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RESEARCH ARTICLE

EFFECT OF HYDROGEN PEROXIDE ON PROTEIN CONTENT AND CATALASE ACTIVITY IN *Cajanus cajan* (L.) Millsp. LEAVES

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ABSTRACT

In this report protein content and catalase (CAT) activity were used to study the effect of hydrogen peroxide (H_2O_2) on leaf senescence in detached Pigeon pea leaves. A decrease in protein content measured as an indicator of leaf senescence, and a drop in CAT activity was observed following treatment with H_2O_2 in Pigeon pea detached leaves compared with control leaves. However after longer incubations CAT activity significantly increased in comparison with day 1 treatment. Protein content and CAT activity were also studied in the leaves treated by 0.025, 0.05, 0.075 and 0.1 mM H_2O_2. The optimal concentration of H_2O_2 in reducing protein content seems to be 0.075 mM, whereas concentration of 0.025, 0.05, 0.075 mM increase the CAT activity while lower and higher concentrations shown the opposite effect. The observed changes revealed that H_2O_2 induces oxidative stress and oxidative damage thereby leaf senescence in the detached leaves of Pigeon pea.

INTRODUCTION

Plants are confronted with exposure to most, if not all biotic and abiotic stresses including strong light, drought, salinity, low or high temperature, air pollutants, herbicides, nutrient deficiency, throughout their lives (Shim *et al.,* 2003; Nahakpam and Shah 2011). Extensive study on oxidative stress has demonstrated that exposure of plants to adverse environmental conditions induces the overproduction of reactive oxygen species (ROS), such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals in plant cells (Hung et al. 2005). Singlet oxygen (O_2) which may arise due to the reaction of O_2 with excited chlorophyll is also considered as a one of the potential ROS (Chen *et al.,* 2011). The accumulation of ROS damages all most all cell components including membrane lipids, chloroplasts, pigments, enzymes, nucleic acids (Verma and Dubey 2003; Upadhyaya 2007) and leads to the death of cells (Liu *et al.,* 2010). Recently, many researchers have focused on the functional aspects of H_2O_2. H_2O_2 is a product of peroxisomal and chloroplastic oxidative reactions (Lin and Kao, 2000). It is the most stable form of the ROS and is capable of rapid diffusion across cell membrane (Upadhyaya *et al.,* 2007). H_2O_2 can also react with superoxide radicals to form more toxic hydroxyl radicals in the presence of transition metals (Hung and Kao, 2005). H_2O_2 is not only is a harmful ROS but also has a role as a signling molecule in pathways of stress signal transduction (Liu *et al.,* 2010). H_2O_2 alters the redox status of surrounding cells where it induces an antioxidative response by acting as a signal of oxidative stress (Upadhyaya *et al.,* 2005). Incubation of detached leaves under H_2O_2 treatment is an ideal system for the rapid induction of leaf senescence (EI-Shora, 2003; Hung and Kao, 2005; Upadhyaya *et al.,* 2007). Detection of lipid peroxidation and protein loss in H_2O_2-promoted senescent leaves (EI-Shora, 2003; Hung and Kao, 2007; Lin and Kao, 2007), suggest that H_2O_2-promoted senescence is mediated through oxidative stress. Leaf senescence refers to the final developmental stage of leaves by which cells undergo programming changes results in hydrolysis of macromolecules such as proteins, chlorophylls, lipids, polysaccharides and DNA, which leads to cell death. Yellowing of the leaves due to chlorophyll breakdown is the most obvious visible characteristic (Smart, 1994; Gan and Amasino, 1997; Gepstein, 2004).

Plants have developed specific antioxidative defence enzymes to control the rapidly increasing ROS under various environmental stress conditions. Antioxidative enzymes work in several ways. For one, they may reduce the energy of free radicals, their by causing it to become stable. The other one, they may also interrupt an oxidizing change reaction to minimize the damage caused by ROS (Pastori and Delrio, 1997). Catalase (CAT) is one of the major antioxidant enzymes and efficiently scavenges H_2O_2 and does not require a reducing substrate to perform the task (Kumari *et al.,* 2006). CAT is a tetramer of four polypeptide chains, each over 500 amino acids long, which is commonly found in nearly all living organisms (Joseph and Jini, 2010). The importance of CAT in scavenging active oxygen generated under stress conditions was also proven by willekens, who depleted CAT gene in transgenic tobacco and confirmed that its leaves were damaged more severely by H_2O_2-promoted senescent leaves compared with normal tobacco leaves (Shim *et al.,* 2003). Most legume species have been found to be either sensitive or moderately tolerant to stress factors although considerable variability in stress tolerance has been reported among and

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within legume species (Garg and Noor, 2008). Pigeon pea (*Cajanus cajan* (L.) mill sp.) is one of the major grain legume (pulse) crops of the tropics and subtropics (Malviya and Yadav, 2010) that can provide fuel wood and fodder for the small scale formers in subsistence agriculture (Egbe and Kallu, 2009). The extract of Pigeon pea is commonly used all over the world for the treatment of diabetes, dysentery, hepatitis and measles, as a febrifuge to stabilize the menstrual period (Wu *et al.,* 2009). The present study has conducted to examine the effect of H_2O_2 on the protein concentration and CAT activity.

MATERIALS AND METHODS

Site Description

Pigeon pea grown in the forms of Biochemistry Department, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad in India were used for during February of 2011. The site is located near about 10 km away from the center of Aurangabad, and the above trees are not under any specific air pollution. The site of the sample area is surrounded by hills on all directions, and characterized by a semiarid climate, with annual temperature in range from 9-40°C and mean annual precipitation of 725 mm. The main climatic characteristics of the sample site are shown in Table 1.

Table 1. Main characteristics of the sample site

Parameter	Value
Annual temperature range	9-40°c
Average annual precipitation	725 mm
Altitude	131 m
Latitude	19° 53' N
Longitude	75° 23' E

Plant Material

A sample of 10 thick mature leaves at equal distance from twig tip was collected from three neem trees. The leaves were procured for each estimation. They were then washed in distilled water and dried just before use.

Chemicals

Phenylmethylsulfonyl fluoride (PMSF), polyvinyl pyrrolidine (PVP) obtained from Himedia Laborateries Pvt Ltd, Mumbai, India and Bovine Serum Albumin (BSA) from Sisco Research Laboratories Pvt Ltd, Mumbai, India. All other chemicals and reagents used were of analytical grade.

Oxidative stress

Detached fresh mature green leaves were submerged in 30 ml of 0.1 mM H_2O_2 solution for 0, 1, 2 and 3 days and in an other experiment 0, 0.025, 0.05, 0.075 and 0.1 mM for 1 day at room temperature in the dark.

Preparation of the Extract

Leaf Samples (0.3 g) were ground in 10 ml of 100 mM phosphate buffer (pH 7.0) using pre-chilled mortar and pestle. The phosphate buffer contained 1 mM EDTA, 1mM PMSF and 1% PVP. The homogenate was filtered through four layers of nylon cloth and the filtrate was centrifuged at 4°C c at 17000xg for 10 min. The supernatant was used for measurements of enzyme activity.

Determination of protein

Protein content was determined by the method of Lowry et al. (1951) using BSA as standard.

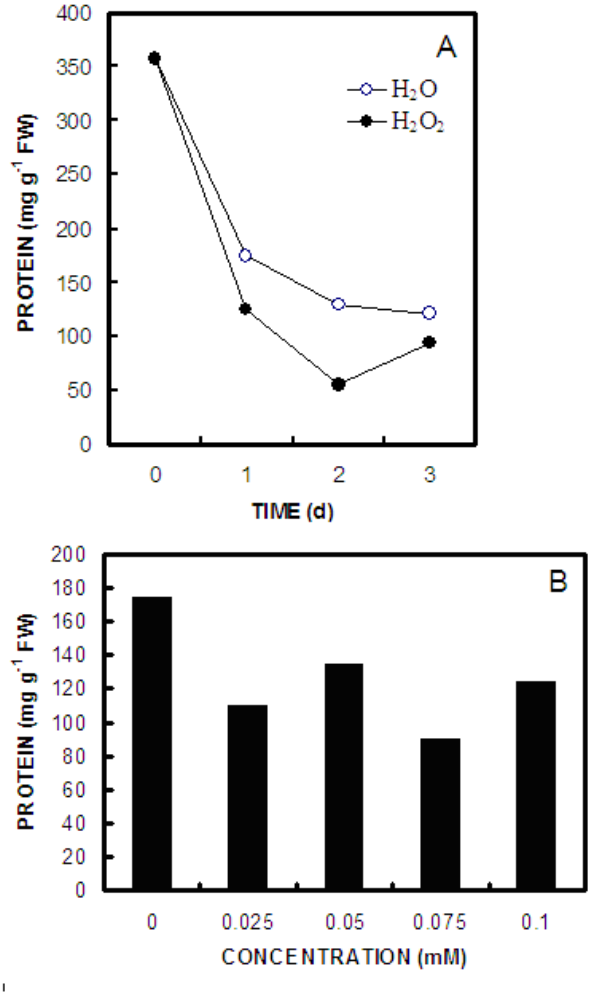

Fig. 1 (A). Effect of H_2O_2 on protein content in detached Pigeon pea leaves. Detached leaves were treated with 1 mM H_2O_2 for 0, 1, 2 and 3 days, respectively. (B) Effect of different concentrations of H_2O_2 on protein content. The experiments were performed three times with two replicates each and a representative one was shown.

Catalase assay

The activity of catalase was assayed as described by Verma and Dubey (2003) with slight modifications. The reaction mixture in a total volume of 2 ml contained 100 mM potassium phosphate buffer (pH 7.0), 400 µl of 6% H_2O_2 and 100 µl leaf extract. Leaf extract was the last component to be added and the decrease in absorbance was recorded at 240 nm (extinction coefficient of 0.036 mM^{-1} cm_{-1}) using a UV-Vis spectrophotometer (***Jasco***-V500, Japan) at 10s intervals up to 1 min. The specific activity of enzyme is expressed as µmol of H_2O_2 oxidised min^{-1} (mg protein)$^{-1}$.

RESULTS

Effect of H_2O_2 on protein content

Protein breakdown has long been considered one of the principal criterions of leaf senescence. Thus, senescence of Pigeon pea leaves in the present study was followed by measuring the decrease in protein content, an indicator of leaf senescence. It was also reported that protein breakdown precedes chlorophyll loss during leaf senescence (Hung and Kao, 2007). The changes in protein content in leaves treated with H_2O_2 in the dark are shown in Fig. 1. The decrease in protein content was evident at 1 day after H_2O_2 treatment compared to control leaves (Fig. 1A). Clearly, H_2O_2 is effective in promoting the senescence of pigeon pea leaves. The optimal concentration of H_2O_2 in reducing protein content seems to be 0.075 mM (Fig. 1B).

Effect of H_2O_2 on CAT activity

CATs constitute the major defense against ROS most consistently associated with senescence of leaves. CAT converts H_2O_2 to form oxygen and water. The changes in the activity of CAT in Pigeon pea leaves were measured in H_2O_2 treated and control leaves in order to access the role of the cell antioxidant system in H_2O_2-induced stress tolerance. When the leaves were excised and floated on water in dark the CAT activity markedly decreased after day 1, and then significantly increased until 3 days. At the 1st day the total CAT activity was 16.7 % of the initial activity, where as after 3 days it was 71.7 %. H_2O_2 retarded the decrease in the CAT activity at 1st day, and then dramatically lowered the activity at day 3 as compared with water treated control leaves. Under H_2O_2 treatment the activity of CAT at 1ST day was 56.7 % of the initial activity, whereas after 3 days it was 46.4 %. The decrease of the CAT activity is positively correlated with the decrease of the protein content during senescence. Testing the effects of different H_2O_2 concentrations clearly demonstrated that a relatively moderate concentrations of 0.025, 0.05 and 0.075 mM increased the CAT activity, while lower and higher concentrations shown the opposite effect.

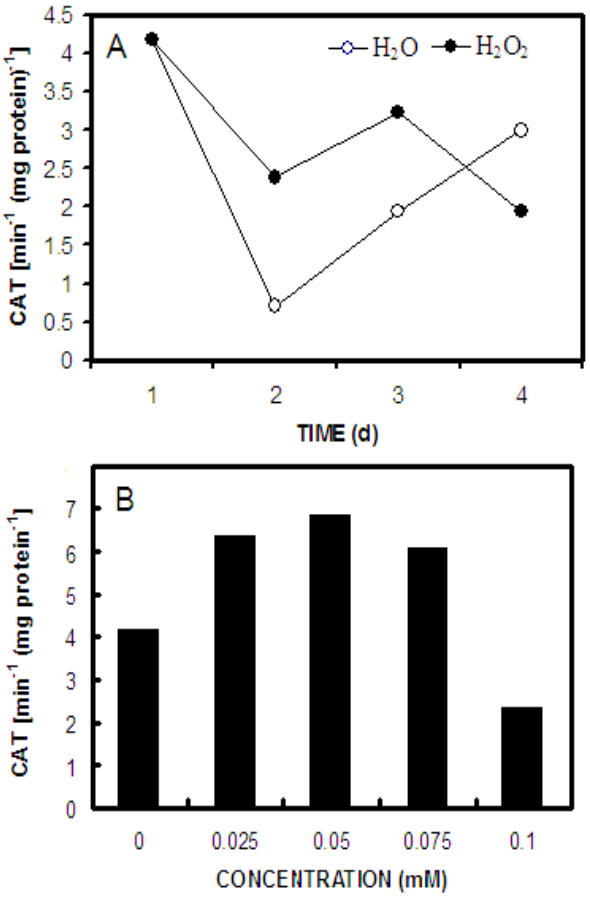

Fig. 2 (A). Effect of H_2O_2 on CAT activity in detached Pigeon pea leaves. Detached leaves were treated with 1 mM H_2O_2 for 0, 1, 2 and 3 days, respectively. (B) Effect of different concentrations of H_2O_2 on CAT activity. The experiments were performed three times with two replicates each and a representative one was shown. The experiments were performed three times with two replicates each and a representative one was shown.

DISCUSSION

Although leaf senescence can generally be defined as a late developmental process leading to cell death, the primary molecular pathway of this program is not known (Gepstein, 2004). Numerous environmental stimuli such as extremes of temperature, drought, ozone, nutrient deficiency, pathogen infection , wounding, and shading, whereas the autonomous factor include age, reproductive development and phytochrome levels can induce leaf senescence . In many systems oxidative stress was found to be involved in the leaf senescence process (Yeh and Kao 1994; Lin and Kao 1998; Navabpour *et al*., 2007) by the increase in lipid peroxidation.
We observed that there was a significant decrease in the content of protein during the H_2O_2 treatment. Testing the effect of different H_2O_2 concentrations clearly demonstrated that a relatively moderate concentration of 0.075 mM is the most effective in inducing protein degradation i.e., leaf senescence in Pigeon pea detached leaves. The protein degradation in senescing leaves may be due to a cytotoxic effect of H_2O_2 on protein degradation could have resulted from the effects of free radicals observed in H_2O_2-promoted senescent leaves (Upadhyaya *et al.,* 2007). Plant cells are equipped with several ROS detoxifying enzymes to protect them against oxidative damage (Hung and Kao, 2005). In order to clarify the protective mechanism of the antioxidant enzymes against H_2O_2 stress, we determined the changes in activation and inactivation of CAT in the leaves of Pigeon pea subjected to H_2O_2 stress. A number of controversial results have been published concerning the CAT activity during H_2O_2-promoted leaf senescence in various plant species. For example, CAT activity decreased in rice (Hung and Kao, 2005), increased in Cucurbita pepo (EI-shora 2003) and almost unchanged in (Moskova *et al.,* 2009) during H_2O_2-promoted leaf senescence. In the present study this enzyme decreased during senescence of Pigeon pea detached leaves under H_2O_2 stress.

In contrast increase in CAT activity under 0.025, 0.05 and 0.075 mM of H_2O_2 suggesting that 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. Apparently the trend of changes in CAT activity during senescence is species specific: its activity increases during senescence in some plants and decreases in others.

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