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Research article

Silicon increases cell wall thickening and lignification in rice (*Oryza sativa*) root tip under excess Fe nutrition

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ABSTRACT

Iron (Fe) as a micronutrients and silicon (Si) as a cell wall element are important in plant cell wall extension and integrity. While the interaction of exogenous Si and excess Fe on root cell wall modifications is known, the effects of these nutritional parameters on the spatial changes in the activities of genes and/or enzymes involved in the lignification of root cell walls are not well studied. Thus, these parameters were investigated in the root apical part (AP) and basal part (BP) of rice (*Oryza sativa* L.) plants supplied with and without Si (1.5 mM) under normal (10 mg/L) and excess Fe (150 mg/L) nutrition for 7 days. Beside growth retardation, excess Fe increased the activities of phenylalanine ammonia lyase (PAL), superoxide dismutase and NADPH–oxidase and *PAL* and cell wall peroxidase (*POD*) genes expression, along with the increased phenols and H_2O_2 contents in the root AP. Furthermore, the increased thickening of endodermal, exodermal and metaxylem cell walls in the root AP by excess Fe was attributed to the enhanced POD activity. *POD* expression, endodermal and exodermal cell wall thickenings were not affected by excess Fe in the root BP. Si application under excess Fe exaggerated the effects of excess Fe on root cell wall thickening, increased POD activity but reduced H_2O_2 content in the root AP. Thus, Si application under excess Fe nutrition promotes earlier initiation of lignin polymerization closer to and toward the root tip and hence restricts the entry of excess Fe into the plant.

1. Introduction

Plant cells are enclosed by a multilayered cell wall consisting of polysaccharides (cellulose, hemicellulose and pectin), cell wall proteins and phenolic compounds. Lignin as a polyphenolic compounds, is an important constituent of the plant cell walls which contributes to the strength and hydrophobicity of this structure, beside its role in xylem sap transport and resistance to biotic and abiotic stresses (Liu et al., 2018). In plant cells, the phenylpropanoid pathway begins by the phenylalanine ammonia–lyase (PAL) activity serves to provide phenolic precursors for the synthesis of cell wall materials (Koch and Schmitt, 2013). The phenolic monoliginols released into the apoplast are polymerized into lignin through the action of peroxidases (POD). Beside, several enzyme systems such as polyamine oxidases and the NADPH–oxidase of the plasma membrane are proposed for the generation of hydrogen peroxide in plant cells (Barceló, 2005). The superoxide radicals generated by NADPH–oxidase can be converted into hydrogen peroxide by the action of superoxide dismutase (SOD). The enzyme NADPH–oxidase is believed to be involved in cell growth, plant development and responses to biotic and abiotic stresses through the signaling pathways associated with the production of reactive oxygen radicals (Marino et al., 2012).

Although iron is an essential element for all plants, but at high concentrations may react with oxygen and generate oxygen free radicals (Curie and Briat, 2003). Iron toxicity is common in the acidic waterlogged rice fields due to the abundance of Fe^{2+} that may pass through the oxidation barrier of the rhizosphere and being absorbed by the roots. A portion of this iron accumulates in roots while the remaining one is transferred to the aerial parts through xylem, after passage through the Casparian strip barrier of the root endodermis. As iron toxicity proceeds, the oxidation capacity of root surfaces decreases and thus it is covered with Fe $(OH)_3$ depositions with inevitable damage to rice plants (Becker and Asch, 2005). Excess Fe may produce intracellular reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals and hydrogen peroxide that may invoke oxidative stress in plants. Our former study in rice leaves has shown that Fe toxicity increases the concentration of hydrogen peroxide and phenols both of which are bio–substrates for some enzymes such as peroxidases

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and polyphenol oxidase (Mehraban et al., 2008). The development of lignified and/or suberized layers like exodermis, endodermis, and other extracellular barriers in roots may limit apoplastic uptake of heavy metals (Adrees et al., 2015) and salinity (Barzegargolchini et al., 2017). Fu et al. (2012) reported the inhibition of root elongation and biomass as well as the formation of reddish iron plaques on root surfaces of rice plants due to Fe toxicity. The growth suppression of both primary and lateral roots due to Fe toxicity has also been reported in *Arabidopsis* (Li et al., 2016). Excess Fe also prevents the development of root border cells in rice while root cap cell walls become thicker (Zhang et al., 2017). Proteome analysis of plasma membranes isolated from the Fe-oversupplied maize roots suggests increased organ capacity for lignification (Hopff et al., 2013). The accelerated maturation of endodermal layer in Cd-stressed sorghum plants has also been suggested to protect shoots from the negative effects of excess Cd (Lux et al., 2011). Fe toxicity may similarly affects the developmental progression of exodermal and endodermal layers in roots, however to date the issue has not been substantiated.

Silicon (Si) is not an essential element for many plants, but it has a high uptake rate in some other plants such as rice, barley and sugar beet (Savant et al., 1997). In rice for example, more than 90% of the absorbed silicon is transferred to leaves while only about 2–3% of it remains in the roots (Ma and Takahashi, 2002); despite of this, limited deposition of Si in roots has an important bearing on the functionality of this organ. Silicon deposition might strengthen root mechanical barrier through hardening the stele and endodermal tissues cell walls in the basal zone however, in the apical and subapical zones it increases cell-wall extensibility (Hattori et al., 2003). Si increases the expression of cell wall related genes such as *PAL* and 4–coumarate: CoA ligase (4CL) in rice plants and subsequently increases monolignol synthesis (Fleck et al., 2011). Increased transcription of ABC transporters and *POD* may facilitate the transfer of monolignols to apoplast where subsequent polymerization of monolignols by the action of putative apoplastic cell wall bound PODs results in the increased lignification of cell walls (Marschner, 2011; Lee et al., 2013). Exogenous Si application is known to increase the suberization and lignification of the exodermis and endodermis in 4–5 cm distances from the rice root tips (Fleck et al., 2011, 2015).

Si application can mitigate symptoms related to Fe toxicity in rice plants as it declined the plant tissue Fe content and increased grain and straw yields as well as the activity of antioxidant enzymes (Chalmardi et al., 2014; Nagula et al., 2016; Dufey et al., 2014). This treatment also decreased the amounts of iron plaques on the root surfaces, restored root elongation growth and resulted in thickened root cell walls in rice plants under Fe toxicity (Fu et al., 2012). In the roots of Cd treated maize plants, Si application stimulated the development of apoplastic barriers and lignification of xylem elements (Vaculik et al., 2012). In spite of reports on the interaction of exogenous Si application with excess Fe nutrition on cell wall modifications especially in roots, there is no report on the effects of these nutritional parameters on the spatial changes in the activities of genes and/or enzymes involved in the lignification of root cell walls in rice or other plants. Thus, in the present study, the expression of phenylalanine ammonia–lyase, peroxidase and protein phosphatase 2C genes, as well as the enzymatic activities of PAL, NADPH–oxidase, superoxide dismutase (SOD) and cell wall peroxidase (POD) were investigated in both root apical (AP) and basal (BP) parts of rice plants grown under excess Fe with and without supplementary Si. The results were further correlated to the structural changes of root anatomy invoked by excess Fe as affected by Si nutrition.

2. Materials and methods

2.1. Plant cultivation

Rice (*Oryza sativa* L. cv Fajr, Iranian rice) seeds were surface sterilized with 3% sodium hypochlorite solution and Tween 20 for 20 min. For germination, rice seeds were imbibed in moistened filter paper, incubated at 25 °C for 10 days and then the healthy seedlings with shoot length of about 3 cm were transferred into a hydroponic culture containing Yoshida nutrient solution (pH = 6 ± 0.2) (Yoshida, 1981). The experiment was carried out in a completely randomized factorial design with three replications and each replication consisted of four plants. The first factor was silicon (Si) treatment (0 and 1.5 mM) which was supplied as sodium silicate and the second factor was iron (Fe) treatments in the form of Fe–EDTA at 10 (as control) and 150 mg/L. Plants were harvested after 7 days and analyzed for molecular, biochemical and morphological parameters in tissue samples taken from two root zones i.e. AP and BP and also from the top first fully expanded leaf. Analyses on roots were carried out at apical part (AP) (0.3–4 cm from the root tip) and basal part (BP) (4–8 cm from the tip) (Gallagher, 2013).

2.2. Determination of genes, RNA extraction and RT–PCR

Gene sequences for rice phenylalanine ammonia–lyase (LOC_Os02g41680), cell wall peroxidase (LOC_Os01g22230) and protein phosphatase 2C (LOC_Os02g05630) were identified using primers designed by Fleck et al. (2011) (Table 1). The sequences for *O. sativa* L. cv Fajr (Iranian rice) derived from gene information in TIGR data base website were compared and approved with Japanese rice (*Oryza sativa* ssp japonica cv. Nipponbare). Rice ELONGATION FACTOR 1 ALPHA (*EF1a*) was used as a control. Protein characteristics of the *O. sativa PAL, POD*, *PP2C* and *EF1a* genes are shown in the supplementary data 1. Extraction of RNA from the AP and BP root zones and leaf were carried out using TRIzol including guanidine thiocyanate (4 mM), sodium citrate (25 mg/pH = 7), sarkosyl 0.5% (w/v) and 2–mercaptoethanol 0.1 M (Chomczynski and Sacchi, 1987). The RT–PCR was performed by BioNEER's, PreMlx AccuPower® RocketScript RT–PCR kit in a total volume of 20 μl with 2 μg RNA and 10 *p*M each of the right and left primers.

2.3. Extraction and activity determination of PAL, NADPH–oxidase, SOD and cell wall peroxidase

To assay PAL activity, tissue samples (0.1 g fresh weight) were homogenized with 2 mL sodium phosphate buffer (200 mM/pH = 6.2), which contained 5% (W/V) PVPP and 2% (V/V) Triton X–100. The crude extract was centrifuged at 15000 g for 20 min at 4 °C. The 15000 g supernatant was then used as an enzyme extract. The reaction mixture consisted of 600 μL of Tris buffer (50 μM, pH = 8.8), 900 μL phenylalanine (2 μM) and the enzyme extract (800 μL), which allowed to proceed for 30 min. Thereafter the production of cinnamic acid from

Table 1
The primer sequences for the expression analyses of the *PAL, POD, PP2C and EF1a* genes in *Oryza sativa* cv. Fajr.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	DNA length	RNA length	Tm
PAL	TCACAAGCTCAAGCACCATC	CTCACCAAGCTTCTTGGCAT	102	102	58
POD	TCACAAGCTCAAGCACCATC	ATCGACACGACACGACGACACT	96	96	60
PP2C	GTGGTGGTCGTCGTCTTCTT	CCCATAACTGAACTTGCCGT	899	190	58
EF1a	GACTTCCTTCACGATTTCATCGTAA	TTTCACTCTTGGTGTGAAGCAGAT	690	103	58

Table 2
Interaction of Fe and Si on growth related parameters of rice plants grown under excess Fe (150 mg/L) supplied with or without Si (1.5 mM) after 7 days of treatments.

Treatments		Root FW	Shoot FW	Total FW	Root DW	Shoot DW	Total DW
		gr					
-Fe	-Si	0.20 ± 0.03a	0.27 ± 0.09b	0.47 ± 0.13b	0.013 ± 0.01a	0.036 ± 0.03b	0.049 ± 0.01b
	+Si	0.19 ± 0.01a	0.32 ± 0.10a	0.51 ± 0.10a	0.013 ± 0.03a	0.041 ± 0.01a	0.054 ± 0.02a
+Fe	-Si	0.13 ± 0.01b	0.23 ± 0.08c	0.36 ± 0.10c	0.009 ± 0.01b	0.031 ± 0.01c	0.040 ± 0.02c
	+Si	0.11 ± 0.02b	0.24 ± 0.09c	0.35 ± 0.11c	0.008 ± 0.02b	0.033 ± 0.02c	0.041 ± 0.01c

Data are shown as mean ± SE. Different small letters in each column indicate significant differences ($P < 0.05$) according to LSD test (n = 3).

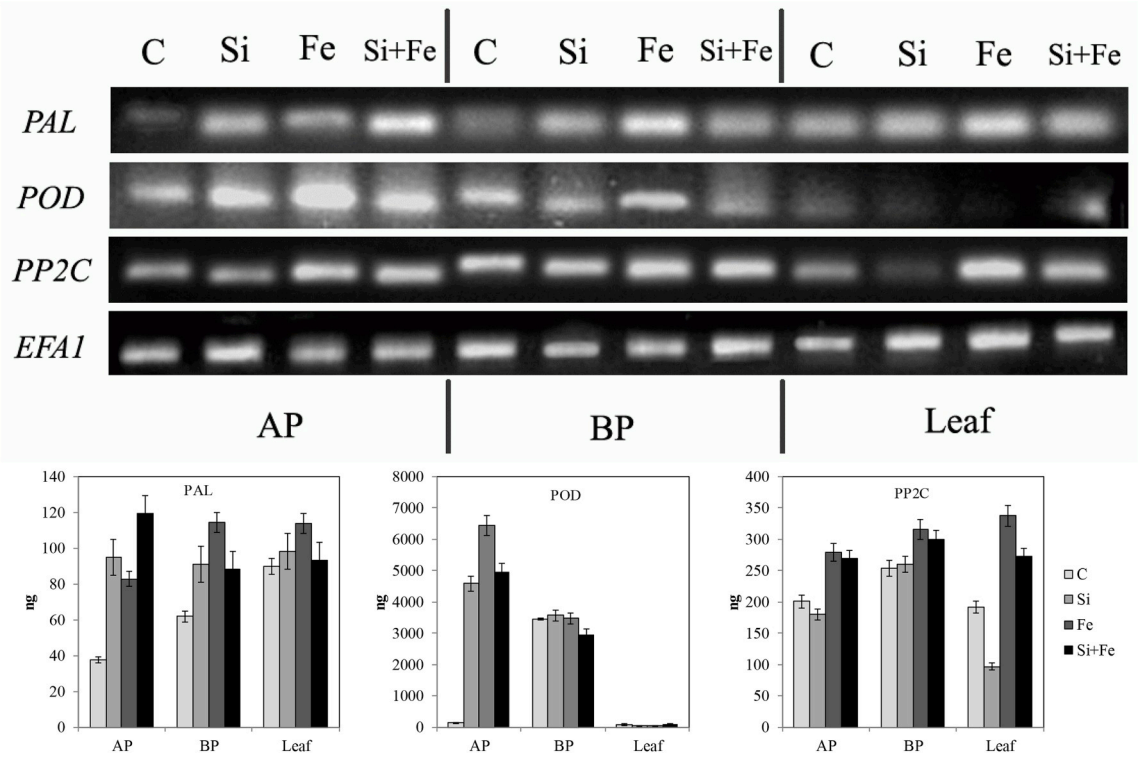

Fig. 1. RT-PCR of *PAL, POD* and *PP2C* genes at root apical part (AP, 0.3–4 cm from tips) and basal part (BP, 4–8 cm from tips) and first fully expanded leaf of rice plants grown for 7 days under control (C), silicon (Si), 150 mg/L iron (Fe) and 1.5 mM silicon plus 150 mg/L iron (Si + Fe) conditions.

phenylalanine was stopped by the addition of 100 μL HCl (2N). In the next step, toluene (2 mL) was added and vortexed for 30 s and the final mixture was centrifuged at 6000 g for 5 min. The enzyme activity was quantified by recording the increase in the absorbance at 290 nm in the toluene phase due to the production of cinnamic acid assuming an extinction coefficient of 20,000 M^{-1} cm^{-1} (Whetten and Sederoff, 1992).

NADPH–oxidase, superoxide dismutase (SOD) and cell wall peroxidase activities were extracted as described by Carter et al. (2007) with minor modification. Briefly, tissue samples (0.05 g) were homogenized with 1 mL Tris buffer (50 mM, pH = 8) containing magnesium chloride (100 mM), sucrose (250 mM), Triton X-100 (5%; V/V), 2- mercaptoanol (10 mM) and PMSF (1 mM). The homogenate was centrifuged at 10000 g for 15 min. The resulting supernatant was then used to measure NADPH–oxidase and SOD activities while the 10000 g pellet was used to assay cell wall peroxidase activity.

The activity of NADPH–oxidase was determined as described by Van Gestelen et al. (1997). The reaction mixture (1 mL) consisted of Tris buffer (50 μM, pH = 8), nitroblue tetrazolium (NBT), NADPH (0.1 mM) and 100 μL of the enzyme extract. Assuming a value of about 12800 M^{-1} cm^{-1} for the extinction coefficient of mono–formazan, the enzyme activity was determined after recording changes in the absorbance at 530 nm due to mono–formazan formation from NBT reduction by superoxide radicals. To confirm the specificity of the color produced by the NADPH–oxidase, 50 units of superoxide dismutase (SOD) were also included in the control reaction.

The activity of superoxide dismutase was determined according to Beauchamp and Fridovich (1971). The 3 mL reaction mixture consisted of monosodic phosphate buffer (50 mg, pH = 7.8), EDTA (0.66 mM), methionine (10 mM), NBT (33 μM), riboflavin (0.133 mM) and the enzyme extract (100 μL). The enzyme activity was determined at 560 nm based on the inhibition of mono–formazan production (NBT reduction) over 10 min. The SOD activity was calculated according to the following equation and finally SOD activity was expressed based on the SOD unit/g FW.

SOD unit = 2 × [1– (Absorption changes with SOD/Absorption changes without SOD)]

For cell wall peroxidase activity assay, the pellet after the extraction of soluble proteins was rinsed 4 times with distilled water. Sodium chloride solution (2 mL; 1 M) was added, then the mixture was vortexed and centrifuged at 15000 g in 4 °C for 15 min. The 15000 g supernatant was used as a source of enzyme. The reaction mixture in a final volume of 3.0 mL consisted of phosphate buffer (25 mM, pH = 6.8), guaiacol (20 mM), H_2O_2 (40 mM) and 10 μL of the enzyme extract. The

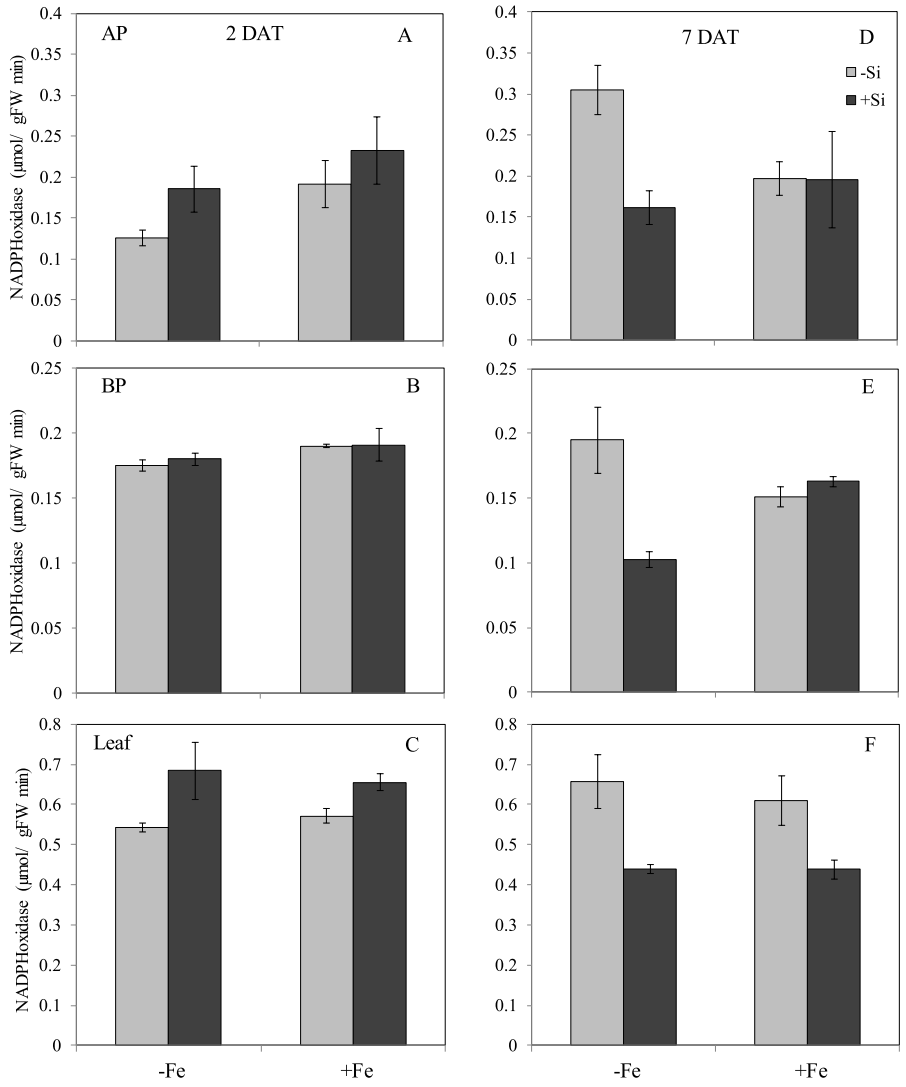

Fig. 2. The activity of NADP oxidase in the root AP (A, D), BP (B, E) and the first fully expanded leaf of rice plants (C, F) grown for 2 (A, B, C) and 7 (E, F, G) days in nutrient media with or without 150 mg/L iron (-Fe, +Fe) and supplied with or without 1.5 mM silicon (-Si, +Si). Data are shown as mean ± SE.

extinction coefficient of tetraguaiacol at 470 nm (26600 M^{-1} cm^{-1}) was used to estimate enzyme activity (Chen et al., 2000; Kar and Mishra, 1976).

2.4. Measurement of total protein, H_2O_2, lipid peroxidation and total soluble phenols

Total protein was extracted according to Markwell et al. (1981) using 1 mL Tris buffer (100 mM, pH = 9) containing EDTA (1 mM), PMSF (1 mM), Triton X–100 (0.1%; V/V) and PVPP (0.06%; W/V) as the extraction medium.

The content of H_2O_2 in plant tissues was determined spectrophotometrically according to Sergiev et al. (1997) using potassium iodide 1 M. The malondialdehyde (MDA) content as an index of lipid peroxidation was measured according to Hodges et al. (1999). Total soluble phenols were quantified spectrophotometrically according to Price and Butler (1977).

2.5. Histochemical analysis

Tissue samples from root AP and BP zones of three replicates plants were fixed in F.A.A solution which consisted of formaldehyde (5 mL: 37%), glacial acetic acid (5 mL) and ethanol (90 mL; 50%). Tissue dehydration and clarification were carried out by immersing specimens in the increasing series of ethanol and xylene solutions. The dehydrated clarified tissues were then embedded in paraffin blocks and trimmed trapezoidal. Tissue sectioning (n = 20; 13 µM thickness) was then carried out using a rotary microtome (DIA PATH). Tissue sections were deparaffinized by xylene and decreasing series of ethanol solutions and they were fixed on slides by Canada Balsam (Ruzin, 1999). Changes in cell wall thicknesses of both endo– and exodermal layers and metaxylem elements were then observed and recorded with a florescent microscope (Smart–FL; magnification 100X) using Digimizer 4.1.1.0 software.

2.6. Statistical analysis

SAS Institute (2009) statistical software (2009) was used for analyzing the data. All data were subjected to ANOVA and a comparison of the means was performed using the Least Significant Difference (LSD) test at the 0.05 probability levels.

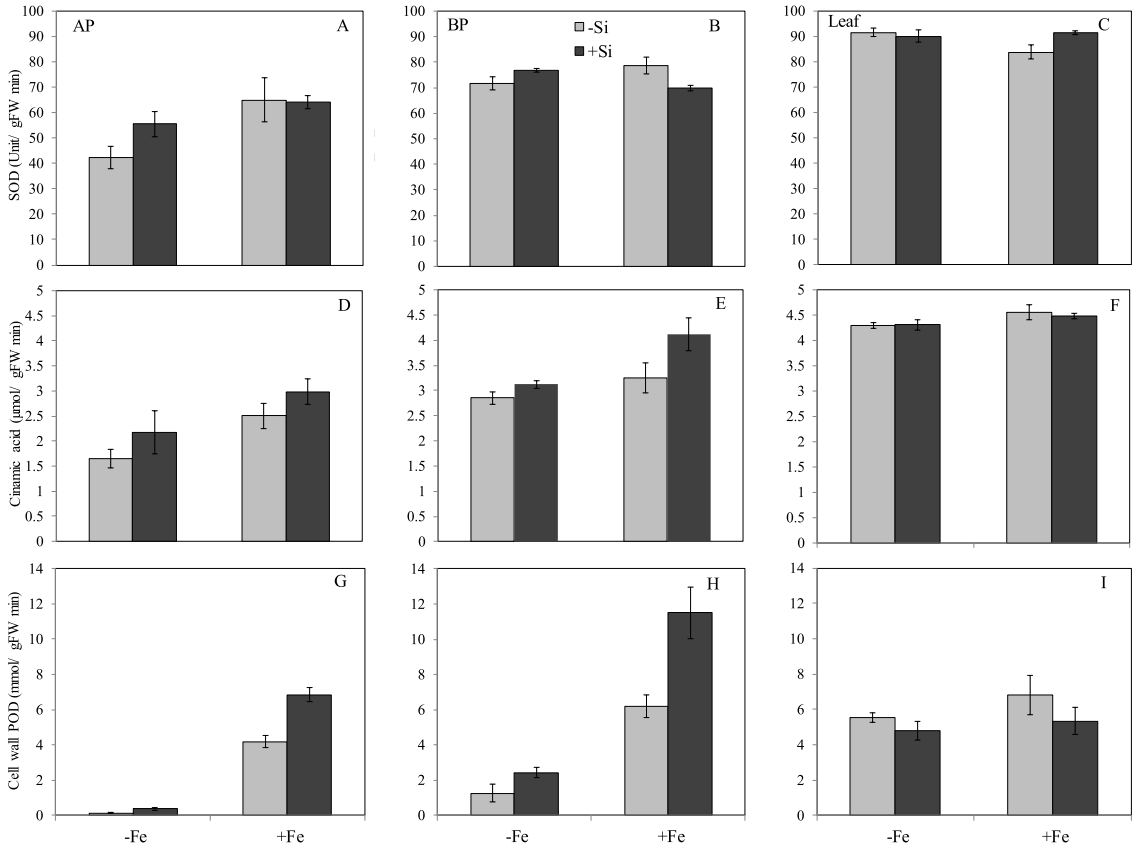

Fig. 3. The activities of SOD (A, B, C), PAL (D, E, F) and cell wall POD (G, H, I) in the root AP (A, D, G), BP (B, E, H) and the first fully expanded leaf of rice plants (C, F, I) grown for 7 days in nutrient media with or without 150 mg/L iron (-Fe, +Fe) and supplied with or without 1.5 mM silicon (-Si, +Si). Data are shown as mean ± SE.

3. Results

3.1. Plant growth

Excess Fe in the root medium reduced both fresh and dry weights of roots and shoots in rice plants. Supplying Si to the nutrient solution increased the growth of –Fe plants, however, Si could not affect growth of plants treated with excess Fe (Table 2).

3.2. PAL, POD and PP2C gene expression

The expression of *PAL* and *PP2C* genes in both AP and BP root zones and leaf increased markedly due to excess Fe (Fig. 1). This treatment however, increased the expression of *POD* only in the root AP. Si application increased the expression of *PAL* and *POD* in root AP-BP and AP zones respectively, but reduced *PP2C* expression in leaf. When Si supplied to plants under excess Fe nutrition the expressions of *PAL* and *POD* were still higher than control (without Fe and Si) root AP-BP and AP, respectively although under this condition *POD* expression decreased in root BP. Plants treated in this way also displayed greater expression of *PP2C* in root AP-BP zones and leaf. Plants supplied with both Fe plus Si however, displayed greatest PAL expression in root AP when compared to plants fed with excess Fe alone.

3.3. The activities of NADPH oxidase, SOD, PAL and cell wall POD

After 2 days in –Si treatments, the NADPH–oxidase activity of the root AP in plants treated with excess Fe increased by about 42%, compared to the controls; however, after 7 days, excess Fe reduced the activity of NADPH–oxidase in both BP and AP root zones (Fig. 2). After 2 and 7 days, Si application did not affect the activity of NADPH–oxidase in both AP and BP root zones of plants supplied with excess Fe. In leaf, the enzyme activity increased after 2 days but decreased after 7 days due to Si application in plants under excess Fe.

In plants without Si application, SOD, PAL and cell wall POD activities significantly increased in AP root zone due to excess Fe nutrition (Fig. 3). Si treatment increased only cell wall POD activity in this zone. In BP root zone without Si nutrition, excess Fe led to the increased PAL and cell wall POD activities when compared to control plants (Fig. 3). In the root BP zone of plants under excess Fe nutrition, Si application resulted in decreased SOD activity but enhanced PAL and cell wall POD activities. In the leaf, excess Fe significantly increased PAL enzyme activity; however, the activities of other studied enzymes remained unaffected following the application of Si (Fig. 3).

3.4. The concentrations of proteins, H_2O_2, lipid peroxidation and total phenols

In plants without Si application, excess Fe did not affect total proteins in both BP and AP root zones (Table 2). Total proteins decreased in both BP and AP root zones in plants under excess Fe nutrition due to Si application. Changes in total proteins in the top first fully expanded leaf were different from that observed in roots. In the absence of Si, the amounts of total proteins increased by about 40% in plants treated with excess Fe. Si application also increased total proteins in plants under excess Fe treatment.

In control plants (without Si nutrition), H_2O_2 content increased by about 43%, 63% and 75% in the root BP and AP and leaf respectively, due to excess Fe treatment (Table 2). Under excess Fe treatment, the application of Si reduced the amount of H_2O_2 in the root AP by about

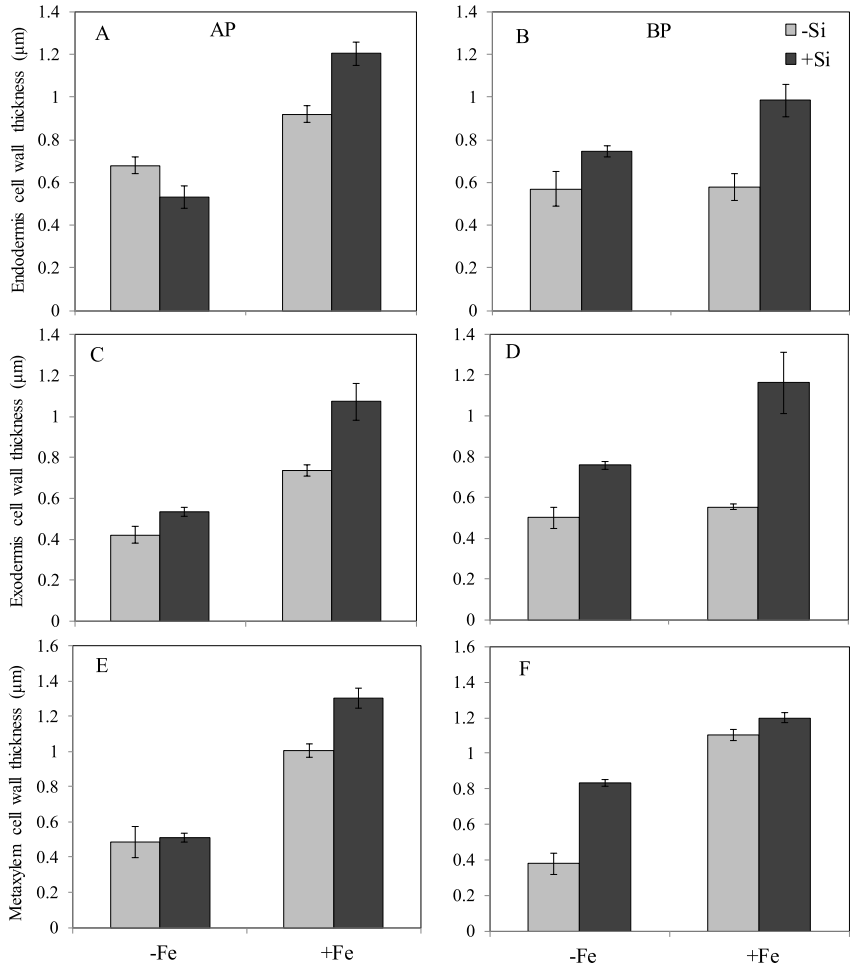

Fig. 4. Cell wall thickness of endodermis (A, B), exodermis (C, D) and protoxylem (E, F) in the root AP (A, C, E) and BP (B, D, F) in rice plants grown for 7 days in nutrient media with or without 150 mg/L iron (-Fe, +Fe) and supplied with or without1.5 mM silicon (-Si, +Si) (data are shown as mean ± SE).

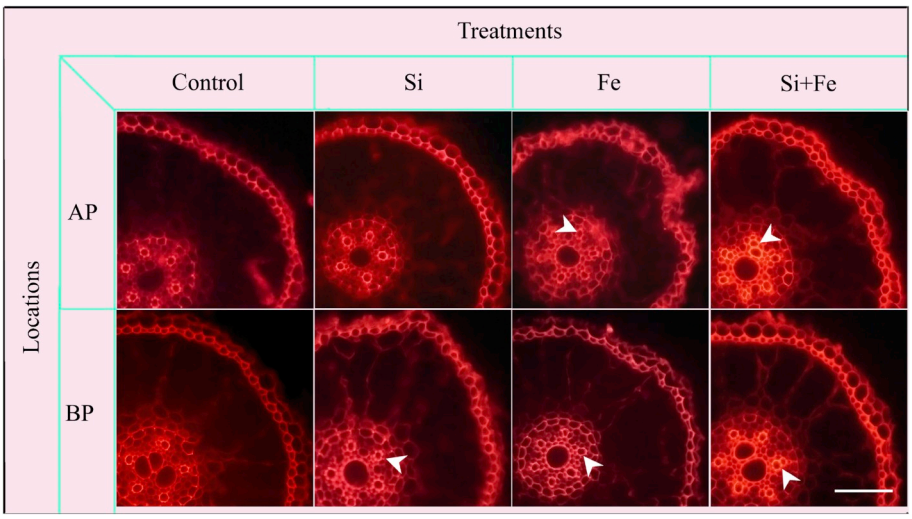

Fig. 5. Cross sections from the root AP and BP of rice plants as seen in the florescent microscope (Smart–FL) showing the distribution of lignified and/or suberized tissues. The plants were grown under control (C), 1.5 mM silicon (Si), 150 mg/L iron (Fe) and 1.5 mM silicon plus 150 mg/L iron (Si + Fe) treatments. The arrow indicates the increased thickening of metaxylem elements (Scale bar = 10 µm). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

15% but it had no significant effect on this parameter in the root BP. In leaf, Si application increased H_2O_2 content irrespective of Fe treatment.

In the absence of Si, lipid peroxidation increased due to excess Fe nutrition by about 51%, 16% and 56% in the root BP and AP and leaves, respectively (Table 2). The application of Si under excess Fe nutrition had no significant effects on lipid peroxidation of root BP and AP however, it could reduce lipid peroxidation in the first fully expanded leaf.

Total phenols increased due to excess Fe nutrition by about 31% in the root AP of plants in the absence of Si (Table 2). This treatment also increased the total phenol contents of root BP but resulted in declined phenol contents of leaves. Under excess Fe nutrition, the application of Si did not affect total phenol contents of the root AP and BP, but decreased this parameter in the leaf.

Table 3
Interaction of Fe and Si on the contents of total proteins, hydrogen peroxide, malondialdehyde and total phenols in the rice root AP, BP and the first fully expanded leaf. Plants were grown under excess Fe (150 mg/L) nutrition supplied with or without Si (1.5 mM) for 7 days and were analyzed.

Treatments		Total protein mg/gFW			H_2O_2 nmol/gFW			Lipid peroxidation nmol malondialdehyde/gFW			Total soluble phenols mg/gDW		
		AP zone	BP zone	Leaf	AP zone	BP zone	Leaf	AP zone	BP zone	Leaf	AP zone	BP zone	Leaf
-Fe	-Si	8.04 ± 0.6a	5.24 ± 0.4b	23.24 ± 6.1c	158.0 ± 13.4d	307.4 ± 49.7b	776.1 ± 80.9d	7.41 ± 0.7c	13.06 ± 0.7b	20.38 ± 1.9b	0.60 ± 0.01b	1.13 ± 0.16b	13.21 ± 1.0a
	+Si	6.33 ± 0.4b	3.79 ± 0.2c	35.01 ± 0.9a	203.4 ± 16.3c	363.6 ± 88.5b	989.5 ± 23.6c	8.93 ± 0.6b	8.27 ± 0.2c	11.44 ± 1.0c	0.30 ± 0.01c	0.57 ± 0.12c	12.36 ± 0.8a
+Fe	-Si	8.84 ± 1.0a	6.78 ± 1.2a	32.73 ± 0.1b	384.2 ± 26.8a	503.6 ± 10.7a	1364.8 ± 52.1b	11.19 ± 0.3a	15.22 ± 0.9a	31.81 ± 3.9a	0.79 ± 0.10a	1.77 ± 0.25a	9.82 ± 1.4b
	+Si	7.31 ± 0.6a	5.31 ± 0.1b	35.02 ± 0.5a	327.8 ± 25.1b	516.7 ± 20.7a	1486.9 ± 48.4a	10.68 ± 0.4a	15.16 ± 1.1a	22.61 ± 0.1b	0.73 ± 0.03a	1.52 ± 0.09a	6.60 ± 2.0c

Data are shown as mean ± SE. Different small letters in each column indicate significant differences ($P < 0.05$) according to LSD test (n = 3).

3.5. Histological changes in roots as affected by Si and Fe treatments

Changes in the extent of lignification-suberization of the root tissues at the BP and AP were observed in cross sections by the emission of red fluorescence signals due to deposition of lignin and/or suberin (Fig. 5). Irrespective of Si application, the thickness of endodermis, exodermis and metaxylem cell walls were significantly higher in the root AP of plants under excess Fe nutrition. In this region, the cell wall thicknesses of endodermis, exodermis and metaxylem increased by 25%, 38% and 23% respectively, in plants fed with Fe plus Si. In the root BP, in the absence of Si the thicknesses of endodermis and exodermis remained unaltered due to excess Fe nutrition but it increased the metaxylem cell wall thickness significantly (Fig. 4A). In the same zone in plants under excess Fe nutrition, Si increased cell wall thicknesses of endodermis, exodermis and metaxylem by 45%, 52% and 15%, respectively (Fig. 4).

4. Discussion

4.1. Physiological effects of excess Fe nutrition

Excess Fe nutrition in the nutrient solution induced Fe toxicity as evidenced by growth retardation of plants (Table 2). The increased thickening of the root cap cell walls in rice (Zhang et al., 2017) and enhanced lignification of maize roots oversupplied with Fe suggest the importance of root cell wall development in the Fe tolerance mechanisms (Hopff et al., 2013). The lignification of root cell walls may block the apoplastic pathways for ion transport and influence plant tolerance to heavy metals stress (Adrees et al., 2015). In this study the rice root BP and AP were examined separately because the apoplastic pathway is typically closed in the former but opened in the latter (Barberon and Geldner, 2014). Furthermore, plants were analyzed after one week exposure to Si, Fe and Si plus Fe to monitor their early physiological responses to these treatments as the identification of these responses opens the ways for their exploitation in subsequent stress tolerance improvement of this important crop species.

In the root AP, both *PAL* gene expression and its enzyme activity increased due to Fe toxicity. Thus, the increase in soluble phenols might have been resulted in this way. PAL is the first enzyme of the phenylpropanoid pathway which provides precursors for lignin biosynthesis (Koch and Schmitt, 2013). In parallel to increased PAL activity, the activities of NADPH- oxidase and SOD increased in the root AP following excess Fe nutrition (Fig. 2). The NADPH- oxidase activity in association with SOD results in H_2O_2 production (Bowler et al., 1994; Foreman et al., 2003), and in congruence, increased SOD activity in the rice root tip following exposure to Fe has also been reported (Zhang et al., 2011). These might justify the accumulation of H_2O_2 in rice plants exposed to excess Fe nutrition (Table 3). It appears that excess Fe treatment of rice plants resulted in increased hydrogen peroxide content of tissues primarily due to greater activities of both NADPH–oxidase and SOD. Hydrogen peroxide can act both as a signaling molecule and a substrate in the apoplastic space and bring about tissue lignification in different root zones, such as endodermis through the action of cell wall PODs (Lee et al., 2013; Sagi and Fluhr, 2006). Interestingly, excess Fe nutrition in rice plants enhanced both *POD* expression and its enzyme activity in the root AP (Figs. 1 and 3), and increased the lignification of exodermal, endodermal and metaxylem elements (Figs. 4 and 5). These evidences suggest the important roles played by cell wall POD in conjunction with NADPH-oxidase and SOD in development of lignified tissues in the rice root AP. Indeed, the need for NADPH–oxidase activity in the modifications and development of plant roots (Foreman et al., 2003), enhanced POD activity in rice root exposed to high Fe concentrations (Zhang et al., 2011), positive correlation between POD activity and rice root lignifications under Ni toxicity (Lin and Kao, 2005) and the onset of programmed cell death in rice root tip border cells exposed to excess Fe (Zhang et al., 2011, 2017) may suggest excess Fe nutrition in rice root AP induces earlier

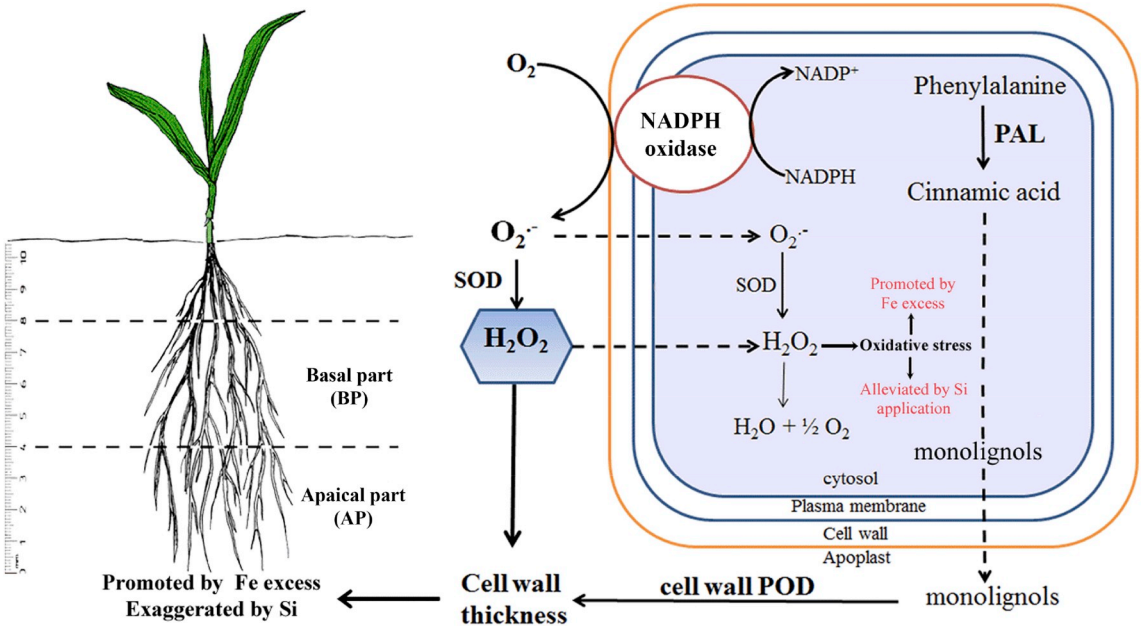

Fig. 6. Cell wall thickening pathway with emphasis on PAL, cell wall POD, NADPH oxidase and SOD activities and the increased H_2O_2 content in different zones of rice plant root treated with excess Fe as affected by Si application.

differentiation of metaxylem elements through programmed cell death while in the endodermal and/or exodermal layers accelerate development of highly lignified and/or suberized cell walls to restrict the entry of excess Fe into the plant. In congruence, the apoplastic movement of Cd into the xylem can also be restricted by the development of the exodermis, endodermis, and other extracellular barriers (Lux et al., 2011).

The underlying events due to excess Fe nutrition in the root BP, were nearly similar to those observed for the AP however, the extent of changes in enzymes activities, hydrogen peroxide and soluble phenol accumulation and tissue lignification were smaller in this zone so that only metaxylem cell walls displayed enhanced thickening due to Fe toxicity (Figs. 4 and 5; Table 3). Thus, the enhanced expression of *PAL* in the root BP and AP zones and *POD* in the root AP as well as their greater enzymes activities under excess Fe nutrition may suggest changes in root differentiation pattern probably by earlier initiation of lignin polymerization closer to and toward the root tip.

In all the investigated tissues, excess Fe nutrition imposed oxidative stress as evidenced by increased H_2O_2 and lipid peroxidation contents with inevitably plant growth declined in both root and shoot in congruence with former studies (Chalmardi et al., 2014; Nagula et al., 2016; Dufey et al., 2014). The accumulation of various ROS forms may cause peroxidation of cell membranes, damage to DNA, proteins, and many macromolecules (Bhattacharjee, 2005) and thus triggers differentiation related events such as programmed cell death in at least some cells in the root tip (Zhang et al., 2017). The increased expression of *PP2C* in rice plants under excess Fe nutrition (Fig. 1) furthermore, is in accordance with the suggested roles for this protein in responses to oxidative stress and programmed cell death in plants (Park et al., 2008; You et al., 2014; Yang et al., 2018).

4.2. Effects of Si application in plants under excess Fe nutrition

The greater expression of *PAL* due to excess Fe nutrition in the root AP was even further enhanced when plants received an additional supply of Si while in the root BP and leaf *PAL* expression even decreased or remained unaltered (Fig. 1). The increased expression of *PAL* in rice roots following Si treatment has also been reported and attributed to the increased capacity for monolignol biosynthesis (Fleck et al., 2011). Meanwhile, Si application did not affect PAL activity and total soluble phenols in both root AP and BP zones of plants under excess Fe nutrition (Fig. 3, Table 2). These data suggest that PAL expression and enzyme activity is regulated mainly by Fe toxicity rather than Si supplementation. The same contention might be drawn for the activities of NADPH oxidase and SOD as well as the tissue H_2O_2 contents.

The application of Si in plants under excess Fe nutrition neither affected NADPH–oxidase activity nor H_2O_2 contents in both root AP and BP zones (Fig. 2; Table 2), and also, Si application did not affect the activity of SOD in the root AP under excess Fe nutrition and even reduced this activity in the BP (Fig. 3). Despite of these, it appears that Si application has a great bearing on cell wall POD activity in the root AP and BP zones exposed to excess Fe nutrition. Parts of this effect might be explained by the enhanced *POD* expression especially in the root AP zone when plants are supplied with Si alone (Fig. 1). Increased expression of *POD* (*LOC_Os01g22230*) encoding a cell wall POD in rice roots has also been reported (Fleck et al., 2011). However, cell wall POD activity increased synergistically in the Fe-stressed root AP-BP zones supplied with Si despite relatively unaltered *POD* expression (Figs. 3 and 1). These imply the possible induction of posttranscriptional regulation of cell wall POD activity under excess Fe plus Si conditions.

The stimulation of cell wall POD activity in Fe-stressed rice roots supplied with Si should have a functional significance. It is already known that Si application restricts Zn and Cd uptake and transport in rice and improves plant tolerance to these heavy metals (Huang et al., 2018). Furthermore, transcriptomal changes in enzymes and/or proteins related to cell wall modifications are prominent in Si treated roots (Haddad et al., 2019). Thus, the increased cell wall POD activity in rice roots exposed to excess Fe plus Si might provide a metabolic sink to counteract deleterious effects of H_2O_2 accumulation from one side (Table 3) and meanwhile attenuates excessive Fe uptake through the development of lignified and/or suberized walls resulting from the polymerization of monolignols (Francoz et al., 2015). In support of this contention we observed increased thicknesses of cell walls especially in the endodermis and metaxylem elements of rice root AP-BP zones fed with excess Fe plus Si (Figs. 4 and 5). Si application may also provide additional binding sites for Fe in the cell walls as suggested by Chalmardi et al. (2014). The positive effects of Si have also been

attributed to increased cell wall thickening and decreased Fe precipitation on the root surfaces (Fu et al., 2012).

For the studied period of experiment, the first fully expanded leaf did not show significant changes in the investigated biochemical parameters in response to excess Fe and Si application except for lipid peroxidation and NADPH oxidase activity (Table 3 and Fig. 2). In this case, lower NADPH oxidase activity and declined lipid peroxidation in plants grown under excess Fe plus Si implied the amelioration of oxidative stress due to Fe toxicity by Si application. Declined expression of *PP2C* as a stress marker (You et al., 2014; Yang et al., 2018) following Si application in leaves from plants fed with excess Fe (Fig. 1) may partly support this contention. Sharper responses of leaves to excess Fe nutrition and Si possibly demands longer exposure of plants to these treatments due to the early development of heavy metal uptake and transport barriers in roots when exposed to heavy metals (Huang et al., 2018).

5. Conclusions

Our study showed that excess Fe nutrition in rice plants imposes marked increase in the activities of PAL and cell wall POD, along with the accumulation of H_2O_2 and the enhancement of root lignifications and/or suberization especially closer to the tip (Fig. 6). Exogenous Si application intensified the above mentioned cell wall related processes and especially this treatment had a great bearing on cell wall POD activity. It seems that Si application restricts Fe uptake through the enhanced formation of apoplastic barriers near the root tip. Longer term studies however, are required to assess the impacts of these treatments on final production and yield.

Author contribution

P. Mehrabanjoubani and B. Barzegargolchini collected data, wrote and prepared the manuscript. A. Abdolzadeh supervised the project, interpreted data and critically read the manuscript. H.R Sadeghipour assisted in enzyme assays and critically reviewed the manuscript. M. Aghdasi advised the project. M.B. Bagherieh-Najjar assisted in parts of molecular analysis. All authors approved the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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