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RESEARCH ARTICLE

Antioxidant responses of Chickpea genotypes exposed to moisture stress

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Abstract

Present investigation was carried on six chickpea genotypes (tolerant and sensitive) imposed to moisture stress at varied growth stages viz. irrigated as and when required (C), sown with one pre-sowing irrigation (WS_{VFP}), stressed at flower initiation (WS_F) and stressed at pod initiation (WS_P). The experiment was conducted in the field area of Plant Breeding and Genetics department, Punjab Agricultural University, Ludhiana, India. Fresh chickpea seeds at 120 DAS, stressed at varied growth stages were used to determine activities of antioxidant enzymes. viz. peroxidase (POD), catalase (CAT), superoxide dismutase (SOD) and glutathione reductase (GR) in order to find out whether these parameters can be used as selection criteria for moisture stress tolerance in chickpea. Results indicated wide variation in stress tolerance amongst chickpea genotypes at varied growth stages. On the basis of antioxidant activity, genotypes GL28151, RSG963 and PDG3 appeared to be more adapted to moisture stress tolerance. Treatment WS_{VFP} showed maximum level of anti-oxidative enzyme activities, followed by stress treatment WS_P and WS_F.

INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most important food legume which had a total global production of 11.6 M tons from 13.2 M ha in 2011 (FAOSTAT 2012). It is one of the most important legume crops in sustainable agriculture system because of its low production cost, wide climate adaptation, use in crop production and atmospheric nitrogen fixing ability (Khamssi *et al* 2010). Due to insufficient, untimely and erratic rainfall in semi-arid and arid areas, the crop often suffers from drought at the end of the cropping season. Drought stress caused increased production of toxic reactive oxygen species such as superoxide radical, hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-). Superoxide dismutase (SOD) is considered to be first line of defence for protection against reactive oxygen species, which catalyzed the superoxide radical (O^{2-}) to O_2 and H_2O_2 which are further quenched by diverse antioxidant enzymes. Peroxidase is among the major enzymes that scavenges H_2O_2 in chloroplasts which is produced through dismutation of O^{2-} catalyzed by superoxide dismutase (Turkan 2005). Water stress strongly enhanced the activities of peroxidase, which could be considered as a response to drought induced oxidative damage, suggesting enzymatic removal of H_2O_2 by peroxidase. Catalase eliminates H_2O_2 by breaking it down directly to form water and oxygen (Gong *et al* 2005). Glutathione has antioxidant properties since the thiol group in its cydtein moiety is a reducing agent and can be reversibly oxidized and reduced .

Plants have developed a series of enzymatic and non-enzymatic antioxidant system to cope with drought stress. This dynamic mechanism comprise of various antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), glutathione reductase (GR) and catalase (CAT) and non enzymatic antioxidants, such as ascorbic acid and reduced glutathione (GSH). Keeping in view, the present investigation was carried out with an objective to understand antioxidative mechanism of drought tolerance by measuring activity of antioxidant enzymes viz. peroxidase, catalase, superoxide dismutase and glutathione reductase in chickpea seeds stressed at varied growth stages.

Material and Methods

Site description: The study was performed at the field area of Department of Plant breeding and Genetics, Punjab Agricultural University, Ludhiana, Punjab, India. Location represents the Indo-Gangetic plains and is situated at 36^0-54'N latitude, 25^0-48'E longitude and at a mean height of 247 meters above sea level.

Plant materials and treatment pattern: The seeds of chickpea (*Cicer arietnum* L.) tolerant (GL28151, RSG963, PDG3) and sensitive (GL22044, GNG1861, PBG1) genotypes selected on the basis of *in vitro* studies conducted under various osmotic concentration levels, were procured from Department of Plant breeding and Genetics, Punjab Agricultural University, India, and grown in experimental field area under varied water stress treatments. Each genotype was grouped in four sets viz., irrigated as and when required (C), sown with one pre-sowing irrigation (WS_{VFP}), stressed at flower initiation (WS_F) and stressed at pod initiation (WS_P). The experiment was laid out in a split plot design with three replications. Fresh chickpea seeds, at 120 DAS stressed at varied growth stages were used to determine activities of antioxidant enzymes.

Extraction and Assay of Antioxidant Enzymes

Developing seeds at podding stage were taken from various stress treatments and control for studying antioxidant enzyme activities. All the enzymes were extracted at 4° C to minimize denaturation and assayed at 30^oC.

Extraction and assay of peroxidase (Kar and Mishra 1976): The enzyme was extracted from the fresh seed samples with 0.1 M potassium phosphate buffer (pH 7.5) containing 1% PVP, 1 mM EDTA and 10 mM β-mercaptoethanol. The extracts were passed through a muslin cloth and centrifuged at 10,000 g for 10 minutes. The reaction mixture contained 3 ml of 0.05 M guaiacol prepared in 0.1 M potassium phosphate buffer (pH 6.5), 0.1 ml of enzyme extract and 0.1 ml of 0.8 M H_2O_2. The reaction mixture without H_2O_2 was measured as a blank. The reaction was initiated by adding H_2O_2 and rate of change in absorbance was recorded at 470 nm for 3 minutes at an interval of 30 seconds. Peroxidase activity has been defined as change in absorbance min^{-1} g^{-1} fresh weight.

Extraction and assay of superoxide dismutase (Nishikami et al 1972): The enzyme was extracted from the fresh seed samples with 0.1M potassium phosphate buffer (pH 7.5) containing 1% PVP, 1mM EDTA and 10mM β-mercaptoethanol. The extracts were passed through a muslin cloth and centrifuged at 10,000 g for 10 minute. To a cuvette, 1.5ml of 0.1M Tris-HCL buffer (pH 8.2), 0.5 ml of 6 mM EDTA, 1ml of 6 mM pyrogallol solution and 0.1 ml of enzyme extract was added. Absorbance was recorded at 420 nm after an interval of 30 seconds upto 3 minutes. A unit of enzyme activity has been defined as the amount of enzyme causing 50% inhibition of auto-oxidation of pyrogallol observed in blank. Superoxide dismutase was expressed as unit enzyme g^{-1} fresh weight.

Extraction and assay of Catalase (Kar and Mishra 1976): The enzyme was extracted with 50 mM sodium phosphate buffer (pH 7.5) containing 1% polyvinyl pyrrolidine. In spectrophotometric cuvette, 1.8 ml of 50 mM sodium phosphate buffer (pH 7.5) and 0.2 ml of enzyme extract was added. The reaction was initiated by adding H_2O_2 and utilization of H_2O_2 was recorded at intervals of 30 seconds for 3 minutes by measuring the decrease in absorbance at 240 nm. Catalase activity was expressed as µmoles of H_2O_2 decomposed min^{-1} g^{-1} fresh weight.

Extraction and assay of Glutathione reductase (Esterbaur and Grill 1978): The tissue (100 mg) was extracted with 2 ml of ice cold 0.1 M Tris HCL buffer (pH 7.5) containing 1mM EDTA, 1% PVP, 10mM β-mercaptoethanol, using pestle and mortar. Homogenate was centrifuged at 10000 g at 4°C for 20 minutes and clear supernatant was used for enzyme assay. To the spectrophotometric cuvette, 0.2 ml of 0.1 M Tris HCl buffer (pH 7.5), 0.1 ml of 1.5 mM $MgCl_2$, 0.1 ml EDTA (0.2 mM), 0.2 ml of 0.5 mM NADPH and 0.2 ml of enzyme extract and then 0.2 ml of 2 mM oxidized glutathione was added. The enzyme activity was estimated as the decrease of absorbance at 340 nm after an interval of 30 seconds upto 3 minutes. Glutathione reductase activity was expressed as µmoles of NADP formed $min^{-1}g^{-1}$ fresh weight.

Statistical Analysis: The data on various parameters were subjected to statistical analysis. Critical difference values were calculated by analysis of variance (ANOVA).

Results

Peroxidase: Peroxidase activity was found to be similar in all the genotypes under control conditions. Noticeable increase was observed in seeds under WS_{VFP}, followed by WS_P and WS_F stress treatments. Higher increase in peroxidase activity was observed in tolerant than sensitive genotypes (Fig 1). Percent increase was highest in RSG963 under stress treatments WS_{VFP} (33.7) and WS_F (26.8), while under WS_P, greater percent increase of 30.3 closely followed by 30.2 was found in GL28151 and RSG963 respectively. Increase in percentage was found least in GL22044 under WS_{VFP} (25.9), WS_F (18.3) and WS_P (22.2) water stress treatments.

Superoxide dismutase: Variation in enzyme activity is shown in Fig 2. Highest activity of SOD was observed in tolerant genotype PDG3 (2.72 unit enzyme g^{-1} fresh weight) whereas lower activity was noticed in sensitive

genotypes GNG1861 (2.50 unit enzyme g^{-1} fresh weight). Pronounced increase was witnessed under various stress treatments, increase being greater in WS_{VFP}, followed by WS_P. In comparison, lesser inclination was found under water stress treatment WS_F. Tolerant genotypes showed greater increase in enzyme activity, when compared to sensitive genotypes. Highest percentage increase was recorded in RSG963 under stress treatments WS_{VFP} (34.7), WS_F (23.2) and WS_P (28.8) among tolerant genotypes, while least increment in percentage within sensitive genotypes was observed in GL22044 under stress treatments WS_{VFP} (22.7), WS_F (10.2) and WS_P (19.5).

Catalase: Under control conditions, (Fig 3) highest enzyme activity among tolerant genotypes was noticed in RSG963 (165.28 ΔA min^{-1} g^{-1} fresh weight) while lowest activity within sensitive genotypes appeared in GL22044 (118.92 ΔA min^{-1} g^{-1} fresh weight). Sharp increase was observed in seeds under WS_{VFP}, followed by WS_P and WS_F stress treatments. More enhanced catalase activity was noticed in tolerant genotypes than sensitive ones. Percentage increase was highest in RSG963 under stress treatments WS_{VFP} (25.3), WS_F (11.4) and WS_P (21.3), among tolerant genotypes, while lesser percentage increase within sensitive genotypes was observed in GL22044 under stress treatments WS_{VFP} (16.5), WS_F (7.60) and WS_P (9.30).

Glutathione reductase: Enzyme activity was less in control, and it enhanced under water stress treatments, magnitude of increase was greater as the level of water stress increased. Under control, (Fig 4) maximum activity was observed in RSG963 (142.38 ΔA $min^{-1}g^{-1}$ fresh weight). Minimum activity was recorded under control in GL22044 (118.28 ΔA $min^{-1}g^{-1}$ fresh weight). Greater inclination in activity of glutathione reductase was observed in water stress treatment WS_{VFP}, followed by WS_P and WS_F. Higher increase in percentage was recorded in tolerant genotypes, than sensitive ones. Among tolerant genotypes, highest increase was observed in RSG963 under water stress treatment WS_{VFP} (41.2) and WS_F (23.7). However, under WS_P, maximum increase in percentage was noticed in PDG3 (36.0), followed by RSG963 (35.5). Among sensitive genotypes, least increase in enzyme activity was found in GL22044 under WS_{VFP} (26.6) and WS_P (22.8), whereas under WS_F, minimum increase in enzyme activity was observed in PBG1 (15.9) followed by GL22044 (16.9). Higher antioxidant enzyme activity lead to better establishment of crop under water stress.

Figures:

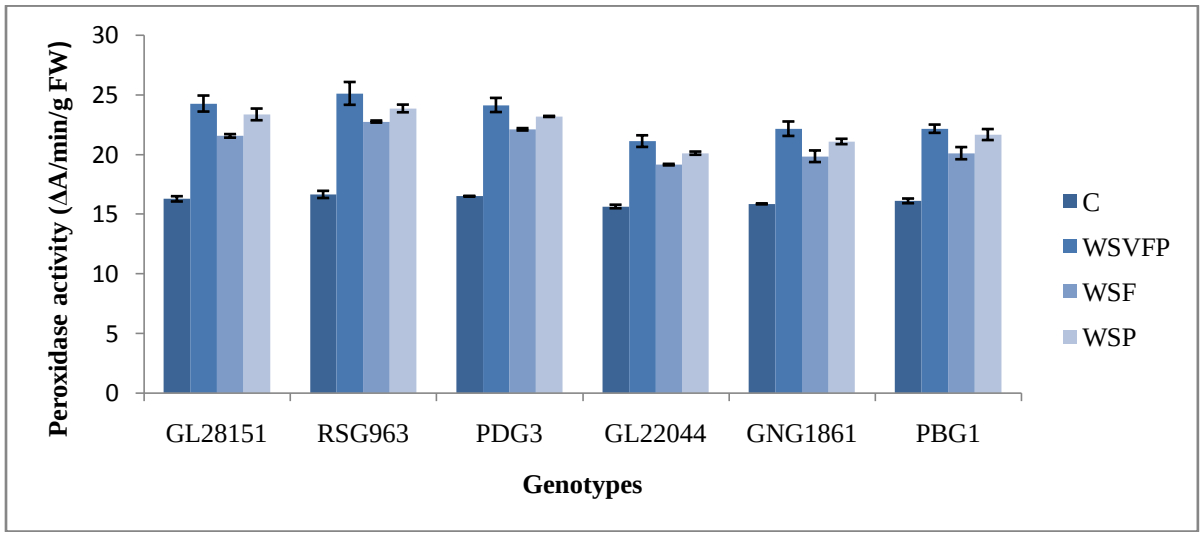

Fig. 1: Peroxidase activity (ΔA$min^{-1}g^{-1}$fresh weight) of various chickpea genotypes in response to moisture stress imposed at vegetative (60 DAS), flower-initiation (90 DAS) and pod-initiation (120 DAS) stages.

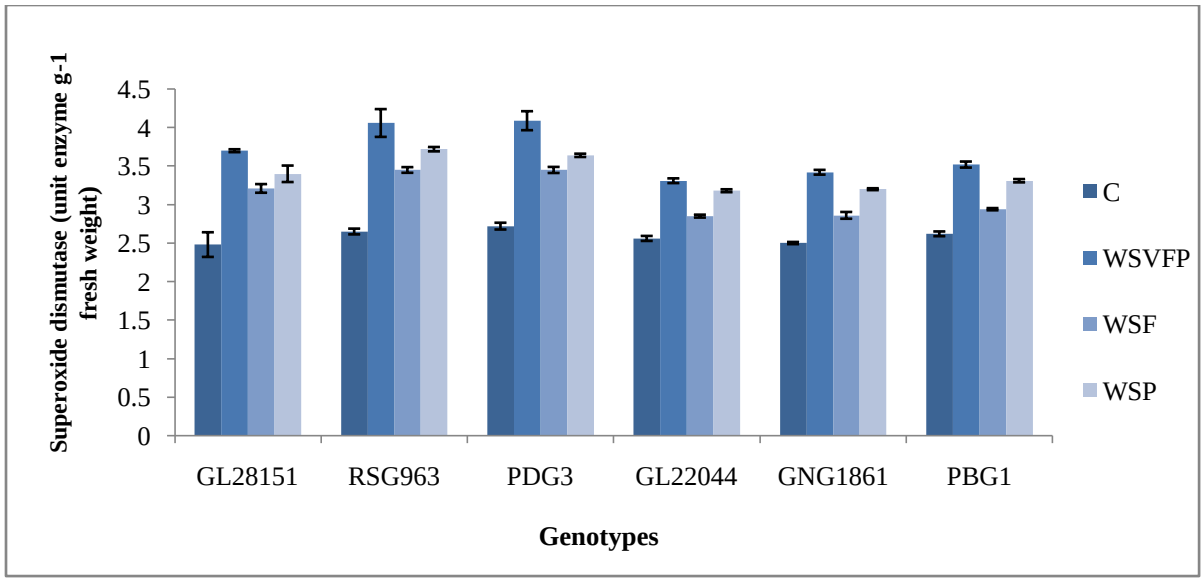

Figure:2 Superoxide dismutase (unit enzyme g^{-1} fresh weight) activity of various chickpea genotypes in response to moisture stress imposed at vegetative (60 DAS), flower-initiation (90 DAS) and pod-initiation (120 DAS) stages.

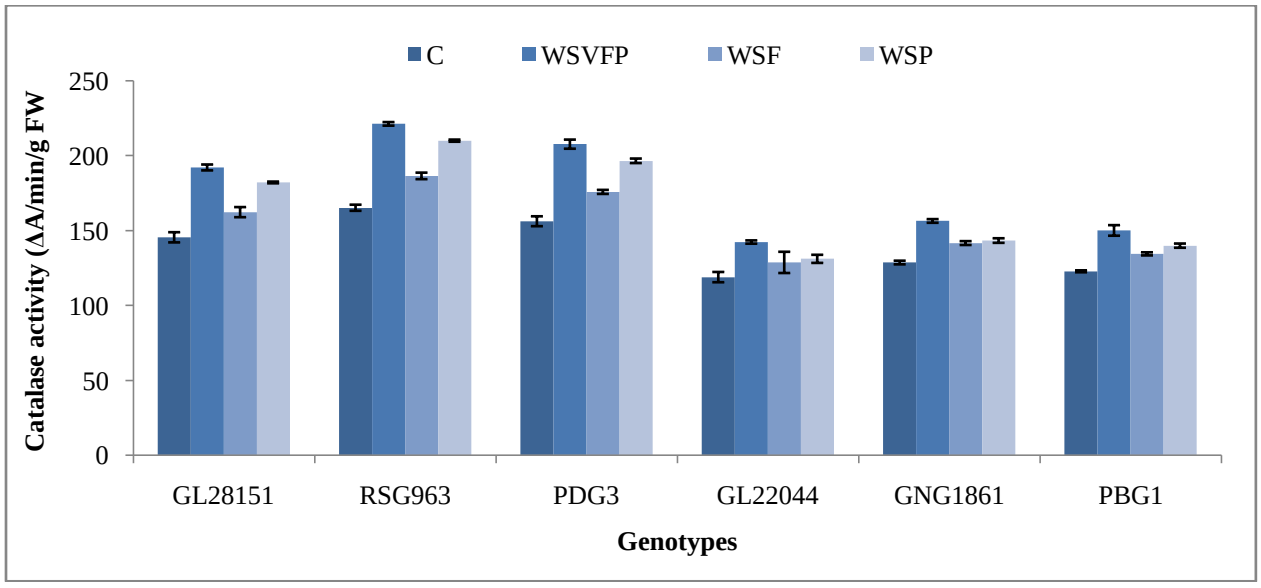

Fig. 3: Catalase (ΔA min^{-1} g^{-1} fresh weight) activity of various chickpea genotypes in response to moisture stress imposed at vegetative (60 DAS), flower-initiation (90 DAS) and pod-initiation (120 DAS) stages.

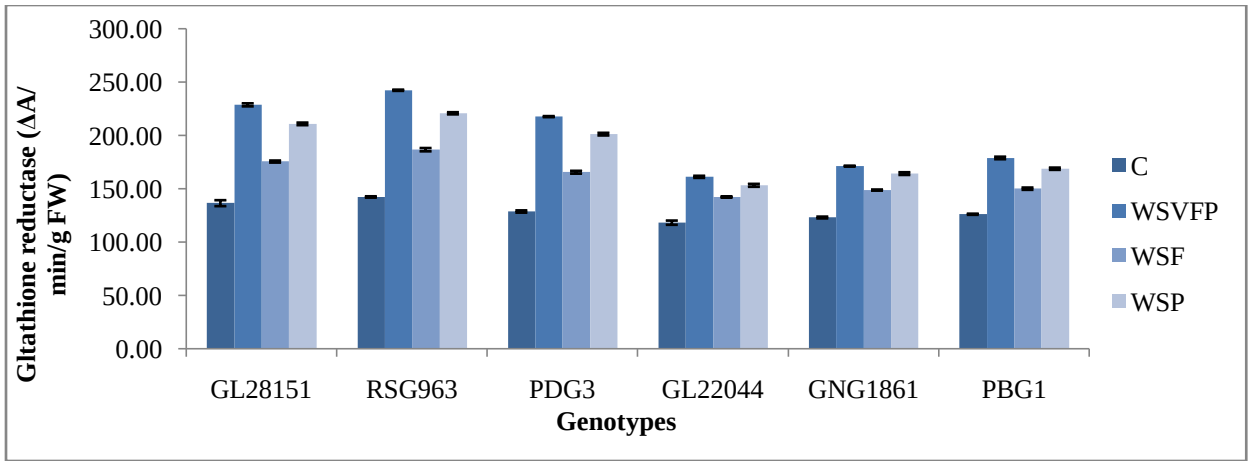

Fig. 4: Glutathione reductase activity (ΔA $min^{-1}g^{-1}$ fresh weight) of various chickpea genotypes in response to moisture stress imposed at vegetative (60 DAS), flower-initiation (90 DAS) and pod-initiation (120 DAS) stages.

Discussion:

Peroxidase is among the major enzyme that scavenges H_2O_2 in chloroplasts which is produced through dismutation of O_2- catalyzed by superoxide dismutase as observed in *Phaseolus acutifolius* and *Phaseolus vulgaris* (Turkan 2005). Peroxidase is found in the cytocol, vacuole as well as in the extracellular space dismutated the H_2O_2 by oxidation of various substrates. Increase in peroxidase activity in response to drought stress has been reported in chickpea (Patel *et al* 2011, Patel and Hemantaranjan 2012). Drought stress strongly enhanced the activities of peroxidase, which could be considered as a response to drought induced oxidative damage, suggesting enzymatic removal of H_2O_2 by peroxidase.

Superoxide dismutase (SOD) is considered to be first line of defence against protection against reactive oxygen species, which catalyzed the superoxide radical (O^{2-}) to O_2 and H_2O_2 which are further quenched by diverse antioxidant enzymes (Wang *et al* 2008), the most important being peroxidase and catalase. Higher SOD activity was observed in chickpea (Patel *et al* 2011, Patel and Hemantaranjan 2012). Raheleh *et al* (2012) observed tolerant chickpea genotypes could decrease damaging effects of drought stress by an increase in antioxidant enzymes activity such as SOD enzymes, in the flowering and podding stages. Sensitive genotypes showed less increase in SOD activity that may be related to the low water potential of this cultivar to remove O_2- under water deficit. The over-expression of superoxide dismutase, if accompanied by enhanced H_2O_2 scavenging systems like catalase and peroxidase enzyme activities has been considered as an important anti-drought mechanism to cope with oxidative stress during water deficit conditions.

Catalase (CAT) is abundant in the glyoxysomes of lipid storing tissues. Catalase eliminates H_2O_2 by breaking it down directly to form water and oxygen reported (Gong *et al* 2005). Catalase activity increased in tolerant chickpea cultivars in comparison to susceptible cultivars (Raheleh *et al* 2012). These results are in accordance with the findings in chickpea (Patel *et al* 2011, Patel and Hemantaranjan 2012). Higher activity of glutathione reductase enzyme activity was observed under drought stress in groundnut (Sharada and Naik 2011). Similar higher inductions in tolerant varieties than sensitive were noticed. Higher increase of glutathione reductase was observed in tolerant than sensitive genotypes Antioxidants assists the plant to withstand from the attack of reactive oxygen species. Similar results were observed in *Medicago sativa* L. (Safarnejad 2004). In contrast to our findings were the results observed in chickpea where glutathione reductase activity remained unchanged during stress (Macar and Ekmekci 2009).

In conclusion, antioxidant defence mechanism has been observed to plays vital role in plant's tolerance against drought stress. This study revealed enhancement in antioxidant enzyme activity with the increase in stress conditions, showing their ameliorative effect under moisture stress. These enzymes help to fight against reactive oxygen species generated under stress conditions. Treatment WS_{VFP} showed maximum level of anti oxidative enzyme activities, followed by stress treatment WS_P and WS_F. Flower initiation stage was least affected among stress treatments, as it might have survived on the irrigation supplied at pre-sowing, vegetative and podding stage.

Stress at podding stage was relatively more damaging as at this stage grain filling occurs and photosynthates are translocated to pods and water availability is necessary for plant functions to be carried out at optimum level. Effect was observed insignificantly variable in terms of peroxidase and catalase activity in sensitive genotypes. However tolerant genotypes could protect their growth and cellular activities under control as well as stress conditions over sensitive genotypes.

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