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Effect of beta-aminobutyric acid (BABA) on enzymatic and non-enzymatic antioxidants of *Brassica napus* L.under drought stress

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Abstract

Water stress is one of the most important environmental factors that affect plant growth and development, and limit plant production. Many regions in Iran suffer from water deficit. To overcome these limitations for improving crop yield, it is important to increase stress tolerance of crops, such as *Brassica napus* L. The broad spectrum protective effect of the non-protein amino acid *β*-aminobutyric acid (BABA) against numerous plant stresses has been well-documented in the literature. This research shows a possibility to increase plant tolerance for drought stress through effective priming of the preexisting defense pathways in rapeseed plant. Pretreatment of plants with BABA increased ascorbate, anthocyanin and flavonoid content, while decreased DHA content in water stress plants. Calcium content was significantly increased in BABA treated plants. In drought stress condition, activity of SOD, APX and POD were elevated over the controls, while CAT activity decreased. In plants which pretreated with BABA and then exposed to drought stress the activity of mentioned enzymes increased.

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Introduction

Water stress is one of the most important environmental factors that affect plant growth and development, and limit plant production (Jiang and Zhang, 2004). The detrimental effect of drought stress is reductions in yield as reported in crops such as rice (*Oryza sativa*) (Brevedan and Egli, 2003), wheat (*Triticum aestivum*) (Cabuslay *et al*., 2002), soybean (*Glycine max*) (Kirigwi *et al*., 2004), and chickpea (*Cicer aerietum*) (Khanna-Chopra, 2004). Many regions in Iran suffer from water deficit. In semi-arid environments, dry-land farming often exposes crops to drought stress. Although some plant species are well adapted to drought, most crops are not. To overcome these limitations for improving crop yield, it is important to increase stress tolerance of crops, such as *Brassica napus* L. Plants have different mechanisms to avoid water deficit. One of these responses is production of abscisic acid (ABA) that, in turn, elevates cytosolic Ca^{2+} concentrations in guard cells leading to stomatal closure (Baisak *et al*., 1994). Decrease in CO2 concentration leads to NADP limitation and under this condition, O2 acts as an alternative electron acceptor resulting in the formation of the molecules are called reactive oxygen species (ROS) (Sairam *et al*., 1995).

Lipid peroxidation and membrane destruction are the harmful effects of ROS. Oxidative damage in the plant tissue is alleviated by a concerted action of both enzymatic and non-enzymatic antioxidant metabolisms. These mechanisms include β-carotenes, ascorbic acid (AA), α-tocopherol, reduced glutathione (GSH) and enzymes including SOD, peroxidase (POX), ascorbate peroxidase (APX), catalase (CAT), polyphenol oxidase (PPO) and glutathione reductase (GR) (Abdul Jaleel *et al*., 2007). There are many reports in previous literature that prove the relation between enhanced antioxidant enzymes activity and increased resistance to water deficit in several plants like, rice (Guo *et al*., 2006), wheat (Khanna-Chopra and Selote, 2007), Kentucky bluegrass (Bian and Jiang, 2009). The non-protein amino acid beta-aminobutyric acid (BABA) enhances Arabidopsis resistance to microbial pathogensand abiotic stresses (Jakab et a., 2005; Ton and Mauch-Mani, 2004; Zimmerli *et al*., 2008; 2001; 2000). BABA acts through potentiating of ABA-dependent signaling pathway and salicylic acid dependent defense mechanism (Cao *et al*., 2009). However, little is known about the role of BABA in antioxidant metabolisms under drought stress. Therefore the objective of this study was to investigate the Influence of BABA on Enzymatic and Non-enzymatic Antioxidants under drought stress.

Materials and methods

Plant material and drought stress applications,

The seeds of *Brassica napus* L. Cv. Madonna were collected from Karaj Agricultural Center. The seeds sterilized with 0.1% sodium hypochlorite solution for 5 min. then the seeds washed two times with deionized water to remove sodium hypochlorite. Four seeds were sown in each pot of 30 cm×30 cm containing perlit. Seedlings were irrigated with Hoagland solution for 28 days. Pots were irrigated with ground water 1-day interval as a control. Mild drought stress (D1), the plants were watered every three days and for Severe drought stress (D2), the plants were watered every four days. Drought was induced by stopping to water 4 week-old plants 1 day after BABA (Sigma-Aldrich) (0, 300μM) treatment by soil drench. At the end of experiment, the leaves and roots of plants were harvested and immediately were frozen in liquid nitrogen and stored at -80°C for the future analysis.

Mineral content

For the determination of the total amounts of Ca^{2+}, dry shoot (0.25 g) was treated with nitric acid, and diluted with distilled water. Total Ca^{2+} contents were directly measured by an atomic absorption spectrophotometer (mg g^{-1}dw).

Non-enzymatic antioxidants

Ascorbic acid (ASA) content, The ASA content was assayed as described by De Pinto (1999). 0.5 g of leaf tissue was homogenized in 10 ml of 5% metaphosphoric acid and centrifuged for 15 min at 1,000×g. For the ASA assay, 300μl of supernatant

was used and the following solutions were added to the extract, 750 µl of potassium phosphate (100 mM, pH 7.2) and 300 µl of distilled water. 300 µl of supernatant was used for the DHAS assay and the following solutions were added, 750 µl of potassium phosphate (100 mM, pH 7.2) and 150 µl of 10 mM dithiotheritol. The samples were incubated at room temperature for 10 min, and then 150 µl of 0.5% N-ethylmaleimide was added. Both samples were vortexed and incubated at room temperature for 10 min. To each sample, 600 µl of 10% (w/v) TCA, 600 µl of 44% (v/v) orthophosphoric acid, 600 µl of 4% (w/v) bipyridyl in 70% (v/v) ethanol and 10 µl of 3% FeCl3 were added. After vortex-mixing, samples were incubated at 40°C in a water bath for 20 min. The samples were vortexed again and incubated at 40°C in a water bath for another 20 min. Absorbance of samples at 525 nm was recorded. A standard curve of ASA and DHAS was used for the calculation of ASA and DHAS concentration.

Anthocyanins

For determination of anthocyanin content by the method of Wanger (1979), frozen tissue samples (0.1 g) were soaked in 10 ml acidified methanol (methanol, HCl 99,1 (v/v)). The tissues were crushed and kept at 25°C for 24 h in the dark. The extract was then centrifuged at 4,000×g for 5 min at room temperature and absorption at 550 nm was read. Coefficient 33,000 mol^{-1} cm^{-1} was used and anthocyanins content was expressed as lmol g^{-1} fw.

Flavonoids

The flavonoids content was determined as described by Krizek *et al.* (1998). Frozen tissue samples (0.1 g) were soaked in 10 ml acidified ethanol (ethanol, acetic acid, 99, 1 (v/v)). The tissues were crushed and kept at 25°C for 24 h in the dark. The extract was then centrifuged at 4,000×g for 5 min at room temperature and absorption at 270, 300 and 330nm was read. Results were expressed as percentage of absorbance (% A).

Enzyme extractions and assays

One gram of frozen leaves was homogenized in 50 mM phosphate buffer (pH 7.2) containing 1 mM EDTA, 1 mM PMSF and 1% soluble PVP. The homogenate was centrifuged at 14000 g for 20 min at 4°C and the supernatant used for assay of the activity of enzymes. The protein content in the supernatant was measured according to the method of Bradford (1976). Bovine serum albumin was used as standard. The activity of CAT was estimated by monitoring the decrease in absorbance of H_2O_2 within 30 s at 240 nm. The assay solution contained 50 mM potassium phosphate buffer (pH 7.0) and 15 mM H_2O_2 and 100µl enzyme extract (Dhindsa and Motowe, 1981). SOD activity was measured by monitoring the inhibition of nitroblue tetrazolium (NBT) reduction at 560 nm (Giannopolitis and Ries, 1977). The reaction solution (3 ml) contained 50 µM NBT, 0.1 mM Na-EDTA, 75 µM of riboflavin, 13 mM methionine, 50 mM phosphate buffer (pH 7.0) and 100µl of enzymes extract. Reaction was carried out in test tubes at 25°C under fluorescent lamp (40) with irradiance of 75 µmol/ms. The reaction was allowed to run for 8 min and stopped by switching the light off. Blanks and controls were run in the same manner but without irradiation and enzyme, respectively. Under the experimental condition, the initial rate of reaction, as measured by the difference in increase of absorbance at 560 nm in the presence and absence of extract, was proportional to the amount of enzyme. One unit of SOD activity was defined as the amount of enzyme that inhibits the NBT photo-reduction by 50%. The activity of APX was measured according to the method of Nakano and Asada (1999). The 3 ml reaction mixture contained 2.5 ml of 50 mM phosphate buffer (pH 7.0), 200 µl of 3% H_2O_2, 200 µl of 0.5 mM ascorbate and 100 µl of enzymes extract. The change in absorbance was determined within 1 min at 290 nm. One unit of APX was defined as the quantity of enzyme required to oxidize 1µmol of ascorbate per min.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Duncan's

multiple range test (DMRT). The values are mean±S.D. for 3 replicate. P ≤0.05 were considered as significant.

Results and discussion

As can be seen in Fig.1, 2, ASA content increased up to 1.5 fold under drought stress. Water stressed plants, pretreated with BABA showed significantly higher ASA content compared to plants under untreated conditions. Mild stress condition showed no significant effect in dehydroascorbate content but severe stress caused a significant increase in the amount of dehydroascorbate. BABA-treated plants showed significant reduction in dehydroascorbate content as compare to non-treated plants. Water deficit was significantly increased anthocyanin content. BABA pretreatment significantly increased anthocyanin content compared with untreated plants (fig 3). Flavonoids absorption at a wavelength of 270 nm significantly increased compared to control plants under stress. but this increase was not significant at a wavelength of 300 and 330 nm. BABA pretreatment significantly increased every three flavonoids compared to untreated plants (fig 4, 5, 6). The leaf CAT activity decreased 2.3-fold and 3.5-fold under mild and severe drought stress. BABA treated plants showed increase in CAT activity to 2-fold and 2.5-fold in mild and severe drought condition, relative to non-treated plants (Figure.7). The SOD activity significantly increased to 1.2-fold, 1.5-fold in the leaves under mild and severe drought stress, respectively. BABA enhanced SOD activity to 1.7-fold, 1.3-fold in the leaves under mild and severe drought stress (Figure.8). The leaf APX activity significantly increased 1.2-fold and 2-fold under mild and severe drought stress, compare to control (Figure.8).The plants treated by BABA, showed significant increase in APX activity under drought stress as compare to non treated plants. Ca^{2+} content increased 1.3 and 1.5-fold under mild and severe drought stress. BABA treated plants showed increase in Ca^{2+} content to 2-fold (Fig 10). BABA has been shown to increased plant tolerance to various environmental stresses (Jakab *et al.*, 2005; Ton and Mauch-Mani, 2004; Zimmerli *et al.*, 2008; 2001; 2000).This study showed that, BABA enhanced the activities of antioxidant enzymes and Non-enzymatic antioxidants. Accumulation of ABA transcription and signaling factors in BABA-treated Arabidopsis might prime Arabidopsis for ABA accumulation (Jakab *et al.*, 2005). According to jiang and zhang report, BABA primes Arabidopsis plants to respond quicker and stronger to biotic and abiotic stresses. Recent studies revealed that BABA enhances mRNA accumulation of abscisic acid (ABA) and ethylene early signaling intermediates (Zimmerli *et al.*, 2008). Pretreatment with BABA enhances the capacity of antioxidant enzymes in canola plants exposed to drought stress. Such changes can bring about drought tolerance, whereby plants continue to function at the low water Potentials. ABA not only regulates water balance by inducing stomatal closure, but also enhances water stress tolerance by induction of antioxidant defense systems. BABA application considered as a mild stress to plants (Wu *et al.*, 2009). Exposure of plants to a mild stress can increase capacity of antioxidant defense systems. In this study, increased accumulation of anthocyanin, ascorbate and flavonoles was found after treatment with BABA. Foliar anthocyanin accumulation was observed in Arabidopsis after BABA treatment (Wu *et al.*, 2009). Anthocyanin acts as modulators of stress signals. It has been shown, BABA stimulate anthocyanin biosynthesis by regulating the expression of CHS (chalcone synthase) and DFR (dihydroflavonol-4-reductase) (Wu *et al.*, 2009). In this study, BABA increased Ca^{2+} content. Probably, BABA activates ABA signaling and ABA stimulates the increases in cytosolic Ca^{2+} by inducing both Ca^{2+}influx from the extracellular space and Ca^{2+} release from intracellular stores (Allen *et al.*, 2000). ABA-induced increases in the activities of a can be prevented by the antioxidant enzymes pretreatments with the Ca^{2+} chelator indicating the involvement of Ca^{2+} in ABA-induced antioxidant defense (Allen *et al.*, 2000).

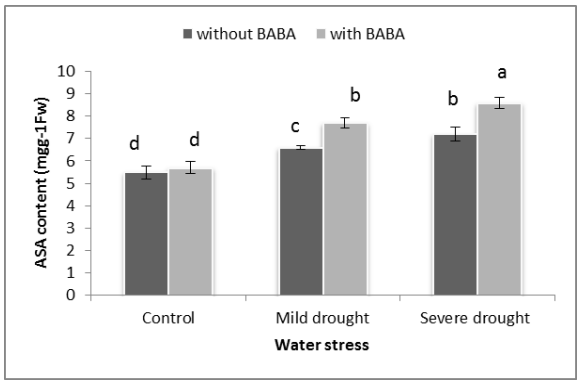

Fig. 1. Effects of drought stress and pretreatment BABA on Ascorbic acid content.

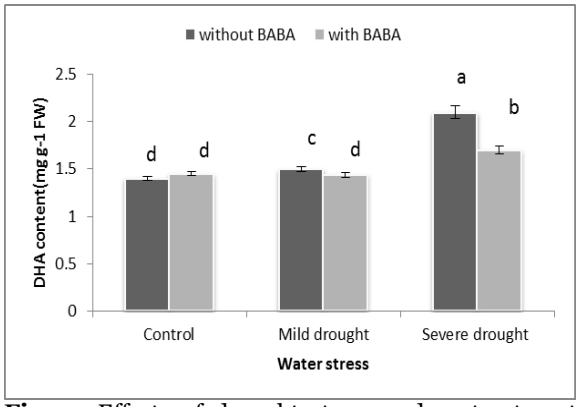

Fig. 2. Effects of drought stress and pretreatment BABA on dehydroascorbate content.

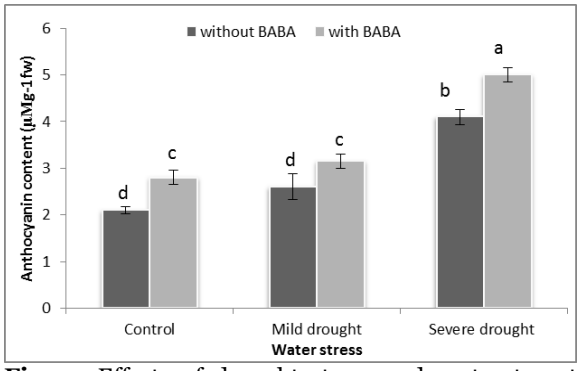

Fig. 3. Effects of drought stress and pretreatment BABA on anthocyanin content.

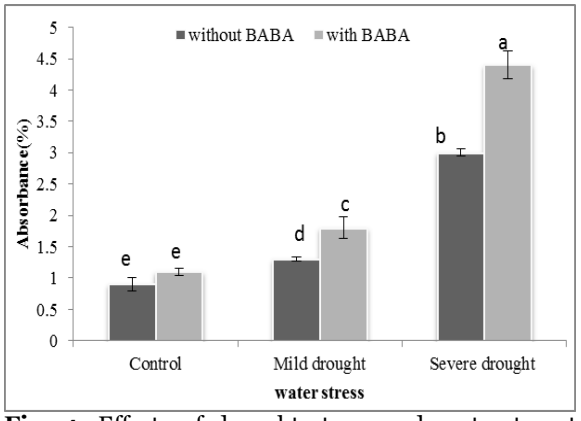

Fig. 4. Effects of drought stress and pretreatment BABA on flavonols absorption (270nm).

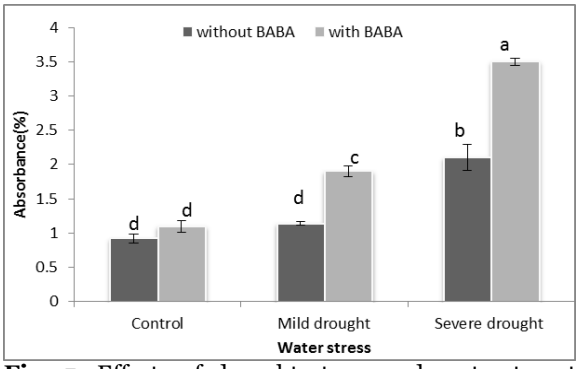

Fig. 5. Effects of drought stress and pretreatment BABA on flavonols absorption (300nm).

Data are means ± SE of three replicates. Different letters indicate the significance of difference at P ≤0.05 levels by Duncan test.

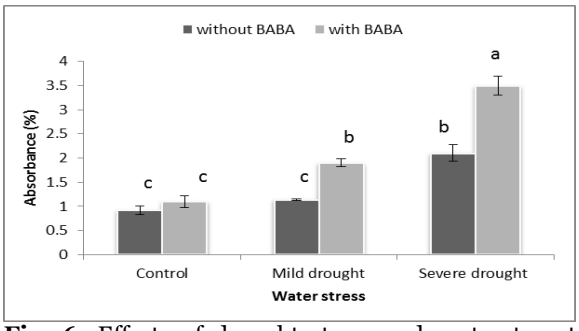

Fig. 6. Effects of drought stress and pretreatment BABA on flavonols absorption (330nm).

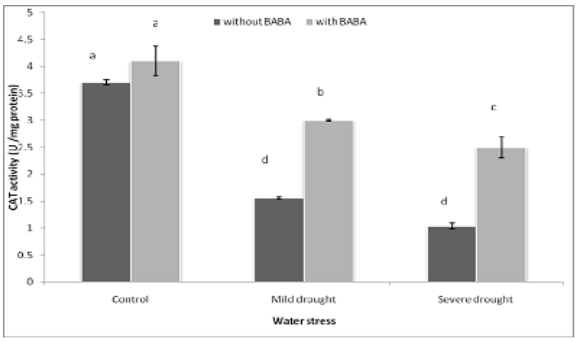

Fig. 7. Effects of drought stress and pretreatment BABA on CAT activity.

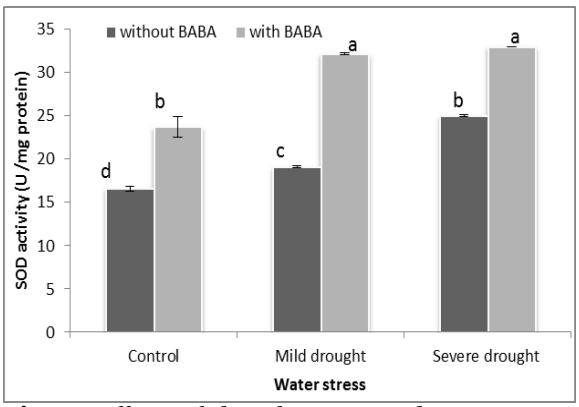

Fig. 8. Effects of drought stress and pretreatment BABA on SOD activity.

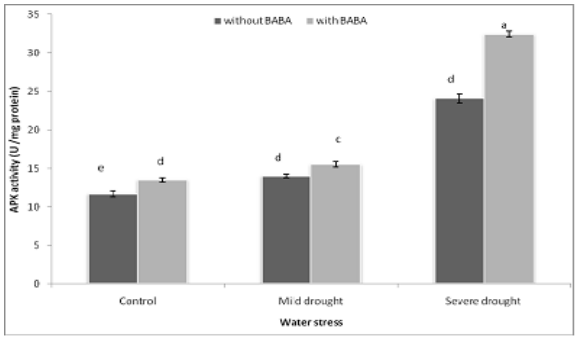

Fig. 9. Effects of drought stress and pretreatment BABA on APX activity.

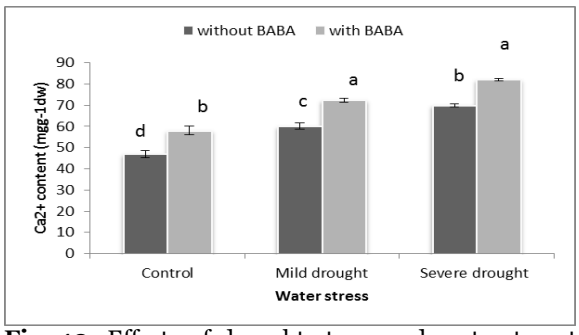

Fig. 10. Effects of drought stress and pretreatment BABA on Ca $^{2+}$ content.

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