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Influence of Boron Priming on the Antioxidant Ability of Alfalfa Seeds

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ABSTRACT

This experiment was designed to determine the relationship between the antioxidant capacity and the vigour of alfalfa seeds during boron priming. Alfalfa seeds were primed with 1.8 % (W/V) concentration of borax solution for 0, 3, 6, 12 and 24 h at 20°C. The results showed that the vigour of alfalfa seeds declined with the extension of priming time, which was closely related to the accumulation of hydrogen peroxide and malonaldehyde contents and the decrease of superoxide dismutase, catalase and peroxidase**.** The radicle might be the most sensitive site to boron priming in alfalfa seeds, but the boron-induced damage might be alleviated during alfalfa seeds' germination.

Key words: Alfalfa, Antioxidant ability, Boron, Seed priming, Seed vigour.

INTRODUCTION

Seed priming is reckoned as an easy, low cost and less risk method to improve the germination, growth and development and productivity of plants (Jisha and Puthur, 2016; Paul and Roychoudhury, 2017), hence it is routinely used by commercial seed companies to improve the vigour of their products (Zhao *et al*., 2018). Furthermore, seed priming can enhance the activities of various antioxidant enzymes and inhibit the occurrence of lipid peroxidation in plants under various unsuitable conditions (Chen and Arora, 2013; Xia *et al*., 2016). Thus it has also been regarded as a promising scheme for resisting biotic and abiotic stress in the field crops (Basra *et al*., 2005; Moulick *et al*., 2018). Therefore, the study and application in the mechanism of seed priming has become the focus in the field of seed science and technology.

Boron, as a vital microelement, is continuously demanded by vascular plants to regulate the various physiological processes in their normal life cycles, *e.g*., the metabolism of carbohydrates and RNA, the integrity of cell wall and plasma membrane, pollen tube growth and germination, floret fertility, anther and seed development (Herrera-Rodriguez *et al*., 2010; Parry *et al*., 2016; Nadeem *et al*., 2019). Current researches showed that boron can effectively improve the seedling emergence and grain yield in many plants, *e.g*., alfalfa (*Medicago sativa)* by Du *et al*. (2009), rice (*Oryza sativa*) by Atique-ur-rehman *et al*. (2014), guayule (*Parthenium argentatum)* by Zhu and Bañuelos (2016), pea (*Pisum sativum*) by Parry *et al*. (2016), wheat (*Triticum aestivum*) by Nadeem *et al*. (2019). Compared to soil application and foliar sprays, boron priming has been regarded as an economical and appropriate option to improve the productivity of various plants (Atique-ur-rehman *et al*., 2014; Iqbal *et al*., 2017). Unfortunately, both boron deficiency and enrichment can astrict the normal vegetative growth and reproductive development of plants (Kaya and Ashraf, 2015; Jr Mattos *et al*., 2017). The alteration of antioxidant enzymes is identified as an important attribute to boron-induced oxidative damage (Molassiotis *et al*., 2006). Nonetheless, there are still only a few reports on the relationship causing by boron priming between the antioxidant activity and seed vigour of plants.

Alfalfa is widely grown in arid and semi-arid regions as an excellent forage crop due to its good quality characteristics and high adaptability (Huang *et al*., 2018; Zhang *et al*., 2019). Additionally, it is also considered to an important source of nectar and pollen for honeybees around the world (Al-Kahtani *et al*., 2017). Consequently, the requirement of alfalfa seeds is also radically augmented with the expansion of their cultivation area, hence the market price of alfalfa seeds and the farmers' enthusiasm in the production of alfalfa seeds are also promoted (Zhang *et al*., 2016). However, the alfalfa seed yield remains at a relatively lower level in most regions around the world, thereby how to solve this problem becomes a worldwide challenge in alfalfa industrial production (Xia *et al*., 2019). Previous studies have showed that the seed yield of alfalfa can be effectively improved by foliar application of boron (Du *et al*., 2009). Moreover, our previous study has also found that the seed vigour of alfalfa can be availably improved by appropriate priming with boron, but the boron toxicity also

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occurs in those primed with high concentrations (Xia *et al*., 2019). Nonetheless, this is still unreported on the relationship between the antioxidant activity and seed vigour of alfalfa causing by boron priming. Thus, the objectives of the present work were to evaluate the changes antioxidant ability in different parts of alfalfa seeds, to uncover the detailed relationship between the antioxidant activity and seed vigour of alfalfa during boron priming.

MATERIALS AND METHODS

Seed source

Alfalfa (variety: Pianguan) seeds were collected by the Forage Seed Laboratory of Shanxi Agricultural University in August 2017 and then sealed in plastic bags and stored at -20°C. The experiment was launched on November 2018.

Boron priming treatments

Alfalfa seeds were soaked in 1.8% concentration (W/V) of borax solution at 20°C for 0, 3, 6, 12 and 24 h. Thereafter, seeds were rinsed two times with deionized water and then air-dried for three days at 25°C and 45% relative humidity in the dark (moisture content reached approximately 10% on fresh weight basis). Each treatment had four replicates.

Germination tests

Germination tests were performed according to the ISTA rules (Anon 2017), the number and length of normal seedlings was counted in each petri dish on day-10 and the seedlings, cotyledons and radicles were collected to analyze their antioxidant capacity. Germination percentage was calculated according to the ISTA rules (Anon 2017). Mean germination time was computed according to Ellis *et al*. (1982). Seedling vigour index were counted according to Abdul-Baki and Anderson (1973). The detailed conditions and operations of tests and the final calculations were descripted in our previous report (Xia *et al*., 2019).

Enzymes extraction and assays

Enzymes were extracted according to Kibinza *et al*. (2006). Seeds, seedlings, cotyledons and radicles of alfalfa (1 g) were ground and homogenized using a mortar and pestle in 3.5 mL of potassium phosphate buffer (0.1 mol L^{-1}, pH 7.8), which contained 0.1 mmol L^{-1} ethylenediaminetetraacetic acid, 2 mmol L^{-1} dithiothreitol, 20 % polyvinylpolypyrrolidone and 1.25 mmol L^{-1} polyethyleneglycol-4000. The resulting supernatant by centrifuging at 15,000 ×g for 15 minutes was stored for analysis of superoxide dismutase (SOD), catalase (CAT) and peroxidase (POD) activities. All steps of the extraction procedure were performed between 1 and 4°C.

SOD activity was assayed according to Rao and Sresty (2000), which was based on its ability to inhibit the photochemical reduction of nitro blue tetrazolium. One unit of the enzyme activity was defined as the amount of enzyme required to result in a 50% inhibition of the rate of NBT reduction at 560 nm.

CAT activity was measured according to Aebi (1986), which was based on the dynamic change in absorbance at 240 nm over one minute due to the decline of extinction of H_2O_2. Supernatant (0.1 mL) was mixed with 3.4 mL phosphate buffer (25 mmol L^{-1}, pH 7.0, mixed with 0.1 mmol L^{-1} EDTA) and 0.2 mL 0.1 mol L^{-1} H_2O_2. The reaction was started by adding H_2O_2.

POD activity was determined according to Kalpana *et al*. (1995), which was based on the change of absorbance recording at 470 nm at 15-second intervals for two minutes. 0.1 mL enzyme extraction was mixed into 0.5 mL O-dianisidine solution (0.2% in methanol, freshly prepared) and 2 mL 0.1 mol L^{-1} sodium-acetate buffer (pH 4.5). The reaction was started by adding 0.1 mL 0.2 mol L^{-1} H_2O_2.

Protein contents were determined by the method of Bradford (1976) using bovine serum albumin (BSA) as the standard.

Analysis of hydrogen peroxide (H_2O_2) and malondialdehyde (MDA) contents

H_2O_2 content of alfalfa seeds, seedlings, cotyledons and radicles were determined according to Patterson *et al*. (1984). The determination was depened on the changes in absorbance values of the titanium peroxide complex at 415 nm, which was calculated from a standard curve of known H_2O_2 concentrations. MDA content of alfalfa seeds, seedlings, cotyledons and radicles were determined according to Bailly *et al*. (1996), which was quantified by measuring the absorbance at 532 and 600 nm. The reaction systems contained 0.5% (w/v) 2-thiobarbituric acid and 20% (w/v) trichloroacetic acid. The homogenate was incubated at 100°C for 15 minutes, then cooled immediately and centrifuged at 4000 rpm for 10 minutes.

Statistical analyses

Data of germination percentage conformed to normal distribution and homoscedasticity, which were respectively tested by the Kolmogorov-Smirnov test and the homogeneity test of variances. Mean difference of comparisons were performed by an analysis of variance according to Panse and Sukhatme (1967), which was conducted using SPSS for Windows ver. 13.0 followed by Duncan's multiple range test (P = 0.05).

RESULTS AND DISCUSSION

Influence of boron priming on seed vigour

Seed vigour was great importance for the seedling establishment, growth and productivity of plants (Eggert and von Wirén, 2016). Germination percentage of alfalfa seeds was significantly (P<0.05) increased after 3 h of boron priming and then declined with the extension of priming time, but there was no significant (P>0.05) difference between the control (primed 0 h) and those primed 6 to 12 h (Table 1). Seed germination was not improved by strong boron priming (Farooq *et al*., 2012) and our previous results also manifested that a germination suppression of alfalfa seeds was caused by a long time of priming with high boron concentration (Xia *et al*., 2019). Mean germination time was usually used to reflect the speed and consistency of seed

germination. However, mean germination time of alfalfa seeds was increased with the extension of priming time and there was significant (P<0.05) difference between the control (primed 0 h) and those primed 6 to 24 h (Table 1). Similarly, previous reports observed that boron priming did not markedly reduce the mean germination time of wheat seeds (Iqbal *et al*., 2017), but suppressed the emergence of rice seeds (Farooq *et al*., 2011). This might be due to the delay in cells activation by a long period of low water potential during priming (Xia *et al*., 2017). Seedling vigour index generally reflected the ability of seedling establishment during plant growth. In this study, seedling vigour index of alfalfa seeds was significantly (P<0.05) declined with the extension of priming time (Table 1), this was similar to our previous results (Xia *et al*., 2019). Excessive boron priming could not provide sufficient energy for seeds in time to complete their germination and seedling growth (Chen and Arora, 2013). In summary, the vigour of alfalfa seeds declined with the extension of priming time in this boron concentration.

Influence of boron priming on lipid peroxidation

Reactive oxygen species were strong oxidizing species that caused oxidative damage to lipids and MDA had been utilized as a suitable biomarker for lipid peroxidation (Xia *et al*., 2016). In this study, H_2O_2 and MDA contents were significantly (P<0.05) increased with the extension of boron priming time in the seedlings, cotyledons, radicles and seeds (Table 2 and 3), suggesting that boron toxicity increased with the extension of boron priming time in the seedlings, cotyledons, radicles and seeds. Similar results were observed in the shoot tips culture of apple rootstock (*Malus domestica*) by Molassiotis *et al*. (2006) and barley (*Hordeum vulgare*) seedlings by Karabal and Yücel (2003). These might be an important reason for the decline in vigour of alfalfa seeds. At the same boron priming time, H_2O_2 and MDA contents of alfalfa seeds were significantly (P<0.05) higher than their seedlings, manifesting that H_2O_2 accumulation and lipid peroxidation induced by boron priming were repaired in alfalfa seeds during their germination. However, H_2O_2 and MDA contents of alfalfa seedlings were significantly (P<0.05) higher than their cotyledons and were significantly (P<0.05) lower than their radicles, which indicated that the radicle of alfalfa seeds suffered more serious boron damage during priming. The radicle was the most vulnerable part of seeds to reactive oxygen species due to locate at the funicular end of most seeds (Xia *et al*., 2017).

Influence of boron priming on antioxidant enzymes activities

Antioxidant enzymes played a crucial role in the protection

Table 1: Changes of germination in alfalfa seeds with boron priming.

Time (h)	Germination index		
	Germination percentage (%)	Mean germination time (h)	Seedlings vigour index
0	74.0±0.94bc	1.59±0.04d	51.99±0.58a
3	83.0±0.67a	1.69±0.01cd	50.77±0.13b
6	77.5±1.00b	1.81±0.03c	38.03±0.12c
12	71.5±1.11c	2.36±0.06b	30.59±0.15d
24	62.5±0.75d	2.71±0.02a	25.26±0.10e

Note: Means in the same column with different small letters are significant difference (P<0.05).

Table 2: Changes of H_2O_2 contents in alfalfa seeds and seedlings with boron priming (nmol g^{-1} DW).

Parts	Time (h)				
	0 (Control)	3	6	12	24
Seedlings	16.25±0.69Ce	25.29±0.57Cd	33.55±0.86Cc	38.78±0.92Cb	47.74±1.16Ca
Cotyledons	13.47±0.31Ce	20.44±0.87Dd	27.37±0.58Dc	35.56±0.54Cb	41.10±0.50Da
Radicles	26.32±1.01Be	34.60±0.52Bd	41.31±0.42Bc	45.09±0.65Bb	55.70±0.82Ba
Seeds	41.00±0.45Ae	46.54±0.59Ad	57.51±0.96Ac	64.28±0.70Ab	69.36±0.83Aa

Note: Means in the same column with different capital letters are significant difference (P<0.05), in the same row with different small letters are significant difference (*P*<0.05).

Table 3: Changes of MDA contents in alfalfa seeds and seedlings with boron priming (µmol mg^{-1} protein).

Parts	Time (h)				
	0 (Control)	3	6	12	24
Seedlings	5.51±0.15Be	6.53±0.19Cd	8.56±0.05Cc	9.28±0.14Bb	10.20±0.17Ba
Cotyledons	4.15±0.04Cd	5.49±0.13Dc	6.45±0.02Db	6.56±0.03Cb	8.32±0.07Ca
Radicles	7.18±0.11Ae	8.52±0.08Bd	10.19±0.06Bc	11.37±0.16Ab	12.63±0.05Aa
Seeds	7.33±0.08Ae	9.40±0.13Ad	10.40±0.03Ac	11.86±0.06Ab	12.49±0.05Aa

Note: Means in the same column with different capital letters are significant difference (*P*P<0.05), in the same row with different small letters are significant difference (P<0.05).

Table 4: Changes of SOD activities in alfalfa seeds and seedlings with boron priming (U mg^{-1} protein).

Parts	Time (h)				
	0 (Control)	3	6	12	24
Seedlings	16.64±0.21Bb	18.69±0.19Ba	13.48±0.14Bc	10.15±0.17Bd	7.26±0.09Be
Cotyledons	10.97±0.26Cb	13.17±0.27Ca	9.65±0.12Cc	8.31±0.05Cd	5.68±0.06Ce
Radicles	19.89±0.28Ab	23.77±0.23Aa	15.29±0.12Ac	12.15±0.13Ad	9.12±0.11Ae
Seeds	6.50±0.16Dc	8.46±0.07Da	7.40±0.13Db	6.15±0.05Dc	5.50±0.15Dd

Note: Means in the same column with different capital letters are significant difference (P<0.05), in the same row with different small letters are significant difference (P<0.05).

Table 5: Changes of CAT activities in alfalfa seeds and seedlings with boron priming (µmol min^{-1} mg^{-1} protein).

Parts	Time (h)				
	0 (Control)	3	6	12	24
Seedlings	11.92±0.12Bb	14.49±0.07Ba	11.75±0.16Ab	8.34±0.14Bc	6.59±0.23Bd
Cotyledons	10.76±0.10Cb	13.32±0.15Ca	10.69±.013Bb	7.95±0.11Bc	6.11±0.10Bd
Radicles	12.94±0.17Ab	15.63±0.15Aa	12.22±0.24Ac	10.26±0.17Ad	7.51±0.16Ae
Seeds	7.26±0.11Db	8.46±0.21Ca	6.41±0.22Cc	5.52±0.13Dd	5.41±0.14Cd

Note: Means in the same column with different capital letters are significant difference (P<0.05), in the same row with different small letters are significant difference (P<0.05).

Table 6: Changes of POD activities in alfalfa seeds and seedlings with boron priming (µmol min^{-1} mg^{-1} protein).

Parts	Time (h)				
	0 (Control)	3	6	12	24
Seedlings	21.29±0.07Bc	23.37±0.17Bb	25.45±0.10Ba	17.32±0.12Cd	15.24±0.13Be
Cotyledons	15.89±0.05Cd	17.45±0.12Db	22.61±0.15Ca	16.78±0.08Cc	13.35±0.13Ce
Radicles	22.49±0.18Ac	24.22±0.17Ab	26.51±0.13Aa	20.61±0.21Bd	16.18±0.15Ae
Seeds	21.26±0.14Bc	22.49±0.18Cb	25.65±0.16Ba	21.47±0.17Ac	16.25±0.04Ad

Note: Means in the same column with different capital letters are significant difference (P<0.05), in the same row with different small letters are significant difference (P<0.05).

against oxidative stress for plants. The enzymes of SOD, CAT and POD were involved in scavenging reactive oxygen species during boron-induced oxidative damage (Molassiotis *et al*., 2006) and their activities were closely related to the vigour of primed seeds (Xia *et al*., 2016). In this study, the SOD and CAT activities reached the highest level in seeds, seedlings, cotyledons and radicles at 3 h of boron priming, but POD activities retained the highest level in the seeds, seedlings, cotyledons and radicles at 6 h of boron priming (Table 4, 5 and 6). These results indicated that the activities of SOD, CAT and POD were improved in the seeds, seedlings, cotyledons and radicles at the initial stage of boron priming, which might be stimulated by a controllable accumulation of H_2O_2 to mild boron toxicity. Nonetheless, the activities of SOD, CAT and POD then declined with the extension of boron priming time (Table 4, 5 and 6), these manifested that the activities of SOD, CAT and POD were all inhibited in the seeds, seedlings, cotyledons and radicles by excessive boron priming. The severe oxidative damage caused by an immoderate accumulation of H_2O_2 might be an important attribute to the depression of SOD, CAT and POD activities in the seeds, seedlings, cotyledons and radicles (Molassiotis *et al*., 2006). At the same boron priming time, the SOD, CAT and POD activities of alfalfa seeds were significantly (P<0.05) lower than their seedlings, cotyledons and radicles (Table 4, 5 and 6), indicating that the scavenging capacity of H_2O_2 was enhanced during seed germination of alfalfa, hence the H_2O_2 and MDA contents of alfalfa seeds were also significantly (P<0.05) higher than their seedlings, cotyledons and radicles (Table 2 and 3). Nevertheless, the SOD, CAT and POD activities of alfalfa seedlings were significantly (P<0.05) higher than their cotyledons and were significantly (P<0.05) lower than their radicles (Table 4, 5 and 6), which was similar to their H_2O_2 and MDA contents (Table 2 and 3). These results showed that the radicle of alfalfa seedlings had a stronger ability to remove the accumulation of H_2O_2 generating by boron toxicity, which might be due to the radicle of alfalfa seeds was more severely attacked by H_2O_2.

CONCLUSION

The enhancement of lipid peroxidation by excessive accumulation of H_2O_2 was an important reason for the decline in the vigour of alfalfa seeds during high strength boron priming, which was closely related to the decrease of SOD, CAT and POD activities. The radicle of alfalfa seeds might be the most serious site of boron-induced damage during priming and the oxidative damage causing by boron

priming was repaired during the germination of alfalfa seeds.

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