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EFFECT OF EXOGENOUS APPLICATION OF H_2O_2 IN ELEUSINE CORACANA PLANTS IS CORRELATED WITH INCREASED ACTIVITY OF ANTIOXIDANT ENZYMES IN A TIME DEPENDENT MANNER

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Keywords: APX, Superoxide Dismutase, Catalase, H_2O_2, Antioxidant, Abbreviations, H_2O_2 - Hydrogen Peroxide, APX - Ascorbate Peroxidase, SOD - Superoxide Dismutase, CAT – Catalase

ABSTRACT

Arid and semiarid regions are characterized by a very stochastic environment, where biotic and abiotic factors exert determinant influences on plant metabolism and restrict distribution and productivity of plants. Adaptation to stress is undoubtedly one of the most complex biological processes. Hydrogen peroxide (H_2O_2) is among the most stable molecule among reactive oxygen species (ROS) acting as a major signaling molecule at lower concentration in plants. It is produced in chloroplasts and mitochondria via electron transport, where oxygen is reduced to superoxide which is further dismutated to H_2O_2 spontaneously or catalyzed by superoxide dismutase. Ascorbate peroxidase: Superoxide dismutase ratio is also known to be a critical factor governing the stress tolerance potential in finger millet plants. H_2O_2 being a toxic byproduct but at certain lower levels prove to be an important messenger in signal perception. Therefore in order to obtain a better knowledge about the physiological mechanism of endurance signal under oxidative stress we used exogenous H_2O_2 for different time intervals. The results suggest that after exogenous signal perception the level of superoxide dismutase increases; leading to H_2O_2 generation which in turns specifically increases the level of enzyme ascorbate peroxidase. This inter related differential expression ratio of SOD/APX can be proposed as a possible tool for bio monitoring the levels of oxidative stress faced by the plants.

I. INTRODUCTION

Plants functioning in an aerobic environment are often subjected to continuous threat from molecular oxygen which is due to toxic reactive oxygen species (ROS). Under any stress stimuli there is a signal generation

concomitantly producing reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), the superoxide anion, and hydroxyl radicals, which in turn damage membranes and enzymes. These reactive oxygen species have the capacity to degrade almost all cell components including membrane lipids, proteins and DNA (Hendry, 1993, Casano et. al., 1997). Toxic hydrogen peroxide is a product of peroxisomal and chloroplast oxidative reactions and can act both as an oxidant and reductant. It is the most stable form of the ROS and is also capable of rapid diffusion across cell membrane (Luis et al., 1992; Upadhyaya et al., 2007). It also changes the redox status of surrounding cells where it initiates an antioxidative response by acting as a signal of oxidative stress (Sairam and Srivastava, 2000). External factors are known to induce $H_2O_2$and other toxic oxygen species production in cellular compartments and result in increased activity of stress metabolizing antioxidant enzymes. Hydrogen peroxide (H_2O_2) is one of the major reactive oxygen species (ROS) in plant tissues. It is produced in chloroplasts and mitochondria via electron transport, where oxygen is reduced to superoxide which is further dismutated to H_2O_2 spontaneously or catalyzed by superoxide dismutase (Asada 1999; Møller & Sweetlove 2010). H_2O_2 production in plant cells is also catalyzed by glycollate oxidase in peroxisomes (Noctor et al. 2002), membrane bound NADPH oxidase (Jiang and Zhang 2003) and oxalate oxidase (Hu et al. 2003). When plants are subject to environment stress, it accumulates and leads to oxidative damage (Asada 1999). Accumulating evidence suggests that H_2O_2 is a key signaling molecule involved in plant response to both biotic and abiotic stresses, such as pathogen attacks, extreme temperatures, drought, excessive radiation, ozone and wounding (Neill et al. 2002; Foyer et al. 1997; Prasad et al. 1994; Orozco-Ca´rdenas et al. 2001; Wohlgemuth et al. 2002).Pre-treatment of plants generally known as priming, a process by exposing plants to some chemical compounds that makes them more tolerant towards environmental stresses, is potentially an important mechanism of induced resistance in plants against biotic stresses.

To test the hypothesis that H_2O_2 induces the level of H_2O_2 metabolizing enzymes in a specific interrelated manner, the present investigation has been undertaken with the aim to study the effect of increasing time duration of H_2O_2 application in the leaves of finger millet plants by performing enzyme kinetic reactions and semi quantitative expression analysis.

II. MATRIAL AND METHODS

2.1 Growth Conditions

Seeds of PR202 and PES400 variety of finger millet were obtained from VPKAS Almorah, Uttarakhand, India. Seeds were washed for 5 minutes with a mild detergent (Tween-20) and were then surface sterilized for 1 minute with 0.5% sodium hypochlorite. Seeds were germinated in pots containing sand, soil and vermi-compost in 1:2:1 ratio. Seedlings were grown in a poly-house under controlled conditions (at 28°C with light intensity of 40µE m^{-2} s^{-1}) for 60 days.

2.2 Exogenous H_2O_2 treatment

In order to identify the supportive role of H_2O_2 in regulating antioxidant enzyme levels for mitigation of deleterious effect of endogenous ROS, 60 day old plants were misted with an aqueous solution containing 50 mM H_2O_2 according to modified protocol of Gechev et al., 2001 and Ishibashi et al., 2011. Samples were collected at

0hr(control), 0.5hr, 1hr, 2hr, 4hr, 6hr and 8hr 16hr 20hr 24hr of time intervals and immediately frozen in Liq. Nitrogen for further analysis.

APX activity: For Ascorbate peroxidase activity measurement the procedure given by Nakano and Asada (1981) was used. 0.4 gm of leaf sample was homogenized in 4.0 ml of chilled homogenization buffer [100mM potassium phosphate buffer (PPB), (pH 7.0) and 0.1mM EDTA] and centrifuged at 12,000 rpm for 30 min at 4 ºC. The supernatant was collected in a fresh super cooled micro centrifuge tube. For enzyme assay, 60µl of supernatant was mixed with 1438 µl of assay buffer [50mM PPB (pH 6.0), 0.1 µM EDTA, 0.5mM ascorbate] in a quartz cuvette and then 2 µl of 0.5M H_2O_2 was added to start the reaction. The decrease in absorbance was measured for three minutes at 290nm; at an interval of every 5 seconds. Protein content in each sample was also measured using Bradford dye binding method.

The specific activity of ascorbate peroxidase was expressed in µmol min^{-1} mg^{-1} protein using the extinction coefficient as 2.8 mM^{-1} cm^{-1} at 290nm.

SOD Activity: Superoxide dismutase (EC 1.15.1.1) activity was determined by the nitro-blue tetrazolium (NBT) photochemical assay method according to Beyer and Fridovich (1987). In this method, 1mL of solution containing 50mM potassium phosphate buffer (pH 7.8), 9.9mM L-methionine, 57 µM NBT and 0.025% (v/v) Triton-X 100 was aliquotted into small glass tubes followed by 20 µL of sample. Reactions were started by adding 10 µL of aqueous riboflavin solution (44 μgmL^{-1}) and placing the tubes in an aluminum foil-lined box with two 20 W fluorescent lamps for 7 min. A parallel control was run where buffer was used instead of sample. After illumination, the absorbance of the solution was measured at 560 nm. A non-irradiated complete reaction mixture served as a blank. SOD activity was expressed as Umg−1 protein. One unit of SOD was defined as the amount of protein causing a 50% decrease of the SOD-inhibitable NBT reduction.

2.3 Protein estimation by Bradford method:

The method based on Bradford (1976) was used for protein estimation.

Reagents:

Dye solution:

Comassie brilliant blue G-250	10 mg
Absolute alcohol	5 ml
Orthophosphoric acid 85 %	10 ml

The volume was made up to 100 ml with distilled water.

Procedure

1. Forty µl of protein samples were taken and final volume was made 300ul by distilled water. 3ml of Bradford dye was added and shaken vigorously.
2. A reagent blank with 0.3 ml water was taken.
3. A stock of BSA (1mg/ml) was prepared and aliquots of representing 10, 20,30,40,50,60,70,80,90,100 ug protein were taken. Final volume of each tube was made up to 0.3 ml.
4. Then reaction mixture was incubated at RT for 5 minutes.

5. Absorbance was recorded at 595 nm against a reagent blank for all samples.
6. Graph was plotted between the Absorbance Vs. amount of protein.
7. The amount of in the sample was determined by extrapolation from the standard curve of BSA.

III. RESULTS AND DISCUSSION

It has been shown by many research groups that H_2O_2 is non-toxic or even beneficial at moderate concentrations but toxic at high concentrations (Desikan et al., 1998; Hossain et al., 2015). The advantage of application by spraying is that plants stay intact and are not mechanically wounded, thus eliminating the difficulties interpreting results obtained from wounded plants (Guan and Scandalios, 2000; Orozco-Càdenas et al, 2001). An increased capacity of the antioxidant system is one of the possible mechanisms responsible for oxidative stress tolerance as demonstrated by the existence of stress-resistant lines with naturally enhanced antioxidant systems (Jiang & Zhang 2002; Fan et al., 2015). The main aim of study was to monitor the H_2O_2 mediated changes in the activity of antioxidant enzymes APX (Ascorbate peroxidase), SOD (Superoxide dismutase) and CAT (Catalase) in finger millet plants. Such an idea is supported by recent evidence that H_2O_2 as a signal molecule plays a key role in antioxidant enzymes mediated plant defense under many abiotic and biotic stress factors **(Neill et al., 2002; Mittler et al., 2004; Maruta et al., 2016)**.

Upon exogenous treatment with H_2O_2, which is a direct substrate of the ascorbate–glutathione cycle, a burst of expression was noticed in enzyme activity of SOD which was consequentially followed by APX, however no significant change was monitored in other H_2O_2 detoxifying enzyme ie. catalase. This initial increase in SOD followed by a higher increase in activity of APX in PR202 may be correlated with an efficient antioxidant system present in this plant, which recognizes external stimuli upon H_2O_2 spray. In a similar study it has been reported that exogenous H_2O_2 can induce tolerance to osmotic stress in cucumber leaves by increasing antioxidant activity, thereby protecting the ultra structure of cellular membranes **(Liu et al., 2010; Sun et al., 2016).**

Both varieties (PR202 and PES400), exposed to exogenous H_2O_2 treatment, showed a temporal increase in activity of both the enzymes in the initial hour of H_2O_2 treatment; however a similar trend in the increase of the SOD enzyme activity was recorded in both the varieties.

The maximum increase in the level of SOD reached at 6hr interval for both the varieties which gradually decreased with further increase in time**(Fig 1)**. SOD activity directly modulates the amount of ROS present at specific time and a higher SOD activity thus is likely to contribute to higher level of ROS under H_2O_2 mediated stress, which was found to be more in 6hr interval for PES400 as compared to PR202 **(Fig 1)**. In contrary, the other variety PR202 shows a lower level of H_2O_2 induces ROS, which is further corroborated by the decreased level of SOD at each time interval as and when compared with PES400, further substantiating it to be a better performing variety. Our results are in agreement with the other results where salt stress was found to increase SOD activity in a salt-tolerant cultivar of tomato and mulberry **(Röhrdanz & Kahl 1998; Harinasut et al., 2003; Mittova et al., 2003)**. Second enzyme tested was ascorbate peroxidase that plays an important role in the fine regulation of ROS concentrations in cells **(Gechev et al., 2006; Hossain, M.S. & Dietz 2016)**. Activity for APX started peaking after 0.5 hrs of H_2O_2 treatment which increased further with time and the APX activity reached its maximum at 8hr interval for both the

varieties **(Fig 2)**. However a increased activity of apx was recorded in PR202 over that of PES200 in all the time intervals. The reason might be that after exogenous H_2O_2 application there might me an initial generation of ROS followed by dismutation of superoxide radical into H_2O_2 by SOD, which peaked at 6 hrs hr interval, this was followed by a gradual enhancement in the levels of ascorbate peroxidase which direct the breakdown of H_2O_2 into water as noticed through the apx activity that peaked at 8hr after H_2O_2 treatment. However the levels of CAT enzymes did not show any significant treatment after exogenous H_2O_2 spray over that of control plants in both the varieties tested **(Fig 3).** As APX and CAT both share a similar substrate for ie. H_2O_2, and as APX has shown major role in converting majority of substrate (Fig 2) therefore the levels of CAT may not have raised significantly. After that there was a gradual decline in the enzyme activity for all the enzymes tested.

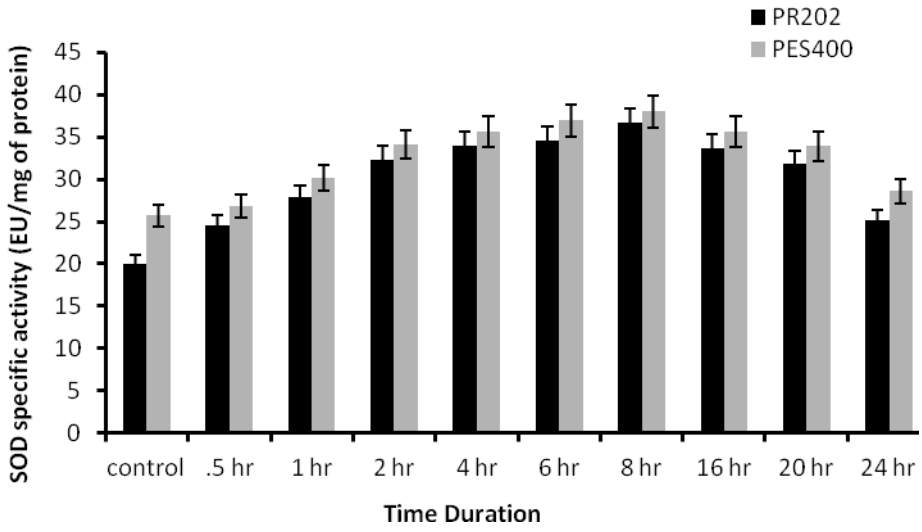

Fig1. Specific activity of SOD after exogenous spray of H_2O_2 in two contrasting varieties

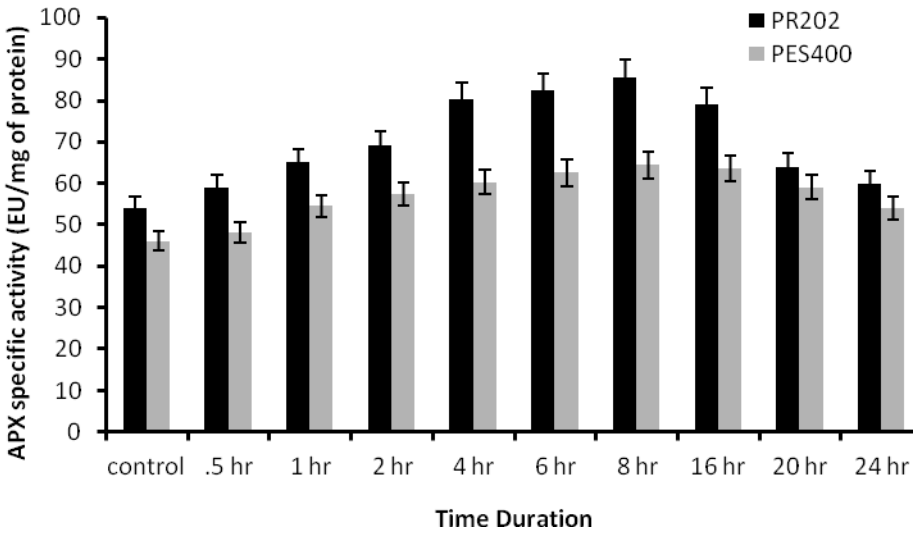

Fig.2 Specific activity of APX after exogenous spray of H_2O_2 in two contrasting varieties

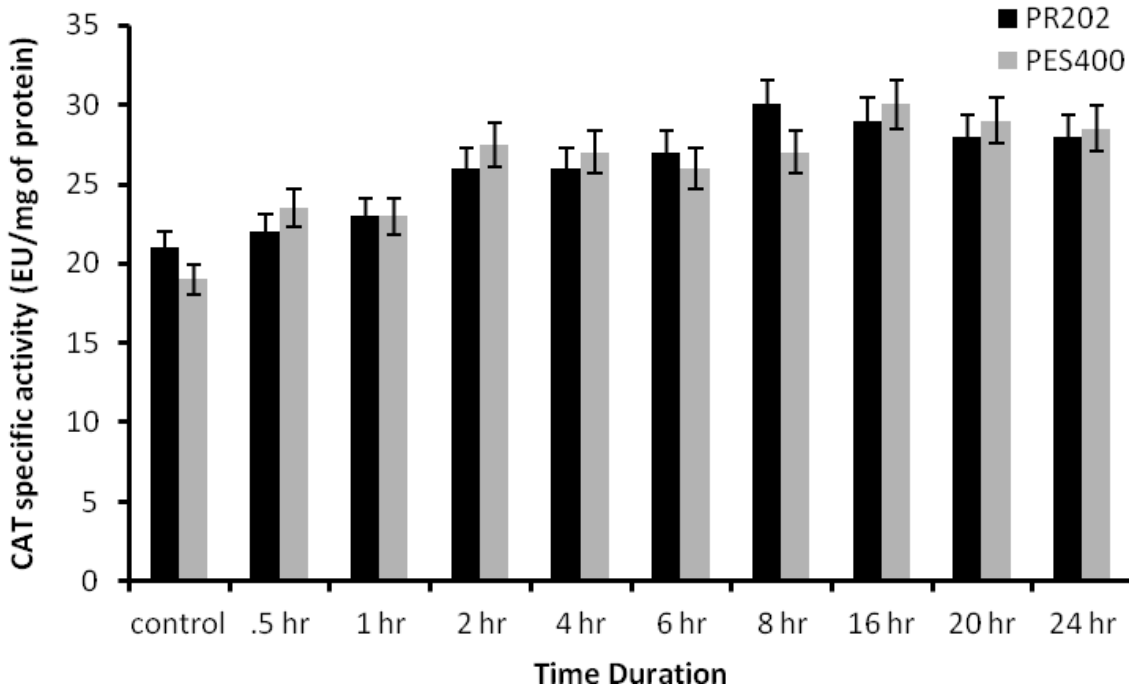

Fig3. Specific activity of CAT after exogenous spray of H_2O_2 in two contrasting varieties

Our results clearly suggest that pre spraying of plants with H_2O_2 may help to alleviate the toxic effect of ROS upto some extent. It was quite noticeable that how a brief application of moderate doses of H_2O_2 can significantly enhance oxidative stress tolerance by elevating the antioxidant status of the plant cell. Such a modulation of the plant antioxidant system may be useful in protecting plants against adverse factors that cause oxidative stress. Furthermore *apx / sod* ratio is found to be a crucial factor in alleviation of oxidative stress induced by exogenous H_2O_2 application. Therefore *sod* and *apx* could very well be selected as a possible tool for bio monitoring the levels of oxidative stress faced by the plants due to high correspondence of its biochemical and expression patterns under oxidative stress.

IV. CONCLUSIONS

Plants possess various intricate mechanisms to mitigate the deleterious effect of environmental stress. Different varieties are invariably different in their physiological and biochemical properties and thus response differently against the external stimuli. Furthermore different levels of stresses also play a critical role in altering the plant physiological conditions. This perception is a critical part which monitors the response time which is a vital factor for plants survival. Here in our experiments two different varieties were accessed for their differential perception for exogenous H_2O_2 levels. Results ascertain that PR202 was the variety that was found to be more tolerant to withstand a prolong stress when exogenously treated with H_2O_2 while upon a similar treatment variety PES400 was found to be much susceptible when compared with PR202.

Delineating the mechanisms of exogenous H_2O_2 induced abiotic stress tolerance will be valuable for identifying biotechnological strategies to improve abiotic stress tolerance in crop plants

Thus bio-prospecting of stress responsive genes could be performed in future using PR202 as a stress tolerant variety.

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