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Alleviation of drought stress effects on red bean by ultrasonication and foliar application of 24-epi-brassinolid

A. Younesian[a], H. Ajam Norouzi[a,*], M. Gholipoor[b]

[a]*Department of Agriculture, Gorgan Branch, Islamic Azad University, Gorgan, Iran.*
[b]*Faculty member of Shahrood University of Technology, Iran.*
*Corresponding author. E-mail: Ajamnorozei@gorganiau.ac.ir

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Abstract

The two-location field experiment was conducted to study the possible alleviation of drought stress effects on red bean by ultrasonication and 24-epi-brassinolid. Locations were Agricultural Research Center in Shahrood, Iran and the other in bean farm, 40 km off Shahrood city in 2015. Experiment factors included irrigation of main plots at three levels of normal irrigation (60 mm evaporation from evaporation pan), mild stress (90 mm evaporation from evaporation pan) and severe stress (120 mm evaporation from evaporation pan). Stress levels were applied after 4-leaf stage and ultrasound waves treatments (in two levels of nonuse of seeds irradiation and use of irradiation for 3 minutes at 32 °C) and 24-epi- brassinolid foliar application (in two levels of nonuse of foliar application and foliar application at a rate of 0.1 mg/L at 50% flowering during two stages) which were located in sub-plots. The evaluated properties included grain yield and contents of superoxide dismutase, peroxidase, catalase, proline and ascorbate. The results showed that with severe water stress (comparison of severe stress and lack of stress), grain yield showed a significant decrease in both experiment sites, however 24-epi-brassinolid foliar application and use of ultrasonic waves in both normal and stress conditions increased the grain yield. The same condition was established for evaluated enzymes. Thus it could be stated that irradiation of ultrasonic waves and 24-epi-brassinolid foliar application for cultivating beans play important role in increment of competitive strength of plant in water deficit condition.

Keywords: Irradiation; Catalase; Superoxide dismutase; Ascorbat; Proline; Proxidase.

Introduction

Red bean (*Phaseolus vulgaris* L.) is an annual legume which has high nutritional value for human being. The production of this plant is highly decreased in arid and semi-arid regions due to drought stress (Szilagyi, 2003). Sing (2007) studied the effects of drought stress on beans and reported that average yield reduction under drought stress was 60% and grain weight loss was 14%. Szilagyi (2003) stated that drought results in reduction of biomass, grain yield, harvest index and grain weight.

Brassinosteroids (BRs) are a class of plant polyhydroxysteroids that have been recognized as a kind of phytohormones and play essential roles in plant development. BRs occur at low concentrations in lower and higher plants. BRs are essential for normal plant growth, reproduction and development. They play critical roles in a variety of physiological responses in plants, including stem elongation, pollen tube growth, leaf bending and epinasty, root growth inhibition, ethylene biosynthesis, proton pump activation, vascular differentiation, nucleic acid and protein synthesis and photosynthesis (Hayat et al., 2010). They also play a significant role in amelioration of various abiotic and biotic stresses, such as cold stress, water deficit, salt injury, oxidative damage, thermal stress, heavy metal stress and pathogen infection. Despite the correlation between oxidative stress and BR level in plants, the physiological rationale for such alteration in BR level is little known (Bajguz and Hayat, 2009).

When water-stressed maize (*Zea mays*) seedlings treated with brassinolide (BL), the activities of SOD, CAT and APX, ascorbic acid and carotenoid contents increased (Li et al., 1998). On the other hand, BRs enhanced the activity of CAT and reduced the activities of peroxidase and ascorbic acid oxidase of osmotic-stressed sorghum (*Sorghum vulgare*) (Vardhini and Rao, 2003).

The ultrasonication-resulted stimulation in germination has been reported for many plants seed including carrot, radish, maize, barley, rice and sunflower (Fl´orez et al., 2006; Yaldagard et al., 2008). The type and amount of ultrasonication effects on seed germination depend on frequency and exposure time and appear to vary widely between the different species and cultivars. Yaldagard et al. (2008) indicated that mild irradiation on barley seeds produced an accelerated germination proportional to an increase in α-amylase activity. Another possible mechanism for such enhancement of seed germination is the mechanical or shear effects due to the large and rapid oscillations in bubble size (microstreaming), which leads to disruption of plants cell walls, thereby increasing water uptake by the cell/seed (Gaba et al., 2008). Machikowa et al. (2013) showed that seeds irradiation with ultrasonic waves will strengthen the seedling of sunflower.

The published reports regarding the possible alleviating effect of ultrasonication on drought-stressed plants at adult-stages of development are scarce, as so far the researches have been focusing on ultrasonication effects on germination and early seedling growth. The present two-location experiment was aimed at exploring whether the ultrasonication and 24-epi-brassinolid foliar application can have any role in ameliorating water deficit effects in red bean.

Materials and Methods

The experiment, as split factorial based on randomized complete block design with three replications, was conducted simultaneously in two locations, one in the research field located in Agricultural Research Center in Shahrood, Iran and the other in bean farm, 40 km off Shahrood city in 2015. The 3 irrigation levels was arranged in main plots and the factorial arrangement of 2 ultrasonication levels and 2 levels of 24-epi-brassinolid foliar application were devoted to subplots. The irrigation levels were normal irrigation (60 mm evaporation from evaporation pan), mild stress (90 mm evaporation from evaporation pan) and severe stress (120 mm evaporation from evaporation pan).

Plants were subjected to drought after complete establishment in the field (4-leaves stage). For ultrasonication, seeds wave irradiated for 3 minutes with constant frequency of 24 kHz of ultrasounic waves at 32 °C, using ultrasonic bath (digital ultrasonic, Model 4820-CD) with; the non-irradiated seeds were considered as control. Immediately, seeds were moved to the farm for planting. The 24-epi-brassinolid was used as foliar application at the rates of 0 (control) and 0.1 mg/L at 50% flowering. The drought levels were attributed to main plots and the factorial arrangement of ultrasonication and 24-epi-brassinolid were devoted to subplots.

Seeds were planted by hand in May 11th. Each plot consisted of 4 planting lines with length of 4 meters. The distance between lines and plants were 60 and 5 centimeters, respectively. It should be noted that farm in both locations has not been used in previous year. The soil-experiment indicated that the type of soil was loam. The studied traits included grain yield, activity of superoxide dismutase, peroxidase, catalase and contents of proline and ascorbate.

Enzyme Measurement

Peroxidase activity was determined at 25 °C with a spectrometer (PD-303UV) at 470 nm using guaiacol as the substrate and H_2O_2 as the hydrogen donor (Ponce et al., 2004). Extracts preparing with 10 grams of leafy vegetables was chopped and then 30 ml of distilled water added during homogenation. The slurry was centrifuged (SIGMA-3K30) at 10000g for 15 min at 4 °C. The supernatant, which contained peroxidase activity, was used as the enzyme source for the experiment. The substrate mixture contained 10 ml of 1% guaiacol, 10 ml of 0.3% hydrogen peroxide and 100 ml of 0.05 M sodium phosphate (pH 6.5) buffer. The reaction cuvette contained 2.87 ml substrate mixture, 0.1 ml crude extract and 0.03 ml treatment solution (essential oils, ascorbic acid and water) in a total volume of 3 ml. (Ponce et al., 2004).

Superoxide dismutase (SOD, EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium (NBT) as described by Becana et al. (1986). The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.8), 0.1 mM EDTA, 14.3 mM methionine, 82.5 mM NBT, 2.2 mM riboflavin and 50 ml enzyme extract. The reaction was initiated by placing the test tubes under 15W fluorescent lamps. The reaction was terminated after 10 min by removing the reaction tubes from the light source. Non-illuminated and illuminated reactions without supernatant served as calibration standards. The absorbance was read at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of NBT reduction under assay conditions (Becana et al., 1986).

Catalase (CAT, EC 1.11.1.6) activity was assayed as described by Chance and Maehly (1955). The reaction mixture consisted of phosphate buffer (pH 6.8), 0.1M H_2O_2 and 1.0ml enzyme extract as a substrate. Changes in absorbance of the reaction solution at 240 nm were sequentially read every 20 s for 1 min. The disappearance of H_2O_2 was detected by titrating the reaction mixture against 0.1 N potassium permanganate solution. The reaction mixture without enzyme was treated as blank. One unit of CAT activity was defined as that amount of enzyme which breaks down 1 mmol of H_2O_2 per minute under the described assay conditions (Chance and Maehly, 1955).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was assayed according to the method of Ramel et al. (2009) by monitoring the rate of ascorbate oxidation at 290 nm (e, 2.8 mM^{-1} cm^{-1}). The reaction mixture (3 ml) contained 1.5 ml of 0.1 M potassium phosphate buffer (pH 6.8), 0.5 ml of 6 mM ascorbate, 0.5 ml of 12 mM H_2O_2 and 0.5 ml of enzyme extract (Ramel, 2009).

Proline was determined by the method of Bates et al. (1973). Fresh leaves were extracted in sulphosalicylic acid, an equal volume of glacial acetic acid and ninhydrin solutions were added to the extract. The sample was heated at 100 ^{o}C and then 5 ml of toluene were added. The absorbance of the toluene layer was read at 528 nm, on a spectrophotometer. Proline (Sigma) was used for the standard curve (Bates et al., 1973). Analysis of variance and mean comparison were performed using PROC ANOVA of SAS (version 9.1.3, 2004).

Results and Discussions

Grain yield

The results of analysis of variance showed that locations were statistically the same for grain yield (Table 1). But the interaction of drought stress and location were significant; the value of grain yield of non-stressed plants was higher in research center than Shahrood farm (data not shown); on the other hand, that of high-stressed plants was higher in Shahrood farm compared to research center. The grain yield was also significantly affected by simple and interactive effects of ultrasonication and brassinolid application; the effect of brassinolid on grain yield was more noticeable on irradiated plants than not-irradiated ones (Figure 1). The ultrasound; additionally, the ultrasonication-resulted increase in grain yield was more remarkable in non-stressed plants than stressed ones (Figure 1), as ultrasonication-drought interaction was significant. These findings indicate that both ultrasonication and brassinolid can alleviate drought stress effects, especially when they are used together.

Water deficit causes oxidative stress which interferes physiological functions of cells. Because of the generation of reactive oxygen species in cell culture, this stress leads to oxidative damages similar to superoxide anion, hydrogen peroxide, hydroxyl radicals. To vanish these toxic species, there is need to very effective antioxidant system (enzymatic and non-enzymatic system) in plant cells (Mozaffari, 2004). It has been reported that the ultrasonic beam affects the biomolecules through structural changes, oxidation and free radical formation, such as superoxide anion, hydrogen peroxide and hydroxyl radicals and so provides the ground for changes in developmental characteristics (Hamed et al., 2008).

Rawling et al. (2001) conducted a trial on soybean under heat radiation and x neutrons treatments. They found that significant genetic variety will be generated under the influence of ray in grain yield, plant height, maturity time and seed size. Besides, certain mutations have been successfully applied by breeders to change the genetic structure of canola and mustard and mutations were identified with favorable economic characteristics such as plant height, number of pods per plant, number of seeds per pod, seed weight, high yield, oil content and disease resistance (Javed et al., 2003).

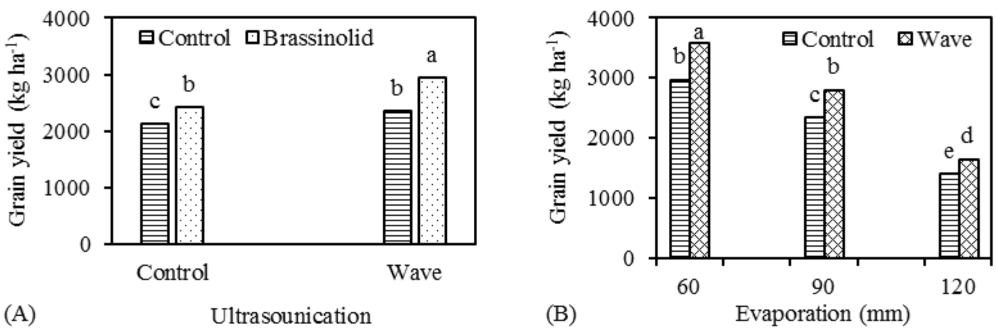

Figure 1. The interactive effects of ultrasonication and brassinolid application (A) and of drought stress and ultrasonication (B) on grain yield.

Superoxide Dismutase (SOD)

Despite location, the simple effects of drought stress, ultrasonication and brassinolid were significant on SOD (Table 1). Among interactions, only interactive effect of drought and ultrasonication was significant; the ultrasonication increased the activity of SOD under drought-stressed but not under non-stressed conditions (Figure 2). The effect of brassinolid appeared to be increasing on SOD (Figure 2).

Ionizing beams enter into tissue and cells react with different atoms and molecules and produce free radicals in cells. Depending on the intensity of the beam, positive or negative changes in morphological, physiological and biochemical processes occur in plants (Kiang et al., 2008). When maize (*Zea mays*) seedlings treated with brassinolide (BL) were subjected to water stress, the activities of SOD, CAT and APX, as well as ascorbic acid and carotenoid contents increased (Li et al., 1998).

Table 1. Mean squares of traits of red bean.

SOV	df	Grain yield	SOD	Proxidase	Catalase	Proline	Ascorbat
Location (L)	1	21993	8199	681*	299*	62	5330*
$Error_1$	4	334731	31882	116	144	236	1240
Drought (D)	2	18543031**	65994**	4066**	6061**	1039**	146502**
D*L	4	894961**	2851	2566**	1222**	205**	7063**
$Error_2$	6	143122	2263	223	284	6	1457
wave (U)	1	2526085**	22396**	2325**	1677**	2652**	5551*
Brassinolid (B)	1	3410201**	9976*	2023**	1367**	776**	19011**
D*U	2	200219*	5526*	1024**	544**	84**	367
D*B	2	125389	3242	294	202	16	1075
B*U	1	373899*	1713	201	338*	21	183
L*U	1	9419	2729	65	60	16	161
L*B	1	1874	1939	92	57	8	1726
B*U*D	2	71404	2622	163	31	22	1389
L*B*U*D	9	56830	1325	109	74	4	307
$Error_3$	40	65999	1725	150	73	10	912

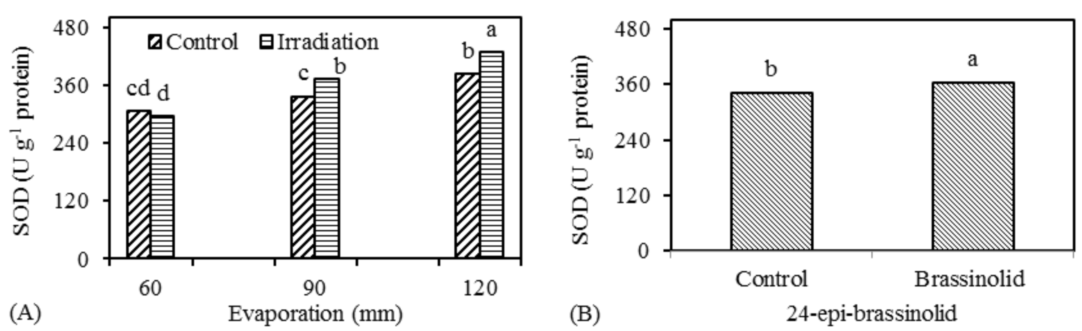

Figure 2. The interactive effects drought stress and ultrasonication (A) and the effects of brassinolid (B) on SOD.

Peroxidase

Results of analysis of variance indicated that locations are different in terms of peroxidase activity (Table 1); its activity was lower in research center that other location (data not shown). The simple effects of factors as well as interactive effects of drought and ultrasonication appeared to be significant on activity of this enzyme (Table 1). Brassinolid caused an increase (13%) in activity of peroxidase (Figure 3). Proxidase activity tended no to be affected by ultrasonication in non-stressed conditions but its activity was positively affected under both moderate and severe stress conditions (Figure 3).

Destructive processes of membrane become activated in stress condition and leads to peroxidation of membrane lipids. Brassino steroids affect the composition of fatty acids and membrane permeability and has positive effect the concentration of solutes. Some researchers have shown that the activity of antioxidant enzymes, especially peroxidase will increase after irradiation to cope with the damage caused by oxidative stress (Hamed et al., 2008). Thus it can be stated that use of radiation in increasing competitiveness strength of plant is effective in the event of stress.

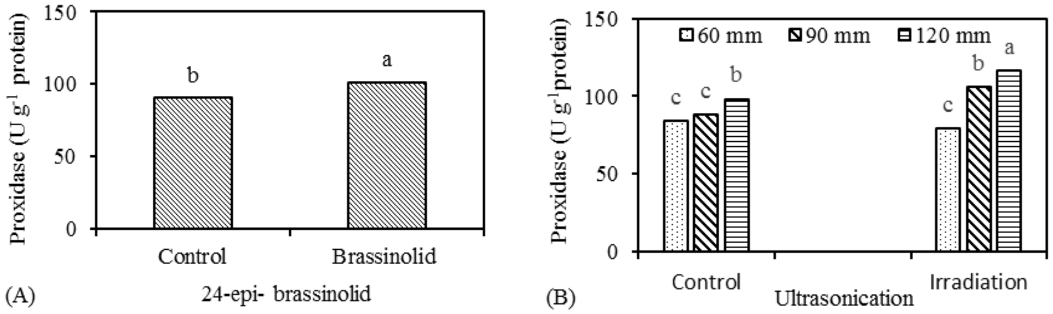

Figure 3. The effect of brassinolid (A) and the interactive effects of ultrasonication and drought (B) on activity of peroxidase.

Catalase

The interactive effects of location and drought stress were significant (Table 1); under severe stress conditions, the activity of catalase was higher research center than other location; but under control and moderate stress conditions, the activity of enzyme was statistically similar in both locations (data not shown). In addition to simple effects of all 3 factors, the interactive effects of drought and ultrasonication and of brassinolid and ultrasonication were found significant (Table 1). The result of mean comparison revealed that only under severe drought conditions, the increasing effect of ultrasonication was significant on catalase activity (Figure 4). In non-irradiated plants, the activity of this enzyme was higher for brassinolid-treated than non-treated conditions; but in irradiated plants, the activity of the enzyme was statistically the same for levels of brassinolid (Figure 4).

Vani and Ennis (2008) examined the seeds of one variety of chickpea under 750, 1000 and 1250 Gray of gamma rays treatments and selection for desirable agronomic traits was implemented in several stages. They found that bushes under the 750 Gray level had higher Thousand Kernel Weight and yield compared to parent. Rice seedlings exposed to saline stress and treated with BR showed a significant increase in the activities of CAT, SOD and glutathione reductase (GR) and a slight increase in APX (Núñez et al., 2003).

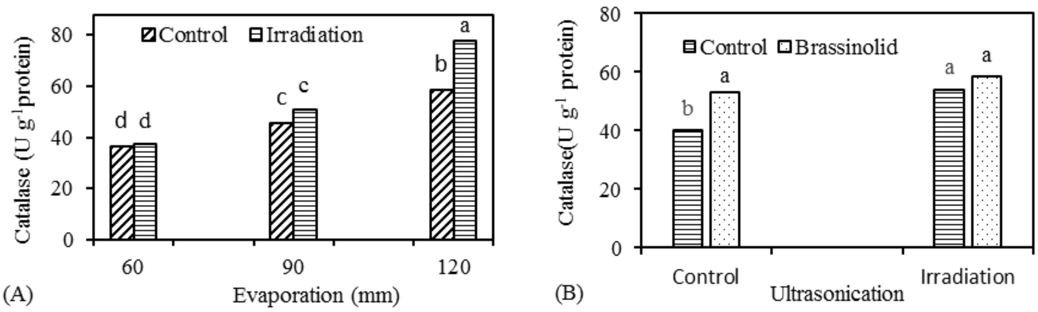

Figure 4. The interactive effects of drought and ultrasonication (A) and of ultrasonication and brassinolid (B) on activity of catalase.

Proline

The interactive effects of drought and location was significant on proline content; under non-stressed conditions, the proline content was lower in research center than Shahrood field; but under stressed conditions, two locations were statistically similar (data not presented). The interactive effects of drought and ultrasonication were also significant; the increasing effect of ultrasonication was more remarkable in stressed than non-stressed conditions (Figure 5). The simple effect of brassinolid was significant on this trait; the proline content was higher in brassinolid-sprayed plant than control (Figure 5).

Plants can absorb water until they have lower water potential than environment. Usually the major part of osmotic regulation may continue through increment of increased concentration of different dissolved substances such common sugars, organic acids, ions especially potassium. Cytosol enzymes in high concentrations of ions are strongly prevented and so ions mostly accumulate within vacuoles, where they are not in contact with cytosol enzymes or intercellular organelles. Because of this type of allocation of compatible solutions which do not interfere with enzyme functions, they accumulate in the cytoplasm for balancing water potential in cells. These types of materials such as glycine betaine, proline and poly L, create a compatible environment for macromolecules, especially proteins (kafi, 1995). Proline accumulation has positive and direct relation with increased resistance to water drought and salinity stress induced in plants (Saneoka, 2004) which was consistent with the results of our study and we observed significant increase in amount of proline in water deficit stress. Similar report is available about water deficit stress on wheat, corn and rice which indicated that proline accumulation in cytoplasm acts like a smotricom in macromolecular structural protection in an environment where ionic equilibrium has been disturbed (Nayyar, 2003). It seems that 24-epi-brassinolid foliar application in present paper has increased osmotic adjustment during stress. Proline increase results in inflation maintenance and reduction of membrane damage in plants and so tolerance to water deficit stress will increase by osmotic adjustment method (Pandey and Agarwal, 1998). Accumulation of soluble sugar into the cells plays important role in osmotic adjustment and helps to decrease water potential of cell and keep much amount of water into the cells and maintain turgor under water stress conditions.

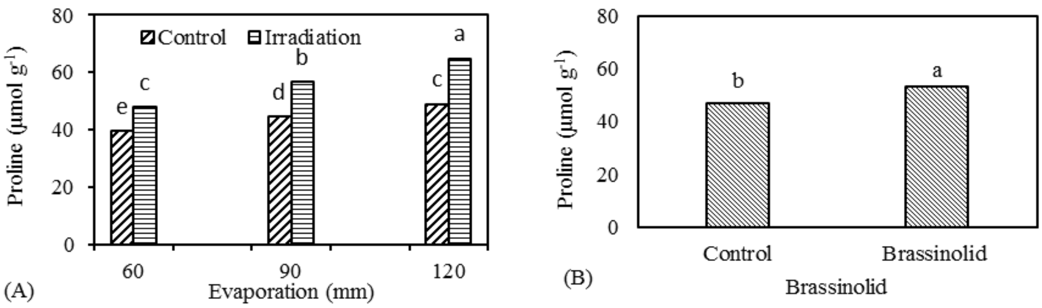

Figure 5. The interactive effects of drought and ultrasonication (A) and the simple effect of brassinolid (B) on proline content.

Ascorbate

The interaction of drought and location appeared to be significant on ascorbat content (Table 1); the value of this trait was statistically the same for moderate stress and control in Shahrood filed and research center, respectively; for other levels of drought stress, locations were statistically different (data not shown). The simple effect of both factors ultrasonication and brassinolid was found to be significant (Table 1); both of them affected ascorbate content positively (Figure 6).

It is now well known that salinity exerts oxidative stress due to the production of variety of active oxygen species (AOS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-) radicals, which cause oxidative damage in plants (McCord, 2000). To scavenge these toxic species, plants develop antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POX), ascorbate peroxidase (APOX), catalase (CAT) and glutathione reductase (GR). Since their activities and transcripts are altered when plants are subjected to stress, changes in the levels of antioxidant enzymes have been used to assess the effect of different stressors including salinity (Filiz et al., 2004).

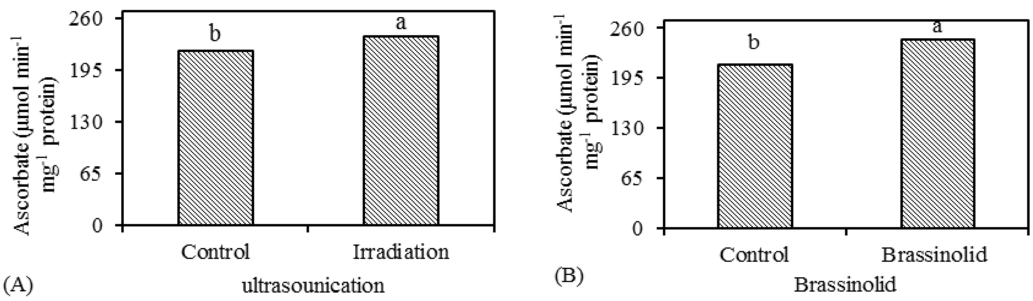

Figure 6. The effect of ultrasonication (A) and brassinolid (B) on ascorbate content.

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