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RESEARCH ARTICLE

EFFECT OF EXOGENOUS HYDROGEN PEROXIDE ON PEROXIDASE AND POLYPHENOL OXIDASE ACTIVITIES IN *Cajanus cajan* (L.) Millsp. DETACHED LEAVES

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ABSTRACT

In this report peroxidase (POD) and polyphenol oxidase (PPO) activities were used to study the effect of hydrogen peroxide (H_2O_2) on leaf senescence in detached pigeonpea (*Cajanus cajan* [L.] Millsp.) leaves. The activities of POD and PPO were observed to be greater in H_2O_2-stressed pigeonpea leaves than in water treated control leaves. However, after longer incubations activities of these enzymes were markedly reduced. The observed changes revealed that exogenous H_2O_2 may induce oxidative stress tolerance by enhancing the activities of POD and PPOs. On the other hand, reduction found in H_2O_2-induced POD and PPO activities at later stages may be due to destruction of these proteins along with other proteins. This study will help to improve the tolerability of plants to environmental stresses by enhancing the expression of POD and PPOs.

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, and 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 radical (O_2), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH), and alkoxyl radicals (RO) in cells (Hung *et al.,* 2005). Singlet oxygen ($^1O_2$) which may arise due to the reaction of O_2 with excited chlorophyll is also considered as one of the potential ROS (Chen *et al.,* 2011). The accumulation of ROS damages almost all cell components including membrane lipids, chloroplasts, pigments, enzymes, nucleic acids, and leads to the death of cells (Verma and Dubey, 2003; Upadhyaya *et al.,* 2007; 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 a harmful ROS but also has a role as a signaling molecule in pathways of stress signal transduction (Liu *et al.,* 2010). Li *et al.* (2010) summarized that H_2O_2 directly regulates the expression of numerous genes associated with plant defence-, cell defence-, and signaling proteins such as kinase, phosphotase and transcription factors. 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., 2007). 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 suggests that H_2O_2-promoted senescence is mediated through oxidative stress (EI-Shora, 2003; Hung and Kao, 2007; Lin and Kao, 2007). Leaf senescence refers to the final developmental stage of leaves by which cells undergo programmed changes which resulting in hydrolysis of macromolecules such as proteins, 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 defense enzymes including peroxidase (POD, EC 1.11.1.7) and polyphenol oxidase (PPO, EC 1.10.3.1) to control the rapidly increasing ROS under various environmental stress conditions such as wounding (Steinite and Levinsh, 2002), pathogen infection (Karthikeyan *et al.,* 2005), and leaf senescence (Kar and Mishra, 1976; Patra and Mishra, 1979). POD is a heam protein, which is a member of oxidoreductases and catalyses the oxidation of a wide variety of organic and inorganic

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substances such as phenolics, cytochrome C and nitrite in the presence of H_2O_2 (Chen *et al.,* 1992; Alokail and Ismael, 2005; Sat, 2008). POD has been implicated in a variety of physiological processes, such as plant growth and development, auxin catabolism, the oxidation of phenolics to form lignin, the cross-linking of hydroxyproline-rich glycoproteins in plant cell walls, as well as biotic and abiotic stress responses (Cipollini, 1998; Fang and Kao, 2000). Parish (1968) suggested that the increase in the activity of POD is one of the most reliable indicators of maturity and senescence, and plays an important role in its regulation. But Ford and Simon (1972) contradicted this theory because POD activity increased several-fold when senescence was delayed in cucumber. PPOs are ubiquitious copper containing enzymes, functions as phenol oxidase in various plant species (Shi et al., 2001). PPO oxidizes phenolic compounds which have been associated with antioxidant activity (Sen and Mukherji, 2009). Because of its involvement in adverse browning of plant products, PPO has received much attention from researchers in the field of plant physiology and food science. Enzymatic browning occurs as a result of the oxidation by PPO, of phenolic compounds to quinones and their eventual (nonenzyme-catalyzed) polymerization to melanin pigments (Yoruk and Marshall, 2003). Even though oxidation of phenols and formation of melanins are normal physiological processes of PPO in plants, the significance of the enzyme activity in living intact plant tissues is not fully understood. Induction in PPO activity was reported during senescence of both attached and detached rice leaves (Kar and Mishra, 1976). However, Patra and Mishra (1979) suggested that the increase in the activities of PPO can not be taken as indicator of senescence because a group of investigated plant species, showed higher PPO activity toward the basal senescent leaves whereas another group showed high activity in middle mature leaves. It suggests that the pattern of change in PPO activity during senescence may be species specific.

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 within legume species (Garg and Noor, 2009). Pigeonpea (*Cajanus cajan* [L.] Millsp.) is one of the major grain legume (pulse) crops of the tropics and subtropics (Malviya and Yadav, 2010). It can provide fuel wood and fodder for the small scale formers in subsistence agriculture (Egbe and Kallu, 2009). The extract of pigeonpea 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). In view of the conflicting reports concerning POD and PPO activities during senescence, the present investigation was designed to assay these enzymes during H_2O_2-promoted senescence of detached pigeonpea leaves.

MATERIALS AND METHODS

Site description

Pigeonpea trees grown in the farms of Biochemistry Department, Dr. Babasaheb Ambedkar Marathwada University, and Aurangabad in India were used. 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.

Plant material

A sample of dark green, thick mature leaves at equal distance from twig tip was collected from three pigeonpea trees just before use. They were then washed in distilled water and dried at room temperature.

Chemicals

Guaiacol, bovine serum albumin (BSA) and cysteine purchased from Sisco Research Laboratories Pvt Ltd, Mumbai, India, catechol obtained from S.D. Fine-Chem Ltd, Mumbai, India, and phenulmethylsulfonyl fluoride (PMSF) and polyvinyl pyrrolidine (PVP) were from Himedia Laborateries Pvt Ltd, Mumbai, India. All other chemicals and reagents used were of analytical grade.

Oxidative stress

Fresh detached pigeonpea mature green leaves were submerged in 30 ml of 0.1 mM H_2O_2 solution for 0, 1, 2 and 3 days.

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 at 17000 x g 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.

POD assay

The activity of POD was assayed as described in Rao *et al.* (1999) with slight modifications. The reaction mixture in a total volume of 2 ml contained 100 mM potassium phosphate buffer (pH 6.5), 200 µl of 16 mM guaiacol, 20 µl of 6% H_2O_2 and 100 µl of leaf extract. Leaf extract was the last component to be added and the increase in absorbance was recorded at 470 nm (extinction coefficient 25.2 mM^{-1} cm^{-1}) using a UV-Vis spectrophotometer (Jasco-V500, Japan) at 10 s intervals up to 1 min. The specific activity of enzyme is expressed as µmol guaiacol oxidized min^{-1} $(mg\ protein)^{-1}$.

PPO assay

The activity of PPO was assayed as described in Saravanan *et al.* (2004) with slight modifications. The reaction mixture in a total volume of 2 ml contained 100 mM potassium phosphate buffer (pH 7.0), 200 µl of catechol and 100 µl of leaf extract. Leaf extract was the last component to be added

and the increase in absorbance was recorded at 420 nm using a UV-Vis spectrophotometer (Jasco-V500, Japan) at 10 s intervals up to 1 min. The specific activity of enzyme is expressed as change in the absorbance of reaction mixture min^{-1} (mg protein)$^{-1}$.

Statistical analysis

Each experiment was repeated three times with two replicates each and the data presented are mean values of independent experiments.

RESULTS

Effect of H_2O_2 on POD activity

PODs constitute the first line of defense against ROS and most consistently associated with senescence of leaves (Veljovic-Jovanovic *et al.,* 2006). PODs catalyze the oxido-reduction between hydrogen peroxide and reductants. They usually use various phenolic substrates for the elimination of H_2O_2 (Lepedus *et al.,* 2005). H_2O_2-mediated changes in POD activity in detached pigeonpea leaves were studied (Fig. 1). When the leaves were excised and floated on water in dark (control) the POD activity markedly increased at day 1, and then the activity was maintained until 3 days. However, water-mediated induction in POD activity further accelerated by H_2O_2 treatment at the first 2 days of incubation. The POD activity in H_2O_2 treated leaves was 2.3-fold higher than the water treated control leaves by day 2. This activity then markedly decreased by day 3. Decline in POD activity at later stages may be due to destruction of these proteins along with other proteins.

Effect of H_2O_2 on PPO activity

PPO enzyme functions as phenol oxidase and it oxidizes phenolic compounds which have been associated with antioxidant activity (Sen and Mukherji, 2009). H_2O_2-mediated changes in PPO activity in detached pigeonpea leaves were studied (Fig. 2). When the leaves were excised and floated on water in dark the PPO activity markedly increased at day 1, but then the activity was significantly decreased until 3 days. Increase in PPO activity (12-fold) induced by water treatment was much more rapid than the increase in POD activity (7.4-fold) by day 1. However, water-mediated induction in PPO activity was further accelerated by H_2O_2 treatement by day 1, maintained by day 2, and then the activity was decreased by day 3. The PPO activity in H_2O_2 treated leaves were 1.1-, 2- and 1.6-fold higher than the water treated control leaves by day 1, 2 and 3, respectively.

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

Table I. 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

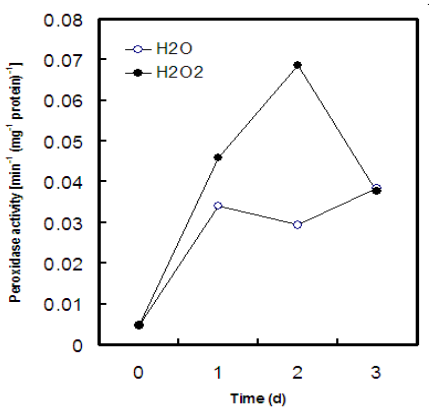

Fig. 1. Effect of H_2O_2 on peroxidase (POD) activity in detached pigeonpea leaves

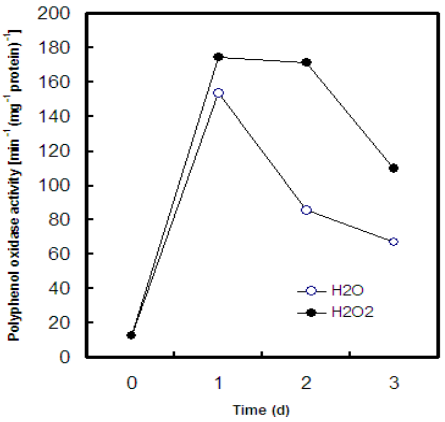

Fig. 2. Effect of H_2O_2 on polyphenol oxidase (PPO) activity in detached pigeonpea leaves

senescence process by the increase in lipid peroxidation and protein loss (Yeh and Kao, 1994; Lin and Kao, 1998; Navabpour *et al.,* 2007). We have shown that H_2O_2 treatment causes oxidative stress in detached pigeonpea leaves by the increase in protein loss (Goud and Kachole, 2011). 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 oxidative stress, we determined the changes in POD and PPO activities in detached leaves of pigeonpea subjected to H_2O_2 treatment. In the present investigation, we noticed that the activities of POD and PPO were higher in H_2O_2-stressed leaves as compared to water treated control leaves. The increased POD activity has been documented under a variety of stressful conditions, such as water deficit (Lin and Kao, 2000; Lee *et al.,* 2009), salinity (Lin and Kao, 2000; Garg and Noor, 2011), ozone (Mohamed and Rangappa, 1993), UV light (Mahdavian *et al.,* 2008), and chilling (Raimbault *et al.,* 2011). It was also reported that H_2O_2 treatment induced POD activity in rice (Hung and Kao, 2005)

and cucumber (Liu *et al.,* 2010) leaves. However, an important question remains unsolved: whether this increased POD activity is simply related to H_2O_2 scavenge or it is involved in H_2O_2-dependent cell wall lignification, reducing leaf growth. The transgenic tobacco plants with suppressed expression of PODs are over responsive to pathogen-mediated oxidative stress (Mitler *et al.,* 1999), whereas plants with overexpressing PODs had increased resistance to it (Aono *et al.,* 1993; Yun *et al.,* 2000). It suggests that PODs play a very important role against various biotic and abiotic stresses. Li *et al.* (2010) summarized that exogenous H_2O_2 treatments prevented the increase of MDA and endogenous H_2O_2 concentration in plants. The content of MDA, a product of lipid peroxidation, has been considered as an indicator of oxidative damage (Li *et al.,* 2010). We have shown that H_2O_2 treatment increased CAT activity in detached pigeonpea leaves (Goud and Kachole, 2011). It suggests that the coordination of POD and CAT activities might play a central protective role in H_2O_2 scavenging process, and the active involvement of these enzymes is related, at least in part, to oxidative stress tolerance in pigeonpea detached leaves subjected to H_2O_2 treatment. PPO enzyme functions as phenol oxidase in higher plants. Kar and Mishra (1973) found that PPO activity was increased during detached rice leaf senescence. H_2O_2 treatment rapidly induced PPO activity at the initial stages, and then markedly reduced at later stages. It suggests that this enzyme serves as an intrinsic tool to restrict H_2O_2-induced oxidative damage in pigeonpea leaves at the initial stage. However, decline in PPO activity at later stages might be due to destruction of these proteins along with other proteins in a similar fashion as PODs did. In parallel the potential role for PPO in plant defence against pests have motivated many studies on PPO in an etiological content (Constabel and Barbehenn, 2008). For example PPO activity has been shown to increase by 300 % in infected water hyacinth leaves compared to that in healthy leaves (Tyagi *et al.,* 2000). It was also reported that transgenic tobacco plants with suppressed expression of PPOs showed greater susceptibility to pathogen mediated-oxidative stress (Thipyapong *et al.,* 2004), whereas plants with over expressing PPOs had increased resistance to it (Li and Steffens, 2002). It suggests that increased PPO activity under various biotic and abiotic stresses may give the plant an enhanced resistance. This would explain why H_2O_2 treatment did not result in the accumulation of H_2O_2 and there was no increase in lipid peroxidation and membrane leakage of leaf tissues. In conclusion, our results suggest that exogenous H_2O_2 treatment may induce by enhancing the activities of POD and PPOs. In addition the activities of these antioxidant enzymes may be a good indicator for selecting stress tolerance genotypes of plants, at least in pigeonpea.

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