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ROLE OF PHYSIOLOGICAL AND ANTIOXIDATIVE ENZYMES IN IMPROVING UNDER DROUGHT STRESS CONDITIONS IN CLUSTER BEAN [CYAMOPSISTETRAGONOLOBA(L.) TAUB].

Ragula Seenaiah[1*], Pinjari Akbar Basha[2], Metla Jyothi[3], Subba Naik Gari Thimma Naik[4]

[1*,3,4]Dept of Botany ,Sri Krishnadevvraya University,Anantapuramu,Andhra Pradesh,India.

[2]Government Degree College , Ananthapurmu (A) akbarbasha.pinjari@gmail.com

[*]**Corresponding Author** - R.Seenaiah

[*]Dept of Botany ,Sri Krishnadevvraya University,Anantapuramu,Andhra Pradesh,India.

skubotanyseenu@gmail.com

Abstract:

Cluster bean [Cyamopsistetragonoloba(L.) Taub, member of the Fabaceae family and drought tolerant plant. The drought stress that occurs during pod filling stage is critical for final pod and fodder yield of cluster bean. The importance of the yield loss, both in terms of quality and quantity would usually depend on the response of cluster bean varieties to drought stress. This provided a detailed description of an experiment or study on the impact of water stress on chlorophyll content, cell membrane injury, and various biochemical parameters in different cultivars of cluster bean genotypes. The percent decrease in total chlorophyll content was higher in certain drought-sensitive genotypes (RGC-936, RGC-1025, HG-365) compared to drought-tolerant genotypes (JG-2, JG-1, GLC-103) at low soil moisture. Membrane injury increased significantly with the intensity of water stress in both cultivars. Drought-tolerant genotypes (RGC-936, RGC-1025, HG-365) exhibited a lower percent membrane injury compared to drought-sensitive genotypes (JG-2, JG-1, GLC-1031) under severe stress. Free proline and proline significantly increased in all genotypes with increasing stress. Antioxidative Enzymes, Superoxide Dismutase (SOD), Catalase (CAT), Glutathione Reductase (GR) SOD activity increased in both cultivars under water stress, with a higher increase in drought-tolerant genotypes at low soil moisture.

Key Words: Cluster bean, drought stress, Membrane injury, Antioxidative Enzymes

1. Introduction

Cluster bean [Cyamopsistetragonoloba(L.) Taub. (Syn. C. psoraliodes)], all and bushy annual herb have a deep rooted system, is a resilient and drought resilient leguminous crop grown on sandy soils of arid and semi-arid regions.. It is cultivated mainly in the rainy season and major

producing states in India are Rajasthan, Haryana, Gujarat, Punjab and to a limited extent in Uttar Pradesh and Madhya Pradesh., This crop is grown in parts of the world, viz. Sudan, Australia , Brazil (Costa 1950), South Africa (Doidge 1952), Pakistan and parts of United States of America (Undersander et al. 1991). Gum obtained from cluster bean seeds is a choice of agrochemical in paper, food, mining, cosmetics, textile, oil and pharmaceutical industries across the world (Hymowitz and Matlock 1963; Pawlik and Laskowski 2006; NRAA 2014). The production list of cluster bean is subjugated by India as leading producers of the crop in the world contributing to around 75–82 % of the total production. The dicotyledonous seed of cluster bean from outer side to the interior consists of three major fractions, viz. the husk or hull (14–17 %), endosperm (35–42 %) and germ or embryo (43–47 %). The galactomannan is found in the endosperm which makes up about 35 % of the dry weight the seed, 80–90 % being pure galactomannan, having 1:2 ratio of galactose to mannose (Das and Arora 1978).

Cluster bean gum in solution behaves as an first-rate thickening power is 10 times higher than that obtained from starch. The gum of cluster bean may promote a protective, pain-relieving and healing effect on gastric ulcers, contribute to lowering cholesterol, and blood sugar levels and also play a positive role in general weight loss and obesity with the promotion of extensiveness and appetite suppression (Sharma et al. 2011).

The water stress imposed at flower initiation stage decrease relative water content, photosynthesis, starch and carbohydrate accumulations (Kuhad and Sheoran 1986) and is most disadvantageous and critical to the growth of plant (Vyas et al. 2001). While free proline and free amino acids showed an increasing trend with increasing water stresses. Even mild water stress at the pre-flowering stage may reduce plant growth and yield significantly (Vyas et al. 1985). Whereas water stress imposed at vegetative and pod formation stages had negative effect on growth, photosynthesis, activity of various enzymes and seed yield of the plant (Vyas et al. 2001). Water stress induced decline in plant water potential and leaf relative water content led to reduction in total chlorophyll, starch and soluble protein contents besides accumulation of free proline to various extents depending upon the genotype and growth stage at which drought was experienced (Shubhra et al. 2004).

Materials and Methods

Key enzymes for absolute biosynthesis sugars, proline transporters, detoxification enzymes. Increased concentrations of reduced sugars provide for plant adaptation stress. Proline in maintaining osmolitic adjustment and adaptation stress and protect membranes proteins from adverse environmental stress increase. Peridaxiase activity with enhanced drought period, plant reduces oxidative injury arising from ROS (Reactive Oxygen Species) especially increasing POD activity during drought stress.

Conformation through Field Experiment;

A field experiment was conducted at the Departmental Botanical garden Sri Krishna devaraya University. Field designed with split plot complete randomized block design and plants were

grown and plots are maintained under optimum temp for 39-48 days in field condition for conducting the experimental study of biochemical's on screened cultivars of RGC-1025, RGC-936, HG365, GC-1031, JG-2, JG-1

Experimental Details

- Design of experiment - RBD
- Number of treatments - 02
- Number of replications - 05
- Number of genotypes - 06
- Plot Area - 6X9 m^2
- Row to Row distance - 30 cm
- Plant to Plant distance - 15 cm
- Replications - 5
- Season -*kharif*(2015)
- SCMR(SPAD) Minolta SPAD-502, Konica, Japan(Nageswara Rao *et al*., (2001)
- Relative Water Content (RWC)(Gonzalez and Gonzanlez-Vilar (2001)

SPAD chlorophyll meter reading (SCMR):

Same leaves that were used to calculate RWC were first used to measure SCMR by using a Minolta SPAD-502, Konica, Japan. SCMR was measured during9.00-11.00 am using the second fully expanded leaf from the top of the main stem described by Nageswara Rao *et al*., (2001), care was taken to ensure that the SPAD meter sensor fully covered the leaf lamina and that interference from veins andmidribs was avoided, totally 5 leaves for each pot and then single value was obtainedfor each pot by averaging the data.

Relative Water Content (RWC):

Leaf relative water content (RWC) was estimated according to the method of(Gonzalez and Gonzanlez-Vilar (2001). RWC was recorded from four leaflets of thefully expanded leaf from the top of the main stem from each pot. Harvested leaf, freshweight were recorded within 15 minutes, then leaf samples were soaked in 20 mldistilled water for 8 hours and blotted for surface drying and water saturated leafweight was recorded. The samples were oven dried at 80°C for 48 hr. The leaf relativewater content was calculated using the following formulaRWCWhere FW is fresh weight, DW is dry weight, and TW are turgid weighted.

Cellular membrane stability (CMS):

Cut out uniform leaf discs with a sharp cork borer from several plants. Transfer the leaf discs to a test tube containing 5 ml of distilled water. Similarly, leaf discs from control plants was excised and transferred to a test tube containing 5 ml of distilled water. Leave the tubes in a shaker for 4 hr at room temperature. Measure the conductivity of the solution by inserting the probe of the conductivity meter into the solution. This represents the ion leakage from the leaf discs (Reading1).

Autoclave the solution containing the leaf discs. After the liquid cools own, measure the conductivity of the solution. This represents the total ions present in the leaf discs.(Reading2). Ion leakage is represented as the percentage the total ions released(Reading1/Reading2 × 100). Compare the percentage of ion leakage between the treated and the control leaves

Sample preparation for the estimation of sugars:

The common procedure was adapted for sample preparation for the biochemical analysis of total soluble sugars, including the total reducing sugars and total free amino acids. Fresh leaf samples (500 mg) collected from different stressed plants and their respective controls were homogenized in 80% ethanol and the final volume was made up to 10ml. The homogenate was centrifuged at 12,000 g for 20 minutes at room temperature, the supernatant was transferred to fresh tubes, while the pellet was re-suspended in 80% ethanol and this step was repeated twice. The supernatant was pooled until the final volume was 25 ml. This supernatant was used for the estimation of total soluble sugars, including the total reducing sugars and total amino acids.

Estimation of total reducing sugars

Total reducing sugars were estimated as per the method proposed by Nelson (1944). To 1 ml of ethanolic extract, 1ml of fresh copper reagent prepared by mixing copper tartarate and copper sulphate solution (25:1v/v) was added. The mixture was heated for 20 min in boiling water bath and cooled. 1 ml of Arsenomolybdate reagent was then added and the contents incubated for 15 min at 100°C, cooled, and the color intensity was read at 500 nm in a spectrophotometer. The reducing sugar content was calculated using the standard graph for D-glucose at a concentration of 0·1 mg ml minus^{-1} and the values were expressed in gm / F.W.

Bio-chemical analysis:

Proline assay:

Estimation of total proline was done according to the method of Bates *et al*.,(1973). Fresh leaf tissue was homogenized using 3ml of 3% sulfo-salicylic acid and centrifuged at 12,000 g at 4°C for 15min. To 2 ml of the supernatant, 2 ml of acid ninhydrin reagent and glacial acetic acid in 1:1:1 ratio was added, the tubes were heated in a water bath at 100°C for 1h and subsequently cooled on ice for 10 min. To the resultant mixture, 4ml of toluene was added and incubated at room temperature for 30 min. The tubes were shaken for 15s and allowed to stand for 10 min to separate the phases. The upper phase was separated and the absorbance was measured at 520 nm using toluene as a blank. L- Proline at the concentration of 20 mg ml^{-1} was taken as a standard.

Lipid Peroxidation Assay (MDA) :

Lipid peroxidation was assayed according to Yagi (1998) homogenize nearly 200 mg of treated and control tissue in 4 ml of 0.1% TCA. Centrifuge the extract at 10,000×*g* for 15 min, then collect the supernatant, and mix 1 ml of supernatant with 2 ml of 20% TCA and 2 ml of 0.5% TBA. Heat the mixture at 95◦C for 30 min in a fume hood and later cool on ice. Read the absorbance of

supernatant at 532 nm and 600 nm. The *A*600 is the nonspecific absorbance and is subtracted from the values for*A*532. Calculate the concentration of MDA using the Beer– Lambert's equation (extinction coefficient of MDA is 155 mM^{-1} cm^{-1}).

Antioxidant Enzyme Assays:

The Anti oxidative enzyme assay was carried out by Elavarthy and Martinprotocols (2010). Fresh leaf tissue was collected from stressed and well-wateredplants Approximately 200 mg of leaf tissue was weighed and ground to a fine powderin liquid nitrogen using a pre-cooled mortar and pestle. The exact weight of eachpowdered sample was determined before it was thoroughly homogenized in 1.2 ml of0.2 M potassium phosphate buffer (pH 7.8 with 0.1 mM EDTA). The samples werecentrifuged at 15,000×*g* for 20 min at 4°C. The supernatant was removed, the pelletresuspended in 0.8 ml of the same buffer, and the suspension centrifuged for another15 min at 15,000×*g*. The combined supernatants were stored on ice and used todetermine different antioxidant enzyme activities.

Superoxide dismutase (SOD; EC 1.15.1.1):

Total Superoxide dismutase activity was assayed using a modified NBTmethod Beyer, and Fridovich, (1987). The 2 ml assay reaction mixture contained 50mM phosphate buffer (pH 7.8) containing 2 mM EDTA, 9.9 mM L-methionine, 55µM NBT, and 0.025% Triton-X100. Forty micro liters of diluted (2×) sample and 20µL of 1mM riboflavin were added and the reaction was initiated by illuminating thesamples under a 15 W fluorescent tube (Giannopolitis, *et al*., (1977). During the 10-min exposure, the test tubes were placed in a box lined with aluminum foil. The boxwith the test tubes was placed on a slowly oscillating platform at a distance ofapproximately 12 cm from the light source. Duplicate tubes with the same reactionmixture were kept in the dark and used as blanks. Absorbance of the samples wasmeasured immediately after the reaction was stopped at 560nm. The enzyme activity(grams per fresh weight) of a sample was determined from a standard curve obtainedby using pure SOD.

Catalase (CAT; EC 1.11.1.6):

Catalase activity was determined according to Aebi (1984). Thedecomposition of H_2O_2 was followed as a decrease in absorbance at 240nm in aUV/Vis spectrophotometer. The 3 ml assay mixture contained 2 ml leaf extract(diluted 200 times in 50 mM potassium phosphate buffer, pH 7.0) and 10 mM H_2O_2.The extinction coefficient of H_2O_2 (40 mM^{-1} cm^{-1} at 240 nm) was used to calculatethe enzyme activity that was expressed in terms of millimoles of H_2O_2 per minute pergram fresh weight).

Ascorbate Peroxidase (APX; EC 1.11.1.11):

Ascorbate peroxidase activity was assayed using a modified method of Nakano and Asada (1981). APX activity was determined from the decrease inabsorbance at 290nm due to oxidation of ascorbate in the reaction. The 1 ml assaymixture contained 50 mM potassium phosphate buffer

(pH 7.0), 0.5 mMascorbate,0.5 mM H_2O_2, and 10 µL of crude leaf extract. H_2O_2 was added last to nitrate thereaction, and the decrease in absorbance was recorded for 3 min. The extinctioncoefficient of 2.8 mM^{-1} cm^{-1} for reduced ascorbate was used in calculating theenzyme activity that gram fresh weight.

Glutathione Reductase (GR; EC 1.6.4.2):

Glutathione reductase activity was assayed according to Smith *et al*., (1988).The increase in absorbance at 412nm was measured when DTNB was reduced toTNB by GSH in the reaction. Ten micro liters of leaf extract were used in the assayalong with 0.75 mM DTNB, 0.1 mM NADPH, and 1 mM GSSG in a total of 1 mlassay volume. GSSG was added last to initiate the reaction and the increase inabsorbance was recorded for 3 min. The extinction coefficient of TNB (14.15 $M^{-1}cm^{-1}$) was used to calculate the activity of GR that was expressed in terms ofmillimole TNB minute per gram fresh weight.

Results

Drought stress modulates the various physiological and metabolic processes of plants (Levitt, 1980). Plant response to drought stress are various; consistent and considerably varies at different directorial levels depending upon the intensity and duration of stress as well as plant species and the stage of development (Chaves et al., 2003). The drought stress that occurs during pod filling stage is critical for final pod and fodder yield of cluster bean. The importance of the yield loss, both in terms of quality and quantity would usually depend on the response of cluster bean varieties to drought stress.

chlorophyll content

The chlorophyll content was measured in control and drought stressed leaves of both cultivars at different soil moisture levels and results were depicted in **Figure A**. The total chlorophyll content was slightly increased with increasing water stress cultivars over controls. However the percent decrease in total chlorophyll content was found to higher in RGC-936, RGC-1025, HG-365 and lower in JG-2, JG-1, and GLC-103 at low soil moisture.

Cell membrane injury

Membranes are main loci affected under water stress. The cell membrane injury was estimated in two cultivars of stressed plants under water stress. The rate of membrane injury was significantly increased with the intensity of water stress in both cultivars and results were depicted in **Figure B** However the percent membrane injury was found to be lesser in drought-tolerant genotypes RGC-936, RGC-1025, HG-365 than in Lesser tolerant genotypes JG-2, JG-1. GLC-1031 under severe stress.

Biochemical parameters:

1.Free proline content

Free proline content was estimated in control and drought stressed leaves of all cluster bean genotypes data were presented in **Figure C**. The free proline content was significantly increased in all cluster bean genotypes with increasing stress over the control plants. However the rate of increase in the free proline content was found to be dependent on stress severity. Nevertheless, a difference in the accumulation of free proline content was observed between the cultivars. However the percent increase of free proline content was comparatively higher in RGC-936, RGC-1025, HG-365 genotypes than JG-2, JG-1, GLC-1031.

2. Proline -5-Corboxylase (p-5-C)

Proline -5-Corboxylase (p-5-C) proline content was estimated in control and drought stressed leaves of all cluster bean genotypes data were presented in **Figure D**. The free proline content was significantly increased in all cluster bean genotypes with increasing stress over the control plants. However the rate of increase in the free proline content was found to be dependent on stress severity. Nevertheless, a difference in the accumulation of free proline content was observed between the cultivars. However the percent increase of free proline content was comparatively higher in RGC-936, HG-365. RGC-1025 genotypes than JG-2, JG-1.

3.Lipid peroxidation

Lipid peroxidation in the leaves of the control and stressed samples were measured in six cluster bean genotypes. The MDA content gradually increased with increase in stress intensity low SMLs in among the cluster bean genotypes. MDA content was significantly more evident in drought-insightful than drought-tolerant genotypes indicate MDA activity is high in RGC-936,RGC-1025,HG-365 hardy tolerant genotypes than low tolerant genotypes JG-2,JG-1, GLC-1031 change in MDA content at the experiment under water stress. **(Figure E.)**

Antioxidative enzymes .

1.Superoxide dismutase

The specific SOD activity was increased in both cultivars of stressed plants under water stress **(Figure F),** but its response patterns were variable with different stress regimes in both cultivars. At low SMLs, the SOD activity was increased in drought-tolerant cultivar compared to drought-sensitive cultivar. In drought-sensitive cultivar there are specifically in stress samples but the pattern of intensity differed in stress levels and the SOD is commonly expressed in all samples. Stress specific and were significantly expressed in drought-tolerant higher in RGC-936, RGC-1025, HG-365 and lower in JG-2, JG-1, GLC-103 at low soil moisture.

2. Catalase

The CAT activity was found to be increased in leaves of all genotypes under water stress. In the stressed leaves CAT activity was increased significantly depending on severity of stress. The enzyme activity is high in RGC-936,RGC-1025,HG-365 hardy tolerant genotypes than low tolerant genotypes JG-2,JG-1, GLC-1031. **(Figure. G).** It indicates catalase could involve in

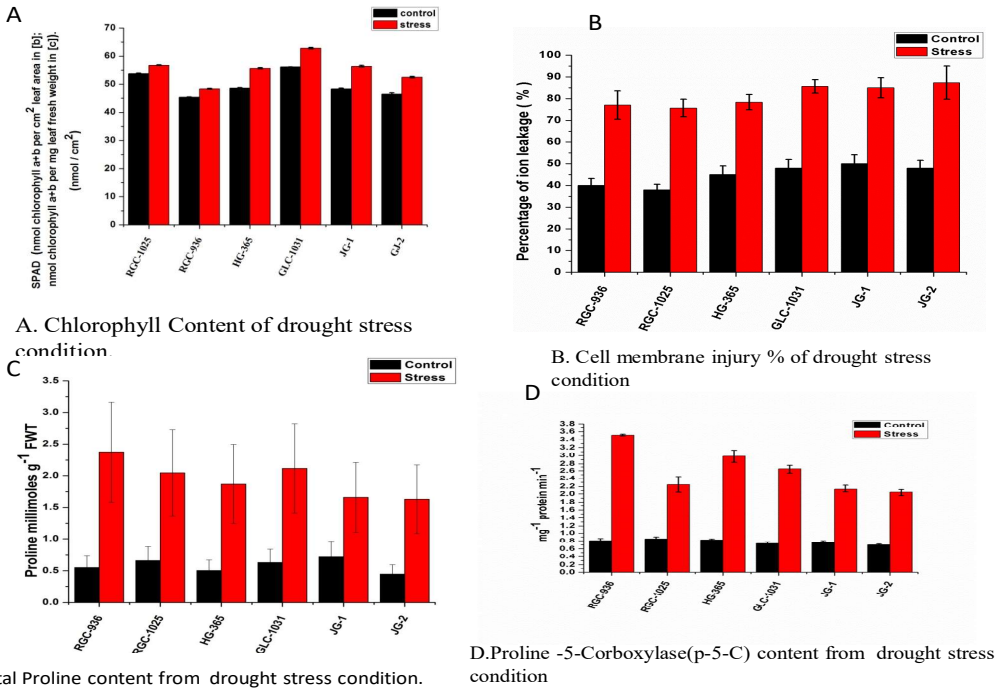
membrane repair/synthesis during post stress. CAT was detected significantly expressed at all level of stress of all genotypes.

3.Glutathione reductase (GR):

The Glutathione reductase (GR) activity was found to be increased in leaves of all genotypes under water stress. In the stressed leaves CAT activity was increased significantly depending on severity of stress. The enzyme activity is high in RGC-936, RGC-1025,HG-365 hardy tolerant genotypes than low tolerant genotypes JG-2,JG-1, GLC-1031. **(Figure.4).** It indicates Glutathione reductase could involve in membrane repair/synthesis during post stress. GR was detected significantly expressed at all level of stress of all genotypes.

4. Ascorbate peroxidase (APX):

The Ascorbate peroxidase (APX) activity was found to be increased in leaves of all genotypes under water stress. In the stressed leaves APX activity was increased significantly depending on severity of stress. The enzyme activity is high in RGC-936,RGC-1025,HG-365 hardy tolerant genotypes than low tolerant genotypes JG-2,JG-1, GLC-1031. **(Figure. I)**. It indicates Glutathione reductase could involve in membrane repair/synthesis during post stress. APX was detected significantly expressed at all level of stress of all genotypes.



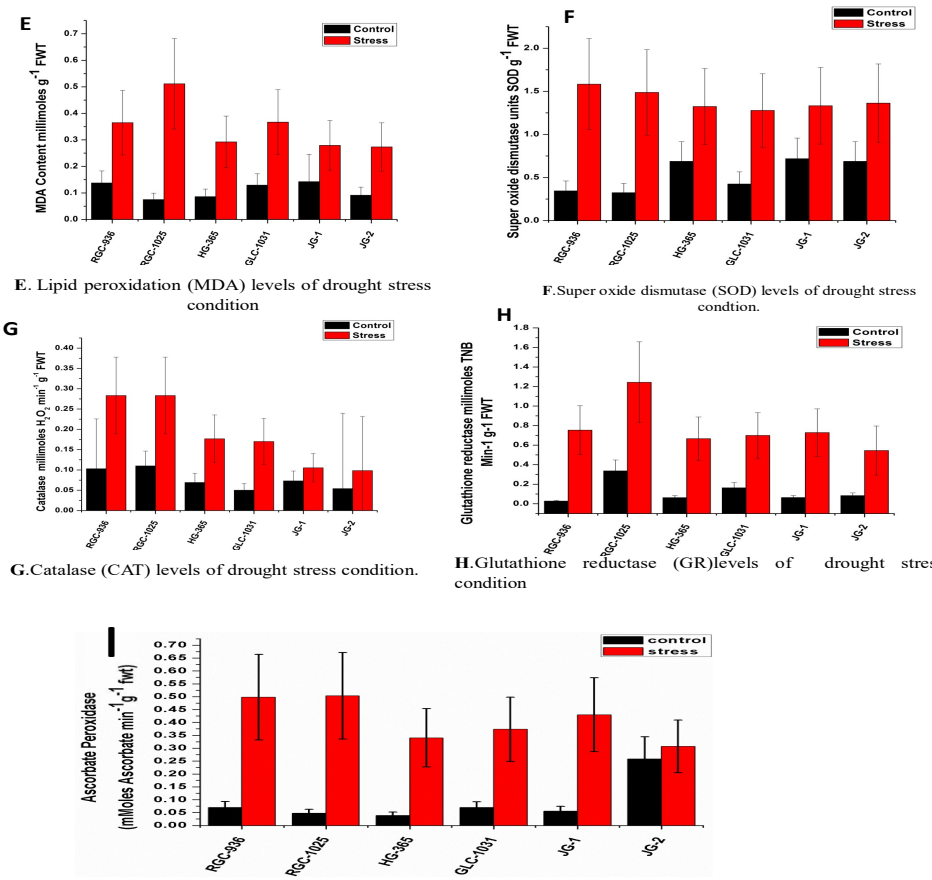
A. Chlorophyll Content of drought stress condition

B. Cell membrane injury % of drought stress condition

C.Total Proline content from drought stress condition.

D.Proline -5-Corboxylase(p-5-C) content from drought stress condition

Figure 1 A-D. Fate of various physiological traits of genotyps under drought stress, A. Chlorophyll, Cell membrane injury, C. Proline, D. Proline-5- Corboxylase (p-5-C). Cluster Bean genotypes: RGC-936, RGC-1025, HG-365, JJ-1, JG-2 and GLC-1031.



E. Lipid peroxidation (MDA) levels of drought stress condition

F.Super oxide dismutase (SOD) levels of drought stress condtion.

G.Catalase (CAT) levels of drought stress condition.

H.Glutathione reductase (GR)levels of drought stress condition

I. Ascorbate Peroxidase (APX)levels of control and drought stress plants.

Figure 1 E-I. Fate of various physiological traits of genotyps under drought stress, E. Lipid peroxidation (MDA), F. Super oxide dismutse (SOD) G. Catalase(CAT), H. Glutathioine reductase (GR), I. Ascorbate peroxidase (APX). Cluster Bean genotypes: RGC-936, RGC-1025, HG-365, JJ-1, JG-2 and GLC-1031.

DISSCION:

The stress factors particularly drought, destructively affect plant growth and development and causes a pointed decrease of plants productivity. Strategies to reduce oxidative injure are a worldwide feature of plant resistance responses. The plant response to water deficit stress would depend on the species inherent “strategy” as well as on the extent and severity of the stress period. The reverse was those moreover found in wheat (Badiani et al. 1990) as well as rice (Sharma and Dubey 2005) where water stress improved SOD activity. Simova-Stoilova et al. (2010) reported increased CAT activity in wheat under drought stress being higher especially in sensitive varieties. In another study, Sharma and Dubey (2005) reported a decrease in CAT activity in rice seedlings following drought stress. It was shown that APX and/or GR activities were enhanced during water stress in wheat seedlings (Keles and Oncel 2002) and alfalfa (Rubio et al. 2002).The specific SOD activity was increased in the shoots of wheat seedlings under stress conditions. Increase in enzyme

activity coincide with a variable individual isoform expression. SOD activity was significantly increased in both mild and severe stress conditions, having non-significant stress wheat group interaction. A significant difference between severe and mild stress was observed in tolerant and susceptible groups for SOD2, whereas in intermediate group there was no significant difference and the maximum activity was obtained in tolerant group at mild (-0.4 MPa) stress. The ability of plants to overcome oxidative stress partly relies on the induction of SOD activity and subsequently on the up-regulation of other downstream antioxidant enzymes (Alscher et al. 2002). According to this fact that SOD processing is known to be substrate inducible) Tsang et al. 1991), an increment in the SOD activity may be attributed to the increased production of the superoxide (O2 •−) as substrate that lead to induced expression of genes encoding SOD. In sunflower seedlings a decrease in SOD activity was detected under water stress (Badiani et al. 1990). The reverse was true in wheat (Badiani et al. 1990; Bakalova et al. 2004) and rice (Sharma and Dubey 2005) where water stress increased SOD activity. Our results are consistent with Badiani et al. (1990) and Bakalova et al. (2004). Higher SOD activity in tolerant group compared with susceptible and intermediate groups can also be explained by less efficiency susceptible group in scavenging of O2 •− under severe stress conditions.

In severe stress condition, CAT showed higher activity as compared with mild stress and control condition . Stress × wheat group and stress × genotype within group interaction for CAT were also significant . Reports on catalase activity under stress condition are heterogeneous. Luna et al 2004. Our results are consistent with works reporting the increased CAT activity in response to osmotic stress in wheat seedling. APX activity showed significant difference for osmotic stress, genotype within group conditions, stress × cluster bean group and stress × genotype within group interactions. Al-Ghamdi (2009) reported that drought acclimated (by cessation of watering for 8 days) wheat seedling exhibited systematic increase in the activity of H2O2 scavenging enzymes, particularly APX and CAT and maintenance of ascorbate redox pool by efficient function of APX enzyme. Effect of water deficit stress was significant on GR activity, measured spectrophotometrically. Stress × cluster bean group interactions and genotype within group were significant. Indicates the mean GR activity values, for treatment combinations. The significant GR activity was obtained for tolerant cluster bean in severe stress conditions. Several authors have reported increased activity of GR in rice seedlings (Sharma and Dubey 2005) and alfalfa (Rubio et al. 2002) under environmental stresses. Lascano et al. (2001) reported high in glutathione reductase (GR) activities in the tolerant cluster bean genotypes and a higher decline in reduced glutathione (GSH), ascorbate content and less oxidative damage than in the susceptible cultivar.

CONCLUSION.

This study aims to address the challenge of drought stress in cluster bean crops in Andhra Pradesh, particularly in the context of the complex genotype × environment interactions. Developing cluster bean genotypes with consistent drought tolerance traits is crucial, and your approach involves identifying key factors and enzymes in biochemical pathways related to drought stress in a diverse set of germplasm. Our emphasis on integrating physiological and biochemical traits for the

selection of drought-tolerant genotypes is noteworthy. This approach recognizes the multifaceted nature of drought stress responses in plants, where a combination of traits may provide a more comprehensive understanding of tolerance mechanisms. Identification of Key Factors and Enzymes: Understanding the biochemical pathways involved in drought stress response can provide insights into the specific mechanisms that confer tolerance. This knowledge is essential for targeted breeding efforts. This study seems to address a critical agricultural issue and has the potential to contribute significantly to the development of drought-tolerant cluster bean genotypes. The integration of physiological and biochemical approaches enhances the comprehensiveness of your research, and the development of reliable tools could have practical applications for farmers and the agricultural industry in Andhra Pradesh and beyond.

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