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Studies on the activities of antioxidant enzymes under induced drought stress in *in vivo* and *in vitro* plants of *Macrotyloma uniflorum* (Lam.) Verdc.

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Abstract
Activities of glutathione reductase, guaiacol peroxidase, catalase and contents of ascorbic acid, Hydrogen peroxide were analysed in *in vitro* and *in vivo* plants of *Macrotyloma uniflorum* under Polyethylene glycol (PEG) induced drought condition. Water stress was induced in *in vitro* plants by supplementing the regeneration medium with PEG (Mol. Wt. 6000) at the concentrations ranging from 5 to 25% while in *in vivo* plants by watering PEG solution with the same concentrations. The activities of glutathione reductase and guaiacol peroxidase were increased under induced drought stress condition in both treated samples. On contrary, catalase activities were decreased. The contents of ascorbic acid and hydrogen peroxide were enhanced correspondingly as the concentration of the PEG increased from 5 to 25%. The role of antioxidant enzymes under water stress condition is discussed. Further, the feasibility of mining the novel genes of drought resistance from the *in vitro* plants of *Macrotyloma uniflorum* is highlighted.

Keywords: Tissue cultured plants, induced oxidative stress, antioxidants, Horse gram.

Water deficit limits plant growth and crop productivity in arid and semi-arid regions more than any one single environment factor [1]. It induces oxidative stress because of inhibition of photosynthetic activity due to imbalance between the light capture and its utilization. Oxidative stress affects physiological processes both at whole plant and cellular levels [2]. Because of the changes in the photochemistry of chloroplast in the leaves of the drought stressed, the generation of reactive oxygen species (ROS) will be enhanced. The accumulation of ROS has to be prevented by plants as soon as possible to maintain the growth and productivity. However, plants have evolved defense mechanisms to overcome the danger posed by the accumulation of ROS. Hence, whenever the plant is exposed to stress condition, either natural or induced, there will be induction of genes involved in the rescue act which is achieved by the synthesis of antioxidant enzymes. Levels of antioxidant enzyme activity and oxidant concentrations are frequently used as indications of oxidative stress in plants [3]. Studies on Sorghum and Wheat revealed that stress tolerant plants are normally endowed with efficient antioxidant defense system [4 and 5]. Identification of novel stress responsive genes in stress tolerant plants has great potential in the transgenic of stress susceptible plants. Over – expression of genes encoding antioxidant enzymes in transgenic plants have been associated with enhanced stress tolerance [6]. *Macrotyloma uniflorum*, commonly known as horse gram, is thus an ideal candidate for mining genes for abiotic stress tolerance, since it is relatively tolerant to drought, salinity and heavy metals. Thus identification of stress induced genes in this crop would provide a logical approach for improving drought tolerance in other related plants [7].

Tissue culture techniques have opened up many new possibilities of crop improvement since responses are well defined under control conditions. It is well established that *in vitro* cultures can induce oxidative stress [8] which consequently makes the defensive mechanism of tissue cultured plants more efficient and effective to sustain their growth under control conditions. Hence, identification of stress genes in the *in vitro* plants of tolerant crops will be of interest. *In vitro* selection can considerably shorten the time for the selection of the desirable traits under selection pressure with minimal environmental interaction and can complement field selection[9]. The present work is an attempt to study the activities of antioxidant enzymes in *in vivo* and *in vitro* plants of *Macrotyloma uniflorum* under induced water stress condition (Fig.1), as they contribute to plants ability to scavenge ROS which in turn determines the levels of tolerance to drought [10].

Seeds of *Macrotyloma uniflorum* (Lam.) Verdc, Var, PHG-9 were procured from GKVK, University of Agricultural Sciences, Bangalore, India. Aseptic seedlings were raised by inoculating surface sterilized seeds on moistened filter paper bridges in culture tubes. Shoot tips excised from one month old seedlings were used as explants and inoculated on L_2 + IBA (2.4 µM) + BAP (8.88 µM). The protocol for regeneration of multiple shoots was published elsewhere [11]. Thus obtained plants were used as tissue cultured control plants. Whereas the plants obtained on L_2 + IBA (2.4 µM) + BAP (8.88 µM) supplemented with PEG (Mol. Wt. 6000) at the concentrations ranging from 5 to 25% served as tissue cultured treated plants. *In vivo* treated plants were obtained by germination of

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seeds presoaked in different concentrations of PEG ranging from 5 to 25% for 24 h and sown in pots containing soil : sand : manure in 1 : 1 : 1 proportion. The seedlings were fed with same concentrations of PEG solution. The control *in vivo* plants were obtained from the germination of seeds without the pretreatment of PEG and seedlings were fed with tap water.

To prepare the enzyme extracts, fresh leaves from one month old seedlings of all the samples were collected and homogenized in 2 ml of extraction buffer having 50 mM EDTA at p^H 7.0. The homogenate was centrifuged at 12000g for 25 min and the supernatant was used as enzyme source. Guaiacol peroxidase (POX) activity was assayed following Chance and Machly [12]. The reaction mixture consisting of 50 mM phosphate buffer (p^H 7.0), 20 mM guaiacol, 10 mM H_2O_2 and 100 µl of enzyme extract was used to measure POX activity. Guaiacol peroxidase activity was measured by the increase in absorbance at 470nm (A_{470}) due to guaiacol oxidation. One unit of POX is defined as the amount of enzyme needed to convert 1 µ mol of H_2O_2 min^{-1} at 25°C. It is expressed as µ mol guaiacol / mon^{-1} g^{-1} FW or activity U/g FW. Catalase enzyme activity (CAT) was assayed by following Aebi [13] method. A reaction mixture having 50 mM sodium phosphate buffer (p^H 7.0) and 50 µl of enzyme extract was used to measure CAT activity. To this 10 mM H_2O_2 was added gradually and its consumption was measured for 2 min. The CAT activity was assayed by following the decline in absorbance of H_2O_2 at 240 nm (A_{240}). One unit of activity is defined as the amount of enzyme that catalyses the oxidation of 1µ mol of H_2O_2 min^{-1} under the assay conditions. It is expressed as µ mol H_2O_2 / mg protein min^{-1} or activity U/g FW.

Glutathione reductase (GR) activity was assayed using Carlburg and Mannervik method[14]. The total volume of the reaction mixture which is having 50 mM Tris – HCl buffer (P^H 7.5), 3mM $Mgcl_2$, 0.5mM GSSG, 0.2mM NADPH and 250 µl of enzyme extract was made up to 1.5ml. GR activity was determined by monitoring the oxidation of NADPH at 340 nm (A_{340}). Content of Hydrogen peroxide (H_2O_2) was determined following Velikova *et al*. method [15]. 500 mg of leaf samples was weighed and homogenized in an ice cold bath with 5 ml of 0.1(w/v) trichloroacetic acid. The homogenate was centrifuged at 10,000g for 15 min and 0.5 ml of the supernatant was added to 0.5 ml of 10mM potassium buffer (p^H 7.0) and 1 ml of 1 M KI. The absorbance of the supernatant was measured at 390 nm. Ascorbic acid activity was determined using Oser method[16]. 0.1 g of leaf was homogenized in 6% TCA. From the homogenate, 4 ml was taken and to this 2 ml of 2% DNPH, 1 drop of 10% thiourea were added. The contents were boiled for 15 min in a water bath and cooled. After cooling, 5 ml of 80% (v/v) H_2SO_4 was added. The absorbance was read at 530 nm.

The results were presented as mean ± standard error based on five replications. The data was subjected to one way ANOVA, significant 'F' ratios between groups means were further subjected to DMRT using SPSS version 15. Probability values < 0.05 were considered significant.

Addition of PEG to nutrient solution produces osmotic stress over a period of 3-4 weeks [17]. The consequence of stress is an increased production of reactive oxygen species [18]. The primary response of the plant to elevated ROS production is the production of antioxidant molecule and elevated activation of antioxidant enzymes [19]. Peroxidases are a large family of enzymes and are associated to changes in physiological processes of plants under stress [20]. POX levels were elevated under induced stress conditions in both treated *in vivo* and *in vitro* plants. The POX activity was higher in *in vitro* plants treated with PEG at 25% showing 231.31 µ/g FW and lowest was observed in 5% of PEG treated *in vivo* plants. Increased in POX activity was reported in several other plants [21 and 22]. This may be due to the increase in contents of POX substrates in response to water stress. The POX substrates such as glutathione, ascorbate and phenolic compounds are scavengers of a ROS [23]. The accumulation of these metabolites could lead to an increase in peroxidase activity in the presence of enhanced levels of H_2O_2 [24]. In the present studies, the level of H_2O_2 was also increased in induced stress condition in both treated samples. Tolerant cultivars decompose toxic H_2O_2 which accumulates at higher levels due to reduction in the rate of CO_2 fixation. H_2O_2 content gradually increased with increasing water stress due to the decreased activity of CAT [25]. The decline in CAT activity is regarded as a general response to many stresses [22]. CAT catalyses the dismutation of hydrogen peroxide into water and oxygen whereas peroxidases decompose H_2O_2 by oxidation of substrates [26]. In the present study, the CAT activity was found decreasing as the concentration of the PEG increased. The activities of CAT were 54.429 U/g to 22.2 U/g FW in 5- 25% PEG treatment in *in vitro* plants as against 51.669 U/g FW and 19.622 U/g FW in *in vivo* plants at the same concentration of PEG respectively. The decrease in CAT activity indicates its inactivation by the accumulated H_2O_2 induced by water shortage and can be explained partly by photo inactivation of the enzyme [24].

Ascorbic acid, another important plant metabolite has been implicated in the regulation of different processes associated with plant growth and development. It has been shown that ascorbic acid maintained the osmotic status of the stressed tissue [27]. In the present study there was an increase in levels of ascorbic acid under induced water stress condition in both treated *in vivo* and *in vitro* plants. However, the levels are almost same in both the samples. Increase in ascorbic acid level might occur as a result of conversion of oxidized ascorbic acid to its reduced form as suggested by Fruton and Simmonds (28) indicating reduction of dehydroascorbic acid to ascorbic acid upon treatment with reduced glutathione and cysteine. Glutathione reductase (GR) is the principal soluble antioxidant enzyme. It catalyses the reduction of oxidized glutathione to reduced glutathione, an important endogenous antioxidant [26]. GR activity was enhanced in both *in vivo* and *in vitro* plants under induced stress conditions. The GR activity was increased to 492-05 µ/g FW under 25% PEG treatment in *in vitro* plants as compared to 479.21 µ/g FW in *in vivo* plants under the same treatment. The enhancement is almost double in both the samples compare to control plants. Increase in ascorbic acid content in induced stressed plants can be explained by the production of reduced glutathione in large quantitives by enhanced GR activity. It was shown that O^{-2} and H_2O_2 generated during water stress might be responsible for the induction of GR [29]. Content of H_2O_2 was increased in both the treated samples indicating correspondingly increased POX and GR levels and decreased CAT levels. The content of H_2O_2 was increased from 9.36 mg/g FW to 22.329 mg/g FW as the concentration of PEG increased from 5 to 25% in *in vitro* plants as against 7.924 mg/g FW to 20.601 mg/g FW in *in vivo* plants. Tolerant cultivars decompose toxic H_2O_2 which accumulates at higher levels due to reduction in the rate of CO_2 fixation which result from retardation in electron transport system or due to reduction in CAT activity [25].

The aforesaid data clearly indicates that *in vitro* plants have better adopting strategies to cope up with the stress conditions than

the *in vivo* plants. It can be concluded that they are the better candidates for identification of stress responsive genes and can be exploited in transgenics technology. Reports of Dertinger *et al*. [30] and Lai *et al*. [31] on the enhancement of activities of antioxidant enzymes in transgenic has strengthen this opinion. The scavenging activity of methonolic extracts of *in vitro* plants of three species of *Dendrobium* on DPPH was significantly higher than the control plants [32]. *In vitro* selection incorporated with molecular and functional genomics can provide an opportunity improve stress tolerance in stress susceptible plants [33].

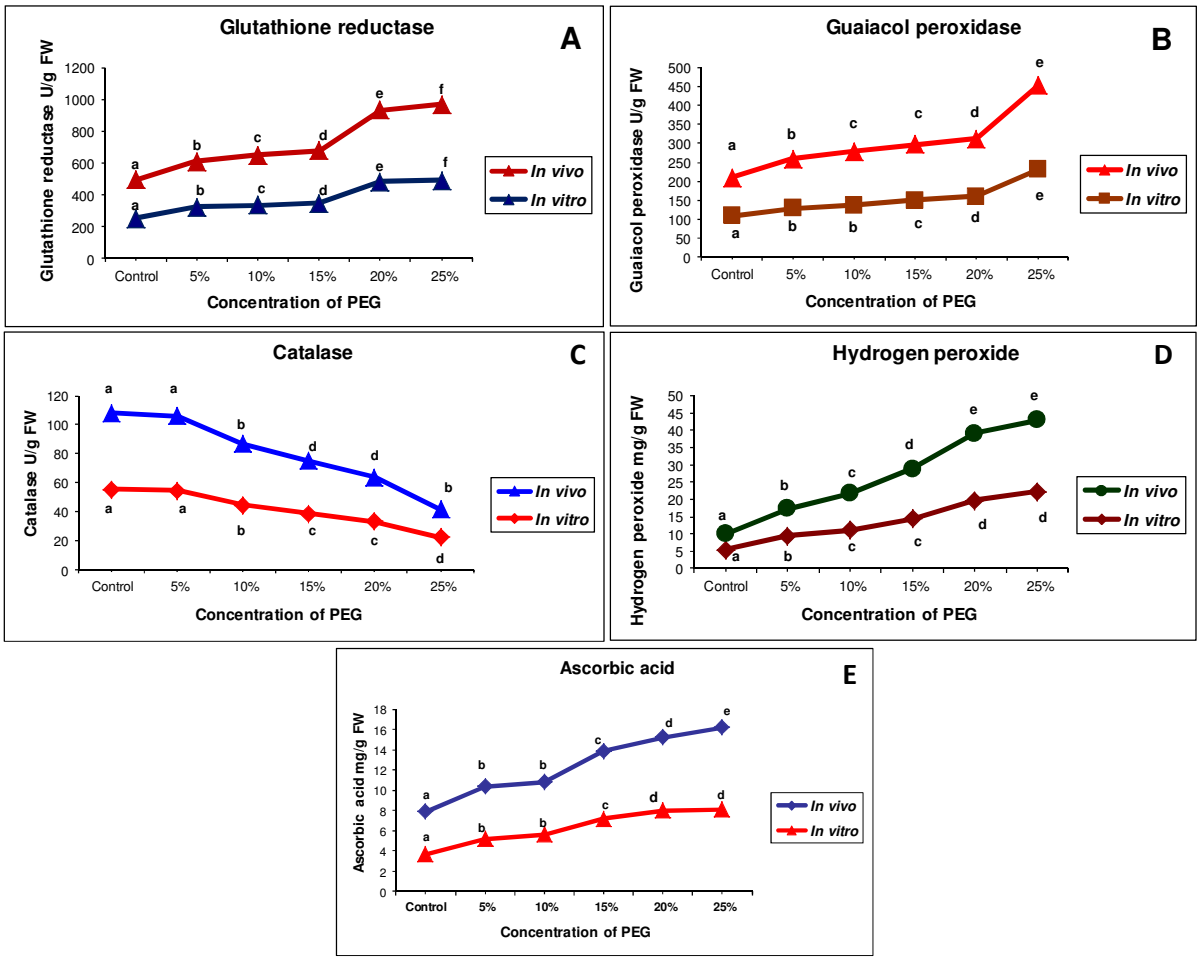

Fig. 1. Effect of induced water stress on activity of glutathione reductase (A), Guaiacol peroxidase (B) and catalase (C) and content of hydrogen peroxide (D) and ascorbic acid (E). The same alphabets followed are not significantly different according to DMRT ($p<0.05$).

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