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# Effect of Boron Toxicity on Oxidative Stress and Genotoxicity in Wheat (*Triticum aestivum* L.)

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**Abstract**
Boron (B) toxicity, which occurs in semi-arid and arid environments, can adversely affect the growth and yield of many plants. The aim of this study was to determine the effects of different concentrations of boric acid (3, 6, 9 and 12 mM) on growth, oxidative stress and genotoxicity parameters in root and shoot tissues of wheat seedlings. Our results indicate that B stress inhibits root and shoot growth of wheat in a concentration-dependent manner, and leads to increases in TBARS and $H_2O_2$ contents in shoot tissue. Moreover, our findings suggest that high concentrations of B may exert a genotoxic effect on wheat. To the best of our knowledge, this is the first report to evaluate the effect of B stress on genotoxicity in both root and shoot tissues of wheat.

**Keywords** Boron · Wheat · Oxidative stress · Genotoxicity · RAPD-PCR

Boron (B) is an essential micronutrient element required for plant growth (Reid 2013), and plays an important role in the maintenance of cell wall-membrane integrity, as well as supporting metabolic functions (Blevins and Lukaszewski 1998; Roessner et al. 2006). On the other hand, the productivity of many agricultural systems is severely affected by insufficient or excessive amounts of B in the soil (Reid et al. 2004). B toxicity generally occurs in semi-arid and arid environments, where B level is high in the soil or in the irrigation water (Nable et al. 1997). Chlorosis, necrosis of leaf tips, and inhibition of root elongation are the main symptoms of B toxicity in many plants (Cervilla et al. 2007; Wang et al. 2010). In addition to these, a number of physiological and metabolic processes such as cell wall development, cell division, stomatal conductance and photosynthesis are known to be affected by toxic levels of B (Papadakis et al. 2004; Reid et al. 2004; Landi et al. 2012).

Reactive oxygen species (ROS), comprising of free radicals such as superoxide anion and hydroxyl radical, as well as non-radical oxygen derivatives like hydrogen peroxide and singlet oxygen, are naturally produced as a by-product of various metabolic processes, including cellular respiration, photorespiration and photosynthesis (Gill and Tuteja 2010; Jia 2011; Sharma et al. 2012). Under normal conditions, ROS are maintained within a physiological range by the antioxidant defense system which consists of enzymatic and non-enzymatic antioxidants (Shen et al. 1997; Wellen and Thompson 2010). However, the equilibrium between production and scavenging of ROS may be disrupted by various stress factors such as drought, salinity, UV radiation and heavy metal pollution, leading a significant increase in intracellular ROS level (Apel and Hirt 2004; Gill and Tuteja 2010). The excessive production of ROS can induce oxidative stress, which results in cellular damage through a number of mechanisms such as lipid peroxidation, protein oxidation, enzyme inactivation and DNA damage (Bartosz 1997; Sharma et al. 2012).

In recent years, many studies have shown that increasing concentrations of B may cause oxidative stress in various plants (Cervilla et al. 2007; Ardıc et al. 2009; Aftab et al. 2010; Kaya and Ashraf 2015). However, even though the B accumulation in root and shoot tissues are generally different from each other (Nable 1988; Kalayci et al. 1998; Cenkci et al. 2009), in most of these studies, oxidative stress parameters have been measured either in shoot or leaf tissues of studied species (Cervilla et al. 2007; Ardıc et al. 2009; Kaya and Ashraf 2015). Consequently, there is limited knowledge regarding whether B can cause an increase in oxidative stress in root tissue (but see, Karabal et al. 2003; Wang et al. 2010).

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On the other hand, various studies have suggested that root and leaf tissues can show different responses to stress conditions (Shah et al. 2001; Bandeoğlu et al. 2004). Determining the oxidative stress parameters in both root and shoot tissues, therefore, may provide valuable information for better understanding of B toxicity in plants.

The random amplified polymorphic DNA (RAPD) technique has been widely applied for the detection of genetic diversity (Ram et al. 2008), the construction of genetic maps (Yin et al. 2001), and the identification of resistance genes (Young and Kelly 1997). Furthermore, it has also been frequently used in ecotoxicological studies to determine the genotoxic effects of various contaminants such as heavy metals (Liu et al. 2009; Cenkci et al. 2010a) and herbicides (Cenkci et al. 2010b; Aksakal et al. 2013). In RAPD studies, the potential genotoxicity is determined by the comparison of band profiles (e.g. appearance and disappearance of bands, changes in band intensity) between toxicant-exposed and unexposed samples (Atienzar et al. 2002; Wolf et al. 2004). It has been suggested that variations in RAPD profiles may be related to DNA alterations such as DNA adducts, point mutations and chromosomal rearrangements induced by toxic agents (Wolf et al. 2004; Atienzar and Jha 2006).

As far as we know, the possible genotoxic effect of B on wheat has been investigated in two studies using RAPD technique (Kekec et al. 2010; Erdal et al. 2014). The findings of these studies suggest that B treatments may cause changes in RAPD profiles of the studied genotypes. On the other hand, the genotoxic effect of B has not been determined for shoot tissue in both studies. Moreover, it is well known that there is a substantial genotypic variation in tolerance to B toxicity in wheat (Nable 1988; Kalayci et al. 1998; Torun et al. 2006). Therefore, more studies are required to clarify the potential genotoxic effect of B on wheat.

The aims of the present study were to determine the effects of B on growth and oxidative stress parameters in root and shoot tissues of wheat seedlings, and to evaluate the possible DNA damage induced by B stress using RAPD technique. We hypothesize that increasing concentrations of B may lead to different levels of oxidative stress and genotoxicity in root and shoot tissues of wheat.

## Materials and Methods

In this study, a Turkish cultivar of wheat (*Triticum aestivum* L.), Kıraç-66, was used. Seeds of this cultivar were sterilized for 10 min with 3% sodium hypochlorite containing one drop of Tween-20 and rinsed with distilled water three times. Surface-sterilized seeds were incubated in distilled water for 24 h at 20°C in darkness. After that, germinated seeds were placed in Eppendorf tubes containing 0.8 mL agar (1%). The narrow end of each tube was cut with a heated knife before placement of seeds to allow the roots to protrude from the tube tip. Eppendorf tubes were then placed in styrofoam floats and let seedlings grow hydroponically in a growth chamber at 22°C under a photoperiod of 16 h (100 µmol $m^{-2}$ $s^{-1}$) for 6 days. A modified nutrient solution (pH 5.6), which consisted of 0.65 mM $KNO_3$, 0.40 mM $CaCl_2$, 0.25 mM $MgCl_2$, 0.08 mM $NH_4NO_3$ and 0.025 mM $H_3BO_3$ (boric acid [BA]), was used to provide essential nutrients. At the end of 7 days of growth, different concentrations of BA (3, 6, 9 and 12 mM) were added to nutrient solutions in order to determine the effects of B on physiological and biochemical parameters in wheat. Control (contains 0.025 mM BA) and B-subjected plants were grown with the same growth conditions for another 5 days (Karabal et al. 2003). The nutrient solutions were changed every 2–3 days and aerated continuously. After the end of the experiment, the root and leaf tissues of 12 days old wheat seedlings were used for further measurements and assays.

After the harvest, photographs of seedlings from each treatment were taken using a digital camera. ImageJ analysis software (version 1.51k) was used to measure the root and shoot length of each seedling. The total seedling length and root/shoot ratio were also calculated. Finally, root and shoot dry weights of each seedling were determined after drying at 70°C for 48 h.

In order to determine the chlorophyll and carotenoid contents, 0.5 g of fresh leaf materials were extracted with 80% acetone and the extracts were centrifuged at 10,000 rpm for 15 min at 4°C. After that, the absorbance of supernatants was measured at 663, 645, and 470 nm using a UV–Vis spectrophotometer (Sumanta et al. 2014).

For determination of $H_2O_2$ levels in root and shoot tissues, samples were homogenized in 10 volumes of (w/v) ice cold 0.1% trichloroacetic acid (TCA). The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C. Subsequently, 0.5 mL of the supernatant was mixed with 0.5 mL of 10 mM potassium phosphate buffer (pH 7.0) and 1 mL of 1 M potassium iodide (KI). After 20 min of incubation at room temperature, the absorbance of the mixtures was measured at 390 nm (Velikova et al. 2000). The content of $H_2O_2$ was calculated by comparison with a standard calibration curve prepared from a range of concentrations of $H_2O_2$ (0–50 µM). The results were expressed as µM $g^{-1}$ FW.

Thiobarbituric acid-reactive substances (TBARS), which is an indicator of lipid peroxidation, was assayed as described by Karabal et al. (2003). Briefly, 0.5 g of root and shoot samples were homogenized in 5 mL ice-cold 0.1% TCA. The homogenates were centrifuged at 10,000 rpm for 20 min at 4°C, and the supernatants were collected for the determination of TBARS content. One mL supernatant of each sample was mixed with 4 mL of 20% TCA and 0.5% TBA and heated at 95°C for 30 min. The reaction was terminated in an ice bath and reaction mixtures were centrifuged

at 10,000 rpm for 10 min at 4°C. Following centrifugation, absorbance of each mixture was measured at 440, 532 and 600 nm with a UV–Vis spectrophotometer (Shimadzu UV-1700). The TBARS content (nmol $g^{-1}$ FW) was calculated by using the formula of Du and Bramlage (1992).

B concentration of root and shoot tissues was determined by azomethine-H method (John et al. 1975). Approximately, 100–200 mg of dried root and shoot samples were put into Teflon vessels containing 10 mL of freshly prepared concentrated $HNO_3$–$H_2O_2$ acid mixture (7:3, v/v). After standing for 10 min, the samples were digested in a microwave system (Berghof Speedwave MWS-3, Germany) at various time intervals and temperatures. The digested samples were then filtered and diluted 10 times with ultrapure water. The azomethine-H solution and buffer masking reagent were prepared according to Banuelos et al. (1992). The reaction mixture consisted of 1 mL sample solution, 2 mL buffer masking reagent and 2 mL azomethine-H solution. The mixtures were mixed thoroughly and incubated at room temperature for 30 min. Finally, the absorbance of mixtures was measured at 420 nm by using a UV–Vis spectrophotometer. The content of B was quantified by using a standard calibration curve prepared from different concentrations of B (0–15 µg/mL).

Genomic DNA was extracted from approximately 250 mg of fresh root and shoot tissues using the CTAB (cetyltrimethylammonium bromide) method described by Doyle and Doyle (1987), with minor modifications. The PCR reactions were performed in a total volume of 25 µL consisting of 25 ng DNA, 1.2 µM primer, 200 µM dNTPs, 2.5 µM $MgCl_2$ and 1 U Taq DNA Polymerase (Thermo). Sequences (5′–3′) from primer 1 to 10 were: AGGCAGAGCA (OPY-8), GGG CCAATGT (OPY-16), GTCCACACGG (OPB-8), CTGGAC GTCA (OPW-7), GGCGGATAAG (OPW-5), AGTCGC CCTT (OPY-15), TTCAGGGCAC (OPW-18), AATCGG GCTG (OPA-4), CTGACCAGCC (OPH-19) and ACCTTT GCGG (Primer 5).

All primers used in our experiments were selected according to published sequences (Liu et al. 2009; Aksakal and Esim 2015). The PCR reactions were repeated three times for each sample and negative controls were used to avoid cross-contamination. Amplification was conducted in a thermal cycler (Bio-Rad iCycler®, USA), programmed as follows: one cycle of 4 min at 94°C followed by 30 cycles of 1 min at 94°C; 1 min at 34°C; and 1 min at 72°C. The extension of all amplified products was completed by a final extension step of 8 min at 72°C. After amplification, PCR products were loaded onto 1.5% agarose gels supplemented with ethidium bromide (0.5 µg/mL). Electrophoresis was carried out in 1X TAE buffer at 70 V for 2 h. A Gene Ruler™ 100 bp DNA Ladder Plus (Fermentas) was used as a standard for molecular size determination. Digital images of the gels were analyzed by using GelJ software (version 2.0). The genomic template stability [GTS (%)] was calculated as follows: $100-(100\times(a/n))$, where "n" is the number of total bands in control and "a" is the number of polymorphic bands detected in B-treated samples. Polymorphism in RAPD profiles was determined with appearance of new bands and disappearance of existing bands as compared to control (Aksakal and Esim 2015).

The data sets were analyzed by one-way ANOVA followed by Tukey HSD test to compare the differences between control and treatment groups. Data normality and homoscedasticity were verified using Shapiro–Wilk and Bartlett's tests, respectively. Furthermore, a two-way ANOVA followed by Holm-Sidak post hoc test was conducted to examine the effects of treatment and tissue type for each tested parameter.

## Results and Discussion

It has been shown that high concentrations of B may adversely affect the root and shoot growth in many plants (Kalayci et al. 1998; Karabal et al. 2003; Wang et al. 2010). In our study, BA treatments significantly reduced the root and shoot growth of wheat in a concentration-dependent manner (Table 1). The results of root/shoot ratios demonstrated that root growth is more sensitive to B toxicity than shoot growth. Furthermore, BA treatments induced a significant reduction in total seedling length at the concentrations of 3 mM and above, while the reduction in total seedling weight was observed at the concentrations of 9 and 12 mM. In addition, the total chlorophyll and carotenoid contents were significantly decreased by the highest concentration of BA treatment (12 mM), as compared to control (Fig. 1a). On the other hand, other BA treatments did not affect the photosynthetic pigment concentrations. Our results are consistent with previous studies showing that B induces dose-dependent decreases in plant growth parameters (Nable 1988; Sakcali et al. 2015; Kaya and Ashraf 2015).

Many stress factors such as UV radiation, drought and high temperature are known to induce the excessive formation of ROS, and it has been demonstrated that lipid peroxidation is one of the main outcomes of ROS-mediated injury (Catalá 2009; Gill and Tuteja 2010). A number of studies in recent years have shown that B stress may also cause increases in ROS production and lipid peroxidation (Ardıc et al. 2009; Aftab et al. 2010; Kaya and Ashraf 2015). However, most of these studies have focused on B-induced oxidative stress in either leaf or shoot tissues. Therefore, there is limited information regarding whether B can lead to an increase in oxidative stress in root tissue (but see, Karabal et al. 2003; Wang et al. 2010). The results of our study demonstrated that $H_2O_2$ content in shoot tissue is markedly ($p<0.05$) enhanced by increasing concentrations of BA. On the other hand, even though a slight increment of $H_2O_2$ was

**Table 1** Effects of different concentrations of BA on growth parameters of wheat (Kıraç-66) seedlings

| Growth parameters | Control | Concentration of BA (mM) | | | | n |
|---|---|---|---|---|---|---|
| | | 3 | 6 | 9 | 12 | |
| Root length (mm) | $246 \pm 7^{d}$ | $203 \pm 7^{c}$ | $165 \pm 7^{b}$ | $132 \pm 6^{a}$ | $124 \pm 8^{a}$ | 267 |
| Shoot length (mm) | $211 \pm 4^{c}$ | $206 \pm 4^{c}$ | $202 \pm 4^{bc}$ | $188 \pm 4^{ab}$ | $182 \pm 4^{a}$ | 267 |
| Total seedling length (mm) | $457 \pm 9^{d}$ | $409 \pm 10^{c}$ | $366 \pm 9^{b}$ | $319 \pm 10^{a}$ | $306 \pm 10^{a}$ | 267 |
| Root/shoot ratio (length) | $1.17 \pm 0.03^{d}$ | $0.98 \pm 0.03^{c}$ | $0.81 \pm 0.03^{b}$ | $0.70 \pm 0.03^{a}$ | $0.67 \pm 0.04^{a}$ | 267 |
| Root dry weight (mg) | $8.1 \pm 0.3^{c}$ | $7.3 \pm 0.3^{c}$ | $6.4 \pm 0.2^{b}$ | $5.5 \pm 0.2^{a}$ | $4.7 \pm 0.2^{a}$ | 256 |
| Shoot dry weight (mg) | $20.8 \pm 0.5^{b}$ | $20.4 \pm 0.6^{ab}$ | $20.3 \pm 0.5^{ab}$ | $19.1 \pm 0.6^{ab}$ | $18.2 \pm 0.6^{a}$ | 256 |
| Total seedling dry weight (mg) | $28.8 \pm 0.7^{c}$ | $27.7 \pm 0.8^{c}$ | $26.7 \pm 0.7^{bc}$ | $24.6 \pm 0.8^{ab}$ | $23.0 \pm 0.8^{a}$ | 256 |
| Root/shoot ratio (dry weight) | $0.39 \pm 0.01^{e}$ | $0.36 \pm 0.01^{d}$ | $0.32 \pm 0.01^{c}$ | $0.29 \pm 0.01^{b}$ | $0.26 \pm 0.01^{a}$ | 256 |

Means ± SE followed by different letters in the same row are significantly different according to Tukey's HSD test ($p < 0.05$). "n" indicates the total number of sample analyzed in corresponding parameter

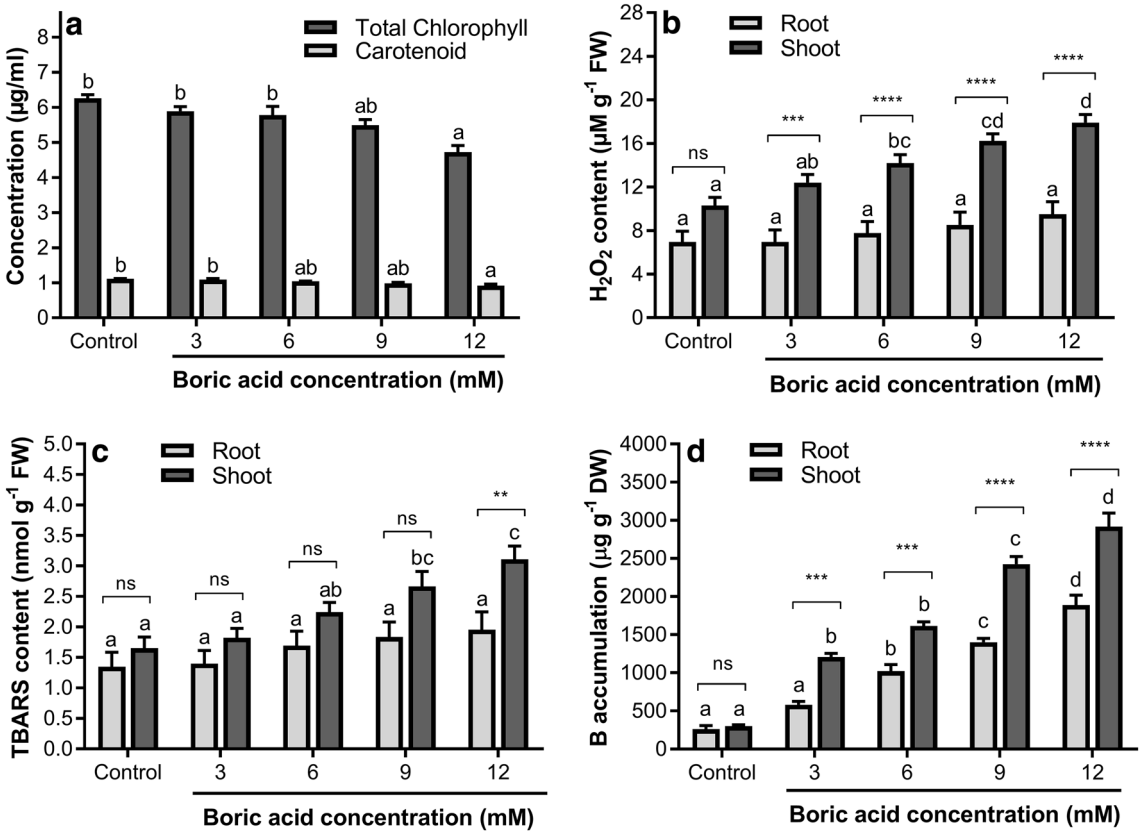


**Fig. 1** Effects of different concentrations of BA on **a** total chlorophyll and carotenoid contents, **b** $H_2O_2$ level, **c** TBARS level, and **d** accumulation of boron. Different letters indicate significant differences at $p < 0.05$ (Tukey's HSD test) between treatments for each tissue category or parameter, while asterisks and ns indicate significance of differences between tissues within the same treatment group according to Holm–Sidak test (*ns* not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$)

observed in root tissue by higher concentrations of BA, no significant difference ($p > 0.05$) was found between treatments (Fig. 1b). Furthermore, significant differences in $H_2O_2$ content were found between root and shoot tissues within the same treatment group. Except for control samples, a higher concentration of $H_2O_2$ was determined in shoot tissues (Fig. 1b). In addition, our results showed that TBARS content in shoot tissue is statistically increased by 9 and 12 mM concentrations of BA. However, none of the BA treatments significantly affected ($p > 0.05$) the TBARS content in root tissue when compared to the control (Fig. 1c). Moreover, no meaningful differences were found for TBARS content between root and shoot tissues within the same treatment group, apart from the 12 mM of BA treatment. The

present results confirm previous reports showing that B toxicity can cause oxidative stress in leaf and shoot tissues (Molassiotis et al. 2006; Cervilla et al. 2007; Ardıc et al. 2009). Our findings also support the results of Karabal et al. (2003), who showed that B stress does not affect the $H_2O_2$ and TBARS contents in roots of barley seedlings. The reason for this difference between root and shoot tissues might be related to different accumulation patterns of B. In our study, even though B accumulation in shoot and root tissues gradually increased with the increase of BA concentrations, B accumulation in shoot tissue was greater than root tissue in all the BA treatments (Fig. 1d).

The RAPD-PCR profiles of root and shoot tissues are shown in Tables 2 and 3 and Fig. 2. Of the 10 decamer oligonucleotide primers tested, all of them gave clear and reproducible bands. Total number of bands produced in the control samples of root and shoot tissues was 91 and 90, respectively. The size of these bands ranged from 280 bp (OPB-8) to 3915 bp (OPY-16) in the control samples. The RAPD profiles showed differences in terms of concentration of treatment and type of tissue for the used primers. For example, some primers resulted in no alteration of amplification products (OPA-4 and OPW-18), while some primers (OPB-8 and Primer 5) gave more complicated

**Table 2** Molecular sizes (base pair) of appearing (+) and disappearing (−) bands, polymorphism ratio and GTS value in the roots of wheat seedlings exposed to different concentration of BA

| Primer | Control | Concentration of BA (mM) | | | |
|---|---|---|---|---|---|
| | | 3 | 6 | 9 | 12 |
| OPA-4 | 7 | ND | ND | ND | ND |
| OPB-8 | 10 | +1500 | +1500; −1100 | +1500 | +1500; −1100 |
| OPH-19 | 9 | ND | ND | +580 | +580 |
| OPW-5 | 11 | ND | +900 | +900 | +900 |
| OPW-7 | 7 | ND | −730 | −730 | −730 |
| OPW-18 | 3 | ND | ND | ND | ND |
| OPY-8 | 12 | ND | ND | −1460 | −1460 |
| OPY-15 | 7 | ND | ND | ND | −900 |
| OPY-16 | 10 | −750 | −750 | −750 | −750; −2100 |
| Primer-5 | 15 | −900; −1100 | −1100 | −1100 | +620; −1100 |
| Total Band | 91 | 4 | 6 | 7 | 11 |
| Polymorphism (%) | 0 | 4.4 | 6.6 | 7.7 | 12.1 |
| GTS (%) | 100 | 95.6 | 93.4 | 92.3 | 87.9 |

*ND* Not determined

**Table 3** Molecular sizes (base pair) of appearing (+) and disappearing (−) bands, polymorphism ratio and GTS value in the shoots of wheat seedlings exposed to different concentration of BA

| Primer | Control | Concentration of BA (mM) | | | |
|---|---|---|---|---|---|
| | | 3 | 6 | 9 | 12 |
| OPA-4 | 7 | ND | −1120 | −330 | −330 |
| OPB-8 | 10 | +1200 | +1200; −1100 | +1200; −1100 | +1200; −1100 |
| OPH-19 | 9 | +932 | +932 | +932 | +932 |
| OPW-5 | 11 | ND | −750 | −750 | −750 |
| OPW-7 | 6 | ND | +726 | +726; +997 | +726; +997 |
| OPW-18 | 3 | ND | ND | ND | ND |
| OPY-8 | 12 | ND | −450 | −450 | −450 |
| OPY-15 | 7 | ND | ND | −1750 | −1750 |
| OPY-16 | 10 | ND | ND | ND | −750; −2100 |
| Primer-5 | 15 | +2000; +2500; −750 | +2000; +2500; −750; −1000 | +2000; +2500; −750 | +2000; +2500; −750; −1000 |
| Total Band | 90 | 5 | 11 | 12 | 16 |
| Polymorphism (%) | 0 | 5.6 | 12.3 | 13.4 | 17.8 |
| GTS (%) | 100 | 94.4 | 87.7 | 86.6 | 82.2 |

*ND* Not determined

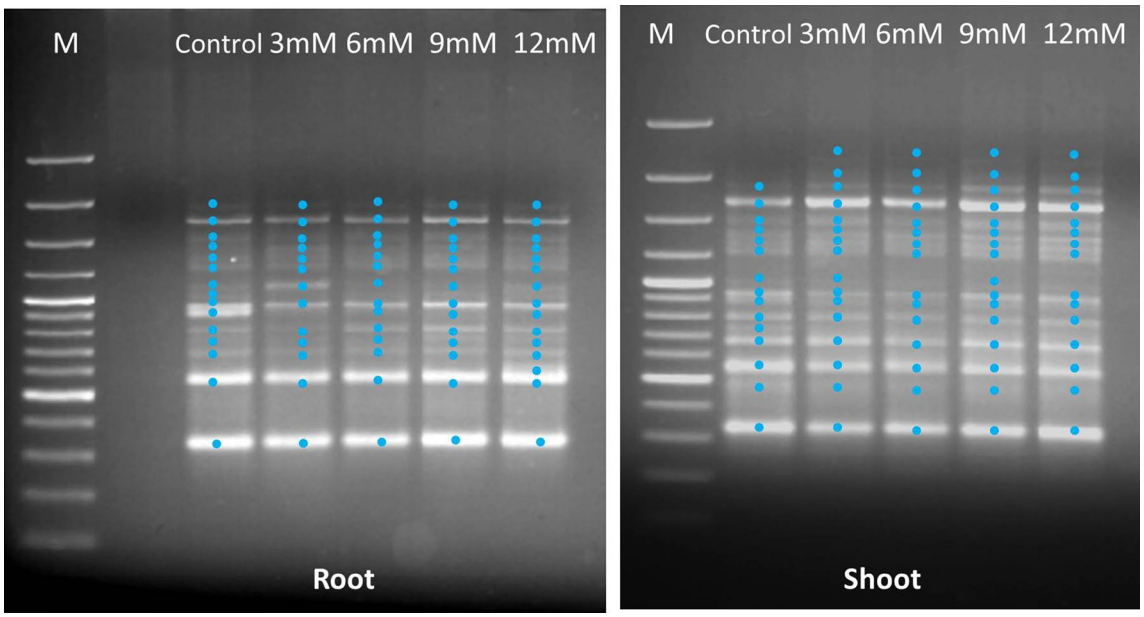


**Fig. 2** RAPD profiles (Primer-5) of genomic DNA from root and shoot tissues of wheat seedlings exposed to different concentration of BA. *M* Molecular size marker (3000, 2000, 1500, 1200, 1000, 900, 800, 700, 600, 500, 400, 300, 200 and 100 bp from top to bottom)

banding patterns (Tables 2, 3). Furthermore, the percentage of polymorphism in both root and shoot samples were increased in a concentration-dependent manner by BA. The values of genomic template stability (GTS %), which demonstrates the quantitative changes in RAPD profiles, were decreased by increasing concentration of the BA in both tissue types (Tables 2, 3). However, the reduction in GTS (%) values of shoot samples was slightly higher than that of root samples. Our data support the results of previous studies suggesting that B toxicity may exert a genotoxic effect on wheat (Kekec et al. 2010; Erdal et al. 2014). On the other hand, remarkable changes in RAPD profiles of studied wheat cultivars were observed at different concentrations of B. These differences may result from the existence of a large genotypic variation in tolerance to B toxicity in wheat (Torun et al. 2006). Moreover, Cenkci et al. (2009) suggested that B toxicity can cause different degrees of genotoxicity on root and leaf samples of bean due to different accumulation rates of B in tissues. Our results also confirmed this observation in wheat. In our study, we found that B content was remarkably lower in root tissue than in shoot tissue (Fig. 1d). This may explain the relatively lower genotoxic effect of B in root tissue of wheat.

In conclusion, the results of present study indicate that: (i) B stress has a negative effect on the growth of wheat seedlings in a dose-dependent manner; (ii) root growth is more sensitive to B toxicity than shoot growth; (iii) B accumulation in shoot tissue is significantly higher than that in root tissue; (iv) B stress leads to increases in oxidative stress parameters such as TBARS and $H_2O_2$ in shoot tissue; and (v) high concentrations of B may exert a genotoxic impact on root and shoot tissues of wheat.

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