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Effects of Drought Stress on Accumulation of Proline and Antioxidant Enzymes in the Different Varieties of Yardlong Beans

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ABSTRACT

The effect of drought stress on biochemical activities included changes in the concentrations of proline and the activity of antioxidant. This study aimed to determine the effect of drought stress on proline activity and antioxidant enzymes catalase (CAT), peroxidase (POX), and superoxide dismutase (SOD) on varieties of yardlong beans. The first factor was the variety of yardlong beans, including Brawijaya Ungu-1 (BU-1), Brawijaya Ungu-2 (BU-2), Brawijaya Ungu-3 (BU-3), Brawijaya Ungu-4 (BU-4), Brawijaya Ungu-5 (BU-5), Brawijaya Ungu-6 (BU-6), Brawijaya-4 (Br-4), and Bagong-2 (Bg-2). The second factor was drought stress level consisting of 50% and 100% field capacity (FC) as a control. The results showed that the concentrations of proline and activity of antioxidant enzymes increased drought stress. BU-4 variety experienced the highest enhancement of proline, and BU-2 variety experienced the highest enhancement of catalase and superoxide dismutase while Br-4 variety experienced the highest enhancement of peroxidase. So BU-4, BU-2, and Br-4 varieties were said to be more tolerant to drought stress, based on proline and antioxidant enzymes accumulation.

Keywords: Antioxidants, drought stress, proline, yardlong beans

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INTRODUCTION

The yardlong bean is generally green, light green, or white green, all with different advantages, but a type of bean not widely known to the public is purple pods yardlong beans. Purple pod yardlong beans contain protein and anthocyanins substances, which are very beneficial to the body.

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The production of yardlong beans is currently low and insufficient for consumption (Kuswanto, 2002). This stems from the decreased production of yardlong beans from one year to another. Low production of yardlong stems from lack of area in which to cultivate plants. Therefore, it is necessary to use marginal lands, such as drylands, acid lands, and/or low fertility lands. The low productivity of yardlong beans also stems from a lack of availability of high yield, drought resistant, and pest resistant varieties.

Drought stress is one of the most endemic environmental factors for crop growth and production, and is the leading cause of crop losses in the world (Bray, 2002; Bruce, Edmeades, & Barker, 2002). The presence of yardlong bean varieties that are tolerant to drought stress and high yield results offer hope of developing yardlong bean crops on dry land.

Drought stress may affect the biochemical activity of the plant, including changes in hormone concentration for example, abscisic acid, proline content (Cárdenas-Avila et al., 2006; Vajrabhaya, Kumpun, & Chadchawan, 2001), and increased antioxidant enzymatic activity (Harinasut, Poonsopa, Roengmongkol, & Charoensataporn, 2003) to destroy reagent oxygen species (ROS).

Proline accumulation occurs in plants with drought stress or a deduction of soil moisture, so the level of tolerance of plants was often associated with a big accumulation of its proline. Mapegau (2010), Mathius *(*2004), Bandurska (2000), Kishor et al. (2005), Cárdenas-Avila et al. (2006), and Esfandiari (2008) research showed that the content of free proline in plants increased with the increasing levels of water stress.

Antioxidants played a significant role as a plant defence against oxidative stress. Oxidative stress, by increasing the level of ROS, can lead to growth inhibition. Plant cells require a mechanism to regulate the concentration of intracellular ROS by *scavenging* ROS (Bailey-Serres & Mittler, 2006). ROS *scavenging* mechanisms were done by plants through antioxidant enzyme catalase, peroxidase, and superoxide dismutase (Harinasut et al., 2003) and non-enzyme antioxidant (ascorbic, glutathione, α-tocopherol, and β-carotene). These compounds played a key role in changing the toxic oxygen compounds into non-toxic compounds. Bailey and Mittler (2006) showed that there was an enhancement in the activity of antioxidant enzymes in plants that experience abiotic stress, such as drought stress, because the plants employ defence mechanisms and increase the activity of antioxidant enzymes to destroy ROS.

This study aimed to determine the effect of drought stress on proline activity and antioxidant enzymes catalase, peroxidase, and superoxide dismutase on different varieties of yardlong beans, and to know which varieties are most tolerant of drought stress, based on the level of accumulation of proline and antioxidant enzymes.

MATERIALS AND METHODS

Time and Place

Planting was done in the greenhouse of Faculty of Agriculture, University of Islam Malang in dry season and aluvial land from March to June 2017, while the proline and antioxidant enzyme analysis was performed at the Laboratory of Science Faculty, Brawijaya University.

Research Methods

The design used a randomised block factorial design. The first factor was eight varieties of yardlong beans with unknown tolerance to drought stress, consisting of six varieties of purple yardlong beans, namely Brawijaya Ungu-1 (BU-1), Brawijaya Ungu-2 (BU-2), Brawijaya Ungu-3 (BU-3), Brawijaya Ungu-4 (BU-4), Brawijaya Ungu-5 (BU-5), Brawijaya Ungu-6 (BU-6), and two varieties of green yardlong beans, namely Brawijaya-4 (Br-4) and Bagong-2 (Bg-2). The second factor was the drought stress, which consisted of 100% and 50% field capacity (FC). Each treatment combination had three samples and was repeated three times. Watering was done every day, maintaining 100% FC until three weeks after planting. The drought test was done after this three week period, and the plant was given threats by reducing the water supply until it reached 50% FC, and this was carried out until its harvest time. Meanwhile, the water content was maintained at 100% FC for the control treatment. Watering occurred every other day, after finding out the water needs by measuring the capacity of the field.

Proline and Antioxidant Enzyme Activity Analysis

Leaf proline analysis was performed using the method of Bates et al. (1973). Extraction was done on every third leaf of all varieties studied. Measurement of proline accumulation and antioxidant enzyme was done on every third leaf , leaves that were not too young (shoots) or too old, and before the generative phase. Antioxidant enzyme activity was tested using three kinds of enzymes-catalase enzyme (CAT), peroxide enzyme (POX), and superoxide dismutase enzyme (SOD).

Estimation of Proline

Approximately 0.5 g of fresh or frozen plant material was homogenized in 10 mL of 3% aqueous sulfosalicyclic acid and filtered through Whatman's No. 2 filter paper. Two ml of filtrate was mixed with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in a test tube. The mixture was placed in a water bath for 1 h at 100°C. The reaction mixture was extracted with 4 mL toluene and the chromophore containing toluene was aspirated, cooled to room temperature, and the absorbance was measured at 520 nm with a Bausch and Lomb Spectrometer 7 IO'. Appropriate proline standards were included for alculation of proline in the sample.

Estimation of Antioxidant Enzyme

Antioxidant enzyme activity was tested using three kinds of enzymes which were the enzyme catalase, peroxidase and superoxide dismutase enzyme.

Five hundred milligrams of frozen material was homogenized in 5 mL of ice-cold 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM PMSF. The extract was centrifuged at 4°C for 20 min at 12,500 rpm. The supernatant was used for enzyme assay.

The activity of enzyme catalase was measured using the method of Chandlee and Scandalios (1984), with modification. The assay mixture contained 2.6 mL of 50 mL of 50 mM potassium phosphate buffer (pH 7.0) 0.4 mL, 15 mM H_2O_2, and 0.04 mL of enzyme extract. The decomposition of H_2O_2 was followed by the decline in absorbance at 240 nm. The enzyme activity was expressed in units 1 mM of H_2O_2 reduction minute^{-1} mg protein^{-1}.

Peroxidase was assayed by the method of Kumar and Khan (1982). Assay mixture of peroxidase contained 2 mL of 0.1 M phosphate buffer (pH 6.8), 1 mL of 0.01 M pyrogallol, 1 mL of 0.005 M H_2O_2, and 0.5 mL of enzyme extract. The solution was incubated for 5 min at 25°C, after which the reaction was terminated by adding 1 mL of 2.5 N H_2SO_4. The amount of purpurogallin formed was determined by measuring the absorbance at 420 nm against a blank prepared by adding the extract after the addition of 2.5 N H_2SO_4 at zero time. The activity was expressed in unit mg^{-}1 protein. One unit is defined as the change in the absorbance by 0.1 min^{-1}mg^{-1} protein.

Crude enzyme extract was prepared, for the assay of superoxide dismutase by the method of Hwang et al. (1999). Extraction (1 g) of fresh tissue was homogenized with 10 mL of ice-cold 50 mM sodium phosphate buffer containing 1 mM PMSF. The extract was filtered through a double-layered cheese cloth. The extract was centrifuged at 12,500 rpm for 20 min at 4°C. The supernatant was saved and made up to 10 mL with the extraction buffer and used for estimation of the SOD enzyme activity. The enzyme protein was determined by the Bradford (1976) method.

Superoxide dismutase activity was assayed as described by Beauchamp and Fridovich (1971). The reaction medium was prepared and to 3 ml of reaction medium, 1 mL of enzyme extract was added. The reaction mixture contained 1.17×10^{-6} M riboflavin, 0.1 M methionine, 2×10^{-5} potassium cyanide, and 5.6×10^{-5} M nitroblue tetrasodium salt (NBT), dissolved in 0.05 M sodium phosphate buffer (pH 7.8). The mixture was illuminated in glass test tubes by two sets of Philips 40 W fluorescent tubes. Illumination started to initiate the reaction at 30°C for 1 h. Those without illumination were saved as blank and kept in the dark. The absorbance was read at 560 nm in the spectrophotometer against blank. Superoxide dismutase activity was expressed in units. One unit is defined as the amount of change in the absorbance by 0.1 h^{-1} mg protein^{-1} under the assay condition (Cherry, 1963).

Data Analysis

Observed quantitative data was statistically analysed using ANOVA with *SPSS software Release 15*. If significant treatment gave a real effect, it was continued by the

Duncan test 5%. Reduction percentage was calculated as follows: % reduction = (Yp –Ys)/Yp × 100 (Choukan, Taherkhani, Ghannadha, & Khodarahmi, 2006), where Yp is the yield under non-stress condition and Ys the yield under stress.

RESULTS AND DISCUSSION

Variations of response to drought stress can be determined by comparing the concentration of proline accumulation in the plants without drought stress to those experiencing drought stress treatment. Proline accumulation in the plants experiencing drought stress showed an increase compared with the controls.

All varieties of yardlong beans that were tested showed an increased accumulation of proline stemming from drought stress, but each variety had a different rate of proline accumulation (Figures 1 and 2). This showed that each variety had a different tolerance level. BU-4 experienced enhanced proline accumulation (166%), which was highly significant with other varieties, while BU-2 showed the lowest enhanced proline accumulation (53%).

Proline was the most stable and least inhibited amino acid when compared to the others which was synthesized in the phloem tissue of plants, roots, and seeds (Shimpson, 2001; Deivanai, 2010). The decrease of the water content induces the plants to produce proline to maintain cell turgor pressure (De Ronde, Van Der Mescht, & Steyn, 2000). In drought stress conditions and a variety of other osmotic stress, some plants had adaptation mechanisms that included the ability to synthesize the compound osmoprotectant, or a suitable solution. Osmoprotectant is a non-toxic solution that can be accumulated to a certain extent without disturbing the metabolism of plants, which usually consisted of several chains of amino acids. Proline accumulation served as a source of cytoplasmic osmoticum

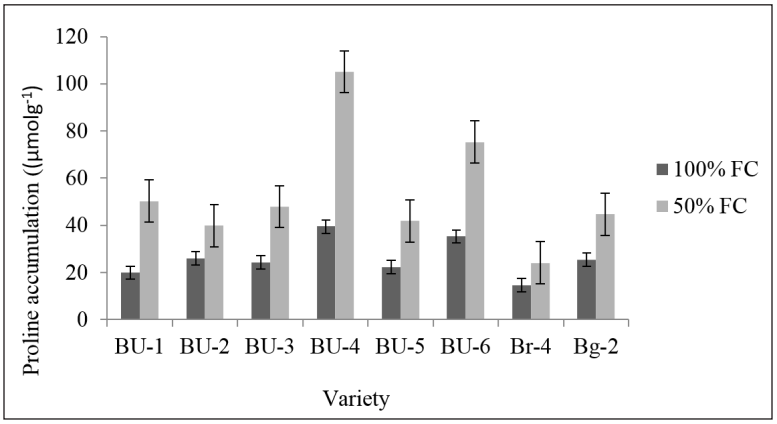


Figure 1. Proline accumulation (µmolg^{-1}) of yardlong beans stemming drought stress
Note: BU-1: Brawijaya Ungu-1, BU-2: Brawijaya Ungu-2, BU-3: Brawijaya Ungu-3, BU-4: Brawijaya Ungu-4, BU-5: Brawijaya Ungu-5, BU-6: Brawijaya Ungu-6, Br-4; Brawijaya-4, Bg-2: Bagong-2

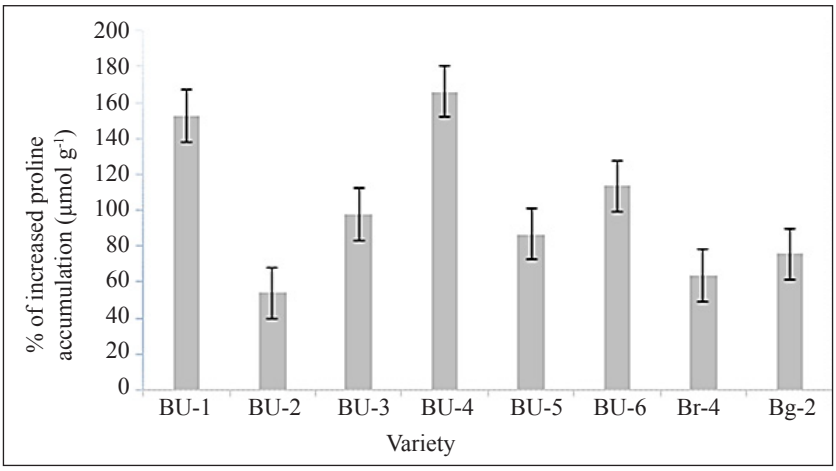


Figure 2. The increased level of proline accumulation of yardlong beans stemming from drought stress
Note: BU-1: Brawijaya Ungu-1, BU-2: Brawijaya Ungu-2, BU-3: Brawijaya Ungu-3, BU-4: Brawijaya Ungu-4, BU-5: Brawijaya Ungu-5, BU-6: Brawijaya Ungu-6, Br-4; Brawijaya-4, Bg-2: Bagong-2

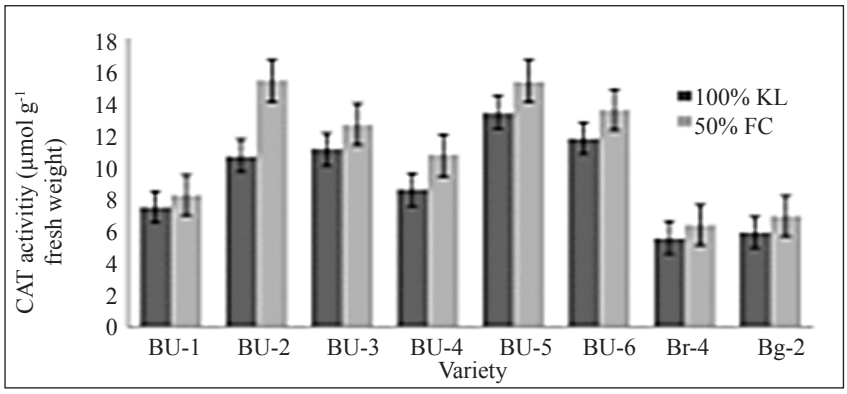
(Liu & Baird, 2003), as a patron and protector of the cytoplasmic enzyme cellular structure (Gibon, Sulpice, & Larher, 2000). Bandurska (2000) research showed that in wheat plants, proline increased ten-fold in plants that experienced drought stress. In a particular research on soybean plants, proline was more common in varieties that were drought-tolerant; produced in a plant's effort to increase the osmotic balance in drought conditions. However, some studies mentioned that the proline accumulation in response to various conditions of osmotic stress was very common in plants (Delauney & Verma, 1993).

The treatment of drought stress caused an increased activity of catalase, peroxidase, and superoxide dismutase enzymes, but the rate of enhancement of antioxidant enzyme activity of each variety was different (Figures 3 and 4).

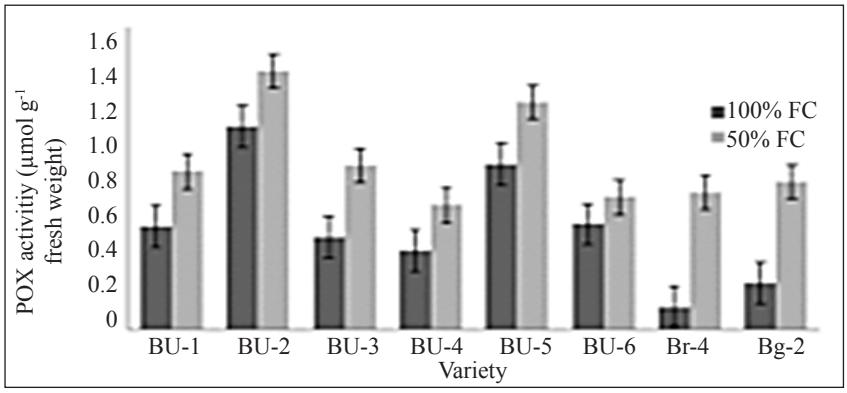
Antioxidant enzymes of catalase, peroxidase, and superoxide dismutase were supposed to play a vital role in the drought stress of the tolerance of pods yardlong beans. The increased activities of antioxidant enzymes in the pod's yardlong beans can inhibit tissue damage that survives in the drought stress condition.

Unyanyar and Cekic (2005) stated that the activity of antioxidant enzymes in plants under drought stress conditions was believed to be an indicator of its level of tolerance in facing drought stress conditions. Detoxification enzymes, which included catalase, peroxidase, and superoxide, were the enzymes that played a significant role in controlling the rate of oxidation and were associated with the tolerance of abiotic stresses.

The enhancement activity of catalase, peroxidase, and superoxide enzyme under the drought stress condition showed the



(a)



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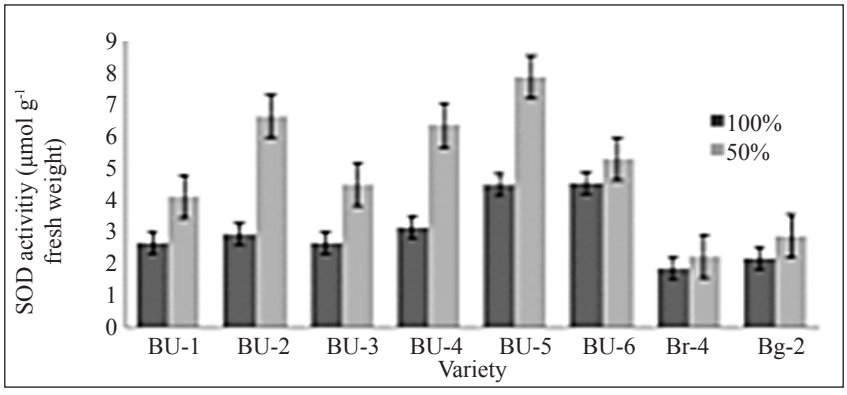
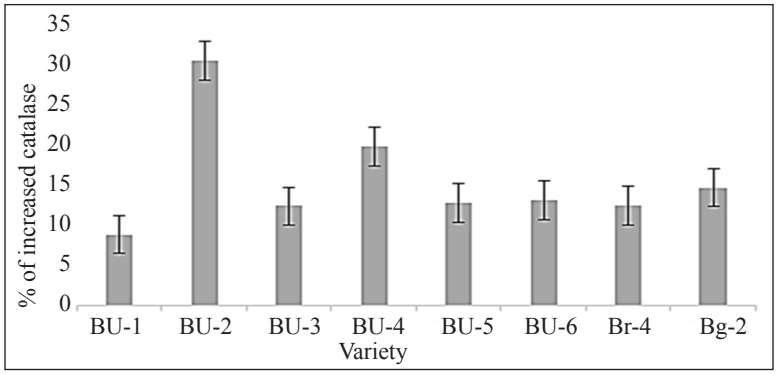


(c)

Figure 3. The enzyme activity of: (a) catalase; (b) peroxidase; (c) and superoxide dismutase enzymes, of yardlong beans in the control condition and experiencing drought stress
Note: BU-1: Brawijaya Ungu-1, BU-2: Brawijaya Ungu-2, BU-3: Brawijaya Ungu-3, BU-4: Brawijaya Ungu-4, BU-5: Brawijaya Ungu-5, BU-6: Brawijaya Ungu-6, Br-4; Brawijaya-4, Bg-2: Bagong-2



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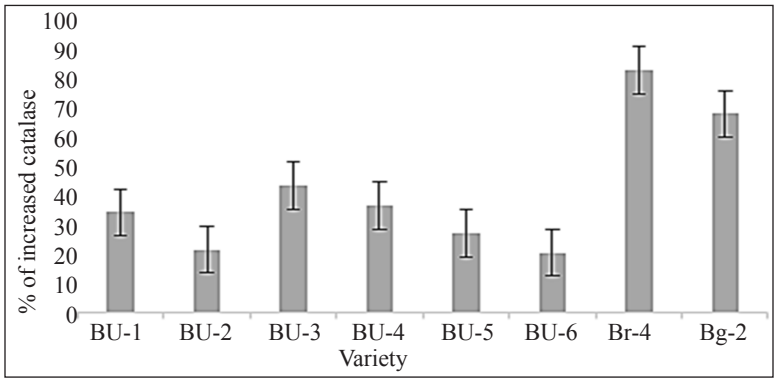

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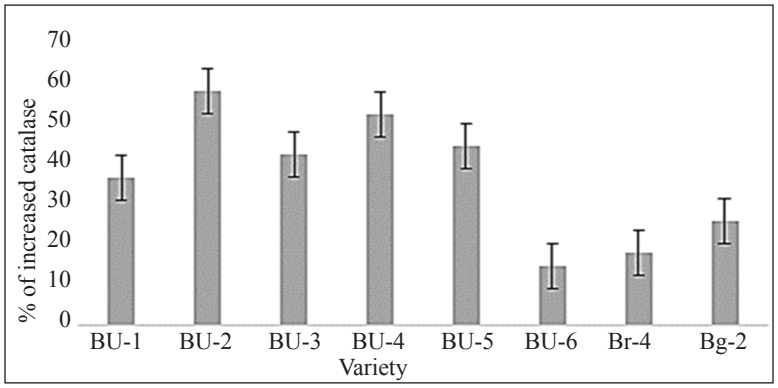

(c)

Figure 4. The enhancement level of: (a) enzyme catalase; (b) peroxidase; and (c) superoxide dismutase activity of yardlong beans stemming drought stress

Note: BU-1: Brawijaya Ungu-1, BU-2: Brawijaya Ungu -2, BU-3: Brawijaya Ungu-3, BU-4: Brawijaya Ungu -4, BU-5: Brawijaya Ungu -5, BU-6: Brawijaya Ungu -6, Br-4; Brawijaya-4, Bg-2: Bagong-2

plant adaptation in the face of increasing ROS stemming from the imposed drought stress conditions. The enhancement activity of those three enzymes showed that they played a key role in the detoxification process of hydrogen peroxide.

The highest enhancement activity of catalase and superoxide dismutase antioxidant enzymes stemming from drought stress was found in BU-2, (30% dan 56%). The highest enhancement activity of peroxidase antioxidant enzymes was in Br-4, i.e., 83%. The activity of catalase, peroxidase, and superoxide dismutase enzymes will be different, depending on tissues and species. This was proved by the drought stress, which increased the activity of peroxidase and superoxide in sprouts J. Oxycedrus (Alguacil, Caravaca, Díaz-Vivancos, Hernández, & Roldán, 2005).

The activity of enzymes catalase, peroxidase, and superoxide dismutase generally increased in plants under drought stress conditions, and, in some cases this activity gave a good indication of the level of tolerance (Criszar et al., 2007). Overexpression of superoxide dismutase increased the tolerance level of oxidative stress. Superoxide dismutase was an important antioxidant enzyme that functioned in cells to prevent the effects of ROS. Superoxide dismutase enzyme dismutated the destruction of ROS and formed H_2O_2, to be detoxified by catalase and peroxidase.

The difference of the enhancement activity of catalase, peroxidase, and superoxide in yardlong beans stemmed from the genetic diversity that also determined its adaptability and responses to the occurring environmental stresses. This indicated that the antioxidant system played a significant role in the tolerance of plants, when facing abiotic stress.

CONCLUSION

All varieties of yardlong beans that were tested showed an increased accumulation of proline and antioxidant stemming drought stress. BU-4 variety experienced the highest enhancement of proline, and BU-2 variety experienced the highest enhancement of catalase and superoxide dismutase while Br-4 variety experienced the highest enhancement of peroxidase. So BU-4, BU-2, and Br-4 varieties were said to be more tolerant to drought stress, based on proline and antioxidant enzymes accumulation.

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