Early induction of phenolic compounds in aluminum-treated roots of *Brassica oleracea* L.

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Abstract

There are evidences showing that adverse impacts of aluminum (Al) on plants growth and development are associated with its effect on plasma membrane and cell wall. Influence of Al on peroxidation of membrane lipids, wall-bound phenolics, lignin, soluble phenolics and the activity of certain enzymes involved in metabolism of phenols, i.e., phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) was measured in red cabbage (*Brassica oleracea* L. cv. Red Dynasty). The plants in their vegetative growth phase were treated with 60 µM $AlCl_3$ in a Hoagland solution, pH 4.5, for 6 h. Aluminum was rapidly absorbed by the plants. The effect of Al on the peroxidation of membrane lipids of both roots and shoots was not significant compared to those of the control plants. The activity of PAL in both roots and shoots and of PPO of roots was significantly higher in Al-treated groups, compared with the control ones. The contents of pectin-bound phenolics as well as lignin contents of plants were significantly enhanced by Al and the increase of phenolics of shoots in Al-treated plants were much more pronounced. While anthocyanin content of the plants increased by Al, total contents of flavonoids were not significantly affected by Al treatment.

Keywords: Brassica oleracea; aluminum; lipid peroxidation; phenolic compounds; phenyalanine ammonia lyase (PAL); polyphenol oxidase (PPO)

Abbreviations:
Al: aluminum; BSA: bovine serum albumin; FAA: formalin-acetic acid-alcohol; MDA: malondialdehyde; PAL: phenyalanine ammonia lyase; PPO: polyphenol oxidase; TBA: thiobarbituric acid; TCA: trichloro acetic acid

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Introduction

Aluminum is one of the most abundant minerals in the soil, comprising approximately 7%, and exists as a stable complex with oxygen and silicate in neutral and weakly acidic soils. When the soil pH is lower than 4.5-5, Al is solubilized in the soil water, absorbed by plant roots and converted to a phytotoxic form (Matsumoto, 2000).

The plant cell wall and membranes are known as sites of determination of Al penetration rate, and Al resistance (Pejchar et al., 2010). Studies of the target of Al action in plant tissues and cells have demonstrated that Al enters and binds to the apoplast (Wissemeier and Horst, 1995) and changes the properties of the plasma

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membrane and cell wall (Sivaguru et al., 2003; Illes et al., 2006). One of the general aspects of Al toxicity is the inhibition of root growth owing to peroxidase-mediated hydrogen peroxide production and/or loss of plasma membrane integrity (Cakmak and Horst, 1991). Al-induced changes in cell wall properties have been suggested as the major factor leading to Al toxicity. It was proposed that binding of Al to the cell wall increases cell wall rigidity and decreases its elasticity (Kopittke et al., 2008). At the biochemical level, different metabolites such as some phenols have been suggested as potential ligands for Al ions (Tolra et al., 2005; Tolra et al., 2009). Notwithstanding the abundance of phenols, data related to their accumulation during Al stress are still rather scarce and/or limited to specific group of phenols such as flavonoids (Tolra et al., 2005; Tolra et al., 2009; Barcelo and Poschenrieder, 2002). Phenolic compounds are known as sources of natural antioxidant due to their ability to scavenge free radicals, give away hydrogen and electron and chelate with metal cations. The phenylpropanoid pathway is involved in many interactions of plants with their abiotic and biotic environments. Phenolic compounds in the plant cell wall and soluble phenolic compounds such as anthocyanins and flavonoids may be able to inhibit Al entrance to cytoplasm and have a role in Al toxicity removal. There is intensive research focused on the effect of high Al concentrations on roots of plants. The present study however, was aimed to clarify responses to 60 µM Al regarding phenolics metabolism in both above- and below-ground parts of red cabbage.

Materials and Methods

Growth conditions and treatment

Red cabbage (*Brassica oleracea* L.cv. Red Dynasty) plants were grown for 1 mount in a modified nutrient solution containing (in m*M*): $(NH_4)_2SO_4$, 0.713; NH_4NO_3, 073; KH_2PO_4, 0.1; K_2SO_4, 0.46; $CaCl_2$, 0.5; $MgSO_4$, 0.41; Fe-EDTA, 0.032; H_3BO_3, 0.046; $CuSO_4$, 0.002; MnSO4, 0.09; Na_2MoO_4, 0.0026; $ZnSO_4$, 0.0091. (pH 6.0). The solution was aerated and was renewed every 5 days. In Al treatments, Al was added as $AlCl_3$ to a final concentration of 60 µM and the pH was adjusted to 4.5. After 6 h of the treatment, the plants in both treated and control group were harvested and their shoots and roots were separated and washed thoroughly with deionized water. The samples were frozen with liquid nitrogen, and kept at -20 °C until used for biochemical analysis. All chemicals were purchased from Sigma (USA).

Aluminum content

Aluminum content of samples was determined by atomic absorption spectrophotometer (ICP-OES, VISTA-PRO, Varian, Australia) after wet-digestion of ash samples.

Histochemical analyses

Aliquots of plant samples were fixed with FAA (17.5 mL of EtOH 80%, 2 mL of formaldehyde 37%, and 0.5–1 mL acetic acid) for 18 h, washed and brought to ethanol 70%. Free hand sections were observed by fluorescent microscope (BH_2, Olympus, Japan). Autofluorescence of phenolic acids and their relative status in cell walls, i.e., whether being bound to pectic substances or polymerized as lignin, was justified according to the change of colors followed by changing pH, using sodium acetate 0.1M (pH 4) and ammonium hydroxide 0.1 M (pH 10.3) (Harris and Hartley, 1976).

Extraction and assay of phenylalanine ammonia lyase (PAL) and wall-bound phenolics

Frozen samples were homogenized in ice-cold K-borate buffer (0.1M, pH 8.8) containing 2 mM 2-mercaptoethanol with a mortar and pestle and centrifuged at 16,000 × g for 15 min at 4 °C. The supernatant was used as a crude enzyme solution. The reaction mixture (total of 2 mL) was composed of 0.5 mL of crude enzyme and 1 mL of extraction buffer (without 2-mercaptoethanol). The reaction started with the addition of 0.5 mL of 4 mM phenylalanine assay, and after 1 h incubation at 37 °C, was stopped with 100 µL of 5M HCl. The mixture was extracted two times with EtOAc. The EtOAc extract was air-dried, re-

dissolved in 5o % MeOH and analyzed by HPLC equipped with an OSD-80 Ts column (15 cm × 6.0 Shimadzu LC – 10AD). Cinnamic acid (product of PAL) was eluted at a flow rate of 0.5 mL/min with a linear gradient of 20-80 % MeOH containing 0.1 % HOAc and was detected at 273 nm.

The enzyme activity was expressed as amounts of CA produced within 1 h/mg of protein in the reaction mixture. Protein concentration was determined by the method of Bradford (1976) using BSA as standard. For preparation of walls and extraction of wall-bound phenolics, samples were homogenized in the buffer and after centrifugation at 1000 × g, the pellets were sequentially washed with 10 volumes (v/w) of 0.5 Mm $CaCl_2$**,** EtOH, $CHCl_3$-MeOH (2:1, V/V) and acetone followed by air-drying. Air-dried materials were designated as wall preparation. Phenolics were liberated from the walls with ammonium oxalate (20 mM, 70 °C) and then with NaOH 0.1 M. After 24 h, phenolics were extracted two times with EtOAc, air-dried, re-dissolved in 5o % MeOH and analyzed by HPLC at 280 nm as described for PAL. Content of caffeic acid, cinnamic acid, ferulic acid, gallic acid, *p*-coumaric acid, and tannic acid were determined using commercially available authentic standards (Ghanati et al., 2005).

Extraction and assay of polyphenol oxidase (PPO)

Polyphenol oxidase was extracted from homogenized samples in freshly prepared 0.25 Mm Na-phosphate buffer (pH 6.8) at 4 °C followed by centrifugation at 15,000 × g for 20 min. Aliquots of supernatant were added to Na-phosphate buffer and freshly prepared 4-methylcatechol at a final concentration of 0.02 M. Enzyme activity was expressed as the increase in absorbance at 410 nm per min per mg protein content of the homogenate (Ghanati et al., 2002).

Determination of lignin

Isolation of cell walls was carried out as described for the determination of wall-bound phenolics. Lignin content was measured via a modified acetyl bromide procedure. In brief, 6 mg of fine powdered, air-dried cell wall preparation was treated with a mixture (total of 2.5 mL) of 2.5 % (w/w) AcBr in HOAc and 0.1 mL of 70 % $HClO_4$ at 70 °C for 30 min, and was shaken at 10 min intervals. After cooling with ice, the digestion mixture was transferred to a 25 mL volumetric flask containing 5 ml NaOH of 2 M and 6 mL HOAc and filled to 25 mL. The lignin content was determined by measuring absorbance at 280 nm using specific absorption coefficient value of 20 $gL^{-1}cm^{-1}$ (Iiyama and Wallis, 1990).

Extraction and assay of total anthocyanin and flavonoids

Frozen shoot samples were homogenized in acidic MeOH (MeOH: HCl, 99:1) with a mortar and pestle followed by centrifugation at 12,000 × g for 15 min. Aliquots of supernatant were kept in dark place overnight and then their anthocyanin contents were determined by measuring absorbance at 550 nm using extinction coefficient value 33000 $cm^{-2}mol^{-1}$. For extraction of flavonoids the frozen shoots were homogenized in acidic EtOH (EtOH: HOAc, 99:1)) with a mortar and pestle, followed by centrifugation at 12,000 × g for 15 min. Aliquots of supernatant were incubated for 10 min at 80 °C. After cooling the samples, flavonoid content was determined by measuring absorbance at 270, 300, and 330 nm using extinction coefficient value 33000 $cm^{-2}mol^{-1}$ (Krizek et al., 1993).

Extraction and assay of lipid peroxidation

The level of peroxidation of membrane lipids was assayed by measuring malondialdehyde (MDA) as final product of lipid peroxidation. Frozen samples were homogenized in 10 % TCA with a mortar and pestle. Aliquots of supernatant (1 mL) were added to 1 mL of thiobarbituric acid (TBA) 0.25 % at 100 °C for 30 min. After cooling them, lipid peroxidation was determined by measuring absorbance at 532 and 600 nm. MDA-TBA complex was calculated from the difference between two absorbance using extinction coefficient 155 mM^{-1} cm^{-1} (Cakmak and Horst, 1991).

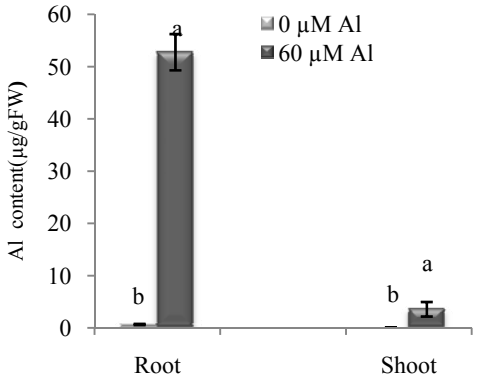

Fig. I. Al content of red cabbage treated with or without Al. Data are presented as the means± SD with n=3. Bars with different letters are significantly different at p≤0.05, according to the Student's t-test.

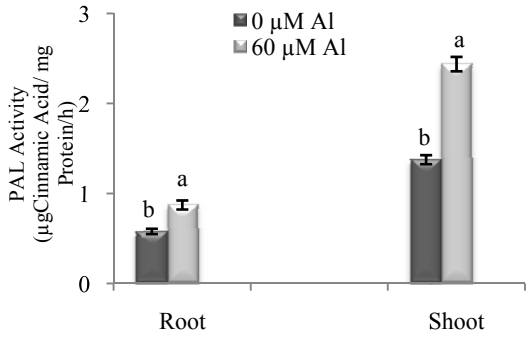

Fig. III. Activity of PAL in red cabbage treated with or without Al. Data are presented as the means ± SD with n=3. Bars with different letters are significantly different at p≤0.05, according to the Student's t-test.

Statistical analysis

All of the experiments were carried out with at least three independent repetition using three samples, and all of the data were expressed as the mean values ± the standard deviation (SD). Statistical analysis was performed using the Student's t-test, and the differences between the treatments were expressed as significant at p≤0.05.

Results

Aluminum was rapidly absorbed by red cabbage plants (Fig. I). Much part of absorbed Al however, was kept by the roots and about 10 % of the absorbed Al was transferred to the shoots (Fig. I). Existence of phenolics in the cell walls of roots and stems was evidenced by their authentic fluorescence in cross sections (Fig. II). Change of the fluorescence color of Al-treated roots and stems after changing pH showed that a part of phenolics in their cell walls were bound to pectin (Fig. II a, b, e, f). In comparison, no remarkable change was observed in cross sections of control plants (Fig. II c, d, g, h). Treatment with Al significantly increased total content of pectin-bound phenolic acids in the plants (Table 1). Increase of wall-bound phenols in shoots of Al-treated plants was more remarkable than those of roots (Table 1). Aluminum significantly increased the activity of PAL in both roots and shoots of treated groups (Fig. III). Similarly, Al

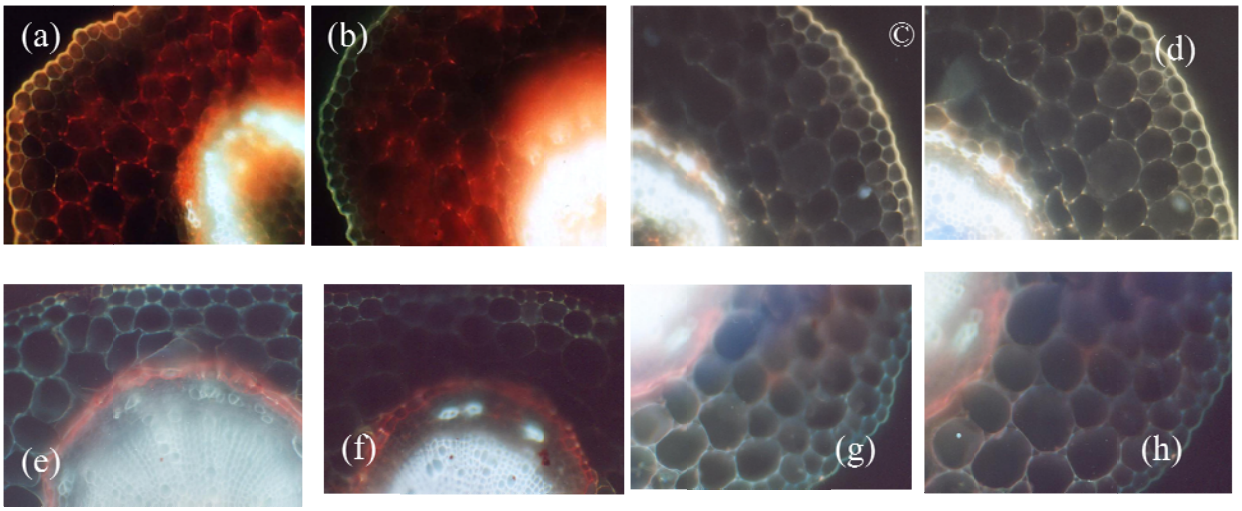

Fig. II. Visualizing of phenolics and their status in the cell walls of red cabbage under fluorescent microscope. a:cross section of shoot of Al-treated plants; b: the same section after changing pH; c and d: cross sections of shoots of the control plants before and after changing pH, respectively; e: cross section of root of Al-treated plants; f: the same section after changing pH; g and h: cross sections of roots of the control plants before and after changing pH, respectively. Magnification of all figures is ×10.

Table 1
Phenolics content of red cabbage treated with or without aluminium

		Phenolic acids (µg. g wall DW^{-1})						
		Gallic acid	Tannic Acid	Ferulic acid	Caffeic acid	*p*-coumaric acid	Cinnamic acid	Total
Root	Ctrl	7.274± 0.06	N	N	N	N	0.396± 0.02	7.670[a]
	Treatment	9.950± 0.06	N	0.133± 0.09	N	N	0.025± 0.01	10.108[b]
Shoot	Ctrl	0.182± 0.08	0.040± 0.03	0.007± 0	N	N	0.072± 0.001	0.301[a]
	Treatment	4.829± 0.09	0.203± 0.01	0.106± 0	0.66± 0.06	0.095± 0.006	0.073± 0.006	5.372[b]

N: not detected; Different letters are significantly different at p≤0.05, according to the Students t-test.

treatment significantly increased the activity of PPO in both shoots and roots of red cabbage (Fig. IV).

Deposition of lignin in roots and shoots of the plants was enhanced by Al (Fig. V). Aluminum treatment increased anthocyanin content of the treated plants, but did not cause significant changes in their total flavonoid content. (Fig. VI).

Treatment of red cabbage with Al for 6 h did not change lipid peroxidation rate of membrane lipids neither in roots nor in shoots of the treated plants, compared with the control (Fig. VII).

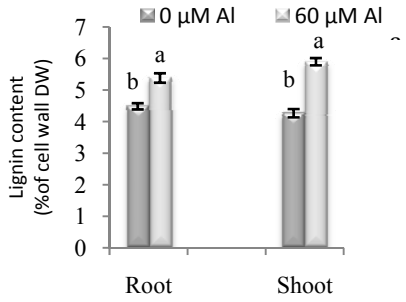

Fig. V. Lignin content of cell walls of red cabbage treated with or without Al. Data are presented as the means± SD with n=3. Bars with different letters are significantly different at p≤0.05, according to the Student's t-test.

Discussion

The molecular mechanisms underlying aluminum toxicity are not yet fully understood, owing to the complexity of aluminum chemistry in aqueous solutions at physiological pH. Although Al itself is not a transient element but it has been suggested that it can result in production of active oxygen species through stimulation of ferrous ions (Cakmak and Horst, 1991). In addition, the binding of Al to the plasma membrane may also result in a disruption of membrane function and enhanced oxidative stress, which stimulates the activation of defense-related signaling cascades (Yamamoto et al., 2003). Active oxygen species have been proposed as a central component of plant adaptation to both biotic and abiotic stresses. They may play two very different roles: exacerbating damage or signaling the activation of defense responses (Dat et al., 2000). Phenolic compounds have been reported to have multiple biological effects, including antioxidant activity and retarding oxidative degradation of lipids (Kahkonen et al., 1999). Coincident with other

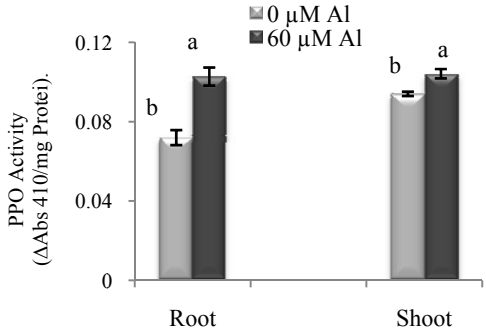

Fig. IV. Polyphenol oxidase (PPO) activity in red cabbage treated with or without Al. Data are presented as the means± SD with n=3. Bars with different letters are significantly different at p≤0.05, according to the Student's t-test.

reports (Sasaki et al., 1996; Fan et al., 2006; Dahajipour Heidarabadia et al., 2011), in our research, treatment of red cabbage with Al resulted in increased accumulation of wall-bound phenolic acids and enhancement of deposition of lignin in the cell walls, compared with the control plants. Relative increase of lignin was more pronounced in walls of shoots than that of the roots (38.5% vs. 20%) of Al treated plants, compared with the control ones. Similarly, wall bound phenols of shoots increased up to 17 folds and of roots increased to 1.3 folds of non-treated plants. Al activation of explicit signaling pathways suggests that Al does not cause a complete shutdown of cell metabolism but moreover acts on specific target sites (Jones et al., 2006). According to the results of the present research only a few parts of absorbed Al was transferred to shoots (~10%), nonetheless it was sufficient to activate phenolics metabolism there. Activation of this metabolic pathway was accompanied by increased activity of PAL as the key enzymes of phenolics biosynthesis pathway. Increased activity of PAL induced by Al has been reported previously (Zakir Hossain et al., 2005; Ye et al., 2011). Aluminum treatment also resulted in increased activity of PPO. This enzyme has various functions: the oxidation of phenolics to quinone and the combination of phenolics to produce falvonoids and also contribute to oxidation of falvonoids (Pourcel et al., 2007). Many of the biological roles of flavonoids are attributed to their antioxidant abilities. They act as scavengers of free radicals and also prevent their formation by chelating metals (Pourcel et al., 2007). Treatment of red cabbage plants with Al did not significantly change total content of flavonoids although to some extent it changed the flavonoids with different absorbance. Anthocyanins are colored phenolic compounds belong to flavonoids and known as useful antioxidants as well. Al significantly increased anthocyanin content of the treated plants, compared to the controls. Altogether, increase of phenolic compounds in terms of wall bound phenolic acids or anthocyanin was sufficient to protect membrane lipids from peroxidation, so that rate of peroxidation of Al-treated plants was identical to those of the control ones.

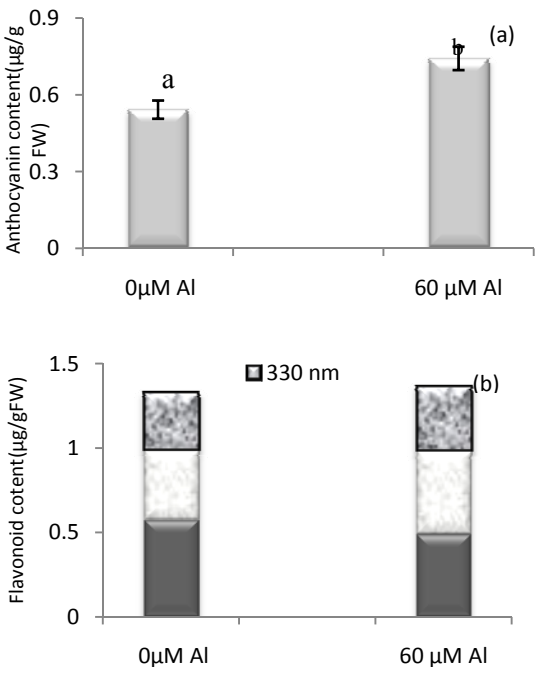

Fig. VI. Anthocyanin (a) and flavonoid (b) contents of the shoots of red cabbage treated with or without Al. Data are presented as the means ± SD with n=3. Bars with different letters are significantly different at p≤0.05, according to the Student's t-test.

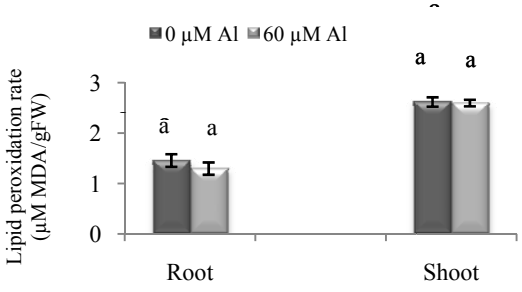

Fig. VII. Lipid peroxidation rate of membrane lipids of red cabbage treated with or without Al. Data are presented as the means ± SD with n=3. Bars with different letters are significantly different at p≤0.05, according to the Student's t-test.

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