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Effects of Bisphenol A and Tetrabromobisphenol A on Chickpea Roots in Germination Stage

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Abstract: In the present study, toxic effects of BPA and TBBPA on chickpea seed germination and some possible biochemical changes in the roots were determined. The seed germination did not significantly change in 10 and 50 mg/L BPA and TBBPA applications. However, seed germination was inhibited by 100 mg/L concentration of both treatments. Similarly, chickpea root development was adversely affected by high concentrations of BPA and TBBPA. Applications of BPA and TBBPA-induced oxidative stress in the roots demonstrating by H_2O_2 formation as well as correlated quantitatively with malondialdehyde (MDA), peroxidase (POD) activity and non-protein SH groups. Toxic effects of BPA and TBBPA on the roots were histochemically determined as well. In terms of stainings, degree of lipid peroxidation rose violently with the concentrations applied, especially in TBBPA. Staining with evans blue stated that the integrity of the plasma membrane of roots was relatively protected in low concentrations of BPA and TBBPA. However, the integrity was not maintained at high concentrations of both chemicals, especially TBBPA. Chickpea has the capacity to tolerate the oxidative stress at 10 and 50 mg/L concentrations of both treatments, but the capacity partly lost at 100 mg/L concentrations of BPA and TBBPA as revealed by POD activity and non-protein SH groups.

Key words: Bisphenol A · Tetrabromobisphenol A · Chickpea · Seed germination · Biochemical effects

INTRODUCTION

Environmental pollutants such as alkylphenolic compounds, polychlorinated biphenyls, pesticides, dioxins and textile dyes have been released into the environment. Most of these chemicals and/or their degradation products are toxic, mutagenic, carcinogen and endocrine disrupting for aquatic and terrestrial life. Bisphenol A (BPA: 2,2-Bis(4-hydroxyphenyl)propane) is an industrially important monomer used in many chemical manufacturing plants throughout the world for the synthesis of polycarbonates, epoxy resins, phenol resins, polyesters, polyacrylates and lacquer coatings on food cans as well as storage vessels [1-3].

Polybrominated flame retardants (PBFRs) are in extensive use in modern society as additives in many products, e.g. computers, other electronic equipment, furniture, cars, contraction materials, sealings etc. The increase in use has been dramatic during the last two decades. Different classes of PBFRs, as well as many metabolites, are today globally distributed in the environment. The most important products today that are accumulated in abiotic as well as biotic systems, are the polybrominated diphenyl ethers (PBDEs), polybrominated biphenyls (PBBs), tetrabromobispenol A (TBBPA; 3,3',5,5'-Tetrabromobisphenol A), pentabromophenol (PBP) and 2,4,6-tribromophenol (TBP). Among these, TBBPA is the major PBFR produced [4]. Due to their similar structure to polychlorinated biphenyls, PBFRs have become a matter of great concern in recent years [4,5]. PBFRs impose a great threat to the environment by being produced, used and distributed in large amounts, being globally transported and expressing bioaccumulative and toxic properties of the same kind as well known envirotoxicants. Actions are now being taken in parts of the world to limit the use of the most threatening products, but the problem will continue to develop for a long time on the basis of the huge amount of these substances already present in the environment [6].

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Plants can rapidly absorb BPA through their roots from water and metabolize it to several glycosidic compounds. The glycosylation of BPA by plants leads to estrogenicity of the parent compound. Two oxidative enzymes, peroxidase and polyphenol oxidase, are closely associated with BPA metabolism [7]. The aim of the present study was to evaluate the effect of endocrine disrupting chemicals BPA and TBBPA on the germination of chickpea seeds and their some biochemical impact on seedling roots.

MATERIAL AND METHODS

For the germination test, *Cicer arietinum* L. cv. Ispanyol seed was used. Analytic grade of BPA and TBBPA were obtained from Sigma-Aldrich Inc. The seeds were treated with 0, 10, 50 and 100 mg/L BPA and TBBPA. Four replicates of 15 seeds for each BPA and TBBPA treatment, or for the control, non-treated seeds, were placed on two layers of filter paper in 120 mm petri dishes. Before the experiment, the seeds were sterilized with 2% sodium hypochloride solution for 15 min and washed with sterile water three times. The filter paper was moistened with distilled water for the controls, or with aqueous solutions of each treatment. Distilled water or application solutions were added periodically maintaining the filter paper wet during the course of the experiment. The petri dishes were incubated in a growth chamber at 27±1°C without photoperiod. The number of germinated seeds was counted every day during 6 days from the start of the test. Seeds were considered to be germinated at the emergence of the radicle (first root). All chemicals used in the study were prepared from the analytical grade.

Treatments were evaluated by counting the number of germinated seeds and measuring the length of roots. Seedling vigor index (SVI) for each treatment was calculated by the total germination percent *x* root length [8].

The content of H_2O_2 was measured according to the method of Sagisaka [9] with the following modification: 0.5 grams of fresh root tissues were ground in 50 mM K-phosphate buffer (pH 7.8). The mixture was centrifuged at 10000 rpm for 10 min. The supernatant was collected. 1.6 mL of the resulting supernatant was mixed with 0.4 mL 50% trichloroacetic acid (TCA), 0.4 mL 10 mM ferrous ammonium sulfate and 0.2 mL 125 mM potassium thiocyanate. The absorbance of the reaction mixture was monitored at 480 nm. Soluble POD activitywas determined by a modification of the method of Hammerschmidt *et al*. [10]. Lipid peroxidation was determined by measuring the level of malondialdehyde (MDA) by method of Zhou [11]. Determination of non-protein SH groups was carried out as described in Cakmak and Marschner [12]. Reduced glutathione (GSH) was used as a standard. Protein content was estimated by Lowry procedure [13], using bovine serum albumin as reference.

Histochemical detection of loss of plasma membrane integrity in root apices was achieved by the method as described by Yamamoto *et al*. [14]. To determine root plasma membrane integrity, the roots were incubated in 5 mL of Evans blue solutions (0.025% (w/v) prepared in 100 μM $CaCl_2$ (pH 5.6) for 30 min. Histochemical detection of lipid peroxidation was conducted with Schiff's reagent [15]. The roots were incubated in Schiff's reagent for 60 min. After that, the stained roots were rinsed with a solution containing 0.5% (w/v) $K_2S_2O_5$ (prepared in 0.05 M HCl) until the root color became light red.

All analysis were carried out triplicate. For statistical analyses we chose the analysis of variance (ANOVA) in Statistical Analysis System (SPSS 11.0 for windows). The significance of differences between mean values were determined by a multiple range test (LSD; Least Significant Difference). For this reason, alpha (α) was preferred to be 0.05, which corresponds to a confidence level of 95%. Correlation analysis (Pearson) was estimated among H_2O_2 content in roots and other parameters analyzed.

RESULTS AND DISCUSSION

There are many studies on the toxic mechanisms of BPA and TBBPA in either mammals or aquatic organisms, although little is known about the effects of BPA and TBBPA on terrestrial plants. Hence, toxic effects of BPA and TBBPA on chickpea seed germination and some physiological changes in roots were determined in this study.

The final germination percentages of the chickpea seeds are represented in Fig. 1A. The germination percentage of control seeds without treatment was estimated as 93.3. The maximum final germinations at 10 mg/L concentrations of BPA and TBBPA were calculated as 94.6% and 95.5%, respectively ($p>0.05$). The minimum germination percentages were recorded for BPA and TBBPA as 83.3 and 85.5, respectively ($p<0.05$). Generally, plants are most sensitive to toxicity during the germination stage. Therefore, seed germination is one of the criteria usually used to screen crops or varieties for their tolerances to toxicity [16]. Ferrara *et al*. [17] reported that 6-d treatment of 10 and 50 mg/L BPA concentrations

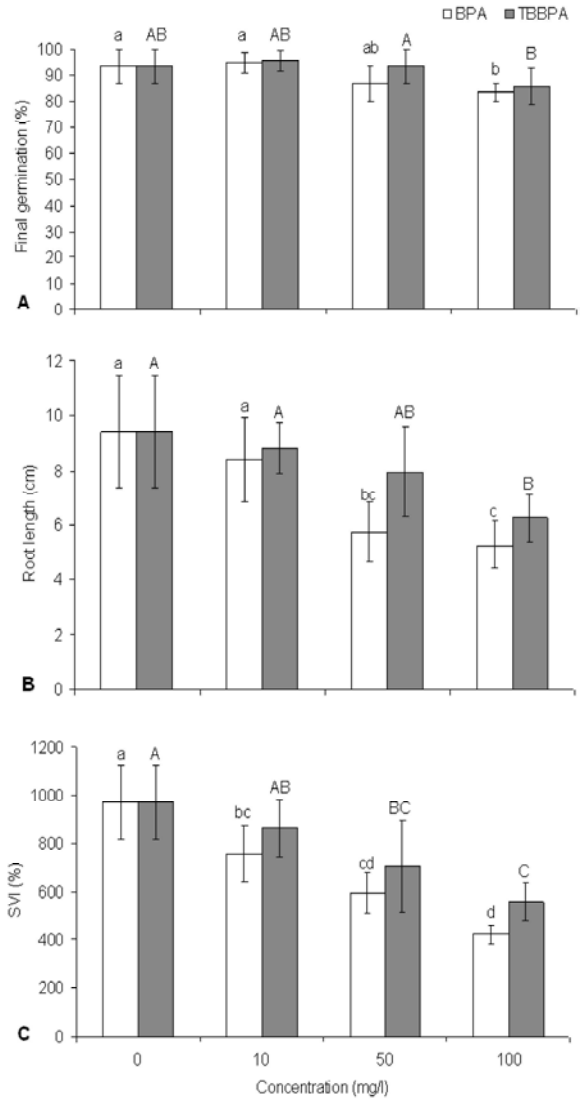

Fig. 1: Effect of different BPA and TBBPA concentrations on (A) seed germination, (B) root growth and (C) seedling vigor index of chickpea after 6-days treatment. Error bars represent the standard deviation of mean. Means with different letters are significantly different from one another according to LSD test ($p<0.05$)

did not inhibit seed germination. According to our results, chickpea seed germination did not significantly change in 10 and 50 mg/L BPA and TBBPA applications. However, seed germination was inhibited by 100 mg/L concentration of both applications.

In both chemicals applied, root developments were adversely affected with respect to control (Fig. 1B). The decreases was not significant for BPA and TBBPA at 10 mg/l as 10.6% and 6.4%, respectively ($p>0.05$). The highest decrease was estimated for BPA and TBBPA at 100 mg/L as 43.8% and 33.5%, respectively ($p<0.05$). However, the capacity of BPA to affect negatively the growth of tomato, durum wheat and lettuce, possibly through inhibition of both cell elongation and cell division, was more evident with progressing seedlings growth [17]. Similarly, chickpea root development was adversely affected by high concentrations of BPA and TBBPA as well. Seedling vigor is defined as the ability of seedlings to emerge rapidly from soil or water [18]. SVIs were gradually reduced by BPA and TBBPA treatments (Fig. 1C).

Oxidative stress is considered an important pathophysiological condition, promoting cell death in a broad variety of disorders [19]. Previous studies suggested that BPA and TBBPA might lead to oxidative stress by inducing reactive oxygen species (ROS) generation [20-22]. Hydrogen peroxide (H_2O_2) is the two electron reduction product of O_2. It is potentially a reactive oxygen, but not a free radical. To verify the identity of ROS involved in BPA and TBBPA-induced oxidative stress, H_2O_2 concentrations were also determined. As can be seen in Fig. 2A, BPA application caused 1.06 ($p>0.05$), 1.33 and 2.0-fold ($p<0.05$) enhancement of H_2O_2 at 10, 50 and 100 mg/L, respectively, compared to control. Similarly, the increase in TBBPA treatments determined as 2.05, 2.39 and 2.98-fold ($p<0.05$) at 10, 50 and 100 mg/L concentrations, respectively, when compared to control. Higher H_2O_2 levels were found in TBPPA applications than BPA applications. Depending upon the obtained data, stimulated H_2O_2 formation clearly demonstrates BPA and TBBPA-induced oxidative stress in roots, although mechanism of triggered ROS production by these chemical remains unclear [23].

Exposure of chickpea to BPA resulted in insignificant increase in POD activity of roots at concentration of 10 mg/L as 1.4-fold ($p>0.05$) (Fig. 2B). POD activities, however, were significantly rised by 50 and 100 mg/L BPA concentrations as 1.9 and 1.6-fold, respectively ($p<0.05$). On the other hand, POD activities were significantly increased by 10, 50 and 100 mg/L TBBPA concentrations as 1.9, 2.5 and 1.9-fold ($p<0.05$), respectively, when compared with control. H_2O_2 is eliminated by various antioxidant enzymes, such as CAT and POD exerting different reaction mechanisms. The most characteristic difference is their co-substrate requirement. CAT can disproportionate H_2O_2 without co-substrates, while POD requires co-substrates to detoxify H_2O_2 [24]. The POD activity treated with 50 mg kg^{-1} TBBPA in wheat was significantly higher than that in the control after the 12-d

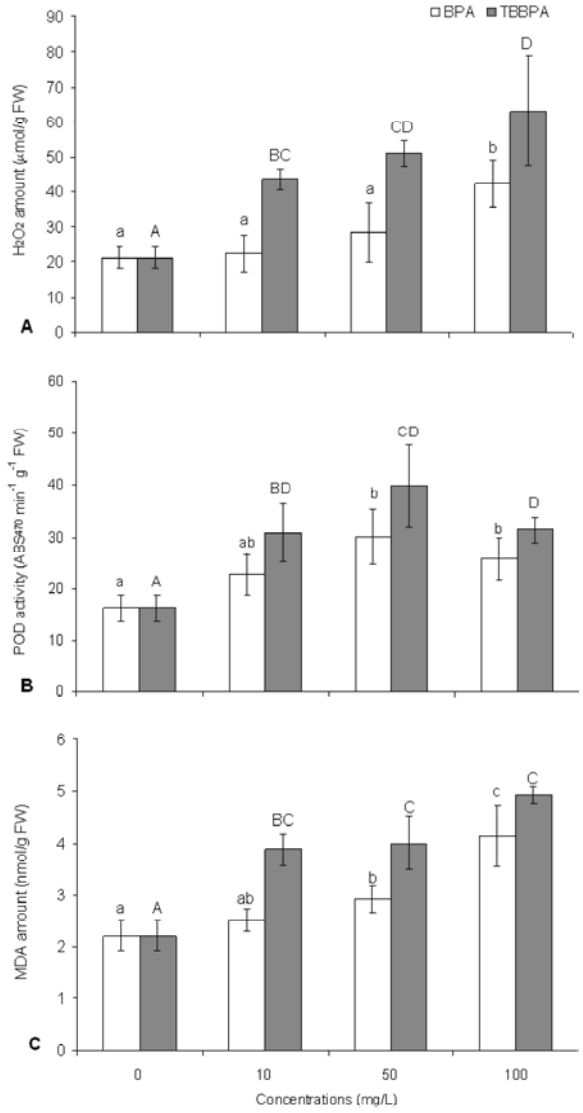

Fig. 2: H_2O_2 amount (A), POD activity (B) and MDA content (C) of chickpea seedling roots at different BPA and TBBPA concentrations after 6 days treatment. Error bars represent the standard deviation of mean. Means with different letters are significantly different from one another according to LSD test ($p<0.05$).

exposure of TBBPA [25]. According to our findings, POD activities were sharply increased by 10 and 50 mg/L BPA and TBBPA treatments, but the decrease was determined at 100 mg/L BPA and TBBPA applications, with respect to 10 and 50 mg/L. However, the capacity would be partly lost at 100 mg/L concentrations of BPA and TBBPA. Correlation analyses showed that there were positive relationship between H_2O_2 contents and POD activities of

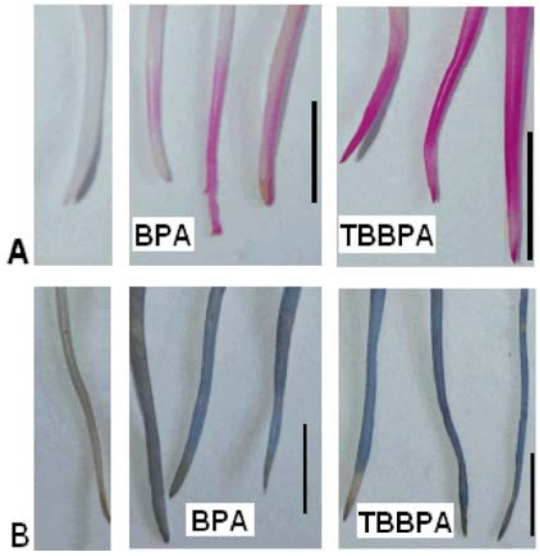

Fig. 3: Histochemical detection of lipid peroxidation (A) and root plasma membrane integrity (B) caused by different BPA and TBBPA concentrations in chickpea roots. At both treatment stages (A and B), roots from left to right indicate: 0 mg/L (without treatment); 10 mg/L; 50 mg/L; and 100 mg/L. Briefly, roots from a particular treatment stage were stained with Schiff's reagent for 60 min (A) or Evans Blue solution (B) for 30 min and then photographed (CANON EOS 450 D). Bars in each graph indicate 5 mm.

the roots at BPA treatment as $r=0.425$; $p=0.169$ and TBBPA treatment as $r=0.680$; $p=0.015$. These indicate that POD is play an important role in oxidative stress caused by BPA and TBBPA treatments and the enzyme is probably associated with BPA metabolism [6] and TBBPA metabolism as well.

Lipid peroxidation refers to the oxidative degradation of lipids. Some studies have revealed that BPA and TBBPA caused lipid peroxidation [22,23]. There was an increase in MDA content of chickpea roots, in parallel to increasing BPA and TBBPA concentrations, as shown in Fig. 2C. The increases in MDA content were found to be significant for BPA and TBBPA concentrations ($p<0.05$), except at 10 mg/L BPA concentration ($p>0.05$). The maximum MDA contents were found in roots exposed to 100 mg/L BPA and TBPPA and rise rates, with respect to control, were estimated to be 87.3% and 122.6% ($p<0.05$), respectively. Statistical analyses showed that there were significant and positive correlation between H_2O_2 contents and MDA contents of the roots at BPA application as $r=0.855$; $p<0.001$ and TBBPA application as

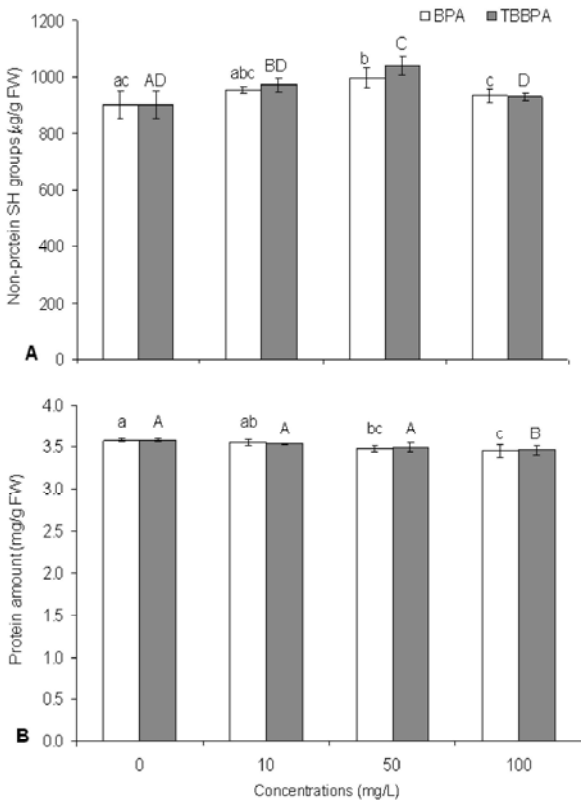

Fig. 4: Effect of different BPA and TBBPA concentrations on the contens of (A) non-protein SH groups and (B) protein on chickpea seedling roots after 6-days treatment. Error bars represent the standard deviation of mean. Means with different letters are significantly different from one another according to LSD test ($p<0.05$).

r=0.693; p=0.012. Both BPA and TBBPA-induced oxidative damage of roots were confirmed by the histochemical staining with Schiff's reagent (Fig. 3A) and Evans Blue (Fig. 3B). With Schiff's reagent, roots in BPA treatment stained lesser (thus less MDA formation, also see Fig. 3A) than TBBPA treatment. In terms of H_2O_2 levels, however, more significant and positive relationship was found in BPA application than TBBPA application. On the other hand, degree of the peroxidation quantitavely and qualitatively became violent rising with the concentrations applied, especially in TBBPA. This may be suggesting that TBBPA is more potent in triggering ROS generation other than H_2O_2. Staining with Evans blue revealed that the integrity of the plasma membrane of roots was protected in low concentrations of BPA and TBBPA while integrity was not maintained at high concentrations of both chemicals, especially TBBPA.

Content of non-protein SH groups in the roots increased following BPA and TBBPA applications. Most of the non-protein SH groups in plants represent glutathione [26]. Glutathione is a ubiquitous thiol-containing tripeptide which plays an important role as an antioxidant to scavenge ROS through the oxidation of GSH to GSSG [22]. Non-protein SH groups in the roots was also increased by BPA and TBBPA treatments (Fig. 4A). In terms of correlation analyses, relationships between H_2O_2 contents and non-protein SH groups were found to be positive ($p>0.05$). The increases at 10, 50 and 100 mg/L concentrations calculated as 5.7% ($p>0.05$), 10.3% ($p<0.05$) and 3.4% ($p>0.05$) for BPA and 7.7%, 15.1% ($p<0.05$) and 3.4% ($p>0.05$) for TBBPA, respectively. However, non-protein SH groups were decreased by BPA and TBBPA at 100 mg/L concentration, with respect to other BPA and TBBPA concentrations. This may be due to severe oxidative stress [22]. Contrary to these, reduction in protein content was observed with a progressive increase in BPA and TBBPA concentrations (Fig. 4B). Protein content in the chickpea roots decreased by 0.8% ($p>0.05$), 2.8% and 3.4% ($p<0.05$) in 10, 50 and 100 mg/L BPA treatments, respectively, when compared with the control. Similarly, the reduction in TBBPA treatment determined as 1.4%, 2.5% ($p>0.05$) and 3.4% ($p<0.05$) for 10, 50 and 100 mg/L, respectively. Correlation analyses showed that there were negative relationship between H_2O_2 contents and protein contents of the roots at BPA treatment as r = -0.735; p=0.006 and TBBPA treatment as r= -0.646; p=0.023. Earlier studies reported that hydroxyl radicals are responsible for destruction of proteins [27,28]. Furthermore, it might be concluded that reduction in protein content is caused by ROS produced as a result of oxidative stress stimulated by BPA and TBBPA.

Results obtained in this study indicated that there were no significant changes in the seed germination at low concentrations of BPA and TBBPA. Concentration-dependant enhancement in H_2O_2 level and lipid peroxidation in chickpea roots was assumed to be resulted from provoked oxidative stress, especially with TBBPA. POD activities were also increased by BPA and TBBPA, but the activity was decreased due to severe oxidative stress by high concentrations of BPA and TBBPA. The present study carried out in petri dishes conditions without soil. Therefore, future researches on effects of the chemicals might be assessed in soil-plant systems, because of the important role of BPA and TBBPA in plant toxicology.

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