# Influence of different iron availability on phosphoenolpiruvate carboxilase and malate dehydrogenase in roots of maize (*Zea Mays* L.) plants grown under iron deficiency

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Abstract

The effect of the different nitrate availability on some enzymatic activities has been evaluated in iron deficient and iron sufficient maize plants (*Zea mays* L.). In order to evaluate if the induction of sensitive to pH enzymatic activities is affected by the variation of the apoplast reaction 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 (+Fe) (added with 80 μM Fe(III)-EDTA) and Fe-deficiency (-Fe) (added with 0.1 μM Fe(III)-EDTA).

As regards 0.4 mM $NO_3^-$ ($NS_2$), independently of iron availability, phosphoenolpiruvate carboxilase and malate dehydrogenase inductions are higher than those recorded for the experiment with 4.0 mM $NO_3^-$. The two activities, for the reaction determined in citosol by $NO_3^-$ uptake, show different responses according to Fe availability. In $NS_1$ the higher nitrate uptake and the contemporaneous $H^+$ incoming cause in (+Fe) plants a decrease of PEP-carboxilase activation and, during the first 24 hours, of malate dehydrogenase. The shifting of the peak of maximum activity shows that iron deficiency conditions, interfering with $e^-$ transport, determinate a slowing down of the enzyme induction, independently of nitrate availability. In $NS_2$, PEPcase is higher under Fe-deficiency and malate dehydrogenase is higher under Fe-sufficiency, both during the first 24 hours.

The different nitrate availability causes a different use of the acid content. In fact, in $NS_1$ citric content, precursor of molecules for the production of phytosiderophores, increased in (-Fe) theses. On the contrary, low nitrate availabilities determined a decrease in acid contents, mostly in (-Fe) theses. This result justifies the higher energy demand to activate membrane carriers under stress conditions for the reduced nitrate availability.

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

## 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 [1], depending on redox potential and pH [2]. 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 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 [3, 4]. 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 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. 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 [5] and activate on the plasma membrane of root cells a high affinity uptaking system for pH dependent $Fe^{3+}$-phytosiderophore [6]. Since iron deficiency induced in maize plants a $H^+$ release [7], the present work was carried out to

point out in iron deficient and sufficient maize plants, the influence of the different nitrate availability on some enzymatic activities that regulate pH-stat mechanisms [8].

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 cotiledones 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 nutrient solution Hoagland.

In order to develop the experiment, four factors were considered: two $NO_3^-$ concentrations and two iron concentrations, obtaining 4 thesis with 3 replications each:

Experiment 1

4.0 mM $NO_3^-$ ($NS_1$)

a) Fe-sufficient (+Fe) thesis, added with 80 μM Fe(III)-EDTA.
b) Fe-deficient (-Fe) thesis, added with 0.1 μM Fe(III)-EDTA.

Experiment 2

0.4 mM $NO_3^-$ ($NS_2$)

c) thesis (+Fe)
d) thesis (-Fe)

Roots were taken from plantlets collected at the $5^{th}$ 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 [9, 10] respectively. Furthermore, the content of organic acids (citric, malic,

axalacetic and succinic acids) and sugars (glucose, fructose and saccarose) were determined by means of gas-chromatographic apparatus (gas chromatograph An 8310 Series, Perkin Elmer) according to [11]. 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 24$^{th}$ hour. At the 48$^{th}$ hour, roots of (+Fe) plants show a decreasing activity of 41,18%, reaching a significantly smaller value than (-Fe) (Fig. 1). In (+Fe) theses the recorded decrease of enzymatic activity at the 24$^{th}$ 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.

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

Malic acid contents decrease in both theses till the 12$^{th}$ hour, with higher values in (+Fe). When in (-Fe) thesis the malate dehydrogenase shows higher activity (Figg. 2, 3), the malic acid contents show a decrease of the 53% and of the 66% at the 6$^{th}$ and the 12$^{th}$ hour respectively, when compared to the initial value. From the 12$^{th}$ to the 24$^{th}$ 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 6$^{th}$ hour, but similar to (+Fe).

Succinic and citric acid contents are generally higher in (-Fe) theses after the 24$^{th}$ hour (Figg. 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 (Figg. 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 (Fig. 6).

Malate dehydrogenase and phosphoenolpiruvate carboxilase activities of (-Fe) theses are the response to the mechanisms that guarantee cell omeostases, connected to carboxilase ions production [12, 13, 14].

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 [12, 15, 16].

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 [16, 17, 18] 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 [19].

In iron sufficient theses, citrate decrease after the 6$^{th}$ 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 [20].

Experiment 2 ($NS_2$)

Phosphoenolpiruvate carboxilase activity shows an increasing trend till the 48$^{th}$ hour for (-Fe) and (+Fe) theses, pointing out significantly different values between the two theses only at the 48$^{th}$ hour (Fig. 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.

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 48$^{th}$ and 72$^{nd}$ hour respectively).

Malate dehydrogenase activity shows a fluctuating and similar trend independently of the theses, resulting higher till the 12$^{th}$ hour in (+Fe) thesis that shows the highest value at the 6$^{th}$ hour (Fig. 8). At the 48$^{th}$ hour, in (-Fe) thesis appears a significally higher value in comparison with (+Fe) thesis with an activity increase of the 51%. From the 48$^{th}$ to the 72$^{nd}$ hour the value is that recorded at the 24$^{th}$ hour (Fig. 8). The malate dehydrogenase trend reflects the variations showed by the nitrate absorbance curves for the same theses in a previous work.

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

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

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

During the whole examined period, (+Fe) thesis shows in $NS_1$ a decreasing trend of total acid contents. In (-Fe) theses the acid levels (Fig. 12), appear to be lower during the first 12 hours when compared to

those of (+Fe) theses. The acid contents show at the 24$^{th}$ hour a 25% increase and the values are higher than those of (+Fe) theses till the 72$^{nd}$ hour, with significant differences of the values (Fig. 12).

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

[12] 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 $Fe^{2+}$ [21, 22] but also to move the cation towards vegetative apexes [23]. In $NS_2$ (Fig. 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 [15], justifies the non detectability of oxalacetic acid in both theses, where $NO_3^-$ deficiency creates stress conditions similar for both (+Fe) and (-Fe) theses.

In $NS_1$, glucose content in (-Fe) plants shows significant decrease from the 6$^{th}$ to the 72$^{nd}$ hour, with higher values when compared to (+Fe) till the 48$^{th}$ hour (Fig. 14). In (+Fe) plants, after the decrease recorded till the 24$^{th}$ hour, the glucose contents of the following stages shows significant increases till the 72$^{nd}$ 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.

In $NS_2$ it is possibile 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) (Fig. 15).

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.

## Conclusion

The induction of pH sensitive enzymatic activities is influenced by the variation of the apoplast reaction determined by the different nitrate availability. The 0.4 mM $NO_3^-$ theses, independently of iron availability, PEPcarboxilase induction is higher than that recorded in the experiment with 4.0 mM $NO_3^-$. In fact, in the former treatment the higher protons extrusion indicated a citosol alcalinization, which is the optimum condition for PEPcarboxilase induction. Nitrate higher availability ($NS_1$) causes a momentaneous acidification in the apoplaste, inducing a decrease in the enzyme activity, higher in (+Fe) theses. MDH activity, which has an optimum pH in acid environment, results in Fe deficient theses highly expressed in $NS_1$ than in $NS_2$, just for the lower alcalinization of citosol. In Fe sufficient theses, MDH, even if with the same trend of Fe deficient theses, during the first 24 hour, is higher in $NS_2$ where the higher uptake of $NO_3^-$ was recorded.

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. PEPcarboxilase is higher in $NS_2$ treatment under Fe deficiency and in $NS_1$ under Fe sufficiency. The shifting of the maximum activity peak shows that the 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 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.

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.



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**Fig. 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. Data are the means of 3 independent experiments ± standard deviation.

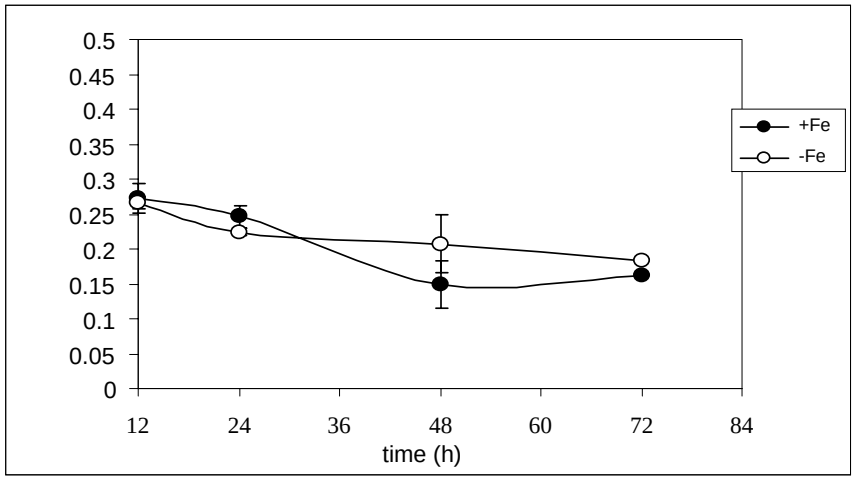

**Fig. 2** Malate dehydrogenase (MDH) activity (U/mg prot.) in maize plants roots (*Zea Mays* L.) grown in nutrient solution with 4.0 mM $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

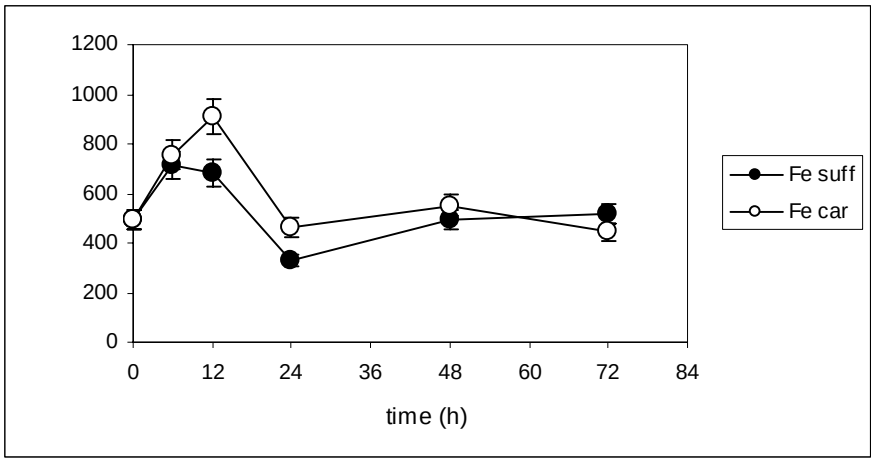

**Fig. 3** Malic acid content (mg 100 $g^{-1}$ f.w.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 4.0 mM ($NS_1$) $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

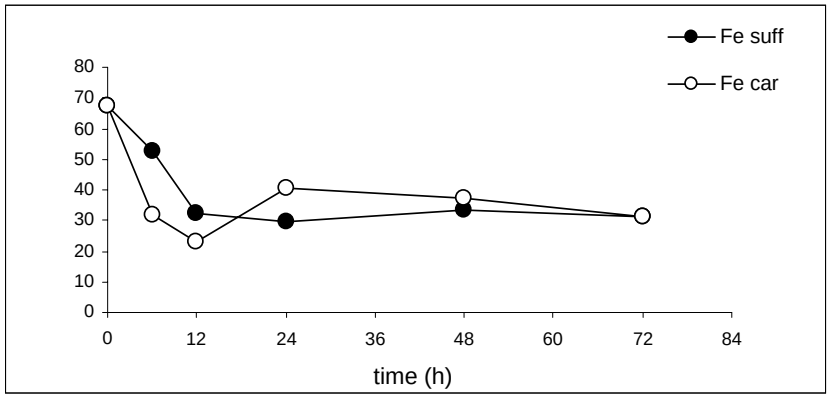

**Fig. 4** Succinic acid content (mg 100 $g^{-1}$ f.w.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 4.0 mM ($SN_1$) $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

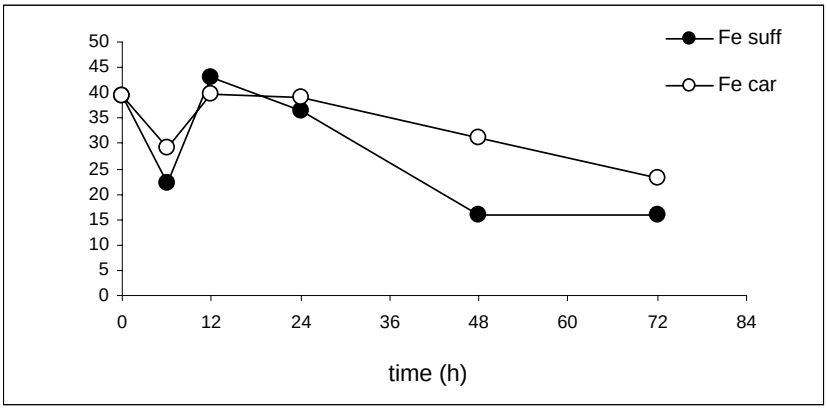

**Fig. 5** Citric acid content (mg 100 $g^{-1}$ f.w.)in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 4.0 mM ($SN_1$) $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

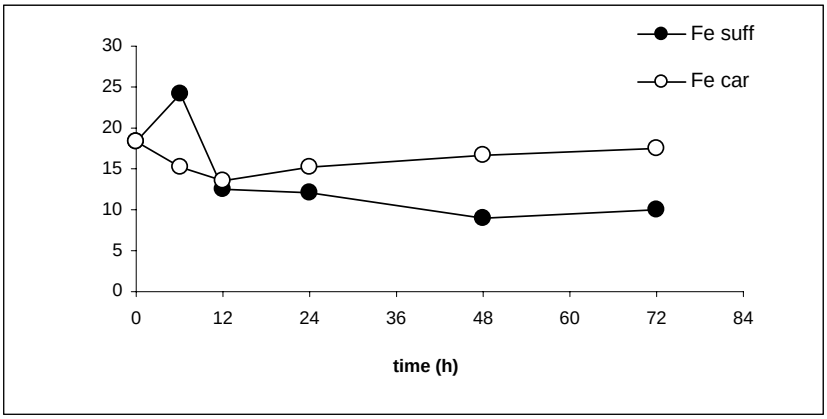

**Fig. 6** Oxalacetic acid content (mg 100 $g^{-1}$ f.w.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 4.0 mM ($SN_1$) $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

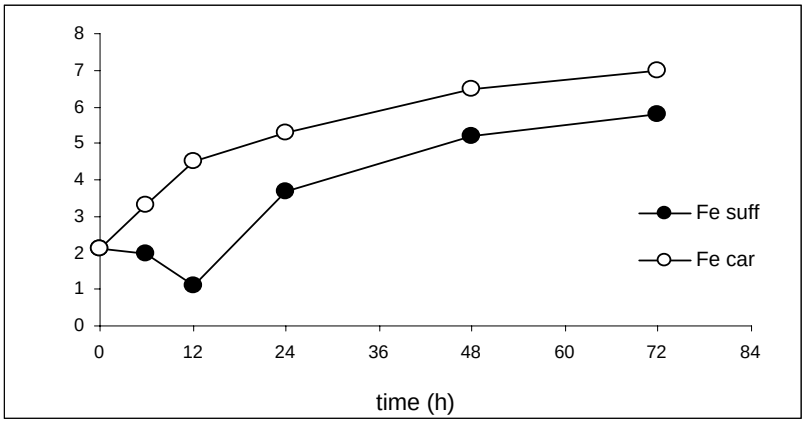

**Fig. 7** Phosphoenolpiruvate carboxilase (PEPcase) activity (U/mg prot.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 0.4 mM $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

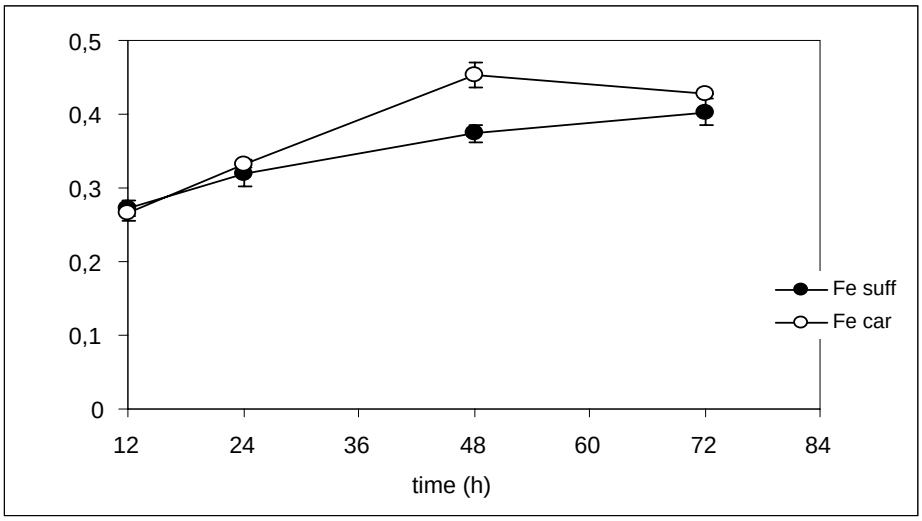

**Fig. 8** Malate dehydrogenase (MDH) activity (U/mg prot.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 0.4 mM $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

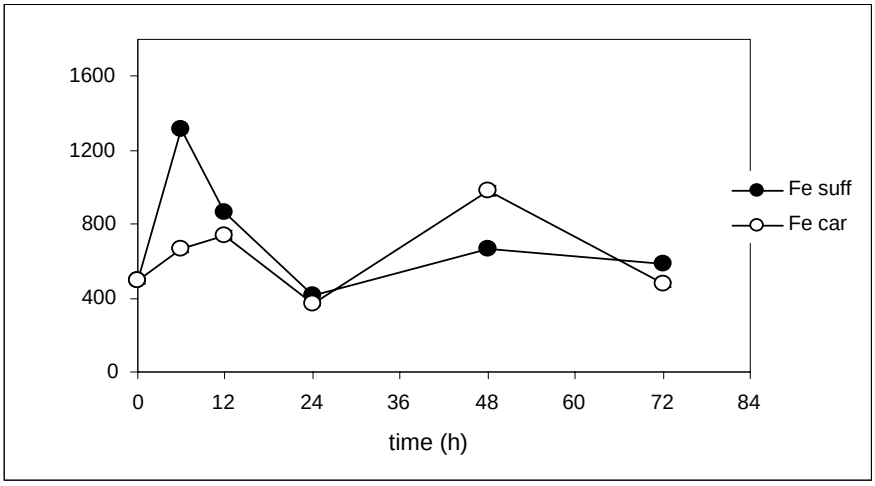

**Fig. 9** Malic acid content (mg 100 $g^{-1}$ f.w.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 0.4 mM $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

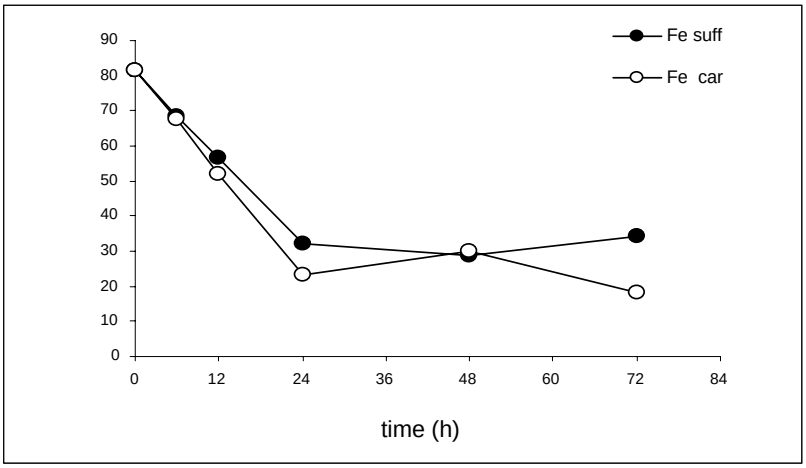

**Fig. 10** Succinic acid content (mg 100 $g^{-1}$ f.w.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 0.4 mM $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

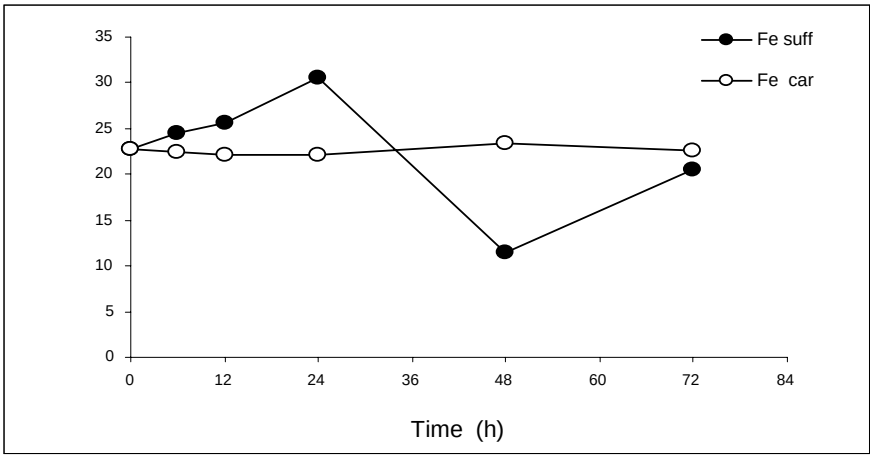

**Fig. 11** Citric acid content (mg 100 $g^{-1}$ f.w.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 0.4 mM $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

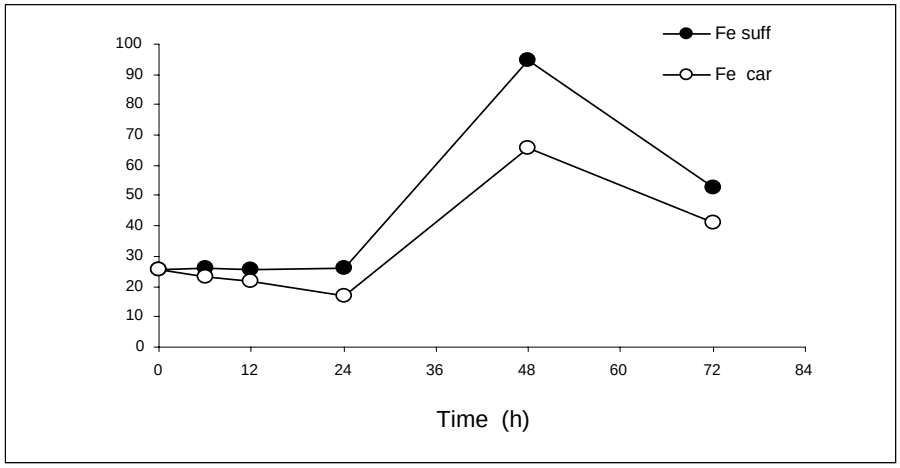

**Fig. 12** Total acid contents (mg/100 g f. w.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 4.0 mM ($NS_1$) $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

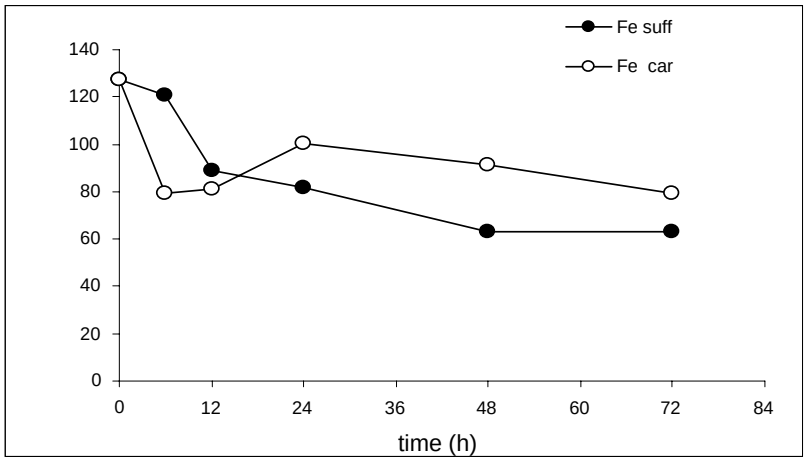

**Fig. 13** Total acid content (mg/100 g f.w.) in roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 0.4 mM ($NS_2$) $NO_3^-$, added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA.

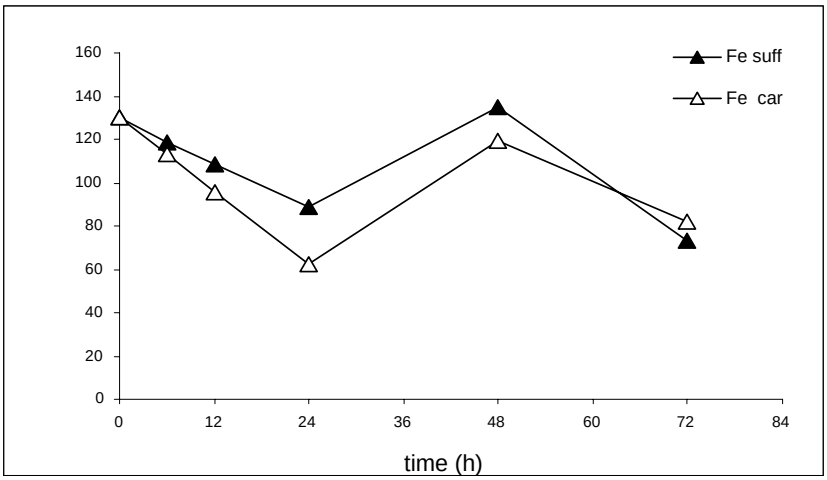

**Fig. 14** Fructose, glucose and saccarose (mg 100 $g^{-1}$ f.w.) contents in the roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 4.0 mM $NO_3^-$ ($NS_1$), added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.

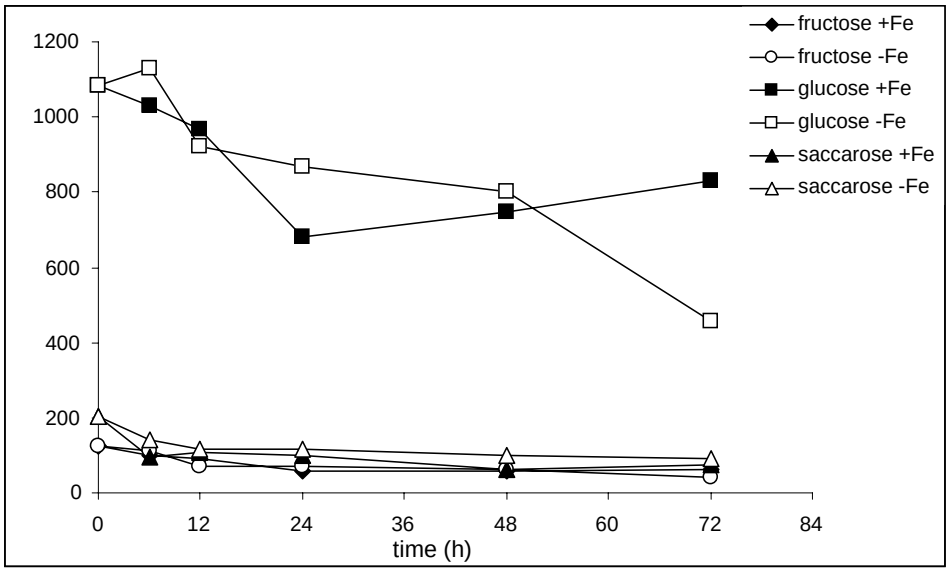

**Fig. 15** Fructose, glucose and saccarose contents (mg 100 $g^{-1}$ f.w.) in the roots of maize plants (*Zea Mays* L.) grown in nutrient solution with 0.4 mM $NO_3^-$ ($NS_2$), added with 80 μM (+Fe) or 0.1 μM (-Fe) Fe-EDTA. Data are the means of 3 independent experiments ± standard deviation.