OPEN ACCESS

International Journal of Applied Sciences and Biotechnology

A Rapid Publishing Journal

ISSN 2091-2609

Indexing and Abstracting

CrossRef, Google Scholar, Global Impact Factor, Genamics, Index Copernicus, Directory of Open Access Journals, WorldCat, Electronic Journals Library (EZB), Universitätsbibliothek Leipzig, Hamburg University, UTS (University of Technology, Sydney): Library, International Society of Universal Research in Sciences (EyeSource), Journal Seeker, WZB, Socolar, BioRes, Indian Science, Jadoun Science, Jour-Informatics, Journal Directory, JournalTOCs, Academic Journals Database, Journal Quality Evaluation Report, PDOAJ, Science Central, Journal Impact Factor, NewJour, Open Science Directory, Directory of Research Journals Indexing, Open Access Library, International Impact Factor Services, SciSeek, Cabell's Directories, Scientific Indexing Services, CiteFactor, UniSA Library, InfoBase Index, Infomine, Getinfo, Open Academic Journals Index, HINARI, etc.

CODEN (Chemical Abstract Services, USA): IJASKD

Vol-3(4) December, 2015

Available online at:

http://www.ijasbt.org

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http://www.nepjol.info/index.php/IJASBT/index

SEM-Biotech Publishing

Impact factor*: **1.422**
Scientific Journal Impact factor#: **3.419**
Index Copernicus Value: **6.02**
IBI Factor 2015**: **4.19**

*Impact factor is issued by Universal Impact Factor. Kindly note that this is not the IF of Journal Citation Report (JCR).
#Impact factor is issued by SJIF INNO SPACE; **Impact factor is issued by INFOBASE INDEX.

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S. Sharma et al. (2015) Int J Appl Sci Biotechnol, Vol 3(4): 642-650
DOI: 10.3126/ijasbt.v3i4.13685

ISSN (Online): 2091-2609
DOI Prefix: 10.3126/ijasbt

International Journal of Applied Sciences and Biotechnology (IJASBT)

Research Article

EFFECT OF DROUGHT ON ANTIOXIDANT SYSTEM AT SEEDLING AND VEGETATIVE STAGE OF *ELEUSINE CORACANA*

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Abstract

Adverse environmental factors like drought stress result in increased levels of ROS that adversely affect the growth and productivity of plants. The alleviation of oxidative damage and increased resistance to environmental stresses is correlated with an efficient antioxidant system. Antioxidant non enzymatic system as well as enzymatic system neutralize the effects of these reactive oxygen species and help plants to survive in stress conditions. The study was carried out with the aim to understand the effect of drought on antioxidant system in seedling and vegetative stages of drought tolerant as well as sensitive genotypes of *Eleusine coracana*. Drought was imposed by withholding water for 8 days at seedling and vegetative stages of drought tolerant ie PRM-6107 and sensitive ie PES-400 genotypes of *E. coracana*. Different enzyme assays and biochemical analysis were performed in leaf samples by following the standard protocols. With the induction of drought, an increment in the level of ascorbate, flavonoid, phenol and proline was recorded in PRM-6107 whereas no significant increase was observed in PES-400. The level of these antioxidants was more in seedling stage than at vegetative stage. Similarly the level of antioxidant enzymes like ascorbate peroxidase, catalase, guaicol peroxidase, superoxide dismutase and glutathione reductase was high in tolerant genotype as compared to sensitive genotype under unstressed condition. Under stress condition, the level of catalase, ascorbate peroxidase and guaicol peroxidase increased at seedling stage while the levels of glutathione reductase and superoxide dismutase increased significantly at vegetative stage of both the genotypes. These results show that PRM-6107 is drought tolerant because the level of antioxidants as well as antioxidative enzymes is high in this genotype in seedling and vegetative stages. Also, the elevated levels of antioxidants and antioxidative enzymes at seedling stage show that plant start combating drought more strongly at seedling stage than at vegetative stage.

Keywords: antioxidant system; drought; seedling stage; vegetative stage; *Eleusine coracana*

Introduction

Abiotic stresses such as extremes of temperature, high light intensity, heavy metals toxicity, salinity, drought and availability of nutrients etc play an important role in growth and development of plants. It may severely affect the yield of plants too. When plant faces any kind of biotic or abiotic stresses, it acts differently to minimize the adverse effects caused by unfavorable conditions. Global warming is one of the major sources of drought stress. Drought affect the plant at molecular, physiological and biochemical level (Mahajan and Tuteja, 2005). Under dehydration conditions in plants, there is an increase in the formation of reactive oxygen species (ROS) such as H_2O_2 (hydrogen peroxide), O_2^{-}. (Superoxide), $HO_2^{\cdot}$ (perhydroxy radicals), $RO^{\cdot}$ (alkoxy radicals) and $OH^{\cdot}$ (hydroxyl) radicals (Arora *et al.*, 2002). The production of ROS molecules takes place within the compartment of different organelles like chloroplast, mitochondria and peroxisomes (Foyer and Noctor, 2003). ROS can act as second messengers involved in the stress signal transduction pathway (Chamnongpol *et al.*, 1998) but excessive ROS production can cause oxidative damages, which adversely affects plants by oxidizing photosynthetic pigments, membrane lipids, proteins and nucleic acids (Yordanov *et al.*, 2000; Hamdoon *et al.*, 2013). The degree of damage by ROS depends on the balance between the product of ROS and its removal by antioxidant scavenging mechanism (Azooz *et al.*, 2009). To cope up with these damages it is necessary to keep the level of reactive oxygen species under control. Therefore plants increase the levels of non-enzymatic and enzymatic antioxidant systems to protect cells from oxidative damages under drought stress (Mittler, 2002). Non-enzymatic antioxidants include flavanoids, phenolics, ascorbate, proline etc. Among non-enzymatic system, ascorbate is the main antioxidant produced by the plant cell. A high ratio of reduced to oxidized ascorbate is essential to eliminate ROS in cells. Ascorbate plays an important role for the detoxification of ROS in vacuoles during drought stress (Koffler *et al.*, 2014). The buffering capacity provided by ascorbate produces stress tolerance in plants (Apel *et al.*, 2004). Phenol and flavanoids also play important role in ROS detoxification under drought stress. These compounds are demonstrated to

have the ability of sequestering anion superoxides and hydroxyl and peroxyl radicals (Harbone *et al.*, 2000). Free proline accumulation takes place in plants subjected to drought stress. It acts as osmolyte for osmotic adjustment during drought as well as stabilizes sub-cellular structures such as membrane and proteins, scavenges free radicals and buffers cellular redox potential under stress (Schafleitner, 2007).

Other defence mechanism that the plant displays against drought stress is the production of antioxidant enzymes like superoxide dismutase, catalase, ascorbate peroxidase, guaicol peroxidase, glutathione reductase etc. Superoxide dismutase brings about scavenging of superoxide anion. It catalyses the dismutation of superoxide to hydrogen peroxide. The intracellular level of hydrogen peroxide is regulated by a wide range of enzymes like catalase and peroxidase. Catalases are peroxisomal enzymes that in contrast to peroxidases do not require a reducing substrate for their activity. Ascorbate peroxidase and glutathione reductase are components of Halliwelt-Asada cycle and bring about H_2O_2 detoxification. Ascorbate peroxidase uses reduced ascorbate as a substrate and catalysis reduction of H_2O_2 to water. Regeneration of ascorbate is carried out by monodehydro or dehydroascrobate reductase together with glutathione reductase. Many studies have reported that activity of antioxidant enzymes can be correlated with the drought tolerance capability of plants like wheat (Hasheminasab *et al.*, 2012; Abdullah and Ghamdi, 2009), rice (Qin *et al.*, 2010; Sharma and Dubey, 2005), chickpea (Mohammadi *et al.*, 2011) and marigold (Sedghi *et al.*, 2012).

With the rising problem of global warming, drought like conditions may prevail. Therefore it is important to select the plants that are able to tolerate such adverse environmental stresses. Finger millet (*Eleusine coracana*) is one such plant that is grown as important food crop in Africa and Asia. It contains more fibers, minerals and vitamins and has eight times more calcium than other cereals. A great degree of adaptability to drought is displayed in various varieties of finger millet. Study of biochemical responses to drought is important to agriculture in order to select crop varieties that can withstand the ill effects of drought and maintain yield. This study becomes important if we understand the response of antioxidant system at different stages in lifecycle of plant *i.e*. seedling, vegetative etc. Therefor**e** the present study was undertaken to understand the effect of drought on antioxidant system at different developmental stages in drought tolerant and sensitive genotypes of finger millet. The study would generate biochemical data of enzymatic as well as non-enzymatic antioxidants that would help in understanding the process of drought tolerance at different developmental stages in lifecycle of plants. Such study will also find use for recommending a particular variety for cultivation in a particular geographic location.

Material and Methods

Collection of Genotypes of E. coracana

Drought tolerant and sensitive genotypes of *Eleusine coracana* i.e. PRM-6107 and PES-400 respectively were collected from G.B.P.U.A&T Pantnagar. Seeds were washed with tween 20 and then surface sterilized with 0.5% sodium hypochlorite. Seeds were then germinated in pots containing sand, soil and vermicompost in 1:2:1 ratio. Seedlings were grown in polyhouse under controlled conditions for 15 days.

Drought Induction

Drought stress was induced at seedling (15 days old plants) and vegetative stage (45 days old plants) by withholding water until soil moisture content declined to 45% of the soil water holding capacity, which occurred after 8 days of withholding water. Control plants were irrigated on alternate days and maintained at 80% of soil water holding capacity.

Analytical Methods

Determination of Proline Content

Proline content was estimated by the method of Bates et.al. (1973). 0.2 gm leaf samples were homogenized in 5ml of 3% sulphosalycylic acid. The homogenate was centrifuged at 10,000 g for 5 min at room temp. 1 ml. of glacial acetic acid and 1 ml of 1 M acid ninhydrin were added to 1 ml of supernatant and kept on boiling water bath at 100^0C for 1 hour. Brick red color developed. The mixture was cooled and 2 ml. of toluene was added to it. It was then transferred to a separating funnel. After thorough mixing, the chromospheres containing toluene separated. Absorbance of samples was recorded at 520 nm against toluene blank.

Determination of Ascorbate Content

Ascorbate was estimated by the method of Roe and Keuther (1943). 0.2 gm of leaf samples were homogenized in 4% TCA. The homogenate was centrifuged at 10,000 rpm for 5 min. at room temp. Supernatant was collected and treated with a pinch of charcoal followed by vortering for 5 min. The mixture was centrifuged at 10,000 rpm for 5 min. at room temp. The volume of 1 ml. aliquot was made up to 2 ml. with 4% TCA and 2% 2,4-dinitrophenyl hydrazine reagent (DNPH) was added in all the tubes. Finally two drops of 10 % thiouria solution was added and incubated at 37^0C for 3 hours. It resulted in the formation of osazone crystals which were dissolved in 2.5ml of 85% H_2SO_4 in cold. The tubes were kept in ice & absorbance was recorded at 540 nm.

Determination of Flavonoid Content

Flavonoid was determined by the modified method of Ordonez *et al.* (2006). 0.5gm leaf samples were homogenized in 5 ml of 80% methanol. Homogenate was

agitated for 5 min. at 70^{0}C and centrifuged at 10,000 rpm for 15 min. 1 ml. of methanolic extract was mixed with 2 ml. of distilled water and 0.5 ml of 2% of $AlCl_3$ was added. The reaction mixture was incubated for 60 min. at room temp for yellow color development, which indicate the presence of flavonoid. Absorbance was taken at 420 nm using UV- visible spectrophotometer.

Determination of Phenolic Content

Phenolic content was estimated by the method of Swains and Hills (1959). 0.5 gm leaf samples were homogenized in 5 ml. of 80% methanol. Homogenate was agitated for 5 min. at 70^{0}C and centrifuged at 10,000 rpm for 15 min. 1 ml. of methanolic extract was mixed with 2 ml. of distilled water and 250µl of Folin- ciocalteu reagent was added to it. Mixture was kept at 25^{o}C for 5 min. and 1 ml. of 10% Na_2CO_3 was added to it followed by 1 ml. of distilled water. The reaction mixture was incubated for 60 min at room temp. Absorbance of developed blue color was measured at 725 nm against blank.

Enzymatic Methods

Preparation of Enzyme Extract

Enzyme extract was prepared by grinding 0.5 g of leaf samples with 50m M sodium phosphate buffer (pH 7.5) containing 0.4 g of 1 mM polyethyleneglycol ,4 gm of 8% (w/v) polyvinylpyrolydone and 50µl of 0.01 % (v/v) Triton X-100. The whole extraction procedure was carried out at 4^{0}C. The homogenate was centrifuged at 10,000 g for 15 min. at 4^{0}C and supernatant was collected which was used for the assay of enzyme activity.

Assay of Ascorbate Peroxidase

Ascorbate peroxidase (APX) (EC 1.11.1.7) was assayed by the method of Nakano and Asada (1981). The reaction mixture comprised of 1 ml of 50m M potassium phosphate buffer (pH 7.0), 50µl of 1 mM EDTA, 50µl of 0.5 mM ascorbic acid, 50µl of 25 H_2O_2 and enzyme source (100µl) at 25^{0}C. Ascorbate oxidation was followed spectrophotometrically by the decrease of absorbance at 290 nm and using the absorption coefficient 2.8mM–1cm–1. The enzyme activity was expressed as nmol $min^{-1}mg^1$ protein.

Assay of Catalase

Catalase (CAT) (EC 1.11.1.6.) activity was determined by the method of Aebi *et al.* (1983) .The reaction mixture comprised of 50mM potassium phosphate buffer (pH7.0) ,0.1mM EDTA containing 10mM H_2O_2, an enzyme source (100µl) in a final volume of 1150µl.The reaction was carried out at 25^{0}C .The activity of enzyme was recorded as increase in absorbance at 240 nm using the absorption coefficient ($36M^{-1}cm^{-1}$) and the enzyme activity expressed as µmol of H_2O_2 $min^{-1}mg^{-1}$ protein.

Assay of Glutathione Reductase

Activity of glutathione reductase (GR) (EC1.6.4.2) was determined according to Halliwell and Foyer (1978), 50 µl of 0.5mM oxidized glutathione (GSSG), 50 µl of 0.2 mM NADPH and enzyme extract (100µl) was added in 1ml of 50mM Tris-HCl buffer (pH7.5) and 50µl of 5mM $MgCl_2$. The reaction was carried out at 25^{o}C. The activity of enzyme was recorded as decrease in absorbance at 340 nm using the absorption coefficient ($6.02mM^{-1}cm^{-1}$). The enzyme activity was expressed as nmol $min^{-1}mg^1$ protein.

Assay of Guaicol Peroxidase

Guaicol peroxidase (GPX) (EC.1.11.1.7.) activity was determined as described by Urbanek *et al.* (1991).The reaction mixture comprised of 1 ml. of 100mM phosphate buffer (pH 7.0) into 0.1mM EDTA, and 100 µl of enzyme extract . Reaction was started by addition of 50 µl of guaicol (5mM) and 50 µl of H_2O_2 (15mM). The increase in absorbance was recorded at 470nm for 3min.The formation of tetraguaicol was quantified using its molar extinction coefficient ($26.6mM^{-1}cm^{-1}$). The enzyme activity was expressed as µmol $min^{-1}mg^1$ protein.

Assay of Superoxide Dismutase

Superoxide dismutase (SOD) was assayed according to the modified method of Kakkar *et al.* (1984). The assay of SOD was based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. Assay mixture contained 1.2ml of 0.025M sodium pyrophosphate buffer (pH 8.3), 0.1ml of 186µM Phenazine methosulphate, 0.3ml of 300µM Nitroblue tetrazolium, 0.5ml of the enzyme preparation and water to make a total volume of 2.8 ml. Reaction was initiated by the addition of 0.2ml of NADH. Reaction mixture was incubated at 30°C for 90 seconds. The reaction was stopped by the addition of 1.0ml of glacial acetic acid. The reaction mixture was shaken with 4.0ml of n-butanol for 10 min. Intensity of the chromogen in the butanol layer was measured at 560nm using a spectrophotometer.

Protein assay

Protein was measured following the procedure of Bradford (1976), using bovine serum albumin as a standard.

Statistical Analysis

The results were expressed as mean ± standard error. Experiments were performed in three replicates. Multiple comparisons were done by using software (STPR2) and Two Way ANOVA was applied. The difference between the means was compared by least significance difference. $P<0.05$ was considered as significant.

Result

The trend in changes of antioxidants as well as anti-oxidative enzymes level was same in both the genotypes. The level of antioxidants and anti-oxidative enzyme was more in seedling stage of both the genotypes. Only the level of SOD was found to be less at seedling stage. At vegetative

stage, there was a very high (nearly 20 times of seedling stage) accumulation of SOD activity in both the genotypes. On comparison of both the genotypes, the level of antioxidants as well as antioxidative enzymes was found to be more in resistant genotype (PRM-6107).

Effect of Drought on Antioxidant Level

The accumulation of antioxidants comprising of ascorbate, proline, phenolics and flavanoids was more in seedling stage than vegetative stage in both the tolerant as well as sensitive genotype. Drought stress enhanced the level of ascorbate by 1.6 times at seedling stage and nearly 1.5 times at vegetative stages of tolerant genotype PRM-6107 (Fig1).

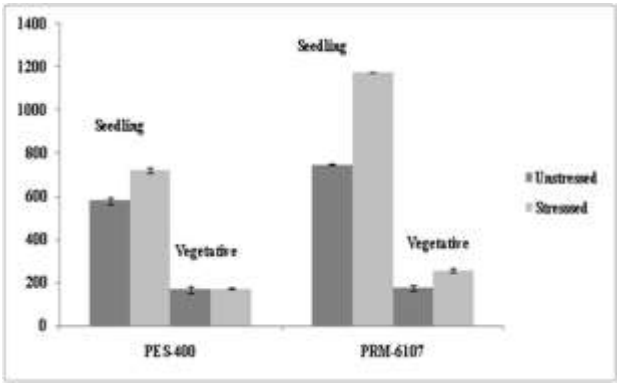

Fig. 1: Effect of drought on Ascorbate content at seedling and vegetative stage. Ascorbate content is expressed in mg/g fresh weight. Values are the mean ± SE for three observations.

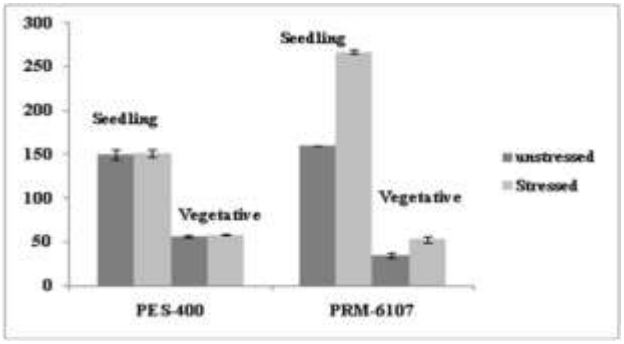

Fig. 2: Effect of drought on Flavonoid content at seedling and vegetative stage stage of E. coracana. Flavonoid content is expressed in mg/g fresh weight. Values are the mean ± SE for three observations.

The levels of ascorbate increased a little at seedling stage and remained relatively unchanged at vegetative stage in sensitive genotype PES-400. Flavonoid content in the leaves of tolerant genotype increased 1.7 times at seedling stage and 1.5 times at vegetative stage in tolerant genotype (Fig2). In sensitive genotype, flavonoid level remained almost unchanged under the influence of drought stress. A slight increase (1.2 times) in the level of phenols was observed in the leaves of tolerant genotype at seedling stage in response to drought stress. An increase of nearly 2.9 times was observed at vegetative stage of the same genotype (Fig. 3). However, no significant change was recorded in the sensitive genotype under water deficit conditions. Drought stress caused an increase in proline content in the tolerant genotype by 2.3 times at seedling stage and 1.2 times at vegetative stage (Fig. 4). The level of proline remained more or less unaltered in the sensitive genotype at both the developing stages.

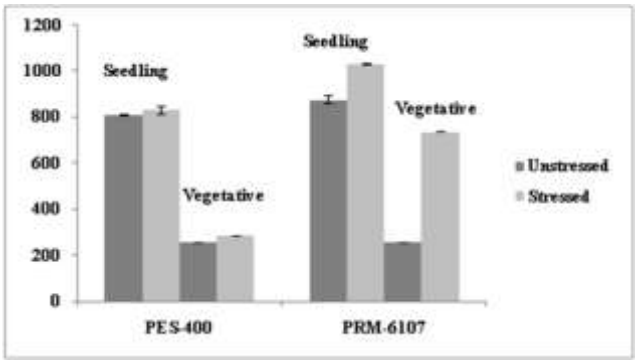

Fig. 3: Effect of drought on Phenolic content at seedling and vegetative stage of E. coracana. Phenol content is expressed in mg/g fresh weight. Values are the mean ± SE for three observations.

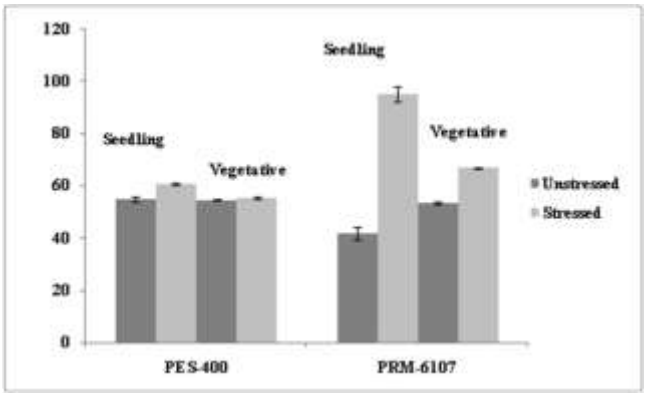

Fig. 4: Effect of drought on Proline content at seedling and vegetative stage. Proline content is expressed in µmol/g fresh weight. Values are the mean ± SE for three observations.

Effect of Drought on Antioxidative Enzymes

In the present study, the antioxidant enzymes activity increased significantly ($p<0.05$) at seedling as well as vegetative stages of PRM6107. However, the increase was more at seedling stage when compared to vegetative stage. There was no significant increase in the activity of antioxidative enzymes in PES400 at both the development stages. Nearly 1.8 times increase in the activity of catalase was recorded in the seedling of PRM6107genotype subjected to water stress conditions. The same genotype under similar conditions showed only 1.5 times increase in the enzyme activity at vegetative stage (Fig5). The sensitive PES400 genotype did not show any significant change in catalase activity under stress in both the developmental stages. With the onset of drought, the activity of ascorbate peroxidase increased nearly 2 times in seedling and 1.2 times in vegetative stage of PRM6107 (Fig6). However, there was no significant change in the activity of enzyme in

PES 400 at seedling as well as vegetative stage. We observed approximately 1.1 times increase in the activity of glutathione reductase in seedling and 1.5 times in vegetative stage in PRM6107 (Fig. 7). No significant change was recorded in the enzyme activity in PES400 in both the developmental stages. Drought stress induced guaicoal peroxidase activity in the different stages of PRM6107. There was a 1.3 times increase in activity in seedling as well as vegetative stage (Fig. 8). In addition, in PES400 genotype, no change in the enzyme activity was observed. Superoxide dismutase activity increased with the onset of drought in the vegetative stage of both the genotype (Fig. 9). The increase was 1.6 times in PRM6107 and 1.1 times in PES400. However, the level of enzyme activity was very low in the seedling stage of both the genotypes and no increase in the activity was recorded at the onset of drought.

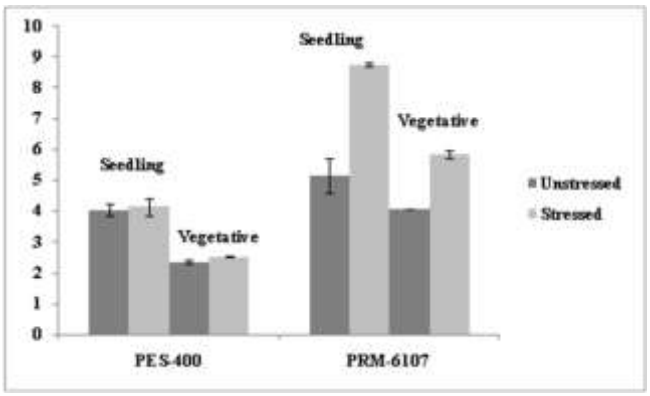

Fig. 5: Effect of drought on Catalase activity at seedling and vegetative stage of E. coracana. CAT activity is expressed as nmol H_2O_2 / mg protein / min. Values are the mean ± SE for three observations.

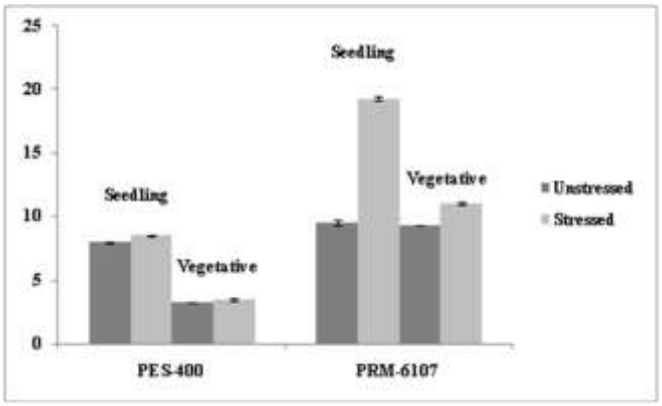

Fig. 6: Effect of drought on Ascorbate peroxidase activity at seedling and vegetative stage of E. coracana. APX activity is expressed as nmol ascorbate/ mg protein/min. Values are the mean ± SE for three observation.

At unstressed condition, the level of antioxidative enzymes was more in seedling stage of tolerant genotype as compared to sensitive genotype. Only level of superoxide dismutase and glutathione reductase was more in vegetative stage. When drought stress was induced, there was an increase in the activity of all these enzymes which together might have provided tolerance to the plant for survival in the adverse environmental condition.

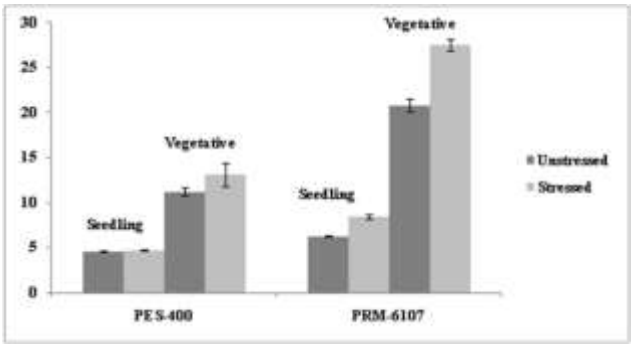

Fig. 7: Effect of drought on Glutathione reductase activity at seedling and vegetative stage of E. coracana . GR activity is expressed as nmol NADPH/mg protein/min .Values are the mean ± SE for three observations.

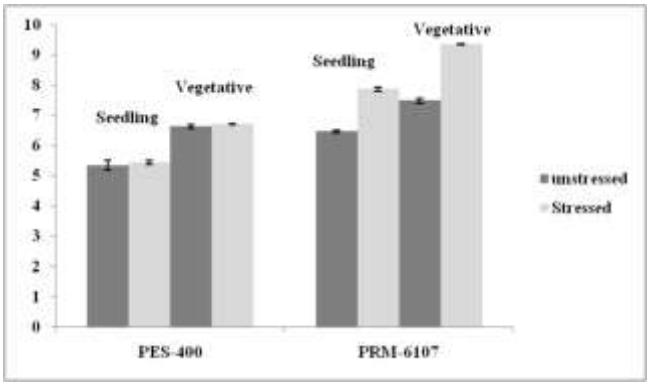

Fig. 8: Effect of drought on Guaicoal peroxidase activity at seedling and vegetative stage of E. coracana . GPX activity is expressed as nmol H_2O_2/mg protein/min. Values are the mean ± SE for three observations.

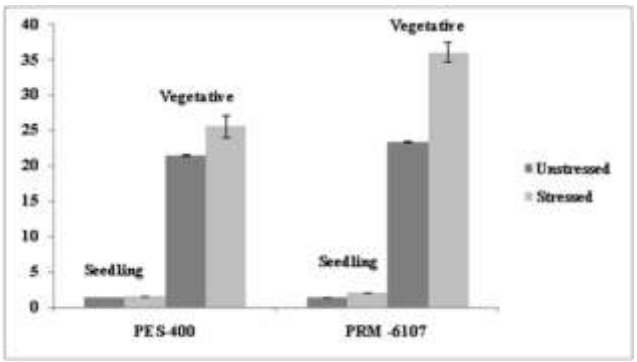

Fig. 9: Effect of drought on Superoxide dismutase activity at seedling and vegetative stage of *E. coracana*. SOD activity is expressed as U/mg protein. Values are the mean ± SE for three observations.

Discussion

Drought stress in plants is caused by loss of water through transpiration and decreased water uptake due to low water availability in the soil. Plant responses to drought by closure of stomata which limits gaseous exchange between the

leaves and the atmosphere and decrease the ratio of CO_2: O_2. This will create oxidative stress in chloroplast and will block electron transport to NADP. As a result, the electrons will be transferred to O_2 leading to generation of reactive oxygen species. Depending on the degree of water deficiency in plants, a large amount of ROS can be generated (Bhargava and Sawant, 2013, Hernandez *et. al*, 2013).

Under drought like condition, various antioxidants and antioxidative enzymes are key players in ROS detoxification. Overproduction of antioxidative enzymes like ascorbate peroxidase(APX), Glutathione reductase(GR), Catalase(CAT), superoxide dismutase(SOD) and Guaicoal peroxidase(GPX) and accumulation of non-enzymatic antioxidants like ascorbate, phenol, proline, flavonoids etc. have been shown to improve oxidative stress tolerance in plants (Xu *et al.,* 2008). Drought may affect the plant at any stage of its lifecycle. There are no reports on how plant respond to drought stress at two important stages of its lifecycle ie seedling and vegetative stages. In the present study, we observed that the plant's antioxidative machinery to respond to drought is more active at seedling stage as compared to vegetative stage.

In many plants, H_2O_2 scavenging systems as represented by APX and CAT, are more important in imparting tolerance against drought induced oxidative stress than SOD (Chen *et al.* ,2010; Hojati *et al.*, 2010). The antioxidative enzyme CAT converts H_2O_2 to water and O_2 (Gill and Tuteja 2010). An alternative mode of H_2O_2 destruction is via peroxidases, which are found throughout the cell and have a much higher affinity for H_2O_2 than CAT. Ascorbate peroxidase uses two molecules of ascorbate to reduce H_2O_2 to H_2O and to form dehydroascorbate (DHA) as the reducing substrate. This reaction generates oxidized glutathione, which in turn is re-reduced to GSH by NADPH the reaction catalyzed by glutathione reductase (GR). According to a report, it was observed that GR activity enhanced in the leaf because GSH concentration increased in leaf of plants. Similarly according to (Sharma and Dubey, 2005) an increase in GR activity in rice originated from enhanced GSH level. Higher POD activity has also been correlated with the relative drought tolerance of crop plants (Gillham and Dodge, 1987). It was reported that *Ctenanthe setosa* was tolerant to the administered drought stress, protecting itself from oxidative damage by increasing POD activity in leaves. According to previous studies it was observed that Guaicoal peroxidase and SOD activity increased in *Brassica napus* L. under drought stress. (Abedi and Pakniyat, 2010). It was also found that activities of SOD and CAT of *Antiaris toxicaria* seeds and axes increased during the initial phase of dehydration, and then decreased with further dehydration, whereas activities of APX and GR decreased with dehydration (Cheng and Song, 2008). Our study showed that at seedling stage, the increase in the activities of the enzymes ascorbate peroxidase, catalase, glutathione peroxidase and guaicoal peroxidase under drought condition was either similar to or significantly more than the increase recorded at vegetative stage under similar condition. Superoxide radicals that are produced in the different compartments of plant cells are rapidly converted to H_2O_2 by the action of SOD. There are reports that SOD activity increased in *Hordeum*, *Armeria* and *Deschampsia* (Smirnoff, 1993) while it decreased in *Corchorus* (Chowdury and Choudhuri, 1985) and did not change in alfalfa (Irigoyen *et al.* , 1992) and sunflower (Smirnoff, 1993) in response to water stress. Also, no significant changes in SOD activity were found in *Triticum aestivum* (Bartoli *et al.*, 1999) and *Lotus corniculatus* leaves (Borsani *et al.* , 2001) under similar condition. In the present study, SOD activity was recorded to be very low at seedling stage in control as well as drought stress condition. It may be that at seedling stage APX and CAT are the main enzymes which are carrying out H_2O_2 scavenging. At vegetative stage, SOD also contributes in detoxification of H_2O_2 as shown by the increase in the enzyme activity when the plant is exposed to drought.

Non-enzymatic antioxidants comprise of ascorbic acid, phenolics, proline, flavonoids, β-carotenes and reduced glutathione (GSH). Proline is the most common osmoprotectant that accumulates in plants in response to water stress. During drought stress, proline acts as an osmolyte as well as a source of nitrogen (Ahmed and Hasan 2011). Therefore, proline accumulation plays an important role in detoxification of ROS and interaction with the hydrophobic residues of proteins and osmotic adjustment (Yildiza and Terzia 2013). Increased proline accumulation was reported in water stressed sorghum (Jaleel *et. al.*, 2008) and can be thought as an adaptation to overcome the stress condition which constantly supplies energy for growth and survival and thereby helps the plants to tolerate stress (Jaleel *et. al.*, 2008). In our study, there was a significant increase in the level of proline with onset of drought in seedling stage of tolerant genotype. However, there was a moderate increase in vegetative stage of the same genotype, suggesting that the response of the genotype to combat drought is more vigorous at seedling stage than at vegetative stage.

Ascorbate is an important plant antioxidant. It is an eliminator of hydroxyl radicals which prevent free radicals chain reactions under drought stress condition. According to previous studies low level of ascorbate was found in response to water stress in *Vigna catjang* (Mukherjee and Chaudhari, 1983). In the present study, drought elevated the levels of ascorbate in seedling of tolerant genotype while there was a little increase was recorded in vegetative stage at similar condition. In sensitive genotype also, a little increase in ascorbate content was observed at seedling stage

only. This further shows that antioxidative response against drought is more active at seedling stage than at vegetative stage.

Phenolic are low molecular compounds in all tissues of higher plants with great significance in plant development. (Bhattacharya *et al.,* 2010). The synthesis and release of phenolics is induced by various biotic and abiotic factors. Stress condition induces accumulation of total phenols, which is positively related to antioxidant capacity in the plant and save it from the adverse effects of drought (Ivani and Oprica, 2013). Drought stress-induced phenolic compounds may increase the antioxidant activity (Singh *et al.*, 2014). In our study, phenolic compound accumulation increased with the onset of drought in vegetative stage of the tolerant genotype. In seedling stage, there was little increase in the level of phenolic compound in similar condition. However, the total content of phenolic compounds at seedling stage was nearly three times higher than at vegetative stage; the level was higher than the level attained by plants at vegetative stage subjected to drought conditions. This again highlights the heightened response of antioxidant system against drought.

Flavonoids, the derivatives of quercetin, act as antioxidants against free radicals, a group of naturally occurring polyphenolic compounds ubiquitously found in plants (Hamdoon *et al.*, 2013). According to a study, under the drought stress condition flavonoids largely accumulate in the proximity of ROS generation centers and maintain the plant homeostasis. It was reported that phenolic and flavonoid content was increased in root and shoot during drought condition in *Dracocephalum moldavica* L. (Halimeh *et al.*, 2013) and phenol content was decreased in nigella plant(Kabiri *et al.*, 2014). In the present research, flavanoid content increased in seedling stage many folds compared to its accumulation in vegetative stage under drought stress suggesting that seedling stage is better equipped to scavenge ROS that are generated during drought conditions.

From the results of our study on the effect of drought on antioxidant system in seedling as well as in vegetative stages of *E. coracana,* it can be concluded that the increase in the level of antioxidant was more at seedling stage compared to vegetative stage when the tolerant genotype was exposed to drought. The sensitive genotype also showed the similar trend but the increase was insignificant. This shows that the plant response to drought begins at the early stage of life cycle of plant. Similarly, the antioxidant enzymes activity also increased more at seedling stage than at vegetative stage. Thus the study shows that the plant has well defined system to counter the adverse environmental conditions like drought at seedling stage too. The defense is more vigorous in this stage as revealed by increased antioxidant levels and antioxidant enzyme activity. Therefore plant (*E. coracana*) can surpass unfavourable condition at seedling stage and the plant variety that carries out this function effectively is considered as drought tolerant. Such varieties can be recommended for growing in the area where drought like condition can be experienced at seedling stage. Studies can also be carried out at this stage to understand the mechanism of drought tolerance in plants at molecular, physiological and genetic level.

Acknowledgement

The authors gratefully acknowledge the grant (Project 1252) received from Uttarakhand State Council of Science & Technology (UCOST), Dehradun, INDIA for carrying out the research work.

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