Introduction

Soils with a relatively high lime concentration are present in about one-third of the earth’s surface (Chen and Barak 1982). This can be a matter of concern for the development of several orchards and vineyards, in which plants must face Fe-deficiency-induced chlorosis, resulting in a decrease in productivity (Tagliavini and Rombolà 2001). However, different varieties of a given plant species may differ appreciably in their sensitivity to calcareous soils (Korcak 1987).

Adverse environmental conditions are reported to induce oxidative stress in plants as a consequence of reactive oxygen species (ROS) production (Foyer et al. 1997). However, relatively little information is available on the relationships between Fe deficiency and secondary oxidative stress, and some data remain controversial (Iturbe-Ormaechea et al. 1995, Ranieri et al. 2001, Lombardi et al. 2003, Chouliaras et al. 2004, Molassiotis et al. 2005). Iron is either a constituent or a cofactor of many antioxidant enzymes and, at the same time, can act as a pro-oxidant through the Fenton reaction (Halliwell and Gutteridge 1984). In its role as an enzyme constituent, Fe is part of catalase (CAT, EC 1.11.1.6), non-specific peroxidases (POD, EC 1.11.1.7), ascorbate peroxidase (APX, EC 1.11.1.11) and Fe superoxide dismutase (Fe-SOD, EC 1.15.1.1).

Superoxide radical (O$_2^-$) is produced at any location where an electron transfer is present and thus in every compartment of the cell. Superoxide dismutases, converting O$_2^-$ to H$_2$O$_2$, constitute the first line of defence against ROS in different plant species under several stress conditions (Alscher et al. 2002,}.
Blokhina et al. 2003). In particular, studies on plants grown in Fe-deprived conditions showed high SOD activity, mainly due to increased Cu/Zn or Mn-SOD isoforms (Iturbe-Ormaeche et al. 1995, Molassiotis et al. 2006).

In addition to SOD activity, the intracellular level of H$_2$O$_2$ is controlled by other enzymes, the most important being CAT and PODs. Peroxidases, by means of their hydroxycilic or peroxidative activity, can regulate both ROS production and scavenging in numerous cell compartments and thus they can be involved in many plant processes, such as growth and biotic/abiotic stress responses (Passardi et al. 2005). In the cell wall, PODs are present as soluble, ionically and covalently bound forms and they can catalyse cross-linking reactions, building a rigid cell wall, or produce ROS-generating wall-loosening reactions, to make it more flexible (Lewis and Yamamoto 1990, Polle et al. 1994, Schweikert et al. 2000). Additionally, PODs are enzymes directly involved in lignin biosynthesis by assembling lignin units through oxidative polymerization (Lewis and Yamamoto 1990). Several works have well documented that some growth conditions are responsible for the increase in cell-wall lignification which, by reducing cell growth, may represent a plant’s adaptation to adverse conditions (Ibir et al. 2001, Lee et al. 2007). With regard to plants grown under Fe deficiency, POD isoforms are differently affected. Ranieri et al. (2001) showed, in Fe-deficient sunflower, a preferential reduction in the activity of those isoforms involved in H$_2$O$_2$ detoxification, rather than in the maintenance of cell-wall structure. According to Molassiotis et al. (2006), increased POD activity may be an important attribute linked to chlorosis tolerance in peach rootstocks.

Approximately 80% of pear cultivars are budded on pear seedlings; the rest are grafted on quince (Cydonia oblonga) rootstock, which induces low vigour of the scion (Kobelus et al. 2007). However, pear cultivars often show symptoms of Fe chlorosis when grafted on quince, above all when grown on calcareous soils (Korçak 1987).

In the present work, we focused our attention on pear (cv. Conference) and quince (clone BA29) genotypes characterized by different levels of tolerance to lime-induced chlorosis. In a previous paper (Donnini et al. 2008), we saw evidence only in cv. Conference (tolerant to the presence of bicarbonate), a strategy of adaptation characterized by a decrease in shoot development that could be finalized to maintain an adequate level of Fe in leaf tissues as a concentration effect. Furthermore, the same cultivar grown in the presence of bicarbonate showed photosynthetic activity similar to that of control plants (Donnini et al. 2009). While in control growth conditions, the photosynthetic products are basically destined for the formation of new tissues, in the bicarbonate supply, these compounds could essentially be targeted to the roots to sustain enzymatic activities involved in Fe uptake, which are energy consuming (Vigani and Zocchi 2009).

In this study, we hypothesize that antioxidant enzymes, which are involved in stress adaptation/tolerance, are activated to different degrees in relation to different chlorosis tolerance. To test this hypothesis, the possibility that Fe deficiency could generate oxidative stress at root level was investigated by checking changes in the activity of some enzymes involved in ROS production/detoxification, as well as the presence of O$_2^-$ and H$_2$O$_2$ at root level. Moreover, by using a specific substrate, syringaldazine, and performing histochemical analysis with safranin/fast green, root lignification was detected in both genotypes to verify whether the difference in the degree of lignification could be related to the different adaptations to such adverse conditions.

Materials and methods

Plant material and culture

One-year-old plants with three primary shoots propagated by microshoots (Pyrus communis L. cv. Conference) or cuttings (Cydonia oblonga Mill. clone BA29) were grown in a hydroponic medium containing (mM) 4 Ca(NO$_3$)$_2$, 4 KNO$_3$, 1.6 MgSO$_4$, 0.8 KH$_2$PO$_4$ and (µM) 37 H$_3$BO$_3$, 7.3 MnCl$_2$, 0.23 CuCl$_2$·2H$_2$O, 0.64 ZnSO$_4$, 0.08 (NH$_4$)$_6$Mo$_7$O$_24$ with either 80 µM Fe(III)Na-ethylenediaminetetracetic acid (EDTA) (+Fe, control) or without Fe (−Fe). Iron shortage was also indirectly achieved by adding 10 mM KHCO$_3$ to +Fe medium (+FeBic). Except for the last treatment (pH 8.30), the initial pH was adjusted to 6.00–6.20 with NaOH. Plants were grown for 21 days under different treatments and the medium was changed weekly. Plants were maintained in a growth chamber with a day/night regime of 16/8 h and with a photosynthetic photon density flux of 200 µmol m$^{-2}$ s$^{-1}$. The temperature was 22°C in the dark with relative humidity (RH) 60% and 26°C in the light with an RH of 80%.

Total chlorophyll determination

Leaf chlorophyll concentration was determined according to Lichtenthaler (1987). Leaf samples were homogenized with 100% acetone, in the presence of Na-ascorbate to prevent pigment oxidation. Extracts were filtered through Minisart SRP15 0.2 µm filters (Sartourius, Goettingen, Germany) and absorbance was determined at 644.8 and 661.6 nm with a Cary 50 Bio UV–visible spectrophotometer (Varian, Walnut Creek, CA, USA).

Root growth determination

Root elongation (in cm) was calculated as the difference between root length at the end of treatment (Day 21) and root length at the starting point (Day 0). Reference roots for growth measurement were marked with tape. Six biological replicates for each genotype and treatment were performed.
SOD extraction and activity assay

Root samples were ground in liquid N2 with 10% (w/w) polyvinylpyrrolidone (PVPP) and homogenized in a medium containing: 50 mM K-phosphate buffer (pH 7.00), 1 mM EDTA, 0.05% (v/v) Triton X-100, 1 mM dithiothreitol (DTT), 1 mM Na-ascorbate and 0.50 mM phenylmethylsulphonyl fluoride (PMSF). After centrifugation at 12,000g for 30 min at 4 °C, the supernatant was collected and dialysed overnight against 2.5 mM Tricine–KOH (pH 8.00). Ascorbic acid was added to the buffer during the extraction procedure. After centrifugation (10,000g for 5 min), the supernatant was washed five times with distilled water. After centrifugation (2,000g for 5 min), the pellet was treated with 1 M CaCl2 for 1 h and centrifuged at 800g for 10 min at 4 °C. The supernatant was the ionically bound fraction, while the pellet was considered the soluble fraction. The first pellet was washed twice with phosphate buffer, twice with distilled water and, after shaking for 1 h at 4 °C in 2% (v/v) Triton-X100 in water, it was rinsed five times with distilled water. After centrifugation (800g for 5 min), the pellet was treated with 1 M CaCl2 for 1 h and centrifuged at 800g for 10 min at 4 °C. The supernatant was then centrifuged at 800g for 5 min, and the second supernatant was considered the soluble fraction. The first pellet was washed twice with phosphate buffer, twice with distilled water and, after shaking for 1 h at 4 °C in 2% (v/v) Triton-X100 in water, it was rinsed five times with distilled water. After centrifugation (2,000g for 5 min), the pellet was treated with 1 M CaCl2 for 1 h and centrifuged at 800g for 10 min at 4 °C. The supernatant was the ionically bound fraction, while the pellet was washed five times with distilled water and incubated overnight at room temperature with 0.3% cellulase, 0.3% macerase and 0.3% cellulolysin in 50 mM Na-acetate buffer (pH 5.50) to obtain, after centrifugation (800g for 10 min), the covalently bound fraction.

Peroxidase activity was assayed in the three fractions by using syringaldazine, a specific substrate analogue of lignin monomer used to test the role of POD in the lignification process (Ranieri et al. 2001, Lee et al. 2007).

The activity of syr-POD was determined by measuring the increase in absorbance at 530 nm of the reaction mixture containing 200 mM Na-K phosphate buffer (pH 6.00), 2 mM syringaldazine, 2.5 mM H2O2 and the protein extract (Pandolfini et al. 1992).

Histochemical detection of H2O2 and O2−

The histochemical detection of H2O2 and O2− in root tissues was performed as described by Hernandez et al. (2001) with some modifications. For H2O2 determination, roots were vacuum infiltrated with 0.1 mg ml−1 3,3′-diaminobenzidine (DAB) in 50 mM Tris-acetate buffer (pH 5.00) and then incubated in the dark at room temperature for 24 h. For O2− determination, roots were vacuum infiltrated with 0.1 mg ml−1 NBT in 25 mM HEPES buffer (pH 7.60) and incubated in the dark at room temperature for 2 h. Controls of H2O2 and O2− (data not shown) were performed by adding to the infiltration buffer 10 mM AA and 10 mM MnCl2, respectively, highly effective inhibitors of the two reactive species (Hernandez et al. 2001). Freshly cut root sections were observed by optical microscope (Leica DMR) and acquired with a Leica DC300F Digital Camera.

Lignin visualization in root tissues

Root segments were treated according to Dell’Orto et al. (2002) with some modifications. After fixing at 4 °C overnight
in 100 mM Na-phosphate buffer (pH 7.00) containing 4% paraformaldehyde (w/v), root segments were dehydrated through an ethanol–tertiary butanol series and embedded in paraffin (Paraplast Plus, Sigma). Serial sections of 5 µm were cut with a microtome, mounted on silanized slides, deparaffinized in xylene and rehydrated thorough an ethanol series. Sections were then stained with the safranin/fast green method according to Johansen (1940), mounted with cover slides, observed by Optical Microscope (Leica DMR) and acquired by Leica DC300F Digital Camera.

Protein determination
Protein content was determined by the Bradford (1976) procedure using the Bio-Rad reagent and bovine serum albumin as a protein standard.

Statistical analysis
All data were subjected to two-way ANOVA (treatment × genotype), using Tukey’s test at $P < 0.05$. The statistical analysis was conducted with Sigma-Stat® 3.1.

Results

Leaf chlorosis symptoms and total chlorophyll content
After growing plants under different treatments (+Fe, −Fe and +FeBic), we observed a high correlation between tolerance to Fe chlorosis reported under field conditions and symptoms of Fe deficiency. In fact, while the absence of Fe caused chlorosis in both genotypes (Figure 1b and e), the presence of bicarbonate caused leaf symptoms only in BA29 (Figure 1f).

Table 1 shows the two-way ANOVA of leaf chlorophyll content expressed on a fresh weight basis. The lack of Fe and the bicarbonate supply conditions resulted in a significant decrease in chlorophyll content in both genotypes. However, the cv. Conference seemed less affected than BA29 when bicarbonate was added to the medium.

Superoxide dismutase activity assay and isoform visualization
Superoxide dismutase activity under +Fe treatment was ~4-fold higher in BA29 roots than in the cv. Conference (Figure 3a). In both genotypes, total SOD activity was strongly increased in the absence of Fe, whereas no appreciable variation was detected in the presence of bicarbonate. In more detail, in comparison with the relative controls (+Fe), cv. Conference and BA29 plants grown in the absence of Fe exhibited a 196 and 284% increase, respectively.

To identify a possible change in the SOD isoform pattern induced by stress conditions, non-denaturing PAGE was

Table 1. Total chlorophyll content of 21-day-old leaves of cv. Conference and BA29 grown in different conditions.

<table>
<thead>
<tr>
<th>Total chlorophyll content (µmol g⁻¹ FW)</th>
<th>Cv. Conference</th>
<th>BA29</th>
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<tbody>
<tr>
<td>+Fe</td>
<td>1.71 ± 0.23a</td>
<td>1.88 ± 0.36a</td>
</tr>
<tr>
<td>−Fe</td>
<td>0.25 ± 0.04d</td>
<td>0.22 ± 0.03d</td>
</tr>
<tr>
<td>+FeBic</td>
<td>1.13 ± 0.17b</td>
<td>0.63 ± 0.06c</td>
</tr>
</tbody>
</table>

Data are means ± SD ($n = 5$). For each parameter the significance of the F-ratio following two-way ANOVA is shown. In the case of significant interaction between treatment and genotype, values followed by different letters are statistically different (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; n.s. = not significant).

Root growth determination
With the aim of verifying the effect of the different treatments on the growth of the two species, the length of young not lignified roots was determined. The results put in evidence a different behaviour between the two species (Figure 2). In fact, while no significant change was observed in cv. Conference roots, a dramatic decrease in root elongation was recorded in BA29 under bicarbonate treatment (−88%, with respect to the control).

Table 1

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Figure 1. Representative pictures of 21-day-old leaves of *P. communis* L. cv. Conference (a–c) and *C. oblonga* Mill. (d–f) grown in different conditions: control (a, d), absence of Fe (b, e) and +FeBic treatment (c, f).
performed. Loading of samples on the gel was performed by keeping the amount of protein constant between treatments for each genotype, but different between genotypes because of their different activity. As shown in Figure 3b, a single SOD isoform was detected in the control roots of the cv. Conference (lane 1). Upon addition of KCN and H$_2$O$_2$ as inhibitors, this isoform was identified as a Cu/Zn-SOD (data not shown). A similar isoform was also detected in −Fe-treated roots; in this condition, an additional isoform at lower molecular weight was detected and identified as Mn-SOD (lane 2). In contrast, in plants grown in bicarbonate, these isoforms were not active and a new faintly stained Mn-SOD isoform was expressed (lane 3). Concerning BA29 (Figure 3c), three different bands were detected only in roots grown in the absence of Fe, one corresponding to Cu/Zn-SOD and two corresponding to Mn-SOD. No isoforms were revealed in the other treatments. Anyway, loading the gel with a higher amount of proteins revealed the same three isoforms for all conditions, but led to an overcharge of the −Fe sample (data not shown).

**Peroxidase activity assay and isoform visualization**

Non-specific POD activity was measured using o-dianisidine as a substrate (Ranieri et al. 1997). The activity under +Fe treatment was similar in both genotypes; however, a different trend was observed under both stress conditions (Figure 4a). The absence of Fe induced in the cv. Conference a decrease in POD activity (−50%, compared with the control), whereas in BA29 it increased by −31%. Conversely, the presence of bicarbonate in the growth medium induced a strong increase (+74%) in the cv. Conference, while in BA29 a decrease of −40% was observed in response to this treatment.

The separation of POD isoforms by non-denaturing PAGE revealed a species-specific difference: in the cv. Conference grown in the presence of Fe, three bands were detected (Figure 4b, lane 1), while in BA29 five bands appeared, two with high and three with low molecular weight (Figure 4c, lane 1). In the cv. Conference, only the bicarbonate treatment induced a generalized increase in the band staining intensity (Figure 4b, lane 3). Conversely, in the BA29 roots grown under −Fe treatment, enhanced intensity of POD isoforms occurred (Figure 4c, lane 2), while under bicarbonate treatment a faint staining of the bands at low molecular weight and the loss of those at high molecular weight was observed (Figure 4c, lane 3).

Concerning APX activity (Figure 5), the absence of Fe induced similar changes in the two species, even if in BA29 the decrease was stronger than in the cv. Conference (−64 and −55%, respectively). On the contrary, the presence of bicarbonate in the growth medium induced a significant decrease (−56%) only in BA29. Concerning APX activity under +Fe condition, in the cv. Conference roots it was approximately fivefold higher than in BA29.
Figure 4. (a) Peroxidase activity (µmol mg prot⁻¹ min⁻¹) assayed by using o-dianisine as substrate in cv. Conference (left) and BA29 (right) roots grown for 21 days in control conditions (+Fe), in the absence of iron (−Fe) and in the presence of bicarbonate (+FeBic). Data are given as means ± SD (n = 5). For each parameter, the significance of the F-ratio following two-way ANOVA is shown. In the case of significant interaction between treatment and genotype, values followed by different letters are statistically different (*P < 0.05; **P < 0.01; ***P < 0.001; n.s. = not significant). Native PAGE for POD isoforms of cv. Conference (b) and BA29 (c) roots. +Fe (lane 1), −Fe (lane 2) and +FeBic (lane 3). An amount of 10 µg of total proteins for each sample was applied.

**Syr-POD determination**

When the soluble, ionically and covalently bound cell-wall fractions were tested for syr-POD activity, a different trend was determined in the tolerant and susceptible genotypes (Table 2).

The activity of syr-POD determined in the soluble fraction increased in both genotypes grown in the presence of bicarbonate although to different extents: while in the cv. Conference an increase of 84% was observed in comparison with the control, in BA29 the increase was dramatically higher (+970%).

In the ionically bound fraction, syr-POD activity was increased only in BA29 roots grown in the presence of bicarbonate, whereas a decrease was observed in response to the absence of Fe. On the other hand, no significant differences were observed in the same fraction of cv. Conference roots. In contrast to what occurred in the soluble and ionically bound fractions, in the covalently bound ones, no significant effects of the different treatments were detected in either genotype.

**Histochemical detection of O₂⁻ and H₂O₂**

Since O₂⁻ is often the first reduced form of oxygen to be generated in plant tissues, we analysed its distribution by treating roots with NBT, which forms a dark blue formazan precipitate in contact with O₂⁻ (Bielski et al. 1980). In whole roots of both genotypes grown under Fe deficiency, this colour appeared intense in the meristematic and elongation zones, whereas it was weaker in the maturation zone (data not shown). The

![Figure 5. Ascorbic POD activity (µmol mg prot⁻¹ min⁻¹) in cv. Conference (left) and BA29 (right) roots grown for 21 days in control conditions (+Fe), in the absence of iron (−Fe) and in the presence of bicarbonate (+FeBic). For each parameter, the significance of the F-ratio following two-way ANOVA is shown. Data are given as means ± SD (n = 5). In the case of significant interaction between treatment and genotype, values followed by different letters are statistically different (*P < 0.05; **P < 0.01; ***P < 0.001; n.s. = not significant).](image-url)

**Table 2. Peroxidase activity of the soluble, ionically and covalently bound cell-wall fractions of cv. Conference and BA29 roots, measured using syringaldazine as electron donor.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity syr-POD</th>
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<tr>
<td></td>
<td>Soluble fraction</td>
</tr>
<tr>
<td></td>
<td>(ΔAbs min⁻¹ mg⁻¹)</td>
</tr>
<tr>
<td>cv. Conference</td>
<td></td>
</tr>
<tr>
<td>+Fe</td>
<td>0.84 ± 0.21c</td>
</tr>
<tr>
<td>−Fe</td>
<td>0.69 ± 0.12c</td>
</tr>
<tr>
<td>+FeBic</td>
<td>5.27 ± 0.42b</td>
</tr>
<tr>
<td>BA29</td>
<td></td>
</tr>
<tr>
<td>+Fe</td>
<td>1.50 ± 0.24c</td>
</tr>
<tr>
<td>−Fe</td>
<td>1.95 ± 0.17c</td>
</tr>
<tr>
<td>+FeBic</td>
<td>16.12 ± 1.04a</td>
</tr>
<tr>
<td>Treatment</td>
<td>**</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
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<td>*</td>
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<tr>
<td></td>
<td>Treat x Gen</td>
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</tbody>
</table>

Syringaldazine POD activity is expressed as ΔAbs₅₃₀ min⁻¹ mg⁻¹ protein, except for the covalently bound fraction, whose activity is reported as ΔAbs₅₃₀ min⁻¹ g⁻¹ DW of residual cell-wall material. Data are means ± SD (n = 5). For each parameter, the significance of the F-ratio following two-way ANOVA is shown. In the case of significant interaction between treatment and genotype, values followed by different letters are statistically different (*P < 0.05; **P < 0.01; ***P < 0.001; n.s. = not significant).
observation of root cross-sections revealed the presence of dark blue precipitate in the stele and in the cortex apoplast of both species under all growth conditions (Figure 6). On the other hand, no staining was observed in the rhizodermis of differently treated roots. However, in BA29 –Fe and +FeBic (Figure 6E and F) and in Conference –Fe (Figure 6B) the blue stain in the cortex apoplast was more evident with respect to the controls; in BA29 –Fe (Figure 6E) it was also present in the cortex cytosol.

The production of H$_2$O$_2$ was revealed after infiltration of the roots with DAB, which reacts with H$_2$O$_2$ in the presence of endogenous PODs to produce a brown polymerization product. Incubation of intact Fe-deficient roots showed a distinct pattern of brown colour. The cells belonging to the region encompassing the end of the elongation zone and the maturation zone appeared brown, but this colour was stronger in the elongation and meristematic zones (data not shown). Cross-sections of primary lateral roots of both genotypes grown without Fe and of BA29 exposed to bicarbonate showed a brown colour in the cortex and, to a greater extent, in the rhizodermis, endodermis and stele (Figure 7b, e and f). Conversely, in the control roots weaker staining was evident in the endodermis, in

![Image of root sections showing blue staining and brown colour in the cortex and stele.](image-url)
the rhizodermis and in the whole stele, while cortex cells were stained only in the apoplast (Figure 7A and D). The distribution of H$_2$O$_2$ followed a similar pattern in cv. Conference roots grown under bicarbonate except for the stele, which appeared completely brown (Figure 7c).

The production of O$_2^-$ and H$_2$O$_2$ was totally suppressed by using MnCl$_2$ and AA, respectively, indicating the specificity of the DAB/NBT staining applied (data not shown).

**Histochemical visualization of lignin**

Microscopic analysis of root sections after safranin/fast green staining allowed lignified cell walls to be visualized as red spots. A significant presence of red stain, consistent with intense lignification, was visible in root sections of BA29 grown in the presence of bicarbonate (Figure 8f); the red colour is clearly localized in the stele and in the endodermis, which appears totally lignified. Red-coloured cell walls were also detected in the sub-epidermis.
Discussion

Under our experimental conditions, the behaviour of the two genotypes was primarily evident by the different induction of Fe chlorosis symptoms (Figure 1). The tolerant cv. Conference showed visual symptoms only in the absence of Fe; on the contrary, in the susceptible BA29, chlorosis was evident in the young leaves growing under both stress conditions. In agreement with the symptoms shown by the plants, the chlorophyll content was more affected in quince than in the cv. Conference by the presence of bicarbonate (Table 1). In addition, Figure 2 shows that this growth condition caused a significant difference also in root elongation, which was negatively affected only in BA29. We suggest that a better growth performance of cv. Conference under both Fe-stress conditions could reside in specific responses to improve Fe acquisition. In the same pear cultivar, our previous results (Donnini et al. 2008) suggested the presence, at the shoot level, of an adaptive strategy to calcareous soils consisting in a lower branching capacity and in a minor elongation of the main stem that could help in maintaining an adequate level of Fe in the same tissues, avoiding its allocation to the new outgrowths of lateral shoots. Taken together, these results suggest the presence, in the cv. Conference, of a combined effect of limited canopy growth and compensation in new root tissue formation, which could reduce sink competition for photosynthates and then optimize plant survival under bicarbonate-induced stress (Marschner 1995). In fact, since Fe is scarcely mobile in calcareous soils, the ability to maintain ample soil volume explored by roots would confer essential benefits. On the other hand, BA29 is affected in a different way by the presence of bicarbonate since it shows a strong depression at root growth level (Donnini et al. 2008). The mechanisms involved in the growth of plant organs are very complex. Among these, factors inducing changes in cell-wall plasticity have been described (Hoson 1993, Cosgrove 1999). Recently, the possibility based on the idea that cell-wall modifications underlying organ growth are affected by ROS production has received support by several reports (Schoepfer 2001, Liszkay et al. 2004, Passardi et al. 2005). It has been suggested that the genesis of ROS in the apoplast can drive the oxidative cross-linking of cell-wall components such as lignin precursors (Boudet 2000), consequently stopping cell elongation. On the other hand, wall-loosening reactions involved in root elongation are also directly linked to the action of ROS (Schoepfer 2001, Liszkay et al. 2003). The hydroxyl radical (OH·) produced in the apoplastic space of plant tissues, for instance, is capable of splitting covalent bonds of wall polymers (Fry 1998). Since the chemical determination of OH· has rarely been attempted because of its extremely short lifetime (Kuchitsu et al. 1995, Liszkay et al. 2004), we tested, in the apoplast of root tissues, the presence of H2O2 and O2−, directly involved in both OH· generation (Schoepfer 2001) and cell-wall plasticity (Halliwell 1978).

The histochemical method we have adopted, though not quantitative, has been chosen since it is generally considered to be one of the most conservative and sensitive methods for plant analysis, being based on a rapid reaction directly at the site of ROS generation (Fryer et al. 2002, Romero-Puertas et al. 2004). Staining of freshly cut root cross-sections in the meristematic, elongation and maturation zones extending for the first 4–5 mm from the tip showed that O2− accumulation preferentially occurs in roots of both genotypes grown in the absence of Fe and in BA29 also in the presence of bicarbonate. In particular, O2− increase is mainly located in the apoplast of stele and cortex (Figure 6). Similar results were obtained using DAB as a probe for H2O2 in the presence of endogenous PODs, although in this case the staining was present in the rhizodermis, in the stele and, to a lesser extent, in the cortex walls (Figure 7). Thus, the information collected by histochemical analysis might suggest that O2− and H2O2 accumulation occurs in root tissues of all chlorotic plants, accounting for the modifications of mechanical properties of the cell wall during stress adaptation.

Reactive oxygen species accumulation detected in BA29 (both treatments) and cv. Conference grown in the absence of Fe could be linked to an imbalance in the antioxidant machinery. Moreover, in cv. Conference +Fe+Bic, the lack of ROS could be due either to their limited synthesis or to their rapid degradation. To test this hypothesis, we have analysed the main enzymes involved in ROS metabolism. Among these activities, the SODs are involved in the responses to Fe deficiency stress, catalysing the dismutation into H2O2 of O2−, formed to a high extent under this condition (Ranieri et al. 1999, Molassiotis et al. 2005). To determine whether differences between the individual SOD isoforms were present, we have also determined SOD activity by using non-denaturating gel staining (Figure 3a–c). Total SOD activity was induced in −Fe roots of both genotypes, associated with Cu/Zn-SOD and Mn-SOD isoforms. However, SOD induction appears not to be sufficient to detoxify O2−, which seems to be accumulated in the root tissues (Figure 6). In BA29 under +Fe+Bic treatment, no increase in total SOD activity was detected, suggesting that (i) the presence of bicarbonate could induce, in the susceptible genotype, a loss of O2− detoxification activity, (ii) the increase in H2O2 (Figure 7) cannot be attributed to increased SOD activity. The H2O2 overproduction could be directly triggered by the rise in other activities, such as NAD(P)H oxidases (Romero-Puertas et al. 2004), pH-dependent PODs (Bestwick et al. 1998,
functioning, probably to counter H$_2$O$_2$ accumulation linked to the absence of Fe, BA29 tries to keep Fe-dependent PODs active. These results seem to suggest that, in the absence of Fe, POD activity was reduced in both genotypes in the absence of Fe and in BA29 also in the presence of bicarbonate (Figure 4), probably as a consequence of insufficient Fe availability for the enzyme, as it contains, in addition to the haem group, another Fe atom. Different behaviour, as regards non-specific PODs, was shown in the two genotypes under –Fe treatment, the activity of these enzymes being diminished only in cv. Conference (Figure 4a). These results seem to suggest that, in the absence of Fe, BA29 tries to keep Fe-dependent PODs functioning, probably to counter H$_2$O$_2$ accumulation linked to the high SOD activity (Figures 3 and 7). In general, though other activities could not be ruled out, the data obtained in the absence of Fe suggest that the presence of ROS in roots of both genotypes could be the consequence of overproduction of these toxic species and, at the same time, of a reduced capacity to detoxify them. An important aspect of this work comes from the behaviour of bicarbonate-treated plants concerning non-specific POD activity. In fact, while an increase in this activity was detected in the tolerant cv. Conference, in BA29 it underwent a reduction (Figure 4), making a contribution to H$_2$O$_2$ accumulation. In general, it can be inferred that in BA29 the presence of bicarbonate negatively affected the enzymes considered in this work. On the other hand, in the cv. Conference +FeBic, only an increase in non-specific POD activity was detected, confirming its higher level of protection in particular against H$_2$O$_2$ accumulation. The specific role of PODs within the lignification process was investigated by using a specific substrate, syringaldazine, on different cellular fractions. There is both histochemical and biochemical evidence that only cell walls that are undergoing lignification are able to oxidize syringaldazine (Imbert et al. 1985, Christensen et al. 1998). While the H$_2$O$_2$ scavenging activity of soluble PODs plays only a detoxification role, both covalently and ionic bound PODs also catalyse the polymerization of lignin precursors and cross-links between extensins and feruloylated polysaccharides (Abeles and Biles 1991, Sato et al. 1993, Polle et al. 1994). In our results, only the bicarbonate treatment induces an increase in syr-POD activity (Table 2). In particular, while in soluble PODs an increase was observed in both genotypes, PODs that are ionic bound to the cell wall underwent an increase only in BA29. Furthermore, histochemical analysis of roots was performed and a staining pattern consistent with an increased process of lignification was observed in particular in BA29 grown with bicarbonate (Figure 8). The increase in syr-POD activity observed in this treatment could contribute to lignin synthesis which, reducing root elongation and consequently nutrient uptake, could be correlated with susceptibility to bicarbonate.

In the present work, POD activity was assayed using dianisidine, which is an aspecific substrate, and syringaldazine, a specific substrate for PODs involved in lignification. This led to the identification of a different effect of bicarbonate on the two genotypes: while in the cv. Conference dian-POD is enhanced, suggesting that this treatment induces an increase in H$_2$O$_2$ scavenging, in BA29 the syr-POD undergoes an increase, playing a role in morphological modification of roots by influencing cell-wall plasticity.

Taken together, these results suggest that, only in BA29 grown in the presence of bicarbonate, can ROS accumulation in root apoplast (Figures 6 and 7) be associated with lignin deposits in external root layers and in endodermis (Figure 8), as the consequence of the shift of PODs towards a lignification role (Table 2). Cell-wall lignification, which provides structural rigidity and durability to plant tissues, can occur as a stress response (Srivastava et al. 2007, Kim et al. 2008) and has been reported to be involved in the mechanism responsible for plant tolerance to salt and drought stress (Ibir et al. 2001, Lee et al. 2007). Under salt stress, the thicker the wall, the more efficiently the cell interior is protected against ion absorption. On the other hand, under drought stress, lignification reduces plant growth, leading to a lower water request. Our results could suggest another picture: we found that under bicarbonate stress the lignification process is much higher in the susceptible than in the tolerant genotype. In any case, while under water and drought stress root lignification could be implicated in the mechanism responsible for plant tolerance, under Fe deficiency, it could further impair mineral nutrition generating higher susceptibility to this constraint. Meanwhile, in the cv. Conference, the allocation of new biomass to the organs involved in nutrient acquisition could assure the exploration of soil and consequently improve Fe uptake.

The results suggest that the tolerance of cv. Conference to the presence of bicarbonate could be ascribed to the ability to feed more appropriately for nutrients by means of: (i) unaffected root growth and (ii) increased Fe uptake activity (Donnini et al. 2009). The absence of a similar adaptive strategy in quince could explain its susceptibility to this growth condition.

Although future work should endeavour to link these aspects with genetic events, the results obtained in this work can contribute to explaining the contrasting phenotypes of these species in response to the presence of bicarbonate in the growth medium.

**Acknowledgments**

We thank Dr Patrizia De Nisi, Dr Gianpiero Vigani and Stefano Mattaini for advice and critical reading of the manuscript. Silvia Donnini was the recipient of a post-doctoral fellowship from MIUR.
Funding
The work was supported by grants from MIUR and the Università degli Studi di Milano (PUR).

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