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Influence of different nitrate and iron availabilities on phosphoenolpiruvate carboxilase and malate dehydrogenase in roots of maize (*Zea mays* L.) plants

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Abstract: The effect of the different nitrate (NO_3) availability on some enzymatic activities has been evaluated in iron (Fe) deficient and iron sufficient maize plants (*Zea mays* L.) in order to evaluate the induction of Fe sensitiveness to enzymatic activities. The apoplast reactions may be altered due to the different nitrate availability. Two experimental tests were done on maize plants grown in nutrient solution with different NO_3 availability and with Fe-sufficiency and Fe-deficiency. Phosphoenolpiruvate carboxilase (PEPcase) and malate dehydrogenase activities, for the reaction determined in citosol, by NO_3 uptake, showed different responses according to Fe availability. The different nitrate availability caused a difference in the acid content. These results justifies the higher energy demand to activate membrane carriers under stress conditions for the reduced nitrate availability.

Keywords: *Zea mays*, iron availability, phosphoenolpiruvate carboxilase, malate dehydrogenase.

تأثير المستويات المختلفة للنترات والحديد المتاح علي فوسفواينولبايروويت كاربوكسيلاز ومماليت ديهايدروجينيس في جذور نبات الذرة

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الملخص: لقد تم تقييم تأثير مستويات مختلفة من نترات النيتروجين ومدى توفرها علي بعض الأنشطة الإنزيمية في نقص وتوفر عنصر الحديد في نبات الذرة وذلك من اجل تقييم اثر حساسية عنصر الحديد على الأنشطة الإنزيمية في النبات ويمكن من خلال التفاعلات خارج جدار الخلية ابوبلاست تغيير ردود الفعل مع اختلاف توفر عنصر النترات وقد تم القيام بتجربتين على نبات الذرة للمستويات مختلفة من عنصر النترات مع وجود عنصر الحديد وأيضا نقص عنصر الحديد في المعاملات المختلفة فوسفواينولبايروويت كاربوكسيلاز ومماليت ديهايدروجينيس ولتحديد ردة فعل الساتيوسول تم تحديد فوسفواينولبايروويت ومماليت ديهايدروجينيس من خلال دراسة النسبة المستخدمة أو الممتصة من التربة من محلول النترات وقد أظهرت النتائج استجابات مختلفة وفقا لتوفر عنصر الحديد .وقد نتج أيضا ظهور مستوى حمضي في نبات الذرة مع اختلاف تواجد عنصر النترات في المحاليل المغذية وهذه النتائج توضح مدى الطلب المتزايد على الطاقة لإعادة تفعيل المواد الغذائية تحت الظروف الصعبة خلال توفر عنصر النترات.

Introduction

Though iron is one of the most abundant elements of the lithosphere (5%), its availability in the soil is linked with a series of balances between ions and free oxides (Cesco et al., 2001), depending on redox potential and pH (Thoiron et al., 1997). This availability is affected by the tendency to hydrolysis of ferric salts, the greater mobility of Fe^{2+} compounds and the chelating action of organic substance. The reduced iron availability in calcareous soils due to high value of pH, interferes with plants development. Due to its implications in redox processes, its deficiency affects the biochemical properties of the nutrients, above all of nitrogen. Nitrate (NO_3^-) assimilation, done by plants by means of a co-transport mechanism through symport in the ratio $2H^+/1\ NO_3^-$, causes pH changes both in the cytosol and apoplast (Aslam and Travis, 2001; Espen, 2001). Under iron deficiency, maize plants follow 'Strategy II' which is above all characterised by the release, independent from rhyzosphere pH, of phytosiderophores, non proteic nitrogen

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chelated compounds with molecular weight of about 320 Da. Studies done on maize suggest that nitrate use needs H^+-ATPase activation and is strictly connected to enzymatic systems, linked with e^- transport and therefore dependent on containing iron compounds (Thoiron et al., 1997). Nitrate uptaking systems are, therefore, induced by anion external concentration that influences H^+-ATPase activity and nitrate reductase. The latter is directly proportional to the nitrate and iron availabilities. Also nitrate internal translocation is linked with such enzymatic systems. Plasma membrane Fe(III)chelate reductase activity seems to be more linked with pH variations, rather than with the micro-nutrient availability.

In maize plants the variation of pH of the soil solution, caused by nitrate uptake, influenced constitutive Fe(III)chelate reductase and plasma membrane H^+-ATPases, as well as nitrate reductase and roots and leaves internal nitrate contents.

In order to uptake iron, maize plants, release phytosiderophores (Takagi et al., 1984) and activate on the plasma membrane of root cells a high affinity uptaking system for pH dependent Fe^{3+}-phytosiderophore (Römheld, 1987).

Since iron deficiency induced in maize plants a H^+ release (Davies, 1986; Landsberg, 1981), the present work was carried out to point out in iron deficient and sufficient maize plants, the influence of the different nitrate availability on sugar and organic acid contents and on phosphoenolpiruvate carboxilase and malate dehydrogenase activities, enzymes that regulate pH-stat mechanisms.

Material and Methods

Maize seeds (*Zea mays* L., Commercial hybrid Cecilia, Pioneer S.p.A.), soaked in distilled water for 24 hours, were put on a metallic net rested on a plastic pot containing 5 L of 0.5 mM $CaSO_4$. Seeds were germinated in the dark, till cotyledons emission at 95% relative humidity (RH) and 27°C temperature. After four days, 10 groups of three plantlets each, were transferred in a growth chamber under controlled conditions (16/8 photoperiod, 20°C temperature and 60-70% RH) and grown in Hoagland nutrient solution.

In order to develop the experiment, four factors were considered: two NO_3^- concentrations and two iron concentrations, with 3 replications each.

Experiment 1 (NS_1)
4.0 mM NO_3^- (NS_1)
a) Fe-sufficient (+Fe), added with 80 μM Fe(III)-EDTA.
b) Fe-deficient (-Fe), added with 0.1 μM Fe(III)-EDTA.

Experiment 2 (NS_2)
0.4 mM NO_3^- (NS_2)
c) Fe-sufficient (+Fe), added with 80 μM Fe(III)-EDTA.
d) Fe-deficient (-Fe), added with 0.1 μM Fe(III)-EDTA.

Roots were taken from plantlets collected at the 5th day from transfer in NS at 6, 12, 24, 48 and 72 hours of growth. Phosphoenolpiruvate carboxilase and malate dehydrogenase activities were determined according to Singal and Sing, 1986; Ritambhare, 2000 respectively. Furthermore, the content of organic acids (citric, malic, oxalacetic and succinic acids) and sugars (glucose, fructose and saccarose) were determined by means of gas-chromatographic apparatus (Gas Chromatograph 8310 Series, Perkin Elmer) according to Sweeley et al. (1963). The conditions were: programmed oven temp 1 130°C, iso time 1 5.0 ramp rate 1 6.0, oven temp 2 260°C, iso time 2 7.0, ramp rate 2 6.0, oven temp 3 280°C, iso time 3 5.0, ramp rate 3 0.0, press psig 18.0, fid sens high, fid zero on, inj temp 250°C, det temp 300°C, relay 1 on, relay 2 off.

All the data obtained from all the analyses are the means of 3 independent experiments ± standard deviation.

Results and Discussion

Experiment 1 (NS_1)

The phosphoenolpiruvate carboxilase activity shows a decreasing trend during the 72 hours period, with similar values between (+Fe) and (-Fe) theses till the 24th hour. At the 48th hour, roots of (+Fe) plants show a decreasing activity of 41.18%, reaching a significantly smaller value than (-Fe) (Figure 1). In (+Fe) theses the recorded decrease of enzymatic activity at the 24th hour agrees with the pH increase of the nutrient solution, from 6 to 7.6 that indicates a temporary acidification of the cytoplasm, due to the activation of the nitrate uptaking system.

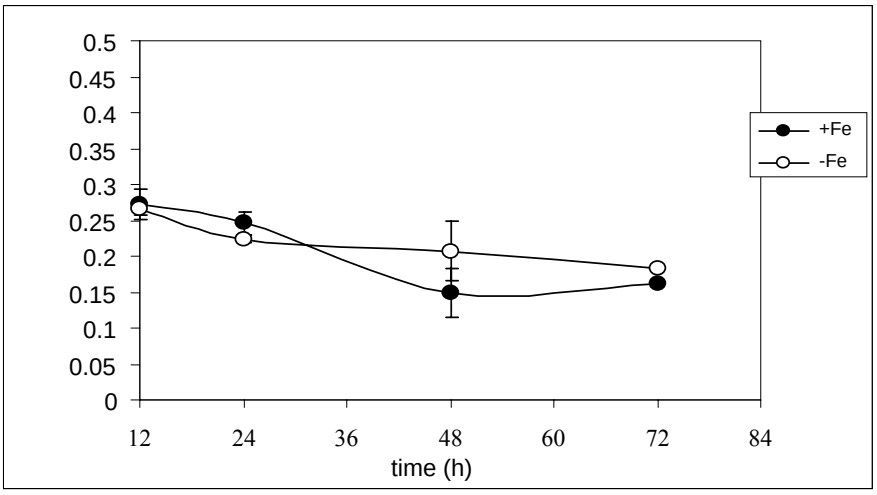

Figure 1. Phosphoenolpiruvate carboxilase (PEPcase) activity (U/mg prot.) in maize plants roots (*Zea mays* L.) grown in nutrient solution with 4.0 mM NO_3^-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

Malate dehydrogenase activity (Figure 2), higher from the 12th hour to the 48th hour in (-Fe) plants, shows a similar trend in the two theses (+Fe and –Fe) with the higher value at the 6th hour for (+Fe) thesis and at the 12th hour for (-Fe) thesis.

Malic acid contents decrease in both theses till the 12th hour, with higher values in (+Fe). When in (-Fe) thesis the malate dehydrogenase shows higher activity (Figure 2, 3), the malic acid contents show a decrease of the 53% and of the 66% at the 6th and the 12th hour respectively, when compared to the initial value. From the 12th to the 24th hour malic acid content, almost doubled, is higher than that of (+Fe) thesis, maintaining till the end a higher level when compared to that recorded at the 6th hour, but similar to (+Fe). Succinic and citric acid contents are generally higher in (-Fe) theses after the 24th hour (Figure 4, 5).

Oxalacetic acid values in (-Fe) theses are always higher and increase when compared to those of (+Fe) theses during the whole experimental period and in agreement with the malate dehydrogenase and phosphoenolpiruvate carboxilase activities (Figure 1, 2, 6). In both theses, the measure of the malate dehydrogenase activity agrees with the variations of the malic acid contents as well as with the oxalacetic acid increase (Figure 6).

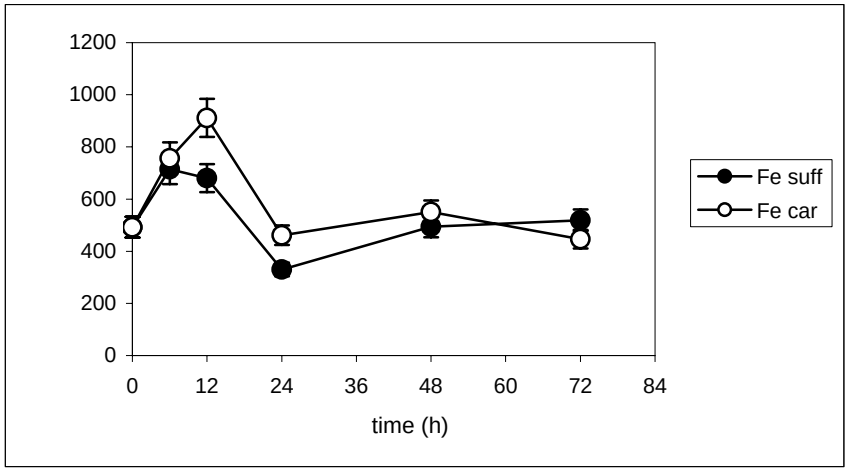

Figure 2. Malate dehydrogenase (MDH) activity (U/mg prot.) in maize plants roots (*Zea mays* L.) grown in nutrient solution with 4.0 mM NO_3^-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

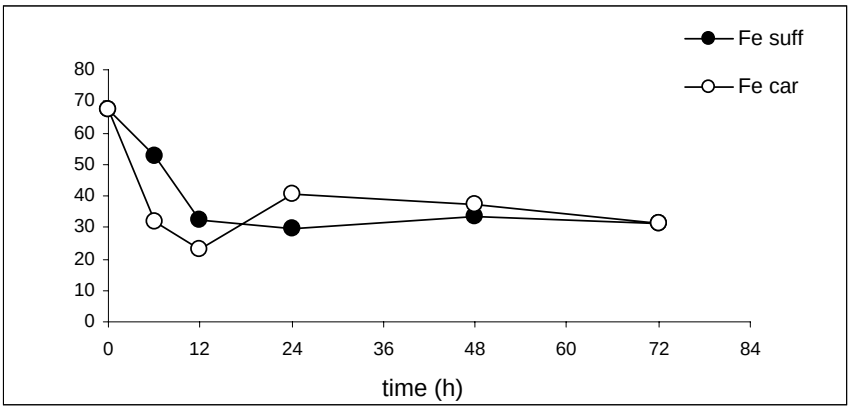

Figure 3. Malic acid content (mg 100 g^{-1} f.w.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 4.0 mM (NS_1) $NO3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

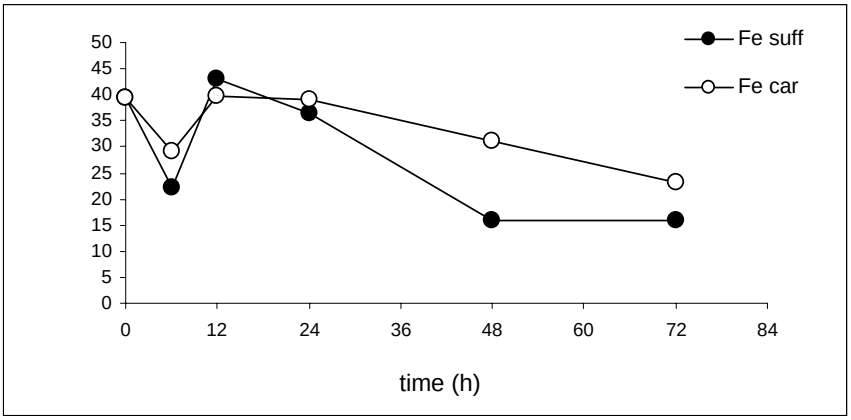

Figure 4. Succinic acid content (mg 100 g-1 f.w.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 4.0 mM (SN1) NO3-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

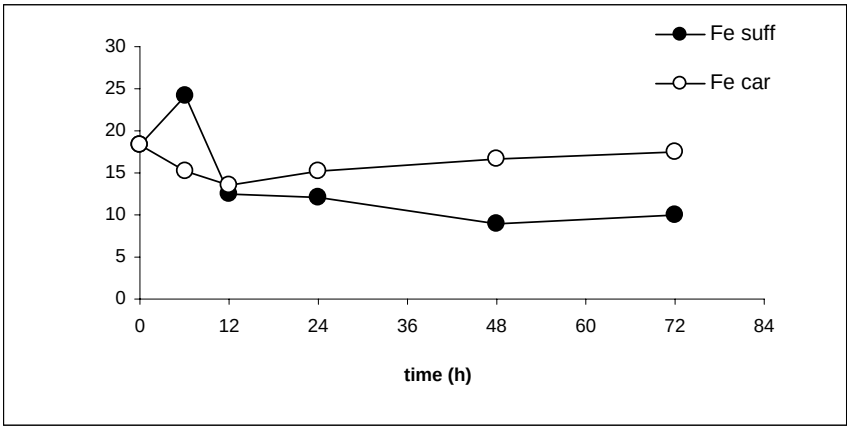

Figure 5. Citric acid content (mg 100 g^{-1} f.w.)in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 4.0 mM (SN_1) NO_3^-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

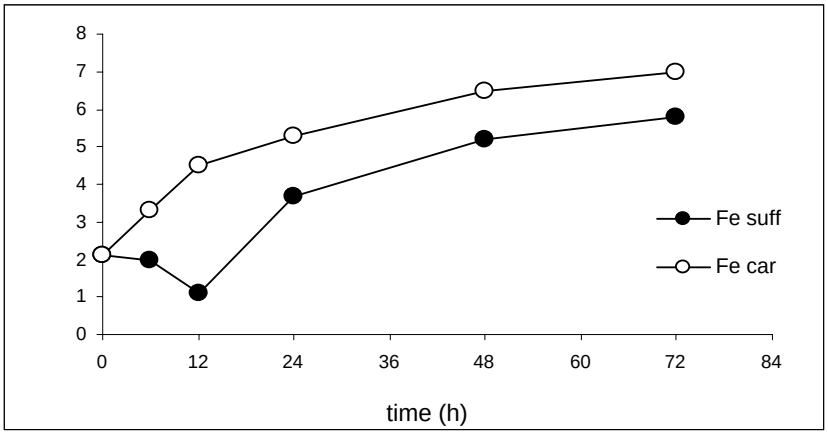

Figure 6. Oxalacetic acid content (mg 100 g^{-1} f.w.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 4.0 mM (SN_1) NO_3^-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

Malate dehydrogenase and phosphoenolpiruvate carboxilase activities of (-Fe) theses are the response to the mechanisms that guarantee cell omeostases, connected to carboxilase ions production (Landsberg, 1986; Bienfait et al., 1989; Rabotti et al., 1995).

The increase of anions production in (-Fe) theses justifies the temporary alcalinization of cytoplasma and PEP carboxilase activation. Furthermore, it links the enzymatic activity to the cell mechanism of stat pH (Landsberg, 1986; Miller et al., 1990; Abadìa et al., 2002).

In iron deficient roots, the malic acid decrease and oxalacetic acid increase might suggest that their utilization is involved, in particular as a mitocondrial organic component, in the production of energy (Abadìa et al., 2002; Andaluz et al., 2001; Lòpez-Millàn et al., 2000) for nutrients assimilation. Furthermore, under iron deficiency the higher production of these acids can be due to the necessity to implement their contents to induce the synthesis of molecules involved in Fe finding, as it has already been observed in other vegetable species (Bialczyk and Lechowski, 1992).

In iron sufficient theses, citrate decrease after the 6th hour, might be referred to its use, above all, to satisfy the

energetic demands. On the contrary, its increases in the iron deficient theses point out its role as a starting molecule involved in the synthesis of phytosiderophores necessary to iron assimilation (Pich et al., 1994).

Experiment 2 (NS_2)

Phosphoenolpiruvate carboxilase activity shows an increasing trend till the 48th hour for (-Fe) and (+Fe) theses, pointing out significantly different values between the two theses only at the 48th hour (Figure 7). The higher expression of this activity in (-Fe) theses might be due to a pH increase in citosol as a response to the higher protons extrusion, due to H^+-ATPase activation and to the lower pH value in the growth medium that is of 4.0 and 3.9 for (+Fe) and (-Fe) theses, respectively.

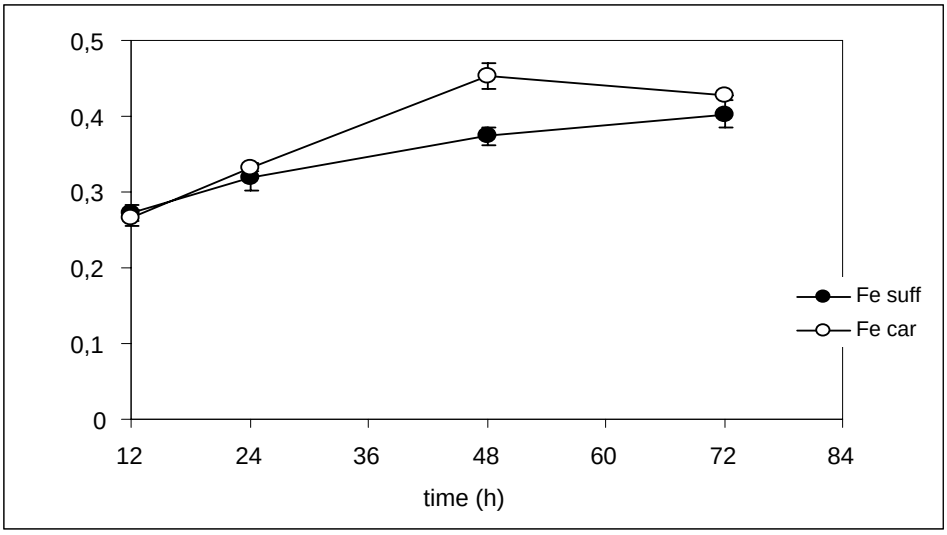

Figure 7. Phosphoenolpiruvate carboxilase (PEPcase) activity (U/mg prot.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 0.4 mM NO_3^-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

The more evident activation of PEPcase in (-Fe) theses might be referred to the higher protons extrusion (growth medium pH = 3.9 and 4.3 at the 48th and 72nd hour respectively).

Malate dehydrogenase activity shows a fluctuating and similar trend independently of the theses, resulting higher till the 12th hour in (+Fe) thesis that shows the highest value at the 6th hour (Figure 8). At the 48th hour, in (-Fe) thesis appears a significantly higher value in comparison with (+Fe) thesis with an activity increase of the 51%. From the 48th to the 72nd hour the value is that recorded at the 24th hour (Figure 8).

Oxalacetic acid contents were not detectable in both theses, while those of malic acid showed a decrease till the 24th hour, with lower values always for (-Fe) theses (Figure 9).

Succinic acid contents, steady in (-Fe) theses, increase at the 24th hour for (+Fe) theses. The significant decrease recorded at the 48th hour in (+Fe) thesis leads the acid levels to lower values than those of (-Fe) (Figure 10). At the 72nd hour, (+Fe) theses show similar values to those of (-Fe).

Citric acid content shows always lower values in (-Fe) theses, decreasing till the 24th hour with significant differences when compared to (+Fe) at the 48th and the 72nd hour. Both theses have similar trend and show the higher value at the 48th hour (Figure 11).

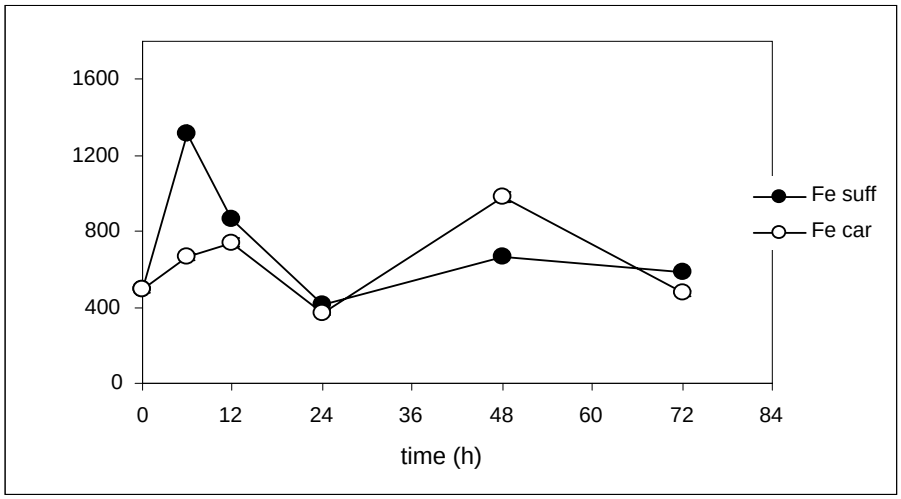

Figure 8. Malate dehydrogenase (MDH) activity (U/mg prot.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 0.4 mM NO_3^-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-FDTA.

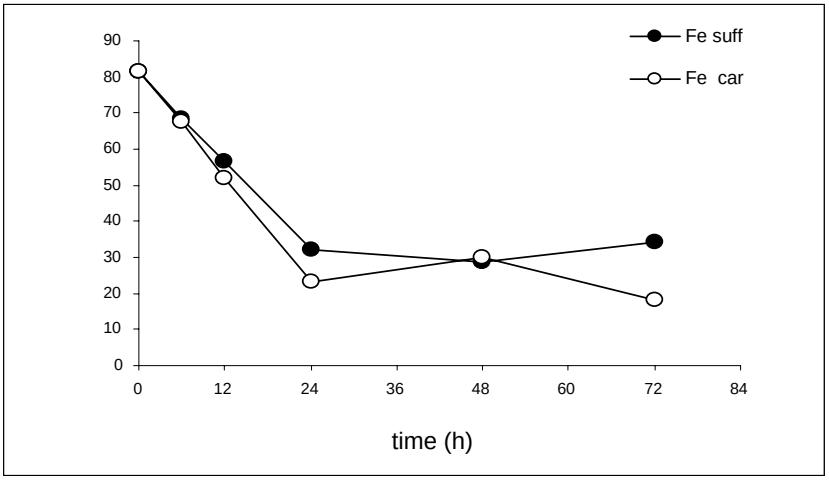

Figure 9. Malic acid content (mg 100 g^{-1} f.w.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 0.4 mM (NS2) NO3-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

During the whole examined period, (+Fe) thesis shows in NS_1 a decreasing trend of total acid contents. In (-Fe) theses the acid levels (Figure 12), appear to be lower during the first 12 hours when compared to those of (+Fe) theses. The acid contents show at the 24th hour a 25% increase and the values are higher than those of (+Fe) theses till the 72nd hour, with significant differences of the values (Figure 12).

In NS_2 the two theses have the same trend, however the values are always higher in (+Fe) (Figure 13).

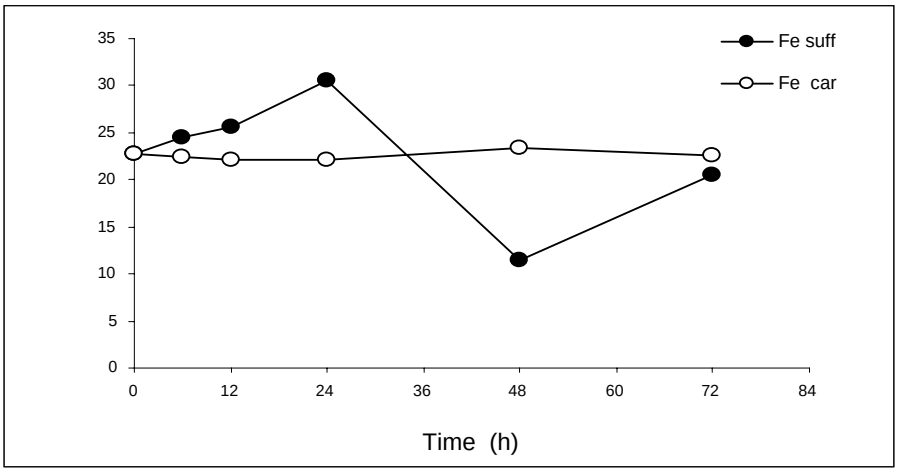

Figure 10. Succinic acid content (mg 100 g^{-1} f.w.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 0.4 mM (NS2) NO3-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

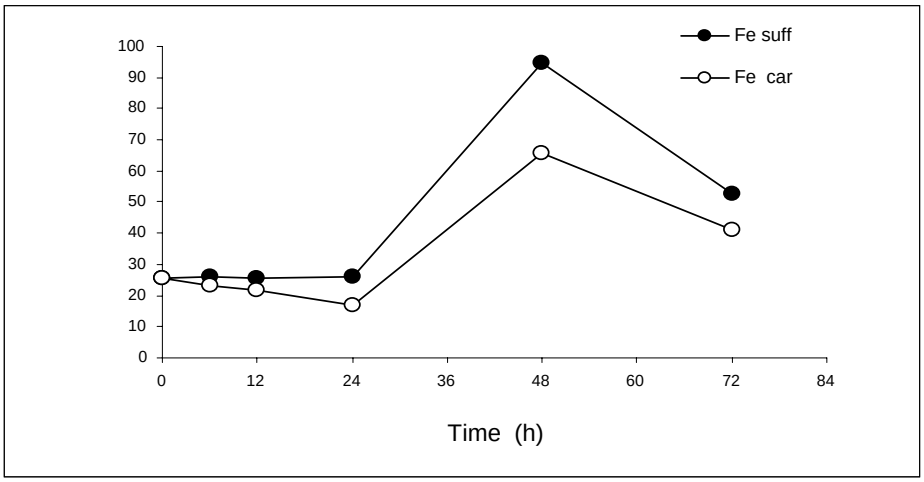

Figure 11. Citric acid content (mg 100 g^{-1} f.w.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 0.4 mM (NS_2) NO_3^-, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

Landsberg (1986) observed in the roots of iron deficient plants an increase of organic acids as a response to balance a possible organic acid extrusion by the roots. We too have found in NS_1 an increase, particularly evident for citric acid, that might be connected to the strategy done by maize for iron uptake, and therefore to the needs to produce phytosiderophores in the Fe deficiency. In this condition citric acid plays a role of a chelating agent for Fe^{2+} (Tiffin, 1966; Pich et al., 1995) but also to move the cation towards vegetative apexes (Bienfait, 1996). In NS_2 (Figure 13) the values of total organic acid show an initial decrease in both theses, with particular evidence in (-Fe) theses. This specific picture might depend on the higher demand of energy necessary to activate the transport through membranes where a doubled activity of the plasma membrane H^+-ATPase plasma membrane was observed when compared to theses with 4.0 mM NO_3^-, showing moreover higher values for (-Fe) plants (date not shown). The higher respiratory activity linked to ATP request necessary to implement plasma membrane H^+-ATPase

pump activity involved in nitrate and iron transport (Miller et al., 1990), justifies the non detectability of oxalacetic acid in both theses, where NO_3^- deficiency creates stress conditions similar for both (+Fe) and (-Fe) theses.

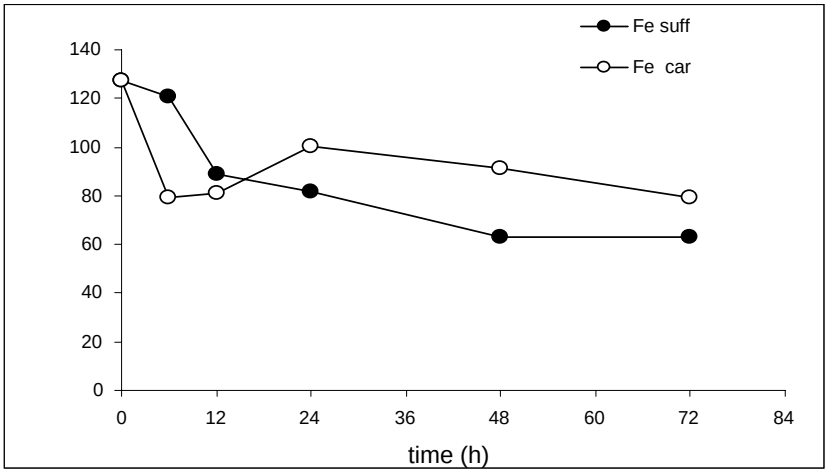

Figure 12. Total acid contents (mg/100 g f. w.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 4.0 mM (NS_1) NO_3^-, added with 80 µM (+Fe) or 0.1 µM (-Fe) Fe-EDTA.

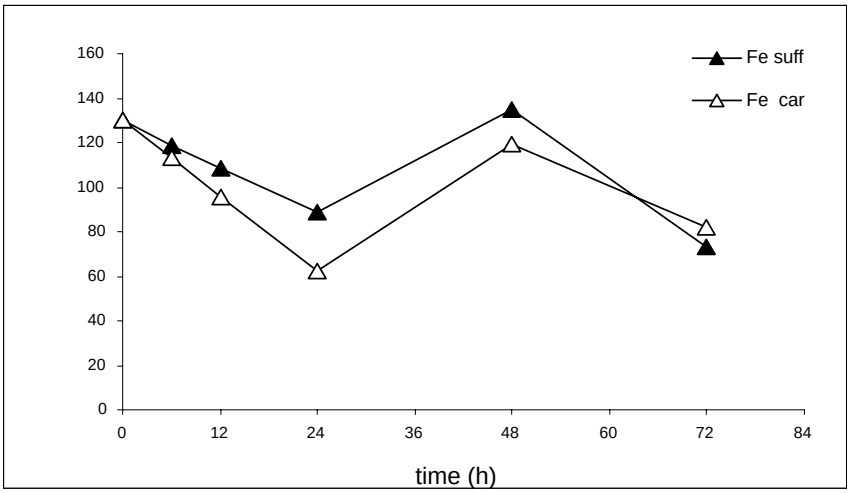

Figure 13. Total acid content (mg/100 g f.w.) in roots of maize plants (*Zea mays* L.) grown in nutrient solution with 0.4 mM (NS_2) NO_3^-, added with 80 µM (+Fe) or 0.1 µM (-Fe) Fe-EDTA.

In NS_1, glucose content in (-Fe) plants shows significant decrease from the 6th to the 72nd hour, with higher values when compared to (+Fe) till the 48th hour (Fig. 14). In (+Fe) plants, after the decrease recorded till the 24th hour, the glucose contents of the following stages shows significant increases till the 72nd hour, with higher values when compared to that recorded for (-Fe) plants. Fructose and saccarose contents show very lower values than those of glucose without significant differences between (+Fe) and (-Fe) theses.

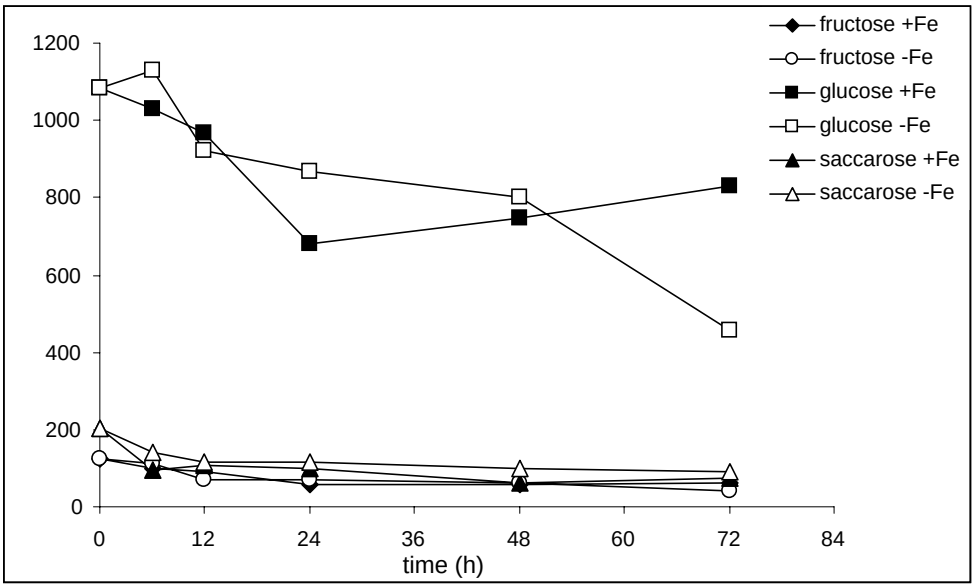

Figure 14. Fructose, glucose and saccarose (mg 100 g^{-1} f.w.) contents in the roots of maize plants (*Zea mays* L.) grown in nutrient solution with 4.0 mM NO_3^- (NS_1), added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

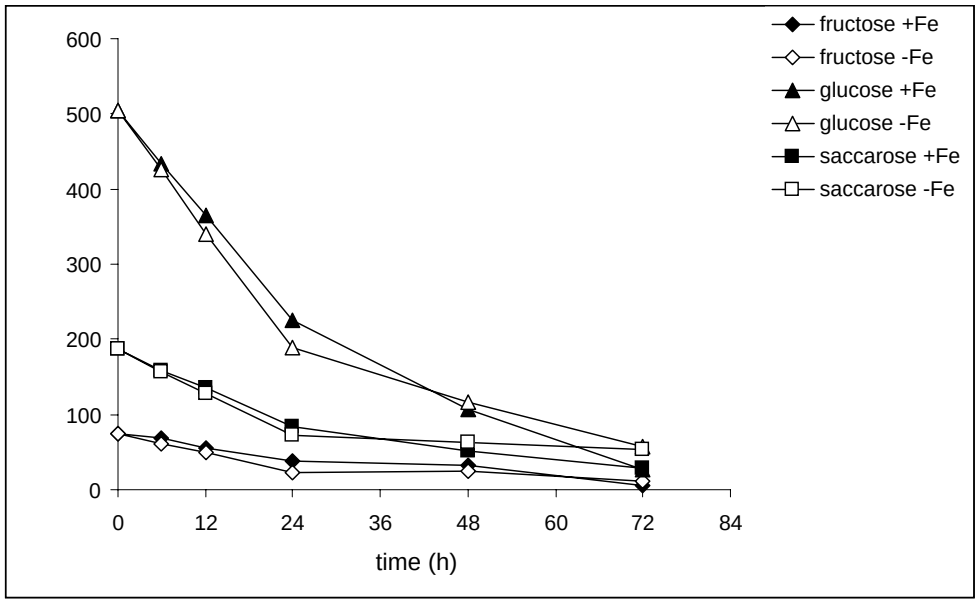

Figure 15. Fructose, glucose and saccarose contents (mg 100 g^{-1} f.w.) in the roots of maize plants (*Zea mays* L.) grown in nutrient solution with 0.4 mM NO3- (NS2), added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

In NS_2 it is possible to observe decreasing values for glucose, saccarose and fructose, in both theses and with lower values in (-Fe) theses when compared to those of (+Fe) (Figure 15, 16).

The higher values of glucose confirm the use of this monosaccaride in the roots as a source of energy above all for (-Fe) theses.

The variations of sugar contents in both experiments agree with those observed for the organic acids, pointing out the use of this compound in relation to the different energy demand in order to

allow the nutrient transport through plasma membrane. They also promote secundary metabolic cycles necessary to effectuate iron acquisition mechanism.

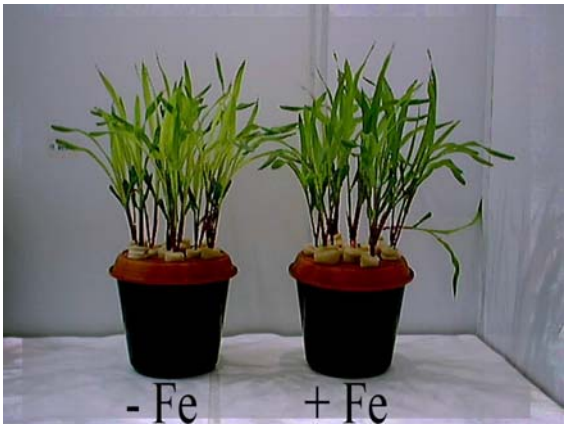

Figure 16. Plants with different availability of iron.

Conclusion

The induction of pH sensitive enzymatic activities is influenced by the variation of the apoplast reaction determined by the different nitrate availability. The different response of the two activities shows to be induced by the reaction caused in the citosol by NO_3^- uptake and with different activities according to Fe availability. In both theses the enzymatic activity, though it has during time the same trend, points out, however, a shifting of the higher activity peak, showing that iron deficiency conditions, interfering with e^- transport, determine a slowing down of the enzyme induction independently of the nitrate availability.

The different nitrate availability implies also a different impact on acid contents. In fact, Fe deficiency in NS_1 increases citrate content, precursor of molecule for phytosiderophores production. On the contrary, low nitrate availabilities determine a decrease in acid contents, mostly in (-Fe) theses, because of the higher energy demand necessary to activate membrane carriers under stress conditions.

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