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Di-*n*-butyl phthalate-induced phytotoxicity in *Hordeum vulgare* seedlings and subsequent antioxidant defense response

A. KUMARI and R. KAUR*

*Department of Botanical and Environmental Sciences, Guru Nanak Dev University
Amritsar-143005, Punjab, India*

Abstract

Di-*n*-butyl phthalate (DBP) is one of the frequently detected phthalates in environmental samples. The effects of phthalates are extensively studied in the animals but the effects on plants are scarce. Therefore, the present study is aimed to envisage the effects of DBP on the antioxidative defense system in *Hordeum vulgare* L. seedlings grown under laboratory conditions for 7 d. The activities of different antioxidative enzymes were enhanced in the shoots. In the roots, the activity of guaiacol peroxidase increased and the catalase activity decreased initially but increased at higher DBP concentrations, whereas the activities of superoxide dismutase, ascorbate peroxidase, and glutathione reductase declined. Furthermore, the content of polyphenols elevated after exposure of seedlings to DBP. The possible reason for these responses of barley seedlings is the oxidative burst, *i.e.*, enhanced production of reactive oxygen species, which were confirmed using confocal microscopy in terms of loss in plasma membrane integrity. DBP also disturbed the normal stomatal morphology of barley seedlings. The study may help to provide insights into the defense of crop plants against phthalate stress.

Additional key words: barley, confocal microscopy, plasma membrane integrity, SEM, stomata

Introduction

Phthalates have prodigious applications which are responsible for their ubiquity in different environmental samples. Phthalates are formed by the reaction of anhydrous phthalic acid with aliphatic alcohol. They are used as plasticizers for the production of cellulose, polyvinyl chloride, and synthetic rubber to impart them flexibility, toughness, and reduce hardness (Gao *et al.* 2019). Phthalates are also used in blood storage bags, intravenous catheters, toys, coatings of drugs, and personal care products, *etc.* (Benjamin *et al.* 2017). In the polymeric matrix, phthalates are incorporated *via* physical bonding rather than covalent bonding which enables their easy release into the environment (Gao *et al.* 2017). Several studies have reported the presence of considerable amounts of phthalates in agricultural soils. The main sources of phthalates are use of agricultural

films, irrigation with waste water, effluents of industries, use of sewage sludge or biosolids for fertilization, use of pesticides, *etc.* (Cai *et al.* 2007, Weschler *et al.* 2008, Guo and Kannan 2012, Ma *et al.* 2013, Wang *et al.* 2013a,b). The increased greenhouse cultivation of edible plants has become a significant source of phthalate contamination (He *et al.* 2015). The concentrations of phthalates in plastic greenhouse soils or in agricultural film covered soils were reported to be several times greater than in other soils (Kong *et al.* 2012, Wang *et al.* 2012). Phthalate accumulation in edible plants affects their quality. For instance, the exposure to di (2-ethyl-hexyl) phthalate (DEHP) decreases the vitamin C content in the fruits of *Capsicum* sp. (Yin *et al.* 2002).

Some of the phthalates have been declared as 'Priority Environmental Pollutants' (Liao *et al.* 2010) and di-*n*-butyl phthalate (DBP) is one of them (Benjamin *et al.* 2017). In our previous studies, we observed that exposure of

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Abbreviations: ABA - abscisic acid; APX - ascorbate peroxidase; CAT - catalase; DBP - di-*n*-butyl phthalate; DEHP - di (2-ethyl-hexyl) phthalate; DEP - diethyl phthalate; EDTA - ethylenediaminetetraacetic acid; ETCs - electron transport complexes; GR - glutathione reductase; GSH - reduced glutathione; GSSG - oxidized glutathione; MDA - malondialdehyde; NBT - nitroblue tetrazolium; PBS - phosphate buffer saline; PI - propidium iodide; POD - guaiacol peroxidase; PS - photosystem; ROS - reactive oxygen species; SEM - scanning electron microscopy; SOD - superoxide dismutase; UHPLC - ultra-high performance liquid chromatography.

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* Corresponding author; e-mail: rajinder.botenv@gndu.ac.in

barley to DBP significantly affected seed germination, growth, and some biochemical indices (Kumari and Kaur 2017, Kumari *et al.* 2019). DBP markedly enhanced the indicators of oxidative stress like malondialdehyde (MDA) and hydrogen peroxide (H_2O_2). Furthermore, stimulation in the content of compatible solutes (proline, soluble sugars, and proteins) was observed in the shoots and roots of barley seedlings under DBP stress. Thus, the current work is an extension of our previous work to assess the potential of the antioxidative defense system against the oxidative stress induced by DBP.

Materials and methods

Plants and experimental setup: The seeds of *Hordeum vulgare* L. cv. VLB-118 were procured from Hill Agricultural Research and Extension Centre, Bajaura, Kullu, India. DBP (CAS: 84-74-2, purity: 99 %) was purchased from Himedia Laboratories (Mumbai, India) and all other chemicals used in the present study were of the analytical grade. DBP has a low water solubility, therefore, for the preparation of the stock solution, the method of Yin *et al.* (2003) was used. Barley seedlings were germinated as previously described by Kumari and Kaur (2017). The barley seeds were surface sterilized using 0.01 % (m/v) mercuric chloride, presoaked in double distilled water and kept in Petri plates lined with Whatman No. 1 filter paper. The seeds were moistened periodically with different concentrations of DBP, viz. 0, 25, 50, 100, 200, 400, 800, and 1600 mg dm^{-3} . The seeds were germinated under a 16-h photoperiod, a temperature of $25 \pm 0.5^\circ\text{C}$, a relative humidity of 75 - 80 %, and an irradiance of $115 \mu\text{mol m}^{-2} \text{s}^{-1}$. The seedlings were harvested on the 7th day and stored at -80°C till analyses.

Extraction and estimation of antioxidative enzymes: The shoots and roots of seedlings were separately pulverized in liquid nitrogen and homogenized in chilled 0.1 M potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 12 000 g and 4°C for 20 min. The supernatant was collected and used for the analysis of activities of superoxide dismutase (SOD, EC 1.15.1.1), guaiacol peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.1.6), ascorbate peroxidase (APX, EC 1.11.1.11), and glutathione reductase (GR, EC 1.8.1.7). For the calculation of activities of antioxidative enzymes per protein unit, the content of the protein was determined using the standard method of the Bradford (1976).

The SOD activity was analyzed by the method of Kono *et al.* (1979). The reaction was initiated by the addition of sodium carbonate buffer (50 mM, pH 10.2), nitroblue tetrazolium (NBT, 96 μM), Triton X-100 (0.6 %, v/v), hydroxylamine hydrochloride (20 mM, pH 6.0) to enzyme extract. SOD activity was recorded as a decrease in absorbance at 540 nm of a complex formed by superoxide and NBT. One unit of SOD activity corresponded to the enzyme concentration required to inhibit the absorbance of formed chromogen at 540 nm by 50 % in one min.

The POD activity was determined using the method

of Putter (1974). For the estimation of POD activity, the reaction mixture was prepared using potassium phosphate buffer (0.1 M, pH 7.0), guaiacol (20 mM), H_2O_2 (12.3 mM), and enzyme extract. The rate of formation of tetra-guaiacol was measured at 436 nm and enzymatic activity was calculated using coefficient of absorbance of $25.5 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit of POD activity corresponded to the amount of the enzyme catalyzing the formation of 1 mol of guaiacol dehydrogenation products per minute.

For determination of the CAT activity, the method given by Aebi (1984) was used. The reaction mixture was consisted of potassium phosphate buffer (0.1 M, pH 7.0), H_2O_2 (150 mM), and enzyme extract. The decrease in absorbance was recorded at 240 nm. The enzyme activity was determined using a coefficient of absorbance of $39.4 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit of CAT activity corresponded to the amount of the enzyme required for liberating 50 % of oxygen from H_2O_2 .

The APX activity was evaluated using the method of Nakano and Asada (1981). APX catalyzes H_2O_2 reduction by causing the oxidation of ascorbate. The reaction solution consisted of potassium phosphate buffer (0.1 M, pH 7.0), ascorbate (5 mM), H_2O_2 (0.5 mM), and enzyme extract. The decrease in absorbance was measured at 290 nm and the enzyme activity was calculated using a coefficient of absorbance of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit corresponded to the amount of the enzyme required to oxidize 1 mol of ascorbate per minute.

The activity of GR was analyzed using the method of Carlberg and Mannervik (1985). The reaction mixture contained potassium phosphate buffer (50 mM, pH 7.6), ethylenediaminetetraacetic acid (EDTA, 3 mM), reduced nicotinamide adenine dinucleotide phosphate (NADPH, 0.1 mM), oxidized glutathione (GSSG, 1 mM), and enzyme extract. The absorbance was recorded at 340 nm. The enzymatic activity was calculated using coefficient of absorbance of $6.22 \text{ mM}^{-1} \text{ cm}^{-1}$. One unit corresponded to the amount of the enzyme required to oxidize 1 mol of NADPH per min.

Polyphenol profiling: The extracts of barley seedlings were prepared in methanol, filtered through 0.22 μm filter membranes and then analyzed for different polyphenols using 130 MPa Shimadzu (Kyoto, Japan) ultra-high performance liquid chromatography (UHPLC) system (NEXERA). UHPLC was coupled with C_{18} column ($150 \times 4.6 \text{ mm}$ and pore size was 5 μm). During analysis, the binary mobile phase consisted of solvent A: 0.01 % (m/v) acetic acid in water and solvent B: 100 % methanol. The analysis of results was performed using Lab Solutions software.

Confocal microscopy: For the determination of cell viability of DBP treated barley roots, method of Sasaki *et al.* (1997) was used with some modifications. The treated roots were rinsed with phosphate buffer saline (PBS) followed by staining with propidium iodide (PI) for 30 min in the dark and room temperature. After staining, the roots were washed in PBS for 3 - 5 times to remove the unbound stain particles from the root surface. The

roots were scanned for confocal fluorescent imaging on *Nikon AIR* laser scanning confocal microscope (*Nikon*, Tokyo, Japan) coupled with built-in *Nikon NIS Element AR* software for the acquisition of confocal images. He-Ne gas laser was used to excite electrons at a wavelength of 535 nm for PI.

Scanning electron microscopy (SEM): For the study of DBP induced stomatal abnormalities, the samples were prepared using the method of Liu *et al.* (2000). For sample preparation, 4–5 mm segments of seedlings were cut followed by fixation in glutaraldehyde (2.5 %, m/v) for 24 h. Then, the samples were dehydrated in graded ethanol series, mounted on the stubs with double sided tape, air-dried, and coated with silver. Abaxial surface of seedling was observed under SEM (*Evo LS 10*, Carl Zeiss, Oberkochen, Germany).

Statistical analysis: The results were analyzed for mean, standard error, and then two-way analysis of variance (*ANOVA*) was used. The differences ($P \leq 0.05$) among means were compared by the honestly significant difference (HSD) using Tukey's multiple comparison test.

Results

The results analyzed using *ANOVA* revealed that DBP exhibited statistically significant effects on the activities of antioxidative enzymes (Fig. 1, Table 1 Suppl.). The increasing concentrations of DBP exerted obvious effects on SOD activity of shoots and roots. In comparison to the control, the activities of SOD significantly increased by 41, 51, 55, 35, 39, 33, and 109 % at 25, 50, 100, 200, 400, 800, and 1600 mg dm⁻³, respectively. In the roots, the SOD activity decreased and the decrease ranged from 48 to 68 % with respect to the control.

A significant increase in the activity of POD was observed in shoots and roots of seedlings as compared to the control. In the shoots, a continuous increase in POD activity was observed, however, at higher concentrations (400–800 mg dm⁻³), the increase in POD activity was not dose-dependent, but remained higher than in control, followed by a decrease at 1600 mg dm⁻³. In the roots, POD activity increased in a dose-dependent manner except at 100–200 mg dm⁻³ DBP. The increase was 57, 81, 24, 22, 132, 182 and 212 % at 25, 50, 100, 200, 400, 800, and 1600 mg dm⁻³, respectively, as compared to the control.

In the shoots, there was a significant concentration-dependent increase in the activity of CAT (except at 50 and 200 mg dm⁻³), and a maximum increase in the activity was observed at 1600 mg dm⁻³. On the contrary, DBP exhibited a significant alteration in the activity of CAT either as an increase or decrease with respect to the control in the roots. The activities decreased at 25, 50, 100, 400 mg dm⁻³, thereafter elevated by 29, 32 and 175 % at 200, 800 and 1600 mg dm⁻³.

The activity of APX increased significantly in the shoots under DBP stress at all the concentrations except at 1600 mg dm⁻³ DBP. The percent increase was dose-

dependent at lower concentrations (100–200 mg dm⁻³). Thereafter, the activity started to decrease but the activity was steadily higher than in control. In the roots, initially the activity of APX increased and then a declining trend was observed. The decline ranged from 53 to 81 % as compared to the control.

In the shoots, the activity of GR significantly increased over the control. The increase in GR activity was concentration-dependent except at 50–100 mg dm⁻³. However, the activity of GR in the roots decreased significantly and the decrease was dose-dependent up to 200 mg dm⁻³ and then it increased but remained less than in the control.

The screening of polyphenols in methanolic extracts of barley seedlings is shown in Table 1. In the present study, the barley seedlings were analyzed for 11 polyphenols *viz.* gallic acid (C₇H₆O₅), caffeic acid (C₉H₈O₄), umbelliferone (C₉H₆O₃), coumaric acid (C₉H₈O₃), ellagic acid (C₁₄H₆O₈), quercetin (C₁₅H₁₀O₇), kaempferol (C₁₅H₁₀O₆), epicatechin (C₁₅H₁₄O₆), catechin (C₁₅H₁₄O₆), chlorogenic acid (C₁₆H₁₈O₉), and rutin (C₂₇H₃₀O₁₆). DBP remarkably altered the content of polyphenols in treated samples with respect to the control. The total polyphenol content decreased initially at 25 mg dm⁻³ followed by an increase up to 50–800 mg dm⁻³ DBP. As concern contribution of individual polyphenols in total polyphenol content, catechin was dominated followed by caffeic acid, chlorogenic acid, epicatechin, kaempferol, umbelliferone, quercetin, ellagic acid, and coumaric acid.

The treated roots were examined for the loss of plasma membrane integrity using propidium iodide as a fluorescent probe. This fluorescent dye is impermeable to the intact cell and is used to differentiate between viable and non-viable cells. The low penetration of propidium iodide in the control showed that the cells were intact and viable, whereas the cells were damaged and became non-viable after exposure to higher concentrations of DBP (Fig. 2).

The dumbbell-shaped stomata of barley are shown on Fig. 3. In comparison to the control, the stomata of DBP treated barley seedlings were deformed. The treatment of DBP also initiated stomatal closure. Moreover, the length of stomata was also reduced, while the width was increased as compared to the control.

Discussion

The ROS is a collective term for radical oxygen species (*i.e.* superoxide, hydroxyl, alkyl, and peroxy radical) and non-radical oxygen species (*i.e.*, hydrogen peroxide, singlet oxygen, ozone, and hypochlorous acid) (Ahmad 2018). They are highly reactive and can mediate the oxidation of cell structure, biomolecules, as well as disturb cell integrity (Kanojia and Dijkwel 2018). Different metabolic processes often produce ROS under normal conditions, but the generation of ROS is observed to be significantly accelerated during abiotic or biotic stress. The cell organelles (chloroplast, mitochondria, and peroxisome) with high metabolic activity are the main sites of ROS generation. In chloroplast, there

are three ways of ROS generation. The first one is the incomplete oxidation of water on the electron donor side of photosystem (PS) II and forms H_2O_2 . The second site is within the electron transport chain, where the reduction of plastoquinone takes place which forms plastoquinone. Plastoquinone can interact with

O_2 to form superoxide radical ($O_2^{\cdot-}$); (Khorobrykh *et al.* 2015). The third site is photosynthesis which involves the well-known electron transport complexes (ETCs) viz. PS I, PS II, and b6f complex (Kanojia and Dijkwel 2018). The direction of electron flow is from excited photosystem centers to NADP that forms NADPH. NADPH enters

Fig. 1. Effect of di-*n*-butyl phthalate on superoxide dismutase (SOD, A), guaiacol peroxidase (POD, B), catalase (CAT, C), ascorbate peroxidase (APX, D), and glutathione reductase (GR, E) activities of shoots and roots of barley seedlings. Results are presented as means $\pm$ SEs, $n = 3$. Different letters indicate significantly different expression at a probability level of 5 % as indicated by two-way ANOVA and honestly significant difference.

Table 1. Effect of different di-*n*-butyl phthalate concentrations [mg dm^{-3}] on content of polyphenols [$\text{mg g}^{-1}(\text{f.m.})$] in barley seedlings (ND - not detected).

DBP	Caffeic acid	Chlorogenic acid	Quercetin	Kaempferol	Epicatechin	Coumaric acid	Ellagic acid	Catechin	Umbelliferone	Total
0	0.108	0.083	0.011	0.024	0.071	ND	ND	1.030	0.015	1.342
25	0.017	0.094	0.006	0.001	0.026	0.001	0.005	0.655	0.035	0.840
50	0.031	0.048	0.026	0.028	0.001	ND	ND	1.215	0.043	1.392
100	0.043	0.055	0.016	0.005	0.025	ND	ND	2.774	0.046	2.964
200	0.039	0.050	0.006	0.019	0.023	ND	ND	2.405	0.018	2.560
400	0.042	0.011	0.009	0.010	0.042	ND	ND	2.000	0.034	2.148
800	0.085	0.001	0.017	0.041	0.041	0.001	ND	2.299	0.020	2.505
1600	0.126	0.049	0.011	0.055	0.079	ND	0.006	0.150	0.038	0.514

into the Calvin cycle and reduces CO_2 which is the final electron acceptor. Whereas, during stress, the normal electron flow gets disturbed which limits CO_2 assimilation and forms $^1\text{O}_2$ and O_2^- .

Similarly, ROS generation takes place in mitochondria during ATP synthesis, and the main ROS generation ETCs are complex I and complex III (Sharma *et al.* 2012). Moreover, the mitochondrial enzymes, 1-galactono- γ -lactone dehydrogenase and aconitase are the key enzymes for the indirect and direct formation of O_2^- respectively (Kanojia and Dijkwel 2018). Beside chloroplasts and mitochondria, peroxisomes also produce ROS during stress and act as a major site for H_2O_2 generation (Smirnoff and Arnaud 2019). During stress, the mechanism behind switching high H_2O_2 generation is the low CO_2 availability inside the plant leaves due to which ribulose-

1,5-bisphosphate carboxylase/oxygenase catalyzes a competitive reaction. In the reaction, O_2 is favored over CO_2 which forms 2-phosphoglycolate (2-PG) instead of 3-phosphoglycerate. To sustain the carbon assimilation, 2-PG is transported to peroxisome, where it is oxidized to form glyoxylate and H_2O_2 by glycolate oxidase (Flügel *et al.* 2017). Thus, to combat with the enhanced accumulation of ROS, plants have a defense grid that relies on endogenous enzymatic and non-enzymatic antioxidants. The enzymatic antioxidative defense system is an intricate, efficient, and includes enzymes like SOD, POD, CAT, APX, GR, *etc.* The non-enzymatic antioxidants are glutathione, ascorbate, carotenoids, tocopherols, polyphenolic compounds, *etc.*

Among the antioxidative enzymes, SOD acts as in a first-line of defense *via* catalyzing the dismutation of superoxide radical to H_2O_2 and O_2 (Berwal and Ram

Fig. 2. Effect of di-*n*-butyl phthalate on cell viability in a control (A), 25 (B), 100 (C), 400 (D), and 1600 mg dm^{-3} (E) using confocal laser scanning microscopy. Fig. 3. Effect of di-*n*-butyl phthalate on stomatal morphology at a control (A), 25 (B), 100 (C), 400 (D), and 1600 mg dm^{-3} (E) using scanning electron microscopy.

2018). In the present study, SOD was observed to increase in shoots, while it decreased in the roots under DBP stress. Cheng and Cheng (2012) reported similar results for the activity of SOD in *Spirodela polyrhiza* under the exposure of diethyl phthalate (DEP) for 7 d and the decline ranges from 22 to 28 %. They reported that the decrease in activity was due to the down-regulation of all isoenzymes of SOD. Ma *et al.* (2013) reported a decrease in the activity of SOD in *Brassica chinensis* under phthalate stress. The activity of SOD was reported to increase in the shoots and roots of mung bean seedlings exposed to DBP (5, 20 and 100 mg kg⁻¹ for 72 h), while a decline was observed at 500 mg kg⁻¹ as compared to the control (Ma *et al.* 2014). In the same study, the effect of DEHP was also observed but the prominent effect was reported in DBP treated seedlings which can be attributed towards greater toxicity of DBP than other phthalates. The activity of SOD under the exposure of DBP in cucumber seedlings increases with increase in the DBP concentration and exposure duration, however, it declines at high concentrations of DBP (100–200 mg dm⁻³) and on the 7th day, the observed decline is 47 % (Zhang *et al.* 2015). In this work, the effects of DBP on SOD activity of shoots and roots could be due to the enhanced level of oxygen radical. The roots are in direct contact with contaminant that is why the effect on SOD activity was more apparent in roots than in shoots.

After the dismutation reaction of O₂^{•-} catalyzed by SOD, the accumulation of H₂O₂ is commonly observed. During the steady state, the content of H₂O₂ in chloroplast is usually up to 0.5 µM and it is elevated to 1 - 1.5 µM under stresses (Corpas *et al.* 2015). H₂O₂ is a moderately active ROS with 2 to 4 µs half-life. However, it is not that much damaging but can form more reactive and damaging ROS like OH[•] via Haber-Weiss reaction (Czarnocka and Karpinski 2018). Therefore, there is a subsequent defense mechanism which involves the depletion of H₂O₂ before it enters to other reactions, and the key antioxidative enzymes are POD, CAT, and APX.

There was an increase in POD activity of shoots and roots under DBP stress. In DBP treated cucumber seedlings, the activity of POD significantly increased after 5 d, while it is decreased in seedlings treated for 3 d (Zhang *et al.* 2015). The activity of POD increases significantly in cucumber seedlings under the exposure to 30 and 50 mg dm⁻³ dimethyl phthalate for 7 d (Zhang *et al.* 2016). PODs are the only antioxidative enzymes which scavenge H₂O₂ in the apoplast, and have been classified as class III peroxidases by Welinder (1992). PODs (glycoproteins) are synthesized in the endoplasmic reticulum and transported via Golgi apparatus to extracellular spaces as well as to vacuoles (Jovanović *et al.* 2018). PODs catalyze the oxidation of phenolics, indoles, hydroquinones, and amines (Kaur 2013). The oxidation reaction catalyzed by PODs leads to the generation of phenoxyl radicals. These radicals are further polymerized or reduced by ascorbate (Jovanović *et al.* 2018).

Activity of CAT followed an increasing trend in the shoots and roots of barley seedlings under DBP exposure. In the various studies, the increase in CAT activity is reported to be correlated positively with H₂O₂ generation

during stress (Choudhary *et al.* 2012, Li *et al.* 2013). Moreover, phthalates are also reported to enhance the production of H₂O₂ remarkably in plants (Bai *et al.* 2009, Zhang *et al.* 2014). CAT is an enzyme which scavenges H₂O₂ produced in peroxisomes under stressed conditions. In the present study, the enhanced activity of CAT is attributed to its indispensable role in the detoxification of H₂O₂. Furthermore, in our previous study, the content of H₂O₂ was also observed to enhance in the shoots and roots of barley seedlings under DBP stress (Kumari *et al.* 2019). The accumulation of H₂O₂ might be because of photorespiratory oxidation or β-oxidation induced by the exposure to DBP (Abbas *et al.* 2018).

In the shoots, APX activity increased initially and then decreased. However, in roots, it was recorded to increase at lower concentrations followed by a decrease at higher concentrations. Ma *et al.* (2014) observed similar results under the exposure to DEHP in the shoots and roots of mung bean seedlings. In *Spirodela polyrhiza* exposed to DEP, the activity of APX is reduced significantly at lower concentrations followed by an increase (Cheng and Cheng 2012). In wheat plants under DEHP exposure, the activity of APX increases as compared to the control (Gao *et al.* 2018). APX catalyzes the reduction of H₂O₂ using ascorbate as an electron donor; it plays an important role in the mitigation of intracellular ROS content (Abbas *et al.* 2018).

In the present study, GR activity was increased in the shoots, while declined in the roots. The activity of GR was also observed to increase under the exposure of DEP in *Spirodela polyrhiza* (Cheng and Cheng 2012). The activity of GR alters significantly under the exposure of other pollutants, *e.g.*, Jiménez-Arias *et al.* (2019) observed an increase in the GR activity in tomato plants under menadione sodium bisulphite stress for 1 d, and the maximum increase after 4 d. The decline of GR activity was also observed in barley seedlings under salinity (Ma *et al.* 2019).

Polyphenols represent a class of secondary metabolites which are categorized as phenolic acids, flavonoids, stilbenes, and lignans. Polyphenols are non-enzymatic antioxidants and play various important roles in UV protection, pollen tube growth, antimicrobial activity, biotic and abiotic stress management, neutralization of free radicals, quenching of singlet oxygen, *etc.* (Dixon *et al.* 1995, Naczk 2004, Yadav *et al.* 2016). During stress, when ROS and free radicals are unable to be subdued by enzymatic antioxidants then, these are usually scavenged by polyphenols. The scavenging mechanisms based on the capacity of the phenol functional group to donate H-atom or transfer single-electron from phenolic antioxidant to the free radical (Wright *et al.* 2001). The content of the phenolic compounds was reported to increase under Cu stress due to increased synthesis of shikimate dehydrogenase enzyme (Díaz *et al.* 2001). The polyphenols biosynthesis was also reported to increase in the crop plants under heavy metal stress (Michalak 2006). Thus, in the present study, an alteration in the content of polyphenols might be a part of coping strategy to deal with oxidative stress induced by DBP in barley seedlings.

The confocal images of treated roots showed the considerable penetration of propidium iodide which may be attributed to the high ROS accumulation that might have led to the oxidative damages and finally cell death. The high accumulation of ROS can trigger programmed cell death (Foyer *et al.* 2005, Shi *et al.* 2015). The staining pattern of treated and untreated roots of barley seedlings clearly showed that exposure to DBP damaged the cell membrane *via* increasing the lipid peroxidation. Hence, the damaged cell membrane might have facilitated PI penetration into the cell which intercalated with DNA or RNA and formed a bright red fluorescent complex (Fig. 2).

The stomata are specialized tiny structures for controlling water and gas exchange. In this study, SEM micrographs of treated samples showed a reduction in size and initiation of stomatal closure. The morphological abnormalities in stomata were clearly visible in the treated samples, while the stomata in the control were compact and properly developed. Absciscic acid (ABA) accumulates during stress which is mainly responsible for the initiation of stomatal closure (Waraich *et al.* 2012). The high content of H₂O₂ was also reported as a possible reason for the initiation of stomatal closure (Yang *et al.* 2006). In this study, the accumulation of both ABA and H₂O₂ might have initiated the stomatal morphological abnormalities and closure in DBP treated samples.

In conclusion, it was observed that the exposure of barley seedlings to DBP caused significant modulations in the activities of antioxidative enzymes and the total polyphenolic content. The reason behind these responses might be the aggravated generation of ROS, which also caused a loss of the plasma membrane as well as disturbed normal stomatal morphology. Thus, the results of the present study will increase understanding crop plant responses to phthalate stress and the roles of enzymatic and non-enzymatic defense systems in the phthalate stress tolerance mechanism.

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