.5, 1 and 2 ppm) were applied as Fe^{3+}EDDHA and for each treatment, four repeats were made. All materials (vessels and pipettes) in contact with plants or nutrient solutions were rinsed with 0.1N HCl followed by 2.0mM Na_2EDTA to remove iron contamination. The pH of the nutrient solution was measured daily. Plants were grown in one-liter vessels. The number of vessels per treatment was four and the number of plants per vessel was one. Four replicates were realized for each of the measured parameters. All plants were grown under controlled growth conditions with a day/night regime of 12/12 h, 26/21°C and light intensity near 5000 lux (fluorescent tubes, 40W/25 Fluora). RH was adjusted to about 65%.

Determination of leaf chlorosis and chlorophyll content

The score of the chlorosis was rated using the following index: (1) green leaves, (2) leaves with slightly yellow margins, (3) distinct yellowing over most of the leaf except in mid-vein, (4) completely bright yellow leaves and (5) largely necrotic leaves (White and Roberson 1990).

The chlorophyll content of the leaves was detected as described in Moran (1982). Leaf discs, 6 mm (28/26 mm^2) in diameter, with equal weights and numbers, were treated with 4 ml of ethanol and *N*,*N*-dimethyl-formamide (at the rates of 1/10 and 1/100 for chlorotic and non-chlorotic leaves, respectively) for 48 h at 4°C. Chlorophyll (*a*, *b*, pchl and total) contents were quantified according to the Moran equations and expressed as μg/ml:

$$C_a = 12.65A_{664} - 2.99A_{647} - 0.04A_{625}$$

$$C_b = -5.48A_{664} + 23.44A_{647} - 0.97A_{625}$$

$$C_p = -3.49A_{664} - 5.25A_{647} + 28.3A_{625}$$

$$C_T = C_a + C_b + C_p$$

where: C_a, C_b, C_p and C_T represent the concentrations of Chl *a*, *b*, protochlorophyll and total chlorophyll. Data were finally calculated and expressed as μg/100 (50) mm^2 leaf area.

Assay of root Fe^{3+} reducing capacity

Roots, with 500 mg in weight, were excised, rinsed in double distilled water and kept in 5 ml of assay solution containing 0.5mM $CaSO_4 \cdot 2\ H_2O$, 5mM Hepes (pH 6.5), 0.5mM Fe^{3+}EDDHA and 0.5mM ortho-phenanthroline. Assays were conducted in the dark with aerobic conditions at 25°C. After 65 min the assay solution was removed and the

concentration of Fe(II) was detected by photometry at 500 nm in comparison to standard curve. Standard assay solution for Fe(II) complexometry (at 510 nm) contained 2 ml of 10% NH_4OH, 4 ml of 0.3% ortho-phenanthroline, 6 ml of acetate-buffer (pH 4.5) and a known volume of $FeCl_3 \cdot 6\ H_2O$ from each of the following concentration: 0.1, 0.2, 0.4, 0.6, 0.8, 1, 1.2, 1.4, 1.6, 1.8 and 2 ppm.

Localization of root reducing and pH response sites

To determine the discrete areas of excised roots active in Fe(III) reduction, the excised section of roots were embedded in Fe(III) reducing capacity assay solution solidified by the addition of 0.75% (W/V) agar-agar. Roots were incubated in the dark at 25°C to allow for the development of characteristic red color.

To determine the locations of pH response along the roots, excised roots were placed on 5 mm thick slabs of agar containing bromocresol purple as a pH indicator. The concentration of bromocresol was so that its pH was adjusted to 5.2 prior to addition of agar, heating and mixture. Roots were then placed in dark at 25°C for the period of 4 h, afterwards results were scored by the development of yellow color.

Statistical analysis

The experimental data were analysed using randomized complete block designs and the mean values were compared to each other according to the Duncan Test at the significant level of $\alpha = 0.01$.

RESULTS

Shoot system

A characteristic of Fe deficiency-chlorosis is yellowing between leaf veins with a typical cavity in the apical part of the leaf. This was first developed in the fourth leaf of plants grown in Fe-free media (Figure 1a, b) and was then observed in the other Fe-treatments (0.125, 0.25, 0.5, 1 ppm Fe) in fifth and sixth leaf. No iron chlorosis was visible in leaves of plants grown in 2 ppm Fe(III) during the period of experiment. The first regreening process started in fifth or sixth leaf in Fe-free treatment, while the fourth leaf remained chlorotic (Figure 1c). The second type of chlorosis, with distinct whitish yellowing over the leaf, appeared in the seventh and eighth leaf and was followed by the second regreening in the ninth and tenth leaf (Figure 1d, e). Plant growth was stunted by the development of the third chlorosis with severe necrotic regions after which no regreening was observed (Figure 1f). The consecutive appearance of chlorosis and regreening were also observed in other treatments. However there was a difference in regreening patterns. In contrast to the plants grown in Fe-free media, in Fe-treated media the regreening process comprised the already chlorotic leaves with crumpling (Figure 1g). The difference between the regreening processes in plants grown in Fe-free and Fe-treated media is shown schematically (Figure 2).

Leaf shapes were influenced by the concentration of Fe(III) EDDHA in the solution media. Leaves in low concentrations were narrow and almond shaped, whereas in high concentrations they were wide and round. Shoot heights and internodes were increased, as the concentration of Fe(III) EDDHA increased in the solution media; however, in the highest concentration (2 ppm Fe) they suddenly decreased and the thickness of shoots increased. Growth of auxiliary buds was also affected by low concentrations of Fe (Figure 1h).

The chlorophyll content of the leaves was influenced by the Fe concentration in culture media. Comparison of contents of chlorophyll *a*, *b*, pchl and total in the seventh leaves of all Fe treatments showed at the same time the lowest amount of chlorophyll in Fe-free medium grown plants. Also, the comparison of total chlorophyll contents in leaves of plants grown in Fe-free and Fe-treated (0.125 ppm Fe) revealed a strong difference between regreening process of Fe-treated and non-treated plants (Figure 3).

Root system

The morphology of the roots was influenced by the Fe concentration in the solution medium. Roots grown in Fe-free medium were thicker and rougher than in the other treatments. As opposed to that, the roots of Fe-treated plants become more soft, thinner and hairy as the concentration of Fe in the solutions increases (Figure 4a). The activity of apical meristem of the main roots was stunted simultaneously to the appearance of the first chlorosis in Fe-free medium, leading to

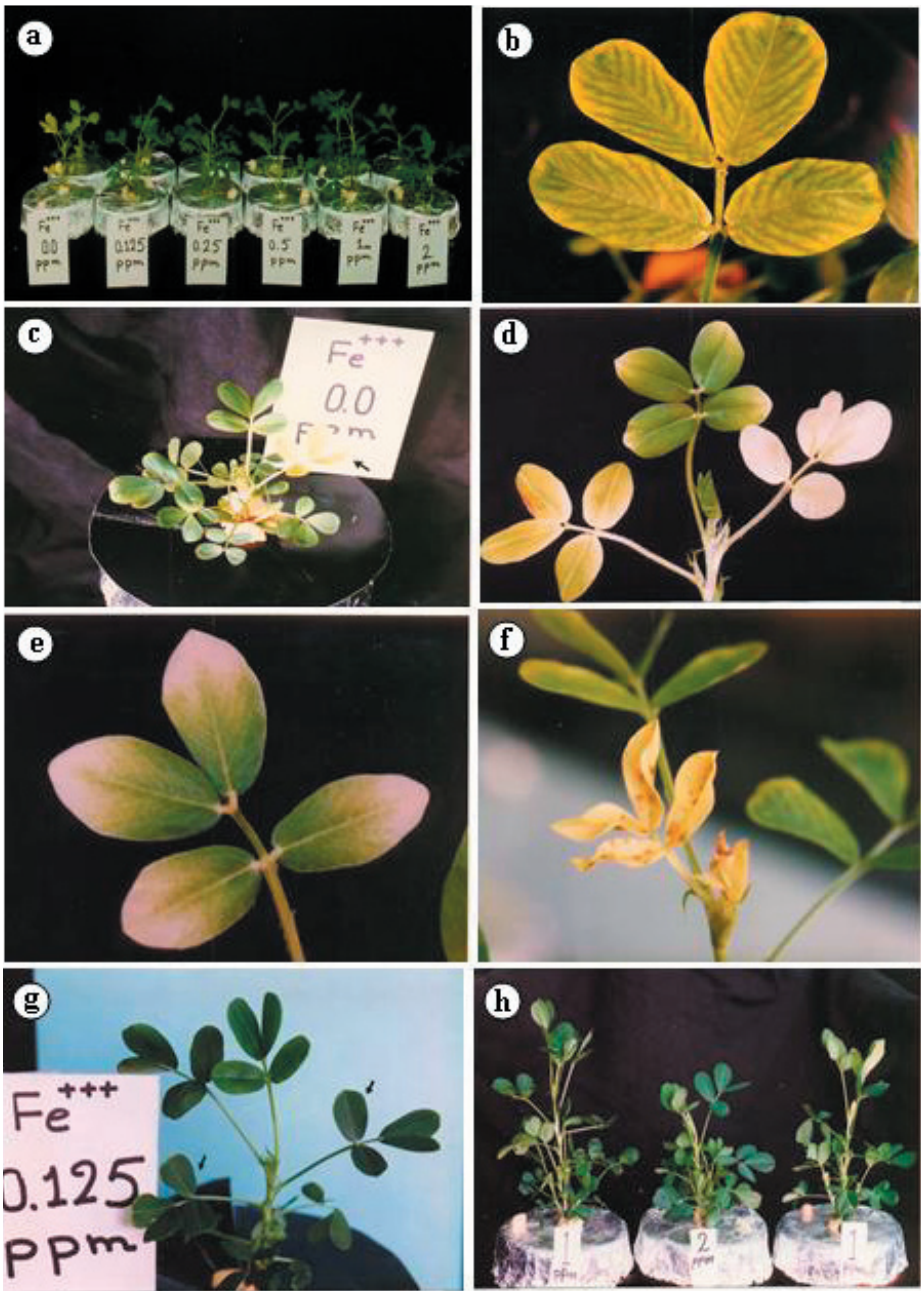

Figure 1. Step-by-step morphological changes in the shoot system: (a) appearance of the first chlorosis in the fourth leaves of plants grown in Fe-free media; (b) a typical iron chlorosis symptoms observed at the first chlorosis stage; (c) the first regreening process developed in the youngest leaves but not in already chlorotic leaves; (d) the second chlorosis and regreening stages with different symptoms; (e) a leaf in the second regreening stage; (f) the third and last chlorosis with necrotic areas in the eleventh leaves of the Fe-free media grown plants; (g) regreening and crumpling in already chlorotic leaves of plants grown in Fe-treated (0.125 ppm) media; (h) shoot growths in plants grown in 1 ppm Fe (left and right) and 2 ppm Fe (middle)

fractures in the cortex layer and the growth of a subapical cluster of auxiliary roots (Figure 4b). Regular dense tertiary roots appeared on the terminal axis of these auxiliary roots at the second chlorosis stage (Figure 4c). Root end malformations were observed at the third chlorosis stage (Figure 4d). These appeared in three types: (a) flaxy roots as a result of root ends bending, (b) lateral roots similar to snake tail at the ends of necrotic roots, and (c) flaxy roots as a result of apical cleavage followed by bending (Figure 4e–g, respectively).

Swelling of the lateral root ends was the latest morphological response of roots to Fe-deficiency

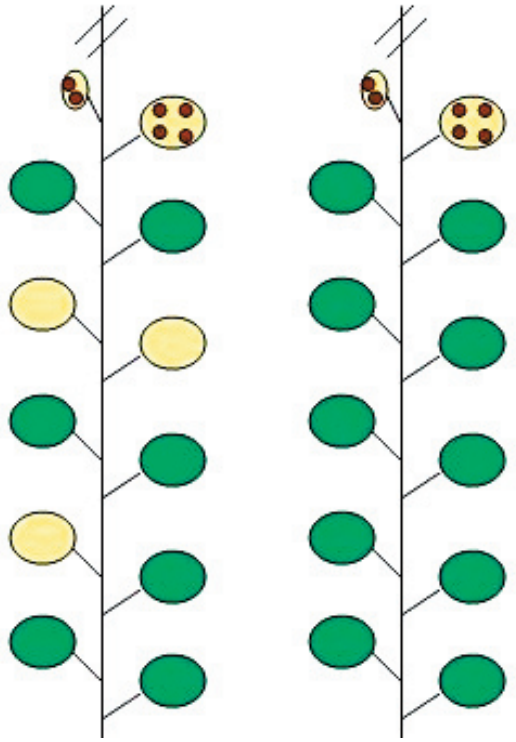

Figure 2. Comparison of regreening process in plants grown in Fe-free (left) and Fe-treated (right) media. The process in Fe-free media grown plants does not include the leaves already chlorotic as compared to Fe-treated media grown plants

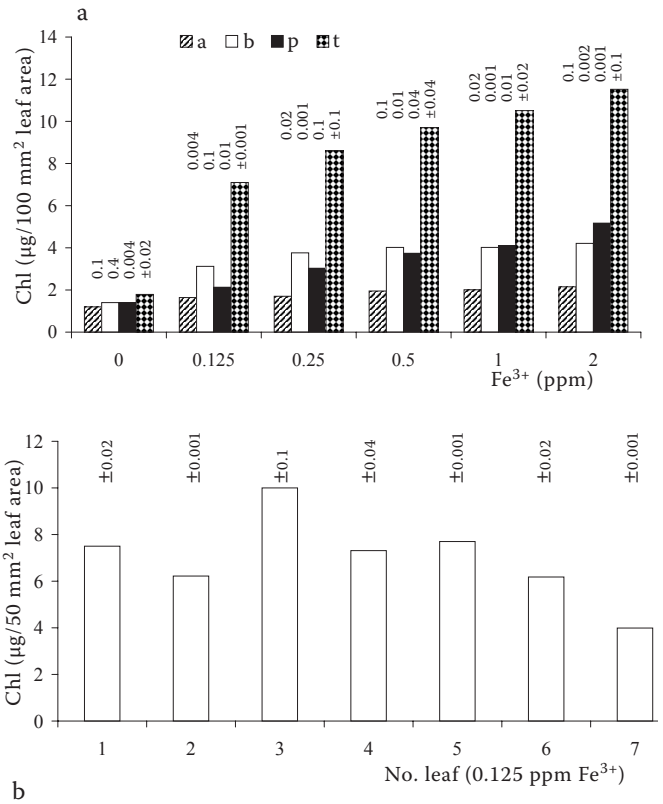

Figure 3. Variations in the chlorophyll contents: (a) chlorophyll (*a*, *b*, pchl and total) contents in the seventh leaves of plants grown under different Fe treatments. Chlorophyll concentrations were assessed by the Moran method and expressed finally as µg/100 mm^2 leaf area; (b) total chlorophyll content in the leaves of plants grown in 0.125 ppm Fe (left) and in Fe-free solutions (right). Chlorophyll concentrations were quantified using the Moran method and expressed as µg/50 mm^2 leaf area. Data presented as the means of four repeats and the mean values compared according to the method at the significance level of $\alpha = 0.01$

stress after which plant growth was stunted leading to death (Figure 4h).

The pH response of Fe-deficient roots was studied by measuring the pH value of the nutrient solutions once a day. The pH value of nutrient solutions of plants grown in Fe-free medium rapidly decreased from 5.75 to 3.30 on day 9, when the first chlorosis was developed in the fourth leaf. The pH of Fe-free solution increased again to 3.91 during the first regreening process in the fifth and sixth leaves. The pH value fell to 2.85 at the second chlorosis in the seventh and eight leaves, increased to 3.22 in the second regreening stage in the ninth and tenth leaves, and finally reached 2.58 at the last chlorosis stage (Figure 5a). As the results show, the highest rate of decrease and increase of pH value occurs in the first chlorosis and regreening stage, while this rate is the lowest at the last chlorosis on day 36. The rhythmic changes in pH of the nutrient solutions were visible, though not considerably, in other Fe-treatments, too. Spraying of chlorotic leaves of Fe-free grown plants with 2 ppm Fe(III) EDDHA twice in a day led to regreening process in these leaves and to a decrease of pH in the nutrient solution, compared with non-sprayed plants (Figure 5b).

Fe^{3+} reduction and pH response sites of roots grown in Fe-free medium were found to cover the

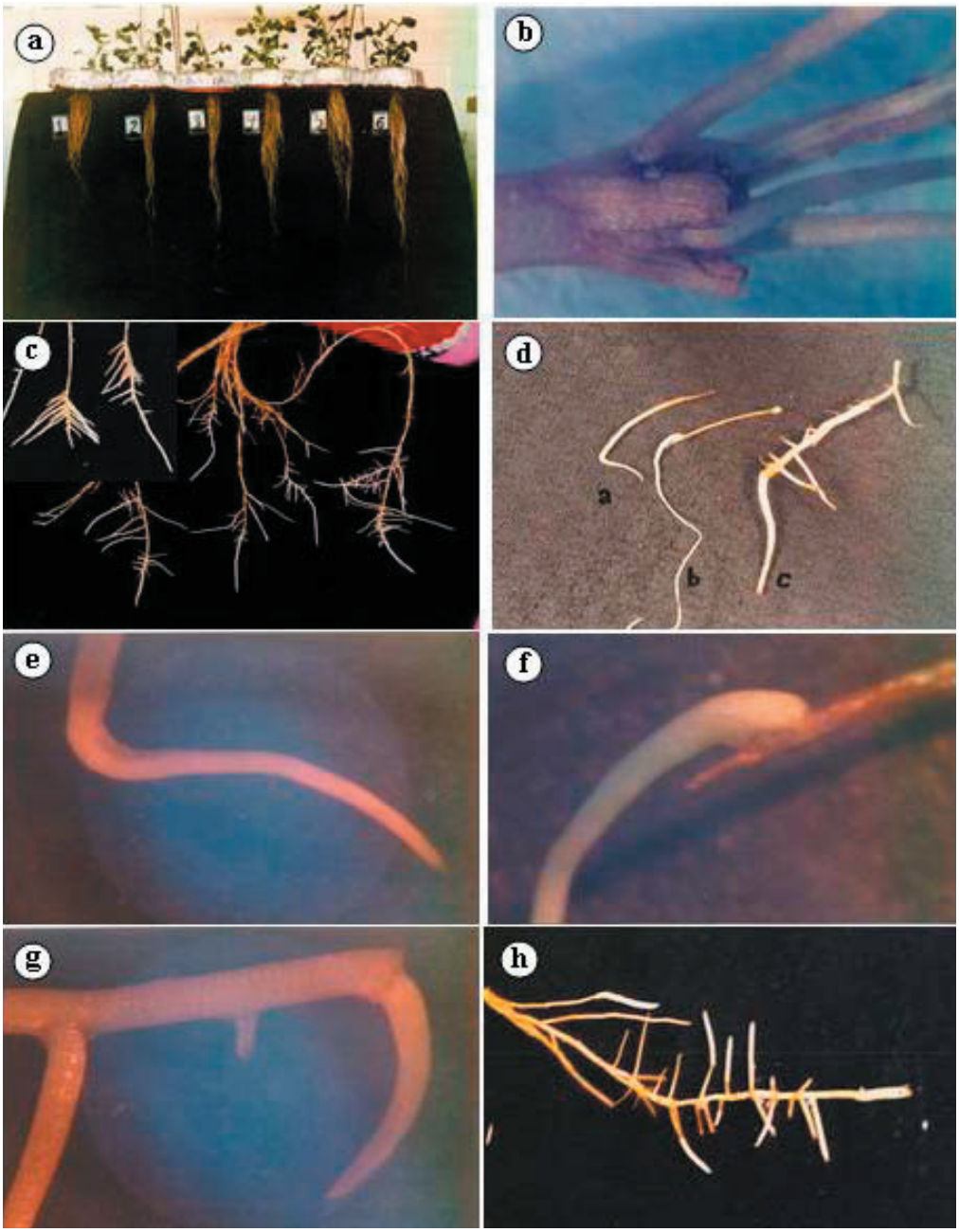

Figure 4. Step-by-step morphological changes in the root system: (a) the length and thickness of the roots grown in different Fe-treatments (from left to right: 0.00, 0.125, 0.25, 0.50, 1 and 2 ppm Fe); (b) main root end fracture and the formation of a dense cluster of lateral roots at the first chlorosis stage; (c) appearance of dense secondary roots on the apical axis of lateral roots at the second chlorosis stage; (d) three types of root end malformations at the third chlorosis stage; (e) a flaxy root as a result of root end bending; (f) a snake tail-shaped root; (g) a flaxy root as a result of root end cleavage; (h) formation of lateral roots with swollen ends at the last stage of plant growth

largest areas of younger, thinner and hairy roots that appeared mostly during the first chlorosis stage. Malformed roots at the last stage of plants growth showed no Fe^{3+} reducing activity and no pH responses (photograph not shown).

Fe^{3+} reducing capacities of roots grown in different Fe-treated media were determined on day 9, together with the appearance of the first chlorosis in plants grown in Fe-free medium. With the decrease of Fe(III) EDDHA concentrations in the solution media, the Fe^{3+} reducing capacity of the roots increased from 13.21 to 67.50 μg Fe^{2+}/g/h (Table 1). In another experiment in which chlorotic leaves were sprayed with 2 ppm Fe once

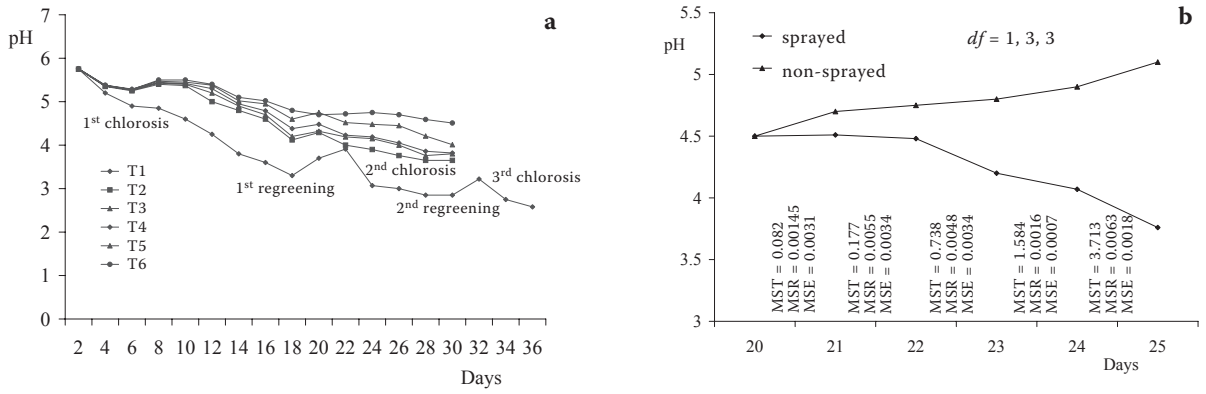

Figure 5. pH responses in the root system; (a) the changes in the pH of the nutrient solutions with different Fe-treatments during the experimental period; pH of the solutions decreased at chlorosis stages and increased in the regreening processes leading to the pH peak formation as shown; (b) compression of pH changes in the nutrient solutions of Fe-sprayed and non-sprayed plants at the first chlorosis stage. Data presented as the means of four replicates and the mean values compared according to the method at the significance level of $\alpha = 0.01$

a day, the reducing activity of roots decreased in the regreening stage, while pH of the nutrient solution increased (Figure 6).

DISCUSSION

Shoot system

Plants grown in iron-deficiency media, particularly in Fe-free solutions, showed three types of chlorosis in their leaves during an experimental period of 36 days (Figure 1). The lack of these symptoms in plants grown in the solution containing the highest concentration (2 ppm) of Fe(III) EDDHA revealed that these chlorosis was due to the effects of Fe-deficiency, but not because of to the other elements.

First and second chloroses were followed by a regreening process in plants grown in both Fe-treated and non-treated media. In plants grown in Fe-free medium regreening did not include the leaves already chlorotic, as compared with Fe-treated media grown plants in which regreening comprised the leaves already chlorotic, too (Figure 2). This may indicate that the signaling pathways for iron transport and localization in plants grown in Fe-free medium may be different from plants grown in Fe-treated media. This suggestion was further confirmed by the observation data obtained from the analysis of total chlorophyll content in the chlorotic leaves before and after regreening process. Iron was already reported to be involved in chlorophyll biosynthesis (Spiller et al. 1982, Tottey et al. 2003).

Table 1. Comparison of Fe^{3+} reducing activities in the roots grown in different Fe concentrations

Fe^{3+} (ppm)	pH of nutrient solution	Root reducing activity (μg Fe^{3+}/g/h)
0.0	3.03	67.50 ± 3.53 a
0.125	3.19	35.54 ± 4.52 b
1.0	4.70	23.53 ± 5.87 c
2.0	5.12	13.21 ± 4.65 d

Means with the same letters are not considerably different

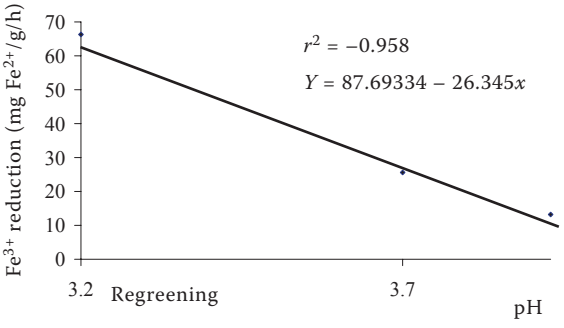

Figure 6. Correlation between Fe^{3+} reducing activity in the roots and the pH of the nutrient solutions

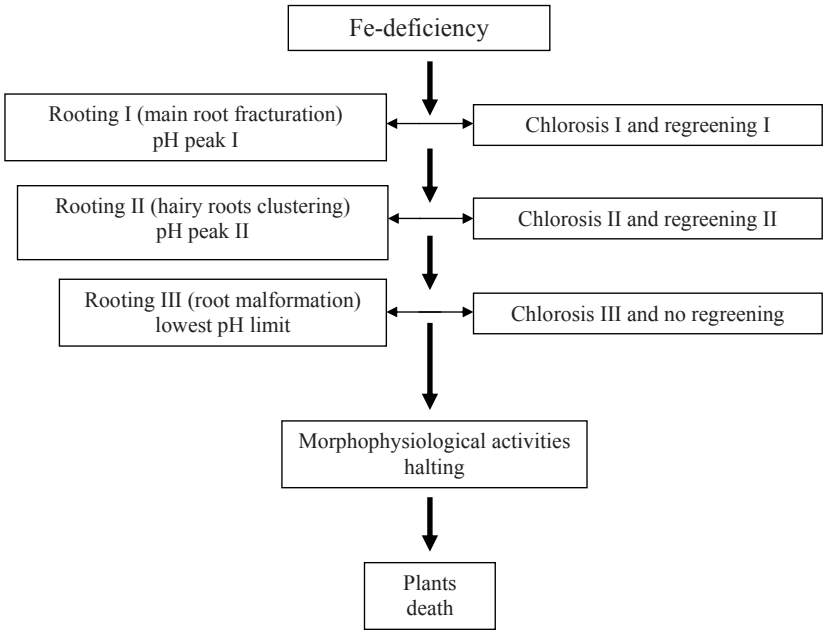

Figure 7. Schematic presentation of step-by-step morpho-physiological changes in the shoot and root systems of plants grown under Fe-deficiency

Root system

The roots of peanut plants became thicker and rougher if the iron stress conditions preceded. The roots of plants grown in iron-free medium were thickest and roughest at the first chlorosis stage (Figure 4). Simultaneously, a dense clusters of auxiliary roots appeared at the necrotic and fractured end of the main root (first rooting), followed by the formation of secondary lateral roots on their apical axis (second rooting) at the second chlorosis stage. A similar result was previously reported in the case of rice (Mori et al. 1991). These changes were followed by formation of flaxy, snake tail-shaped and swollen lateral roots (third rooting) at the third chlorosis stage. In sunflower roots, these types of malformations appear as first symptoms of iron deficiency (Romheld and Marschner 1981). In peanut plant, the changes in the morphology of the root system were classified into three types; they occurred step-by-step and finally led to the halting of plant growth. In lupines and peas grown in high HCO_3^- concentrations, root malformations markedly decreased the growth of the shoots and finally led to the death of the plants (White and Robson 1990).

A rhythmic pH response was found in the roots grown in Fe-deficient media. This was especially remarkable in plants grown in Fe-free media (Figure 5). In general, the pH of the solutions decreased during chlorosis and increased during regreening process. Two types of pH peaks (I and II) and one lowest limit were found, consistent with different stages of morphological responses in shoot and root systems (Figures 5 and 7).

Fe^{3+} reducing activity was found to depend on the Fe concentration in the solution media. It increased during chlorosis stage and decreased during regreening process (Table 1, Figure 6). In previous studies, it was reported that such reduction occurs enzymatically in the cytoplasmic membrane in the root cells of peanut plant (Romheld and Marschner 1983). pH responses and Fe^{3+} reductions located over the large areas of youngest roots, but did not include flaxy, snake tail-shaped and swollen roots. This result is in agreement with the report by Romera et al. (1991) in which the morphological changes and physiological activities in peach halted in the swollen roots (Romera et al. 1991).

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