Assessment of Toxicological Effects of Di-n-butyl phthalate to a Cereal Crop (*Hordeum vulgare* L.)

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***Abstract*—Phthalates have been declared as emerging environmental pollutants and their wide array of applications made them ubiquitous in almost all of the environmental matrices. However, a number of studies have focused on phthalates induced toxicity in animal experimental models, while limited attention has been given to phthalates induced toxicity to cereal crops. Therefore, the present study investigates the phytotoxic responses of exogenous exposure of di-n-butyl phthalate (DBP) in a cereal crop *i.e.* barley. The barley was exposed to different concentrations of DBP *viz*. 0, 25, 50, 100, 200, 400, 800 and 1600 mg/L for 7 days under controlled conditions. The exposure of DBP significantly altered different biochemical indices of barley seedlings which are attributed to the disturbance of normal physiological mechanisms of seedlings. It was observed that the barley seedlings were affected adversely in response to DBP stress. The exposure of DBP showed the stimulatory effect on carbohydrate, malondialdehyde (MDA), proline and hydrogen peroxide (H_2O_2) content of seedlings. The study also revealed that the roots were more sensitive to DBP stress than shoots of barley seedlings.**

***Index Terms*—toxicity, emerging pollutants, di-n-butyl phthalate, *Hordeum vulgare* L., biochemical responses**

I. Introduction

Presently, the sustainable crop production is one of the major need of mankind to meet their increasing food demands. The release of various anthropogenic pollutants poses a serious threat to sustainable crop production and also raises a concern about food security. Therefore, various agricultural management strategies have been adopted to improve crop production [1], [2]. Among them, plastic mulching (PM) is one of the most successful strategies [3]-[5]. The use of plastic mulching has been increasing in the developing countries for the agriculture [6]-[8]. No doubt, there is a number of advantages of using PM such as an increase in soil temperature, reduction in weeds, insect pests, maintenance of soil moisture, high crop yields, efficient use of nutrients etc. but there are also many disadvantages [3]. Among them, high pollution load or contamination of soil with phthalates; increase in CO_2 emission are the major ones [9]-[11]. The contamination of agricultural soil with phthalates was already well reported by many researchers. The other contributors of phthalates contamination in the agricultural soil are irrigation with waste water, paint spraying, incineration of plastic garbage, chemical/organic fertilizers, pesticides, leaching from plastic waste (due to the absence of covalent bond), application of sewage sludge or biosolids for fertilization *etc.* [12]-[14]. The dialkyl or alkyl/aryl esters of 1, 2-benzenedicarboxylic acid are known as phthalates which have a wide array of applications but especially known for their use as plasticizer [15]. The phthalates used as plasticizer in polymeric products like polyvinyl chloride (PVC), different industrial plastic products as well as in various non-polymeric products like in as adhesives in solvents, personal care products, cosmetics, paints *etc.* [16]-[18]. The absence of covalent bond is the main contributing factor for the emission of phthalates to different environments [19]. Thus, from the agricultural soil, the accumulation of phthalates in crop plants has been reported in the literature. The accumulation of phthalates in crops lead to their biomagnification at different trophic levels of the ecosystem [20]. In animals, phthalates are reported to induce a number of toxic effects like endocrine disruption, carcinogenicity, mutagenicity, teratogenicity, reproductive toxicities *etc.* The toxicity studies have emphasized mainly on phthalates induced toxicities in animals and less literature is available on phytotoxicity of phthalates. Therefore, the present study is focused on di-n-butyl phthalate induced biochemical toxicities to a cereal crop i.e. barley. Barley (*Hordeum vulgare* L.; 2n=14) is one of the oldest consumed cereal rabi crops which is a member of family Poaceae. In this study, DBP was chosen as test compound because it is frequently detected phthalate in the agricultural soil after diethylhexyl phthalate (DEHP) and also listed among priority environmental contaminants by United State Environmental Protection Agency [21], [22].

II. Materials and Methods

A. Chemicals and Experimental Material

DBP (CAS: 84-74-2, purity: 99%) was purchased from Himedia Laboratories Private Limited (India) and other

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chemical were used of analytical grade. The healthy seeds of *Hordeum vulgare* L. var. VLB-118 were procured from Hill Agricultural Research and Extension Centre Bajaura, Kullu (H.P.) India.

B. DBP Treatments

For the treatment of DBP, the stock solution (1600 mg/L) was prepared using ethanol, tween-20 and distilled water in required proportion [23]. The stock solution was further used for the preparation of working solutions were consisted of 0, 25, 50, 100, 200, 400, 800, 1600 mg/L of DBP through serial dilution.

C. Germination Procedure

The procedure for the germination of seedlings was same as previously described by Kumari and Kaur (2017) [24]. Briefly, seeds were surface sterilized using 0.01% mercuric chloride ($HgCl_2$) for 1 min. followed by drying in the folds of filter paper and presoaking in double distilled water. The seeds were kept in Petri plates lined with Whatman filter paper no. 1 and then moistened with different concentrations of DBP periodically. Petri plates were kept in the seed germinator at 25 ± 0.5^{o}C and photoperiod of 16 h. The seedlings were harvested on the 7^{th} day and analyzed for following indices.

D. Pigments Content

The plant sample was extracted in 80% acetone for the determination of pigments [25]. The calculation of chlorophyll a, chlorophyll b and total chlorophyll content using the equations given Arnon (1949), while carotenoids content was determined using the equation given by Lichtenthaler and Wellburn (1985) [25], [26].

E. Carbohydrate Content

Anthrone reagent method was used for the estimation of carbohydrate content [27]. The chopped shoots and roots of barley seedlings were hydrolyzed with 2.5 N hydrochloric acid (HCl) for 3 h in boiling water bath. The hydrolyzed sample solution was neutralized with sodium carbonate (Na_2CO_3) until the effervescence ceases and volume raised up to 100 ml with distilled water and then centrifuged. Anthrone reagent was added to the supernatant and kept in boiling water bath for 8 min. After cooling, the absorbance was observed at 630 nm. The carbohydrate content was estimated using glucose as standard.

F. MDA Content

MDA content provides the extent of cell membrane damage under abiotic or biotic stress and was determined by using the method of Heath and Packer (1968) [28]. The roots and shoots were homogenized in triacetic acid (TCA, 0.1%) and centrifuged at 10000 rpm for 5 min. thiobarbituric acid (TBA) (0.5% in 20% TCA) solution was added to supernatant followed by heating in water bath at 95^{o}C for 30 min. After cooling, the absorbance was recorded at 532 and 600 nm.

G. Protein Content

The protein content of seedlings was estimated using Bradford method (1976) [29]. The roots and shoots were homogenized in potassium phosphate buffer (pH=7.0) and were centrifuged at 12000 rpm for 20 min at 4^{o}C temperature. Bradford reagent was added to the resultant solution and absorbance was recorded at 595 nm. Bovine Serum Albumin (BSA) was used as standard.

H. Proline Content

The content of proline was estimated in plant samples using the method proposed by Bates *et al.* (1973) [30]. The shoots and roots were homogenized in aqueous sulfosalicylic acid (3.0%) and the homogenate was filtered through Whatman filter paper number 1. The reaction mixture was consisted of filtrate, acid ninhydrin and glacial acetic acid and the reaction mixture was heated for 1 h at 100^{o}C. The reaction was terminated placing reaction mixture test tube in ice bath. Toluene was added followed by vigorous mixing which resulted into the chromophore formation. The absorbance of chromophore was observed at 520 nm. Proline was used as standard.

I. Hydrogen Peroxide (H_2O_2) Content

H_2O_2 content was determined using the method of Alexiera *et al*. (2001) [31]. The shoots and roots were extracted using 0.1% TCA. The reaction mixture was prepared by mixing leaf extract, 100 mM potassium-phosphate buffer and potassium iodide (1.0 M). Then, the reaction mixture was kept in dark for 1 h and absorbance was recorded at 390 nm. The content of hydrogen peroxide was determined using H_2O_2 as standard.

J. Statistical Analysis

The data were analyzed for mean, standard error using one-way and two-way analysis of variance (ANOVA). The differences ($p\leq0.05$) among means were compared by honestly significant difference (HSD) using Tukey's test.

III. Results and Discussion

A. Effect on Pigments

The exposure of DBP significantly affected the content of photosynthetic and accessory pigments in barley seedlings when treated for 7 days under controlled conditions.

1) Effect on photosynthetic pigments

The effect of DBP on photosynthetic pigments was shown in Fig. 1(a-c). The chlorophyll a was declined at initial concentrations of DBP and then increased at higher concentrations The decrease/increase in chl a content at different concentrations of DBP was not significantly ($p\leq0.05$) different from each other except the chl a content at concentrations 100 mg/L and 1600 mg/L of DBP. Similar trends were obtained in case of chl b and total chl. But the treatment of DBP was significantly ($p\leq0.05$) affected the content of chl a, chl b and total chl because values of F-ratio$_{(7,64)}$ were 2.82, 2.60 and 3.30 respectively which was greater than critical value. In a study, it was observed that the chlorophyll content at different concentrations was not significantly different from each other in water celery treated with benzyl-butyl

phthalate (BBP) for 28 days [32]. The cotton seedlings showed the similar trend of increase or decrease in chl content under the exposure of DBP [33]. The exposure of BBP and DBP also affected the content of chl a, chl b and total chl in duckweed for 7 and 15 days of treatment [24]. The effect on photosynthetic pigments under the stress of DBP might be due to the damage to chloroplast. The present results were also supported by Zhang *et al*. (2015) and they revealed that DBP degraded the grana, stroma as well as thylakoid membranes of the chloroplast [34]. The chlorophyll degradation is commonly reported under abiotic stress. The chlorophyll degradation in the present study might be due to the increase in the activity of chlorophyllase enzyme which is a chlorophyll degrading enzyme [35].

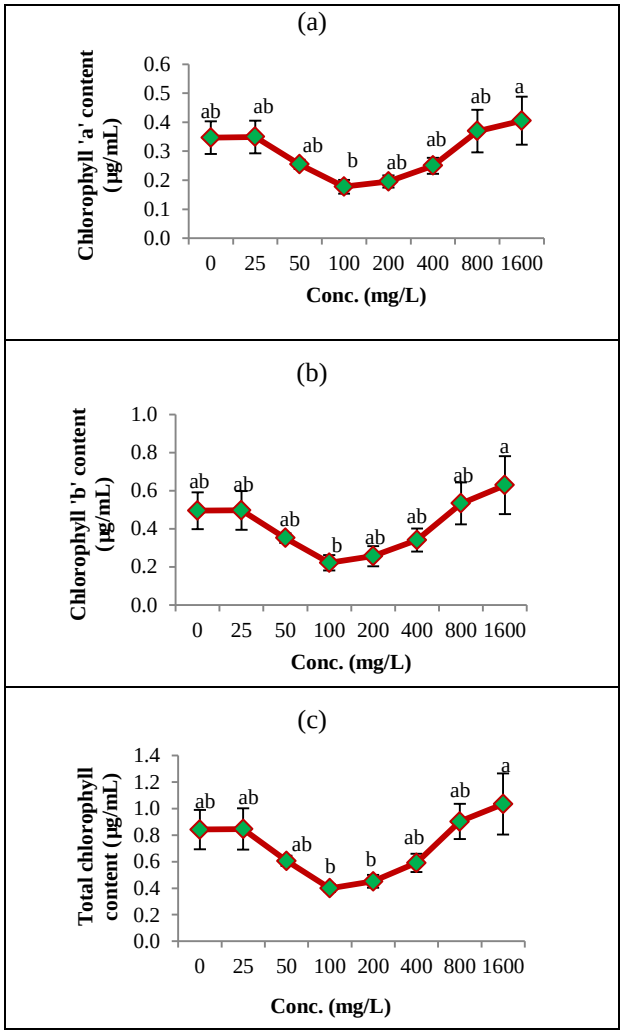

Figure 1. (a-c) Effect of DBP on chl a, chl b and total chl content of barley seedlings.

Note: Same letter means does not significantly differ at p≤0.05.

2) Effect on accessory pigments

The effect of DBP on accessory pigment was shown in Fig. 2. The treatment of DBP significantly (p≤0.05) enhanced the carotenoids content. In plants, carotenoids play role in light harvesting or photoprotection and also provide photostability to the chlorophyll by quenching singlet oxygen [36]. Giant duckweed under the exposure of BBP for 15 days showed enhancement in carotenoids content [23]. Carotenoids act as accessory pigments due to their role as light-harvesting complexes along with chlorophyll and main physiological roles are photoprotection and light harvesting [37]. A number of studies reported the increase in carotenoids content under abiotic stress. In the present study, DBP stress might have induced the damaging effects to barley seedlings by generating Reactive Oxygen Species (ROS). To minimize the damaging effects of ROS there might be an increase in carotenoids content in response to DBP exposure.

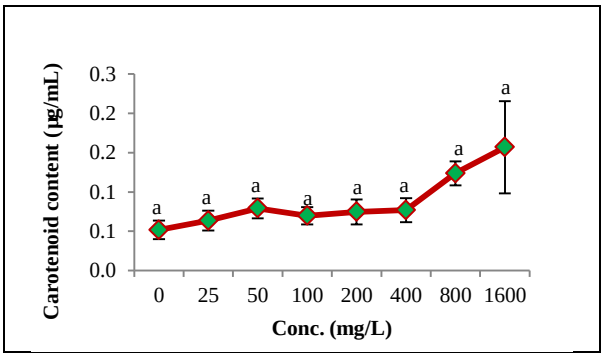

Figure 2. Effect of DBP on carotenoids content of barley seedlings.

Note: Same letter means does not significantly different at p≤0.05.

B. Carbohydrate Content

The effect of DBP on the shoots and roots of barley seedlings was presented in Fig. 3. There was an increase in the content of carbohydrate content in shoots and roots of seedlings. However, the increase is much more prominent in roots as compared to shoots. In case of roots, the percent increase in carbohydrate content was 88.62% at 25 mg/L of DBP, while the percent increase in case shoot was 29.33% at 25 mg/L. The content of carbohydrate showed a positive correlation with the concentrations of DBP in *Potamogeton maachianus* [38]. The carbohydrate content of mung bean also observed to enhance under the exposure of DBP and DEHP (at 500 mg/kg). The study also revealed that increasing stress of phthalates enhanced the accumulation of the storage compounds during the germination of mung bean as its early defense strategy to cope up the stress *via* synthesizing the required protective enzymes or solutes [39]. The abiotic stress can also trigger the release of monomeric forms (e.g. glucose, fructose) from polymeric forms like starch and fructans (carbohydrate) to facilitate fast and reversible osmotic adjustment. Moreover, these monomeric units can repolymerize themselves after the removal of stress [40]. Therefore, the increased content of carbohydrate might be the possible early defense mechanism of barley seedlings to deal with the stress induced by DBP.

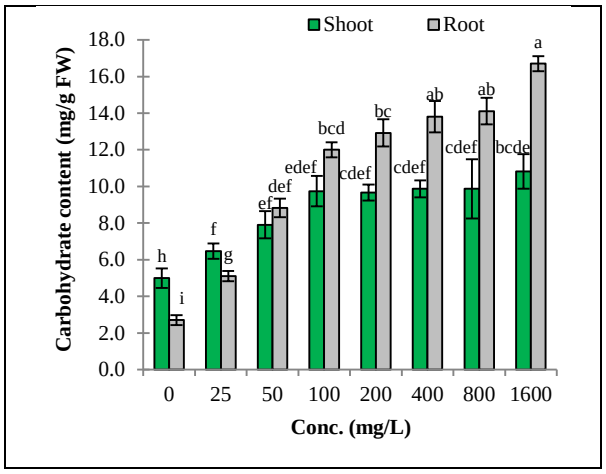

Figure 3. Effect of DBP on carbohydrate content of barley seedlings.

Note: Same letter means does not significantly different at p≤0.05.

C. MDA Content

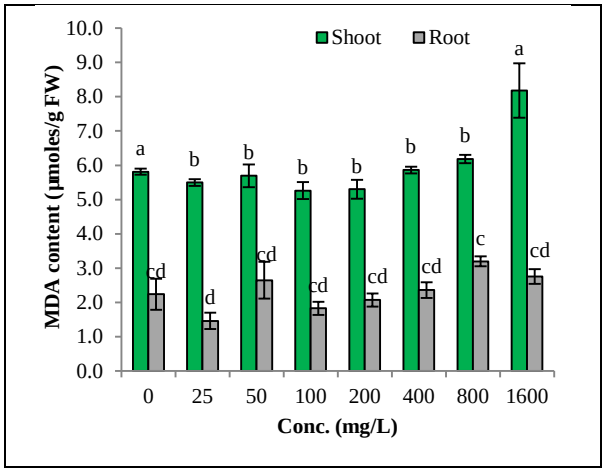

Figure 4. Effect of DBP on MDA content of barley seedlings.

Note: Same letter means does not significantly different at p≤0.05.

MDA is an end product of lipid peroxidation and reported as one of the most reliable index of oxidative stress induced by the variety of abiotic stressors [41]. In the present study, the MDA content of shoots and roots of barley seedlings was increased (Fig. 4). However, at lower concentrations of DBP the MDA was observed to decline as compared to control in case of the shoots. In shoots, the percent increase in MDA content was 0.86%, 6.41%, 40.79% at 400, 800, 1600 mg/L of DBP with respect to control. Similarly, in roots the percent increase was 5.50%, 42.80%, 23.10% at 400, 800, 1600 mg/L of DBP as compared to control. The percent increase in MDA content was more prominent in roots at higher concentrations of DBP than shoots but at higher concentration (1600 mg/L) it was found to decline. Here, an increase in root MDA content in response to DBP showed more sensitivity than shoot of barley seedlings. The trends of MDA content in shoots and roots of barley were also supported by the study of Ma *et al*. (2013) who studied the effect of DBP and DEHP in *Brassica chinensis* L. [39]. They also confirmed that it would be due to the direct contact of roots to the phthalates. Zhang *et al.* (2015) observed a significant increase in MDA content under the exposure of DBP for 3 days in cucumber seedlings [34]. The exposure of DBP has also increased MDA content in two freshwater algae [42].

D. Protein Content

The protein content of shoots and roots of barley seedlings was significantly (p≤0.05) increased (Fig. 5). The protein content in shoots was initially declined at lower concentrations and then observed to increase but the decrease/increase was not significantly different (p≤0.05) from each other treatment concentrations of DBP. In roots, the content of protein was observed to increase and was significantly different (p≤0.05) as compared to control. During the exposure of DBP to Bok choy (*Brassica rapa* subsp. *chinensis*), it was reported that there was an increase in Peroxidase 21 precursor and superoxide dismutase proteins, while four proteins were reported to disappear [43]. In another previous study, the exposure of DBP enhanced the level of three proteins and these were acyl-(acyl-carrier-protein) desaturase, ferredoxin-nitrite-reductase and root phototropism protein 3 in Chinese cabbage [44]. However, there were three other proteins which declined or disappeared under the exposure of DBP in the same plant. Moreover, the exposure of di-ethyl phthalate also elicited the biosynthesis of various heat shock proteins in radish [45]. The increase in protein content was also reported to increase under exposure of a heavy metal in *Coronopus didymus* [46]. Therefore, the present increasing trends of protein content in roots might be due to their more sensitivity towards DBP which led to the expression of stress proteins. It could also be the response of roots to the increased levels of free radicals. On other hand, the initial decrease in protein content of shoots may be due to degradation of some proteins and exposure of higher concentrations of DBP might have up-regulated the level of genes association with the expression of stress related proteins.

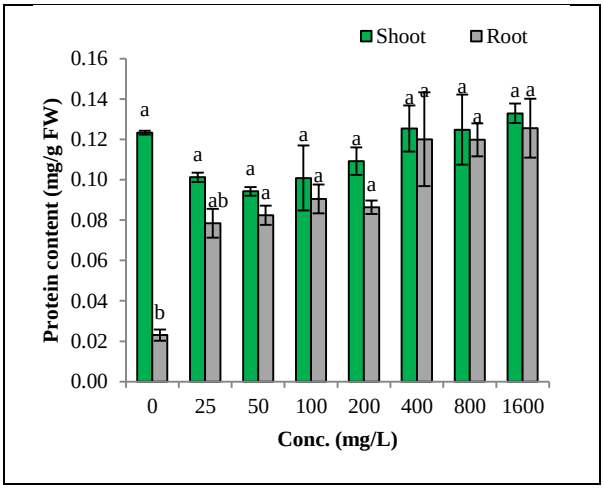

Figure 5. Effect of DBP on protein content of barley seedlings.

Same letter means does not significantly different at p≤0.05.

E. Proline Content

The proline content of shoots and roots of barley seedlings under DBP stress was shown in Fig. 6. The content of proline was enhanced significantly ($p \leq 0.05$, 0.01) in the shoots of seedlings and percent increase was ranged 2.52 to 19.63% as compared to control. Similarly, the content of proline in roots showed significant ($p \leq 0.05$, 0.01) stimulatory effects with the increasing concentrations of DBP and also more remarkable than shoots of seedlings. In a previous study, proline content showed increasing trends with the increase in concentrations and exposure of DBP treatment in cucumber seedlings [34]. The exposure of DBP and DEHP was also recorded to enhance the content of proline in shoots and roots of mung bean seedlings [39]. Proline is one of compatible osmolytes/solutes which are highly soluble organic compounds. In plant, the higher accumulation of compatible solutes do not disturb/interfere the normal cellular mechanisms [40]. Moreover, proline was reported to play number of physiological functions like stabilization of proteins, protection of enzymes, scavenging of free radicals, involvement in vital physiological processes *viz.* respiration and photosynthesis by providing NAD^+ and $NADP^+$ Accumulation of proline in plants was considered to act as a consequence to climate change as well as abiotic stresses [47], [48].

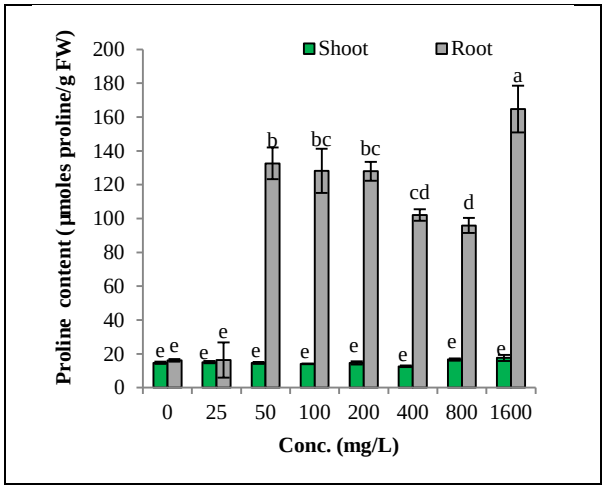

Figure 6. Effect of DBP on proline content of barley seedlings.

Note: Same letter means does not significantly different at $p \leq 0.05$.

F. Hydrogen Peroxide Content

The effect of DBP on H_2O_2 content of shoots and roots of barley seedlings is shown in Fig. 7. The content of H_2O_2 was observed to increase in shoots and roots of barley seedlings under the stress of DBP. In a previous study, an increase in the content of H_2O_2 was observed with increase in concentrations of DBP and prolonged treatment duration of DBP [34]. As earlier stated that DBP stress might led to generation of various ROS and H_2O_2 is also one of them. However, the excess accumulation of H_2O_2 in plant reflects the level of oxidative damage induced by abiotic/biotic stress. From number of studies, it has been proven that H_2O_2 plays an important role in plants under severe abiotic/biotic stresses [49]. Therefore, in response to DBP induced oxidative stress there might be increase in the content of H_2O_2 in shoot and root of seedlings.

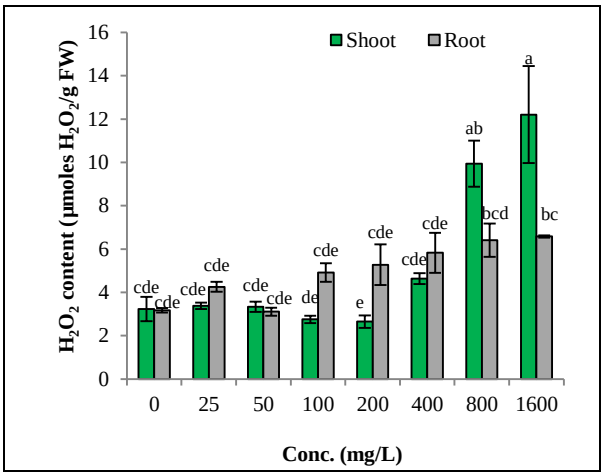

Figure 7. Effect of DBP on H_2O_2 content of barley seedlings.

Note: Same letter means does not significantly different at $p \leq 0.05$.

IV. Conclusions

From the present study, it can be elucidated that DBP has affected the biochemical indices of barley seedlings to a considerable extent. Obviously, the treatment of DBP might have led to the production of reactive oxygen species which is responsible for oxidative stress to the barley seedlings. Therefore, the oxidative stress in seedlings could be the plausible reason for the enhanced content of carotenoids, carbohydrate, MDA, protein, proline and H_2O_2 in seedlings. However, the DBP induced biochemical consequences were prominent in roots of seedlings which reflected the more sensitivity of roots than shoots. This study will be helpful in understanding the toxicity of DBP to cereal crops which can contribute to the risk assessment of DBP in terrestrial ecosystem. However, further studies are still required to explore the other toxicities like response of antioxidative enzymes, polyphenol and hormonal profiling under the exposure of DBP to barley seedlings.

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