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Integrated responses of a wild species *Parietaria judaica* (L. 1753) to direct and induced iron deficiency growth conditions

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National and International poster presentation

- P. Tremolada, N. Guazzoni, L. **Tato**. *A model for the evaluation of vegetation to milk transfer of Persistent Organic Pollutants (POPs): the Cow model*, XX Congresso SITE 2010 - "Sapienza" Università, Roma 27-30 September 2010

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- Integrated responses of *Parietaria judaica* to iron deficiency conditions

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- *Cetacei in mar Mediterraneo* - Dott. Marco Colla Università degli studi di Milano 20-gen-10

- *Mineral Fertilization of Fruit Trees: Basic knowledge and dissemination* - Prof. Volker Roemheld University Hohenheim – Stuttgart DI.PRO.VE - Università degli studi di Milano 06-set-10
- *Utilisation of Halophyte species for saline soil rehabilitation and valorisation* - Prof. Chedly Abdelly - Laboratoire des plantes Extremophiles - Centre de Biotechnologie de Borj Cedria – Tunisia DI.PRO.VE - Università degli studi di Milano 25-nov-10
- *Strategies of salt tolerance in halophites* - Prof. Chedly Abdelly - Laboratoire des plantes Extremophiles - Centre de Biotechnologie de Borj Cedria – Tunisia DI.PRO.VE - Università degli studi di Milano 25-nov-10
- *La fluorescenza della clorofilla come metodologia di studio degli stress nelle piante* - Dott.ssa Lucia Guidi - Dip.Biologia delle Piante Agrarie - Università di Pisa DI.PRO.VE - Università degli studi di Milano 27-gen-11
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M.Sc. Liliana M. Tato attended the three year PhD studies with diligence, perseverance and responsibility. She has shown great interest in the topics addressed demonstrating ability in critical analysis towards the issues faced during the research activities. She has acquired a very good knowledge of the eco physiological basis inherent the issue studied as well as the physiological and biochemical techniques for their study. She has independently set collaborations with other research groups in order to investigate specific aspects of the research, thus acquiring new analytical techniques. She attended for a short period the Instituto di Scienze delle Produzioni Alimentari (ISPA), Consiglio Nazionale delle Ricerche (CNR), Bari – Italy under the supervision of Prof. Vincenzo Lattanzio applying the HPLC technique to the study of phenolic compounds; and the Dipartimento di Agraria – Università Mediterranea di Reggio Calabria under the supervision of Prof. Maria Rosa Abenavoli, where she acquired the root image analysis technique (winrhizo) to study the modification of root morphology and architecture. Therefore I express an excellent opinion regarding the work carried out by Liliana Tato.

to my mother who taught me the art of never giving up

to Fernando, Ramiro, Clara and Gregorio
who supported and put up with me in this adventure

ABSTRACT

Iron (Fe) is an essential micronutrient in plants as it takes part in major metabolic pathways such as photosynthesis and respiration and is linked to many enzymes that accomplish many other cellular functions (DNA synthesis, nitrogen fixation, hormone production). Fe deficiency, a major abiotic stress, reduces crop yields, especially in calcareous soils in which the solubility of Fe is extremely low because of the high soil pH.

Parietaria judaica is a wild sinantropic Strategy I plant that implements many integrated mechanisms allowing to successfully complete the life cycle in highly calcareous environments.

In this work the main mechanisms by which *P. judaica* overcomes the low bioavailability of Fe have been identified. *P. judaica* was subject to direct and induced Fe deficiency growth conditions in hydroponic systems. Strategy I biochemical mechanisms of FeII-reduction and rhizosphere acidification were studied, as well as low organic acids and phenolics in root exudates. It was suggested that the accumulation and exudation of phenolic compounds plays a central role in the adaptive strategy of *P. judaica* to cope with Fe deficiency conditions.

Key enzymes of primary and secondary metabolism were assayed in order to identify the metabolic rearrangement that occurs under Fe deficiency conditions. The data analysed confirm that under Fe deficiency the metabolic rearrangement takes place by modifying allocation of carbon skeletons between primary and secondary metabolism. It was observed that secondary metabolism constitutes the main concern under this stress condition as *P. judaica* sustains the supply of substrates using non oxidative ways.

Phenolic composition was characterized by HPLC analyses in *P. judaica* focusing on phenolic composition changes due to low Fe availability. Phenolic compounds found in *P. judaica* belong mainly to the mono- and di- caffeoylchinic acids group. The chlorogenic acid was resulted the most sensible component under Fe deficiency stress.

Morphological and architectural modifications of root system were also analysed. *P. judaica* changes its root system according to the experimental treatment imposed. The differences found in direct and induced Fe-Deficiency conditions were oriented to increase the root contact surface with the medium. In particular, the root architecture reflected the plant nutritional status.

Comparing the data obtained from high bicarbonate and highly alkaline buffer conditions it was observed that *P. judaica* has no problems to acquire Fe in highly alkaline environments, suggesting that in a highly chalky environment the availability of bicarbonate itself constitutes the real factor of stress.

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"[…] *porque todos son sentencias sacadas de la mesma experiencia, que es la madre de las ciencias todas* [….]*"*

Miguel de Cervantes Saavedra, 1605

(from *El Ingenioso Hidalgo don Quijote de la Mancha* I, Chapter XXI)

"The most beautiful think we can experience is the mysterious. It is the source of all true art and all science. […]*"*

Albert Einstein, 1931

(from *The world as I see It*, Forum and Century, 84:193-194)

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CHAPTER 1

General background: Ecophysiology of plant iron nutrition.

Iron (Fe) is an essential element that participates in major biological processes both in plants and animals. Due to their reversible redox characteristic Fe ions were readily incorporated for use in biological processes early in evolution playing a key role in metabolic pathways such as respiration, photosynthesis, and nitrogen fixation. Although Fe is a ubiquitous element in circumneutral and well oxygenated environments it is found mostly in Fe(III) oxidize forms that have very low solubility and are not readily available for plants. As a consequence, plants have evolved two main strategies to acquire Fe: a reduction-based strategy (Strategy I) in dycotiledoneous and monocotyledoneous non *poacea* plants and a chelation strategy (Strategy II) in graminaceous ones.

Strategy I plants are able to uptake only Fe(II) from the soil, so they are forced to reduce Fe(III) before carrying it inside the cell. The reduction-based mechanism of Fe acquisition relay on the combined function of three plasma membrane proteins: H^+-ATPase, Fe-chelate reductases (FCR) and Iron Regulated Transporter (IRT). Fe deficiency induces the activation of these root plasmalemma enzymes/ion transporter as well as many other morphological and physiological responses at root level aiming at facilitate the mobilization of Fe (Schmidt, 2003; Marschner et al. 1986).

Inside the cell the accumulation of Fe(II) possess also limitations as it catalyzes the generation of hydroxyl radicals that are potent oxidation agents resulting in oxidative damage of cellular components such as DNA and lipids. Thus, plants have to deal with a dual effect of Fe: on one hand the metal reduced form Fe(II) is essential for life, but on the other hand free Fe(II) is toxic. Therefore, to ensure Fe acquisition and avoiding toxicity, plants have to tightly control uptake, utilization, and storage of Fe in response to its environmental availability.

A further limitation for Fe availability occurs in alkaline environments such as calcareous soils. In fact, Fe deficiency is a common nutritional disorder in crops growing on calcareous soils which account for 30% of world cultivated lands. In calcareous soils high pH decrease even more dramatically the Fe solubility. Moreover, it was reported that the presence of high levels of bicarbonate negatively affects either the FCR activity and/or IRT (Lucena et al. 2007).

Fe deficiency manifests itself primarily as an interveinal yellowing of the leaves known as chlorosis. Chlorosis is related to a diminution in chlorophyll due to the lack of Fe and leads to substantial agricultural losses and decreases in nutritional quality of many crops of great economic interest. Crop chlorosis is generally a result of both limited Fe bioavailability and cultivation of susceptible genotypes (Hansen et al. 2006).

Furthermore, plants are the primary source of Fe for humans therefore a decrease in the amount of Fe in agricultural products has important implications for human health. Understanding the mechanisms of Fe uptake and regulation provide useful insights for producing plants tolerant to low Fe availability and able to increase their Fe content.

1.1. **Iron in the soil**

Fe is the most abundant element in the Earth as a whole and the fourth most abundant element in the crust. (Taylor and Konhauser, 2011).

Iron can exist in a range of oxidation states, from –2 to +6, but the most common are Fe(II) (ferrous iron) and Fe(III) (ferric iron). In soils three main sources of Fe can be recognized:

- Fe contained in minerals;

- Fe in soil solution;

- Fe bound to organic matter.

Fe availability in soils depends on a complex relation of the above components.

In primary minerals Fe can be found mostly as ferromagnesian silicates such as olivine, augite, hornblende and biotite (Schwertmann and Taylor, 1989). Primary minerals release Fe(II) and Fe(III) through weathering processes of dissolution and oxidation. Fe(II) rapidly oxidizes to Fe(III) in the presence of water and atmospheric oxygen. Furthermore, free Fe forms a range of oxides, hydroxides and oxyhydroxides such as hematite, goethite and ferrihydrite. Most soils are oxic environments so Fe is mainly

found as Fe(III) oxides that confer to the soil typical colors varying from brown to yellow-red. Fe(III) oxides are stable, precipitates and have extremely low solubility. On the contrary, soluble Fe(II) oxides forms are found in anoxic and reducing environments. In an aqueous or moist environment, exposed to oxygen from Earth's atmosphere, Fe(II) has a half-life of several minutes as it rapidly oxidizes to Fe(III) (Stumm and Morgan 1996).

Fe oxides solubility is basically the result of the equilibrium between dissolution and precipitation processes. The hydrolysis of Fe oxides is fully dependent on the pH solution. As show in figure 1 the concentration of all Fe species at a physiological pH (6-7) is far below the amount of Fe required by plant for optimal growth that ranges from 10^{-4} to 10^{-9} M (Guerinot and Yi, 1994).

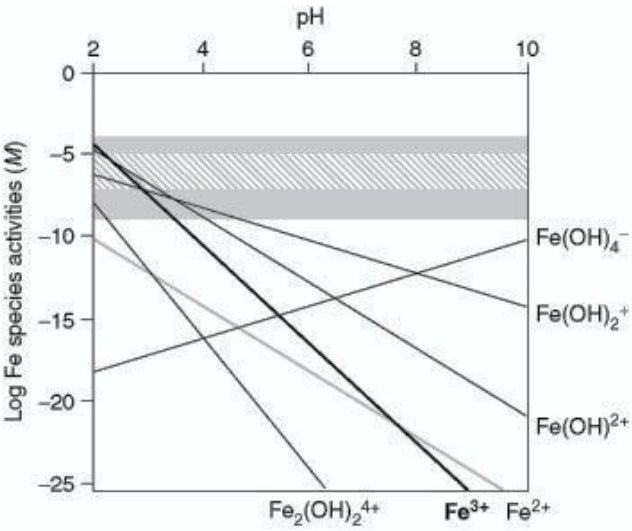

Figure 1. Solubility diagram of the different Fe(III) species obtained by hydrolysis an iron oxide (goethite) as a function of pH. Black lines indicates Fe(III) species, while the grey line designates Fe(II). On the top grey bands indicates the range of concentration require for optimal growth of plants. Dashed area represents the optimal concentration for microbes. (from Robin et al., 2008)

From figure 1 it can be seen that:

- Fe solubility is inversely related to the pH of the soil solution;

- total soluble Fe from different species reaches a minimum at about pH 8, a value close to that of calcareous soils;
- in most Fe species for each unit increase in pH the solubility decreases from hundred to thousand fold
- Fe concentrations for optimal plant growth would be met at a very acidic condition that is a pH value of 3.5

Many other factors influence Fe solubility as redox conditions, mineral particle size, humic substances, chelating agent's content and the presence of bicarbonate.

Under oxidizing conditions the activity of the readily available Fe(II) species are higher than the Fe(III) species at physiological pH and its concentration. However when the redox potential (*pe*) drops by 1 unit Fe(II) hydroxides concentration increases by two orders of magnitude. As mentioned before, the Fe species concentrations are anyway too low to supply available Fe to plants (Lindsay and Schwab, 1982). Conversely, under reducing conditions, where *pe* is very low, the concentration of soluble Fe(II) increases reaching high levels and becomes toxic. Typical reducing environments are flooded soils.

The particle size of Fe minerals much affected its solubility: smaller the size of the particles, greater is the solubility of the Fe species. This is important because in well oxygenated environments nanometric particle sizes of Fe main minerals are very abundant (Cornell and Schwertmann, 2003). Moreover particle sizes greatly affect the kinetic of dissolution of Fe oxides (Schwertmann, 1991) also in the presence of organic ligands (Kraemer, 2004).

The presence of organic matter considerably influences Fe solubility by complexation or chelation of Fe(III). The organic ligands are organic molecules that can bind to, and form a stable complex with metals wherein the metal is surrounded by the ligand or ligands. The organic ligands are called chelators or chelating agents while the metal complex is called chelate. Chelating agents usually originate from the activity of soil microrganisms, plants exudates and humic substance. Many organic

molecules containing unsaturated bonds, oxygen, nitrogen or sulphur, are likely to play the role of chelators of transition metals. Van Hess and Lundström (2000) have reported that more than 95% of Fe in soil solution was found to be organically bound. Compounds of very different nature act as chelating agents from low molecular weight organic compounds such as citric acid to complex substance like polyphenols or humic acids. Chelating agents play an important role in providing available Fe for plants through increasing its mobilization and bioavailability.

Calcareous soils contain sufficient $CaCO_3$ and other carbonates. Because of the high values of soil solution pH,calcareous soils almost always fall in the alkaline range due to equilibrium reactions between Ca and bicarbonate. Although salinity and high percentage of exchangeable Na (sodic soils) are alkaline, the soils dominate by HCO_3^- are the most alkaline ones (Läuchli and Grattan, 2012). The concentration of Fe expressed both on a dry weight leaf basis or on the amount of Fe per leaf frequently decreases in chlorotic leaves, although the Fe concentration can sometimes be the same or even higher in chlorotic leaves as compared with green ones (iron chlorosis paradox) (Morales *et al.*, 1998; Römheld, 2000; Nikolic and Römheld, 2002).

The presence of free soil carbonate promotes the formation of poorly soluble Fe phosphates impairing the availability of Fe. The solubilization of $CaCO_3$ forms in calcareous soil solution leads to a high concentration of HCO_3^- ions. It was reported for sunflower, soybean and cucumber that addition of HCO_3^- at neutral pH could result in an inhibition of the activity of the key plasmalemma enzyme (FCR) involved in Fe uptake (Toulon et al., 1992). On the contrary it was demonstrate that Fe reducing capacity increases when HCO_3^- was added at high pH values (Dofing et al., 1989; Romera et al., 1992). Despite contrasting findings that have been obtained for different species it is clear that between HCO_3^- and Fe uptake mechanism an interaction occurs. Thus, in calcareous soil Fe unavailability is not only due to high pH and buffering conditions but also to an interaction of HCO_3^- ion with the cell biochemistry.

In Harmsen et al. (2005) bioavailable Fe has been defined “as the portion of total Fe that can be easily assimilated by living organisms.” As

mentioned above Fe bioavailability depends on many different factors that include inorganic and organic chemical processes, physical and biological processes. Furthermore it must be taken into account that different organisms vary in their acquisition pathways and capabilities, other than usually perform strategies to inhibit the growth of other species limiting nutrient competition (allelophaty). Variation between organisms may also result in differences of the soil areas in which each organism interacts with its environment, the so-called bioinfluenced zone, thereby altering the availability of the nutrient. For plants, this bioinfluenced zone typically corresponds to the rhizosphere (Harmsen et al., 2005). Soil microbial activity plays an important role in favouring plant Fe uptake. Typically soil microbes produce siderophores that is, relatively small molecules that have high affinity and chelate Fe. These effective chelators increase Fe bioavailability for plants. In fact, it has been widely evidenced that Fe bioavailability depends on the plant/microbe mutualistic interactions.

Through root exudations plants provide a plenty of organic substances into rhizosphere supporting microbial communities that produce siderophores. A quite complex interaction settles among plant and microorganisms that lies in the equilibrium between competition for Fe, as plant and microorganism need it for growing, and enhancing Fe bioavailability (Lemanceau et al., 2009). Recent studies have suggested that in Fe deficiency treatments the number of microbes that produce siderophores increase as a result of the enhanced root exudation of phenolics by the plants (Jin et al., 2007).

1.2. **Iron in plant physiology**

The biological importance of Fe lies on its electronic structure which can undergo reversible changes of its oxidation state in a wide range of redox potentials (*pe*). This ability confers to Fe a central role in all those biochemical reactions that involve transfer of electrons and variations of redox potential, as well as bind in a reversible way many ligands. The common biological ligands for Fe are oxygen, nitrogen and sulfur with which it form complexes of coordination.

The major classes of Fe-containing proteins are:

- Fe-containing heme proteins; heme is a prosthetic group composed of a porphyrin ring structure with a central Fe^{2+} atom. There are many heme enzymes that catalyzed crucial biological processes such as DNA synthesis, chlorophyll synthesis, sulfur assimilation, ethylene synthesis. Heme is also found in cytochromes of the respiratory and photosynthetic chains, catalases, superoxide dismutase, oxidase and peroxidases that are involved in detoxification of Reactive Oxygen Species (ROS).

- Fe-S proteins that are characterized by the presence of Fe-S clusters. In these clusters Fe is bond to S in different arrangement (Fe-S, 2Fe-2S, 4Fe-4S, 3Fe-4S proteins) that are special redox centers. These proteins participate in various oxidation-reduction reactions. Fe-S proteins could accomplish also catalytic functions as aconitase which catalyzed the transformation of citrate in isocitrate.

- proteins for Fe storage; Ferritin is a globular protein that can store from 2000 to 4500 atoms of Fe(III).

Fe is almost never found in free form inside the cell. Once absorbed by epidermal or cortical root cells it is complexed with organic acids mainly citric and malic acids or other organic molecules which prevents Fe precipitation and avoid oxidative damages. In fact, once inside the cell, the redox capabilities of Fe can also be the basis of potential toxicity resulting from the Haber-Weiss-Fenton sequence that leads to the generation of hydroxyl radical (OH •) subsequent to the formation of superoxide (O_2^-•) following the one-electron reduction of dioxygen (O_2) by ferrous iron:

$$Fe^{+2} + O_2 \rightarrow Fe^{+3} + O_2^- \bullet$$

$$2\,O_2^- \bullet + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{+2} + H_2O_2 \rightarrow OH\bullet + OH^- + Fe^{+3}$$

Figure 2. Haber-Weis-Fenton sequence. Fe(II) can react with O_2 to form hydroxyl radicals and other reactive oxygen species.

The hydroxyl radical is highly reactive and can damage virtually all types of macromolecules: carbohydrates, nucleic acids (mutations), lipids (lipid

peroxidation) and amino acids.

The accumulation and transport of ionic Fe is improbable as Fe has a low solubility at physiological pH.

The acquisition of Fe starts in the apoplast of the root epidermal cells. Fe inside the apoplast must undergo a reduction step before being transported through the plasmamembrane. It has been observed that as much as 75% of Fe in the roots is precipitated in the apoplast as hydroxide or phosphate salts (Bienfait et al. 1985) forming an apoplastic Fe pool. In addition Fe is attached to the apoplast since the negatively charged carboxyl groups of the cell walls serve as a cation sink. This Fe pool becomes important under Fe deficiency condition. It has been demonstrated that apoplastic Fe decreases when plant are grown under Fe starved conditions suggesting also the involvement of root exudates in facilitating Fe mobilization (Zhang et al. 1991, Jin et al. 2007).

Once inside the cell Fe is bound by chelating agents to prevent its oxidation and as a consequence its precipitation and generation of hydroxyl radicals. Even if many organic acids and amino acids are potentially Fe chelators, NA seems to have a privileged function as Fe-chelator (Hell and Stephan, 2003). Fe–chelator complexes then move through the symplast into the stele along the diffusion gradient. At the pericycle, Fe is loaded into the xylem, and moves towards the shoot through the transpiration stream. When Fe is release into the xylem it is generally agreed that is reoxidized to Fe(III) and it is probably transported in the xylem as Fe(III)-citrate (Rellán-Álvarez et al. 2011). The mechanism by which Fe is unloaded from the xylem to the leaf tissues is not yet clear, but it has also to be mediated by transporters (Fe-citrate, Na-Fe or other complexes) and the photoreduction of the Fe-transporters seems to play an important role (Bienfait and Scheffers 1992). In leaves the plasmalemma activity of FCR has been demonstrated supporting the opinion of an enzymatic Fe reduction. The distribution of Fe in leaf cells is again most likely mediate by NA. To prevent the toxic effect of overload Fe in the leaf, Fe is stored in ferritin.

Different groups of plant have very different optimum requirements of Fe from 0.5 to 50 ppm in higher plants. The general Fe content of green

plants tissues is of about 2 μmol g^{-1} of dry matter (Marschner 1995) which is quite lower in comparison of macronutrients, but represent the highest quantity among micronutrients.

1.3. **Iron acquisition in dycotiledoneous plants: the Strategy I**

As pointed out before to face the problem of acquiring Fe dycotyledoneous and monocotyoledoneous non *poaceae* plants activate a reduce-based mechanism performed by, the so called Stategy I

In general, Strategy I comprises physiological, developmental and metabolic reactions, adapting the plant to changing levels of available Fe sources. In Strategy I two main specific processes are involved, the reduction of Fe(III) chelates at the root surface and the transport of Fe(II) across the root plasma membrane, as these plants can only take up Fe(II). Other non specific mechanisms involved in Strategy I are the extrusion of H^+ and exudation of organic acids and phenolics into the rhizosphere. Moreover, depending on Fe availability, morphological and architectural root changes accompanied the activation of the previous mechanism.

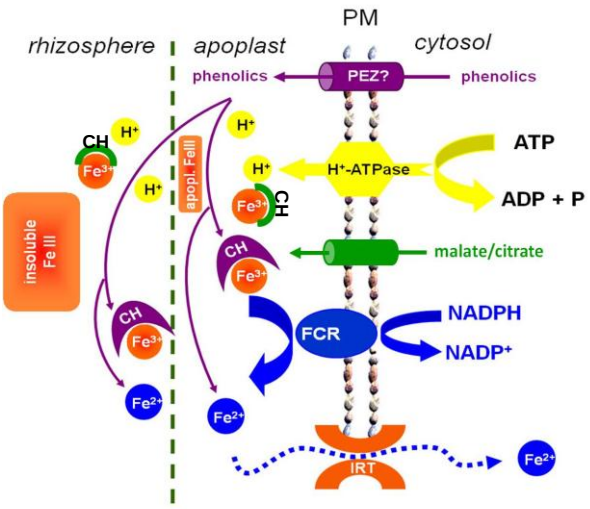

Figure 3. Schematic representation of Strategy I mechanisms for Fe acquisition. IRT= Iron Regulated Trasporter; FCR= ferric-chelate reductase; H^+-ATPase= proton pump ATP dependent; PEZ= phenolic efflux zero transporter; CH= chelator

1.3.1. *Reduction of Fe(III) chelates: a pre-condition to Fe acquisition.*

In all plants investigated so far FCR activity increased in response to Fe starvation and it has been observed that its activity can achieve an increased of as much as 10-20 fold respect to plants grown in complete nutritive substrates (López-Millán et al. 2000 and references therein). The transmembrane FCR acts transferring electrons through the plasma membrane that reduce Fe(III) to Fe(II) in the apoplast thus permitting its transport into the cell.

A FCR responsible of the reduction step of Fe(III) to Fe(II) has been elucidated from the identification of the gene, *FRO2,* in *Arabidopsis thaliana* (Yi and Guerinot, 1994; Robinson et al. 1999). FRO2 belongs to a superfamily of flavocytochromes that transport electrons across membranes.

As shown in figure 4 FCR is constituted by 8 transmembrane helices 4 of which present structural homology with the flavocytochrome b family. An important water soluble domain is localized between helices VIII and IX inside the membrane and contains NADPH, FAD and oxidoreductase activity. Between helices V and VII, probably two heme groups, are bind to conserved histidines. Flanking this assemble is located in the outer side of the membrane helix IV while in the inner side there is helix VI. The structural homology with flavocytochrome b suggest a similar mechanism of electron transport: NADPH is oxidized in the cytoplasm and electrons are transported through a flavine up to heme groups, when electrons arrive to the external surface of the membrane the reduction of F(III) to the Fe(II) occurs.

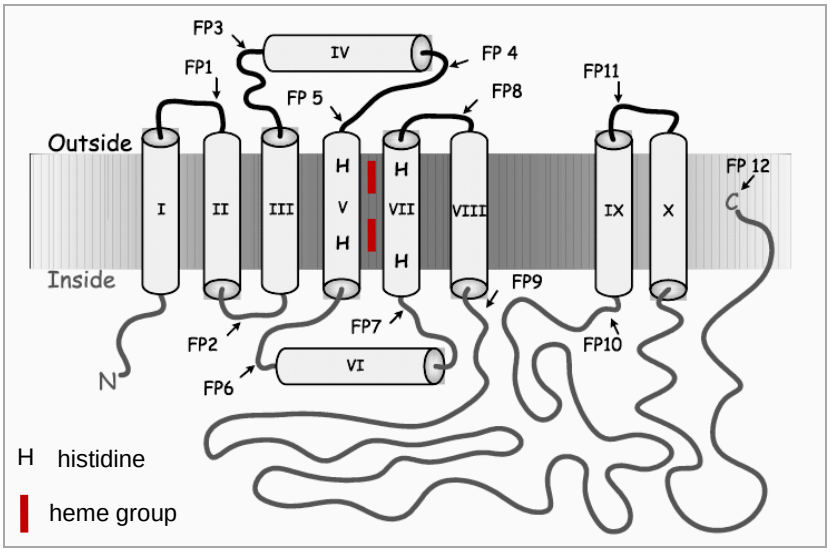

Figure 4. Topology model of FRO2 take from Schagerlöf et al. (2006). Arrows indicate the localization of different fusion points.

In *Arabidopsis* other genes encoding isoforms of FCR were identified. Eight genes have shown conserved the domain where FAD and NADPH are bound to. Each isoforms seems to be expressed preferentially at different levels in the plant (leaves, roots, in photosynthetic tissues). These isoforms do not compensate from one another. FRO2 is responsible for Fe reduction at root level and mutants that lack in *FRO2* gene do not show any FCR activity induced in other isoforms. Genes encoding for FCR has also been identified in other Strategy I species as pea, tomato and cucumber, their expression varies among species suggesting that FROs may have roles in Fe distribution in the plant (Jeong and Connolly, 2009 and references therein).

In agreement with the idea that reduction of Fe is the rate-limiting step in Fe uptake by Strategy I plants (Grusak 1990), overexpression of *FRO2* confers tolerance to growth on low Fe media. Moreover, overexpression of *Arabidopsis FRO2* in soybean resulted in enhanced root ferric reductase activity and tolerance to Fe deficiency induced chlorosis (Vasconcelos et al. 2006).

1.3.2. *Fe(II) transport inside the root cell.*

Once reduce Fe(II) is transported into the root by means of an IRT generally localized in the plasmalemma. The IRT was first discovered as an homolog of a wide family of multigene metal transporters (ZIP, zinc-regulated transporter iron-regulated transporter like protein) in *Arabidopsis* (IRT1), and even if it can transport other divalent metals like Mn, Zn, and Cd shows a high affinity for Fe (Kobayashi and Nishizawa, 2012). IRT1 plays a pivotal role in the regulation of plant Fe homeostasis, as demonstrated by the severe chlorosis and lethality of an *irt1-1* knockout mutant (Vert et al. 2002, Henriques et al. 2002). Consistently, *IRT1* gene is highly expressed in epidermal cells and the underlying cortex of Fe starved roots. Hence the Fe absorption dependent on IRT1 permits proper growth and development under iron limited conditions (Vert et al. 2002).

The Zip family transporters in *Arabidopsis* contain 15 members (including IRT1) that can be divided in 4 groups according to the alignment of the predicted amino acid sequences. The high variety of transporters in *Arabidopsis* have been explained as a consequence of the high number of different membranes that cations have to cross to be distributed through the plant, suggesting that different ZIP protein could have different localizations and specific functions. Furthermore, it is not to be excluded that many ZIP proteins could exhibit functional redundancy (Mäser et al. 2001). IRT2, for example, is very similar to IRT1 in its aminoacidic sequence, it is also expressed in the external layer cells of Fe starved roots. However, it was observed that up-regulation of *irt2* gene in knockout mutants of *irt1* under Fe deficiency conditions was not sufficient to recover from the chlorotic phenotype (Vert et al. 2002).

The predicted topology of most ZIP transporters has eight transmembrane domains and a similar orientation such that the amino and carboxy terminal ends are located on the extracellular surface. The difference of the various proteins is due to different lengths of the loop region (called variable region) between domains III and IV (Eide and Guerinot 1998). ZIP transporters have no similarities to other families of metal transporters.

Plants induce or repress various genes related to Fe homeostasis in response to Fe deficiency or Fe surplus. The molecular mechanism of control expression of *IRT1* has been elucidated quite recently. IRT1 gene expression in root epidermis is transcriptionally promoted by a transcriptor factor know as FIT (Fe-deficiency-induced transcription factor) in *Arabidopsis* and FER in tomato which are positive regulators of root Fe deficiency responses (Colangelo and Guerinot 2004, Ling et al. 2002). FIT/FER play a decisive role in positively regulating various Fe deficiency inducible genes, including *IRT1* and *FRO2*. Since *FIT* overexpression did not induce downstream genes under Fe sufficiency conditions it was suggested the existence of interacting partners that are expressed or activated as Fe deficiency response (Colangelo and Guerinot 2004, Jakoby et al. 2004). It was observed that Fe deficiency responses are transcriptionally regulated by a coexpression of different genes with *FIT*.

IRT1 and *FRO2* expression showed also a post-trascriptional regulation as the restoration of a Fe sufficiency medium induced a reduction in IRT1 protein accumulation and FRO2 activity in roots (Connolly et al. 2002). A work has reported that monoubiquitination of IRT1 at two lysine residues controls the subcellular localization, vacuolar sorting and degradation (Barberon et al. 2011). Many other studies have reported a large variability in genes involved in Fe deficiency depending on the specific response, function and localization. Other transcription factors identified are POPEYE (PYE) and BRUTUS (BTS) that play an important role at root morphology and growth level. Both PYE and BTS may act inversely; PYE regulates positively growth, elongation and swelling under Fe deficiency conditions while on the contrary BTS may repress them (Long et al. 2010).

Other group of transporters belonging to the NRAMP (natural resistance-associated macrophage protein) family of integral membrane proteins was found in *Arabidopsis*. It was demonstrated the involvement of atNRAMP proteins in divalent metal transport (Curie et al. 2000) and their localization either in the plasma membrane and intracellular vesicles (Kobayashi and Nashizawa, 2012).

Fe regulation and homeostasis involves a large number of genes that

expressed themselves at different localizations and with different modes; multiple pathway signaling and negative feed-backs loops. This complexity might confer the necessary flexibility to cope with an ever changing environment.

1.3.3. Acidification of the rhizosphere: the proton pump H^+-ATPase

In strategy I plants, Fe deficiency is generally associated with an increased extrusion of protons mediated by plasmalemma H^+-ATPases. In Arabidopsis 12 isoforms of H^+-ATPase are known from which AHA2 is involved in rhizosphere acidification whilst AHA7 seems related with the radical hairs development.

The active extrusion of H^+ is implicated in mineral nutrition among many other physiological functions such as control of the stomatal aperture, cell elongation, plant development, organ movement, and intracellular pH homeostasis, although evidence for the direct involvement of H^+-ATPases in some of these roles is scarce (Sondergaart et al. 2004). In particular, regarding Fe nutrition the activation of this enzyme constitutes a key mechanism to Fe uptake by non-graminaceous plants and is tightly interrelated with the processes described in the previous paragraphs.

The plasma membrane H^+-ATPase is a universal electrogenic H^+ pump, which uses ATP as energy source to pump H^+ across the plasma membranes into the immediate vicinity of root surface (apoplast and rhizosphere). In general, the key function of this enzyme is to keep pH homeostasis of plant cells and generate an H^+ electrochemical gradient providing the driving force for the active influx and efflux of ions and metabolites across the plasma membrane. Uptake of cations into plant cells is thus driven by ATP-dependent proton pumps. The resulting proton motive force typically comprises a membrane potential of about −150 mV, and a pH difference of 2 units (which contributes to another −120 mV to the proton motive force) (Mäser et al. 2001).

As protons accumulate outside the cell, the pH of the apoplast decreases to reach values of 5–6 that are markedly more acidic than the cytoplasmic pH (Palmgren 2001). The acidification of the apoplast and the rhizosphere

is an essential step in Strategy I species since the solubility of Fe increases up to 1000-fold for each unit pH decrease. Thus, this process can have a huge impact on Fe activity in the close proximity of the roots (Olsen et al. 1981). The extrusion of H^+ promotes the solubilization of Fe(III) and also balances the negative charges of the cell wall preventing repulsions of chelates. The H+ extrusion has also been associated to morphological changes in roots (López-Millán et al. 2000).

Plasma membrane H^+-ATPases are found throughout the plant in every cell type investigated so far. However, certain cell types have much higher concentrations of H^+-ATPases than others. In general, cell types with abundant H^+-ATPases are specialized in an active adsorption of solutes from their surroundings. In some strategy I species the increased acidification of the rhizosphere under Fe deficiency conditions occurs because an increase in protein abundance that are predominantly localized in epidermal cells being differentiated as transfer cells (Dell'Orto et al. 2002).

Plant plasma membrane H^+-ATPases is a single polypeptide of around 100 kDa that belongs to the large P-type H^+-ATPases of cation pumps (Palmgren 2001). The enzyme has about 20% of its mass in the membrane, less than 10% is facing the non-cytoplasmic side and the largest mass is in four cytosolic domains, altogether accounting for about 70% of the mass (Palmgren and Harper 1999).

The primary structure of *Arabidopsis* AHA2 predicts ten transmembrane helices (figure 5). In the cytoplasmic region there are 3 well distinct domains: A, the actuator domain; P, the phosphorylation domain and N, the nucleotidic binding domain that is fused with the P domain and contains the ATP binding site. Furthermore, there is a C-terminal domain (R) that exerts a post-translational auto-inhibitory regulation. In the inactive state the R-domain might indeed be close to or partially folded on the membrane and the activation of the enzyme depends on the phosphorylation of the penultimate Thr residue and the subsequent binding of 14-3-3 proteins that causes the displacement of the domain, removing the inhibition.

The H^+-ATPases are encoded by a gene family of about 10 members in

Arabidopsis thaliana and other species (Arango et al. 2003). Depending on the gene, expression is either restricted to particular cell types or widespread in the plant, with the possibility of more than one gene being expressed in a given cell type at the same developmental stage, thus excluding the characterization of a single isoform from plant material. A study carried out in cucumber has demonstrated a transcriptional regulation of a root plasma membrane H^+-ATPase (Santi et al. 2005).

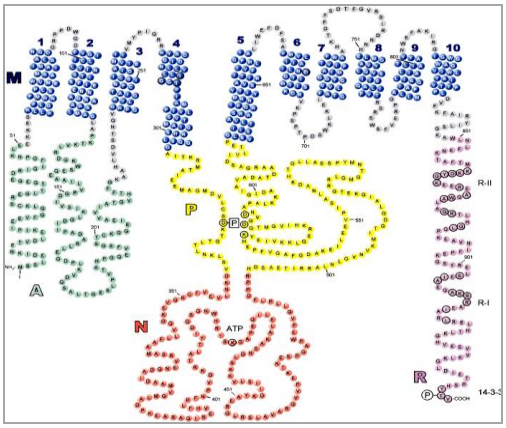

Figure 5. Schematic presentation of the AHA2 plasma membrane H^+-ATPase. The various domains (A, P, N, and R) of the enzyme are indicated by colored residues (from Palmgren 2001)

Not all Strategy I species perform a high acidification and in those species in which acidification is very scarce the response depends on many factors such as the balance of cation/anion uptake, the composition of root exudates and the type of nitrogen nutrition.

In conclusion, plants contain a complex regulation network of genes which encode uptake, chelation, transport, sub-cellular distribution and storage of Fe. Understanding these processes is the prerequisite for their manipulation in order to breed in the future high-quality nutritious crops.

1.3.4. *The role of exudates in Fe acquisition*

Root exudates are considered as one of the main root products, which are well known to influence nutrient solubility and uptake when plants are subject to stress. Root exudates are involved in plant nutrition through direct or indirect mechanisms. In the first case, some compounds act directly on nutrients making them more available while, in the second case, exudates act on soil microbial communities generating mutualistic associations and determining the structure of microbial community in its area of influence.

The quantity and quality of root exudates depends on the specie, the age of an individual plant and external factors like biotic and abiotic stress. The composition of root exudates can be complex, and often ranges from mucilage, root border cells, extracellular enzymes, simple and complex sugars, phenolics, amino acids, vitamins, organic acids, nitrogenous macromolecules such as purines and nucleosides to inorganic or gaseous molecules such as HCO_3^-, OH^-, H^+, CO_2 and H_2. Many of these compounds have metal reductant or/and chelating abilities and can enhance Fe availability in the apoplast and rhizosphere (Ohwaki and Sugahara, 1997, Jin et al. 2007, Cesco et al. 2010, Rodríguez-Celma et al. 2011, Mimmo et al. 2012). These organic compounds can often be divided in two main classes: LOAs, which include amino acids, organic acids, sugars, phenolics and an array of secondary metabolites; and high-molecular weight compounds like mucilage and proteins. The exudation of a vast array of compounds is an important metabolic feature of plant root considering that a high portion of root carbon (70%) can be release into the rhizosphere (Neumann and Römheld 2007).

Root exudation of various chemical molecules into the rhizosphere is largely dependent on the nutritional status of the plant, with some species exuding organic acid anions in response to P and Fe deficiency. The accumulation of low molecular weight organic acids (LOAs) has been recorded in many species as a response to Fe deficiency and may be involved in solubilization of iron from the soil. Landsberg (1981) has demonstrated that mono and dicots plant species released much more organic acids. The phenolic compounds are also important components of

root exudates and among their numerous functions they enhance the availability of iron to the roots (Julian et al. 1983, Jin et al. 2007, Cesco et al. 2010).

In Fe deficiency conditions it has been demonstrate that dicots accumulate and exudates organic acids, mainly malate and citrate (de Vos et al. 1986, López-Bucio et al. 2000, Abadía et al. 2002). Organic acids due to their carboxylic groups can chelate metals in soil solution and thus are involved in mobilization and uptake of nutrients such us P and Fe. Furthermore when soil pH is high, as in calcareous soils, mobilization of Fe(III) by malate and citrate are very slow as the chelates they form are quite instable (Jones et al. 1996). However, in that condition it was suggested a coordinate action between organic acids and the acidification performed by the H^+-ATPase (Jones, 1998). Several reports have indicated that cations were simultaneously released during the excretion of organic acids from roots. For example, wheat and lupin released K^+ (Ryan et al. 1995) and H^+ (Neumann and Römheld 1999) during the excretion of malate and citrate, respectively. Cation transport might be necessary for maintaining a transmembrane electrical potential difference during the release of organic acids (anion). LOAs are likely to cross the plasma membrane as multivalent anion. The pathways by which organic acids cross the plasma membrane of root cells are not well characterized and little is known of the molecular mechanisms that regulate the exudation of organic acids from roots. It has been suggested two main mechanisms of transport through the plasma membrane: diffusion as the gradient is favorable and anion channels that seems are directly related with the proton extrusion. Recently, a citrate-permeable channel in the plasma membrane of proteoid roots from white lupin has been characterized (Zhang et al. 2004).

Many phenolic compounds have been identified in root exudates (Dakora and Phillips 2002, Jin et al. 2007, Cesco et al. 2010). Lan et al. has recently observed in a proteomic analysis a strong induction in phenylpropanoid pathways in *Arabidopsis* under Fe deficiency conditions. Phenolic compounds exhibited multiple functions in root exudates. Regarding Fe nutrition Jin et al. have demonstrate their role in mobilization of Fe apoplastic deposits (Jin et al. 2007).

1.3.5. *Morphological changes induced by Fe deficiency*

The bioavailability of nutrients in the soil solution influences root growth, root proliferation and specific functional responses that in part depend on the prevalent nutrient status of the plant. Nitrogen (N), phosphorus (P), iron (Fe) and sulfur (S) are among the nutrients that have been reported to alter post-embryonic root developmental processes. Changes in root architecture can mediate the adaptation of plants to soils in which nutrient availability is limited by increasing the total absorptive surface of the root system. The development of root systems is usually highly asymmetric and reflects the ability of roots to adjust their growth and development to environmental factors (Forde and Lorenzo 2001). Furthermore, the morphological response is species specific even if a general trend could be drawn the variability among different species could be very high.

Low Fe availability induces morphological changes both at macroscopic and microscopic level. Changes observed in root epidermal cell under Fe deficiency include an increase in root hair by modulating the length, position and abundance. Root hairs are the extensions of single epidermal cells and comprise as much as 77% of the total root surface area of cultivated crops, forming the major point of contact between the plant and the rhizosphere (Parker et al. 2000) and are involved in root secretion of compounds. It was seen that root hairs in plants under Fe deficiency occupied zones that did not have this structures in sufficient Fe conditions (Schmidt et al. 2000). Moreover, low Fe availability frequently leads to the formation of branched root hairs (Müller and Schmidt 2004) through a signaling cascade that probably involves auxin and ethylene (Schmidt et al. 2000, Schmidt and Schikora 2001). P deficiency induce similar changes in epidermal cells but their responses are mediated by different signal transduction pathways (Schmidt and Schikora 2001)

In many species it was observed a subapical swelling as a response to low Fe availability that has been associated with an increase activity of the tips to acquire Fe. In Strategy I plants it was also found the development of rhizodermal transfer cells (Schikora et al. 2003; Santi and Schmidt

2008). Transfer cells are found in all taxonomic groups and have unique structural features: an invaginated secondary wall coated by the plasma membrane leading to an up to 20-fold area enlargement and is enriched in a set of transporters. Abundant mitochondria are also disposed near the plasma membrane of transfer cells. In these cells it was found a high metabolic activity that has been attributed to an increase activity of the Fe acquisition mechanism.

Changes in root elongation and in the number of lateral roots were observed in Fe deficient roots. In a recent work on *Arabidopsis* it was point out that symplastic content of Fe triggers the local elongation of lateral roots by inducing an Auxin promoter. The changes in root morphology are directly related with hormone regulation.

All root modifications previously pointed out are oriented to increase the contact surface of the root with the soil, its primary source of nutrients.

The study of root system architecture modifications provides interesting inputs regarding the tight relationship between root development and soil resource. Root system architecture (RSA), the spatial configuration of a root system in the soil, is used to describe the shape and structure of root systems. Its importance in plant productivity lies in the fact that major soil resources are heterogeneously distributed in the soil, so that the spatial disposition of roots will substantially determine the ability of a plant to ensure edaphic resources. Therefore, studies revealing the extent and nature of the genetic variation of RSA have profound implications for improving water- and nutrient-use efficiency of crops or for enhancing their productivity under abiotic stresses or suboptimal soil conditions.

1.4. **The Iron deficiency in crops**

According to FAO 39% of rural land areas are affected by mineral deficiency and low fertility that constrain crop production (Cramer et al., 2011). Iron deficiency is a problem in crop production worldwide but particularly in plants grown on calcareous soils (Vose, 1982). Calcareous soils represent the 30% of the earth land surface. Their total extent has

been estimated by FAO at 800 million hectares worldwide 57 million hectares of which in Europe. In the near future to cope with the increasing demand of food, agriculture must be extended to marginal areas, many of which include calcareous soils.

Figure 6. Soybeans varietal differences in iron deficiency chlorosis tolerance.

Iron deficiency in plants causes a nutritional disorder that results in dramatic yield losses and decreases the nutritional quality of crop products. The main Fe deficiency symptom in plants is chlorosis that consists in the yellowing of top leaves in the intervenial blade zones. Iron deficiency symptoms manifest themselves in the younger leaves due to the relative immobility of Fe in the plants. When Fe nutritional deficiency persist the leaves yellowing extends to the veins and the new leaves appears completely yellow. Low Fe availability results in substantial crop losses and reducing quality of crop products. Many differences in genotypic characteristic exist among crop species, and also among varieties of the same species, regarding their responses to low Fe availability. These differences are mainly base on the capacity to mobilize and acquire Fe from the rhizosphere.

Crop Fe deficiencies have been reported for many plant species and geographical regions. The most frequent problems correspond to the cultivation of sensitive crop species in arid and semi-arid regions with calcareous soils for crop such as soybean (*Glycine max*), peanut (*Arachis hupogaea*), dry bean (*Phaseolus vulgaris*), sorghum (*Sorghum bicolor*) and rice (*Oryza sativa*). Fe deficiencies have also been reported in corn (*Zea mays*), wheat (*Triticum aestivum*) and oat (*Avenae sativa*) but only in particular conditions. The following crops have been also found to be

especially responsive to Fe low bioavailability: alfalfa, asparagus, barley, beans (white), beets, broccoli, brussel sprouts, cabbage, cauliflower, celery, peach, pear, kiwifruit, the grapevine riparia, citrus, grass, oats, peanuts, rye, spinach, strawberry, sudangrass, tomato, and turf.

In areas where Fe deficiency is manifested, the deficiencies generally occur in patches rather than uniformly across a field. Chlorotic patches often occur in fields where high soil pH is homogeneous, indicating that alkalinity is not the only factor controlling the availability and uptake of Fe (Hansen et al. 2006).

Many interactions exist between different mineral especially between P and Fe. In fact the appearance of chlorosis depends on the P/Fe ratio rather than the single amounts of one or another nutrient.

Because plants are a primary food source for humans, the nutritional state of plants is also of central importance to human health. In particular, Fe content in plants becomes important in those populations whose diet is primarily vegetal. Understanding of Fe homeostasis has been successfully applied to generate crops that are tolerant of Fe deficiency or whose edible parts are more nutritious. Creating designer crops with enhanced nutrient uptake could also aid agriculture to reduce the need for fertilizer application decreasing consistently the managing costs of agriculture.

1.5. **Wild plant strategies to low nutritional deficiencies: the case of iron deficiency**

A Plant strategy is defined as a set of interlinked adaptations that arose as a consequence of natural selection and promotes growth and successful reproduction in a given environment (Craine, 2009). The traits associated with plant resource strategies are important for the vegetative growth of individual plants and determine the acquisition, allocation, and loss of resources that support successful growth under a particular set of environmental conditions. These traits range from biochemistry of the cell to tissue construction, to plant effects on the abiotic environment.

Many plants are well adapted to leave successfully in low-nutrient

environments. The traits observed in plants performing a low-nutrient strategy are related with:

- increasing the acquisition of nutrients;

- decreasing the requirement of nutrients;

- decreasing losses of nutrients.

Increasing the acquisition of nutrients implies the activation of strategies of mining and scavenging of nutrients. Mining strategies comprise all those mechanism oriented to mobilize the not yet available resources. In previous paragraphs the mining mechanisms that are activated by plants to make available the Fe present in the soil are presented: the reduction-based mechanism of Fe acquisition (par. 1.3.1-1.3.3.), the exudation of organic compounds (par. 1.3.4.), and the scavenging strategies that mainly consist in exploring the soil and hence directly related to root morphology (par. 1.3.5.).

Decreasing nutrient requirements does not necessary mean decrease the nutrient requirement per plant but rather the reduction of nutrient to produce a given unit of biomass. Species that display low nutrient strategies shifts the nutrient flow to those activities considered primary while on the contrary all purposes of secondary importance to growth are restricted in the limited nutrient. In many wild species flowering and produce seed or produce resistant structures may have priority over vegetative growth.

Decreasing losses of nutrients refers mainly to the turnover of biomass. Generally low nutrient strategy species reduce the loss rate of biomass having increase longevity of leaves and roots.

These observations match well with physiological traits observed in non-crop plants that are plants that have been developed through natural selection of all coordinated adaptations of roots, leaves, and support structures.

The acclimation of plants to abiotic stress conditions is a complex and coordinated response involving hundreds of genes. Study how plants sense and acclimate to abiotic stress conditions is crucial to develop plants and

crops with enhanced tolerance to abiotic stresses.

1.6. **Parietaria judaica: a wild species tolerant to high calcareous environments.**

Parietaria judaica (L. 1753) – of which *Parietaria diffusa* (M. et K. 1823) or *Parietaria ramiflora* (Moench 1794) are synonyms – belongs to the Urticacea family. It is a wild perennial sinantropic plant.

Figure 7. *Parietaria judaica*. **A** spontaneous growth, **B** and **C** detail of *P. judaica* morphology

Parietaria judaica stands 0.2 until 1.0 m tall, displaying variable habits depending on the environmental characteristics, from spreading to decumbent-erect. The leaves are alternate from lanceolate to oval and 2-8 cm long. Both leaves' surfaces are covered with fine white hairs. It reproduces sexually and asexually. Flowering season is up to ten month. Seeds germinate over a wide range of temperatures from 10 to 27°C, with 75% of germination seeds at 20°C in light and dark conditions. Seeds are dispersed by wind (anemochorous). Most scientific literature concerns studies on immunology and biomedicine due to its high pollen allergeny (Colombo et al. 2003; Barranca et al. 2010) and medical properties (Uncini Manganelli et al. 2005).

Studies in ethnobotanics have identified many active principles in *Parietaria* such as flavonoids. At the moment a detailed chemical characterization of *Parietaria j.* has not been carried out, anyway it is well known that it has a high content in KNO_3 as well as polyphenols (Donnini et al., 2012).

Even though it is consider an “indiffent plant” due to its ability to grow and complete successfully its life cycle in both acid and alkaline substrates, it constitute the widespread flora in calcareous hostile environments, as wall cracks exposed to sun without showing any sign of chlorosis. As a dicot, *Parietaria* encompasses all the inducible metabolic changes of Strategy I Fe efficiency (dell’Orto et al. 2003). However the extreme efficiency by which it adapts to stress conditions suggested a great metabolic flexibility. In particular the high production of phenols almost certainly plays an important role within its adaptive strategy.

1.7. **Aim of the research**

The study of wild plants responses to calcareous conditions could provide interesting insights as they are well adapted to the environment. 30% of all cultivated soils are calcareous and considerable crop losses result from the cultivation in these types of soils.

The natural adaptation of wild plants to the environment occurs on the entire plant at different levels so the adaptive response always involves different traits. More diversified and flexible is the response more possibilities of adaptive success are.

This study focusses on the integrated responses of a wild calcicole plant *Parietaria judaica* to different alkaline conditions. The main purpose of the study is to characterized the diversified responses at morphological, physiological and biochemical level.

Another goal is to distinguish the response due to the presence of a high concentration of bicarbonate in the growing medium from a response due only to a high pH of the growing condition. In this regard two different high alkaline buffer treatments were performed: one with CaCO3/NaHCO3 which mimics the calcareous environment and other with a high alkaline organic buffer (Tricine).

The main Strategy I responses were recorded: reduction by means of the iron chelate reductase activity; acidification through the activation of the plasmalemma H+-ATPase; the content of low organic acids (LOAs) and phenolics compounds in root tissues and exudates.

Activation of Strategy I requires an increase production of energetic metabolites as ATP and $NAD(P)H^+$, LOAs, and secondary metabolites such as phenolic compounds. This requirement implies a general metabolic arrangement of the cell.

Further responses to Fe starvation are concerned with alterations in root morphology and root architecture, often leading to an increase in the absorptive surface area. Depending on the species, such an increase can be achieved by the formation of extra root hairs, development of clusters of secondary lateral roots (proteoid roots), or the formation of transfer

cells in the rhizodermis.

Morphological changes of the root system as a response to direct or induced Fe deficiency were archived using an image system analysis with the aim to identifying the patterns of morphological change.

The study of the metabolic rearrangement in a wild species (*Parietaria judaica)* grown in different conditions of iron availability could provide interesting insights in order to recognize the adaptive traits of a spontaneous plant coping with a highly calcareous environment.

References

Abadía J., López-Millán A.F., Rombolà A., Abadía A., 2002. *Organic acids and Fe deficiency: a review*, Plant and Soil, 241:75–86

Arango M., Gévaudant F., Oufattole M., Boutry M., 2003. *The plasma membrane proton pump ATPase: the significance of gene subfamilies*, Planta, 216(3):355-65

Barberon M., Zelaznya E., Robert S., Conéjéro G., Curie C., Frim J., Verta G., 2011. Monoubiquitin-dependent endocytosis of the IRON-REGULATED TRANSPORTER 1 (IRT1) transporter controls iron uptake in plants, PNAS,108(32):E450-E458

Barranca M., Fontana S., Taverna S., Duro G., Zanella-Cleon I., Becchi M., De Leo G., Alessandro R., 2010. *Proteomic analysis of Parietaria judaica pollen and allergen profiling by an immunoproteomic approach*, Biotechnology Letters, 32(4):565-570

Bienfait H.F., de Weger L.A., Kramer D., 1987. *Control of the development of iron-efficiency reactions in potato as a response to iron deficiency is located in the roots*, Plant Physiology, 83:244–247

Bienfait H.F., Scheffers M.R., 1992. *Some properties of ferric citrate relevant to the iron nutrition of plants*, Plant and Soil, 143:141–144

Bienfait H.F., van den Briel W., Mesland-Mul N.T., 1985. *Free Space Iron Pools in Roots*, Plant Physiology, 78(3):596–600

Cesco S., Neumann G., Tomasi N., Pinton R., Weisskopf L., 2010. *Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition,* Plant Soil , 329:1–25

Colangelo E.P., Guerinot M.L., 2004. *The Essential Basic Helix-Loop-Helix Protein FIT1 Is Required for the Iron Deficiency Response*, The Plant Cell, 16(12):3400-3412

Colombo P., Bonura A., Costa M., Izzo V., Passantino R., Locorotondo G., Amoroso S., Geraci D., 2003. *The Allergens of Parietaria*, International Archives of Allergy and Immunology, 130(3):173–179

Connolly E.L., Campbell N.H., Grotz N., Prichard C.L., Guerinot M.L., 2003. *Overexpression of the FRO2 ferric chelate reductase confers tolerance to growth on low iron and uncovers posttranscriptional control*, Plant Physiology, 133:1102–1110

Connolly E.L., Fett J.P., Guerinot M.L., 2002. *Expression of the IRT1 metal transporter is controlled by metals at the levels of transcript and protein accumulation*, Plant Cell, 14:1347–1357

Cornell R.M., Schwertmann U., 2003. The iron oxides: Structure, Properties, Reactions, Occurrences and Uses, Wiley-VCH, Weinheim, New York

Curie C., Alonso J.M., Le Jean M., Ecker R., Briat J-F., 2000. *Involvement of NRAMP1 from Arabidopsis thaliana in iron transport*, Biochemical Journal, 347:749–755

de Vos C.R., Lubberding H.J., Bienfait H.F., 1986. *Rhizosphere Acidification as a Response to Iron Deficiency in Bean Plant*, Plant Physiology , 81(3):842-846

Dell'Orto M., Pirovano L., Villalba J.M., Gonzales-Reyes J.A., Zocchi G, 2002. *Localization of the plasma membrane H^+-ATPase in Fe-deficient cucumber roots by immunodetection*, Plant and Soil, 241:11–17

Dell'Orto M., Santi S., De Nisi P., Cesco S., Varanini Z., Zocchi G., Pinton R., 2000. *Development of Fe-deficiency responses in cucumber (Cucumis sativus L.) roots: involvement of plasma membrane H+-ATPase activity*, Journal of Experimental Botany, 51:695–701

Dell'Orto M., De Nisi P., Pontiggia A., Zocchi G., 2003. *Fe Deficiency Responses in Parietaria diffusa: A Calcicole Plant*, Journal of Plant Nutrition, 26(10-11):2057-2068

Dofing S.M., Penas E.J., Maranville J.W., 1989. *Effect of bicarbonate on iron reduction by soybean roots*, Journal of Plant Nutrition, 12(6):797-802

Donnini S., De Nisi P., Gabotti D., Tato L., Zocchi G., *Adaptive strategies of Parietaria diffusa (M.&K.) to calcareous habitat with limited iron availability*, Plant, Cell and Environment, 35(6):1171–1184

Eide D.J., Guerinot M.L. 1998. *The ZIP Genes: a Family of Eukaryotic Metal Ion Transporters*, 5th Internet World Congress for Biomedical Sciences, Ontario-Canada, december 7-16

Forde B., Lorenzo H., 2001. *The nutritional control of root development*, Plant Soil, 232:51-68

Grusak M.A., Welch R.M., Kochian L.V., 1990. *Does iron deficiency in Pisum sativum enhance the activity of the root plasmalemma iron transport protein?*, Plant Physiology, 94:1353–1357

Guerinot M.L., Yi Y., 1994. *Iron: Nutritious, Noxious, and Not Readily Available*, Plant Physiology, 104:815-820

Hansen, N.C., Hopkin B.G., Ellswoth, J.W., Jolley V.D., 2006. *Iron nutrition in field crops*, in L. L. Barton and J. Abadía (eds.), *Iron Nutrition in Plants and Rhizospheric Microorganisms,* Springer, 23–59

Harmsen J., Rulkens W.H. and Eiasakers H.J.P. (2005*). Bioavailability, concept for understanding to tool for predicting?* Land Contamination and reclamation, 13(2):161-171

Hell R., Stephan U.W., 2003. *Iron uptake, trafficking and homeostasis in plants*, Planta, 216:541–551

Henriques R., Jásik J., Klein M., Martinoia E., Feller U., Schell J., Pais M.S., Koncz C., 2002. *Knock-out of Arabidopsis metal transporter gene IRT1 results in iron deficiency accompanied by cell differentiation defects*, Plant Molecular Biology, 50:587–597

Jakoby M., Wang H.-Y., Reidt W., Weisshaar B., Bauer P., 2004. *FRU (BHLH029) is required for induction of iron mobilization genes in Arabidopsis thaliana*, FEBS Letters, 577(3):528–534

Jin C.W., Li G.X., Yu X.H., Zheng S.J., 2010. *Plant Fe status affects the composition of siderophore-secreting microbes in the rhizosphere*, Annals of Botany, 105: 835–841.

Jin C.W., You G.Y., He Y.F., Tang C., Wu P., Zheng S.J., 2007. *Iron Deficiency-Induced Secretion of Phenolics Facilitates the Reutilization of Root Apoplastic Iron in Red Clover*, Plant Physiology, 144(1):278–285

Jones D.L., 1998. *Organic acids in the rhizosphere - a critical review*, Plant and Soil, 205:25–44

Jones D.L., Darah P.R., Kochian L.V., 1996. Critical evaluation of organic acid mediated iron dissolution in the rhizosphere and its potential role in root iron uptake, Plant and Soil, 180(1):57-66

Julian G., Cameron J., Olsen R.A., 1983. *Role of chelation by ortho di hydroxy phenols in iron absorption by plant roots*, Journal of Plant Nutrition, 6:163–75

Kobayashi T., Nishizawa N.K., 2012. *Iron Uptake, Translocation, and Regulation in Higher Plants*, Annual Review of Plant Biology, 63:131-152

Kochian U., Lucas W.J. 1991. *Do plasmalemma oxidoreductases play a role in plant mineral ion transport?*, In: Crane FU, Morre DJ, Low HE (eds.) *Oxidoreduction at the plasma membrane: relation to growth and transport*, Madison, Wisconsin, USA: Soil Science Society of America, 189–205

Kraemer, S.M., 2004. Iron oxide dissolution and solubility in the presence of siderophores, Aquatic Science, 66:3-18

Lan P., Li W., Wen T.-N., Shiau J.-Y., Wu Y.C., Lin W., Schmidt W., 2011. *iTRAQ Protein Profile Analysis of Arabidopsis Roots Reveals New Aspects Critical for Iron Homeostasis*, Plant Physiology, 155(2):821-834

Landsberg, E.C., 1981. *Organic acid synthesis and release of hydrogen ions in response to Fe-deficiency stress of mono - and dicotyledonous plant species*, Journal of Plant Nutrition, 3:579–91

Läuchli A., Grattan S.R., 2012. Soil pH extreme, in Shabala S. (ed.), Plant stress physiology, CAB International, UK

Lemanceau P., Expert D., Gaymard F., Bakker P.A.H.M., Briat J.-F., 2009. *Role of Iron in Plant-Microbe Interactions*, Advances in Botanical Research, Advances in Botanical Research, 51:491–549

Lindsay W.L., Schwab A.P., 1982. *The chemistry of iron in soils and its availability to plants*, Journal of Plant Nutrition, 5(4-7):821-840

Ling H.-Q., Bauer P., Bereczky Z., Keller B., Ganal M., 2002. *The tomato fer gene encoding a bHLH protein controls iron-uptake responses in roots*, PNAS, 99(21):13938–13943

Long T. A., Tsukagoshi H., Busch W., Lahner B., Salt D.E., Benfey P.N., *The bHLH Transcription Factor POPEYE Regulates Response to Iron Deficiency in Arabidopsis Roots*, The Plant Cell, 22(7):2219-2236

López-Bucio J., Nieto-Jacobo M.F., Ramírez-Rodríguez V., Herrera-Estrella L., 2000. *Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils*, Plant Science, 160(1):1–13

López-Millán A.F., Morales F., Abadía A., Abadía J., 2000. *Effects of Iron Deficiency on the Composition of the Leaf Apoplastic Fluid and Xylem Sap in Sugar Beet. Implications for Iron and Carbon Transport*, Plant Physiology, 124(2):873-884

Lucena C., Romera F.J., Rojas C.L., García M.J., Alcántara E., Pérez-Vicente R., 2007. *Bicarbonate blocks the expression of several genes involved in the physiological responses to Fe deficiency of Strategy I plants*, Functional Plant Biology, 34(11):1002–1009

Marschner H., Römheld V., Kissela M., 1986. *Different strategies in higher plants in mobilization and uptake of iron*, Journal of Plant Nutrition, 9(3-7):695-713

Marschner H.,1995. *Mineral nutrition of higher plants*, Academic Press, London

Mäser P., Thomine S., Schroeder J.I., Ward J.M., Hirschi K., Sze H., Talke I.N., Amtmann A., Maathuis F.J.M., Sanders D., Harper J.F., Tchieu J., Gribskov M., Persans M.W., Salt D.E., Kim S.A., Guerinot M.L., 2001. *Phylogenetic Relationships within Cation Transporter Families of Arabidopsis*, Plant Physiology, 126(4):1646-1667

Mimmo T., Terzano R., Medici L., Lettino A., Fiore S., Tomasi N., Pinton R., Cesco S., 2012. *Interaction of root exudates with the mineral soil constituents and their effect on mineral weathering*, EGU General Assembly, 22-27 April Vienna - Austria., p.11494

Morales F., Grasa R., Abadía A., Abadía J., 1998. *Iron chlorosis paradox in fruit trees*, Journal of Plant Nutrition, 21:815-825

Müller M., Schmidt W., 2004. *Environmentally induced plasticity of root hair development in Arabidopsis*, Plant Physiology, 134:409–419

Neumann G., Römheld V., 1999. *Root excretion of carboxylic acids and protons in phosphorus-deficient plants*, Plant and Soil, 211:121–130

Neumann G., Römheld V., 2001. *The Release of Root Exudates as Affected by the Plant's Physiological Status*, in: Willig S., Varanini Z., Nannipieri P. (eds), *The Rhizosphere: Biochemistry and Organic Substance at the Soil-Plant Interface: Biochemistry and Organic Substance at the Soil-Plant Interface*, Marcel Dekker, New York, pp. 41-93

Nikolic M., Roemheld V., 2002. *Does high bicarbonate supply to roots change availability of iron in the leaf apoplast?*, Plant Soil, 241:67-74

Ohwaki Y., Sugahara K., 1997. *Active extrusion of protons and exudation of carboxylic acids in response to iron deficiency by roots of chickpea (Cicer arietinum L.)*, Plant and Soil 189: 49–55, 1997

Olsen R.A., Clark R.B., Bennett J.H., 1981. *The Enhancement of Soil Fertility by Plant Roots*, American Scientist, 69(4):378-384

Palmgren M.G., 2001. *Plant Plasma Membrane H^+-ATPases: Powerhouses for Nutrient Uptake*, Annual Review of Plant Physiology and Plant Molecular Biology, 52:817–45

Palmgren M.G., Harper J.F., 1999. *Pumping with plant P-type ATPases*, Journal of Experimental Botany, 50:883–893

Parker J.S., Cavell A.C., Dolan L., Roberts K., Grierson C.S., 2000. *Genetic interactions during root hair morphogenesis in Arabidopsis*, The Plant Cell, 12:1961–1974

Rellán-Álvarez R., El-Jendoubi H., Wohlgemuth G., Abadía A., Fiehn O., Abadía J., Álvarez-Fernández A., 2011. Metabolite Profile Changes in Xylem Sap and Leaf Extracts of Strategy I Plants in Response to Iron Deficiency and Resupply, Frontiers Plant Science, 2: 66

Robin A., Vansuyt G., Hinsinger P., Meyer J.M., Briat J.F., Lemanceau P., 2008. *Iron Dynamics in the Rhizosphere: Consequences for Plant Health and Nutrition*, in: Sparks D.L. (ed), Advances in Agronomy, 99:183-225

Robinson N.J., Procter C.M., Connolly E.L., Guerinot M.L., 1999. *A ferric-chelate reductase for iron uptake from soils*, Nature, 397:694-697

Rodríguez-Celma J., Lattanzio G., Grusak M.A., Abadía A., Abadía J., López-Millán A.F., 2011. *Root Responses of Medicago truncatula Plants Grown in Two Different Iron Deficiency Conditions: Changes in Root Protein Profile and Riboflavin Biosynthesis*, Journal of Proteome Research,10(5):2590–2601

Römheld V., 2000. *The chlorosis paradox: Fe inactivation as a secondary event in chlorotic leaves of grapevine*, Journal of Plant Nutrition, 23:1629-1643

Romera F.J., Alcántara E., de la Guardia M.D., 1992. *Effects of bicarbonate, phosphate and high pH on the reducing capacity of Fe-deficient sunflower and cucumber plants*, Journal of Plant Nutrition, 15(10):1519-1530

Ryan P.R., Delhaize E., Randall P.J., 1995. *Malate Efflux From Root Apices and Tolerance to Aluminium Are Highly Correlated in Wheat*, Australian Journal of Plant Physiology, 22(4):531-536

Santi S., Cesco S., Pinton V.Z.R., 2005.*Two plasma membrane H+-ATPase genes are differentially expressed in iron-deficient cucumber plants*, Plant Physiology and Biochemistry, 43:287–292

Santi S., Schmidt W., 2008. *Laser microdissection-assisted analysis of the functional fate of iron deficiency-induced root hairs in cucumber*, Journal of Experimental Botany, 59:697–704.

Schagerlöf U., Wilson G., Hebert H., Al-Karadaghi S., Hägerhäll C., 2006. *Transmembrane topology of FRO2, a ferric chelate reductase from Arabidopsis thaliana*, Plant Molecular Biology, 62:215–221

Schikora A., Schmidt W., 2002. *Formation of transfer cells and H+-ATPase expression in tomato roots under P and Fe deficiency*, Planta, 215:304–311

Schmidt W., 2003. *Iron solutions: acquisition strategies and signaling pathways in plants*, Trends in Plant Science, 8(4): 188–193

Schmidt W., Michalke W., Schikora A., 2003. *Proton pumping by tomato roots. Effect of Fe eficiency and hormones on the activity and distribution of plasma membrane H+-ATPase in rhizodermal cells*, Plant, Cell and Environment, 26:361–370

Schmidt W., Tittel J., Schikora A., 2000. *Role of hormones in the induction of iron deficiency responses in Arabidopsis roots*, Plant Physiology, 122:1109-1118

Schwertmann U., Taylor R.M., 1989. *Iron oxides*, in: Dixon J.B, Weed S.B., *Mineral in soils Environments*, Soil society of America, 379-438

Sondergaard T.E., Schulz A., Palmgren M.G., 2004. *Energization of Transport Processes in Plants. Roles of the Plasma Membrane H^+-ATPase*, Plant Physiology, 136(1):2475-2482

Stumm, W., Morgan, J.J., 1996. *Aquatic Chemistry, Chemical Equilibria and Rates in Natural Waters*, John Wiley & Sons, New York, NY

Taylor K.G., Konhauser K.O., 2011. *Iron in Earth Surface Systems: A Major Player in Chemical and Biological Processes*, Elements, 7:83-88

Toulon V., Sentenac H., Thibaud J.B., Davidian J.C., Moulineau C., Grignon C., 1992. *Role of apoplast acidification by the H^+pump*, Planta, 186 (2):212-218

Uncini Manganelli R.E., Zaccaro L., Tomei P.E., *Antiviral activity in vitro of Urtica dioica L., Parietaria diffusa M. et K. and Sambucus nigra L.*, Journal of Ethnopharmacology, 98(3):323–327

van Hees P.A.W, Lundström U.S, 2000. *Equilibrium models of aluminium and iron complexation with different organic acids in soil solution*, Geoderma, 94(2–4):201–221

Vasconcelos M., Eckert H., Arahana V., Graef G., Grusak M.A., Clemente T., 2006. *Molecular and phenotypic characterization of transgenic soybean expressing the Arabidopsis ferric chelate reductase gene, FRO2*, Planta, 224:1116–1128.

Vert G., Grotzb N., Dédaldéchamp F., Gaymard F., Guerinot M.L., Briat J.-F., Curie C., 2002. *IRT1, an Arabidopsis transporter essential for iron uptake from the soil and for plant growth*, Plant Cell, 14:1223–1233.

Vose P.B., 1982. *Rationale of selection for specific nutritional characters in crop improvement with Phaseolus vulgaris L. as a case study*, Plant and Soil, 72 (2-3):351-364

Yi Y., Guerinot M.L., 1996. *Genetic evidence that induction of root Fe(III) chelate reductase activity is necessary for iron uptake under iron deficiency*, The Plant Journal, 10(5):835–844

Zhang F., Römheld V., Marschner H., 1991. *Role of the Root Apoplasm for Iron Acquisition by Wheat Plants*, Plant Physiology, 97(4):1302–1305

Zhang W.-H., Ryan P.R., Tyerman S.D.,2004. *Citrate-Permeable Channels in the Plasma Membrane of Cluster Roots from White Lupin*, Plant Physiology, 136:3771-3783

CHAPTER 2

Adaptive strategies of Parietaria diffusa (M.& K.) to calcareous habitat with limited iron availability*

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ABSTRACT

The study of native plants growing in hostile environments is useful to understand how these species respond to stress conditions. Parietaria diffusa (M.& K.) is able to survive in highly calcareous soils and extreme environments, such as house walls, without displaying any chlorotic symptoms. Here, we have investigated the existence of Strategy I complementary/alternative mechanism(s) involved in Fe solubilization and uptake and responsible for Parietaria's extraordinary efficiency. After assessing the specific traits involved in a calcicole-behaviour in the field, we have grown plants in conditions of Fe deficiency, either direct (-Fe) or induced by the presence of bicarbonate (+FeBic). Then, the growth performance, physiological and biochemical responses of the plants were investigated. The study shows that in Parietaria +FeBic, the classical responses of Strategy I plants are activated to a lower extent than in -Fe. In addition, there is a greater production of phenolics and organic acids that are both exuded and accumulated in the roots, which in turn show structures similar to 'proteoid-like roots'. We suggest that in the presence of this constraint, Parietaria undergoes some metabolic rearrangements that involve PEP-consuming reactions and an enhancement of the shikimate pathway.

Key-words: bicarbonate; calcareous soils; Fe deficiency; organic acids; phenolics.

2.1. **Introduction**

In an ecosystem, the colonization by a specific plant species affects biodiversity. However, there are few studies about the physiological properties of species that determine their success during ecological competition and therefore their environmental distribution. The chemico-physical characteristics of the soil are often important factors that strongly control plant distribution and growth.

It is well known that the presence of bicarbonate and high pH in the soil cause a diminished bioavailability of some nutrients, mainly Fe, inducing in plants a common nutritional disorder named chlorosis (Lindsay 1984). Given that more than 30% of the soils in the world are calcareous, it is

quite common that crops must face this problem (Chen and Barak 1982). Plants show different susceptibility to soil factors inducing Fe deficiency, so that a different degree of stress and a great variability exists in response to Fe deprivation. A variability in Fe acquisition capacity has been found in spontaneous vegetation (Nelson 1992), and native plants can be assigned to Fe-efficient and Fe-inefficient classes on the basis of the presence and diffusion in pedological environments inducing a low Fe availability. In particular, the so called calcicole species seem to strongly colonize calcareous habitats better than other species (i.e. calcifuge plants), demonstrating a better efficiency in obtaining Fe under these unfavourable environments. In fact, it has been demonstrated that calcicole native dicotyledonous plants are more Fe-efficient than calcifuge ones, showing a higher Fe^{3+}-chelate reductase activity, a response assigned to the Strategy I (Schmidt and Bartles 1998). Strategy I is a complex Fe uptake mechanism developed by all plants, with the only exclusion of the Poaceae, which belong to Strategy II (Römheld and Marschner 1986; Morrissey and Guerinot 2009; Abadía *et al*. 2011). This Fe uptake mechanism is based on the reduction of external Fe^{3+} to Fe^{2+} through the induction of a Fe^{3+}-chelate reductase enzyme localized at the plasma membrane of the rhizodermal cells (Robinson *et al*. 1999; Waters, Blevins and Eide 2002; Li, Cheng and Ling 2004). Once reduced, the Fe^{2+} ion is transported inside the roots by a carrier (IRTs) belonging to the ZIP family of transporters (Guerinot 2000; Varotto *et al*. 2002; Vert *et al*. 2002; Henriques *et al*. 2002). The capacity to grow in Fe-poor environments is not restricted only to the induction of the two above mentioned mechanisms, but other activities can be induced during Fe deficiency stress. Amongst these, several biochemical and morphological adaptations occur in Strategy I plants. One of the most important is the enhanced capacity to decrease the rhizospheric pH through the activation of a plasma membrane-localized P-type H^+-ATPase (Zocchi and Cocucci 1990; Palmgren 2001; Santi and Schmidt 2009). Proton extrusion is useful both to increase Fe availability by increasing the solubility of Fe compounds (Dell'Orto *et al*. 2000), as well as to set up a favourable transmembrane electrical potential (negative inside) for cation uptake. However, the induction of the reduction processes and the enhanced extrusion of protons under Fe deficiency needs an increased rate of

NAD(P)H and ATP regeneration, which is achieved through the acceleration of metabolism, in particular glycolysis. Amongst the metabolic activities which are increased under Fe deficiency, phospho*enol*pyruvate carboxylase (PEPC) seems to play a pivotal role (Zocchi 2006 and references therein). In fact, the enzyme is active in several anaplerotic reactions to replenish tricarboxylic acid cycle intermediates, to provide carbon skeletons to sustain the synthesis of amino acids and for the pH-stat mechanism (De Nisi and Zocchi 2000; López-Millán *et al*. 2000; Zocchi 2006).

In some Strategy I species also the release of organic compounds such as phenolics, flavins, sugars and organic acids could help in the solubilisation of Fe containing compounds (Welkie 2000; Curie and Briat 2003; Jin *et al*. 2007; López-Millán *et al*. 2009). Considering the reducing and complexing proprieties of phenolic compounds, it is widely accepted that the mechanism by which they can regulate Fe mobility in the rhizosphere might play an important role (Jin *et al*. 2007; Tomasi *et al*. 2008; Cesco *et al*. 2010). Recently, a few studies have explored the role of secondary metabolic pathways in plant response to Fe deficiency. An example is the shikimate pathway, which is responsible for the synthesis of phenolic compounds. Some of the key enzymes catalyzing this pathway include shikimate dehydrogenase (SDH), shikimate kinase (SK), and phenylalanine ammonia lyase (PAL) (M'Sehli *et al*. 2009; Lan *et al*. 2011). From a morphological point of view, the appearance of swollen tips, secondary lateral roots and root hairs increase the surface of contact between roots and soil, favouring the search and acquisition of nutrients (Landsberg 1996; Schmidt and Bartels 1996). Induction of these different responses, in addition to reduction and transport processes, would increase plant efficiency in solving the problem of Fe deficiency. The more a plant is able to diversify its response, the more efficient will be the result.

In alkaline and calcareous environments, Fe solubility and availability are drastically reduced along with other nutrients (for instance P) (Guerinot & Yi 1994; von Wandruszka 2006). This means that in these conditions, relatively low amount of soluble nutrients might be available for plant uptake and therefore for use in various physiological functions, affecting photosynthesis, growth, competition and survival of the species. However,

heavy calcareous sites are colonized by plants with morpho-physiological characteristics of resistance to these particular conditions. *Parietaria diffusa*, a spontaneous species also named “pellitory of the wall”, is a dicot belonging to the Urticaceae family, widespread in the Mediterranean area, United States and Australia. It is able to grow on walls, debris and substrates with a very high carbonate concentration without showing any Fe deficiency symptom (Fig. 1). For this reason *Parietaria* could represent a model plant to understand the ecophysiological traits determining the calcicole behaviour of plants and the mechanisms of resistance developed by them to face with these adverse conditions, particularly concerning Fe deficiency.

Figure 1. Spontaneously growing *Parietaria diffusa* (M&K).

In a previous work (Dell’Orto *et al*. 2003) we have characterized in part the biochemical and physiological responses of *Parietaria* under Fe deficiency conditions, typical of a calcareous habitat, in order to clarify whether it behaves as a Strategy I plant. In the present study some secondary responses to Fe deficiency were further investigated, with the aim to determine the existence of alternative or complementary mechanism(s) to the typical Strategy I, which may be responsible for *Parietaria*’s extraordinary efficiency in Fe acquisition. In particular, to identify the specific traits of tolerance involved in a calcicole-behaviour, an analysis on *Parietaria* plants collected directly from walls with

different pH values has been carried out. Then, *Parietaria* plants have been grown in hydroponics under Fe deficiency, either direct or induced by the presence of bicarbonate, and the morphological, physiological and the biochemical traits of the plants have been investigated.

2.2. **Materials and methods**

2.2.1. *Plant Material*

2.2.1.1. FIELD SAMPLING

Parietaria diffusa (M.&K.) plants were sampled in three different sites near the University of Milan (Italy). Sampling areas were chosen *a priori* based on the presence of *Parietaria* as the unique species. Samples were collected by breaking the walls and the substrates surrounding roots were also collected and solubilized in distilled water in order to determine the pH (pH-meter PHM 240 pH/Ion Meter).

2.2.1.2. HYDROPONIC CULTURE

Cuttings of *Parietaria judaica* were obtained from a mother plant and put in an aerated half strength nutrient solution for 1 week to radicate. Once rooted, plants were transferred to 10 L plastic pots (40 plants/pot) containing (i) full nutrient solution plus 100 µM Fe(III)-EDTA (+Fe), pH 6.2; (ii) full nutrient solution without Fe (-Fe), pH 6.2; (iii) full nutrient solution with 100 µM Fe(III)-EDTA, 15 mM $NaHCO_3$ and 0.5 g L^{-1} $CaCO_3$ (+FeBic), which brought the pH to 8.3. Addition of 0.5 g L^{-1} $CaCO_3$ is useful to keep the bicarbonate concentration constant to approximately 15 mM throughout the experiment. The composition of the full strength solution was as reported in Donnini *et al*. (2010). Plants were grown for 9 days under different treatments in a growth chamber under 16/8 h light/dark regime, 27/21°C, 65-75% relative humidity and with a PPDF of 200 µmol m^{-2} s^{-1}.

2.2.2. *Collection of root exudates*

Root exudates were collected according to Gries *et al.* (1995). Five plants from each treatment were transferred to 250 ml of distilled water, with the pH adjusted to 6.2, containing 10 mg L^{-1} of Micropur™ to prevent decomposition of organic matter by microorganisms. Root exudates were collected over a 24 h period in a growth chamber under continuous aeration and freeze dried. The lyophilized material was resuspended in distilled water and filtered through a 0,45 μm Millipore Millex-HN; both phenolics and organic acid concentrations were then determined. For each treatment five replicates were performed.

2.2.3. *Determination of phenolic compounds*

Determination of phenolics concentration in the roots was performed using two different extraction solutions; distilled water or methanol. Sampled roots were homogenized in 1 volume (w/v) of extraction solution and the homogenate centrifuged at 10 000 *g* for 10 min. Extracts were filtered through a 0,45 μm Millipore Millex-HN. The concentration of phenolics in both roots and exudates was determined spectrophotometrically at 750 nm with the Folin-Ciocalteau reagent according to the method of Swain and Hillis (1959), using gallic acid as a standard. For each treatment five replicates were performed.

2.2.4. *Organic acids assay*

For the quantitative determination of organic acids, roots were collected, carefully rinsed in distilled water and homogenized in the presence of 5 mL of 10% (v/v) perchloric acid and centrifuged at 10 000 *g* for 15 min. The pH was brought to 7.5 with 0.5 M K_2CO_3 to neutralize the acidity and to precipitate the perchlorate. Extracts were clarified by centrifugation at 15 000 *g* for 15 min. Citric and malic acid concentration in both roots and exudates was determined enzymatically, using specific kits from Boehringer Mannheim and according to the manufacturer's instructions. The recovery of both organic acids was more than 90% as determined by

the use of an internal standard (Rabotti, De Nisi and Zocchi 1995). For each treatment five replicates were performed.

2.2.5. *Biomass measurement and leaf chlorophyll determination*

Growth of shoots and roots was calculated as the difference between the end (9 d) and the beginning of treatments. FW and DW were determined at the end of treatments. Three independent experiments in triplicate (n=9) were performed.

Chlorophyll concentration was determined according to Lichtenthaler (1987). For each treatment five replicates were performed.

2.2.6. *Determination of Fe and P*

For the determination of apoplastic Fe, plants from each treatment (n=5) were transferred to a beaker with 0.5 mM $CaSO_4$ under vigorous aeration. After 10 to 15 min, plants were placed with their root system in 40-ml tubes with 21 ml of 10 mM MES, 0.5 mM $Ca(NO_3)_2$, 1.5 mM 2,2'-bipyridyl (pH 5.5) at 25°C. Tubes were covered with a cotton plug and N_2 was bubbled through the solution. After 5 min, 1 ml of 250 mM $Na_2S_2O_4$ was added from a syringe. The A_{520} of the solution (A_{520} of 1 mM Fe[bipyridyl]$_3$ = 8.650) was determined on 2 ml aliquots as reported by Bienfait, van den Briel and Mesland-Mul (1985). After removal of apoplastic Fe the amount of Fe and P accumulated in the same plant was determined. For this, roots and leaves were collected separately, carefully rinsed with distilled water and oven-dried at 55°C, ground to powder and mineralized in 1M HNO_3. Final volumes were adjusted to 1 mL by 0.1 M HNO_3. Fe was determined by ICP-OES (Sequential ICP-OES AX Liberty, Varian) and P by ICP-MS (Varian 820-MS).

2.2.7. *Fe reduction and acidification in vivo*

Visualization and localization of Fe reduction and acidification activities of the roots were performed using the agar technique as reported by

Donnini *et al.* (2009). Root segments were extensively washed until the pH in the solution was stable at pH 6.1 and embedded in 0.75% (w/v) agar containing 100 µM Fe(III)-EDTA and 100 µM bathophenanthroline disulfonate (BPDS) for reduction activity or 0.006% bromocresol purple (pH indicator) for acidification activity. The experiment was repeated three times with the same results.

2.2.8. *Fe reduction by phenolics exuded and accumulated in the roots*

The phenolics' ability to reduce Fe(III)-EDTA was measured spectrophotometrically using the BPDS reagent (Chaney, Brown and Tiffin 1972). 75-100 µg of phenolics prepared according to the method described above were incubated for 120 min in 1 ml of a solution containing 100 µM Fe(III)-EDTA and 100 µM BPDS in the dark at 26°C under shaking. The absorbance at 535 nm was determined as reported by Donnini et al. (2009). The experiment was repeated three times.

2.2.9. *Preparation of plasma membrane vesicles*

Roots of 9-d-old plants were excised, rinsed in distilled water and homogenized in a mortar at 2–4°C in a buffer containing 50 mM MOPS-BTP pH 7.5, 330 mM sucrose, 5 mM EDTA, 1 mg mL^{-1} bovine serum albumin (BSA), 5 mM dithiothritol (DTT), 2 g mL^{-1} PMSF, 10 % (w/v) diethyldithiocarbamic acid sodium salt (DIECA). After filtration through four layers of gauze, the homogenate was centrifuged at 13 000 *g* for 15 min and the supernatant centrifuged at 100 000 *g* for 30 min to obtain a microsomal fraction. The plasma membrane enriched fraction was prepared using the two-phase partitioning procedure as reported by Larsson, Sommarin and Widell (1994). The enrichment in plasma membrane was determined assaying vanadate-sensitive H^+-ATPase (EC 3.6.1.35) (PM), nitrate-sensitive H^+-ATPase (EC 3.6.1.3) (tonoplast) and azide-sensitive H^+-ATPase (EC 3.6.1.34) (mitochondria), respectively, according to Rabotti and Zocchi (1994).

2.2.10. *H^+-ATPase assay*

H^+-ATPase activity in plasma membrane preparations was determined in a medium with the following composition: 250 mM sucrose, 50 mM KCl, 25 mM MOPS-BTP pH 6.5, 1 mM ATP, 0.25 mM NADH, 1 mM PEP, 15 µg mL^{-1} lactate dehydrogenase (EC 1.1.1.27), 30 µg mL^{-1} pyruvate kinase (EC 2.7.1.40), 0.015% (w/v) Lubrol and 10–20 µg plasma membrane protein (omitted in blanks). The reaction was started by the addition of 1 mM $MgSO_4$. NADH oxidation was followed at 340 nm. Three independent experiments in triplicate (n=9) were performed.

2.2.11. *FC-R assay*

The NADH-dependent Fe(III)-reductase activity of plasma membrane preparations isolated from roots was determined at 26°C in a medium containing 250 mM sucrose, 15 mM Mops-BTP pH 7, 0.25 mM $K_3Fe(CN)_6$, 0.25 mM NADH and 0.01% (w/v) Lubrol and the reaction was started by the addition of 10-20 µg of plasma membrane protein (omitted in blanks). The absorbance change at 340 nm was monitored. Three independent experiments in triplicate (n=9) were performed.

2.2.12. *Soluble protein extraction and PEPC assay*

Roots of 9-d-old plants grown under the different treatments were harvested, rinsed and homogenized in a buffer (1 mL g^{-1} FW) containing 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10% (v/v) glycerol, 1 mM EDTA, 14 mM ß-mercaptoethanol, 1 mM PMSF and 10 µg mL^{-1} leupeptin. The homogenate was filtered through four layers of gauze and centrifuged at 13 000 *g* for 15 min and the supernatant was again centrifuged at 100 000 *g* for 30 min. Phospho*enol*pyruvate carboxylase (PEPC) (EC 4.1.1.31) was determined as in De Nisi and Zocchi (2000). The reaction was started by the addition of 20-50 µL of soluble fraction (omitted in blanks) and the absorbance change at 340 nm was monitored. Three independent experiments in triplicate (n=9) were performed.

2.2.13. *Western blot analysis of PEPC and H^+-ATPase*

Soluble and plasma membrane proteins (15 µg) were loaded on a discontinuous SDS-polyacrylamide gel (3.75% (w/v) acrylamide stacking gel and 8% (w/v) acrylamide separating gel). After SDS-PAGE, western blot analyses were performed as reported in De Nisi and Zocchi (2000) for PEPC and in Dell'Orto et al. (2000) for the H^+-ATPase, respectively. Two different antisera were used; one raised against the central domain of PM H^+-ATPase of *Arabidopsis thaliana* (a kind gift from Dr R. Serrano) and a second one raised against a PEPC isoform of sorghum (a kind gift from Dr J. Vidal). The experiment was repeated three times with the same result.

2.2.14. *Shikimate pathway enzyme extraction and assay*

For 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS, EC 4.1.2.15) 0.2 g fresh roots were homogenized in 100 mM potassium-phosphate buffer (pH 8.0) containing 1.4 mM ß-mercaptoethanol. The homogenate was centrifuged at 15 000 *g* for 15 min at 4 °C and DAHPS activity was assayed on soluble fraction as reported by Sánchez-Rodríguez *et al*. (2011).

Shikimate dehydrogenase (SDH) and shikimate kinase (SK) extraction were performed according to Díaz, Barceló and Merino (1997).

SDH (EC 1.1.1.25) was assayed at 25°C by monitoring the reduction of $NADP^+$ at 340 nm according to Chaudhuri and Coggins (1985). The assay mixture contained 20-50 µL of soluble fraction (omitted in blanks) in 100 mM Na_2CO_3 pH 10.6, 4 mM shikimic acid and 2 mM $NADP^+$.

SK (EC 2.7.1.71) was assayed at 25°C by coupling the release of ADP to the oxidation of NADH using pyruvate kinase (EC 2.7.1.40) and lactate dehydrogenase (EC 1.1.1.27) as coupling enzymes according to Krell *et al*. (2001). Shikimate-dependent oxidation of NADH was monitored at 340 nm. The assay mixture contained 20-50 µL of soluble fraction (omitted in blanks) in 50 mM triethanolamine hydrochloride/KOH buffer at pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, 1.6 mM shikimic acid, 5 mM ATP, 1 mM PEP, 0.1 mM NADH, 30 µg mL^{-1} pyruvate kinase, and 15 µg mL^{-1} lactate dehydrogenase.

The enzyme 5-enol-pyruvyl-shikimate-3-phosphate (EPSP) syntase (EC 2.5.1.19) was extracted and assayed as reported by Forlani, Nielsen and Racchi (1992). EPSP synthase activity was measured in a medium containing 100 mM Hepes NaOH pH 7.4, 1 mM shikimate-3-phosphate (S3P), 1 mM PEP, 0.5 mM ammonium heptamolybdate and 60 μL of enzyme. After incubation, the reaction was stopped by addition of a solution containing malachite green and samples were read at 660 nm against blanks in which S3P had been omitted.

Phenylalanine ammonia-lyase (PAL) (EC 4.3.1.5) was obtained by homogenizing 0.20 g fresh tissue in 15 mL of an extraction medium containing 20 mM ß-mercaptoethanol, 0.1 M sodium borate buffer pH 8.8 and 5% (w/v) PVPP. After filtration through four layers of gauze, the homogenate was centrifuged at 12 000 *g* for 20 min, 4 °C. The enzyme activity was determined by adding 1 mL of the protein extract (omitted in blanks) to a reaction medium according to Cahill and Mc Comb (1992). All the enzymes were assayed by three independent experiments in triplicate (n=9).

2.2.15. *Protein determination*

Protein content was determined by the Bradford (1976) procedure using BSA as a standard.

2.2.16. *Oxygen consumption*

Apical root segments (2 cm) were excised under water at room temperature from plants illuminated for several hours. Root O_2 consumption rates were measured from the decrease in O_2 concentration in an aqueous phase with a Clark-type O_2 electrode (YS1 Analytical control) at 25° C. Calibration was made from the difference in signal between aerated water and Na-dithionite saturated water. The effects of KCN (2mM) and salycilichydroxamic acid (SHAM) (2mM) were determined as reported by Vigani, Maffi and Zocchi (2009). Five replicates for each treatment were performed.

2.2.17. *Statistics*

All statistical analyses were conducted with Sigma-Stat® 3.1. Means were compared by *t* test at P< 0.05 in all cases.

2.3. **Results**

2.3.1. *Organic compounds in roots and exudates*

Table 1. Water and methanol soluble phenolics, malic acid and citric acid content in *Parietaria* roots and exudates. Plants were collected either from the field or from the hydroponic culture. Growth conditions are reported as in Materials and Methods section. Data are means ± S.D. (n = 5). In the case of significant differences (P<0.05) values are marked with different letters.

Sample	water-soluble phenolics (mg g^{-1} FW)	methanol-soluble phenolics (mg g^{-1} FW)	malic acid (μg g^{-1} FW)	citric acid (μg g^{-1} FW)
Parietaria in the field				
pH 7.19	3.54 ± 0.29 c	7.85 ± 0.17 b	596.91 ± 8.15 c	188.06 ± 5.37 b
pH 7.78	3.79 ± 0.14 b	8.17 ± 0.07 b	635.83 ± 5.27 b	224.30 ± 9.22 b
pH 8.3	4.40 ± 0.21 a	8.22 ± 0.18 a	696.96 ± 7.23 a	387.21 ± 10.15a
Parietaria in hydroponic				
+Fe (pH 6.2)	0.13 ± 0.02 c	0.17 ± 0.02 c	343.71 ± 11.46 c	75.05 ± 9.68 c
-Fe (pH 6.2)	0.39 ± 0.03 b	0.67 ± 0.05 b	370.22 ± 12.18 c	366.67 ± 4.31 b
+FeBic (pH 8.3)	0.93 ± 0.07 a	3.77 ± 0.03 a	1127.4 ± 29.74 a	623.98 ± 7.66 a
Exudates in hydroponic				
+Fe (pH 6.2)	0.028 ± 0.002 c	-	1.68 ± 0.21 c	0.33 ± 0.09 c
-Fe (pH 6.2)	0.033 ± 0.003 b	-	7.67 ± 0.48 a	5.89 ± 0.21 b
+FeBic (pH 8.3)	0.045 ± 0.005 a	-	5.17 ± 0.91 b	10.87 ± 0.29 a

Table 1 shows the results for *Parietaria* roots sampled both in the field and in hydroponic culture. Concerning the first ones, we observed a correlation between the increase in pH of the substrates surrounding roots and phenolics accumulation in the root. A similar trend was also observed for malic and citric acid concentrations, which increased in the

roots when the pH of the growing medium increased.

In *Parietaria* plants grown in hydroponic culture, the absence of Fe induced an accumulation of phenolics in the roots (about 3- and 4-fold when extracted in water and methanol, respectively). Also in the presence of bicarbonate, an increase in phenolics concentrations occurred in the roots (7- and 22-fold when extracted in water and methanol, respectively). Both malic and citric acid concentrations were found to increase (8% and 5-fold, respectively) as Fe in the hydroponic culture was removed. However, when bicarbonate was added to the +Fe solution, the concentrations of organic acids were significantly higher (3.3- and 8.3-fold increases for malic and citric acid, respectively). Concerning phenolics concentrations in the exudates, an increase in –Fe plants (+18%) and in plants grown in the presence of bicarbonate (+61%) was detected.

The malic acid concentration in exudates of plant exposed to –Fe condition was significantly higher than in plants grown in the presence of bicarbonate, whereas for citric acid the major increase was found in the +FeBic treatment (Table 1). With respect to the relative controls, the malic acid concentration increased by 4.6-fold and 3-fold in –Fe and +FeBic treatments respectively, whereas citric acid concentration increased by about 18-fold and 33-fold in –Fe and +FeBic, respectively.

2.3.2. *Biomass measurement*

Plant growth was influenced by the stress conditions and a significant decrease in shoot length was found both in the absence of Fe (-31%) and in the presence of bicarbonate (-55%). Root length also decreased when bicarbonate was added to the medium (-74%), while the –Fe treatment did not cause significant decreases in this parameter (Fig. 2A). The fresh weight (FW) of the shoot was reduced when plants were exposed to Fe deficiency (-26%) and bicarbonate (-40%). No significant differences were *found in root FW (Fig. 2B). The plant dry weight (DW) showed the same* trends (Fig. 2C).

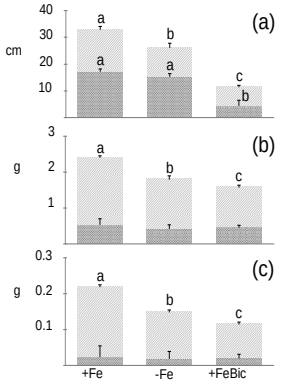

Figure 2. Shoot and root lenght (a), fresh weight (b) and dry weight (c). The clear part of the histogram corresponds to parameters determined on the shoots; the dark area indicates the parameters determined on the roots. Three independent experiments in triplicate (n=9) were performed. In the case of significant differences (P<0.05) values are marked with different letters.

2.3.3. *Leaf chlorophyll determination*

Fig. 3 shows the chlorophyll concentration expressed on a FW basis. The total lack of Fe resulted in a larger decrease in leaf chlorophyll concentration with respect to the bicarbonate supply (-34% and -20%, respectively).

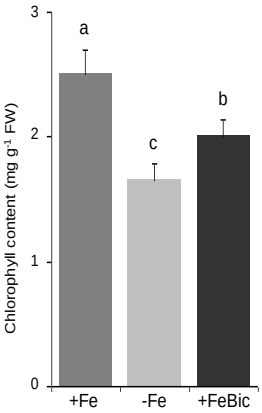

Figure 3. Change in the leaf chlorophyll concentration (mg g^{-1} FW) in plants grown for 9 days in control condition (+Fe), Fe deficiency (-Fe) and in the presence of bicarbonate (+FeBic). Data are the means ± S.D. (n = 5). In the case of significant differences (P<0.05) values are marked with different letters.

2.3.4. *Fe and P determination*

Table 2 reports the amount of apoplastic Fe in the roots from different growth conditions. The Fe trapped in the free space of roots grown in the presence of bicarbonate increased 4.9-fold, while in -Fe a decrease (-19%) was found. In the same plants, a decrease in Fe concentration was detected only in the tissues of –Fe roots, while at the shoot level the decrease was observed in both –Fe and +FeBic treatments (-70% and -53%, respectively). Concerning P concentration, a different trend was observed: while in the roots a slight decrease was found in absence of Fe and in the presence of bicarbonate (-17% and -26%, respectively), no significant difference was detected at the leaf level.

Table 2. Fe and P concentration (μmol g^{-1} DW) in leaves and roots of plants grown for 9 days in control condition (+Fe), iron deficiency (-Fe) and in the presence of bicarbonate (+FeBic). Data are means ± S.D. (*n*=5). In the case of significant differences (*P*<0.05) values are marked with different letters.

	Fe			**P**	
	shoot	root simplast	root apoplast	shoot	root
+Fe	4.44±0.23 a	1.13±0.09 a	0.80±0.03 b	503.14±32.25 a	518.41± 41.44 a
-Fe	1.34±0.11 c	0.86±0.14 b	0.65±0.01 c	499.32±17.52 a	430.08±26.17 b
+FeBic	2.08±0.19 b	1.09±0.11 ab	3.91±0.25 a	449.95±29.54 a	384.94±13.97 b

2.3.5. *Root Fe reduction and medium acidification in vivo*

In order to obtain a preliminary overview of the induction of Fe deficiency responses, the reduction and the acidification capacities were carried out *in vivo* by embedding the roots in agar (Fig. 4).

The reduction activity was detected by using BPDS, which forms a stable red complex only with Fe^{2+} (Römheld, Müller and Marschner 1984), indicating that reduction of Fe^{3+} has occurred. Fig. 4A shows that plants grown in presence of Fe did not develop any colour, and conversely in –Fe treated plants a high reduction activity was present, mainly localized in the apical and sub-apical zones (Fig. 4B). *Parietaria* plants grown with bicarbonate showed the formation of a red colour less intense than –Fe treated plants (Fig. 4C). However, in this treatment the reduction activity appeared to be more diffuse along the root length. Medium acidification was determined *in vivo* by the change in the colour of the pH indicator bromocresol purple and it is shown in Fig. 4D-F. The pictures reported in the figure were taken approximately 2 h after agar embedding and show an acidification activity only in –Fe and +FeBic conditions (Fig. 4E and 4F). It is noteworthy the difference with the localization of the reduction activity, since the acidification appeared more diffuse in the –Fe treated

roots and more localized in the roots of bicarbonate-treated plants, particularly in the zone where the proteoid-like roots were present.

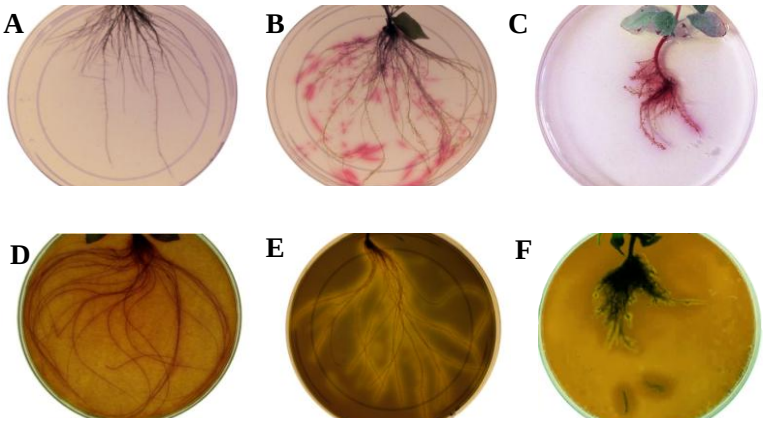

Figure 4. Visualization of Fe reduction (A, B, C) and acidification (D, E, F) activities of the whole root system. Roots were embedded in 0.75% agar medium and the reduction was determined, after about two hours, as the Fe^{2+}-$(BPDS)_3$ complex formation (red), whereas the acidification was detected as pH changes of the indicator bromocresol purple (yellow). From left: 9-day-old root grown under control condition (A, D), Fe deficiency (B, E), and +FeBic treatment (C, F). The experiment was repeated three times with similar results.

2.3.6. *Fe reduction ability by phenolics*

Table 3 shows the results obtained testing the Fe reduction ability by both exuded and root accumulated phenolics. In particular, even if an activity was detected for all the root extracts, higher values were found for the +FeBic treatment. Regarding the exuded phenolics, an increase in Fe reduction ability was detected for both –Fe (+17%) and +FeBic (+32%) with respect to the control.

Table 3. Fe(III) reduction activity by phenolics extracted from *Parietaria* roots and plant exudates. Reduction is expressed as μmol Fe mg^{-1} phenolics h^{-1}. Data are means ± S.D. (n = 3). In the case of significant differences (P<0.05) values are marked with different letters.

	Root extracts		**Plant exudates**
	Water-soluble	Methanol-soluble	
+Fe	0.10 ± 0.01 b	0.15 ± 0.02 b	122.12 ± 6.54 c
-Fe	0.09 ± 0.01 b	0.18 ± 0.06 b	142.91 ± 3.28 b
+FeBic	0.34 ± 0.03 a	0.36 ± 0.11 a	161.57 ± 3.81 a

2.3.7. *Strategy I response activities in plasma membrane preparations and root soluble extracts*

The assay of marker enzyme activities (vanadate-sensitive, nitrate-sensitive and azide-sensitive ATPases) carried out on plasma membrane preparations isolated from roots of differently treated plants indicate that the H^+-ATPase activity was scarcely sensitive to nitrate and azide (less than 6% inhibition), while it was almost completely inhibited by vanadate (ca. 90%), indicating an enrichment in plasma membrane in the preparations used (Table 4).

On these enriched preparations we have analyzed the Fe^{3+}-chelate reductase and the H^+-ATPase activities (Table 4). Both Fe-deficiency conditions induced an increase in FC-R activity in plasma membrane preparations from roots of *Parietaria diffusa*; in particular, in –Fe treatment the enhancement was approximately 2.3-fold with respect to control plants, while in the presence of bicarbonate the increase in FC-R activity was only approximately +51%. The H^+-ATPase activity showed the same trend: both –Fe and +FeBic treatments showed an increased activity with respect to the control, but lower for the bicarbonate treatment (+95% and +45%, respectively).

Table 4. Enzymatic activities of typical Strategy I responses in root plasma membrane preparations and root soluble extracts from control (+Fe), Fe-deficient (-Fe) and bicarbonate (+FeBic) treated plants. For the H^+-ATPase, the marker enzyme activities in plasma membrane-enriched fractions are reported. Data are the means ± S.D. (*n*=9). In the case of significant differences (*P*<0.05), values are marked with different letters.

Enzymes	+Fe	-Fe	+FeBic
H^+-ATPase nmol $min^{-1}mg^{-1}$ prot	237.92 ± 12.31 c	463.09 ± 32.86 a	344.01 ± 28.65 b
Vanadate-sensitive	218.18 ± 3.04	418.35 ± 2.17	324.63 ± 15.01
Nitrate-sensitive	14.30 ± 2.33	24.45 ± 1.92	21.70 ± 3.15
Azide-sensitive	7.16 ± 0.48	12.96 ± 0.47	5.84 ± 0.29
FC-R nmol min^{-1} mg^{-1} prot	189.96 ± 16.21 c	435.59 ± 22.17 a	287.03 ± 16.04 b
PEPC nmol min^{-1} mg^{-1} prot	79.93 ± 11.96 b	138.71 ± 13.18 a	56.06 ± 7.91 c

PEPC activity in root soluble extracts was differently affected in plants grown under Fe absence and bicarbonate supply conditions. In fact, an increase (+74%) in PEPC activity was observed only in -Fe, while in the presence of bicarbonate supply a slight decrease was induced (-30%) (Table 4).

Western blot analysis revealed an increased level of the plasma membrane H^+-ATPase protein in plants grown under both stress conditions. However, the increase was more evident under Fe-free nutrient solution than under bicarbonate supply, confirming the results obtained both *in vivo* and *in vitro* (cfr. Fig. 4 and Table 4).

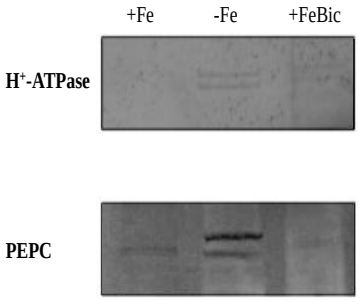

Figure 5. Western blot analysis of H^+-ATPase and PEPC carried out on root plasma membrane preparations and root soluble extracts from plants grown for 9 days in control conditions (+Fe), Fe deficiency (-Fe) and in the presence of bicarbonate (+FeBic), respectively. Plasma membrane or soluble proteins (15 μg) were loaded on a discontinuous SDS-polyacrylamide gel (8%). After SDS-PAGE, proteins were electrophoretically transferred to PVDF membrane filters and two different antisera were used; one raised against the central domain of PM H^+-ATPase of *Arabidopsis thaliana* (1:10 000) and the second one raised against a PEPC isoform of sorghum (1:1000). The experiment was repeated three times with the same results.

Polyclonal antibodies raised against sorghum PEPC were also used to assess the presence of this enzyme in root soluble extracts and to evaluate the expression level under stress conditions. The Western blot results are shown in Fig. 5. In the control lane the antibody reacted only against a polypeptide with an apparent molecular mass of 103 kDa.

However, in Fe-deficient condition two polypeptides corresponding to 103 and 108 kDa, respectively, were detected, in agreement with previous studies (De Nisi and Zocchi 2000). No appreciable bands were evident in the lane corresponding to root soluble extracts grown under bicarbonate supply. These results are in good agreement with the activities presented in Table 4.

2.3.8. *Shikimate pathway enzymes*

Since shikimic acid is a precursor in the biosynthesis of aromatic compounds, and consequently of phenolics, some enzymatic activities with a key role in the shikimate pathway were assayed (Fig. 6). In comparison with the control, Fe deficiency, especially when induced by bicarbonate, led to a significant increase in the activity of these enzymes. DAHPS, which is the key enzyme controlling the carbon flow towards phenolics metabolism, increased significantly when plants were

grown in the presence of bicarbonate (+33% with respect to the control). The same was true for SDH activity (+41%) in agreement with the fact that plants grown under bicarbonate were characterized by a higher phenolics content. The SK activity, which converts the SDH's product in 3-phospho-shikimate, was affected in both Fe-deficient conditions (Fig. 6). The activity of EPSP synthase in extracts from –Fe roots did not significantly differ from that found in the control (Fig. 6). On the contrary, in presence of bicarbonate, the EPSP synthase activity increase about 3-fold with respect to the +Fe treatment.

Finally, the PAL activity, the first enzyme in the phenylpropanoid biosynthetic pathway, was also increased significantly in both stress conditions, but more in +FeBic (+38% and +92%, respectively).

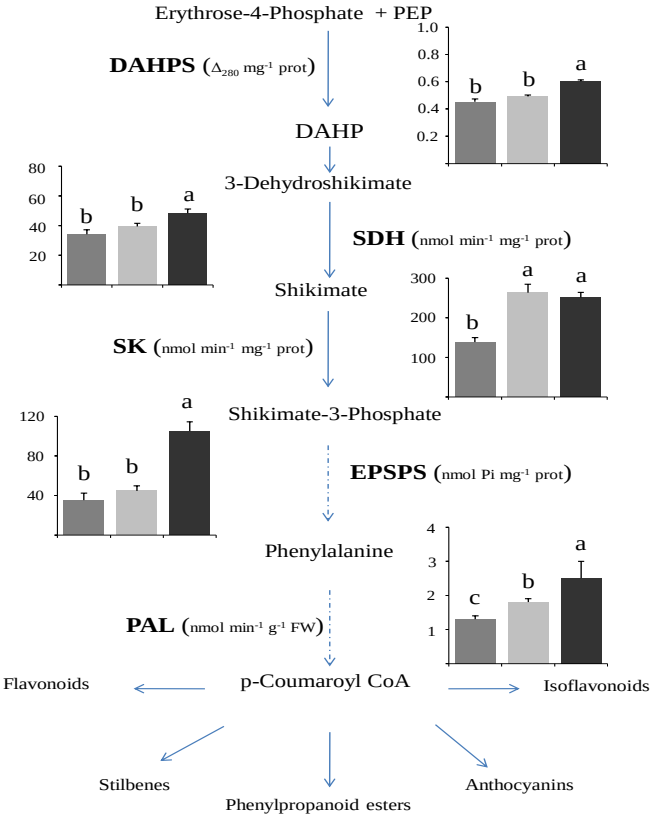

Figure 6. Enzyme activities of shikimate pathway in extracts of roots from control (+Fe), Fe-deficient (-Fe) and bicarbonate (+FeBic) treated plants. Data are the means ± S.D. (*n*= 9). In the case of significant differences ($P<0.05$) values are marked with different letters. DAHPS, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; SDH, shikimate dehydrogenase; SK, shikimate kinase; EPSPS, 5-enol-pyruvyl-shikimate-3-phosphate syntase; PAL, phenylalanine ammonia-lyase.

2.3.9. *Oxygen consumption*

In order to obtain an overview of mitochondrial activity, the O_2 consumption rate of apical root segments was determined. Fig. 7 shows that O_2 consumption rate decreases in roots of Fe-deficient (-40%) with respect to those from Fe sufficient and bicarbonate treated plants. After KCN addition, the total O_2 consumption decreased by -17 % and -32% in control and +FeBic roots, respectively, whereas in the absence of Fe the decrease was larger, -57%. The presence of SHAM, which inhibits the alternative oxidase, caused a greater decrease in the consumption of O_2 in control and +FeBic conditions (-91% and -94%, respectively), while in -Fe roots decrease was ca. 100% (Fig. 7).

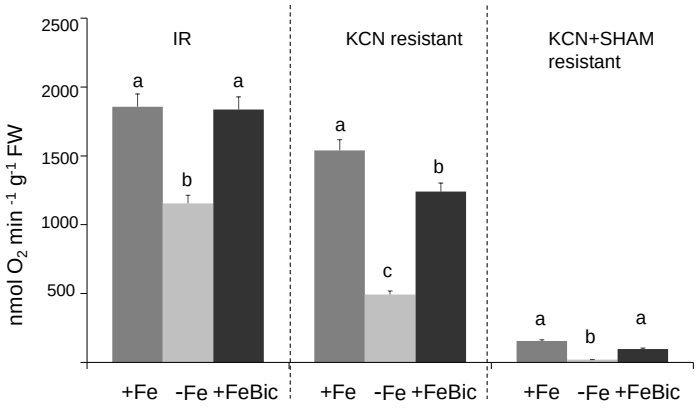

Figure 7. Oxygen consumption rate in excised apical root segments from plants grown for 9 days in control condition (+Fe), Fe deficiency (-Fe) and in the presence of bicarbonate (+FeBic). Data are the means ± S.D. (n = 5). In the case of significant differences ($P<0.05$) values are marked with different letters. IR, initial rate; SHAM, salycilichydroxamic acid.

2.4. **Discussion**

A limited nutrient availability generally affects plant growth more than photosynthesis, enabling the allocation of photosyntates to the production of C-based secondary metabolites, such as phenolics (Leradau and Coley 2002). Accordingly, when root phenolics were quantified in *Parietaria* plants collected directly from the walls (Table 1), a correlation between their amount and the pH of the substrates was found. These data might suggest that plants respond to the pH variation through metabolic rearrangement, modifying the allocation of carbon skeletons between primary and secondary metabolism. Interestingly, in the same roots a simultaneous increase in the organic acid concentration occurs (Table 1); these compounds are reported to be accumulated and exuded not only under Fe deficiency, but also under other nutrient deficiencies (Lin *et al*. 2011; Widodo *et al*. 2010).

Subsequently, to investigate growth and physiological behaviour particularly concerning Fe uptake, rooted cuttings of *Parietaria* were grown in hydroponic medium and submitted to direct (-Fe) or induced Fe deficiency in the presence of bicarbonate (+FeBic). This last condition would mimic the natural environment where plants grow showing a stable phenotype. In fact, while the absence of Fe resulted in a significant decrease in the chlorophyll concentration causing the classical symptoms of chlorosis, in the presence of bicarbonate chlorophyll was less affected, showing weak or no symptoms of chlorosis (cfr. Fig. 1 and Fig. 3). However, under the latter condition a more significant decrease in shoot growth was observed, probably as an adaptive strategy to maintain an adequate level of Fe (and chlorophyll, as well) in the tissues (Fig. 2 and Table 2), preserving the photosynthetic activity as it has already been suggested for pear plants (Donnini *et al*. 2009). At root level, the -Fe condition induced the proliferation of lateral roots (Moog *et al*. 1995; Dasgan *et al*. 2002; Jin *et al*. 2008), while the bicarbonate supply induced a shorter root system, with the appearance of structures similar to “proteoid roots” (Purnell 1960), maintaining the same root mass as the control plants. Proteoid roots, developing from secondary and tertiary lateral roots, provide an enhanced surface of contact between plant and soil which is useful for the release of nutrient-solubilising compounds and

for the uptake of nutrients from the rhizosphere (Dell'Orto *et al*. 2003; Lambers *et al*. 2003). The presence of such structures only in plants grown with bicarbonate suggests that this should not be a specific response to Fe deficiency but to a more general condition of low nutrient availability: indeed P deficiency has been found to be induced in calcareous soils as well (Guerinot & Yi 1994). In 9-d-old *Parietaria* we found a slightly decrease in P concentration in both Fe deficiency conditions (Table 2).

Preliminary findings obtained by testing *Parietaria* in the field prompted us to investigate both the accumulation and exudation of organic compounds. In particular, as suggested by Jin *et al*. (2007), phenolics could play an important role in the mobilization of Fe accumulated in the highly negatively charged cell wall, probably through chemical chelation and reduction operated by phenolics themselves. We found an increase in the accumulated and exuded phenolics in both conditions of Fe deficiency that was higher in the presence of bicarbonate. However, the release of phenolics into the exudates in these conditions, which has been attributed mainly to proteoid roots tissues (Weisskopf *et al*. 2006), appears to be scarce when compared with the amount accumulated in the roots (Table 1). Table 3 shows the capacity of these phenolics to reduce Fe(III)-EDTA *in vitro*, demonstrating that they could effectively influence the reduction and then the mobility of Fe-chelates both in the apoplast and in the external medium. The major Fe reduction efficiency detected under bicarbonate supply might be ascribed to relative abundance of specific compounds (results not shown), since the reducing capacity has been attributed to the presence of particular groups or double bonds in the molecular structure (Bors *et al*. 1990).

The analysis of some key enzymes of the shikimate pathway shows a good correlation between the concentration of phenolics and an increase in the activities of several enzymes in their biosynthetic pathway. In fact, an enhanced amount of phenolic compounds, as well as increases in the activities of DAHP synthase, SDH, SK and PAL, was found in both Fe-deficient conditions, and the increase was larger under the bicarbonate treatment with the exception of SK (see Table 1 and Fig. 6). These results are also in agreement with data by Lan et al. (2011) who find a robust

increase in the amount of enzymes linked to the phenylpropanoid pathway induced by Fe deficiency. In the presence of bicarbonate, we found also an increase in the EPSP synthase, the sixth enzyme of the prechorismate pathway, whose over-expression has been associated to the tolerance to the herbicide glyphosate, which blocks the synthesis of aromatic compounds (Amrhein *et al*. 1983; Comai, Sen and Stalker 1983; Reinbothe, Nelles and Parthier 1991). Interestingly, resistance to glyphosate has been reported in *Parietaria* (Protopapadakis 1985).

When organic acids are measured in both roots and exudates, a significant increase was detected in roots grown under both stress conditions. Under Fe, but also P deficiency, organic acid excretion would increase plant efficiency, through a decrease in the pH of the medium and consequently a solubilisation of Fe and P. Our data show that in the presence of bicarbonate, citric acid is the main exuded organic acid (Table 1), as already reported in other species (Massonneau *et al*. 2001; Jelali *et al*. 2010). On the other hand, it has been reported that malate is poor at mobilising micronutrients from the soil, whilst citrate is capable of mobilising significant quantities (Jones and Darrah 1994). Furthermore, Langlade *et al*. (2002) reported that the ratio of malate to citrate is higher only in the apex and juvenile proteoid roots.

P. diffusa, a Strategy I plant species (Dell'Orto *et al*. 2003), responds to Fe deficiency by inducing the FC-R and H^+-ATPase activities (Fig. 4B and 4E and Table 4), the last one attributable to an increase in the protein expression (Fig. 5). An increase in Strategy I enzymatic activities were also observed in +FeBic, although to a minor extent, which could be attributed to the presence of Fe in the medium, that might in part satisfy the plant requirements. Interestingly, the results obtained *in vivo* show that when roots are grown with bicarbonate, the reduction activity appears widely distributed along the roots (Fig. 4). We suggest that for this treatment, the FC-R activity measured in the plasmalemma fraction and known to be localized mainly at apical and sub apical root zones (see –Fe treatment) could be only a part of the total Fe reduction activity detected *in vivo*; the release of reductants (e.g. phenolics) could be responsible for the activity which appears to be present along the whole root length.

In the present work, an increase in the PEPC activity was observed only in –Fe; surprisingly, the bicarbonate supply in the presence of Fe induces a decrease in this activity and the protein expression in root soluble extracts shown by Western blot analysis confirms these results (Fig. 5). Another intriguing result obtained in *Parietaria* is the different presence of polypeptides reacting with the antibody. In fact, only in the absence of Fe we found both the 103 and 108 KDa as reported in the PEPC literature (see De Nisi and Zocchi 2000 and references therein). Phospho*enol*pyruvate (PEP) is a substrate for the reactions catalyzed by both PEPC and DAHP synthase. We suggest that in the presence of bicarbonate, the PEP produced by glycolysis could be in part channelled into the shikimate pathway and converted into phenolics, which are abundant in this condition.

In a mural habitat, the pH buffered to 7.2-8.3 causes precipitation of Fe along with other nutrients. In this condition the Strategy I activities might become inadequate to assure Fe supply if not by means of an extreme effort by H^+-ATPase activity to counteract the buffering effect of bicarbonate. In this case, the Fe pool stored in the root apoplast could represent a valid extracellular reserve; the more a plant will be able to mobilize such pools, the more it will be competitive in these growing conditions. We show that *P. diffusa* responds to the presence of bicarbonate through an *over*production of phenolics and organic acids. Furthermore, data obtained through an experimental approach in hydroponic culture, suggest that this behaviour could be related to a metabolic shift involving particularly the PEP-consuming reactions. In the absence of Fe, *Parietaria* would need more energy for the activation of the Strategy I responses. However, in this condition, the primary source of energy (mithochondrion) is impaired (Fig. 7, see also Vigani, Maffi and Zocchi 2009). It has been shown that in Fe-deficient cucumber roots the rate of glycolysis and the PEPC activity are increased to produce the substrates useful to sustain FC-R and H^+-ATPase activities (Espen *et al*. 2000; De Nisi and Zocchi 2000; Zocchi 2006). The PEP produced could then be addressed to the sustenance of biosynthesis of both phenolics and organic acids. In the presence of bicarbonate, Fe is present in the external and apoplastic solution even if in unavailable forms, and the

metabolic choice of *Parietaria* might be the activation of the shikimate pathway to produce phenolics, useful to Fe desorption from the cell wall. In the presence of Fe and bicarbonate, the mitochondrial electron transport chain is unaffected in comparison with the control (Fig. 7), remaining the primary mechanism to produce energy; furthermore, since the mitochondria are not significantly affected, the TCA cycle could better produce organic acids, explaining the increase in these compounds seen in this condition.

The diversion from primary metabolism for a part of the PEP pool is in agreement with the decrease in the PEPC activity observed in the presence of Fe and bicarbonate. This enzyme is negatively regulated by L-malate (Chollet, Vidal and O'Leary 1996), whose concentration increases under this condition and, as proposed by a recent work (De María *et al.*, 2006), by shikimate, which has been shown to exert a putative inhibitory effect on the PEPC, reinforcing the hypothesis.

In conclusion, while *Parietaria* grown in the absence of Fe shows metabolic responses similar to other Strategy I plants growing in the same condition, under +FeBic this species is able to diversify its response by increasing alternative pathways for Fe solubilisation. In calcareous soils, where Fe is present but scarcely soluble and more likely "trapped" in the cell wall, the aptitude of the secondary metabolism to assume a relevant role could be responsible for the *Parietaria*'s exceptional efficiency. We summarize the results of this and previous studies (Dell'Orto *et al.* 2003) by proposing a model for metabolic rearrangement in *Parietaria* (Fig. 8).

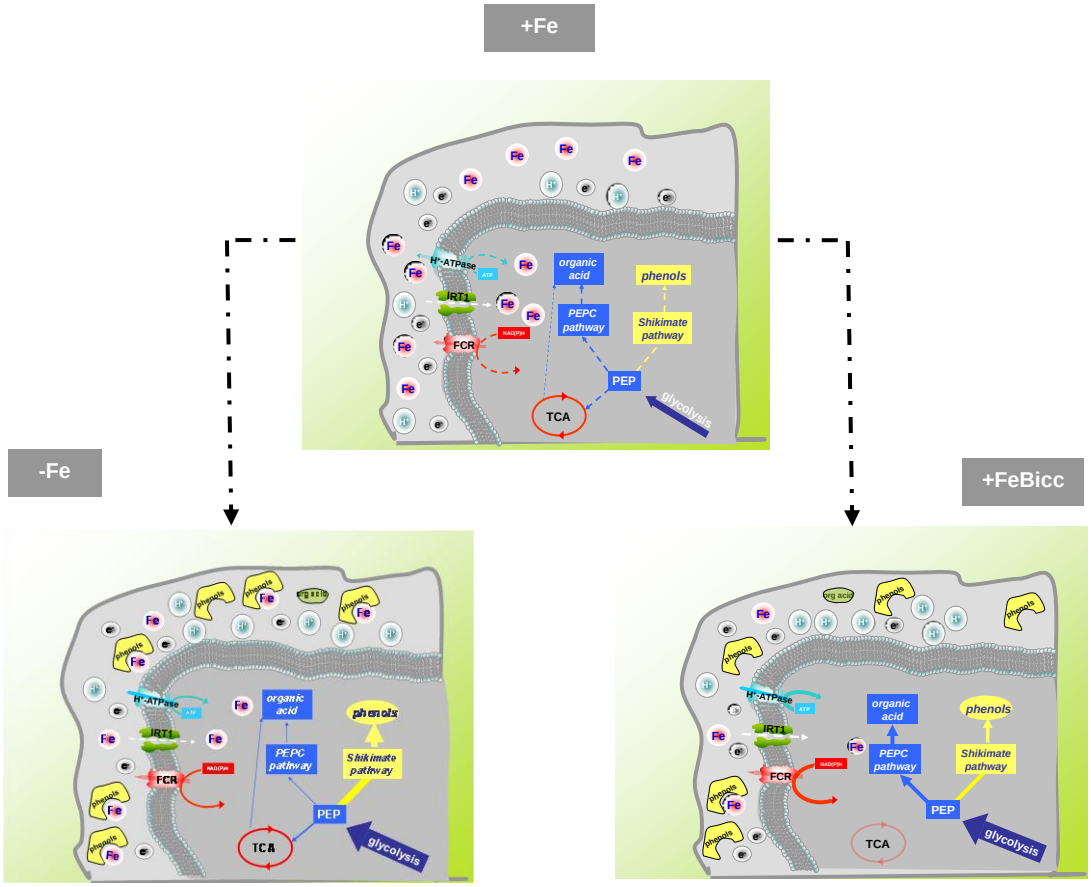

Figure 8. Schematic representation of metabolic changes occurring in roots of *P. diffusa* growing in control conditions (+Fe), Fe deficiency (-Fe) and in the presence of bicarbonate (+FeBic). Dotted lines indicate the metabolic pathways and Strategy I activities in the control condition (+Fe). In –Fe and +FeBic panels, the arrow thickness would indicate the change in the metabolic flux and Strategy I activities. FC-R, Fe^{3+}-chelate reductase; IRT1, Iron Transporter 1; PEP, phospho*enol*pyruvate; PEPC, phospho*enol*pyruvate carboxylase.

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References

Abadía J., Vázquez S., Rellán- Álvarez R., El-Jendoubi H., Abadía A., Álvarez-Fernández A. and López-Millán A.F. (2011) Towards a knowledge-based correction of iron chlorosis. *Plant Physiology and Biochemistry* **49**, 471-482.

Amrhein N., Joharming D., Schab J. and Schulz A. (1983) Biochemical basis for glyphosate-tolerance in a bacterium and a plant tissue culture. *FEBS letters* **157**, 191-196.

Bienfait H.F., van den Briel W. and Mesland-Mul N.T.(1985) Free space iron pools in roots. Generation and mobilization. *Plant Physiology* **78**, 596-600.

Bors W., Heller W., Michel C. and Saran M. (1990). Flavonoids as antioxidants: determination of radical scavenging efficacies. *Methods in Enzymology* **186,** 343-355.

Bradford M.M. (1976) A rapid and sensitive method for the quantization of micrograms quantities of protein utilizing the principle of protein-dye binding. *Anaytical Biochemisty* **72**, 248–254.

Cahill D.M. and Mc Comb J.A. (1992) A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of *Eucalyptus calophylla* (field resistant) and *E. marginata* (susceptible) when infected with *Phytophthora cinnamomi*. *Physiological and Molecular Plant Pathology* **40**, 315–332.

Cesco S., Neumann G., Tomasi N., Pinton R. and Weisskopf L. (2010) Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. *Plant and Soil* **329,** 1-25.

Chaney R.L., Brown J.C. and Tiffin L.O. (1972) Obligatory reduction of ferric chelates in iron uptake by soybeans. *Plant Physiology* **50,** 208-213.

Chaudhuri S. and Coggins J.R. (1985) The purification of shikimate dehydrogenase from *Escherichia coli*. *Biochemestry* **226**, 217–223.

Chen Y. and Barak P. (1982) Iron nutrition of plants in calcareous soils. *Advances in Agronomy* **35**, 217-240.

Chollet R., Vidal J. and O'Leary M.H. (1996) Phospho*enol*pyruvate carboxylase: a ubiquitous, highly regulated enzyme in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47,** 273-298.

Comai L., Sen L.C. and Stalker D.M. (1983) An altered aroA gene-product confers resistance to the herbicide glyphosate. *Science* **221**, 370-371.

Curie C. and Briat J.F. (2003) Iron transport and signaling in plants. *Annual Review of Plant Biology* **54,** 183-206.

Dasgan H.Y., Römheld V., Cakmak I. and Abak K. (2002) Physiological root resposes of iron deficiency susceptible and tolerant tomato genotypes and their reciprocal F1 hybrids. *Plant and Soil* **241,** 97-104

De María N., Becerril J.M., García-Plazaola J.I., Hernández A., De Felipe M.R. and Fenández-Pascual M. (2006) New insights on glyphosate mode of action in nodular metabolism: role of shikimate accumulation. *Journal of Agricultural and Food Chemistry* **54,** 2621–2628.

De Nisi P. and Zocchi G. (2000) Phosphoenolpyruvate carboxylase in cucumber (*Cucumis sativus* L.) roots under iron deficiency: activity and kinetic characterization. *Journal of Experimental Botany* **352,** 1903–1909.

Dell'Orto M., Santi S., De Nisi P., Cesco S., Varanini Z., Zocchi G. and Pinton R. (2000) Development of Fe deficiency responses in cucumber (*Cucumis sativus* L.) roots: involvement of plasma membrane H^+-ATPase activity. *Journal of Experimental Botany* **51**, 695-701.

Dell'Orto M., De Nisi P., Pontiggia A. and Zocchi G. (2003) Fe deficiency responses in *Parietaria diffusa*: a calcicole plant. *Journal of Plant Nutrition* **26**, 2057-2068.

Diáz J., Barceló R. and Merino F. (1997) Changes in shikimate dehydrogenase and the end products of the shikimate pathway, chlorogenic acid and lignins, during the early development of seed lings of *Capsicum annuum*. *New Phytologist* **136**, 183–188.

Donnini, S., Castagna A., Ranieri A. and Zocchi G. (2009) Differential responses in pear and quince genotypes induced by Fe deficiency and bicarbonate. *Journal of Plant Physiology* **166,** 1181-1193.

Donnini S., Prinsi B., Negri S., Vigani G., Espen L. and Zocchi G. (2010) Proteomic characterization of iron deficiency responses in *Cucumis sativus* L. roots *BMC Plant Biology* **10,** 268.

Espen L., Dell'Orto M., De Nisi P. and Zocchi G. (2000) Metabolic responses in cucumber (*Cucumis sativus* L.) roots under Fe-deficiency: a ^{31}P-nuclear magnetic resonance in-vivo study. *Planta* **210,** 985-992.

Forlani G., Nielsen E. and Racchi M.L. (1992) A glyphosate-resistant 5-enol-pyruvil-shikimate-3-phosphate synthase confers tolerance to a maize cell line. *Plant Science* **85,** 9-15.

Gries D., Brunn S., Crowley D.E. and Parker D.R. (1995) Phytosiderophore release in relation to micronutrient metal deficiencies in barley. *Plant and Soil* **172**, 299–308.

Guerinot M.L. (2000) The ZIP family of metal transporters. *Biochimica Biophysica Acta* **1465**, 190-198.

Guerinot M.L. & Yi Y. (1994) Iron: nutritious, noxious, and not readily available. *Plant Physiology* **104**, 815-820.

Henriques R., Jásik J., Klein M., Martinoia E., Feller U., Schell F., Pais M.S. and Koncz C. (2002) Knock-out of *Arabidopsis* metal transporter gene *IRT1* results in iron deficiency accompanied by cell differentiation defects. *Plant Molecular Biology* **50(4-5)**, 587-597.

Jelali N., M'Sehli W., Dell'Orto M., Abdelly C., Garsalli M. and Zocchi G. (2010) Changes of metabolic responses to direct and induced fe deficiency of two *Pisum Sativum* cultivars. *Environmental and Experimental Botany* **68,** 238-246.

Jin C.W., You G.Y., He Y.F., Tang C., Wu P. and Zheng S.J. (2007) Iron deficiency-induced secretion of phenolics facilitates the reutilization of root apoplastic iron in red clover. *Plant Physiology* **144**, 278-285.

Jin C.W., Chen W.W., Meng Z.B. and Zheng S.J. (2008) Iron-deficiency-induced increase of root branching contributes to the enhanced root ferric chelate reductase activity. *Journal of Integrative Plant Biology* **50**, 1557-1562.

Jones D.L. and Darrah P.R. (1994) Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. *Plant and Soil* **166**, 247-257.

Krell T., Maclean J., Boam D.J., Cooper A., Resmini M., Brocklehurst K., Kelly S.M., Price N.C., Lapthorn A.J. and Coggins J.R. (2001) Biochemical and X-ray crystallographic studies on shikimate kinase: the important structural role of the P-looplysine. *Protein Science* **10**, 1137–1149.

Lambers H., Cramer M.D., Shane M.W., Wouterlood M., Poot P. and Veneklaas E.J. (2003) Structure and functioning of cluster roots and plant responses to phosphate deficiency. *Plant and Soil* **248**, 43-59.

Lan P., Li W., Wen T-N., Shiau J-Y., Wu Y-C., Lin W. and Schmidt W. (2011) iTRAQ protein profile analysis of Arabidopsis roots reveals new aspects critical for iron homeostasis. *Plant Physiology* **155**, 821-834.

Landsberg E.C. (1996) Hormonal regulation of iron-stress response in sunflower roots: a morphological and cytological investigation. *Protoplasma* **194**, 69-80.

Langlade N.B., Messerly G., Weisskopf L., Plaza S., Tomasi N., Smutny J., Neumann G., Martinoia E. and Massonneau A. (2002) ATP citrate lyase: cloning, heterologous expression and possible implication in root organic acid metabolism and excretion. *Plant, Cell and Environment* **25**, 1561-1569.

Larsson C., Sommarin M. and Widell S. (1994) Isolation of highly purified plat plasma membranes and separation of inside-out and right-side-out vesicles. *Methods of Enzymology* **228**, 451-469.

Lerdau M. and Coley P.D. (2002) Benefits of the carbon-nutrient balance hypothesis. *Oikos* **98,** 534-536.

Li L., Cheng X. and Ling H.Q. (2004) Isolation and characterization of Fe(III)-chelate reductase gene LeFRO1 in tomato. *Plant Molecular Biology* **54**, 125-136.

Lichtenthaler H.K. (1987) Chlorophyll and carotenoids: pigments of photosynthetic biomembranes. *Methods of Enzymology* **148**, 350–82.

Lin Z.H., Chen L.S., Chen R.B., Zang F.Z., Jiang H.X., Tang N. and Smith B.R. (2011) Root release and metabolism of organic acids in tea plants in response to phosphorus supply. *Journal of Plant Physiology* **168**, 644-652.

Lindsay W.L. (1984) Soil and plant relationships associated with iron deficiency with emphasis on nutrient interactions. *Journal of Plant Nutrition* **7**, 489-500.

López-Millán A.F., Morales F., Abadia A. and Abadia J. (2000) Effects of iron deficiency on the composition of the leaf apoplastic fluid transport. *Plant Physiology* **124**, 873–884.

López-Millan A..F, Morales F., Gogorcena Y., Abadia A. and Abadia J. (2009) Metabolic responses in iron deficient tomato plants. *Journal of Plant Physiology* **166,** 375-384

Massonneau A., Langlade N., Leon S., Smutny J., Vogt E., Neumann G. and Martinoia, E. (2001) Metabolic changes associated with cluster root development in white lupin (*Lupinus albus* L.): relationship between organic acid excretion, sucrose metabolism and energy status *Planta* **213,** 534–542.

Moog P.R., van der Kooij T.A., Bruggemann W., Schiefelbein J.W. and Kuiper P.J. (1995) responses to iron deficiency in Arabidopsis thaliana: the turbo iron reductase does not depend on the formation of root hairs and transfer cells. *Planta* **195**, 505-513.

Morrissey J. and Guerinot M.L. (2009) Iron uptake and transport in plants: the good, the bad, and the ionome. *Chemical Review* **109**, 4553-4567.

M'sehli W., Dell'Orto M., Donnini S., De Nisi P., Zocchi G., Abdelly C. and Gharsalli M. (2009) Variability of metabolic responses and antioxidant defence in two lines of *Medicago ciliaris* to Fe deficiency. *Plant and Soil* **320**, 219-230.

Nelson S.D. (1992) Response of several wildland shrubs and forbs of arid regions to iron-deficiency stress. *Journal of Plant Nutrition* **15**, 2015-2023.

Palmgren M.G. (2001) Plasma membrane H^+-ATPases: powerhouses for nutrient uptake. *Annual Review of Plant Biology* **52**, 817-845.

Protopapadakis E. (1985) Changement de la flore adventice des vergers d'agrumes en Crète sous la pression du dèsherbage chimique. *Agronomie* **5**, 833-840.

Purnell H.M. (1960) Studies of the family Proteaceae. 1. Anatomy and morphology of the roots of some Victorian species. *Australian Journal of Botany* **8**, 38-50.

Rabotti G. and Zocchi G. (1994) Plasma membrane-bound H^+-ATPase and reductase activities in Fe deficient cucumber roots. *Physiologia Plantarum* **90**, 779-785.

Rabotti G., De Nisi P. and Zocchi G. (1995) Metabolic implications in the biochemical responses to iron deficiency in cucumber (*Cucumis sativus* L.) roots. *Plant Physiology* **107**, 1195–1199.

Reinbothe S., Nelles, A. and Parthier, B. (1991) N-(Phosphonomethyl) glycine (glyphosate) tolerance in *Euglena gracilis* acquired by either overproduced or resistant 5-*enol*pyruvylshikimate-3-phosphate synthase. *European Journal of Biochemistry* **198**, 365–373.

Robinson N.J., Procter C.M., Connolly E.L. and Guerinot M.L. (1999) A Ferric-chelate reductase for iron uptake from soils. *Nature* **397**, 694-697.

Römheld V. and Marschner H. (1986) Evidence for a specific uptake system for iron phytosiderophores in roots of grasses. *Plant Physiology* **80**, 175-180.

Römheld V., Müller C. and Marschner H. (1984) Localization and capacity of proton pumps in roots of iron. *Plant Physiology* **76**, 603-606.

Sánchez-Rodríguez E., Moreno D. A., Ferreres F., del Mar Rubio-Wilhelmi M., Ruiz J. M. (2011) Differential responses of five cherry tomato varieties to water stress: changes on phenolic metabolites and related enzymes. *Phytochemistry* **72**, 723–729.

Santi S. and Schmidt W. (2009) Dissecting iron deficiency-induced proton extrusion in Arabidopsis roots. *New Phytologist* **183**, 1072-1084.

Schmidt W. and Bartels M. (1996) Formation of root epidermal transfer cells in *Plantago*. *Plant Physiology* **110**, 217-225.

Schmidt W. and Bartles M. (1998) Orientation of NADH-linked ferric chelate (turbo) in plasma membranes from roots of *Plantago lanceolata*. *Protoplasma* **203**, 186-193.

Swain T. and Hillis W.E. (1959) The phenolic constituents of *Prunus domestica*. 1. The quantitative analysis of phenolic constituents. *Journal of the Science of Food and Agriculture* **10**, 63–68.

Tomasi N., Weisskopf L., Renella G., Landi L., Pinton R., Varanini Z., Nannipieri P., Torrent J., Martinoia E. and Cesco S. (2008) Flavonoids of white lupin roots partecipate in phosphorous mobilization from soil. *Soil Biology and Biochemistry* **40**, 1971-1974.

Varotto C., Maiwald D., Pesaresi P., Jahns P., Salamini F. and Leister D. (2002) The metal ion transporter IRT1 is necessary for iron homeostasis and efficient photosynthesis in *Arabidopsis thaliana*. *Plant Journal* **31**, 589-599.

Vert G., Grotz N., Dédaldéchamp F.,Gaymard F., Guerinot M.L., Briat J.F. and Curie C. (2002) IRT1, an Arabidopsis Transporter Essential for Iron Uptake from the Soil and for Plant Growth. *Plant Cell* **14**, 1223-1233.

Vigani G., Maffi D. and Zocchi G. (2009) Iron availability affects the function of mitochondria in Fe-deficient cucumber roots. *New Phytologist* **182**, 127-136.

von Wandruszka R. (2006) Phosphorus retention in calcareous soils and the effect of organic matter on its mobility. *Geochemical Transactions* **7,** 6

Waters B.M., Blevins D.G. and Eide D.J. (2002) Characterization of FRO1, a pea ferric-chelate reductase involved in root iron acquisition. *Plant Physiology* **129**, 85-94.

Weisskopf L., Tomasi N., Santelia D., Martinoia E., Langlade N.B., Tabacchi R. and Abou-Mansour E. (2006) Isoflavonoid exudation from white lupin roots is influenced by phosphate supply root type and cluster-root stage. *New Phytologist* **171**, 657-668.

Welkie G.W. (2000) Taxonomic distribution of dicotyledonous species capable of root excretion of riboflavin under iron deficiency. *Journal of Plant Nutrition* **23**, 1819-1831.

Widodo B., Broadley M.R., Rose T., Frei M., Pariasca-Tanaka J., Joshihashi T., Thomson M., Hammond J.P., Aprile A., Close T.J, Ismail A.M. and Wissuwa M. (2010) Response to zinc deficiency of two lines with contrasting tolerance is determined by root growth maintenance and organic acid exudation rates and not by zinc-transporter activity. *New Phytologist* **186,** 400-14.

Zocchi G. (2006) Metabolic changes in iron-stressed dicotyledonous plants. In *Iron nutrition in plants and rhizospheric microorganisms* (eds L.L. Barton and J. Abadía), pp 359-70. Springer, Dordrecht.

Zocchi G. and Cocucci S. (1990) Fe uptake mechanism in Fe-efficient cucumber roots. *Plant Physiology* **92**, 908–911

CHAPTER 3

Comparison of different alkaline pH growth conditions (bicarbonate and organic buffer media) on *Parietaria judaica* in a time course analysis.

ABSTRACT

Fe deficiency-induced chlorosis, otherwise known as lime-chlorosis, is a major nutritional disorder in crops growing in calcareous soils that lead to huge losses in terms of quantity and quality of products. Calcareous soils represent 30% of the Earth land surface and are characterized by high pH values. The high alkalinity of calcareous soils causes a dramatic decrease in Fe bioavailability. Nevertheless, many wild species are well adapted to live (i.e. calcicole species) in calcareous environments overtaking the low bioavailability of Fe. The adaptation of a species most frequently depends on more than a single trait as natural selection operate on the whole organisms. Hence the set of traits shaped by natural selection in wild species represent a successful strategy to live in a given environment, thus identify the main functional traits involved in tolerance to Fe low availability of a wild species could offer interesting inputs for breeding programs in agriculture.

Parietaria judaica is a wild sinantropic plant that grows successfully in highly calcareous environments without showing any symptom of chlorosis. As a dicot, *P. judaica* encompasses all the inducible metabolic changes of a Strategy I plant at biochemical, physiological and morphological level. Moreover, other non-specific responses are induced in *P. judaica,* such as tissue accumulation and root exudation of organic acids and phenolic compounds. Probably these non-specific responses confer to the high efficiency demonstrated in the acquisition of Fe. In the present work, in a 7 day time course, the root accumulation and exudation of the main low molecular weight organic acids and phenolic compounds was analysed in *P. judaica* grown in hydroponics under direct or induced Fe deficiency conditions. Two different alkaline conditions were applied in order to distinguish the effect due to the high pH from the responses induced by the presence of high concentrations of calcium carbonate. It was also analysed the activity of two key enzymes of primary metabolism - PEPC and G6PDH. The great efficiency exhibited by *P. Judaica* in high calcareous environments suggested high metabolic flexibility that enables to shift the metabolic fluxes in order to adapt the plant's physiology to environmental conditions.

3.1. **Introduction**

In plants iron (Fe) is required for a wide range of biological functions like photosynthesis, respiration and chlorophyll biosynthesis. Notwithstanding Fe is the second most abundant metal in the Earth's crust it is not readily available for plants due to its low solubility. Actually, in well aerated soils at pH 7.0 Fe^{3+} and Fe^{2+} concentrations are less than 10^{-15} M, a level far below that required for plants for optimal growth (Marschner, 1995; Kim and Guerinot, 2007). Furthermore, in well aerated soils Fe occurs principally in oxidized forms - Fe(III) - that cannot be taken up by most plants, which can acquire just Fe(II). Accordingly, plants must be able to render these elements available in order to survive and compete successfully.

Dicots and monocots plants (except Poaceae) have developed active metabolic adaptations to cope with low Fe availability, known as Strategy I. In Strategy I, a ferric chelate reductase (FCR) located at the root cell plasma membrane converts FeIII-chelates to Fe(II), and a Fe regulated transporter (IRT1) moves the ferrous ion across the plasma membrane into the cell. Moreover, in various species a H^+ pumping activity, carried out by the plasma membrane H^+-ATPase, is increased under Fe deficiency. The extrusion of H^+ outward the cell acidifies the apoplast and the rhizosphere incrementing Fe solubility and generating an electrochemical gradient useful to drive the cation uptake (Rabotti and Zocchi, 1994; Dell'Orto et al., 2000; Santi et al., 2005; Santi and Schmidt, 2008, 2009). The activation of the Strategy I mechanism enhances the request of ATP and $NAD(P)H^+$ for H^+ extrusion and reduction processes, respectively, resulting in a general metabolic rearrangement. Among the metabolic changes that occur under Fe starvation, an increased activity of many glycolytic and TCA enzymes and of PEPCase was found, indicating a rising in carbohydrate catabolism (Vigani et al. 2011). Fe deficiency induces also an accumulation of low molecular weight organic acids (LOAs), mainly malate and citrate, in roots, xylem sap, leaf apoplastic fluid and whole leaves. Organic acid accumulation in Fe-deficient plants can improve long-distance Fe transport (López-Millán et al. 2000; López-Millán et al. 2001), and since the production of organic acids is protogenic, it could also contribute to the control of cytosolic pH and

feed the increased activity of the plasma membrane (PM) H^+-ATPase (Zocchi, 2006).

In addition, many works have shown increased root exudation of a variety of organic compound as a response to Fe deficiency, to increase the ferric ion solubility and/or support the reducing capacity of Fe(III) on the root surface (Dakora and Phillips, 2002; Tomasi et al., 2008; Cesco et al., 2010). Phenolic compounds have been reported as the main components in root exudates in Fe deficiency conditions and it has been suggested their implication in Fe uptake (Jin et al., 2007).

It is well known that the presence of carbonates in soils cause a decrease in the solubility of Fe and other micronutrients reducing their exchangeable capacity with plants (Lindsay and Schwab, 1982; Boxma, 1972; Loeppert, 1986). In fact, at high soil pH there are several essential elements, notably phosphate, Fe and manganese (Mn) that are scarcely present in soluble or easily available form. Fe deficiency-induced chlorosis, otherwise known as lime-chlorosis, is a major nutritional disorder in crops growing in calcareous soils. Calcareous soils represent 30% of the Earth land surface, are characterized by high pH values and may contain high concentrations of $HCO3^-$ ions in the soil solution. On one hand alkaline pH dramatically reduces Fe solubility, but on the other it has been suggested that the presence of $HCO3^-$ interferes with the Fe uptake physiological processes. It was found higher Fe content in roots of plants grown under calcareous condition respect to those grown on neutral and non-calcareous conditions, indicating that low Fe availability in soils is not a problem *per se,* but rather a question of Fe uptake from the apoplast (Mengel, 1994). In the soil solution $HCO3^-$ certainly acts buffering the rhizosphere and the root apoplast thus neutralizing the H^+ extruded by H^+-ATPase. Furthermore, some studies have shown that alkaline pH and especially high $HCO3^-$concentrations strongly inhibit the activity of the FCR which is a necessary step in Fe acquisition of Strategy I plants (Kosegarten et al., 2004). A study conducted on graminaceous species (sorghum, barley and maize) grown at different concentrations of $HCO3^-$ suggested that the impaired Fe acquisition observed at high concentration of $HCO3^-$ is attributable to the root growth inhibition (Alhendawi et al., 1997). Studies conducted on wild plants grown on

calcareous soils have suggested that chlorosis is caused by the immobilization of Fe in non-active forms rather than by a decrease of its total concentration in leaves or roots (Zohlen and Tyler, 2000; Zohlen, 2002). Lucena et al. (2007) working on *Arabidopsis* and other dicotyledonous crop species (pea, tomato and cucumber) have suggested that high contents of $HCO3^-$ could cause Fe deficiency symptoms by inhibiting the expression of several Fe acquisition genes. The exact mechanism by which $HCO3^-$ acts was not yet completely elucidated. Anyway, many plants species, despite the low concentration and availability of Fe in calcareous soils, can develop successfully without showing signs of chlorosis.

Parietaria judaica is a wild sinantropic plant that grows successfully in high calcareous environments such as walls and is highly Fe-efficient.

Figure 1.*Parietariajudaica* grown in a rock crack.

Just few works have been done on *P. judaica* regarding its adaptation to limestone. A previous study has confirmed that *P. judaica* exhibits the typical Strategy I response to Fe deficiency: increased FCR activity; high capacity to acidify the rhizosphere through the extrusion of H^+. Anatomical and morphological changes have also been observed in response to low Fe availability. *P. judaica* grown under low Fe availability develops proliferation of secondary roots and a "proteoid" root like feature, which are typical modifications induced in some Strategy I plants (Dell'Orto et al. 2003). Other non-specific responses of *P. judaica* to Fe deficiency are the accumulation and exudation of sugars, amino acids and

phenols.It seems that the high Fe efficiency of *P. judaica* lies on secondary mechanisms such as morphological modifications and exudation (Dell'Orto et al. 2003). Donnini et al. (2012) found an enhanced activity of enzymes directly involved in the polyphenols biosynthesis pathway (SDH and PAL); a significant increase in low molecular weight organic acids (citric and malic) both in root tissues and exudates when plants were grown under the presence of bicarbonate.

In Fe-starved conditions a common metabolic response concerns the increase of PEPC activity that plays a key role both in the pH stat control and in maintaining a high rate of glycolysis (Vigani and Zocchi, 2009).
G6PDH catalyzes the first step in the pentose phosphate pathway that, among other roles, provideserythose-4 phosphate (E4P), one of the substrates required by the shikimate pathway, which in turn leads to the synthesis of phenols. Furthermore, G6PDH has been suggested to be involved in the cell redox homeostasis (Singh et al. 2012).

In the present work the responses of *P. judaica* grown in hydroponics under direct and induced Fe deficiency conditions were analyzed. In particular two high alkaline treatments were set up: one containing $CaCO_3$ and $NaHCO_3$ having a pH value of 8.3 in order to mimic a calcareous soil condition and another one buffered with Tricine at a pH 8.3. These alkaline treatments were carried out in order to separate the effects due to a calcareous medium (presence of HCO_3^- and Ca^{2+}in solution) from those of the high pH on Fe acquisition. Determination of citric and malic acid and phenolic compound content in roots and exudates was conducted in a time course, as well as the assay of PEPCase and G6PDH activities.

3.2. **Materials and methods**

3.2.1. *Plant material*

Cuttings of *P. judaica* were taken from a mother plant and transplanted in an aerated half nutrient solution for 10 days. Rooted plant cuttings were then transferred to 10 L plastic tanks (40 plants per tank) with four different hydroponic conditions: +Fe (control, full nutrient solution adjusted to pH 6.2), -Fe (full nutrient solution in the total absence of Fe,

brought to pH 6.2), +Bic (full nutrient solution with addition of 0.5 gL^{-1} $CaCO_3$ and 15 mM $NaHCO_3$ which brought the pH to 8.3), Trc (full nutrient solution buffered with Tricine and adjusted to pH 8.3). Where required pH was adjusted with NaOH. The treatment buffered with Tricine was introduced to distinguish the low availability of Fe due just to high pH from the effect of the presence of bicarbonate on Fe availability.

Treatments were carried out for 7 days in a growth chamber under 16/18 h light/dark regime with cool-white light 200 μmol m^{-1} s^{-1}, 27/21°C temperature range, 65-75% relative humidity. Plant samples were collected at first, third, fifth and seventh day of the overall treatment.

3.2.2. *Collection of root exudates*

Fifteen 7-d-old plants for each treatment were transferred to 250 mL distilled water with 10% (v/v) Micropur (Katadyn, Minneapolis, MN) to prevent microbial activity. Root exudates were collected for a period of 24 h under continuous aeration. The exudate solution collected for the total phenolic compound determination was acidified to pH 3.5-4.0 with HCl before being freeze dried. For all the other measurements the exudate solution was freeze dried without adjusting the pH. The lyophilized material was resuspended in 3 mL distilled water and filtered through 0.45 μm Millipore Millex-HN.

3.2.3. *Enzymatic determination of organic acids*

Root samples were collected, carefully rinsed in distilled water and homogenized in 10% (v/v) perchloric acid following a 1:1 ratio g/mL and centrifuged at 10,000 *g* for 15 min. The pH was brought to 7.5 with 0.5 M K_2CO_3 to neutralize the acidity and to precipitate the perchlorate. Extracts were successively centrifuged at 15,000 *g* for 15 min and the supernatant was recovered. Citric and malic acid concentration in both root extracts and exudates was determined enzymatically as follows: Citric acid was assayed by coupling its conversion to oxaloacetate by citrate lyase (CL, EC 4.1.3.6). In the presence of malate dehydrogenase (MDH, EC 1.1.1.37) and lactate dehydrogenase (LDH, EC 1.1.1.27), the

oxaloacetate and its decarboxylation derivative, pyruvate, are reduced to L-malate and L-lactate by reduced nicotinamide adenine dinucelotide (NADH). The amount of NADH oxidized to NAD+ in these reactions is proportional to the amount of citrate present.

Malic acid was analysed by the enzymatic conversion of L(−)malate to oxaloacetate in the presence of NAD^+ and L(−)malate dehydrogenase (MDH). In order to favour the reaction towards the production of NADH the enzyme glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1) is used.

Variations in NAD/NADH+ amounts were detected by monitoring the absorbance changes at 340 nm and were performed at 26°C and in a final volume of 1 mL. At least three independent assays were performed for each treatment.

3.2.4. *Determination of phenolic content*

Phenolic content in root tissues was determined in two different extraction media: 100% distilled water and 100% methanol. Root samples from the different treatments were homogenized in the extraction medium with a volume ratio 1:1 (w/v). The homogenates were centrifuged at 10,000g for 15 min and the supernatants collected. The amount of phenolics in root extracts and exudates was determined spectrophotometrically at 750 nm with the Folin-Ciocalteau reagent using gallic acid as a standard (Donnini et al., 2012). Three replicates of three independent experiments were performed for each treatment (n=9).

3.2.5. *Determination of apoplastic Fe*

Apoplastic Fe was determined in a time course at 1,3, 5 and 7 days. Roots from 5 plants per treatment were transferred to a beaker with 0.5 mM $CaSO_4$ under vigorous aeration. After 10-15 min, roots were transferred to 40-ml tubes with 21 ml of 10 mM MES, 0.5 mMCa $(NO_3)_2$, 1.5 mM 2,2'-bipyridyl (pH 5.5) at 25°C. Tubes were covered with a cotton plug and N_2 was bubbled through the solution. After 5 min, 1 mL of 250 mM $Na_2S_2O_4$ was added. The A_{520} of the solution (A_{520} of 1 mM

Fe[bipyridyl]$_3$ = 8.650) was determined on 2 mL aliquots as reported by Bienfait et al. (1985). The aliquots employed for the determinations were returned to the tube and left for 1h in the dark. The determination was carried out every 1.30 h until a constant value was obtained.

3.2.6. *Soluble enzyme extraction and assay of PEPCase and G6PDH*

Roots sampled from different treatments were cut, rinsed in distilled water, and homogenated at 2-4°C in a buffer solution (1:1 mL/g) in the presence of 20% (w/w) PVPP. The buffer solution contained: 50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10% glycerol, 1mM EDTA, 14 mM β–mercaptoethanol, 1mM PMSF and 10 mg mL^{-1}leupeptin. The homogenates were filtered through 4 layers of gauze and centrifuged at 13,000g at 4°C for 15 min. Supernatants were centrifuged at 100,000g at 4°C for 30 min. The final supernatants were collected and dialyzed for 3h at 4°C against 2 L of leupeptine-free buffer solution. The dialyzed extracts were used for enzymatic assays of *Phosphoenolpyruvate carboxylase* (PEPC) and *Glucose-6-phosphate dehydrogenase* (G6PDH). *PEPC* (EC.4.1.1.31) activity was determined as in De Nisi and Zocchi (2000). The reaction was started by adding an aliquot of soluble enzyme extract. *G6PDH* (EC 1.1.1.49) was assayed according to Rabotti et al. (1995). The reaction was started by adding an aliquot of soluble extract.

3.2.7. *Statistical analysis*

Values are expressed as means ±SD. One way ANOVA analysis was used to test any statistical difference among data. Differences at $P<0.05$ were considered to be significant. Statistical differences have been expressed by different letters. All statistical analyses were carried out with SPSS v.15.0.1 (SPSS Inc.)

3.3. **Results**

3.3.1. *Malic and citric acid content in root tissues and exudates*

Table 1 shows the contents of LOAs in roots at 1, 3, 5 and 7 days of treatment.

A **Malic acid content in roots (μg g^{-1}FW)**

	+Fe	-Fe	Bic	Tric
T1	264.66±20.32 c	177.49±12.67 e	452.24±47.58 a	289.34±20.75 c
T2	221.88±19.23 cd	150.78±11.73 e	400.81±35.51 b	259.01±17.37 d
T3	111.17±9.69 ef	157.98±13.62 ef	348.65±32.57 c	241.79±23.78 d
T4	105.89±8.45 f	167.76±18.84 e	307.05±20.62 c	148.70±10.94 e

B **Citric acid content in roots (μg g^{-1}FW)**

	+Fe	-Fe	Bic	Tric
T1	748.11±70.27 b	470.55±35.87 d	611.63±54.90 bc	682.76±45.20 bc
T2	183.03±15.98 f	377.66±35.76 e	764.84±74.38 b	787.29±70.26 b
T3	90.89±10.34 f	452.72±46.72 de	1148.72±102.39 a	963.80±82.58 ab
T4	101.23±9.56 f	525.34±49.84 d	1346.56±128.83 a	1024.35±99.87 b

Table 1. Time course of the amount of LOAs in root tissues in +Fe, -Fe, Bic and Tric treatments. **A.** content of malic acid; **B.** content of citric acid. T1, T2, T3 and T4 correspond to sampling at 1, 3, 5 and 7 days. Data are the means ± SD (*n*=7). Statistical differences (*P*< 0,05)are indicated with different letters.

Regarding malic acid content (Table 1 A), values gradually diminished during the treatment in +Fe, Bic and Tric condition). The highest decreases were registered for +Fe (control) and Tric, in which nearly 60% and 50% decrease was registered, respectively, comparing with their initial amounts at the beginning of the treatment. In bicarbonate

condition (Bic) a 32% decrease was recorded with respect to the initial content at the beginning of the treatment. In –Fe condition the absolute values did not present significant differences.

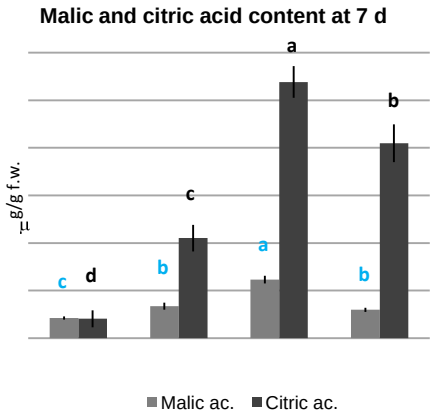

Figure 1. Comparison of malic and citric acid root contents expressed as μg g^{-1}FW at the end of the treatment for +Fe, -Fe, Bic and Tric treatments. Error bars indicate standard deviation (*n*=7). Different letters indicate significant difference (*P*<0.05). Significant differences were calculated for each individual compound and are indicated in different colours.

Citric acid content in roots shows a different trend. While in control condition citrate diminished by 86% after 24 h (T1) remaining then constant, the other treatments showed a significant increase. Bic and Tric conditions exhibit the highest accumulation of citric acid (2 and 1.5 fold respectively) at the end of the treatment compared with their contents at T1.

Figure 1 compares the malate and citrate content at the end of the experiment. All stressed treatments (-Fe, Bic, Tric) show an increase of malic and citric contents with respect to the control (+Fe). The accumulation of malic and citric acids is considerably higher in the presence of bicarbonate (Bic), 3-fold and 13-fold, respectively, compared to the control (Fig. 1). In Fe starved roots (-Fe), malate accumulation increases by 60% respect to the control. Both alkaline treatments increase citric acid 13-fold and 10-fold,respectively, compared with the control. In

direct Fe deficiency condition (-Fe) citrate accumulate by 5 fold respect to the control (Fig. 1).

Table 2 A and B shows the concentrations of malic and citric acid in exudates collected for 24 h from roots of plants submitted to the Fe-deficient treatments for 1, 3, 5 and 7 days.

A

Malic acid in root exudates ($\mu g\ g^{-1} FW\ d^{-1}$)

	+Fe	-Fe	Bic	Tric
T1	n.d.	n.d.	n.d.	n.d.
T2	n.d	0.96±0.12 de	n.d.	n.d.
T3	0.87±0.10 e	6.05±0.74 ab	3.47±0.51 c	1.22±0.25 d
T4	1.68±0.28 d	7.67±0.88 a	5.17±0.65 b	2.83±0.24 c

B

Citric acid in root exudates ($\mu g\ g^{-1}$ FW d^{-1})

	+Fe	-Fe	Bic	Tric
T1	n.d.*	0.53±0.10 f	n.d.	0.42±0.09 f
T2	n.d	1.15±0.21 e	1.96±0.27 d	1.06±0.12 e
T3	n.d	4.73±0.50 b	3.47±0.35 c	2.27±0.25 d
T4	0.33±0.04 f	5.89±0.49 b	10.87±0.84 a	5.96±0.37 b

* n.d. no detectable

Table 2. Time course of the amount of LOAs in root exudates in +Fe, -Fe, Bic and Tric treatments. **A.** malic acid; **B.** citric acid. T1, T2, T3 and T4 correspond to sampling at 1, 3, 5 and 7 days. Data are the means ± SD (*n*=7). Statistical differences (*P*< 0,05)are indicated with different letters.

Malic acid content in exudates increases in all treatments respect to the control. Malic acid was detected since T3 sampling for almost all the treatments. The highest increases in malic concentration were recorded for -Fe and Bic (3-4 fold at T4). –Fe roots respond first with respect to the other treatments exuding malic acid at T2, increasing consistently at T3 while the successive amounts at T4 and T5 remain constant.

Similarly, citric acid content in exudates exhibits a strong increase in all treatments. Bic condition increased 33-fold while –Fe and Tric showed an 18-fold increment with respect to the control. In general, citrate could be detected earlier in exudates in comparison to malate. In –Fe and Tric conditions citric acid was found in exudates after 24 h of treatment (T1).

3.3.2. *Phenolic compounds in root extracts and exudates*

Table 3 A and B shows the results concerning the total phenolics measured in root water and methanol extracts. Water soluble contents of phenolics are quite variable along the time course and no significant correlation could be found between concentration and time for any of the treatments. At the end of the treatment (T4) direct and induced Fe deficiency treatments exhibit a higher accumulation of water soluble phenolics respect to the control. Bicarbonate (Bic) condition induces an accumulation of phenolics of about +75% compared with the control while –Fe and Tric treatments increase by 40% their phenolic content without any significant difference between them.

The methanol soluble phenolics showed a positive correlation between concentration and time only in Bic condition, in which the accumulation of phenolics exhibits a 2-fold increase at the end of the treatment. Fe starved treatment showed no variations of contents in the time course, while the control condition decreased its content in phenolics, slightly less than 50%, at the end of the treatment. Phenolic compounds determined in methanol extracts showed a consistent increase in all stressed conditions at final sampling (T4) when compared with control. Bicarbonate treatment accumulated 4-fold more phenolics than control while –Fe and Tric increased 2-fold. Similarly to what registered for water extracts, Fe starved and Tric roots exhibit similar phenolic contents.

The content of phenolics in root exudates collected in +Fe, -Fe, Bic and Tric is shown in Table 4.

A **Water soluble phenolic compounds in roots (mg g^{-1}FW)**

	+Fe	-Fe	Bic	Tric
T1	1.09±0.08.f	2.076±0.12 ab	1.24±0.07 e	2.15±0.11 ab
T2	1.64±0.09 d	1.93±0.08 bc	1.65±0.10 d	1.84±0.07 c
T3	1.08±0.10 f	2.23±0.14 a	1.31±0.09 e	0.94±0.10 f
T4	0.97±0.09 f	1.37±0.11 e	1.71±0.12 cd	1.36±0.10 e

B **Methanol soluble phenolic compounds in roots (mg g^{-1}FW)**

	+Fe	-Fe	Bic	Tric
T1	1.13±0.11 cd	1.28±0.12 c	1.05±0.12 cd	1.16±0.08 cd
T2	1.20±0.13 cd	1.13±0.11cd	1.50±0.10b	1.22±0.08cd
T3	0.74±0.06 e	1.02±0.09 d	1.22±0.09 c	0.66±0.05 e
T4	0.60±0.07 e	1.08±0.01d	2.21±0.13 a	1.09±0.99 d

Table 3. Content of phenolic compounds in roots of *P. judaica* along a time course for +Fe, -Fe, Bic and Tric treatments. **A.** concentration in root extracts with water; **B.** concentration in roots extracts with methanol. Values are expressed as the means ± standard deviations. Different letters indicate the significant differences (P=0.05) among values.

Total phenolic compounds in root exudates (mg g^{-1}FW d^{-1})

	+Fe	-Fe	Bic	Tric
T1	0.021±0.001 f	0.113±0.012 d	0.052±0.007 e	0.140±0.025 c
T2	0.020±0.002 f	0.213±0.021 c	0.093±0.004 d	0.124±0.081 c
T3	0.031±0.001 f	0.272±0.022 b	0.112±0.014 c	0.107±0.023 c
T4	0.030±0.002 f	0.314±0.026 a	0.126±0.011 c	0.115±0.012 c

Table 4. Time course of total phenolics in exudates from control (+Fe), Fe starved (-Fe), bicarbonate (Bic) and alkaline organic buffer (Tric) treated plants. Amounts are averages coupled with standard deviation. Different letters indicate the significant differences (*P*=0.05) among values (*n*=9).

The results obtained are one order of magnitude higher respect to a previous work (Chap. 2, par. 2.3.1. Table 1).In fact, the method for collecting exudates has been modified in the present study. Phenolics are more stable at low pH and precipitate at high pH so, to avoid loss of material and to maintain the structure of compounds, the collected solutions were acidified before being freeze dried.

Phenolic compounds have been detected in exudates at the first sampling date (T1) in all treatments. At T1 higher amounts of phenolics were found in all stress treatments respect to the control; -Fe and Tric show 5 fold and 7 fold increases, respectively.Exudates from –Fe and Bic conditions showed a gradual increase in phenolic content over the time course, reaching a maximum level at the end of the treatment which resulted 3- and 24-fold higher with respect to the beginning of the treatment. In +Fe and Tric conditions the concentrations remained constant along the treatment.

All stress treatments exhibit a higher content of phenolics with respect to the control at the end of the treatment (T4). The highest content of phenolics was found in -Fe exudates that exhibit a 11-fold increase with respect to the control at the end of the treatment. Bic and Tric treatment increased 4-fold respect to the control at the end of the treatment and showed no significant differences in the phenolic content of exudates between them.

3.3.3. *Root apoplastic Fe*

The results of root apoplastic Fe content obtained along the time course for +Fe, -Fe, Bic and Tric treatments are shown in Table 5.

The initial amount of apoplastic Fe of rooted plants was determined (T0) before imposing the treatments (1.41 ± 0.17 μmol g^{-1}FW). In the control condition after 1 d of treatment a surprisingly 2-fold accumulation of Fe was registered, than rapidly decreased reaching a value comparable with that found at T0. In Fe starved conditions the content of Fe in the apoplast decreased until reaching zero at T3 (5 d). In agreement with previous results (cfr. Chapter 2, par.2.3.4.), the Bic condition exhibited a strong accumulation of Fe, reaching a 2.7-fold increase at T3 respect to

T0 and remaining almost constant until the end of the treatment (T4). In Tric condition an increase of 23% was recorded at the end of the treatment. No significant difference was found after 7 d (T4) between control and Tric condition, while in bicarbonate the accumulation was of 4-fold with respect to the control at the end of the treatment.

Apoplastic Fe content (μmol g^{-1}FW)

	+Fe	-Fe	Bic	Tric
T1	3.04±0.25 b	0.98±0.15 d	1.30±0.15 c	0.90±0.10 d
T2	1.39±0.23 c	0.42±0.24 f	2.85±0.24 b	0.61±0.052 e
T3	1.04±0.12 cd	0.03±0.002 g	3.86±0.37 a	0.62±0.063 e
T4	0.94±0.05 cd	0.04±0.006 g	3.45±0.41 ab	1.11±0.19 g

Table 5. ApoplasticFe concentration (μmol g^{-1} DW) in roots of plants grown for 1, 3, 5, 7 days in control condition (+Fe), iron deficiency (-Fe), in the presence of bicarbonate (+FeBic) and alkaline organic buffer (Tric) treated plants. Data are means ± S.D. (n=5). In the case of significant differences ($P<0.05$) values are marked with different letters.

3.3.4. *PEPCase and G6PDH activity*

PEPCase is considered a key enzyme under Fe deficiency conditions, both in terms of pH stat regulation and as a driving force for glycolysis (De Nisi and Zocchi, 2000). PEPCase activity showed a general and gradual decrease in all treatments (Tab. 6A) during the time course: the decrease was sharper in Tric condition, leading to a low enzymatic activity at the end of the treatment. In -Fe condition the PEPCase activity decreased at the beginning of the treatment, then reaching a constant value.

Table 6B shows the results of the time course for the G6PDH activity.

In control condition G6PDH activity at the end of the time course was about 21-fold higher respect to the activity at the beginning of the treatment. In –Fe and Bic conditions it decresed by 8- and 6-fold, respectively, in relation to their initial activity rates.

A **PEPCase activity (nmol $min^{-1}mg^{-1}$prot.)**

	+Fe	-Fe	Bic	Tric
T1	126.07±10.90 bc	118.74±10.56 bc	168.86±15.20 a	143.85±11.73 ab
T2	96.61±8.25 c	94.02±9.34 c	129.46±13.00 b	117.57±10.94 b
T3	67.24±6.84 d	82.68±6.79 c	70.00±5.24 cd	78.79±6.51 c
T4	62.54±4.70 d	90.80±8.90 c	83.77±5.77 c	24.61±3.70 e

B **G6PDH activity (nmol $min^{-1}mg^{-1}$prot.)**

	+Fe	-Fe	Bic	Tric
T1	2.64±0.30 h	4.07±0.37 g	3.70±0.30 g	7.26±0.75 f
T2	35.82±2.96 b	32.03±2.74 b	2.48±0.32 h	9.46±0.89 e
T3	57.68±4.28 a	10.00±0.97 e	19.20±2.13 d	5.49±0.54 g
T4	56.00±4.30 a	6.23±0.75 f	24.00±2.36 c	7.65±0.81 f

Table 6. Enzymatic activities measured in dialysed root soluble extracts from +Fe, -Fe, Bic and Tric treated plants. **A.** PEPC (phosphoenolpyruvate carboxylase) and, **B.** G6PDH (glucose 6-phosphate dehydrogenase). Data are expressed as the means ± SD (n=9). Enzyme activity is expressed as nmol $min^{-1}mg^{-1}$prot. Significant differences are labelled with different letters (P<0.05).

In Tric condition G6PDH activity did not significantly change during the treatment. Comparing this enzyme activity at 7 d (T4) among treatments it can be observed that in direct (-Fe) and induced Fe deficiency (Bic, Tric) conditions G6PDH activity decreses by 9-, 2- and 7-fold, respectively.

3.4. **Discussion**

The levels of LOAs in plants vary greatly among different tissues and different species. However, increase in their content and exudation by

roots has been reported by several works as a response to environmental stresses and in particular to nutritional constrains such as Fe deficiency (Abadía et al. 2002; Jelali et al. 2010; Rellán-Alvarez et al. 2011; Jimenez et al. 2011). In many Fe-efficient genotypes of different crop species, Fe uptake efficiency has been associated to the ability to accumulate organic acids, mainly malic and citric acid (Lopez Bucio et al. 2000). Different functions of organic acids could explain their accumulation in Fe deficient roots: control of cytoplasmic pH by the pH Stat mechanism (Reid and Smith, 2002); control of apoplastic pH (López-Bucio et al. 2000); acting as chelating agent of Fe in apoplast and rhizosphere; acting as chelating agent of Ca to solubilize P in the soil (Tyler and Strom, 1995) and to capture excessive Ca inside the cell (Bush et al. 1995); Fe plant translocation through the formation of different species of FeIII-citrate (Rellán-Álvarez et al. 2011).

In agreement with previous works (Zocchi, 2006; López-Millan et al. 2009 and references therein), a strong accumulation of malic and citric acids was recorded at the end of the treatment in roots and root exudates of *P. judaica* subject to direct or induced Fe deficiency. In both alkaline conditions (Bic and Tric) *P. judaica* performs a clear response of accumulation of citrate. During the time course plants grown in the presence of an adequate supply of Fe (+Fe) exhibited a consistent decrease in malate root content. These data are well correlated with the decreasing activity of PEPC observed along the treatment. Also in the presence of bicarbonate (Bic) and tricine (Tric) the trend for malic acid content was correlated with the relative PEPCase activity.

A previous study (Chapter 2, par. 2.3.7.) has shown an increased H^+-ATPase activity in *P. judaica* roots grown in the absence of Fe, suggesting the need for a cytosolic pH-Stat mechanism. In fact, in the present work, Fe deficient roots exhibited increased PEPC activity respect to the control since the T3 sampling, and accordingly, a strong accumulation of malate occurs. An even higher accumulation of malic and citric acids (fig. 1) occurred in plants grown in the presence of bicarbonate, probably due to both the enhanced specific PEPC activity and the high availability of the substrate HCO_3^-which needs to be removed. Actually, in a previous work (Donnini et al. 2012) a low PEPC activity was reported in bicarbonate condition but the assay was carried

out in non-dialyzed extracts. In the present work the enzyme activity assays were conducted on dialyzed root extracts in order to reduce the high malic acid concentration, which is known to inhibit the PEPC activity (De Nisi and Zocchi, 2000).

Furthermore, in Bic condition the growth rate is significantly lower with respect to control, -Fe and Tric conditions (data not show). Under active growth conditions TCA provides many precursors of anabolic metabolism such as amino acids, so the replenishment of TCA becomes necessary and it could occur through the contribution of cytosolic malate. The lower growth rate in Bic–treated plants could result in a lower consumption of malate. In Tric condition, growing rates are similar to the control and the organic acids levels in roots and exudates are compatible with a low Fe availability (tab.1). In these plants probably there is no necessity to exert a strong control on cytosolic pH, in addition mitochondrial activity should not be impaired so organic acids could be supplied by TCA.

It has been well documented that roots exude a variety of organic compounds that play multiple functions in plant nutrient acquisition. An increase in organic acid root exudation has been reported in several dicotyledonous species under Fe deficiency and their involvement in mobilization of Fe has been documented as well (Farrar and Jones, 2000). Some studies have pointed out the enhanced rate of exudation of citric, malic and oxalic acids in calcicole species with respect to calcifuges ones and ascribe their efficient acquisition of P and Fe to this ability (Ström et al. 2005).

P. judaica greatly increase the malic and citric acid contents in exudates when subject to direct or induced Fe deficiency (tab. 2A and B). Both malate and citrate once exuded may undergo complexation with target metals as Fe(III), improving its low solubilisation particularly in calcareous soils (López-Bucio et al. 2000, Abadía et al. 2011). Citrate demonstrates better metal extraction capabilities than malate in alkaline medium. Citric acid shows a higher concentration than malate in exudates produced by *P. judaica* under alkaline conditions (Bic and Tric), while in direct Fe deficiency (-Fe) malate presents a higher amount than citric acid. In –Fe and Tric conditions small quantities of citric acid were

detected after 24 h (T1) of treatment (tab. 2A and B) suggesting the predominant role of citric acid in the response to Fe deficiency.

In calcareous soils the alkaline pH decreases the availability of many nutrients other than Fe, in particular P constitutes the main limitation since it precipitates as $Ca_3(PO_4)_2$. Citric acid can also chelate Ca, enhancing Ca-P mineral dissolution (Ström et al. 2005) thus increasing P availability. Thus, the high content of citric acid in Bic exudates at the end of the treatment (33-fold higher than control) could occur in response of both Fe deficiency and low P availability due to high Ca concentrations. In Tric exudates the concentration of citric acid does not show significant difference with –Fe and the amount of malic acid is 3-fold lower: in this last condition the low availability of Fe and P is just a consequence of pH, lacking the effect of $CaCO_3$ on P sequestration.

In –Fe conditions the predominance of malate exudation could be attributable to a metabolic state. -Fe plants activate the strategy I mechanisms, increasing ATP and reducing power requirements that cannot be fulfilled through the respiratory chain which is impaired by the lack of Fe. In fact, the impaired mitochondrial electron chain results in a low turnover of NAD^+/NADH (Vigani and Zocchi, 2009), thus Fe starved plants have to face the problem of refilling NAD^+. The synthesis of citric acid produces $NADH+H^+$, so in a high reductive environment is probably inhibited. In this reductive medium malate could represent a good compromise even if citrate is a better chelating agent.

It was widely demonstrated that environmental stresses induce the accumulation of phenolic compounds in plants (Dixon and Pavia, 1995). Phenolics have been reported to have multiple biological effects including antioxidant and chelating activity. The antioxidant activity of phenolics is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donators, and singlet oxygen and free radical scavengers. Furthermore, as phenolics have a metal chelation potential (Rice-Evans et al. 1997) they can contribute through root exudates to facilitate Fe acquisition by plants (Lehman et al. 1987; Dakora and Phillips, 2002; Jin et al. 2006; Cesco et al. 2010).

P. judaica growing under directed or induced low Fe availability shows a high accumulation of phenolic compounds both in root tissues and root

exudates.No correlation has been found between soluble phenolics concentrations in roots and the progress of treatment. At the end of the treatment however, the concentration of both polar and non-polar phenolics in roots was increased in all stress conditions. The highest amounts of phenolics were recorded in Bic-treated roots, respectively 70% and nearly 4-fold of polar and non-polar phenolics in comparison with their initial contents (tab.3A). This could be explained because a plant growing on a high calcareous condition has to face cumulative stress, due to the low availability of Fe and P as a consequence of the alkaline pH and to the precipitation of P caused by the high content of Ca. Lack of both Fe and P in plants induces high levels of ROS which indicate an oxidative stress (Tewari et al. 2007; Hajiboland, 2007). Many phenolics have antioxidant activities and oxidative stress conditions promote their accumulation. The lower concentration of phenolics in Tric-treated roots with respect to Bic could be due to a lower stress regarding P, as above explained discussing the organic acid content. In –Fe and Tric treatments the concentrations of polar and non-polar phenolics in root tissues increase but do not show significant differences in their values despite the fact that in Tric treatment *P. judaica* have to cope with the additional problem of P reduced availability respect to –Fe condition. However, as it was pointed out before, the content of citric acid in Tric roots doubles respect to Fe starved roots (tab. 1B) favoring also P availability.

Although organic acids have been recognized to play an important role in iron deficiency response, phenolic compounds have been detected earlier in exudates of all treatments. This fact could suggest on one hand that phenolics exudation is a default strategy of *P. judaica* species, and one the other hand, it could confirm their central role in the environmental stress responses.

Phenolic concentrations in root exudates of *P. judaica* plants subject to direct and induced Fe deficiency are higher than in the control; moreover they significantly increase along the time course. In Bic condition the initial amount of phenolics exuded is lower respect to –Fe and Tric but it increases along the time course reaching concentration equivalent to Tric treatment. In Bic and Tric conditions the content of phenolics at the end of the treatment are similar (4-fold compared to the control) but the

highest concentration of phenolics was found in Fe starved condition (10-fold respect to the control). In alkaline conditions the exudation of phenolics most probably achieve the acquisition of Fe as it is present in solution and it is also immobilized in the apoplast (cap. 3, par. 3.3.3) so the plant could reduce the amount of exudates. Comparing the amounts of organic acids (tab. 2) and phenolic compounds (tab. 4) in exudates it can be noted that the latter presents values one order of magnitude higher, this fact is in accordance with many works that reported phenolic compounds as the main components in root exudates (Jin et al. 2007). Jin et al. (2007) have found that when phenolics were removed, the FCR activity and the acidification increased suggesting that they are not involved in the reutilization of apoplastic Fe mediated by phenolics. However, the acidification of the apoplast as well as the rhizosphere could confer structural stability to phenolic compounds.

The root apoplastic Fe has been suggested to be an important Fe source for plants as 75% of total Fe in roots is located in the apoplast (Bienfait et al. 1985). The amount of apoplastic Fe in direct and induced Fe deficiency is shown in Table 5. Fe starved roots show a decrease in apoplastic Fe along the time course, reaching a null value at T3. These data are in agreement with findings of Zhang et al. (1991) who found a reduction in the amount of apoplastic Fe as plants were transferred from full nutrient growing solution to Fe starved one. In fact in Fe-deprived conditions the Fe immobilized in the apoplast is the only available source of Fe for the plant. Jin et al. (2007) have demonstrated the involvement of phenolic exudates in the mobilization of apoplastic Fe. The fast depletion of apoplastic Fe in –Fe roots is supported by the high levels of phenolics found in exudates (tab. 4). In alkaline conditions (Bic and Tric) the amount of apoplastic Fe increases due to the high pH which dramatically decreases Fe solubility and induces Fe precipitation. In addition, it has been pointed out that high apoplastic pH depresses FCR activity reducing, as a consequence, the uptake of Fe (Kosegarten et al. 2004). Bicarbonate (Bic) condition shows a higher content of Fe in the apoplast respect to the organic alkaline buffer treatment (Tric). This result could be explained considering the low growth rate exhibited by Bic plants (data not show) causing a lower consumption rate of apoplastic Fe.

Two enzymes of the primary metabolism were assayed along the treatment: PEPC and G6PDH (Tab. 6 A and B). Many papers have reported the increased activity of PEPC under Fe starved growing conditions (De Nisi and Zocchi, 2000; Andaluz et al. 2002; López-Millán et al. 2009; Donnini et al. 2010) indicating its key role in Fe-efficient responses. It has been proposed that enhanced activity of PEPC acts in the homeostasis of cytosolic pH and as a driving force to maintain a high rate of glycolysis by consuming PEP, other than producing malate (Zocchi, 2006). The increase in PEPC activity could then maintain an adequate rate of glycolysis required to provide energy supply and hold up reduce based mechanism to acquire Fe. In Fe starved roots a decrease in PK activity was shown (Thimm et al. 2001; Donnini et al. 2010) so PEP seemingly does not follow the TCA pathway directly.

It was found a decreasing activity of PEPC along the time course in all treatments. This could be probably due to the narrow localization of the increased PEPC activity to the external layers of cortical cells of root apices where also the H^+-ATPase activity is particularly enhanced (Dell'Orto et al. 2002) and the nutrient uptake takes place: as root grows and tissues differentiate, the active portion, in which the PEPC activity is increased, becomes a smaller percentage of the total root biomass, resulting in a dilution effect. Nevertheless, after 7 days under both direct (-Fe) and bicarbonate-induced Fe deficiency the PEPC activity is significantly higher than under normal Fe supply, according to what found in various Fe-deficient species. Regarding the Bic-treated plants, the presence of HCO_3^-represents a further alkalinisation of the cytosol and PEPC activity could decrease the HCO_3^-concentration, giving a further contribution in controlling the cytosolic pH. On the contrary, in alkaline Tric treated roots, PEPC activity is very low at the end of the time course, suggesting that PEP is probably consumed at an adequate rate mainly via TCA and shikimate pathway to support the mechanism to acquire Fe. In fact in Tric condition Fe can be acquired by the plant and the mitochondrial activity is not impaired.

G6PDH is the first enzyme of the oxidative pentose phosphate pathway (OPPP) and is involved in the supply of C5 sugar phosphates formed in the cytosol that can be used as primary substrates for nucleotide synthesis or aromatic aminoacids via the shikimate pathway. G6PDH is also a key

enzyme in modulating the redox state of the cell thus preventing oxidative stress (Singh et al. 2012). In fact, NADPH is a strong inhibitor of G6PDH as well as ATP (Singh et al. 2012).

In control condition a consistent increase in G6PDH activity was recorded along the time course, while all stress treatments exhibited a reduced activity respect to the control at the end of the experiment (Tab. 6). In particular, the lowest activity was recorded under –Fe condition probably due to a high $NADPH/NAD^+$ ratio, resulting from the impairment of mitochondrial activity and consequent lowered reducing power turnover and energy supply in form of ATP; in fact both the respiratory chain and the TCA cycle, having Fe-containing proteins, are deeply affected under Fe deficiency (Vigani and Zocchi, 2009; Vigani, 2012). The temporary increase in G6PDH activity at T2 could be explained considering that until the second sampling –Fe roots have not depleted the apoplastic source of Fe yet (Tab. 5). Surprisingly also Tric-treated plants, showed very low G6PDH activity along the treatment, without any significant difference with –Fe condition at the final sampling (T4).There is no reason to suppose that $NAD(P)H/NAD(P)^+$ ratio should be high, as mitochondrial activity could not be impaired. A possible inhibition of G6PDH by tricine buffer cannot be excluded. As in Tric treatment phenolic content in roots and exudates are high and some shikimic enzymes are activated (Chap. 4 par. 4.2.4.), we assume that probably the supply of E4P does not come from OPPP in roots but from photosyntates from the shoot. In Bic treatment the activity of G6PDH at the end of the treatment is decreased by 60% respect to the control but it is still higher than under –Fe and Tric treatments, moreover it increases by 60% along the time course with respect to its initial value.

The results of the present work show a great flexibility in *P. judaica* responses to Fe deficiency. Low organic acids and phenolic compounds definitely play a pivotal role in the adaptive strategy performed to cope with Fe deficiency. Both these compounds carry out potentially multiple functions, so *P. judaica* can modulate their amounts depending on the status and different requirements of the plant. The responses of *P. judaica* to both alkaline treatments have shown that *P. judaica* can be defined as a Fe-efficient plant. Furthermore, it has been shown that in calcareous treatment the presence of HCO_3^- and Ca^{2+}operates in a direct

and indirect way over the Fe bioavailability. As a direct effect, HCO_3^- raises the pH decreasing Fe solubility; additionally HCO_3^- ion buffers the attempt to acidify the apoplast reducing the FCR activity. An indirect effect could be due to the high content of Ca^{2+} that is preferentially chelate by citrate respect to Fe.

References

Abadía J., López-Millán A.F., Rombolà A., Abadía A., 2002. *Organic acids and Fe deficiency: a review*, Plant and Soil, 241(1):75-86

AbadíaJ., VázquezS., Rellán-ÁlvarezR., El-JendoubiH., AbadíaA., Álvarez-FernándezA., López-MilláNA.F., 2011. *Towards a knowledge-based correction of iron chlorosis*, Plant Physiology and Biochemistry, 49(5):471–482

Alhendawi R.A., Römheld V., Kirkby E.A., Marschner H., 1997. *Influence of increasing bicarbonate concentrations on plant growth, organic acid accumulation in roots and iron uptake by barley, sorghum, and maize* Journal of Plant Nutrition, 20(12): 1731-1753

Andaluz S., Rodríguez-Celma J., Abadía A., Abadía J., López-Millán A.F., 2009.*Time course induction of several key enzymes in Medicagotruncatula roots in response to Fe deficiency*, Plant Physiology and Biochemistry, 47(11–12):1082–1088

Andjelković M., Van Camp J., De Meulenaer B.,Depaemelaere G., Socaciu C., Verloo M., Verhe R., 2006. *Iron-chelation properties of phenolic acids bearing catechol and galloyl groups*, Food Chemistry, 98:23–31

Bienfait H.F., van den Briel W., Mesland-Mul N.T., 1985.*Free space iron pools in roots. Generation and mobilization*, Plant Physiology, 78:596-600

Boxma R., 1972. *Bicarbonate as the most important soil factor in lime-induced chlorosis in the Netherlands*, Plant and Soil, 37(2):233-243

Bush D.S., 1995. *Calcium regulation in plant cells and its rolein signaling*, Annual Review of Plant Physiology and Plant Molecular Biology, 46:95–122

Cesco S., Neumann G., Tommasi N., Pinton R., Weisskopf L., 2010. Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition, Plant and Soil, 329:1-25

Dakora F.D., Phillips D.A., 2002. Root exudates as mediators of mineral acquisition in low nutrient environments, Plant and Soil, 245:35-47

Dell'Orto M., Santi S., De Nisi P., Cesco S., Varanini Z., Zocchi G.,2000 *Development of Fe deficiency responses in cucumber (Cucumissativus L.) roots: involvement of plasma membrane H+-ATPase activity*, Journal of Experimental Botany, 51:695–701

Dell'Orto M., Pirovano L., Villalba J.M., González-Reyes J.A., Zocchi G., 2002. *Localization of the plasma membrane H^+-ATPase in Fe-deficientcucumber roots by immunodetection*, Plant and Soil, 241(1):11-17(7)

De Nisi P., Zocchi G., 2000. *Phosphoenolpyruvate carboxylase in cucumber (Cucumissativus L.) roots under iron deficiency: activity and kinetic characterization*, Journal of Experimental Botany, 352:1903-1909

Dixon R.A., Paiva N.L., 1995. *Stress-lnducedPhenylpropanoid Metabolism*, The Plant Cell, 7:1085-1097

Donnini S., De Nisi P., Gabotti D., Tato L., Zocchi G., 2012. *Adaptive strategies of Parietariadiffusa (M.K.) to calcareous habitat with limited iron availability*, Plant Cell and Environment,35:1171-1184

Donnini S., Prinsi B., Negri A.S., Vigani G., Espen L., Zocchi G., 2010. *Proteomic characterization of iron deficiency responses in Cucumissativus L. roots*, BMC Plant Biology ,10:268

Farrar J.F., Jones D.L., 2000.*The control of carbon acquisition by roots*, New Phytologist, 147(1):43–53

Hajiboland R., 2007. *Effect of Micronutrient Deficiencies on Plant Stress Responses*, in: Hyderabad A.P., Parvaiz A. (eds.) *Abiotic Stress Responses in Plants*, Springer, New York, pp. 389-330

Jelali N., Wissala M., Dell'Orto M., Abdelly C., Gharsalli M., Zocchi G., 2010. *Changes of metabolic responses to direct and induced Fe deficiency of two Pisumsativum cultivars*, Environmental and Experimental Botany, 68(3):238–246

Jiménez S.,Ollat N., Deborde C., Maucourt M., Rellán-Álvarez R., Moreno M.Á., Gogorcena Y., 2011. *Metabolic response in roots o of Prunusrootstock submitted to iron chlorosis*, Journal of Plant Physiology, 168(5):415–423

Jin C.W., He Y.F., Tang C.X., Wu P., Zheng S.J., 2006. *Mechanisms of microbial enhanced iron uptake in red clover*, Plant Cell and Environment, 29:888–897

Jin C.W., You G.Y., He Y.F., Tang C., Wu P., Zheng S.J., 2007. *Iron deficiency-induced secretion of phenolics facilitates the reutilization of root apoplastic iron in red clover*, Plant Physiology, 144: 278-285.

Kim S.A., Guerinot M.L., 2007. *Mining iron: Iron uptake and transport in plants*, FEBS Letters, 581:2273–2280

Kosegarten H., Hoffmann B., Rroco E., Grolig F., Glüsenkamp K.H., Mengel K., 2004. *Apoplastic pH and FeIII reduction in young sunflower (Helianthus annuus) roots*, PhysiologiaPlantarum, 122(1):95–106

LehmannR.G., Cheng H.H., HarshJ.B., 1987. Oxidation of phenolic acids by iron and manganese oxides, Soil Science Society of American Journal, 51:352-356

Lindsay W.L., Schwab A.P., 1982. *The chemistry of iron in soils and its availability to plants*, Journal of Plant Nutrition, 5(4-7):821-840

Loeppert R.H., 1986. *Reactions of iron and carbonates in calcareous soils*, Journal of Plant Nutrition, 9(3-7):195-214

López-Bucio J., Nieto-Jacobo M.F., Ramírez-Rodríguez V.,Herrera-Estrella L., 2000. *Organic acid metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils*, Plant Science, 160:1–13

López-Millán A.F., Morales F., Gogorcena, Y., Abadía A., Abadía J., 2009. *Metabolic responses in iron deficient tomato plants, Journal of Plant* Physiology, 166(4):375–384

López-Millán A.F., Morales F., Abadía A., Abadía J., 2000. *Effects of iron deficiency on the composition of the leaf apoplastic fluid and xylem sap in sugar beet. Implications for iron and carbon transport*, Plant Physiology, 124:873–884

López-Millán A.F., Morales F., Abadía A., AbadíaJ., 2001. *Iron deficiency-associated changes in the composition of the leaf apoplastic fluid from field-grown pear (Pyruscommunis L.) trees*, Journal of Experimental Botany, 52:1489–1498

Marschner, H., 1995. *Mineral Nutrition of Higher Plants*, Academic Press, London

Mengel K., 1994. *Iron availability in plant tissues-iron chlorosis on calcareous soils*, Plant and Soil, 165(2):275-283

Rabotti G., De Nisi P., Zocchi G., 1995. *Metabolic implications in the biochemical responses to iron deficiency in cucumber (Cucumissativus L.) roots*,Plant Physiology, 107:1195–1199

Rabotti G., Zocchi G., 1994. *Plasma membrane-bound H+-ATPase and reductase activities in Fe-deficient cucumber roots*, PhysiologiaPlantarum, 90:779–85

Reid R., Smith F.A., 2002. *The cytoplasmic pH Stat*, in: Rengel Z. (ed.), *Handbook of Plant Growth: pH as the Master Variable*, Marcel Dekker, New York, pp. 47-67

Rellán-Álvarez R., Andaluz S., Rodríguez-Celma J., Wohlgemuth G., Zocchi G., Álvarez-Fernández A., Fiehn O., López-Millán A.F., Abadía J., 2010, *Changes in the proteomic and metabolic profiles of Beta vulgaris root tips in response to iron deficiency and resupply*, BMC Plant Biology, 10:120

Rice-Evans C.A., Miller N.J., Paganga G.,1997.*Antioxidant properties of phenolic compounds*, Trends in Plant Science, 2:152-159

Santi S., Cesco S., Varanini Z., Pinton R., 2005. *Two plasma membrane H+-ATPase genes are differentially expressed in iron-deficient cucumber plants*, Plant Physiology and Biochemistry, 43:287-292

Santi S., Schmidt W., 2008. *Laser microdissection-assisted analysis of the functional fate of iron deficiency-induced root hairs in cucumber*, Journal of Experimental Botany, 59:697–704

Santi S., Schmidt W., 2009. *Dissecting iron deficiency-induced proton extrusion in Arabidop-sis roots*, New Phytologist,183:1072–84

Singh S., Anand A., Srivastava P.K., 2012.*Regulation and properties of glucose-6-phosphate dehydrogenase: A review*, International Journal of Plant Physiology and Biochemistry, 4(1):1-19

Ström, L., Owen A.G., Godbold D.L., Jones D.L., 2005. *Organic acid behavior in calcareous soil implication for rhizosphere nutrient cycling*, Soil Biology and Biochemistry, 37:2046-2054

TewariR.K., KumarP., SharmaP.N., 2007. *Oxidative Stress and antioxidant Responses in Young Leaves of Mulberry Plants Grown Under Nitrogen, Phosphorus or Potassium Deficiency*, Journal of Integrative Plant Biology, 49(3):313−322

Thimm O., Essigmann B., Kloska S., Altmann T., Buckhout T.J., 2001. *Response of Arabidopsis to iron deficiency stress as revealed by microarray analysis*, Plant Physiology,127:1030-1043.

TylerG., StromL., 1995. *Differing organic acid exudationpatterns explain calcifuge and acidifugebehaviour ofplants*, Annales of Botany, 75:75–78

ViganiG., 2012. *Discovering the role of mitochondria in the iron deficiency-induced metabolic responses of plants*, Journal of Plant Physiology, 169:1– 11

Vigani G., Zocchi G., 2009. *The fate and the role of mitochondria in Fe-deficient roots of Strategy I plants*, Plant Signaling andBehaviour, 4:375-379

Vigani, G., Donnini, S., Zocchi, G., 2011. *Metabolic adjustment under Fe deficiency in roots of Dicotyledoneous plants*, in: Dincer, Y. (ed.), *Iron deficiency and its complications*, Nova Science, New York, pp. 1-27.

Zhang F.S., Romheld V., Marschner H., 1991. *Role of the root apoplasm for iron acquisition by wheat plants*, Plant Physiology, 97:1302–1305

Zocchi G., 2006. *Metabolic changes in iron-stressed dicotyledonous plants*, in: Barton L.L., Abadía J. (Eds.), *Iron nutrition in plants and rhizospheric microorganisms*, Springer, Dordrecht, pp. 359–370

Zohlen A., Tyler G., 2000. *Immobilization of tissue iron on calcareous soil: differences between calcicole and calcifuge plants*, Oikos, 89(1): 95–106

Zohlen A.,2002. *Chlorosis in wild plants: is it a sign of iron deficiency?*, Journal of Plant Nutrition, 25(10):2205-2228

CHAPTER 4

Low iron availability and phenolic metabolism in a wild plant species (*Parietaria judaica* L.)*

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ABSTRACT

Plant phenolics encompass a wide range of aromatic compounds and functions mainly related to abiotic and biotic environmental responses. In calcareous soils, the presence of bicarbonate and a high pH cause a decrease in iron (Fe) bioavailability leading to crop yield losses both qualitatively and quantitatively. High increases in phenolics were reported in roots and root exudates as a consequence of decreased Fe bioavailability suggesting their role in chelation and reduction of inorganic Fe(III) contributing to the mobilization of Fe oxides in soil and plant apoplast. Shikimate pathway represents the main pathway to provide aromatic precursors for the synthesis of phenylpropanoids and constitutes a link between primary and secondary metabolism. Thus the increased level of phenolics suggests a metabolic shift of carbon skeletons from primary to secondary metabolism. *Parietaria judaica*, a spontaneous plant well adapted to calcareous environments, demonstrates a high metabolic flexibility in response to Fe starvation. Plants grown under low Fe availability conditions showed a strong accumulation of phenolics in roots as well as an improved secretion of root exudates. *P. judaica* exhibits enhanced enzymatic activities of the shikimate pathway. Furthermore, the non-oxidative pentose phosphate pathway, through the transketolase activity supplies erythrose-4-phosphate, is strongly activated. These data may indicate a metabolic rearrangement modifying the allocation of carbon skeletons between primary and secondary metabolism and the activation of a non-oxidative way to overcome a mitochondrial impairment. We suggest that high content of phenolics in *P. judaica* play a crucial role in its adaptive strategy to cope with low Fe availability.

Keywords: iron deficiency, metabolic rearrangement, *Parietaria judaica*, phenolic compounds, shikimate pathway

4.1. **Introduction**

Iron (Fe) is an essential element for life. Due to its redox characteristics Fe plays an important role in cell metabolism. In particular in mitochondrial and photosynthetic electron transport chains it is directly

related with energy-yielding processes. Iron is one of the most abundant elements in the Earth's crust but, in spite of this, in oxygenated environments it is found in ferric forms (Fe(III)), principally oxides and hydroxides that are very stable and scarcely soluble at neutral pH, reaching a concentration far below that required for optimal plant growth [1]. In alkaline soils, mainly calcareous ones, Fe solubility dramatically decreases and it is not readily taken up by the plant, leading to consistent crop yield losses both qualitatively and quantitatively. Accordingly, plants have evolved different strategies to face Fe acquisition. In general Fe acquisition involves two principal strategies in plants: a scavenging strategy and a mining one. Scavenging strategy concern those mechanisms implemented for soil exploration and results in changes in root growth, morphology and architecture, as well as in increased in root hairs density. Mining mechanisms are aimed at mobilizing non bioavailable forms of Fe and include three chemical processes, reduction, acidification and chelation. Obviously these two strategies operate together.

Dicots and non Poaceae monocots have evolved a reduction based mining mechanism (Strategy I) localized at the root cell membrane to cope with low Fe availability [2]. Strategy I includes: a Fe specific mechanism of reduction via a transmembrane Fe(III) Chelate Reductase (FC-R), which reduce ferric to ferrous ions that are transported inside the cell by a Fe regulated transporter (IRT); and a non-specific mechanism of protonation by means of the H^+-ATPase that extrudes protons acidifying the rhizosphere and consequently increasing Fe solubility [3,4,5]. In addition plants exude a large variety of substances even further acidifying the rhizosphere and acting as organic complexing agents that can contribute to mobilize minerals in soil solution [6]. In many species phenolics have been recognized as an important component of root exudates [7, 8]. Phenolics are a wide group of hydroxylated aromatic compounds found only in microorganisms and plants. They are secondary metabolites that show a huge diversity of structures, from rather simple structures, as phenolic acids, through polyphenols, for instance flavonoids, that comprise several groups, to polymeric compounds such as tannins or lignin. Phenolics play multiple chemical and biological functions in plants mainly related with adaptation to environmental changes [9-12]. They represent a clear example of metabolic plasticity as plants are able to

respond to external stresses rapidly inducing their biosynthesis in a reversible way [13]. Countless works reported the increase of phenolic content in plants tissues, roots and root exudates as a response to different biotic and abiotic stresses [14-19]. Among these it has been registered an induction of phenolics secretion by roots under Fe-deficient condition [17, 20, 21]. A multiple role of phenolics as mediators in Fe acquisition has been suggested. Phenolics could operate in three ways: by directly improving Fe solubility mainly due to their reductant and chelating properties; by mediating the reutilization of root apoplastic Fe and by their allelopathic activity influencing rhizospheric microbial communities to produce siderophores and auxin [22, 23]. Hence phenolics can indeed affect nutrient availability [19, 24].

Several studies have underlined that under abiotic stress plants have to face the trade-off in carbon skeletons allocation between growth, maintenance and defence [25-27].

The shikimate pathway is the common route to provide aromatic precursors for further secondary metabolites. It also represents a link between primary and aromatic secondary metabolism. It was estimated that 60% of total plant biomass is composed of molecules that have traversed the shikimate pathway [25]. Successfully coping with a stress conditions such as Fe deficiency presupposes a fine metabolic rearrangement arising from a contrasting physiological requirements. In fact, plants have to manage, on one hand, with an increased demand of ATP and NADH required for Fe acquisition by Strategy I mechanisms concomitant to the impaired mitochondrial transduction of energy and, on the other hand, the carbon skeleton flux shifting towards the production of secondary metabolites.

Parietaria judaica (L. 1753) is a wild perennial and sinantropic dicot that from a nutritional point of view behave as an “indifferent” plant. It grows in both acidic and alkaline soils but it usually represents the most widespread flora in highly calcareous and hostile environments such as wall cracks exposed to the sun. In these calcareous environments *P. judaica* does not show any symptom of chlorosis which is the primary symptom of Fe deficiency. Previous data showed that *P. judaica* activates all Strategy I mechanisms as a response to Fe deficiency but the degree of

activation does not explain the efficiency through which this species adapts to low Fe bioavailability. Significant root morphological modifications have also been observed in Fe-deficient growth conditions [28, 29]

High content in phenolics is a characteristic of *P. judaica* species; nevertheless it has been observed a significant increase of them in roots and exudates when subject to Fe deficiency [29]. In this work we grew *P. judaica* in different direct or induced Fe-deficient hydroponic conditions: a control full nutrient solution (+Fe), a minus Fe solution (-Fe) deprived of Fe, an alkaline full nutrient supplemented with $NaHCO_3$ and $CaCO_3$ that brings the pH to 8.3 and mimics a calcareous environment, an alkaline full nutrient solution buffered with Tricine (Tric) adjusted to pH 8.3. Tric treatment was performed in order to discriminate the response caused only by the pH effect from that due to the presence of the bicarbonate ion. We focused our attention on the mechanisms through which *P. judaica* shifts carbon skeletons from primary to secondary metabolism. We also investigated how primary metabolism rearranges to supply the substrates for shikimate pathway (erythrose 4-phosphate, E4P and phosphoenolpyruvate, PEP) considering that under Fe deficiency there is a greater demand of energy and reducing power as a consequence of the activation of proton extrusion and Fe(III) reduction, and on the other hand an impaired mitochondrial NADH turnover.

4.2. **Results**

4.2.1. *Root modifications*

Previous works have reported modifications in root morphology in *P. judaica* grown in Fe deficiency conditions pointing out to the presence of an increased number of secondary roots as well as the onset of clusters of short and thick rootlets [28]. After 7-d treatment we observed results similar to those obtained before. In fact in all treatments we found a shorter but more branched radical system in terms of both adventitious and lateral roots with respect to control. Anyway, it can be noted a different branching pattern in -Fe, Bic and Tric treatment (Fig. 1). –Fe roots develop a lot of very short sketches of lateral roots (Fig.1 B). In particular, we observed a more similar root system in Bic and Tric plants

even though shoots show a quite different response. In fact, Bic shoots exhibit a slowdown in growth and more chlorotic leaves respect to Tric (Fig. 1C and 1D).

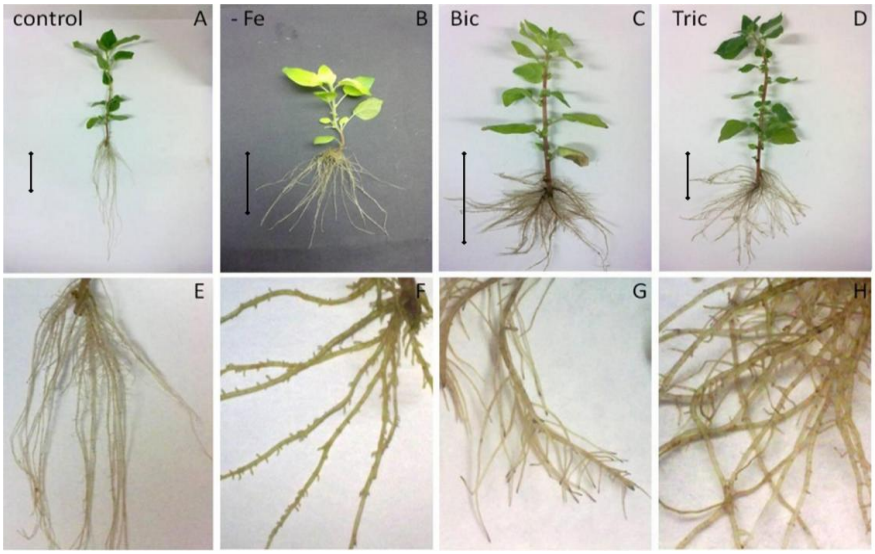

Figure 1. *P. judaica* plants obtained after 7d of treatment. A, B, C, D are images of the whole plant in control, Fe starvation (-Fe), bicarbonate (Bic) and alkaline organic buffer (Tric) condition; E, F, G, H are detailed images of the respective root systems. The bar beside the images A, B, C, D indicate the scale.

4.2.2. *Phenolic compounds in root and exudates*

Phenolic concentration was measured in roots and exudates of 7-d-old plants. The content of soluble phenolics found in root extracts of all stress treatments show a higher values increased by 40-50 % respect to +Fe but no significant differences were found between -Fe, Bic and Tric conditions. Total phenolic content obtained through methanolic extraction also show an increase in polar phenolics in comparison with +Fe of nearly 60% for -Fe and Tric treatments while in Bic we found a remarkable increase (nearly 4-fold compared to the control and more than 1-fold respect to the other treatments). Furthermore, it can be

noted that in Bic condition the content of polar phenolics is higher than that of soluble ones (Fig. 2 A). Total phenolic content found in field samples collected from alkaline substrates are comparable to those measured in samples grown in hydroponic samples at high bicarbonate content (data not show). In root exudates (Fig. 2 B) a noteworthy increase in phenolics in all stress conditions (5- to 8-fold) with respect to +Fe was detected; while Bic and Tric conditions show comparable phenolic content in exudates, -Fe plants extrude a significant higher amount.

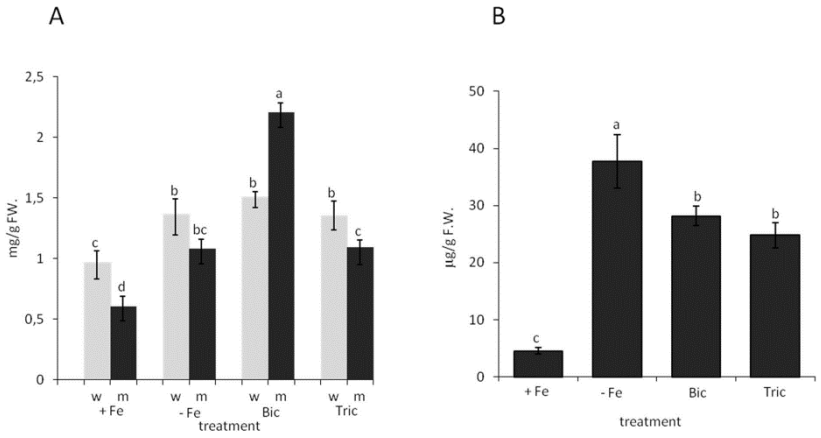

Figure 2. A. Total phenolic content in roots extract with water (w) and methanol (m), B. Total phenolics content in root exudates. Significant differences ($P<0.05$) are marked with different letters. FW= fresh weight.

4.2.3. *Responses of primary metabolism*

Fe deficiency induces Strategy I mechanisms to facilitate Fe acquisition, among these FC-R and H^+-ATPase activities, which require a constant production of energetic substrates. As glucose is the main and most immediate source of energy in plant metabolism, some key enzymatic activities belonging to the glycolytic pathway have been determined.

Table 1 shows the results obtained assaying the hexokinase (HK), glyceraldehide 3-phosphate dehydrogenase (GAPDH) and phosphoenolpyruvate carboxylase (PEPC) activities on root soluble extracts.

Enzymes	+Fe		-Fe		Bic		Tric	
HK	67.31 ± 5.20	c	119.76 ± 10.95	a	88.99 ± 8.75	b	81.85 ± 7.69	b
GAPDH	105.60 ± 10.40	d	205.93 ± 15.00	a	143.27 ± 7.80	c	170.63 ± 12.50	b
PEPC	62.54 ± 4.70	b	90.80 ± 8.90	a	83.77 ± 5.70	a	24.61 ± 3.70	c

Table 1. Enzymatic activities of Hk (Hexokinase), GAPDH (glyceraldehyde 3-phosphate dehydrogenase) and PEPC (phosphoenolpyruvate carboxylase) measured in dialysed root soluble extracts from +Fe, -Fe, Bic and Tric treated plants. Data are expressed as the means ± SD (*n*=12). Enzyme activity is expressed as nmol $min^{-1}mg^{-1}$ prot. Significant differences are labelled with different letters.

HK catalyzes the first step of glycolysis and has been shown to be involved in a complex regulatory mechanism related with shoot and root growth [30]. GAPDH is the first enzyme of the so called energy harvesting glycolytic steps. HK and GAPDH catalysed reactions that generates H^+ and could play an important role both in equilibrating the cytosolic pH and in supplying protons for the H^+-ATPase [31, 32].

All stress conditions induce an activation of both HK and GAPDH activity confirming the increasing rate of carbohydrate catabolism. -Fe root extracts show activity increase of about 78% for HK and 95% for GAPDH with respect to the control. In bicarbonate supply and tricine buffer conditions HK activity enhancement respect to the control was of 30-20% and no statistical difference was found between them. Regarding GAPDH in Tric treatment, the activity was increase by +62% with respect to the control whereas Bic condition shows a moderate increase (+36%).

PEPC is responsible for the assimilation of available HCO_3^- into oxalacetate which is further converted in malate. All Fe deficiency conditions show different PEPC activity. -Fe and Bic extracts exhibit an increase of 45% and 34% in PEPC activity, respectively. On the contrary, Tricine buffered condition shows a marked reduction of PEPC activity (about -60%) with respect to the control.

Western blot analyses performed for GAPDH and PEPC are shown in Fig. 3. The results obtained using antibodies against GAPDH reveal more intense bands in all treatments respect to the control. PEPC Western blot results confirmed previous works [29, 33]; all treatments show at least two bands, even more or less evident, corresponding to an apparent molecular mass of 103 kDa and 108 kDa, respectively. A more intense band at 108 kDa was detected -Fe condition with respect to the control, while in Bic treatment it is comparable with control and less intense with respect to -Fe condition. Also in Bic condition the 103 kDa band appears more pronounced than the 108 kDa band. Overall Tric condition show the lower band intensity respect to the other stressed treatments.

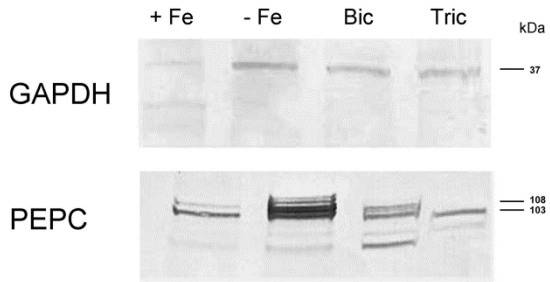

Figure 3. Western blot analysis of GAPDH and PEPCase carried out on dialysed root extracts from plants growing for 7 d in control (+Fe), Fe starvation (-Fe), bicarbonate supply (Bic) and alkaline tricine buffered (Tric) condition. The experiment was conducted twice with the same results.

4.2.4. *Shikimic pathway enzymes*

As shikimic acid is a precursor of the biosynthesis of aromatic compounds and consequently phenolics, some enzymatic activities belonging to the shikimate pathway [3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS); shikimate dehydrogenase (SDH); shikimate kinase (SK)] and phenylalanine ammonia-lyase (PAL, the first enzyme of phenylpropanoid pathway) were assayed. Table 2 reports the enzymatic activities analysed.

Enzymes	+Fe		-Fe		Bic		Tric	
SKDH	4.38 ± 0.71	c	24.08 ± 1.98	a	14.38 ± 1.34	b	15.26 ± 0.99	b
SK	72.15 ± 9.41	c	105.93 ± 8.20	b	170.00 ± 15.76	a	102.23 ± 11.03	b
PAL	4.77 ± 0.26	c	8.88 ± 0.39	a	8.80 ± 0.59	a	5.92 ± 0.35	b

Table 2. Enzymatic activities of SKDH, SK and PAL assayed in root extracts of control, -Fe, Bic and Tric treated plants. Data are the means ± SD (*n*=12). Enzyme activity is expressed as nmol min^{-1} mg^{-1} prot for SKDH and SK. For PAL one unit of enzyme activity was defined as the amount of PAL that produced 1 µmol of cinnamic acid within 1 h and was expressed as A290 µmol cinnamic acid/mg protein h^{-1} for PAL. Differences among data with statistical significance ($P < 0.05$) are expressed with different letters.

DAHPS is a key enzymatic step in the shikimate pathway since it catalyses the first reaction which condenses phosphoenolpyruvate (PEP) and erythrose 4-phosphate (E4P) giving 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP). Several studies have indicated the existence of DAHPS isoenzymes in plants. Their expression is influenced by many kinds of environmental stimuli, thus modulation of the whole DAHPS activity seems to be the result of differential isoenzyme expression [34, 35]. Unfortunately, DAHPS activity could not be assayed due to the commercial unavailability of the substrate E4P (as mentioned in Materials and Methods). Western blot analysis of DAHPS shows an immunodecoration in correspondence with a polypeptide with the apparent mass of 45 kDa for all treatments (Fig.4). In the absence of Fe a more intense band is present with respect to the control. Surprisingly, Bic condition shows a much weakened band notwithstanding its high content in phenolics. Tric root extracts present an intermediate condition between the control and Bic treatment.

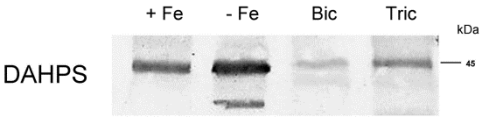

Figure 4. Western blot analysis of DAHPS carried out with root extracts of 7 d plants from control (+Fe), -Fe, Bic and Tric treatments.

Shikimate dehydrogenase (SKDH) catalyzes the reversible NADPH-dependent reaction of 3-dehydroshikimate to shikimate. In plants SKDH acts as a bifunctional enzyme that catalyzes also the former shikimate step. A significant enhancement of SKDH activity was observed in all treatments respect to the control. In the absence of Fe a 5-fold increase in SKDH activity was shown, while Bic and Tric root extracts exhibit an increase of about 3-fold without significant differences between them. SK is the fifth enzyme of the shikimate pathway and catalyses the specific phosphorylation of the 3-hydroxyl group of shikimic acid using ATP as a co-substrate. SK activity was affected in all treatments showing a significant increase compared with the control. Bicarbonate supply condition shows the highest SK activity (2.5-fold), while –Fe and Tric treatments increase by about 40-50% with respect to the control and do not show significant differences with each other. The ubiquitous higher plant enzyme phenylalanine ammonia-lyase (PAL) is a key biosynthetic catalyst in phenylpropanoid assembly. PAL is the first enzyme in phenylpropanoid pathway and catalyses the non-oxidative deamination of L-phenylalanine to trans-cinnamic acid. As most natural phenolic compounds derived from trans-cinnamic acid PAL is considered to play a pivotal role in channeling carbon flux from primary metabolism to phenolic synthesis. In all stressed growing conditions PAL activity increases. In –Fe and Bic treated plants PAL exhibit a significant enhancement (+86%). A moderate increase of 22% was recorded in the Tric condition. Western immunoblot analysis performed using antibodies raised against PAL is shown in Fig. 5.

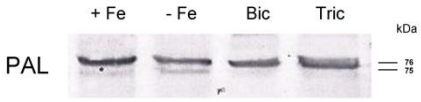

Figure 5. Immunological detection of PAL in rootextracts of 7 d-old plants from control (+Fe), -Fe, Bic and Tric treatments.

In purified extracts of roots grown in all treatments two isoforms of apparent molecular mass of 76 and 75 kDa were identified. The 76 kDa polypeptide appears more intense than the 75 kDa one in all lines, moreover Tric treatment extract exhibits a wider marked band. The 75 kDa isoform is more evident in –Fe samples.

4.2.5. *Oxidative and non-oxidative pentose phosphate pathway activation (OPPP and non-OPPP)*

Glucose 6 phosphate dehydrogenase (G6PDH) catalyzes the first oxidative reaction of the OPPP converting glucose 6-phosphate in 6- gluconolactone producing NADPH. G6PDH is the rate-limiting step of OPPP. This enzyme is regulated by $NADP^+$ availability. It has been speculated to be an important source of NADPH in non photosynthetic tissues and to be involved in maintaining the redox potential necessary to protect cell against oxidative stress [36]. Transketolase (TK) is considered a key enzyme of the non-oxidative reactions that links OPPP to glycolysis and catalyzes a series of reversible interconversion reactions of sugars. Through TK activity erythrose 4-phosphate and xylulose 5-phosphate can be obtained from glyceraldehyde 3- phosphate (GAP) and fructose 6-phosphate.

The enzymatic activities of G6PDH and TK, belonging to OPPP and non-OPPP, respectively and involved in the supply of initial substrates for the shikimate pathway were also analyzed (Tab. 3). Under Fe deficiency conditions (-Fe, +Bic, Tric) the following can be observed with respect to control: a) a lower G6PDH activity; b) a remarkable increase in TK activitiy (-Fe, Bic and Tric activities were enhanced by about 10-, 7- and 3-fold, respectively).

Enzymes	+Fe		-Fe		Bic		Tric	
G6PDH	56.68 ± 4.30	d	6.23 ± 0.75	a	24.00 ± 2.36	b	7.65 ± 0.81	c
TK	13.30 ± 1.24	d	139.88 ± 15.24	a	89.25 ± 8.71	b	35.48 ± 3.12	c

Table 3. OPPP Glucose 6-phosphate dehydrogenase (G6PDH) and non-OPPP Transketolase (TK) enzymatic activities assayed in root extracts dialysed and purified respectively in control, -Fe, Bic and Tric treated plants. Data are the means ± SD (n=12). Enzyme activity is expressed as nmol min^{-1} mg^{-1} prot. Statistical differences ($P < 0.05$) are expressed with different letters.

Figure 6 shows the immunological localization of a band corresponding to the apparent molecular weight of 74 kDa. This datum is in agreement with what reported in literature [37]. In fact TK is an homotetramer constituted of two dimers of about 74kDa. A very marked band, more intense respect to the control is observed in –Fe treated plants line. In Fe deficiency induced by bicarbonate a more noticeable band in comparison with the control is also detected, while the Tric treatment is comparable with the control.

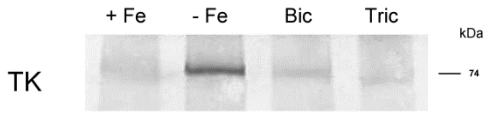

Figure 6. Western blot analysis of TK carried out on root extracts of 7 d plants from control (+Fe), -Fe, Bic and Tric treatments. (The antibody (*tkt3*) was raised in rabbits, and the primary antibody dilution was 1:5000;)

4.3. **Discussion**

Plants in natural environment must face continuously with abiotic and biotic stresses. Plant responses to environmental constrains are complexes and involve multiple traits. Anyway, the primary response takes place at metabolic level. Metabolic responses to abiotic stresses concerned interactions and interferences with many molecular pathways. Iron chlorosis is one of the limiting factors in areas of alkaline calcareous soils. Along the evolution, calcicole wild plants have shaped their adaptive responses to these complexes set of environmental stresses. For this reason studying wild plants responses could provide interesting insights useful to increase our knowledge on how plants can overcome these constraints. Increase in phenolic compounds is one of the general stress responses in plants resulting in a shift of carbon flux from primary to secondary metabolism. Moreover, under stress conditions, plants have to cope with the efficient distribution of limited resources between physiological processes and the activation of mechanisms to acquire the depleted nutrients. The metabolic flexibility is therefore the main feature to cope successfully with environmental limitations. A limited availability of nutrients may determine root growth, root proliferation and specific functional responses that depend on genetic makeup as well as the prevailing nutrient status of the plant [38, 39].

P. judaica can grow in highly calcareous environments and displays diversified responses to Fe deficiency that allow it to adapt efficiently. Under Fe deficiency conditions general root morphological modifications have been described in different species. Morphological changes induced by Fe deficiency include swelling of root tips and formation of lateral roots, root hairs, and transfer cells that increase the root surface in contact with the external medium thereby increasing the Fe uptake capability [40-42]. Consistently with previous works [28, 29] it was observed in *P. judaica* after 7-d of direct or induced Fe deficiency treatments a proliferation of lateral roots that enhance the total absorptive root surface. Albeit the increase of root surface it can be recognized a different branching pattern among the differently treated plants. Lack of Fe induces a high appearance of lateral roots primordia all along the adventitious roots (Fig. 1B and 1F) while Bic and Tric treatments showed shorter root systems with well developed lateral roots distributed in patches (Bic) or uniformly (Tric), respectively (Fig. 1G and 1H). These variances are probably due to the different metabolic status of the plants. In –Fe plants have to face with an absolute lack of Fe that poses severe energetic problems [43]. In fact, on one hand mitochondria have been indicated as the main victim of Fe starvation [44] and on the other hand the biosynthesis of chlorophyll decreases dramatically [45]. The Fe-starved plant has to allocate its restricted energetic resources among searching for available Fe and physiological maintenance of other vital processes. In fact, at root level –Fe plants produce just rootlets that constitute the most active portion of the root system (Fig.1B and 1F). In this way the plant maximizes the response per unit biomass and energy. In Fe alkaline growth conditions Fe bioavailability becomes very low so plants address their efforts to acquire the nutrient increasing the root surface and finally succeeding in retrieve it. However, under bicarbonate supply shoot features denote a slight chlorosis with respect to alkaline tricine buffer condition (Fig.1C and 1D) suggesting a specific stress caused probably by the presence of the bicarbonate ion itself.

Phenolic compounds fulfill an important role in the adaptive response to Fe deficiency carrying out important antioxidant and chelating properties [10, 28, 46, 47]. In particular, it was suggested their role in re-mobilizing apoplastic Fe [17]. The results of total phenolics content obtained both in

extracts and exudates show a significant increase in all direct or induced Fe starved conditions compared with the control. These data confirm in *P. judaica* the high increase in these compounds as a response to an external stress. We found the highest content of total phenolics in bicarbonate treated root tissues. Furthermore, in Bic condition the total phenolic content extracted with methanol was higher than that obtained with water (Fig. 2A). This might be attributable to an increased root lignification occurring in these samples (data not show). The release of phenolics through root exudates was greater in -Fe condition while a diminution of about 25% was shown in alkaline conditions (Fig. 2 B). In fact, under Fe starvation plants activate exudation and maintain this response as they do not receive any feedback from Fe uptake. In Bic and Tric conditions Fe is present in the solution in a non available form and precipitates in the apoplast (data not show), so these Fe deposits could be mobilized as the root exudes phenolics. These data suggest that phenolics play a pivotal role in the adaptive strategy of *P. judaica* to cope with low Fe availability.

The activation of reduction processes and the increase in proton extrusion under Fe deficiency requires an increase in ATP, NAD(P)H and H^+. Accordingly, an enhancement in the rate of glycolysis could fulfil this demand [32]. Several enzymes of glucose catabolism have been shown to increase their activity [48-52]. In *P. judaica* we assayed HK and GAPDH which are protogenic enzymes. It was found an increased activity of both enzymes in all treatments, especially in –Fe condition confirming the results found in other species [48].

PEPC catalyzes the fixation of bicarbonate to form oxalacetate/malate. It was pointed out as one of the enzyme that highly increases its activity under Fe deficiency [33, 53, 54]. PEPC plays a central role in Fe deficiency performing a double function: it is involved in the pH stat mechanism in the cytosol and it acts as a driving force for glycolysis [32] consuming PEP that is an inhibitor of phosphofructokinase (PFK). PEPC is responsible for the production of organic acids which are protogenic and can also replenish the TCA cycle. In both direct and induced Fe deficiency treatments the glycolytic enzyme HK, and GAPDH increased significantly their activity, but particularly in -Fe in which they almost double their activity (Table 1). The difference found in glycolytic enzymes and PEPC

activities could be looking at the root growth as dry weight (data not shown), while Bic plants have slow down their root growth, Tric roots grow at a comparable rate as the control. This fact could be interpreted as that the energetic substrates resulting from the increased rate of glycolysis are used in Bic treated plants mainly for adaptive processes such as the reduction based mechanism, phenolics and organic acids synthesis, whereas in Tric condition the improved glycolytic activities could be committed both to the increase in biomass and to the adaptive processes. Also in -Fe roots we registered a diminution of growth so in this case the highest rate of carbohydrates degradation through the glycolytic pathway indicates the effort carried out by plants to cope with serious limitations in the energy production due to impaired Fe-dependent mitochondrial activity. In Tric condition PEPC activity is lower than in the control suggesting that probably the consumption of PEP, that is channelled into the shikimate pathway and that forms pyruvate to enter in the TCA - considering that the mitochondrial activity is not impaired - is enough to run glycolysis at an adequate rate (Fig. 7).

As primary metabolism is more closely related with growth and development, there is increasing evidence that accumulation of phenolics in plant tissues is a common adaptive response. However, primary metabolism is the source of precursors for secondary metabolism so they are tightly linked.

Shikimic pathway is the main way to synthesize aromatic aminoacids, among which phenylalanine (Phe) is a common precursor of numerous phenolics compounds [34]. It has been pointed out that Phe-derived phenolics can constitute up to 30% of organic matter in some species [55].

Actually, around 20% of the total carbon fixed by photosynthesis in land ecosystems is incorporated into lignin, being the second main constituent of plant biomass after cellulose [56]. Furthermore, shikimate pathway represents the bridge through which carbon flux shifts from carbohydrate metabolism to secondary metabolism [57].

Figure 7. General overview of reactions linking primary and secondary metabolism in *P. judaica* roots growing for 7 d in control (+Fe), Fe starvation (-Fe), bicarbonate supply (Bic) and alkaline tricine buffered (Tric) condition.

PAL plays a central function at the branch point of phenylpropanoid derivative metabolism and is considered to be one of the key enzymes in the biosynthesis of flavonoids. PAL is also an important enzyme in plant stress response. Its biosynthesis is stimulated by pathogen attack, tissue wounding, UV irradiation, low temperature, or low levels of nitrogen, phosphate, or Fe [18].

Assays conducted on some key enzyme activities of the shikimate pathway and PAL in *P. judaica* match well with the observed high content and accumulation of phenolics. In almost all cases a high activity of SK, SKDH and PAL corresponds to a high content of phenolics, except for SKDH

which only under Bic condition showed a significant increase (Table 2). The Western blot analysis of DAHPS and PAL reveals a different regulation of the enzymatic activity depending on the treatment condition. While -Fe treated plants always show an increase in protein expression, in Bic condition protein expression is usually low despite a high enzymatic activity. In Bic condition it was recorded a significant slowdown in plant growth so it is reasonable to assume that also protein synthesis was inhibited.

An interesting question concerns the way to supply the primary substrates for shikimate pathway: E4P and PEP. PEP is obtained by glycolysis the rate of which was observed to be accelerated in all stress conditions. E4P can be supplied through two ways: OPPP and non-OPPP. G6PDH and TK could be seen as the representative enzymes of these two ways, respectively. The first reaction in the oxidative branch of the OPPP catalyzed by G6PDH, is the rate limiting step under physiological conditions. NADPH is a potent competitive inhibitor of the enzyme. Thus, the ratio of $NADP^+$/NADPH regulates the pathway. As the $NADP^+$ level rises, the flux through the pathway increases. The non oxidative branch of the pathway is regulated primarily by substrate availability.

Our results provide an interesting scenario. In all treatments an inverse correlation between G6PDH and TK activities was found. These results suggest a distinct change in the way of supplying substrates for the shikimate pathway. This behavior could be explained considering that the respiratory chain is less efficient under Fe deficiency leading, probably, to a lower NAD(P)H turnover [44]. Thus, the shift to a non-OPPP step could be favored, instead of producing more reducing power, through the OPPP. In addition, the non-OPPP way preserves carbon skeletons by avoiding decarboxylation.

In conclusion, *Parietaria judaica* is a Strategy I plant that implements many other mechanisms that allow it to successfully complete the life cycle in highly calcareous environments. The synthesis of phenolic compounds plays a central role in the adaptive strategy. We could observe that secondary metabolism constitutes the main concern under this stress condition as *P. judaica* sustains the supply of substrates using non

oxidative ways. The data we analysed confirm that under Fe deficiency the metabolic rearrangement takes place by modifying allocation of carbon skeletons between primary and secondary metabolism.

We also observe that *Parietaria judaica* has no problems to acquire Fe in high alkaline environments and we suggest that in a high carbonate environment the availability of bicarbonate itself constitutes the real factor of stress.

4.4. **Materials and Methods.**

4.4.1. *Plant material*

Cuttings of *P. judaica* from a mother plant have been obtained and transplanted in an aereated half nutrient solution for 10 days to radicate. Plants were successively transferred to 10 L plastic tanks (40 plants per tank) with four different hydroponic conditions: +Fe (control, full nutrient solution adjusted to pH 6.2), -Fe (full nutrient solution but total absence of Fe brought to pH 6.2), +Bic (full nutrient solution with addition of 0.5 gL^{-1} $CaCO_3$ and 15 mM $NaHCO_3$ which brought the pH to 8.3), Trc (full nutrient solution buffered with Tricine and adjusted to pH 8.3). Were required pH was adjusted with Na(OH). The treatment buffered with Tricine was introduced to distinguish the low availability of Fe due just to a high pH from the effect of the presence of bicarbonate.

Treatments were carried out for 7 days in a grown chamber under 16/18 h light/dark regime with cool-white light 200 μmol m^{-1} s^{-1}, 27/21°C temperature range, 65-75% relative humidity.

The composition of the full solution was as follow: (mM) 0.75 K_2SO4, 0.65 $MgSO_4$, 0.5 KH_2PO_4, 2 $Ca(NO_3)_2$, 0.1 FeIII-EDTA, (μM) 10 H_3BO_3, 1 $MnSO_4$, 0.5 $CuSO_4$, 0.5 $ZnSO_4$, 0.05 $(NH_4)_6Mo_7O_{24}$.

4.4.2. *Collection of root exudates*

Fifthteen 7-d-old plants for each treatment were transferred to 250 mL distilled water with 10% (v/v) Micropur (Katadyn, Minneapolis, MN) to prevent microorganisms' activity. Root exudates were collect for a period

of 24 h under continuous aeration. The collected exudates were acidified to pH 3.5-4 with HCl and freeze dried. The lyophilized material was resuspended in 3 mL distilled water and filtered through 0.45 μm Millipore Millex-HN.

4.4.3. *Determination of phenolic content*

Phenolic content in root tissues was carried out following two different extraction media: 100% distilled water and 100% methanol. Root samples of the different treatments were homogenized in the extraction solution with a volume ratio 1:1 (w/v). The homogenates were centifuged at 10,000g for 15 min. The supernatants were collected. The amount of phenolics in root extracts and exudates was determined spectrophotometrically at 750 nm with the Folin-Ciocalteau reagent using gallic acid as a standard [29]. Seven independent determinations were performed for each treatment.

4.4.4. *Soluble enzyme extraction and assay*

Roots sampled from different treatments were excised, rinsed in distilled water, and homogenate at 2 - 4 °C in one mL of buffer solution per gr of root F.W. with 20% (w/w) PVPP. Buffer solution contained: 50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 10% glycerol, 1mM EDTA, 14 mM ▫▫mercaptoethanol, 1mM PMSF and 10 mg mL^{-1} leupeptin. The homogenates were filtered through 4 layers of gauze and centrifuged at 13,000g at 4 °C for 15 min. The supernatants were centrifuged at 100,000g at 4°C for 30 min. The new supernatants were dialysed for 3 h at 4°C against 2 L of the same buffer solution without leupeptine. The dialysed extracts were used for enzymatic assays of *Phosphoenolpyruvate carboxylase* (PEPC), *Glucose-6-phosphate dehydrogenase* (G6PD), *Hexokinase* (HXK) and *Glyceraldehyde-3-phosphate dehydrogenase* (GAPDH).

PEPC (EC.4.1.1.31) activity was determined as in [33]. The reaction was started by adding an aliquot of soluble enzyme extract. *G6PD* (EC 1.1.1.49) and GAPDH (EC 1.2.1.12) were assayed according to [48]. In G6PDH assay the reaction was started by adding an aliquot of soluble extract, while in GAPDH assay the reaction started with the addition of 1

μg/mL of 3-phosphoglycerate kinase. *HXK* (EC 2.7.1.1.) activity was determined as in [58]. The reaction was triggered by adding an aliquot of soluble extract.

All the enzymatic activities were assayed by monitoring the absorbance change at 340 nm and were performed at 26°C and in a final volume of 1 mL. Three independent assays were performed for each treatment.

4.4.5. *Shikimate pathway enzyme extraction and assay*

Shikimate dehydrogenase (SKDH, EC 1.1.1.25), *shikimate kinase* (SHK, EC 2.7.1.71) enzyme extraction and assay were performed as described in [29]. Determination of shikimate pathway enzymes was carried out at 26°C by monitoring the variation in absorbance at 340 nm. Three independent enzymatic assays were performed for each treatment.

3-deoxy-D-arabino-heptulosonate-7-phosphate synthase (DAHPS) and Phenylalanine ammonia lyase (PAL) extraction and assay

DAHPS (EC 4.1.2.15) and PAL (PAL, EC 4.1.1.5) extraction was performed as follows: fresh root samples were homogenized in 100 mM potassium-phosphate buffer (pH 8.0) containing 1.4 mM 2-mercaptoethanol at a ratio of 1:1 (v/w) and 20% (w/w) PVPP was added. The homogenate was centrifuged at 15,000 g for 15 min at 4°C. The supernatant was purified through a Sephadex G-25M column (Pharmacia LKB, Uppsala) previously equilibrated with the same buffer.

PAL activity was assayed in purified extracts according to [59]. The absorbance was measured at 290 nm before and after incubation. One unit of enzyme activity was defined as the amount of PAL that produced 1 μmol of cinnamic acid within 1 h and was expressed as μmol cinnamic acid mg^{-1} prot. h^{-1}. *DAHPS* activity could not be determined because of the commercial unavailability of substrate erythrose-4-phosphate.

4.4.6. *Transketolase enzyme extraction and assay*

Transketolase (TK, EC 2.2.1.1.) extraction and activity were determined in accordance to [37]. The homogenation was performed in ice, with the addition of 20% (w/w) of PVPP and leupeptine as protease inhibitor. The

activity assay was performed by measuring the concentration decrease in NADH at 340 nm. The assay was carried out as in [37] in a final volume of 1 mL.

4.4.7. *Protein determination*

Protein was estimated according to [60] using BSA as a standard.

4.4.8. *Polyacrylamide gel electrophoresis and Western blot analysis of enzymes*

Enzymes were loaded on a discontinuous SDS-PAGE electrophoresis (3.75% stacking gel, 8% separating gel). After SDS-PAGE Western blot analyses were performed as in [33]. Polyclonal antibodies raised against PEPC [33], GAPDH [61], TK [37], DAHPS [62] and PAL [63] were used. Antibodies were diluted as indicated in the literature. Membranes were incubated at room T for 2 h with 1:25,000 diluted secondary antibodies (alkaline phosphatase-conjugated anti-rabbit IgG, Sigma).

4.4.9. *Statistical Analysis*

One way ANOVA analysis was used to test any statistical difference among data. Differences at $P < 0.05$ were considered to be significant. Statistical differences have been expressed by different letters. All Statistical analysis was carried out with SPSS v.15.0.1 (SPSS Inc.)

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References

[1] M.L Guerinot, Y. Yi, *Iron: nutritious, noxious and not readily available*, Plant Physiol. 104 (1994) 815-820.

[2] H. Marschner, *Different strategies in higher plants in mobilization and uptake of iron*, J. Plant Nutr. 9 (1986) 695-713.

[3] M. Dell'Orto, S. Santi, P. De Nisi, S. Cesco, Z. Varanini, G. Zocchi, *Fe-deficiency response in cucumber (Cucumis sativus L.) roots: involvement of plasma membrane H+-ATPase activity*, J. Exp. Bot. 51 (2000) 695-701.

[4] M. Dell'Orto, L. Pirovano, J.M. Villalba, J.A. Gonzales-Reyes, G. Zocchi, *Localization of the plasma membrane H+-ATPase in Fe-deficient cucumber roots by immunodetection*, Plant Soil 241 (2002) 11-17.

[5] A. Schikora, Schmidt, W., *Formation of transfer cells and H^+-ATPase expression in tomato roots under P and Fe deficiency*, Planta 215 (2002) 304-311.

[6] H.P. Bais, T.L Weir, L.G. Perry, S. Gilroy, J.M Vivanco, *The role of root exudates in rhizosphere interactions with plants and other organisms*, Annu. Rev. Plant Biol. 57 (2006) 233-266.

[7] S. Haettenschwiler, P.M. Vitousek, *The role of polyphenols in terrestrial ecosystem nutrient cycling*, Trends Ecol. Evol. 15 (2000) 238-243.

[8] T. Mimmo, R. Terzano, L. Medici, A. Lettino, S. Fiore, N. Tommasi, R. Pinton e S. Cesco,. *Interaction of root exudates with the mineral soil constituents and their effect on mineral weathering*, Geographical Reserch Abstracts (2012) 14.

[9] K.S. Gould, C. Lister, 2006 *Flavonoids functions in plants*, in: *Flavonoids: Chemistry*, Biochemistry and Applications, Andersen Ø. , K.R. Markham (eds), CRS press, Boca Raton, pp. 397-441.

[10] F.D. Dakora, D.A. Phillips, *Root exudates as mediators of mineral acquisition in low nutrient environments*, Plant Soil 245 (2002) 35-47.

[11] A.J. Parr, P. Bolwell, *Phenols in the plant and in man. The potential for possible nutritional enhacement of the diet by modifying the phenols content or profile*, J. Agric. Food Chem. 80 (2000) 985-1012.

[12] V. Lattanzio, A. Cardinali, V. Linsalata, *Plant Phenolics: A Biochemical and Physiological Perspective*, in: Cheynier V. , P. Sarni-Machado , S. Quideau (eds), *Recent Advances in Polyphenol Research*, Wiley-Blacwell, 2012, pp. 3-39.

[13] K.L. Melten, E.T. Ascheoug, R.M. Callaway, *Plant behavioural ecology: dynamic plasticity in secondary metabolites*, Plant Cell Environ. 32 (2009) 641-653.

[14] N.M.M. Saviranta, R.Julkunen-Tiittoo, E. Oksanen, R.O. Karjalainen, *Red clover (Trifolium pratense L.) isoflavones: root phenolic compounds affected by biotic and abiotic stress factors*, J. Sci. Food Agric. 90 (2010) 418-423.

[15] E. Sánchez-Rodríguez, D.A. Moreno, F. Ferreres, M.M. Rubio Wilhelmi, J.M. Ruiz, *Differential responses of five cherry tomatoe varieties to water stress: changes on phenolic metabolites and related enzymes*, Phytochemistry 72 (2011) 723-729.

[16] M.M. Oh, H. N. Trick, C.B. Rajashekar, *Secondary metabolism and antioxidants are involved in environmental adaptation and stress tolerance in lettuce*, J. Plant Physiol. 166 (2009) 180-191.

[17] C.W. Jin, C.Y. You, Y.F. He, C.Tang, P. Wu, S.J. Zheng, *Iron deficiency-induce secretion of phenolics facilitates the reutilization of root apoplastic iron in red clover*, Plant Physiol. 144 (2007) 278–285.

[18] R.A. Dixon, N.L. Paiva, *Stress-Induce Phenylpropanoid Metabolism*, Plant Cell 7 (1995) 1085-1097.

[19] S. Cesco, G. Neumann, N. Tommasi, R. Pinton, L. Weisskopf, *Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition*, Plant Soil 329 (2010) 1-25.

[20] H. Marschner, *Root-induced changes in the availability of micronutrients in the rhizosphere*, in Waisel, Y., A. Eshel , U. Kafkafi (eds.), *Plant Roots: the Hidden Half*, Marcel Dekker, New York, 1991, pp. 503-528.

[21] S. Susin, Abián J., Peleato M.L., Sánchez-Baeza F., Abadía A., Gelpi E., Abadía J., *Flavin excretion from roots of iron-deficient sugar beet (Beta vulgaris L.)*, Planta 193 (1994) 514-519.

[22] N. Slabbert, *Complexation of tannins with metal ions, in: Hemingway*, R.W., PE Laks (eds.) *Plant polyphenols, Synthesis, properties, significance*, Plenum press, New York, 1992 pp. 421-436.

[23] C.W. Jin, G.Y. You, S.J. Zheng, *The iron deficiency-induced phenolics secretion plays multiple important roles in plant iron acquisition underground*, Plant Signal. Behav. 3 (2008) 60–61.

[24] H. Hu, C. Tang, Z. Rengel, *Influence of phenolic acids on phosphorus mobilisation in acidic and calcareous soils*, Plant Soil 268 (2005) 173–180.

[25] D.A. Herms, W.J. Mattson, *The dilemma of plants: to grow or defend*, Quarter. Rev. Biol. 63 (1992), 283-335.

[26] R. Matyssek, J. Koricheva, H. Schnyder, D. Ernst, J.C. Munch, W. Oßwald , H. Pretzsch,. *Balance Between Resource Sequestration and Retention: A Challenge in Plant Science*, in: Matyssek R., H. Schnyder, W. Oßwald, D. Ernst, J.C. Munch , H. Pretzsch (eds.) *Growth and Defence in Plants*, Series Ecological Studies, 2012 pp. 3-24.

[27] G.R. Cramer, K. Urano, S. Delrot, M. Pezzotti, K. Shinozaki, *Effects of abiotic stress on plants: a systems biology perspective*, BMC Plant Biol. 11 (2011) 163.

[28] M. Dell'Orto, P. De Nisi, A. Pontiggia, G. Zocchi, *Fe deficiency responses in parietaria diffusa: a calcicole plant*, J. Plant Nutr. 26 (2003) 2057-2068.

[29] S. Donnini, P. De Nisi, D. Gabotti, L. Tato, G. Zocchi, *Adaptive strategies of Parietaria diffusa (M.K.) to calcareous habitat with limited iron availability*, Plant Cell Environ. 35 (2012) 1171-1184.

[30] W. Xiao, J. Sheen, J.Ch. Jang, *The role of hexokinase in plant sugar signal transduction and growth and development*, Plant Mol. Biol. 44 (2000) 451-461.

[31] W. Sakano, *Revision of biochemical pH-stat: involvement of alternative pathway metabolisms*, Plant Cell Physiol. 39 (1998) 467-473.

[32] G. Zocchi, *Metabolic changes in iron-stressed dicotyledonous plants*, In: L. Barton L.L., J. Abadía, *Iron nutrition in plants and rhizospheric microorganisms*, Springer, Dordrecht, 2006, pp. 359-370.

[33] P. De Nisi, G. Zocchi, *Phosphoenolpyruvate carboxylase in cucumber (Cucumis sativus L.) roots under iron deficiency: activity and kinetic characterization*, J. Exp. Bot. 352 (2000) 1903-1909.

[34] K.M. Hermann, *The shikimate pathway as an entry to aromatic secondary metabolism*, Plant Physiol. 107 (1995) 7-12.

[35] B. S. Winkel, *Metabolic channelling in plants*, Annu. Rev. Plant Biol. 55 (2004) 85-107

[36] N.J Kruger, A. von Schaewen, *The oxidative pentose phosphate pathway: structure and organisation*, Curr. Opin. Plant Biol. 6 (2003) 236–246.

[37] G. Bernacchia, G. Schwall, F. Lottspeich, F. Salamini, D. Bartels, *The transketolase gene family of resurrection plant Craterostigma plantaginum: differential expression during the rehydration phase*, Embo J. 14 (1995) 610-618.

[38] B. Forde, H. Lorenzo, *The nutritional control of root development*, Plant Soil 232 (2001) 51-68.

[39] J. López-Bucio, A. Cruz-Ramírez, L. Herrera-Estrella, *The role of nutrient availability in regulating root architecture*, Curr. Op. Plant Biol. 6 (2003) 280–287.

[40] V. Römheld, H. Marschner, *Iron deficiency stress induced morphological and physiological changes in root tips of sunflower*, Physiol. Plant. 53 (1981) 354–360.

[41] E.C Landsberg, *Transfer cell formation in the root epidermis: A prerequisite for Fe-efficiency?*, J. Plant Nutr. 5 (1982) 415–432.

[42] M. Muller, W. Schmidt, *Environmentally induced plasticity of root hair development in Arabidopsis*, Plant Physiol. 134 (2004) 409–419.

[43] O. Thimm, B. Essigmann, S. Kloska, T. Altmann , T.J. Buckhout, *Response of Arabidopsis to Iron Deficiency Stress as Revealed by Microarray Analysis*, Plant Physiol. 127 (2001) 1030-1043.

[44] G. Vigani, G. Zocchi, *The fate and the role of mitochondria in Fe-deficient roots of Strategy I plants*, Plant Signal. Behav. 4 (2009) 375-379.

[45] A. Castagna, S. Donnini, A. Ranieri, *Adaptation to iron-deficiency Requires Remodelling of Plant Metabolism: An Insight in Chloroplast Biochemistry and Functionality, Salinity and Water Stress*, Tasks for Vegetation Sciences 44 (2009) 205-212.

[46] R.G. Lehmann, H.H. Cheng , J.B. Harsh, *Oxidation of phenolic acids by iron and manganese oxides*, Soil Sci. Soc. Am. J., 51 (1987) 352-356.

[47] C.W. Jin, Y.F. He, C.X. Tang, P. Wu, S.J. Zheng, *Mechanisms of microbial enhanced iron uptake in red clover*, Plant Cell Environ. 29 (2006) 888–897.

[48] G. Rabotti, P. De Nisi, G. Zocchi, *Metabolic implications in the biochemical responses to iron deficiency in cucumber (Cucumis sativus L.) roots*, Plant Physiol. 107 (1995) 1195-1199.

[49] L. Espen, M. Dell'Orto, P. De Nisi , G. Zocchi, *Metabolic responses in cucumber (Cucumis sativus L.) roots under Fe-deficiency: a 31P-nuclear magnetic resonance in vivo study*, Planta 210 (2000) 985-992.

[50] J. Li, X. Wu, S. Hao, X. Wang, H. Ling, *Proteomic response to iron deficiency in tomato root*, Proteomics, 8 (2008) 2299–2311.

[51] S. Donnini, B. Prinsi, A.S. Negri, G. Vigani, L. Espen, G. Zocchi, *Proteomic characterization of iron deficiency responses in Cucumis sativus L. roots*, BMC Plant Biology 10 (2010) 268.

[52] R. Rellán Álvarez, S. Andaluz, J. Rodríguez-Celma, G. Wohlgemuth, G. Zocchi, A. Álvarez-Fernández, O. Fiehn, A.F. López-Millán, J. Abadía, *Changes in the proteomic and metabolic profiles of Beta vulgaris root tips in response to iron deficiency and resupply*, BMC Plant Biol. (2010) 10:120.

[53] A.D. Rombolà, W. Brüggemann, A.F. López-Millán, J. Abadía, M. Tagliavini, B. Marangoni, P.R. Moog, *Biochemical mechanisms of tolerance to Fe-deficiency in kiwifruit (A. deliciosa)*, Tree Physiol. 22 (2002) 869-875.

[54] T. Slatni, G. Vigani, I. Ben Salah, S. Kouas, M. Dell'Orto, H. Gouia, G. Zocchi, C. Abdelly, *Metabolic changes of iron uptake in N2-fixing common bean nodules during iron deficiency*, Plant Sci. 181 (2011) 151-158.

[55] H. Maeda, N. Dudareva, *The shikimate pathway and aromatic amino acid biosynthesis*, Plants. Ann. Rev. Plant Biol. 63 (2012) 73-105.

[56] F.J. Ruiz-Dueñas, Á.T. Martínez, *Microbial degradation of lignin: how a bulky recalcitrant polymer is efficiently recycled in nature and how we can take advantage of this*, Microbial Biotechnology 2 (2009) 164–177.

[57] V. Tzin, G. Galili, *New insights into the shikimate and aromatic amino acids biosynthesis pathways in plants*, Molecular Plant, 3 (2010) 956-972.

[58] G. Zocchi, P. De Nisi, M. Dell'Orto, L. Espen , P.M. Gallina, *Iron deficiency differently affects metabolic responses in soybean roots*, J. Exp. Bot. 58 (2007) 993-1000.

[59] Cahill, D. M., J. A. McComb, *A comparison of changes in phenylalanine ammonia-lyase activity, lignin and phenolic synthesis in the roots of Eucalyptus calophylla (field resistant) with E. marginata (suceptible) when infected with Phytophthora cinnamomi*, Physiol. Mol. Plant Pathol. 40 (1992) 315-332.

[60] M.M. Bradford, *A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding*, Anal. Biochem. 72 (1976) 248-254.

[61] I. Wawer, M. Bucholc, J. Astier, A. Anielska-Mazur, J. Dahan, A. Kulik, A. Wysłouch-Cieszynska, M. Zaręba-Kozioł, E. Krzywinska, M. Dadlez, G. Dobrowolska , D. Wendehenne, *Regulation of Nicotiana tabacum osmotic stress-activated protein kinase and its cellular partner GAPDH by nitric oxide in response to salinity*, Biochem. J. 429 (2010) 73-83.

[62] L. Orcaray, M. Igal, A. Zabalza , M. Royuela, *Role of Exogenously Supplied Ferulic and p-coumaric acids in mimicking the mode of action of acetolactate synthase inhibiting herbicides*, J. Agric Food Chem. 59 (2011), 10162-10168.

[63] Y. Osakabe, K. Nanto, H. Kitamura, S. Kawai, Y. Kondo, T. Fujii, K. Takabe, Y. Katayama , N. Morohosh,. *Immunocytochemical localization of phenylalanine ammonia-lyase in tissues of Populus kitakamiensis*, Planta 200 (1996) 13-19.

CHAPTER 5

Characterization of polyphenols in tissues and exudates of *Parietaria judaica* undergoing direct and induced iron deficiency growth conditions

ABSTRACT

Parietaria judaica is a sinantropic perennial weed that displays a high adaptation to live in very disturbed environments such as wall cracks exposed to the sun. In these highly calcareous environments *Parietaria* does not show any iron (Fe) deficiency symptom. As a Strategy I plant *P. judaica* activates all Strategy I mechanisms to acquire Fe, anyway it seems that its high content of phenolic compounds in roots and exudates could play a central role in its adaptive response regarding the low Fe bioavailability. In this work a HPLC characterization of phenolic compounds of *P. judaica* was carried out on samples collected in the field, showing that the most abundant phenolics are some mono- and di-caffeoylquinic derivatives. It was also examined the effect of direct and induced Fe deficiency of plants grown in hydroponics in different conditions on phenolic composition, both quantitatively and qualitatively, in roots and exudates. HPLC analysis revealed that the increase in phenolic compounds induced by Fe starvation, bicarbonate and alkaline buffer treatments in roots had similar HPLC profiles, but showing quantitative differences among the treatments. On the contrary, HPLC profiles of root exudates have shown altered profiles respect to the corresponding root tissues and demonstrate both qualitative and quantitative differences in phenolic compounds composition among root exudates from different growth conditions.

5.1. **Introduction**

Iron (Fe) is an essential micronutrient for major metabolic processes in plants and the energy-yielding electron transfer reactions of respiration and photosynthesis (Guerinot and Ying 1994).Despite its abundance in the Earth's crust, it is not readily available for plants under oxygenated conditions. The major factor affecting acquisition of Fe by plants is soil pH. In fact, high pH makes Fe, which is mainly present in its oxidized form in well aerated soils, less available leading the concentration of free Fe in soil solution far below that required for optimal growth (Guerinot and Yi 1994). Plants have developed two main strategies to cope with Fe acquisition: Strategy I, in non graminaceous plants, that is based on the reduction of Fe(III) to Fe(II) which is then transported into the cell by a

specific transmembrane transporter (IRT1) and Strategy II, in grasses, where particular FeIII chelators (phytosiderophores) are exuded and subsequently transported inside the cell (Römheld 1987, Curie and Briat 2003, Schmidt 2003). In addition to these biochemical mechanisms, Strategy I also comprises physiological, developmental and metabolic mechanisms aimed at adapting the plant to changing levels of available Fe resources.

In calcareous environments, where pH ranges from 7.5 to 8.5, Fe bioavailability decreases even more dramatically as it is found in highly insoluble forms. In fact, in calcareous soils crops most frequently display disorders causing the so called lime-induced chlorosis that lead to yield losses both quantitatively and qualitatively (Chen and Barak, 1982, Römheld 1987, Kim and Guerinot 2007, Diaz et al. 2012).

Parietaria judaica (L. 1753) is a wild perennial and sinantropic dicot. Under a nutritional point of view *P. judaica* is an "indifferent" plant as it grows in both acidic and alkaline soils. However *P. judaica* usually represents the most widespread flora in highly calcareous and hostile environments such as wall cracks exposed to the sun. *P. judaica* in such environments displays phenotypic changes but does not manifest any chlorotic symptoms. Previous data have shown that *P. judaica* under Fe deficiency conditions activates Strategy I mechanisms: Fe(III)-chelate reductase and H^+-ATPase activities increase as well as the content of low weight organic acids (LOAs) and phenolic compounds in root exudates (Dell'Orto et al. 2003, Donnini et al 2012).

Phenolics are a wide group of aromatic compounds found only in microorganisms and plants. Their multiple chemical and biological functions were recognized long ago and involve radical scavenging, UV protectants, insect repellents, antimicrobial activity, pigments, allelophaty and structural functions among others (Hahlbrock and Scheel 1989, Lattanzio et al. 2009). Several classes of phenolics have been categorized on the basis of their basic skeleton: C_6 (simple phenols, benzoquinones), C_6-C_1 (phenolic acids and aldehydes), C_6-C_2 (acetophenones, phenylacetic acids), C_6-C_3 (hydroxycinnamic acids, coumarins, phenylpropanes, chromones), C_6-C_4 (naphthoquinones), C_6-C_1-C_6 (xanthones), C_6-C_2-C_6 (stilbenes, anthraquinones), C_6-C_3-C_6 (flavonoids,

isoflavonoids, neoflavonoids), $(C_6\text{-}C_3\text{-}C_6)_{2,3}$ (bi-, triflavonoids, proanthocyanidin dimers, trimers), $(C_6\text{-}C_3)_2$ (lignans, neolignans), $(C_6\text{-}C_3)_n$ (lignins), $(C_6)_n$ (catechol melanins, phlorotannins), $(C_6\text{-}C_3\text{-}C_6)_n$ (condensed tannins) (Harborne 1980). Phenolic acids are simple phenols that are found usually in a bound form, occurring often in the form ofesters, glycosides and bound complexes.

Countless works reported the increase in the amount of phenolic compounds in plants and root exudates as a response to different environmental stresses (Cesco et al. 2010, Treutter2006). Recent studies have suggested a central role of the adjustment of secondary metabolism in the adaptive response to Fe mobilization (Jin et al. 2007). The metal chelating ability of polyphenols is related to the presence of ortho-dihydroxy polyphenols. Notwithstanding many authors have considered the chelating function of some polyphenols as a minor mechanism (Rice-Evans et al 1997). Andjelkovic´ et al. (2006) in an experimental study have demonstrated the Fe biding capacity of dicaffeoylquinic acids (chlorogenic acid in particular).

Caffeoylquinic acid (CQA) derivatives are a class of conjugated polyphenolic compounds broadly distributed in plants. The structures ofCQAs are constituted of one or more caffeic acid molecules linked to one or two quinic acid molecules through ester bonds(Fig. 1).

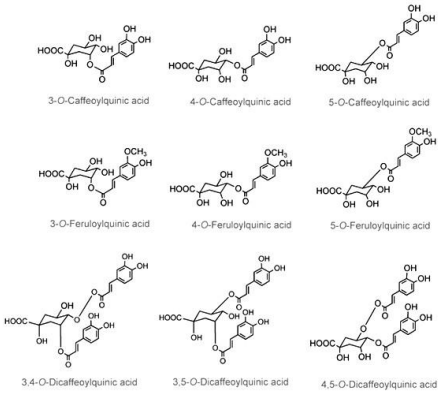

Figure 1. Major caffeoylquinic and dicaffeoylquinic isomers. 5-*O* caffeoylquinic acid isomer is known as chlorogenic acid.

This family of compounds has strong antioxidant properties displaying high cytoprotection activity against ROS, and some of them could display interesting Fe chelating activity. A biomedical research conducted on model cell lines (U937) has suggested that the antioxidantactivity of a series of caffeic acid esters and flavonoids is largely mediated by a Fe-chelating mechanism: the catechol ring was identified as playing a pivotal role in this mechanism. In fact, if catechol group is absent,the presence of hydroxy groups did not promote any antioxidant activity (Sestili et al. 2002).

Various caffeoylquinic derivatives have been identified in root exudates of different species (Badri and Vivanco 2009) but the studies were principally oriented to evaluate their role as chemical mediators in allelopathy and interactions with soil microbes (Bais et al. 2006).

In the present work the phenolic compounds present in roots and leaves of field samples of *P. judaica* were characterized by HPLC analysis. It was also analyzed the effect of direct and induced Fe deficiency of plants grown in hydroponics in different conditions on phenolic composition, both quantitatively and qualitatively terms, in roots and exudates, with the aim at identifying the responses of secondary metabolism induced by low Fe availability.

5.2. **Materials and Methods**

5.2.1. *Parietaria judaica culture*

- Field samples: plants were sampled in an urban area of Milan. Samples were collected by breaking the walls and the substrates to obtain whole roots.

- Hydroponic culture: cuttings of *Parietaria* were placed to radicate in aerated half-strength nutrient solution for 10 days. Rooted plants were then transferred to 10 L plastic tanks (40 plants per tank) with four different conditions: +Fe (control, full nutrient solution adjusted to pH 6.2), -Fe (full nutrient solution but total absence of Fe brought to pH 6.2), +Bic (full nutrient solution with addition of 0.5 gL^{-1} $CaCO_3$ and 15

mM $NaHCO_3$ which brought the pH to 8.3), Trc (full nutrient solution buffered with Tricine and adjusted to pH 8.3).The pH was corrected with Na(OH) were required. The alkaline treatments were performed in order to distinguish the low availability of Fe due just to a high pH from the effect caused by bicarbonate. The composition of the full solution was prepared as in Dell'Orto et al. (2003).

Treatments were carried out for 7 days in a growth chamber under 16/18 h light/dark regime with cool-white light 200 μmol m^{-1} s^{-1}, 27/21°C temperature range, 65-75% relative humidity.

5.2.2. *Collection of root exudates*

Fifteen 7-d-old plants for each treatment were transferred to 250 mL distilled water with 10% (v/v) Micropur (Katadyn, Minneapolis, MN) to prevent microorganisms' activity. For the collection of exudates, plants were kept during 24 h under continuous aeration in a growth chamber at the same conditions of the previous culture. The collected exudates were acidified to pH 3.5-4.0 with HCl to maintain the structural stability of phenolic compounds and freeze dried. The lyophilized material was resuspended in 3 mL methanol and filtered through 0.45 μm Millipore Millex-HN. The filtered solution was then analysed for total phenolic content and HPLC determination.

5.2.3. *Isolation and characterization of phenolic compounds in Parietaria judaica*

For qualitative and quantitative determination of phenolic compounds in plant tissues of *Parietaria,* roots and shoots (1 g) were first boiled for 3 min in 30 ml MetOH-EtOH (1:1) and then refluxed for 30 min (2x). The solution was then recovered and concentrated under vacuum. As no aqueous fraction was detected the concentrated solution was filtered through 0.45 μm Millipore Millex-HN. The filtered solutionswere analyzed for total phenolic content and HPLC determination of phenolic compounds.

HPLC analyses were performed with a Hewlett Packard Series 1100 liquid

chromatograph equipped with a binary gradient pump G1312A, a G1315A spectrophotometric photodiode array detector was set at 325 nm, and G1316A Column with the thermostat set at 45°C. The Hewlett Packard Chem Station (Rev. A. 06.03) software was used for spectra and data processing. An analytical Phenomenex (Torrance, California, USA) Luna C18 (5μ) column (4.6 x 250 mm) was used throughout this work. The solvent system consisted of (A) MetOH and (B) acetic acid-water (5/95, v/v). The elution profile was as reported by Lattanzio and Van Sumere (1987). The flow rate was 1 ml/min. Samples of 25 μl were applied to the column by means of a 25 μl loop valve. UV absorption spectra were acquire in the range of 235 to 450 nm.

HPLC-MS/MS analyses were performed on a QTrap MS/MS system, from Applied Biosystems (Foster City, CA, USA), equipped with an ESI interface and a 1100 series micro-LC system comprising a binary pump and a microautosampler from Agilent Technologies (Waldbronn, Germany). ESI interface was used in positive ion mode, with the following settings: temperature (TEM) 350 °C; curtain gas (CUR), nitrogen, 30 psi; nebulizer gas (GS1), air, 10 psi; heater gas (GS2), air, 30 psi; ion spray voltage + 4500 V. Full scan chromatograms were acquired in the mass range 100 – 800 amu, MS/MS chromatograms were acquired at collision energy of 20V. LC conditions were as for HPLC-DAD analyses.

5.2.4. *Determination of total phenolic compounds*

The amount of phenolic compounds in root extracts and exudates was determined spectrophotometrically at 750 nm with the Folin-Ciocalteau reagent using caffeic acid as a standard. The method was adapted according to Cicco et al. (2009). Three replicates of three independent determinations were performed for each treatment (*n*=9).

5.2.5. *Estimation of total ortho-dihydroxy phenolic compounds content (Arnow's reagent)*

One mL of extract sample was place in a test-tube and1 mL 0.5 N HClwas added. Tube was well mixed and then1 mL Arnow's reagent (10 g sodium nitrite and 10 g sodium molybdate made up to 100 ml with distilled

water) was added resulting in a yellow colour. After mixing, 1 mL1 N NaOH was added turning to red color. The solution was brought to a final volume of 5 mL with distilled water. Blank was performed without the sample. Absorbance was read at 500 nm. The content of each sample was calculated and expressed as $\mu g g^{-1}$fresh weight. Chlorogenic acid was used for the standard curve prepared in a range of 0-0.15 mg/mL. Three replicates of three independent determinations were performed for each treatment (*n*=9).

5.3. **Results and Discussion**

5.3.1. *Phenolic characterization of Parietaria judaica collected from the field*

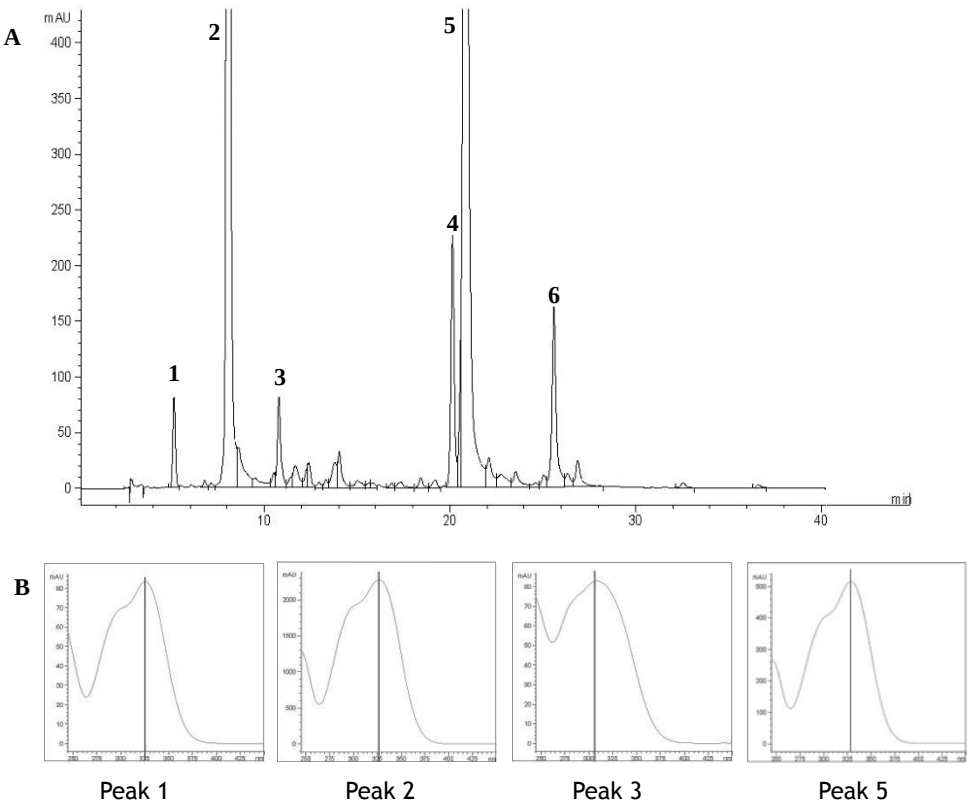

Figure 1. A. HPLC-DAD chromatogram of methanolic extracts of root samples of *Parietaria judaica*collected from the field, B. UV spectra of the main peaks.

The characterization of field samples of roots and leaves of *P. judaica* were analyzed by HPLC-DAD and MS/MS. Figure 1A shows the HPLC profile of *P. judaica* roots collected from the field. Six peaks were identified in roots according to retention times and UV absorption spectra. The main peaks were also analysed by MS/MS (Tab. 1). Peaks 2 and 5 exhibit UV spectra with a maximum at 325-328 nm and a shoulder at 295-310 nm which is consistent with caffeoylquinic acids spectrum (Fig. 1B). Peak 2 showed a parent molecular mass *m/z* at 355 and characteristic MS^2 ion at *m/z* 181.1 indicative of the presence of caffeic acid moiety (Tab. 1). Peak 5 showed a deprotonated molecular ion at *m/z* 517 and a fragmentation at *m/z* 355 consistent with the chlorogenic moiety and *m/z* 181.1 diagnostic of the presence of caffeic acid. Peaks 2 and 5 were identified as 5-*O*-caffeoylchinic acid (chlorogenic acid) and 3,5-*O*-dicaffeoylchinic acid, respectively. Peaks 2 and 5 represent the most abundant constituents in roots and account for 40% and 48% of the total content of phenolics respectively.

Peak	R_t (min)	λmax (nm)	MS (*m/z*)	MS^2 (*m/z*)	Phenolic cc (μg g^{-1} FW)	Identification
Field root					**7493.60**	
1	5.139	325	-	-	85,16	3-*O*-Caffeoylquinic ac.
2	8.051	325	355	181.1, 163.1, 145.1, 135.1	3006.27	5-*O*-Caffeoylquinic ac. (Chlorogenic acid)
3	10.789	310	-	-	118.81	1,3-*O*-Dicaffeoylquinic ac. (?)
4	20.139	325	-	-	314.83	4,5-*O*-Dicaffeoylquinic ac.
5	20.837	330	517	355.2, 337.2, 319.2	3571.35	3,5-*O*-Dicaffeoylquinic ac.
6	25.605	325	-	-	286.89	3,4-*O*-Dicaffeoylquinic ac.
Field leaves					**5734.74**	
1	5.321	320	-	-	373.63	3-*O*-Caffeoylquinic ac.
2	7.331	328	-	-	307.00	
3	8.068	325	-	-	1335.42	Chlorogenic ac.
4	12.448	330	-	-	3056.00	Caffeic ac. derivative
5	17.423	315	-	-	358.43	Coumaric ac. derivate
6	22.656	330	-	-	345.35	Quercetine 3 glucoside
7	25.609	313	-	-	86.04	Coumaric acid derivative

Table 1. Retention time (R_t), wavelengths of maximum absorption in visible region (λmax), mass spectral data, total concentration and concentration of main components identified in field samples of roots and leaves of *Parietaria judaica*. Data acquisition parameters were performed as indicated in paragraph 5.2.3.

Peak 3 presents UV spectra with a maximum at 310 nm and very slight shoulder. Considering the UV spectra and retention time the compound could be identified as a coumaric acid derivative but present in a very low content (1.5% of total phenolic content).Other minor compounds identified are: 3-*O*-Caffeoylchinic acid known also as isochlorogenic acid (peak 1), 4,5-*O*-Dicaffeoylchinic acid (peak 4) and 3,4-*O*-Dicaffeoylchinic acid (peak 6) that together represent less than 10% of total phenolic compounds. The main constituents of field roots extracts of *P. judaica* are represented Chlorogenic acid and 3,5-*O*-Dicaffeoylchinic acid that constitute together about 87% of the total phenolic compounds present in root extracts (Tab. 1).

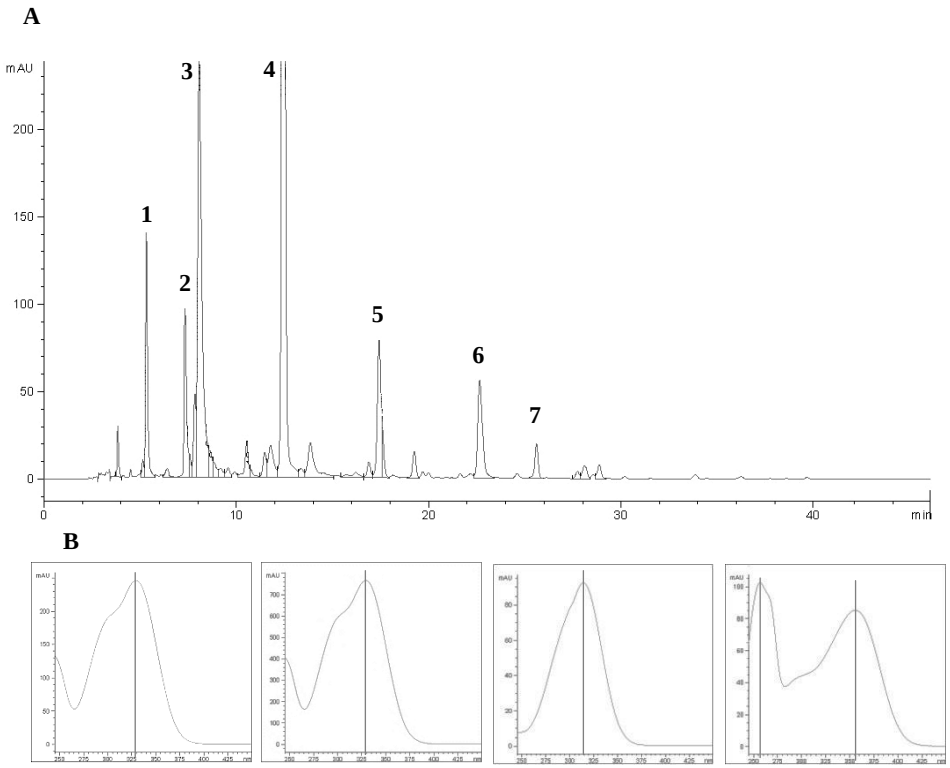

Figure 2. **A.**HPLC-DAD chromatograms of methanol extracts of field samples of leaves of *Parietaria judaica* and **B.**UV spectra of the main peaks.

HPLC profile of methanolic leaf extracts is shown in figure 2A. In leaves 7

peaks were identified by considering both retention times and UV spectra. Peak 3 and 4 represent the main components in leaves. Peak 3 was identified as Chlorogenic acid and accounted for a 23% of the total phenolic compounds. Peak 4 is the principal compound and constitutes the 53% of total phenolic compounds in leaves. Considering the retention time (Tab.1) and UV absorption spectra (Fig.2B) peak 4 is not in the sequence of mono or dicaffeoylquinic acids. Instead, it could be a conjugated Caffeic acid derivative. Other hydroxycinnamic compounds were identified: peak 5 which presents UV spectra consistent with a coumaric derivative, peak 1 probably corresponding to 3-*O*-Caffeoylquinic acid and peak 7 which was identified by a standard as 3,4-*O*-Dicaffeoylquinic acid and is present in a very low concentration. All hydroxycinnamic acids in leaves accounted for 93% of the total phenolic compounds. The remaining 7% correspond to a flavonoid (peak 6). Peak 6 presents UV absorption spectra with a maximum at 270 and 370 that correspond to quercetine. The retention time suggests that peak 6 could be a quercetine-3-glucoside.

In leaves as in roots phenolic compounds composition is represented mainly by phenolic acids that belong to the hydroxycinnamic class. Even though, the HPLC profiles of leaves and roots are not similar. In leaves the highest concentration is attributable to a caffeic acid conjugate not yet identified whereas in roots the major component corresponds to 3,5-*O*-Dicaffeoylquinic acid. Chlorogenic acid constitutes an important component in both leaves and roots. In quantitative terms a higher amount of total phenolic compounds of about 23% was found in roots respect to leaves.

5.3.2. *Phenolic compounds in roots from different hydroponic treatments (+Fe, -Fe, Bic and Tric)*

HPLC qualitative and quantitative analyses were carried out in root extracts and root exudates collected from plants undergone direct or induced Fe deficiency growth conditions.

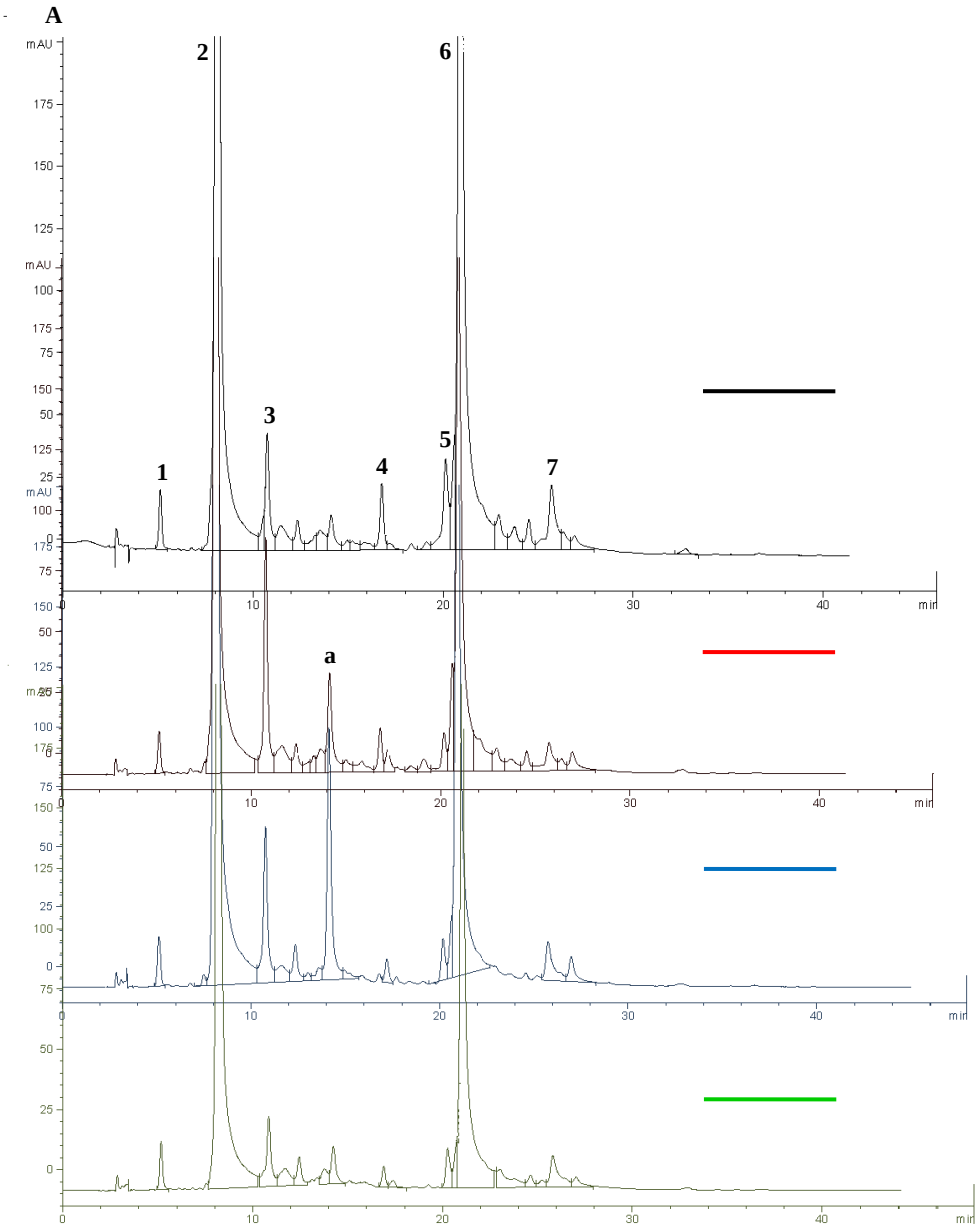

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Figure 3. A. Comparison of HPLC-DAD chromatograms of root samples of *Parietaria judaica* subject to direct or induced Fe deficiency conditions. Control condition is represented by the black trace=control; red trace=Fe starvation (-Fe); blue trace=bicarbonate treatment (Bic) and green trace=alkaline organic buffer condition (Tric).

continued

B

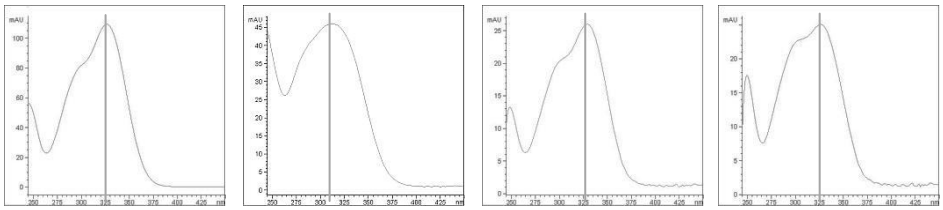

Figure 3.B. UV absorption spectra of some peaks of HPLC chromatogram roots grown in different hydroponic conditions.

Many studies have characterized root exudates in different species (Badri and Vivanco 2009) but no work has been done regarding the profile differences in phenolic composition in both roots and exudates as a consequence of low Fe availability.

In Figure 3 a comparison of HPLC profiles of root tissues of *P. judaica* grown in control conditions (+Fe), Fe starvation (-Fe), calcium bicarbonate condition (Bic) and a high alkaline organic buffer condition (Tric) is shown.

Root extracts of different treatments have shown very similar HPLC profiles among each other. By comparing retention times and UV spectra 8 peaks (1, 2, 3, a, 4, 5, 6 and 7) were detected. The most abundant phenolic compounds identified in roots grown in different hydroponic treatments belong to the family of hydroxycinnamic acids derivatives, mainly mono and dicaffeoylquinic acids. In control roots (+Fe)(Fig. 3) no significant difference were found in the HPLC profiles respect to field roots (fig. 1A). All peaks correspond in the different treatments except for the compound at peak 4 in +Fe condition. Peaks 2 and 6 were identified as Chlorogenic acid and 3,5-*O*-Dicaffeoylquinic acid.Peak 1 is present in all root extracts in low quantities and was identified as 3-*O*-Caffeoylquinic acid. Chlorogenic acid represents the most abundant compound found in roots in all treatments accounting for 57% to 69% of the respective total phenolic content (tab. 2). Minor peaks were

identified as dicaffeoylquinic acids isomers: peak 3 corresponds to 1,3-*O*-Dicaffeoylquinic acid, peak 5 corresponds to 4,5-*O*-Dicaffeoylquinic acid and peak 7 to 3,4-*O*-Dicaffeoylquinic acid. Peak **"a"** is present only in Bic and –Fe condition. UV-absorption spectra (fig. 3B) and retention time (tab. 2) indicate that it could be identified as a caffeic derivative. In Bic peak "a" exhibit a higher amount but it constitute only a 4% of its total phenolic compounds (tab. 2). Peak 4 is present in control and –Fe roots it correspond to a low amount and considering the UV-absorption spectra could be identified as a caffeic derivative.

In roots under direct or induced Fe deficiency the total phenolic content increases. Higher increase was detected in roots subject to bicarbonate condition (Bic) with an increase of 73% respect to the control. The main components of the phenolic composition are Chlorogenic (peak 2) and 3,5-*O*-Dicaffeoylquinic acid (peak 6) together accounting for about 80-90% of the total phenolic contents in all conditions. However the ratio between the main compounds (Chlorogenic/3,5-*O*-Diccafeoylquinic acid) is slightly higher in -Fe, Bic and Tric conditions respect to the control, revealing a relative augmentation in chlorogenic acid. In comparison with the phenolic components of the control (+Fe), the increase in total phenolic content in stress treatments is principally due to the increase of Chlorogenic acid (peak 2) and the supposed 1,3-*O*-Dicaffeoylquinic acid (peak 3). Several studies have reported the increase in Chlorogenic acid in response to different abiotic stresses such as wounding(Cantos et al. 2001), increased UV-B irradiation and insect attacks (Izaguirre et al. 2007). Moreover, Fe deficiency, as an abiotic stress for plants, was shown to affect the expression and the activity of certain peroxidase isoenzymes and to induce secondary oxidative stress in dicotyledonous species (Ranieri et al., 2001). The increase in Chlorogenic acid could be explained because of its role as antioxidant and free radical scavenger (Niggeweg et al. 2004).

Table 3 summarizes the total phenolic content in roots estimated through the Arnow reagent which determines the content in *ortho*-dihydroxy phenolic compounds.

Peak	Treatment				Identification
	+Fe	-Fe	Bic	Tric	
1	7.15	7.65	10.18	10.36	3-*O*-Caffeoylquinic ac.
2	487.43	592.23	1028.91	659.78	Chlorogenic ac.
3	20.16	58.92	47.29	26.89	1,3-*O*-Dicaffeoylquinic ac. or Caffeic ac.
a	-	31.64	72.13	-	Caffeic derivative
4	10.71	10.57	-	5.30	probably Caffeic derivative
5	21.20	11.35	10.11	12.66	4,5-*O*-Dicaffeoylquinic ac.
6	285.81	206.21	272.15	254.08	3,5-*O*-Dicaffeoylchinic ac.
7	21.30	206.21	19.19	20.59	3,4-*O*-Dicaffeoylquinic ac.
Total	**853.76**	**933.49**	**1477.65**	**1002.78**	

Table 2. Quantitative HPLC determination of the concentrations of the main phenolic components express in μg g^{-1}FWin roots of *Parietaria* grown under different treatments (+Fe, - Fe, Bic, Tric).

The results match well with those obtained by HPLC, showing an increase in all treatments. The highest value was obtained in Bic condition with an increase of 87% respect to the control while in –Fe and Trc conditions the augmentation is moderate (+28% and +17%) respectively.

Treatment	Total *ortho*-dihydroxyl phenolics (*Arnow reagent*) (μg g^{-1}FW)
+Fe (control)	1216.87
-Fe	1568.61
Bic	2278.58
Tric	1424.57

Table 3. Total *ortho*-dihydroxy phenolic content in roots of *Parietaria* grown in different treatments (+Fe, -Fe, Bic, Trc) estimated with the Arnow reagent.

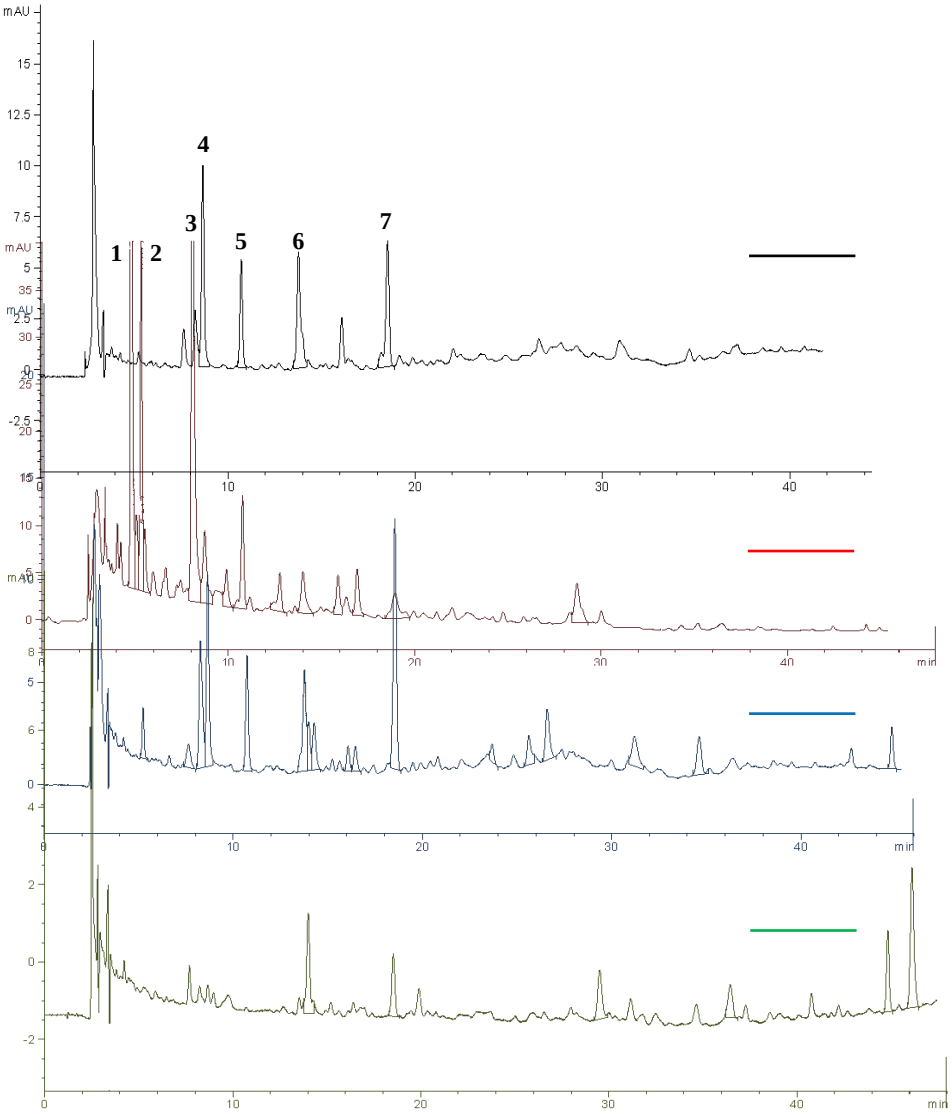

Figure 4. Comparison of HPLC-DAD chromatograms of root exudatesof *Parietaria judaica* under control conditions (+Fe) black line, Fe starvation (-Fe) red line, bicarbonate treatment (Bic) blue line and alkaline organic buffer condition (Tric) green line.

In Figure 4 the HPLC profiles of root exudates collected from *Parietaria judaica* under control conditions (+Fe) and subjected to Fe deprivation (-Fe) and induced Fe deficiency conditions (Bic and Trc) are shown.

Among all chromatograms 7 main peaks were detected. The results obtain so far by the HPLC analysis show exudate profiles dissimilar to those obtained in root extracts. All peaks found in control root exudates (+Fe) presented UV spectra with maximum absorption at 325 nm and a shoulder between 295-310 nm and retention times range from about 8 to 18 minutes, these characteristic are compatible with hydroxycinnamic acids derivatives. MS/MS analysis could not be performed as exudate samples contain very low concentrations of phenolics.

Peak 2 and 3 could be probably Neochlorogenic and Chlorogenic acid, respectively. Peak 4 and 5 are present in control, -Fe and Bic exudates in very little amounts. Peak 5 (R_t 10.724) is also present in root tissues of all treatments (fig. 3) and could correspond to 1,3-*O*-Dicaffeoylquinic acid. Peak 6 and 7 are present in all exudates but constitute a major component in Bic ones.

The amount of *ortho* hydroxyl substituted phenolic compounds is presented in table 4. The highest content of *ortho* hydroxyl phenolics was found in exudates from Fe-deprived roots (3-fold respect to the control) whereas Bic and Tric exudates show similar values compared to the control. This result confirms the higher exudation of phenolics by –Fe roots respect to the other treatments (cfr. Chapter 3, par. 3.3.2.). Bic and Tric amounts, on the contrary, are lower respect to those obtained by the Folin Ciocalteu method.

Treatment	Total *ortho*-dihydroxyl phenolics in root exudates (*Arnow reagent*) ($\mu g\ g^{-1}$ FW)
+Fe (control)	49
-Fe	161
Bic	52
Tric	38

Table 4. Amount of *ortho*-dihydroxy phenolics in root exudates.

Fe deficiency-induced secretion of phenolics is an important part of a plant's adaptive strategy as it plays multiple functions in favoring Fe acquisition. The increased secretion of phenolics in Fe deficiency conditions is involved in the mobilization of apoplastic and soil Fe (Jin et al. 2008). Furthermore, exudation of phenolics into the rhizosphere influences selectively some microbial soil species that produce either siderophores or auxins that in turn support Fe acquisition by the plant (Jin et al. 2008). The higher content of phenolics found in the exudates collected from Fe-deprived roots represents an exacerbated response. The complete absence of Fe — previous work demonstrated that –Fe roots have no apoplastic Fe after 3rd day of treatment (cf. Chapter 3, par.3.3.3.) — probably avoids a feedback signal to modulate the response keeping activated at high levels the mechanisms to scavenge Fe. In Bic and Tric treatments probably the response is modulated as Fe is present, although in a not readily available form, thus when the plant exudes phenolics Fe can be mobilized through chelation or reduction from the apoplast or the growth medium.

5.4. **Conclusions**

Phenylpropanpids are usually found in plants as derivatives of the three hydroxicinnamic acids: caffeic, *p*-coumaric and ferulic acid. In *Parietaria judaica* the main phenolic constituents identified are mono and dicaffeoyl quinic acids derivatives, in particular Chlorogenic acid and 3,5-*O*-Dicaffeoylquinic acid. Notwithstanding qualitative differences, most of the phenolic compounds identified present a catechol group that has been pointed out as a strong antioxidant and a potential metal chelator (Arts et al. 2003). Chlorogenic acid is known to be involved in responses to different biotic and abiotic stresses in several plant species probably through its ability to interact with ROS (Niggeweg et al. 2004). In roots of *P. judaica* subject to low Fe availability the accumulation of phenolics was mainly due to the increase of the chlorogenic acid component. HPLC profiles have shown similarities in root phenolic composition while exudates presented different profiles from their producer roots. This could suggest a directed and active mechanism related to phenolic exudation so far.

References

Andjelkovic´M., Van CampJ., De MeulenaerB., DepaemelaereG., SocaciuC., VerlooM., VerheR., 2006. *Iron-chelation properties of phenolic acids bearing catechol and galloyl groups*, Food Chemistry, 98:23–31

Arts M.J., Dallinga J.S., Voss H.-P., Haenen G.R., Bast A., 2003.*A critical appraisal of the use of the antioxidant capacity (TEAC) assay in defining optimal antioxidant structures*, Food Chemistry, 80:409-414

Badri D., VivancoJ.M., 2009. *Regulation and function of root exudates*, Plant, Cell and Environment, 32:666–681

Bais H.P.,Weir T.L.,Perry L.G.,Gilroy S., Vivanco J.M., 2006. *The Role of Root Exudates in Rhizosphere Interactions with Plants and Other Organisms*, The Annual Review of Plant Biology, 57:233–66

CantoniO., 2002. *Plant-derived phenolic compounds prevent the DNA single-strand breakage and cytotoxicity induced by tert-utylhydroperoxide via an iron-chelating mechanism*, Biochemical Journal, 364:121-128

Cantos E., Espin J.C., Tomas-Barberan F. A., 2001. Effect of woundingon phenolic enzymes in six minimally processed lettuce cultivars upon storage, Journal of Agricultural and Food Chemistry, 49:322–330

Cesco S., Neumann G., Tomasi N., Pinton R., Weisskopf L., 2010. *Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition*, Plant and Soil, 329(1-2):1-25

Chen Y., Barak P., 1982. *Iron Nutrition of Plants in Calcareous Soils*, Advances in Agronomy, 35:217–240

Cicco N., Lanorte M.T., Paraggio M., Viggiano M., Lattanzio V., 2009. *A reproducible, rapid and inexpensive Folin–Ciocalteu micro-method in determining phenolics of plant methanol extracts*, Microchemical Journal, 91:107–110

Curie C., Briat J.-F., 2003. *Iron Transport and Signaling in Plants*, Annual Review of Plant Biology, 54:183-206

Dell'Orto M., De Nisi P., Pontiggia A., Zocchi G., 2003. *Fe DeficiencyResponses in Parietaria diffusa: A Calcicole Plant*, Journal of Plant Nutrition, 26(10-11):2057–2068

Díaz I., Delgado A., de Santiago A., del Campillo M.C., Torrent J., 2012. *Iron deficiency chlorosis in plants as related to Fe sources in soil*, EGU General Assembly 2012, 22-27 April, Vienna - Austria, p.4454

DonniniS., De NisiP., GabottiD., TatoL., ZocchiG., 2012. *Adaptive strategies of Parietaria diffusa (M.&K.) to calcareous habitat with limited iron availability*, 35(6):1171–1184

Guerinot M.L., Yi Y., 1994. *Iron: Nutritious, Noxious, and Not Readily Available*, Plant Physiology, 104(3): 815–820

Hahlbrock K., Scheel D., 1989. *Physiology and Molecular Biology of Phenylpropanoid Metabolism*, Annual Review of Plant Physiology and Plant Molecular Biology, 40:347-369

Harborne J.B., 1980. *Plant phenolics*, In: Bell E.A., Charlwood B.V. (eds.), *Enciclopedia of Plant Physiology*, New Series, Vol. 8, Secondary Plant Products, Springer-Verlag, Berlin, Germany, pp. 329-402

Imsande J., 1998.*Iron, sulfur, and chlorophyll deficiencies: a need for an integrative approach in plant physiology*, Physiologia Plantarum, 103:139-144

Izaguirre M.M., Mazza C.A., Svatos A., Baldwin I.T., Ballare C.L., 2007.*Solar ultraviolet-B radiation and insect herbivory trigger partially overlapping phenolic responses in Nicotiana attenuate and Nicotiana longiflora*, Annals of Botany, 99:103–109

Jin C.W., You G.Y., Zheng S.J., 2008.*The iron deficiency-induced phenolics secretion plays multiple important roles in plant iron acquisition underground*, Plant Signaling & Behavior, 3(1):60-61

Kim S.A., Guerinot M.L., 2007. *Mining iron: Iron uptake and transport in plants*,FEBS letters, 581(12):2273–2280

Lattanzio V., Kroon P. A., Quideau S., Treutter D., 2009. *Plant Phenolics - Secondary Metabolites with Diverse Functions*, in: Daayf F., Lattanzio V. (eds), *Recent Advances in Polyphenol Research*, Volume 1, pp.1-35.

Marschner H., 1995. *Mineral nutrition of plants*, Academic. 2nded - San Diego, CA (USA).

Niggeweg R., Michael A. J., Martin C., 2004.*Engineering plants with increased levels of the antioxidant chlorogenic acid*, Nature Biotechnology, 22:746–754

Ranieri A., Castagna A., Baldan B., Soldatini G.F., 2001.*Iron deficiency differently affects peroxidase isoforms in sunflower*, Journal of experimental Botany, 52(354):25–35

Rice-Evans C.A., Miller, N.J., Paganga, G., 1997. *Antioxidant properties of phenolic compounds*, Trends in Plant Science, 2:152–159.

Römheld V., 1987. *Different strategies for iron acquisition in higher plants*, Physiologia Plantarum, 70(2):231–234

Schmidt W., 2003. *Iron solutions: acquisition strategies and signaling pathways in plants*, Trends in Plant Science, 8(4):188-193

Sestili P., DiamantiniG., BediniA., CeroniL., Tommasini I., TarziaG., Treutter D., 2006. *Significance of flavonoids in plant resistance: a review*, Environmental Chemistry Letters, 4(3):147-157

VermerrisW., NicholsonR., 2006. *Phenolic Compound Biochemistry*, Springer Netherlands

Whipps J. M., 1990. *Carbon economy*, in: Lynch J. M. (ed.)*The rhizosphere*, John Wiley and Sons, Chichester – West Sussex (UK), pp. 59-97

CHAPTER 6

Morphological and architectural modifications of the root system of *Parietaria judaica* in response to different Fe deficiency conditions*

** this work was carried out in collaboration with Prof. Maria Rosa Abenavoli and Dr. Agostino Sorgonà - Dipartimento di Agraria - Università Mediterranea di Reggio Calabria*

ABSTRACT

Parietaria judaica is a calcicole wild plant that exhibits a high adaptation to Fe low bioavailability environments. As a Strategy I plant Fe-deficiency induces in *P. judaica* roots all the biochemical mechanisms aimed at favouring Fe availability and acquisition. The high efficiency by which *P. judaica* deals with Fe-deficiency was linked to the diversified and integrated responses it can perform. Among the most critical environmental factors, nutrient availability can profoundly shape root architecture. Root morphological plasticity could play an important role in *P. judaica* adaptability.

In this study we have analyzed how changes in growth parameters root mass ratio (RMR), root finesness (RF) and root density tissue (RDT) of the root of *P. judaica* have been able to determine the RLR pattern in response to different experimental treatments.

The changes in root morphology and architecture of *P. judaica* subject to Fe-deficiency direct or induced by the presence of bicarbonate, were examined, focusing on the relationship between the shape and the function of the root.

6.1. **Introduction**

Root architectural plasticity, the ability to exhibit morphological and physiological responses to a changing environmental factor, may play an important role in plant adaptation to heterogeneous environments (Sultan, 2000; Pigliucci, 2001). Root branching is essential to increase the surface area of the root system, enabling the plant to tap more distant reserves of water and nutrients and improve soil anchorage.

Plasticity may increase overall resource acquisition efficiency by allowing a plant to respond dynamically to temporally and spatially limited resources (Hutchings and de Kroon, 1994; Huang and Eissenstat, 2000). Consequently, root architectures among and between species vary greatly in form and subsequently in function and appear to be correlated with adaptation and productivity in specific environments (Fitter et al. 1991).

Within soil, nutrients are distributed irregularly both in space and time Caldwell, 1994) and, consequently, plants have developed mechanisms to modify their root morphology and physiology in response to availability of nutrients. Such plant plasticity is strongly associated with many functional root traits including root elongation (Cahill and Casper, 2000) and its spatial influence (Casper et al., 2003), root architecture, and uptake capacities among others.

Studies on plasticity of functional root traits involved in nutrient acquisition have focused mainly on root length, a morphological parameter that best describes the ability of the root to explore the soil (Ryser, 1998). Root proliferation in response to nutrient deficiency has been the most investigated functional root trait.

Parietaria judaica is a wild species that grows in calcareous soils characterized by high concentrations of bicarbonate and a alkaline pH. Despite the bioavailability of nutrients, of Fe in particular, is a limiting factor for plant spreading in such soils, *P. judaica* is well adapted to these environments and does not show typical symptoms of chlorosis due to Fe-deficiency. Previous work has suggested a physiological strategy that allows *P. judaica* to adapt to Fe low availability integrating the typical biochemical responses of a Strategy I plant with root exudation of organic compounds, changes in root morphology and a rearrangement of its metabolism (Chap. 4).

Reports of morphological changes of the root system in response to varying Fe availabilities mostly refer back to earlier work and describe exclusively adaptations to Fe deficiency (Römheld and Marschner, 1981; Landsberg, 1986). Fe deficiency also induces the ectopic formation of root hairs (Schmidt et al., 2000) by modulating the length, position, and abundance of root hairs (Perry et al., 2007). Moreover, low Fe availability frequently leads to the formation of branched root hairs (Müller and Schmidt, 2004).

In this work changes in the root morphology and architecture of *P. judaica* subject to direct or induced Fe-deficiency conditions were analysed, focusing on the relationship between the shape and the function of the root.

6.2. **Materials and Methods**

6.2.1. Plant material and growth conditions

Plant material and growth conditions were performed as reported in Chapter 2 par. 2.2.1.2. All treatments were carried out in hydroponics.

6.2.2. Root morphology and biomass allocation

After 7d of treatment 5 plants of each treatment were collected randomly. Shoot and root were separated. Shoot dry weight (W_S, g) was measured after drying in oven at 70°C for 48 h. The roots were divided into two orders: shoot-borne roots and 1st-order lateral. Each root was stained with 0.1% (w/v) toludine blue O for 5 min and then scanned at a resolution of 300 dpi (WinRhizo STD 1600, Instruments Régent Inc., Canada) for determining the length of the shoot-borne root (L_T, cm), the total length of the 1st-order laterals(L_l, cm), the volume of the shoot-borne root (V_T, cm^3), and the total volume of 1st-order laterals (V_l, cm^3) lateral roots by the WinRhizo Pro v. 4.0 software package (Instruments Régent Inc.). Then, dry weights of the shoot-borne root (W_T, g) and the total dry weight of 1st-order lateral roots (W_l, g) were measured after drying in oven at 70°C for 48 h. The total root dry weight (W_R, g) was the sum of W_T and W_l, and the plant dry weight (W_P, g) was obtained by the sum of W_R and W_S. Based on the measurements above, the following parameters were calculated for each root order:

$$RLR = L/W_P \ (cm\ g^{-1}) \quad (1)$$

$$RMR = W/W_P \ (g\ g^{-1}) \quad (2)$$

$$SRL = L/W \ (cm\ g^{-1}) \quad (3)$$

$$F = L/V \ (cm\ cm^{-3}) \quad (4)$$

$$TD = W/V \ (g\ cm^{-3}) \quad (5)$$

where RLR is the root length ratio, which expresses the root order's potential for the acquisition of below-ground resources; the RMR is the root mass ratio, which indicates the relative biomass allocated to the root; the SRL, F and TD are the specific root length, fineness and tissue density, respectively, which represent the structural root parameters. *L*,

W and *V* indicated the length, the dry weight and the volume of each root order, respectively. As reported by Ryser and Lambers (1995), the following relationships can be obtained among the above parameters:

$$RLR = RMR \times SRL \quad (6)$$

$$SRL = F/TD \quad (7)$$

The number of the 1st-order laterals (N_l) was counted directly from the images of each root order. The average length of the 1st-order laterals [$L_l = L_l/N_l$] (cm) was also calculated.

6.2.3. Statistical analysis of data

The effects of the different treatments on the root parameters calculated were tested by two-way ANOVA. The data were checked for deviations from normality and homogeneity of variances prior to analysis. Tukey's post hoc test comparison was applied to test the effect of each treatment at $P<0.05$.

In order to correct for allometric effects (Coleman et al., 1994), the ln-transformed plant dry weight ($\ln W_P$) was used as a covariate in analysing the parameters of root morphology and biomass allocation when significant correlations between $\ln W_P$ and these root parameters were found. The data were checked for deviations from normality and homogeneity of variances prior to analysis and the necessary transformations were carried out.

The effects of different treatments on the number and average length of the 1st-order lateral roots were tested by two-way ANOVA. The data were checked for deviations from normality and homogeneity of variances prior to analysis.

Statistical analysis was conducted using the Systat v. 8.0 software package (SPSS Inc., Evanston, IL, USA).

6.3. **Results and Discussion**

In Figure 1 the effect of the different experimental treatments both on the morphology and the radical architecture of seedlings of *P. judaica* are shown. The parameters that determine these alterations were quantified using an image analysis system (Fig. 2).

The length of the entire root system does not show a significant reduction in response to different experimental treatments (Fig. 2.A.), although it is possible to observe a lower total length in the presence of both Bic(-39%) and Tric (-53%). These results are in agreement with what has been previously observed (Chap. 2) for these treatments.

These responses were also observed concerning the total root area (Fig. 2.B.). Bic and Trictreatments showed a decrease in the absolute total root area value respect to the control and –Fe conditions, though there are no statistical differences among mean values of different treatments.

Regarding the dry weight of the whole root system no statistically significant differences were found between different experimental treatments (Fig. 2C), although it has been observed an increase (+61%) in dry weight in roots of *P. judaica* exposed to Fe starvation. Therefore, the length, the surface area, the dry weight of the whole root system of *P. judaica* seemed not to be affected by direct or induced Fe-deficiency conditions compared to the control. Anyway, these parameters tend to appear lower when the plants are grown in bicarbonate (Bic) or organic alkaline conditions (Tric).

A long and extended root system allows the plant a greater acquisition of soil resources; especially those present at low concentrations or scarcely available (Marschner 1995; Tinker and Nye, 2000) such as phosphate (P) (Lambers et al., 2006) and Fe (Maschner, 1995). Therefore, data obtained from the whole root system morphology of *P. judaica* seem to suggest a better adaptation to direct Fe deficiency respect to that induced by both alkaline conditions (Bic and Tric). Although these results appear to be positively related to the values of the shoot dry weight (data not shown), no relation is found with the content of chlorophyll, which remains lower in –Fe condition respect to the alkaline conditions (Chap. 2 par. 2.3.3.).

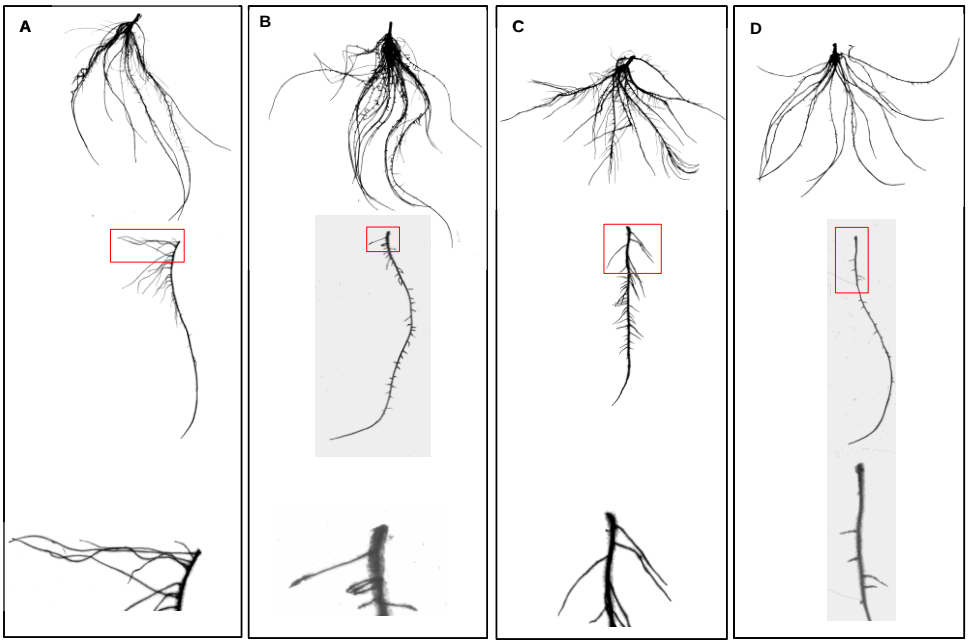

Figure 1. – Root architecture and morphology of *Parietaria judaica* grown under the following treatments: **A.** full nutrient solution at pH 6.5 (+Fe, control); **B.** Fe starved solution at pH 6.5 (-Fe); **C.** high bicarbonate solution at pH 8.5 (Bic); **D.** alkaline organic buffer solution at pH 8.5 (Tric). The images at the top and at the center of each panel are, respectively, the entire root system and a single shoot-borne root. The red box indicates the portion of shoot-borne root that has been magnified. At the bottom of the figure there is the enlarged shoot-borne root zone.

These apparently contradictory observations could be explained by studying the functional role of other traits of the root morphology. In particular, such approach provides two additional levels of information. The first one considers some absolute root morphological parameters per unit plant biomass or per unit total root volume, in particular the root length ratio (RLR) and especially how this relative parameter is defined by its components. The second level of information is supplied by the study of the spatial configuration of the root system (root architecture) that considers the types of roots and their distribution in the whole root system.

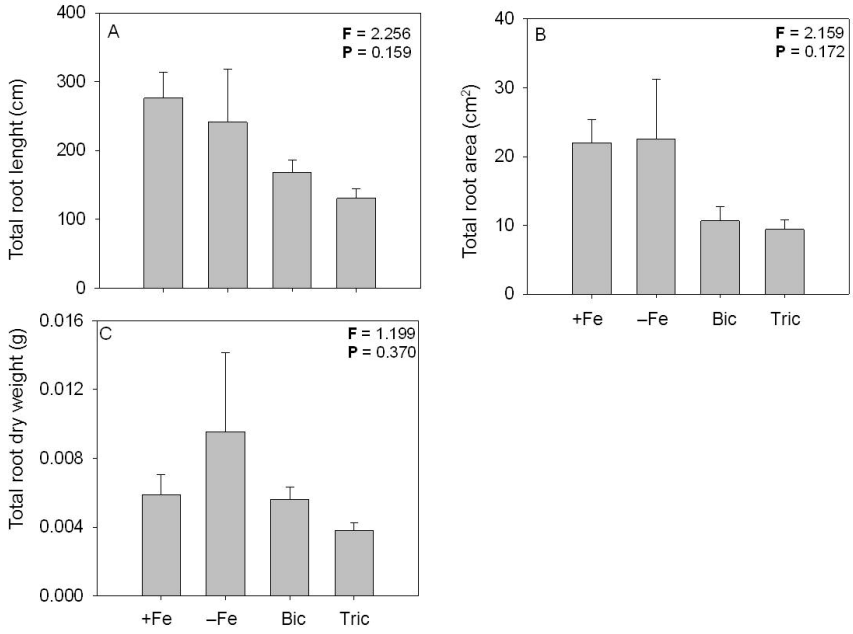

Figura 2. Morphological analysis of the whole root system of *P. judaica* grown in *Fe, -Fe, Bic, and Tric conditions. F and P correspond to the F-test value and the probability value respectively. No significant differences were found among means ($p<0.05$; Tukey test). *n*=3.

Many authors have considered the root length ratio (RLR) as a morphological trait associated with the ability of the plant to acquire the nutrients from the soil (Boot and Den Dubbelden 1990; Ryser and Lambers, 1995; Ryser 1998; Ryser and Eek 2000; Sorgonà et al. 2007). This approach would avoid the "allometric effects" (Coleman et al., 1994) or the "*apparent plasticity*" (Weiner, 2004), especially when dealing with the variations of the root length in response to the edaphic conditions of the soil.

The results obtained showed that the RLR of *P. judaica* resulted not significantly affected by the different experimental treatments (Fig. 3A). This suggests that *P. judaica* while preserving the RLR in response to direct or induced Fe-deficiency maintained a long root system able to explore a greater volume of soil. The correlation between these results

and the morphological chlorophyll content (Chap. 2, par. 2.3.3.) supports the hypothesis of the functional role of a long root system in a medium characterized by a low bioavailability of Fe. In addition, the RLR depends on the allocation of biomass (root mass ratio, RMR) and structure (fineness, RF and density of root tissue, RDT) parameters that consitute its major components (Ryser and Lambers 1995). Therefore, plants could increase the length of their root system by increasing the allocation of biomass (RMR) towards the root or by increasing the fineness (RF) and/or by reducing the density of tissue (RDT). In fact, it has been demonstrated that the variation of the RLR under conditions of nutritional deficiencies could be attributable to the variation of its components, RDT and RF, (Ryser and Lambers, 1995, Hill et al., 2006); while, under conditions of low levels of nitrate the RMR becomes a determinant component in the RLR variation (Sorgonà et al. 2007). In *Plantago maritima* it was observed that the increase in the root fineness/root tissue density ratio and the reduction of RMR are able to cancel the negative effect of salinity on the root length (Rubinigg et al., 2003).

The analysis of *P. judaica* RMR, RF and RDT root parameters was carried out to determine the RLR pattern in response to different experimental treatments.

The whole root system showed in –Fe condition a weak non statistically significant increase in RMR (+25%), accompanied by a slight reduction of RF (-15%) and a greater increase in RDT (+34%) which results in a slight reduction of the RLR with respect to the control (Fig. 3). On the contrary, the presence of bicarbonate causes an increase, non statistically significant, of the RMR (+63%) and RF (+77%) which, associated with a significant increase in RDT (+170%), produces a weak RLR increase compared with the control (+5%; Fig. 3).

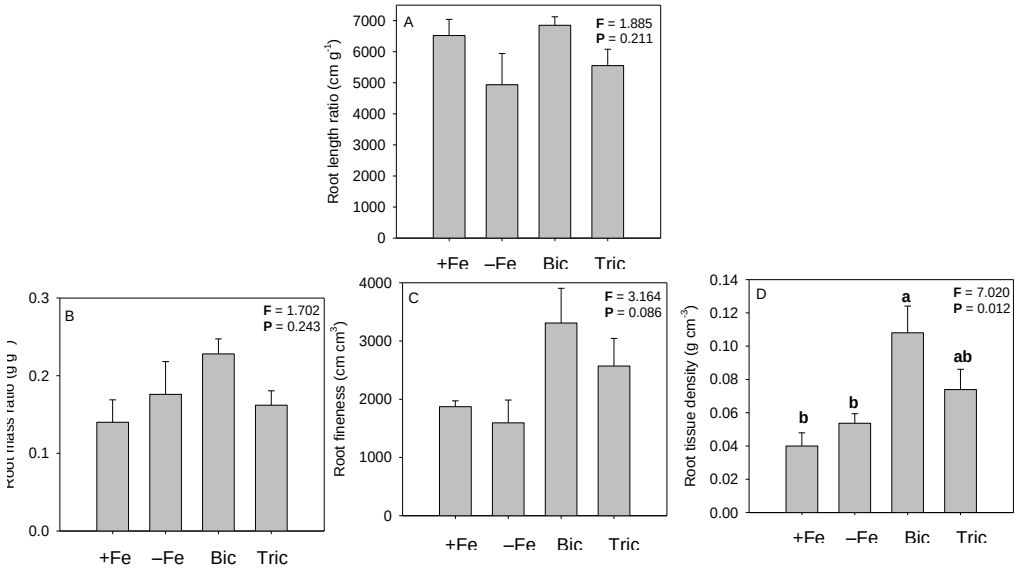

Figure 3. Root lenght ratio and its components (root mass, fineness and tissue density ratio) of the whole root system of *P. judaica* in control (+Fe), direct Fe deficiency (-Fe), high bicarbonate (Bic) and high alkaline organic buffer (Tric) conditions. F and P correspond to the F-test value and the probability value respectively. Different letters correspond to significant differences among means ($p<0.05$; Tukey test), $n=3$.

Finally, Tric treatment has shown a pattern of RMR, RF and RDT variation intermediate between –Fe and Bic conditions (Fig. 3). It could be concluded that the observed responses of the components of the RLR of *P. judaica* are diversified depending on the experimental conditions imposed. However they are aimed at maintaining unchanged the RLR with respect to the control. In particular, in direct Fe deficiency conditions, *P. judaica* allocates greater biomass in the root keeping unchanged the values of RF and RDT respect to the control. On the contrary, in high bicarbonate condition (Bic) all components (RMR, RF and RDT) registered an increase, more consistent in terms of tissue density (RDT).

The intra-root analysis could provide a first indication of the root architecture. This analysis takes into account the number and types of roots that constitute the whole root system and the branching pattern which determines the root architecture.

In Figure 1 is shown that *P. judaica* root system consists essentially of "*shoot-borne*" roots (any root developed from shoot tissues), according to the nomenclature proposed by Zobel and Waisel (2010), from which lateral root emerge. This structure is due to the cutting system of breeding. In direct Fe deficiency conditions (-Fe), the total length of the "*shoot-borne*" roots is greater than in the control (+40%), while decreases in the presence of both Bic (-55%) and Tric (-16%) treatment (Fig. 4A). The number of "*shoot-borne*" roots per root system is greater in -Fe (+52%), while it does not appear modified by the alkaline conditions (Bic and Tric) compared to the control (Fig. 4C).

A general decrease of the mean length of the "*shoot-borne*" roots was recorded. In Bic condition a higher statistically significant decrese was found (-49%) whilst a non statistically significant decrease was registered in Tric (-26%) and –Fe conditions with respect to the control (Fig. 4E).

Regarding lateral roots, in Tric condition a statistically significant reduction of lateral total length was found compared with the control (-82%). A similar trend, although non statistically significant, was also observed in –Fe and Bic conditions (-56% and -70% respectively) (Fig. 4B). The number of lateral roots in *P. judaica* is modified by the different experimental conditions, although not significant difference was found. In particular, it is slightly reduced in the presence of bicarbonate (-13%), highly reduced in Tric condition (-37%) and slightly increased in Fe-starved condition (+13%) (Fig. 4D). The mean total length of lateral roots is significantly reduced in Tric (-70%) as well as in -Fe (-52%); while in Bic condition it did not show a significant difference with respect to the control (Fig. 4F).

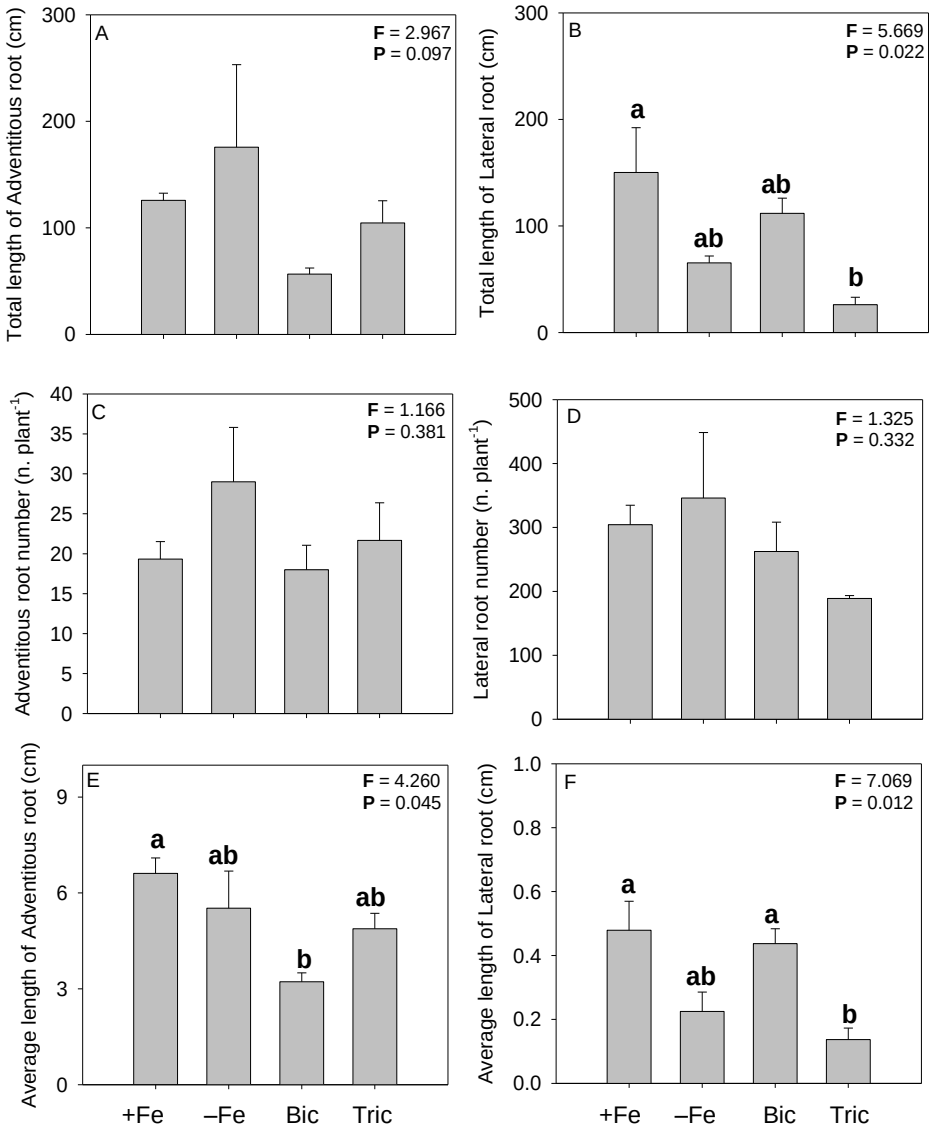

Figure 5 shows the results regarding the root system branching *P. judaica*, according to the nomenclature proposed by Dubrovsky and Forde (2012)

for the quantitative analysis of the development of lateral roots. In particular, the length of the region defined as "zone of branching", between the formation of the first lateral root up to the point of attachment to the stem, is significantly reduced by the presence of bicarbonate (-55%) and to a lesser extent by the other experimental treatments (-34% in Fe-deficiency, in Tric -31%) (Fig. 5A). In agreement with Dubrovsky and Forde (2012), this region was used to calculate the density of branching, which appears increased significantly in the presence of bicarbonate (+61%) while it seems unchanged by Fe deficiency and by tricine (Fig. 5B).

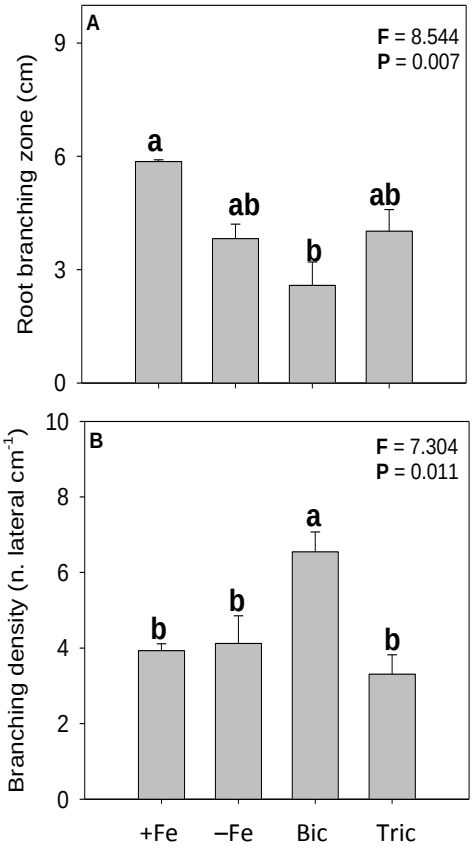

Figure 5. Root branching analysis of *P. judaica* in +Fe, -Fe, Bic and Tric grown conditions. F and P correspond to the F-test value and the probability value respectively. Different letters correspond to significant differences among means ($p<0.05$; Tukey test), $n=3$.

The results obtained regarding the intra-root morphology indicate that different treatments induce different modification in the root branching pattern in *P. judaica*. Thus, different types of root systems can be defined in comparison with the control:

1. The root system subject to direct Fe deficiency (-Fe) appears to be characterized by a greater total length of the *shoot-borne* roots attributable to a greater number of this type of roots rather than an increase in their average length (Fig. 1, images at the top of the panel). Furthermore, roots of this type develop a greater number of shorter lateral roots distributed along the main axis, with a similar density to the control, resulting in a reduction of their total length (Fig. 1 images placed at the centre and at the bottom of the panel);
2. The root system in Bic condition has shown a strong reduction of the total length of the *shoot-borne* roots attributable to a reduction of their average length, as the number of these roots is similar to the control (Fig. 1, images in top panel). The lateral root length shows a lesser reduction with respect to –Fe condition. In fact although the number of lateral roots in Bic condition is lower compared to –Fe condition the average root length is higher (Fig. 1 images placed at the centre and at the bottom of the panel);
3. The root system developed in Tric condition shows similar average branching parameters with respect to the –Fe condition.

The length contribution (%) of the different root types (*shoot-borne* and lateral roots) to the whole root system length was assessed in order to better understand the effects of the direct and induced Fe deficiency treatments on the root architecture of *P. judaica*.

In agreement with the results of intra-root morphology, Figure 6 shows that the root architecture in -Fe and in Tric are preponderantly characterized by the development of *shoot-borne* roots, while, in Bic condition a higher development of lateral roots prevailed.

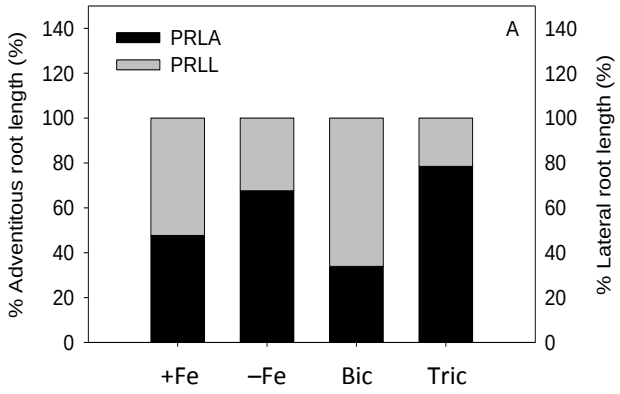

Figure 6. Root architecture of *P.judaica* in +Fe, -Fe, Bic and Tric grown conditions. F and P correspond to the F-test value and the probability value respectively. Different letters correspond to significant differences among means ($p<0.05$; Tukey test), $n=3$.

The diversification of morphological responses of the root system of *P. judaica* to direct (-Fe) and induced Fe deficiency (Bic and Tric) can be summarized as follows:

a) In all stress treatments the morphological component of the root of *P. judaica* remains relatively unchanged compared to the control;

b) The root systems developed by +Fe and –Fe plants are characterized by a preponderance of *shoot-borne* roots while those grown in the presence of bicarbonate (Bic) showed a proliferation of lateral roots;

c) In Bic condition the allocation of biomass (higher RMR) increases. This increment in biomass is distributed in a fine but high density root tissue (RDT);

d) The intra-root morphological traits and those related to the components of the root length have shown to be more plastic to the different experimental treatments compared to the morphological parameters of the entire root system.

6.4. **Conclusion**

In the treatments imposed, two nutritional stress conditions can be distinguished on the basis of the pH. On one hand we have a condition of pH 6.2 in which Fe starvation is imposed; on the other hand a high alkalinity condition, induced by bicarbonate or tricine, where a high pH induce a low availability of Fe and other nutrients such as phosphate, zinc, manganese.

In Fe-starved conditions, the plant must combine two fundamental needs: the "search of the nutrient" and "the ability to acquire it".

The root system developed in direct Fe-deficiency (-Fe) is characterized by long and numerous "*shoot-borne*" roots that have a greater number of short lateral roots. This could be a good strategy implemented by *P.judaica* for adapting to a Fe-starved condition. In fact, it was observed that the "*shoot-borne*" roots function in anchoring but also in the colonization of deep soil layers and show a higher longevity than the finer lateral roots (Canadell et al. 1996, Pregitzer et al. 1997, Gill et al. 2002, Gill and Jackson 2008).

In strongly alkaline conditions, such as that induced by bicarbonate, the adaptive response of the plant is focusing especially to make nutrients soluble. In order to facilitate the solubility of nutrients, the plant adopts physiological mechanisms, such as acidification through extrusion of H^+ (Zocchi and Cocucci, 1990; Palmgren, 2001; Santi and Schmidt, 2009) and the release of exudates (Welkie, 2000; Curie and Briat, 2003, Jin et al. 2007, López-Millán et al. 2009). From the morphological point of view, however, the proliferation of lateral roots and the increase in tissue density and length of root hairs represent important traits to explore a larger volume of soil and therefore acquire nutrients. In this context, the root system with a high number of lateral roots, as observed in *P. judaica* in the presence of bicarbonate (Bic), could exert a decisive role in the process of exudation, since it has been observed that lateral roots or their formation points (Jones, 1998; Darwent et al. 2003; López-Bucio et al. 2003) are the most active root types in exuding organic compounds.

Moreover, the number of tips may have an important role in the processes of exudation. In fact, they have been observed to be the areas of greatest release of organic compounds (Badri and Vivanco, 2009; Jones et al. 2009 and reference therein). The root system of *P. judaica* in Bic condition maintains the number of apices of both adventitious and lateral roots similar to that found in the control, suggesting that conservation of such a root system may have a role in adapting to alkaline environments. Another morphological characteristic observed in Bic condition is the high fineness associated with root segments with a high tissue density. A fine root system might better explore the soil at a close distance. This factor is crucial for the less mobile or poorly available nutrients typical of calcareous soils, when the the "depletion zone" of the nutrient is exceed through the root growth. Furthermore, the presence of a high density of tissues in the root system of *P. judaica* exposed to bicarbonate correlates well with the adaptive success coping with changes in nutrient availability (Wahl et al., 2001; Hill et al. 2006), with the activation of the secondary metabolism (Chap. 2), as such morphological trait was associated with rich sclerophyll and lignin elements (Wahl et al. 2001; Hummel et al. 2007). Finally, the increase in the root tissue density determines a greater ability of the plant to transport water and also a consequent high tensile strength that protects it against mechanical stress (Wahl et al. 2001). This functional role could be important for *P. judaica* as in calcareous soils the shortage of water and the mechanical strength constitute important factors of stress.

More complex seems the response of the root morphology and architecture in Tric condition, in which a greater reduction of all morphological parameters of the whole root system was found (total length, surface area and dry weight) compared to the other treatments. In addition, as previously reported, the pattern of responses of the root parameters RMR, RF and RDT appears intermediate with respect to –Fe and Bic conditions. In Tric-treated roots a greater root fineness was found that is a sign of a good soil exploration The lower number of lateral roots in Tric treatment could suggest an easier Fe acquisition respect to the bicarbonate condition in agreement with previous observations (Chap. 3 and 4).

These results show a morphological diversification of the root response of *P. judaica* to direct or induced Fe deficiency growth conditions in hydroponic, which, however, should also be validated in soil conditions, where the morphological and architecture root response could be more complex.

References

Badri D.V., Vivanco J.M., 2009. *Regulation and function of root exudates*, Plant, Cell & Environment, 32(6):666–681

Boot R.G.A., den Dubbelden K.C., 1990. *Effect of nitrogen supply on growth, allocation and gas exchange characteristics of two perennial grasses from inland dunes*, Oecologia, 85(1):115-121

Cahill Jr J.F., Casper B.B., 2000. *Investigating the relationship between neighbor root biomass and belowground competition: field evidence for symmetric competition belowground*, Oikos, 90(2):311–320

Caldwell M.M., 1994. *Exploiting nutrients in fertile soil microsites*, in: *Exploitation of environmental heterogeneity by plants*, Accademic Press, San Diego, pp. 325-347

Canadell J., Jackson R.B., Ehleringer J.B., Mooney H.A., Sala O.E., Schulze E.-D., 1996. *Maximum rooting depth of vegetation types at the global scale*, Oecologia, 108(4):583-595

Casper B.B., Schenk H.J., Jackson R.B., 2003. Defining a plant's belowground zone of influence, Ecology, 84(9):2313-2321

Coleman J.S., McConnaughay K.D.M., Ackerly D.D., 1994. *Interpreting phenotypic variation in plants, Trends in Ecology and Evolution*, 9:187–191

Curie C., Briat J.F., 2003. *Iron transport and signaling in plants*, Annual Review of Plant Biology, 54:183-206.

Darwent M.J., Paterson E., McDonald A.J.S., Tomos A.D., 2003. *Biosensor reporting of root exudation from Hordeum vulgare in relation to shoot nitrate concentration*, Journal of Experimental Botany, 54(381):325-334.

Dubrovsky J.G., Forde B.G., 2012. *Quantitative Analysis of Lateral Root Development: Pitfalls and How to Avoid Them*, The Plant Cell, 24(1):4-14

Fitter A.H., 1991. *Characteristics and functions of root systems*, in: *Plant roots: the hidden half*, 2:1-29

Gill R.A., Burke I.C., Lauenroth W.K., Milchunas D.G., 2002. *Longevity and turnover of roots in the shortgrass steppe: influence of diameter and depth*, Plant Ecology, 159(2):241-251

Gill R.A., Jackson R.B., 2008. *Global patterns of root turnover for terrestrial ecosystems*, New Phytologist, 147(1):13–31

Hill J.O., Simpson R.J., Moore A.D., Chapman D.F., 2006. Morphology and response of roots of pasture species to phosphorus and nitrogen nutrition, Plant and Soil, 286(1-2):7-19

Huang B., Eissenstat D.M., 2000. *Linking Hydraulic Conductivity to Anatomy in Plants that Vary in Specific Root Length*, Journal of American Society of Hoticultural Science, 125(2):260-264

Hummel G.M., Naumann M., Schurr U., Walter A., 2007. *Root growth dynamics of Nicotiana attenuata seedlings are affected by simulated herbivore attack*, Plant, Cell & Environment, 30(10):1326–1336

Hutchings M.J., de Kroon H., 1994. *Foraging in Plants: the Role of Morphological Plasticity in Resource Acquisition*, Advances in Ecological Research, 25:159–238

Intra-and interspecific variation in root length, root turnover and the underlying parameters

Jin C.W., You G.Y., He Y.F., Tang C., Wu P., Zheng S.J., 2007. *Iron deficiency-induced secretion of phenolics facilitates the reutilization of root apoplastic iron in red clover*, Plant Physiology, 144: 278-285.

Jones D.L., 1998. *Organic acids in the rhizosphere – a critical review*, Plant and Soil, 205(1):25-44

Jones D.L., Nguyen C., Finlay R.D., 2009. *Carbon flow in the rhizosphere: carbon trading at the soil-root interface*, Plant and Soil, 321(1-2):5-33

Lambers H., Shane M.W., Cramer M.D., Pearse S.J., Veneklaas E.J., 2006. *Root Structure and Functioning for Efficient Acquisition of Phosphorus: Matching Morphological and Physiological Traits*, Annals of Botany, 98(4):693-713

Landsberg, E-Ch., 1986. *Function of rhizodermal transfer cells in the Fe stress response mechanism of Capsicum annuum L.*, Plant physiology, 82(2):511-517.

López-Bucio J., Cruz-Ramírez A., Herrera-Estrella L., 2003. *The role of nutrient availability in regulating root architecture*, Current Opinion in Plant Biology, 6:280–287

López-Millán A.F., Morales F., Gogorcena, Y., Abadía A., Abadía J., 2009. *Metabolic responses in iron deficient tomato plants, Journal of Plant* Physiology, 166(4):375–384

Marschner H. 1995. *Mineral nutrition of higher plants* (2nd ed.), Accademic Press, London

Müller M., Schmidt W., 2004. Environmentally induced plasticity of root hair development in Arabidopsis, Plant Physiology, 134(1):409-419

Palmgren M.G., 2001. *Plasma membrane H^+-ATPases: powerhouses for nutrient uptake*, Annual Review of Plant Biology, 52:817-845

Perry P., Linke B., Schmidt W. 2007. *Reprogramming of root epidermal cells in response to nutrient deficiency*, Biochemical Society Transactions, 35:161-163

Pigliucci M., 2001. *Phenotypic Plasticity: Beyond Nature and Nurture*, Johns Hopkins University Press, Baltimore

Pregitzer K.S., Kubiske M.E., Yu C.K., Hendrick R.L., 1997. *Relationships among root branch order, carbon, and nitrogen in four temperate species*, Oecologia, 111(3):302-308

Römheld V., Marschner H., 1981. *Rhythmic iron stress reactions in sunflower at suboptimal iron supply*, Physiologia Plantarum, 53(3):347-353.

Rubinigg M., Posthumus F., Ferschke M., Elzenga J.T.M., Stulen I., 2003, *Effects of NaCl salinity on 15N-nitrate fluxes and specific root length in the halophyte Plantago maritima L.*, Plant and Soil, 250(2):201-213

Ryser P., 1998. *Intra-and interspecific variation in root length, root turnover and the underlying parameters*, In: *Inherent variation in plant growth. Physiological mechanisms and ecological consequences*, Backhuys Publishers, Leiden, The Netherlands, pp. 441-465

Ryser P., Eek L., 2000. *Consequences of phenotypic plasticity vs. interspecific differences in leaf and root traits for acquisition of aboveground and belowground resources*, American Journal of Botany, 87(3):402-411

Ryser P., Lambers H., 1995. *Root and leaf attributes accounting for the performance of fast- and slow-growing grasses at different nutrient supply*, Plant Soil, 170: 251–265

Ryser P., Lambers H., 1995. *Root and leaf attributes accounting for the performance of fast- and slow-growing grasses at different nutrient supply*, Plant and Soil, 170(2):251-265

Santi S., Schmidt W., 2009. *Dissecting iron deficiency-induced proton extrusion in Arabidop-sis roots*, New Phytologist, 183:1072-84

Schmidt W., J. Tittel, A. Schikora, 2000. *Role of hormones in the induction of iron deficiency responses in Arabidopsis roots*, Plant Physiology, 122(4):1109-1118

Sorgonà A., Abenavoli M.R., Gringeri P.G., Cacco G., 2007. *Comparing Morphological Plasticity of Root Orders in Slow- and Fast-growing Citrus Rootstocks Supplied with Different Nitrate Levels*, Annals of Botany, 100:1287–1296

Sultan S.E., 2000. *Phenotypic plasticity for plant development, function and life history*, Trends in Plant Science, 5(12):537–542

Tinker P.B., Nye P.H., 2000, *Solute movement in the rhizosphere*, Oxford University Press, New York

Wahl S., Ryser P., Edwards P.J., *Phenotypic Plasticity of Grass Root Anatomy in Response to Light Intensity and Nutrient Supply*, Annals of Botany, 88(6):1071-1078

Weiner J., 2004. *Allocation, plasticity and allometry in plants*, Perspectives in Plant Ecology, Evolution and Systematics, 6(4):207–215

Welkie G.W., 2000. *Taxonomic distribution of dicotyledonous species capable of root excretion of riboflavin under iron deficiency*, Journal of Plant Nutrition, 23:1819-1831

Zobel R.W., Waisel Y., 2010. *A plant root system architectural taxonomy: A framework for root nomenclature*, Plant Biosystems, 144 (2): 507–512

Zocchi G., Cocucci S., 1990. *Fe uptake mechanism in Fe-efficient cucumber roots*, Plant Physiology, 92:908–911

CHAPTER 7

General Conclusions

— Summary of findings and future research directions —

Plants, due to their sessile nature, must continuously face the changing environmental conditions. In order to cope with constant and diverse challenges, plants must adjust their physiology, growth and development. Plant responses to abiotic and biotic stresses are complex and involve multiple traits. Anyway, the primary response takes place at the metabolic level. Metabolic responses to abiotic stresses concern interactions and interferences involving many molecular pathways.

One of the most important issues for plants is to maintain an adequate nutrient supply under fluctuating environmental conditions. In fact, soil nutrients are typically less than 1% by weight readily available for plant uptake. Roots have the primary function of acquiring nutrients thus their phenotypic plasticity to low resource environment is crucial in plant adaptation. To acquire nutrients successfully plant performs two main strategies: scavenging and mining. Mining strategies are aimed at mobilizing nutrients from unavailable forms to available soil solution forms for plant uptake and encompass biochemical and physiological mechanisms implemented by roots such as acidification, reduction or chelation. Scavenging strategies are achieved by morphological and architectural root changes mainly oriented to increase the soil exploration. Scavenging and mining strategies act simultaneously performing an integrated response.

Iron (Fe) is an essential micronutrient for plants and animals which fulfills critical catalytic roles in a plethora of proteins in main metabolic pathways such as photosynthesis and respiration. Despite highly abundant in soils, Fe exhibits a very low bioavailability due to the scarce solubility of its oxidized forms. In calcareous soils, as well as in high pH environments, Fe solubility decreases even more dramatically. In fact, in chalky soils Fe deficiency is the cause of a common plant nutritional disorder known as lime-induced chlorosis which leads to a considerable yield loss.

Along the evolution, calcicole wild plants have shaped their adaptive responses to this nutritional environmental stress. For this reason studying wild plants responses could provide interesting insights useful to increase our knowledge on how plants can overcome Fe deficiency constraints.

Parietaria judaica, a wild sinantropic plant, can grow in highly calcareous environments without showing chlorosis symptoms. *P. judaica* displays diversified responses to Fe deficiency that allow it to adapt efficiently. In this work the mining and scavenging strategies performed by *P. judaica* in response to direct and induced Fe deficiency conditions were studied.

Physiological, metabolic and morphological root responses were analyzed.

It was demonstrated (Chapter 2) that *P. judaica*, as a Strategy I plant species, responds to direct and induced Fe deficiency by inducing the FC-R and H^+-ATPase activities, and increasing the release through exudates of low organic acids, mainly citric acid, and phenolics. A significant increase in organic acids and phenolics was detected also in root tissues of plants grown under Fe deficiency stress conditions. Multiple functions related to Fe deficiency responses have been attributed to these compounds. Citric acid is protogenic and inside the cell could be involved in cytosolic pH control, in feeding the H^+-ATPase proton pump and plant Fe transport, while as exudate is a metal chelator, acidifies the rhizosphere. Phenolic compounds are versatile molecules. They are involved in antioxidant and reduction activities and can also act as metal chelators. Thus, inside the cell they probably play a protective role against the ROS formed under stress condition, whereas in the exudates they are involved in Fe mobilization, as source of carbon for soil microorganism and allelopathy.

At metabolic level an increase in the PEPC activity was observed only in Fe starved condition while in the presence of bicarbonate the low PEPC activity was attributed to the inhibition exerted by the high concentration of malic acid and of shikimic acid. It was suggested that in the presence of bicarbonate, the PEP produced by glycolysis could in part be channelled into the shikimate pathway and converted into phenolics, which are abundant in this condition. In agreement with the high amounts of phenolics an increase in key enzymes of the shikimate pathway was found, as the first enzyme of the phenylpropanoid pathway (PAL). It was thus suggested that *P. judaica* responds through a metabolic shift involving particularly the PEP-consuming reactions.

Through a time course (Chapter 3) it was analysed the efficiency of *P. judaica* responses to Fe deficiency. A highly alkaline organic buffered treatment was added to distinguish the effect of the high pH on Fe availability from the responses induced by the presence of high concentrations of bicarbonate. Low organic acids and phenolic compounds definitely play a pivotal role in the adaptive strategy performed by *P. judaica* to cope with Fe deficiency. However, it was demonstrated that the biosynthesis of phenolic constitutes a preferential way in *P. judaica* responses, as they were found in greater amounts and were detected first than low organic acids in exudates. Both these compounds carry out potentially multiple functions, so *P. judaica* can modulate their contents depending on the status and different requirements of the plant. The responses of *P. judaica* to both alkaline treatments have confirmed that it

is a Fe-efficient plant. However, whilst in organic buffered alkaline condition *P. judaica* has no problems to acquire Fe and displays a non-stressed shoot phenotype, in the presence of high concentrations of bicarbonate the growth rate diminishes significantly and the plant shows a stressed shoot phenotype even if non-chlorotic. These findings suggest that in a highly calcareous environment the availability of bicarbonate itself constitutes a real factor of stress. In calcareous treatment the presence of HCO_3^- and Ca^{2+} could operate in a direct and indirect way over Fe bioavailability. As a direct effect, HCO_3^- raises the pH decreasing Fe solubility and, additionally, HCO_3^- ion buffers the attempt to acidify the apoplast reducing as a consequence the FCR activity. It was also suggested that an indirect effect of bicarbonate treatment could be due to the high content of Ca^{2+} that is preferentially chelated by citrate respect to Fe, making the chelating action of citrate less effective in acquiring Fe.

Increase in phenolic compounds is one of the general stress responses in plants, resulting in a shift of carbon flux from primary to secondary metabolism. Moreover, under stress conditions, plants have to cope with the efficient distribution of limited resources between physiological processes and the activation of mechanisms to acquire the depleted nutrients. The metabolic flexibility is therefore the main feature to successfully cope with environmental limitations.

It was then investigated the activity of some key enzymes of the primary metabolism and the way by which *P. judaica* supplies the primary substrates to sustain the high rate of biosynthesis of phenolic compounds (Chapter 4). The results obtained provide an interesting scenario that suggests a distinct change in the way to supply substrates for the shikimate pathway. The inverse correlation between G6PDH (the first enzyme of OPPP) and TK (an enzyme of the non-OPPP) activities found in Fe starved condition may indicate a metabolic rearrangement by modifying the allocation of carbon skeletons between primary and secondary metabolism. Moreover, the activation of a non-oxidative way could overcome a mitochondrial impairment due to the scarcity of Fe. The shift to a non-OPPP way to obtain erythrose-4-phosphate — a primary substrate in the shikimate pathway — could be favored as a consequence of a reduced turnover of NADH, so avoiding an overproduction of reducing power through the OPPP when Fe lacks. In addition, the non-OPPP way preserves carbon skeletons by preventing decarboxylation, thus constituting a carbon saving strategy.

The effect of direct and induced Fe deficiency on the composition of

phenolic compounds of *P. judaica* was investigated by HPLC analyses on roots and exudates (Chapter 5). Field samples have shown that the most abundant phenolics are some mono- and di-caffeoylquinic derivatives. The analyses carried out on samples from plants grown in hydroponics in different conditions revealed similar HPLC profiles in roots but showed quantitative differences among the treatments. On the contrary, HPLC profiles of root exudates have shown altered profiles respect to the corresponding root tissues and demonstrate both qualitative and quantitative differences in phenolic compounds composition among root exudates from different growth conditions. The phenolic compounds identified present a catechol group that has been pointed out as a strong antioxidant and a potential metal chelator. Furthermore, chlorogenic acid — the main component — is known to be involved in the responses to different biotic and abiotic stresses in several plant species, probably through its ability to interact with ROS acting as a potent antioxidant. It was shown its ability to chelate Fe, thus avoiding the catalysis of Fenton reaction. In roots of *P. judaica* subject to low Fe availability the accumulation of phenolics was mainly due to the increase of the chlorogenic acid component. Root exudates presented different HPLC profiles from their producer roots so far. This could suggest an addressed and active mechanism related to phenolic exudation.

Under Fe deficiency general root morphological modifications have been described in different species. Morphological changes induced by Fe deficiency include swelling of root tips and formation of lateral roots, root hairs, and transfer cells that increase the root surface in contact with the external medium, thereby increasing the Fe uptake capability.

Changes in root morphology were studied in *Parietaria judaica* under direct or induced Fe deficiency treatments (Chapter 6).Through imaging system analyses it was demonstrated a morphological and architectural diversification of the root system as a response to the direct or induced Fe deficiency conditions. In stress conditions a proliferation of lateral roots that enhance the total absorptive root surface was registered. Albeit the increase of root surface a different branching pattern among the differently treated plants was observed. These differences are probably due to the different metabolic status of the plants. Minus Fe plants produce just rootlets that constitute the most active portion of the root system. In this way the plant maximizes the response per unit biomass and energy. In Fe alkaline growth conditions Fe bioavailability becomes very low, although Fe is still present, so plants address their efforts to acquire Fe increasing the root surface that can succeed in

retrieve it. However, under bicarbonate supply root morphology denoted a higher stress condition as the number of lateral roots and tissue density were greater. Moreover shoot features presented a slight chlorosis with respect to alkaline organic buffer condition suggesting a specific stress caused probably by the presence of the bicarbonate ion itself.

In conclusion, the results obtained so far suggest that *Parietaria judaica* responds to Fe deficiency performing integrated responses which indicate a high plasticity at physiological, biochemical and morphological level. Secondary metabolism plays a pivotal role in Fe acquisition but further research are needed to investigate:

- the direct involvement of dicaffeoylquinic acids in antioxidant and chelating activities regarding plant Fe deficiency;

- the action of dicaffeoylquinic acids on Fe apoplastic deposits;

- the role of dicaffeoylquinic acids as promoters of mutual interactions on soil microorganism;

- a metabolomic study to confirm the primary–to-secondary metabolism shifts proposed

- the regulation of the main shift (PEP and G6PDH) between primary and secondary metabolism.