African Journal of Biotechnology Vol. 11(82), pp. 14828-14836, 11 October, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.1680
ISSN 1684–5315 ©2012 Academic Journals

Full Length Research Paper

Secondary metabolism responses in two *Pisum sativum* L. cultivars cultivated under Fe deficiency conditions

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Accepted 5 July, 2012

The present study was carried out to investigate the Fe deficiency effect on the secondary metabolism responses in two *Pisum sativum* cultivars characterized by different tolerance to Fe deficiency. Previous study investigating the physiological responses to Fe deficiency in these two pea cultivars showed that Kelvedon was more tolerant than Lincoln. Both cultivars were grown in the absence or presence of Fe with the addition of bicarbonate for twelve days. Higher concentrations of phenols and flavonoids were observed in Fe-deficient tissues of both cultivars; however, the increase was greater in the tolerant cultivar than in the susceptible one. The activity of shikimate pathway enzymes tested was more enhanced in the tolerant cultivar. In addition, lipid peroxidation and H_2O_2 concentrations were more increased in the susceptible cultivar when compared with the tolerant one. Peroxidase activity was increased in the tolerant cultivar grown under bicarbonate supply, while a considerable diminution was observed in the susceptible one, suggesting the involvement of this antioxidant enzyme in the tolerance of pea to Fe deficiency. The lignifying peroxidases activity was more decreased in Lincoln than in Kelvedon, especially in the presence of bicarbonate. Our data suggest that the tolerance of Kelvedon was related to its ability to modulate the phenolic metabolism pathway and to enhance the antioxidant potentials.

Key words: Iron deficiency, bicarbonate, phenolic metabolism, antioxidative enzymes, *Pisum sativum*.

INTRODUCTION

Iron is a micronutrient of high importance to plant, mediating vital growth and development processes (Jiménez et al., 2009). However, in soils with a relatively high concentration of bicarbonate, Fe is inaccessible to the plant which induces Fe chlorosis (Bavaresco et al., 2006). This phenomenon, known as lime-induced Fe chlorosis is considered as one of the most important abiotic factors and represents a major constraint for the majority of legumes (Bavaresco and Poni, 2003).

Oxidative stress can be activated in response to different environmental conditions, such as high and low temperatures, exposure to UV rays, nutrient deficiency, drought, herbicides and pathogen attack (Imlay, 2003). Among these factors, mineral nutrient deficiency (K, Mg, B, Cu, Zn, Fe and Mn) that may modulate the activities of antioxidant enzymes remains of major importance (Shuang et al., 2008). When scarcely present and when present at toxic levels, Fe can lead to oxidative stress in plants as a consequence of reactive oxygen species (ROS) production such as superoxide ($O2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\bullet}$) (Chou et al.,

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2011). ROS can seriously alter normal cellular metabolism through oxidative damage to lipids, proteins and nucleic acids. In this context, the importance of the equilibrium between ROS production and scavenging is principal (Van et al., 2008).

Phenolic compounds may be increased or *de novo* synthesized in plants in response to various biotic and abiotic stresses (Ranieri et al., 2001; Molassiotis et al., 2006). Among these stress factors, Fe deficiency in Strategy I plants has been shown to increase the synthesis and release of phenolics (Donnini et al., 2011). Phenolic compounds such as phenolic acids, flavonoids and anthocyanins play an important role in scavenging free radicals (Passardi et al., 2005). These compounds constitute the most abundant class of plant secondary metabolites and share a common origin in the phenylpropanoid biosynthetic pathway (Diaz et al., 2001). In this context, some of the key enzymes catalyzing the biosynthesis of polyphenols include shikimate kinase (SK), shikimate dehydrogenase (SKDH) and phenylalanine ammonia-lyase (PAL). The antioxidant activity of phenolics is mainly due to their redox properties, acting as reductants and chelators of Fe(III) when released in the rhizosphere (Ali et al., 2006).

Peroxidases are involved in the biosynthesis of lignin from cinnamyl alcohols, and thus have a role in phenolic metabolism (Lattaziaoa et al., 2011; Heidarabadia et al., 2011). PODs, by means of their hydroxylic or peroxidative activity, can regulate both production and scavenging of ROS in cell compartments and thus they can be involved in many plant processes, such as growth and biotic/abiotic stress responses (Jin et al., 2007). 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 (Jbir et al., 2001, Chaoui and El Ferjani, 2005; Lee et al., 2007). According to Molassiotis et al. (2006), increased POD activity may be an important attribute linked to chlorosis tolerance in peach rootstocks.

Many studies have focused on the secondary metabolism responses of plants to Fe deficiency (Ranieri et al., 1999; Espen et al., 2000; Zancan et al., 2008, Ciccod 2009). However, relatively little information is available on the relationships between Fe deficiency and secondary oxidative stress, and some data remain controversial (Molassiotis et al., 2005). With this aim, the purposes of the present work were therefore to investigate in two cultivars of *Pisum sativum* (cv. Kelvedon and Lincoln) with different tolerance to Fe-deficiency, the former being tolerant and the latter sensitive: (i) the effect of Fe-deficiency on plant biomass production, (ii) phenols and flavonoids accumulation, together with the activity of some shikimate pathway enzymes (SK, SKDH and PAL) and, (iii) the changes in POD activity, as well as the activity of lignifying peroxidases; coniferyl alcohol peroxidase (CAPX) and NADH oxidase.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of *P. sativum* cultivars (cv. Kelvedon and cv. Lincoln) were selected at the Tunisian National Institute of Agronomic Research, INRAT. The two cultivars were selected on the basis of their different tolerance to Fe deficiency, which was appraised in previous work (Jelali et al., 2011). Seeds were germinated for 6 days at 19°C in Petri dishes with filter paper constantly moistened with 0.1 mM $CaSO_4$. Six-day-old seedlings were then transferred to a half strength aerated nutrient solution for 7 days. After that seedlings having similar length were selected and cultured as groups of 10 plants in 10 L of full strength aerated nutrient solution. The composition of the nutrient solution was: 1.25 mM Ca $(NO_3)_2$, 1.25 mM KNO_3, 0.5 mM $MgSO_4$, 0.25 mM KH_2PO4, 10 µM H_3BO_3, 1 µM $MnSO_4$, 0.5 µM $ZnSO_4$, 0.05 µM $(NH4)_6MO_7O_{24}$ and 0.4 µM $CuSO_4$. Three treatments were established for twelve days as follows: presence of 30 µM Fe(III)-EDTA (control, C), direct Fe deficiency (absence of Fe, DD) and presence of 30 µM Fe(III)-EDTA + 0.5 g L^{-1} $CaCO_3$ + 10 mM $NaHCO_3$ (induced Fe deficiency, ID). The pH was adjusted to 6.0 with NaOH for both C and DD treatments, while it reached pH 8.2 in the ID treatment. $NaHCO_3$ and $CaCO_3$ were added to the nutrient solution in the ID treatment to simulate the effect of a calcareous soil. Aerated hydroponic cultures were maintained in a growth chamber with a day/night temperatures of 25/18°C, a 16-h photoperiod, a photon flux density of 200 µmol m^{-2} s^{-1} and a relative humidity of 70%. The solution was renewed every 6 days. It is worth mentioning that with the exception of the studies carried out on plants grown in the field; most of the studies performed in hydroponic culture used a total iron depletion growth condition which can be the origin of a great stress to plants (Donnini et al., 2009). We therefore chose to introduce an induced Fe deficiency by the addition of bicarbonate to the nutrient solution in order to mimic the field conditions and to allow plants to grow and adapt.

Plant growth determination

Six plants of each cultivar (Kelvedon and Lincoln) collected from the replicates of each treatment were harvested after 12 days of treatment. Roots were briefly rinsed with distilled water. Root and shoot dry weights were determined at 65°C.

Total phenols

Total phenols were determined using the Folin–Ciocalteu (F–C) reagent following to the method of Singleton et al. (1965) slightly modified by Marigo (1973). Twenty-five microliter (25 µl) of root or leaf extract was placed in a reaction test tube to which 1.5 ml of water and 100 µl of (F–C) reagent were added. The test tube was allowed to stand for 6 min, and then 300 µl Na_2CO_3 (20%) was added. After 20 min at 40°C, absorbance was measured at 750 nm versus the prepared blank. Total polyphenol content was calculated from the calibration curve using caffeic acid as a standard. All samples were analyzed in triplicate.

Total flavonoids

Total flavonoids content was measured by a colorimetric assay (Kim et al., 2003). Aliquots of diluted sample or standard solution were mixed with 2 mL of distilled H_2O and 0.15 mL of $NaNO_2$ (5%). After 5 min, 0.15 mL of $AlCl_3$ (10%) was added. The mixture was allowed to stand for another 5 min, and then 1 mL of the NaOH was added. The final volume was adjusted to 2 mL with distilled water.

The reaction solution was well mixed and kept for 15 min, and the absorbance was determined at 415 nm against the blank where the sample was omitted. Total flavonoid content was calculated using the standard rutin curve and expressed as µg of rutin g^{-1} FW. All samples were analyzed in triplicate.

Shikimate pathway enzyme extraction and assay

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 (Krell et al., 2001). Shikimate-dependent oxidation of NADH was monitored at 340 nm. The assay mixture contained 20 to 50 mL 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 mg mL^{-1} pyruvate kinase and 15 mg mL^{-1} lactate dehydrogenase.

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

The extract enzyme preparation for phenylalanine ammonia-lyase PAL (EC. 4.3.1.5) was obtained by homogenizing fresh leaf and root tissues (1 g) in a medium containing 15 mM β-mercaptoethanol, 20 mM Tris–HCl (pH 7.8), 20% glycerol, 1 mM phenylmethyl sulfonyl fluoride (PMSF) and 1% (v/v) Triton X-100. PAL (EC 4.3.1.5) activity was determined in 100 mM Tris–HCl, pH 8.8, containing 11 mM L-phenylalanine and protein extract (Raag et al., 1984). The formation of trans-cinnamic acid was monitored at 290 nm. PAL activity is defined as the amount of enzyme forming 1 nmol of trans-cinnamic acid from the substrate phenylalanine per minute at 30°C. All these enzyme activities were assayed by two independent experiments in triplicate (n = 6).

Hydrogen peroxide

Concentration of H_2O_2 was determined by measuring the complex titanium-peroxide (Brennan and Frenkel, 1977) as described by Ranieri et al. (2001). Fresh leaf and root tissues (0.5 g) were homogenized in cold 100% acetone at 1:2 ratio (w/v) and centrifuged for 10 min at 10 000 g, then aliquots of 20% $TiCl_4OH$ in concentrated HCl were added to the supernatant. After the addition of NH_4OH (0.2 ml/l of sample) in order to precipitate the titanium-peroxide complex, samples were centrifuged at 10 000 g for 5 min. The resulting pellet was washed five times in acetone and resuspended in 2 N H_2SO_4. The absorbance of the solution was spectrophotometrically determined at 415 nm against a blank containing H_2O_2 instead of sample extract. H_2O_2 content was calculated using a standard curve of known H_2O_2 concentrations from 0.1 to 1 mM. Three replicates for each treatment were performed.

Lipid peroxidation

Fresh samples (200 and 500 mg) for roots and were homogenized in 2 ml of 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 15 000 g for 10 min at 4°C. A 0.5 ml aliquot of the supernatant was mixed with 1.5 ml of 0.5% thiobarbituric acid (TBA) prepared in 20% TCA and incubated at 90°C for 20 min. After stopping the reaction in an ice bath, samples were centrifuged at 10 000 g for 5 min. The supernatant absorbance at 532 nm was then measured. After subtracting the non-specific absorbance at 600 nm, MDA concentration was determined using the extinction coefficient 155 mM^{-1} cm^{-1} (Hernández and Almansa, 2002). Three replicates for each treatment were performed.

POD extraction and enzymatic assay

All operations were carried out at 4°C. Two grams of young fresh leaves and 1 g of roots were ground in liquid N_2 with 10% (w/w) polyvinylpolypyrrolidone (PVPP) and homogenized in a medium containing: 0.1 M Tricine-KOH buffer (pH 8.0), 10 mM dithiotreitol (DTT), 10 mM $MgCl_2$, 50 mM KCl, 1 mM EDTA, 0.1% Triton X-100 and 50 µg ml^{-1} phenylmethylsulphonyl fluoride (PMSF). After centrifugation at 14 000 *g* for 30 min at 4°C, the supernatant was dialyzed overnight and then tested for enzyme activity. Dianisidine 0.1% in methanol : dioxane (1:1) was used as phenolic reducing substrate and the absorbance was measured by a spectrophotometer at 460 nm (Ranieri et al., 1997). The activity is expressed as µmol min^{-1} mg^{-1} protein. POD activities were assayed by two independent experiments in triplicate (n = 6).

CAPX activity representing the hydrogen peroxide-dependent oxidation of coniferyl alcohol by peroxidase was determined according to the method of Pedreno et al. (1989) by measuring the decrease in absorbance at 260 nm using an extinction coefficient of 2.2 l /mmol/cm. The reaction mixture contained: 0.1 mmol/l coniferyl alcohol, 0.5 mmol/l H_2O_2, 100 mmol/l phosphate buffer (pH 7.0) and enzyme extract.

NADH oxidase activity was determined in an assay mixture containing 100 mmol/l sodium acetate (pH 6.5), 1 mmol/l $MnCl_2$, 0.5 mmol/l p-coumaric acid, 0.2 mmol/l NADH and enzyme extract. The reaction was monitored by following the decrease in absorbance at 340 nm with an extinction coefficient of 6.2 l / mmol/cm (Ishida et al., 1987).

Protein determination

Protein concentration was determined by using the BioRad reagent and BSA as a standard (Bradford, 1976).

Statistical analysis

Variance analysis of data (one-way ANOVA) was performed using the SPSS 10.0 program, and means were separated according to Duncan's test at $p \leq 0.05$. Data shown are means of three (total phenols and flavonoids, H_2O_2, and MDA concentrations) six (plant DW, POD, lignifying peroxidases and shikimate pathway enzyme activities) replicates for each treatment.

RESULTS

Plant dry weight

At the end of the treatment, Fe deficient plants of the tolerant cultivar showed no significant difference in its shoot biomass production despite the slight decreases (less than 10%) observed under ID (Figure 1a). On the contrary, shoot (DW) of the susceptible cultivar plants exhibited a noticeable decline (-25 and -33%, respectively under DD and ID). Concerning root growth, both direct and induced Fe deficiency resulted in a significant increase of root DW in both cultivars. The increase was higher in the tolerant cultivar than in the susceptible one, and it was more pronounce under ID

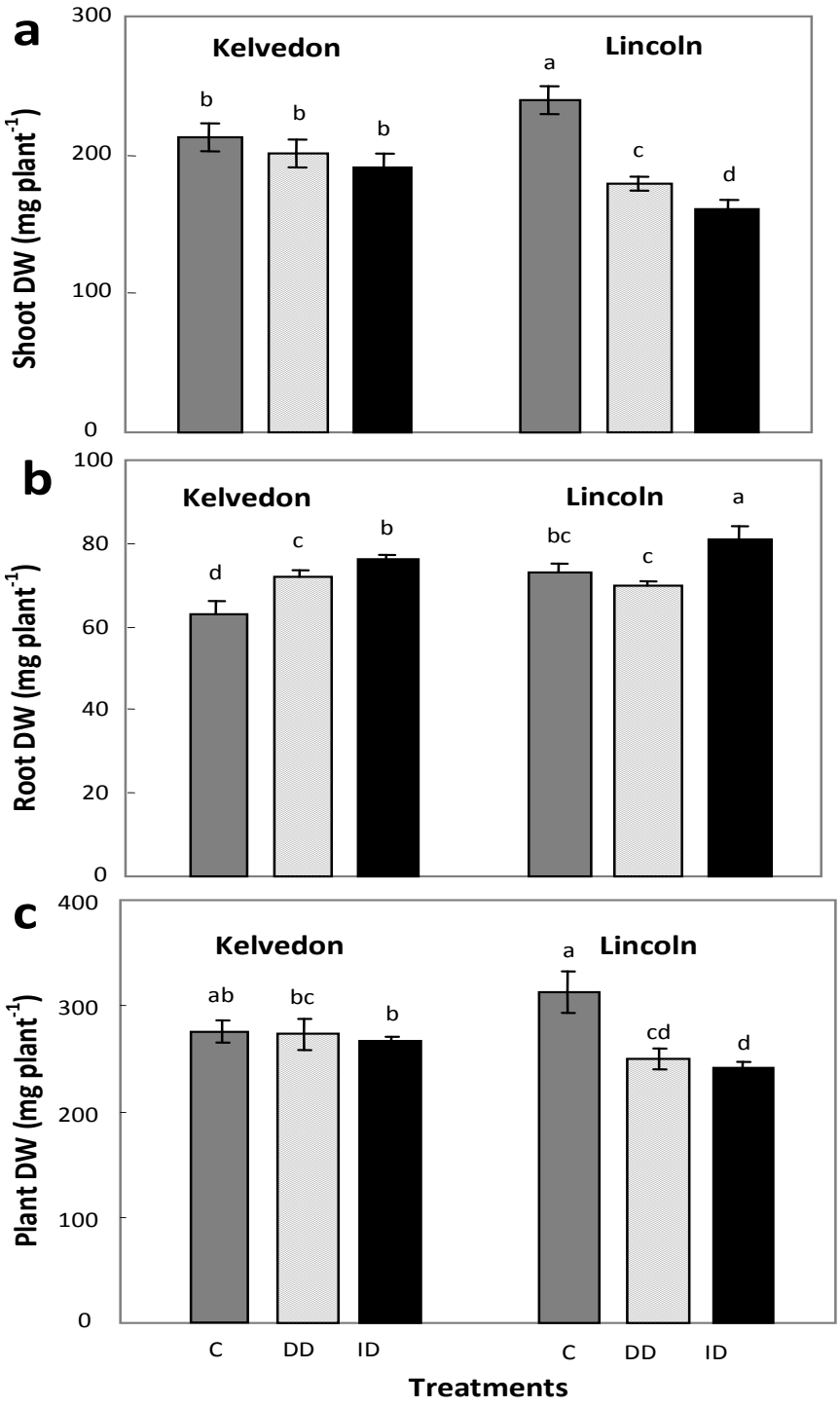

Figure 1. Dry weight in shoots, roots and whole plant of two *P. sativum* cultivars ('Kelvedon': tolerant and 'Lincoln': susceptible) cultivated in the presence of Fe (C: control), in the absence of Fe (DD: direct deficiency) or in the presence of Fe plus bicarbonate (ID: induced deficiency).

(Figure 1b). At the whole plant level, the tolerant cultivar DW was not affected by Fe deficiency, as it was observed in shoots, whereas that of the susceptible one showed a significant decrease (Figure 1c).

Total phenolics and flavonoids concentrations

Our results show a significant increase in phenols and flavonoids concentration in Fe-deficient tissues of

Table 1. Effect of Fe deficiency on total phenolics and flavonoids concentrations in leaves (L) and roots (R) of two *P. sativum* cultivars ('Kelvedon': tolerant and 'Lincoln': susceptible) cultivated in the presence of Fe (C: control), in the absence of Fe (DD: direct deficiency) or in the presence of Fe plus bicarbonate (ID: induced deficiency).

Parameter		Kelvedon			Lincoln		
		C	DD	ID	C	DD	ID
Total phenolics	L	210± 14^{d}	342 ± 5^{a}	275± 12^{b}	202± 4^{d}	280± 3^{b}	235 ± 8^{c}
(µg g^{-1} FW)	R	115 ± 4^{d}	256 ± 6^{a}	166 ± 9^{b}	105 ± 8^{d}	159 ± 10^{b}	131 ± 6^{c}
Total flavonoids	L	103± 9^{d}	160 ± 6^{a}	125± 2^{b}	98± 5^{d}	130± 8^{b}	112 ± 2^{c}
(µg rutin g^{-1} FW)	R	53 ± 2^{d}	118 ± 8^{a}	82 ± 3^{b}	51 ± 5^{d}	74 ± 6^{b}	60 ± 3^{c}

Values are means ± SE and differences between means were compared using Duncan's test at $P<0.05$.

P. sativum. A higher concentration was found in leaves than in roots in both cultivars. Notably, the increase in these compounds, phenols and flavonoids was greater in the tolerant cultivar roots grown under DD (2.2 and 2.1-fold, respectively) than in the susceptible one (1.5 and 1.4-fold, respectively) and it was more pronounce in the absence of Fe (DD) (Table 1).

Shikimate pathway enzymes

As shikimic acid is a precursor of the aromatic compounds biosynthesis and consequently of phenolics; some enzymatic activities with a key role in the shikimate pathway were assayed in *P. sativum* tissues. As shown in Table 2, SK, SKDH and PAL activities were higher in leaves than in roots, irrespective of the treatment and the cultivar. The direct Fe deficiency (DD) and to a lesser extent the bicarbonate treatment (ID) increased significantly the activity of these enzymes for both cultivars (Table 2). The highest activities were recorded in the tolerant cultivar in agreement with the fact that these plants were characterized by higher phenolic concentration (Table 2).

Indeed, the SK activity, which converts the SKDH's product in 3-phospho-shikimate, was particularly high in the susceptible cultivar under DD (2.7 and 2.1-fold, respectively in leaves and roots) (Table 2). The same trend was noted for SKDH activity in both cultivars.

PAL activity, the first enzyme in the phenylpropanoid biosynthetic pathway, was also increased significantly in both cultivars, but more in plants of the tolerant cultivar grown in the absence of Fe (2.7 and 2.1-fold, respectively in leaves and roots) (Table 2).

Lipid peroxidation

Lipid peroxidation measured by the accumulation of MDA-TBA complex was also assayed in plant tissues (leaves and roots). As shown in Table 3, MDA concentration increased in both cultivar tissues. Though, a greater accumulation was observed in leaves of the susceptible cultivar under bicarbonate-induced Fe deficiency (1.3-fold increase of the respective control), the same trend was noted for DD with a less accentuated accumulation of MDA concentration in the tolerant cultivar as compared to the susceptible one (Table 3).

Hydrogen peroxide

Since H_2O_2 concentration was considered an indicator of oxidative damage (Mittler, 2002), its production was determined in both tissues. As shown in Table 3, H_2O_2 concentration was genotype and treatment dependant showing a significant increase in both cultivars under ID, with a higher accumulation observed particularly in the susceptible cultivar (1.9 and 2.1-fold, respectively in leaves and roots) (Table 3). Moreover, under DD, H_2O_2 concentration remained close to the control values in the tolerant cultivar, whereas it was significantly increased in tissues of the susceptible one (1.6 and 1.9-fold, respectively in leaves and roots) (Table 3).

Peroxidase activity

Under sufficient Fe supply, POD activity was similar in both cultivars tissues (Table 3). In the absence of Fe (DD), no significant difference was observed in leaves of both cultivars as compared to the control. Conversely, the presence of bicarbonate in the growth medium resulted in an increase in the tolerant cultivar (2.5-fold), while a decrease of 50% was observed in the susceptible one (Table 3). The same trend was observed at the root level. Indeed, no significant difference was observed between both cultivars subjected to DD. In the presence of bicarbonate (ID), POD activity was notably stimulated in the tolerant cultivar (2.4-fold respect to the control) (Table 3).

CAPX and NADH oxidase activities

To investigate the effect of Fe deficiency in the

Table 2. Effect of Fe deficiency on SK, SDH and PAL activities in leaves (L) and roots (R) of two *. sativum* cultivars ('Kelvedon': tolerant and 'Lincoln': susceptible) cultivated in the presence of Fe (C: control), in the absence of Fe (DD: direct deficiency) or in the presence of Fe plus bicarbonate (ID: induced deficiency).

Parameter		Kelvedon			Lincoln		
		C	DD	ID	C	DD	ID
SK (nmol min^{-1} mg^{-1}prot)	L	102 ± 4^{e}	245 ± 10^{a}	223 ± 8^{f}	98 ± 6^{e}	160 ± 9^{c}	148 ± 3^{d}
	R	53 ± 2^{d}	149 ± 13^{a}	130 ± 4^{b}	49 ± 11^{d}	105 ± 6^{c}	92 ± 14^{c}
SDH (nmol min^{-1} mg^{-1}prot)	L	72 ± 3^{d}	142 ± 3^{a}	120 ± 8^{b}	68 ± 5^{d}	91 ± 13^{c}	85 ± 2^{c}
	R	51 ± 6^{cd}	86 ± 4^{a}	64 ± 2^{b}	47 ± 4^{d}	70 ± 2^{b}	55 ± 3^{c}
PAL (nmol min^{-1} g^{-1}FW)	L	210 ± 6^{d}	311 ± 12^{a}	287 ± 10^{b}	199 ± 7^{d}	244 ± 14^{c}	236 ± 16^{c}
	R	115 ± 10^{d}	225 ± 9^{a}	200 ± 11^{b}	108 ± 15^{d}	165 ± 8^{c}	154 ± 13^{c}

Values are means ± SE and differences between means were compared using Duncan's test at P<0.05.

Table 3. Effect of Fe deficiency on H_2O_2 and MDA concentration, and POD activity in leaves (L) and roots (R) of two *P. sativum* cultivars ('Kelvedon': tolerant and 'Lincoln': susceptible) cultivated in the presence of Fe (C: control), in the absence of Fe (DD: direct deficiency) or in the presence of Fe plus bicarbonate (ID: induced deficiency).

Treatment		Kelvedon			Lincoln		
		C	DD	ID	C	DD	ID
H_2O_2 (µmol g^{-1}FW)	L	1.23 ± 0.11^{c}	1.46 ± 0.21^{bc}	1.53 ± 0.10^{b}	1.10 ± 0.20^{c}	1.78 ± 0.4^{a}	2.1 ± 0.1^{a}
	R	1.40 ± 0.20^{c}	1.85 ± 0.25^{bc}	1.96 ± 0.10^{b}	1.33 ± 0.13^{c}	2.56 ± 0.10^{a}	2.80 ± 0.20^{a}
MDA (nmol g^{-1} FW)	L	13.50 ± 0.20^{e}	14.75 ± 0.10^{d}	15.10 ± 0.5^{c}	13.20 ± 0.40^{e}	16.35 ± 0.10^{b}	17 ± 0.20^{a}
	R	10.60 ± 0.40^{d}	12.9 ± 0.80^{c}	13.80 ± 1.10^{c}	10.20 ± 0.80^{d}	17.4 ± 0.20^{b}	19.5 ± 1.40^{a}
POD (U mg^{-1} protein)	L	92 ± 3^{cd}	86 ± 3^{d}	227 ± 10^{a}	96 ± 2^{bc}	104 ± 4^{b}	48 ± 2^{d}
	R	104 ± 5^{c}	119 ± 8^{b}	253 ± 15^{a}	98 ± 12^{c}	109 ± 10^{bc}	55 ± 2^{d}

Values are means ± SE and differences between means were compared using Duncan's test at P<0.05.

Table 4. Effect of Fe deficiency on CAPX and NADH oxidase activities in roots of two *P. sativum* cultivars ('Kelvedon': tolerant and 'Lincoln': susceptible) cultivated in the presence of Fe (C: control), in the absence of Fe (DD: direct deficiency) or in the presence of Fe plus bicarbonate (ID: induced deficiency).

Treatment	Kelvedon			Lincoln		
	C	DD	ID	C	DD	ID
CAPX (U g^{-1}FW)	48 ± 4^{a}	27 ± 1^{c}	36 ± 4^{b}	44 ± 2^{a}	29 ± 2^{c}	23 ± 2^{d}
NADH oxidase(U g^{-1} FW)	286 ± 14^{a}	139 ± 8^{c}	190 ± 11^{b}	280 ± 10^{a}	144 ± 6^{c}	120 ± 5^{d}

Values are means ± SE and differences between means were compared using Duncan's test at P<0.05.

P. sativum roots lignification, the activity of CAPX and NADH oxidase was determined (Table 4). In the absence of Fe (DD), no significant difference was noted between both cultivars as compared to the control (Table 4). On the contrary, the presence of bicarbonate in the growth medium induced a significant decrease in both cultivars. The reduction was more pronounced in Lincoln than in Kelvedon (25 and 48%, respectively for CAPX) and (34 and 57%, respectively for NADH oxidase) as compared to the control.

DISCUSSION

Iron deficiency has been generally shown to affect plant growth in several plant species (Rombolà et al., 2005; Pestana et al., 2005). As shown above in our results, significant differences in the pattern of plant growth were found to depend on the treatment and cultivar. Subsequently, in a previous work (Jelali et al., 2011), a significant decrease of bivalent iron content was found in Fe deficient plants of both cultivars with a higher

reduction noted in the sensitive cultivar grown under ID. This result indicated that growth under bicarbonate supply could induce some adaptive mechanisms in the tolerant cultivar.

In addition to these physiological mechanisms, several other biochemical mechanisms such as the exudation and/or accumulation of organic compounds (flavins, organic acids and phenolics) remain of major importance (Abadía et al., 2002). In this work, we found that total phenols concentration increased in roots and exudates leaves under Fe deficiency conditions, and it was higher in the tolerant cultivar grown in the absence of Fe (Table 2). Phenolics compounds are also reported to be accumulated under other nutrient deficiencies (Widodo et al., 2010; Lin et al., 2011). The observed stimulatory effect of Fe deficiency on the total phenol content led us to investigate the activities of three enzymes belonging to the shikimate pathway, involved in phenolic synthesis: SK, SKDH and PAL. Our results showed that Fe deficiency caused enhanced activities of these enzymes, which was higher in the tolerant cultivar (Kelvedon) when compared with the susceptible one (Lincoln). These results are also in agreement with those of Lan et al. (2011) who showed an increase in the activity of enzymes involved in the phenylpropanoid pathway in *Arabidopsis* induced by Fe deficiency. The activation of phenylpropanoid pathway by biotic stress is well established (Dixon and Paiva, 1995). However, the ability of a wide range of abiotic stresses to stimulate phenylpropanoid pathway is less widely appreciated, these abiotic stresses include high light (Beggs and Wellman, 1994), low temperature (Solecka et al., 1999), copper stress (Diaz et al., 2001), phosphate deficiency (Trull et al., 1997) and pollutant ozone (Guidi et al., 2005).

The MDA and H_2O_2 concentrations were measured in tissues as an indicator of oxidative stress (Hernández et al., 2001). Both parameters increased significantly in Fe-deficient leaves and roots of both cultivars. This accumulation was greater in the sensitive cultivar than in the tolerant one, notably under ID (Table 3). The lower level of lipid peroxidation in the tolerant cultivar suggested, therefore, that it was better protected from oxidative damage under Fe starvation. Our results are in agreement with those recently found in sunflower (Ranieri et al., 2003), maize (Tewari et al., 2005) and peach (Molassiotis et al., 2006) showing an increase in MDA and H_2O_2 concentration under Fe deficiency conditions. An increase in lipid peroxidation was also observed in leaves of pea plants grown under cadmium stress (Sandalio et al., 2001). Under salinity conditions, Martí et al. (2011) recently showed an increase in H_2O_2 concentration in roots and leaves of pea plants treated for 14 days, while no significant effect was noted for lipid peroxidation. Under boron toxicity, no significant increase was observed in lipid peroxidation of tomato roots (Cervilla et al., 2009)

The H_2O_2 overproduction could be directly triggered by the increase in some activities, such as SOD, NAD(P)H oxidases (Romero-Puertas et al., 2004), pH-dependent PODs (Bestwick et al., 1998) or other extracellular PODs (Wojtaszek, 1997). Total POD activity that contributes to the plant defence system against oxidative stress was also determined. An increase of this enzyme activity was observed in both cultivars grown in the absence of Fe (Table 3). Although, other activities could not be ruled out, these data suggested that the presence of H_2O_2 in leaves and roots of both cultivars under DD could be the consequence of increased production of this toxic species and, at the same time, of an insufficient capacity to detoxify it (Donnini et al., 2011). It is noteworthy that an important aspect of this work comes from the behavior of bicarbonate-treated plants. In fact, while an increase in this activity was detected in the tolerant cultivar, there was a reduction in the susceptible one (Table 3), giving a strong contribution to H_2O_2 accumulation. These results suggested that under bicarbonate supply, the tolerant cultivar tried to keep Fe-dependent PODs functioning, probably to counteract H_2O_2 accumulation. In general, it can be inferred that in the susceptible cultivar, the presence of bicarbonate led to a drastic decrease in POD activity as compared to the tolerant one, indicating that the H_2O_2 scavenging mechanism was less effective in the former. It turned out that the active involvement of this antioxidant enzyme was related, at least in part, to the tolerance to Fe-deficiency-induced oxidative stress.

The specific role of PODs within the lignification process was investigated by determining the activity of lignifying peroxidases coniferyl: CAPX and NADH oxidase. In the present work, we noted a significant difference in the activity of both enzymes between both cultivars only in the bicarbonate treatment as compared to the control (Table 4).

Our results showed that under ID, the lignification process was much higher in the susceptible cultivar than in the tolerant one. Cell-wall lignification, which provides structural rigidity and durability to plant tissues, can occur as a stress response and has been reported to be involved in the mechanism responsible for plant tolerance to salt and drought stress (Jbir et al., 2001, Lee et al., 2007). Under boron toxicity, Cervilla et al. (2009) indicated that high levels of boron supply induce a higher lignin deposition in roots of Kosaco cultivar but not in Josefina cultivar. This phenomenon suggests that lignification is not an essential factor reducing root growth in tomato plants; however, it proves the existence of high genotypic variation in response to boron toxicity at the root level.

Conclusion

The present work demonstrates that the different Fe deficiency tolerance found in both pea cultivars

(Kelvedon and Lincoln) can be ascribed to the different aptitude to modulate the secondary metabolism pathways. Kelvedon has shown to be less susceptible to bicarbonate-induced Fe deficiency than Lincoln, as indicated by phenols accumulation and shikimate pathway enzymes (SK, SKDH and PAL) responses, in addition to POD and lignifying peroxidases activities.

ACKNOWLEDGEMENT

This work was funded by the Tunisian Ministry of Higher Education, Research and Technology (LR02CB02).

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