Change in activity of antioxidative enzymes in leaves of *Acacia retinodes, Biota orientalis* and *Casuarina equisetifolia* under heat stress condition

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Abstract

Terminal heat stress causes an array of physiological, biochemical and morphological changes in plants, which affect plant growth and development. Heat stress is one of the major abiotic stresses in agriculture worldwide. This study was carried out to investigate the effects of heat stress on soluble protein, catalase (CAT), and peroxidase (GPX) activities in three plant species (*Casuarina equisetifolia*, *Acacia retinodes* and *Biota orientalis*). Plants were randomly divided into two groups (the first group for heat stress treatment and the second for control) and heat stress treatments were applied at 36°C, 38°C, 40°C, 42°C and 44°C for 3h. Heat stress imposed significantly increased soluble protein content and CAT and GPX concentration in the three plants. These results suggest that CAT and GPX activities play an essential protective role against heat stress in *Casuarina equisetifolia*, *Acacia retinodes* and *Biota orientalis*. Antioxidants act as a major defense against radical mediated toxicity by protecting the damages caused by free radicals. An increase was observed in GPX and CAT activity of three plant species under stress conditions throughout the experiment. Results showed that CAT acts as the major antioxidant enzyme in *Casuarina equisetifolia* and *Acacia retinodes* leaves under oxidative stress condition and GPX was more important in *Biota orientalis*. So activity of these enzymes in stress condition can be used as an index for tolerance assessment.

KEYWORDS: heat stress, CAT, GPX, soluble protein, *Casuarina equisetifolia*, *Acacia retinodes*, *Biota orientalis*

Introduction:

A remarkable diversity in trees' vitality is noticed between urban trees even at the level of the same row, thus demonstrating stress adaptation expressions with certain subjects rather than others (Rejeb *and al.*, 1999). Osmotic stresses (draught, salinity and heat) are considered to be the most important abiotic factors responsible of urban trees heterogeneous vitality (Tomiczek, 2003; Ledoigt and Coudret, 1992 in Khelifa *and al.*, 2011).

Abiotic stress is the major factor that affects productivity of plants. Along with the tissue injury in response to various stresses, oxidative stress has been implicated as one of the underlying agents causing damage (Allen, 1995). Under conditions of temperature stress, plants require less energy, resulting in an excess of photons in the electron transport system in photosystem II. The excess electrons are transferred to oxygen molecules, thus causing the accumulation of toxic reactive oxygen species (ROS) like superoxide radical, hydrogen peroxide, hydroxyl radical, alkoxyl radical and singlet oxygen (Khan and Panda, 2002; Panda, 2002). These reactive oxygen species have the capacity to degrade almost all cell components including membrane lipids, proteins and DNA (Hendry, 1993, Casano *and al.*, 1994). 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 capable of rapid diffusion across cell membrane (Del Rio *and al.*, 1992).

Abiotic stresses are known to induce H_2O_2 and other toxic oxygen species production in cellular compartments and result in acceleration of leaf senescence through lipid peroxidation and other oxidative damage. H_2O_2 being a strong oxidant can initiate localized oxidative damage in leaf cells leading to disruption of metabolic function and loss of cellular integrity is resulting in senescence promotion. It also changes the redox status of surrounding cells where it initiates an antioxidative response by acting as a signal of oxidative stress (Lin and Kao, 1998; Sairam and Srivastava, 2000).

Several endogenous defense mechanisms, including enzymatic and non enzymatic, act in the cells to provide protection against oxidative damage. Important enzymes that scavenge ROS include superoxide dismutase, peroxidase and catalase (Noctor and Foyor, 1998 in Ijaz, 2012) and non-enzymatic metabolites like ascorbic acid (Athar *and al.*, 2008), salicylic acid (Gautam and Singh, 2009), proline and quercitol (Rached-Kanouni *and al.*, 2012) and low concentration of H_2O_2 (Wahid *and al.*, 2007) that quench these oxygen radicals and protect membranes from injurious effects of ROS (Foyer and Noctor, 2003).

Nevertheless, plant antioxidant response is dependent on exogenous parameters such as plant development environment leading to resistance or sensitivity (Xu and Huang, 2004).

The purpose of the present study was to contribute to a better understanding of the physiological responses of *Biota orientalis*, *Acacia retinodes* and *Casuarina equisetifolia* plants to heat stress. We investigated the influence of five types of heat stress on the contents of proteins, catalase (CAT), peroxidase (GPX) in the plants differing in heat tolerance. We also investigated how plants recovered from the heat stress and search for some elements in relation with stress tolerance.

Material and methods:

Plant materials

Enzymes (catalase and peroxydase) were extracted from *Acacia retinodes*, *Casuarina equisetifolia and Biota orientalis* leaves. To minimize stress related differences in enzymes biosynthesis, all the plant species were grown in the same farm and in the same natural environment (Constantine, East-Algeria), during the year 2011 to 2012.

Stress treatments

Plants were randomly divided into two groups (the first group for heat stress treatment and the second for control) and heat stress treatments were applied at 36°C, 38°C, 40°C, 42°C and 44°C for 3h. After each stress treatment, leaf samples were harvested and immediately frozen in liquid nitrogen for subsequent analyses. The plants for control were grown under natural environment.

Extraction of Antioxidants

To extract antioxidant enzymes, 0.5 g of leaves were ground using a tissue grinder in 8 ml of cooled phosphate buffer (pH 7.0, containing 1% (w/v) polyvinylpyrrolidone) and 0.2 g quartz sand in test tubes that were placed in an ice bath. The homogenate was centrifuged at 15000 xg for 20 min at 4°C. The supernatant was used for assays of enzyme activity (catalase and peroxydase).

Proteins

For the quantification of soluble protein content, Coomassie blue dye-binding assay was used (Bradford, 1976). Bovine serum albumin (BSA) was used for the preparation of the standard curve.

Catalase (CAT)

Activities of catalase (CAT) were measured using the method of Chance and Maehly (1955) with modification. The CAT reaction solution (3 ml) contained 50 mM phosphate buffer (pH 7.0), 15 Mm H_2O_2 and 0.1 ml enzyme extract. Reaction was initiated by adding enzyme extract. Changes in absorbance of the reaction solution at 240 nm were read every 20s. One unit CAT activity was defined as an absorbance change of 0.01 unit min^{-1}.

Peroxidase (GPX)

Activities of peroxidase were measured using the method of Chance and Maehly (1955) with modification. For guaiacol peroxidase acivity assay the reaction mixture (3.0 ml) contained 0.1 M phosphate buffer (pH 6.80), guaiacol (30 mM), H_2O_2 (30 mM) and 0.3 ml enzyme extract. Changes in absorbance of reaction solution at 470 nm were determined every 20s. One unit GPX activity was defined as an absorbance change of 0.01 unit min^{-1}. The activity of each enzyme was expressed on a protein basis.

Statistical analysis

The one way ANOVA and Newman-Keuls multiple range tests was performed as compare means to determine differences between existed peroxidase and catalase in five different elevations of temperature.

Results and discussion:

Plant growth and yield are adversely affected by abiotic stresses such as high or low temperatures, drought, salinity etc. Among abiotic stresses, heat stress influences photosynthesis, cellular and subcellular membrane components, protein content in cell and antioxidant enzyme activity; thereby significantly limiting crop production (Georgieva, 1999). Heat stress also induces oxidative stress in plants caused by the generation and accumulation of superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-), which are commonly known as reactive oxygen species (ROS) (Breusegem *and al.,* 2001).

Plants have the ability to acquire thermotolerance by exposure to a gradual increase in temperature (heat acclimation) (Hong *and al*., 2003), which often occurs in the naturel environment. Understanding mechanisms of heat acclimation will facilitate the development of new strategies for crop improvement under high temperature stress.

Cellular membranes are among the most sensitive components of a plant cell to heat stress (Raison *and al.,* 1980). Heat stress may disrupt membranes and leads to the leakage of organic and inorganic solutes (electrolytes) from the cell (Levitt, 1980). Electrolyte leakage of cells is widely used to evaluate membrane thermostability (Marcum, 1998). The ability of maintaining high membrane thermostability under heat stress is positively correlated to whole-plant tolerance to heat stress.

Plants produce a family of proteins called heat shock proteins (Hsps) in response to either rapid heat shock or a gradual increase in temperature (Al-Neimi and Stout, 2002).

It's important to quantify the total protein for evaluated the specific activity to each enzyme. The values of protein for each treatment are represented in Table 1. All the three plants had significant differences ($P < 0.05$) in their content in total protein compared to the control. Among the three plant species, the highest total protein was detected in *C. equisetifolia and B. orientalis* at 38°C and in *A. retinodes* at 40°C.

Table 1. Comparison of total protein (mg/g) among tree plants

	Temperature					
Name of plant	Control	36°C	38°C	40°C	42°C	44°C
C. equisetifolia	9.36c	10.07b	11.88a	10.44b	9.04c	9.03c
A. retinodes	8.12c	9.04c	9.88b	11.64a	10.95b	10.42b
B. orientalis	8.63d	11.21a	11.55a	10.56b	9.45c	8.04d

Heat stress is a major factor limiting the productivity and adaptation of crops, especially when temperature extremes coincide with critical stages of plant development. The rate of temperature change and the duration and degree of high temperatures all contribute to the intensity of heat stress. Where heat stress occurs, it is important that plants possess a certain degree of heat tolerance to survive the stress period. In addition, plant response to heat stress depends on the thermal adaptation, the duration of the exposure and the stage of growth of the exposed tissue (Chen *and al*., 1982).

Temperature which maximum Hsps were synthesized changes according to species and the optimum temperature that species grow in plants. Plants synthesize Hsps proportionally with severity of heat until maximum level. Hsp synthesis is completely induced for surviving with maximum activation of other protection mechanisms at near deadly temperatures. However plants probably synthesize middle level Hsps at mild heat stress conditions at first, but if heat stress continues they synthesize more Hsps (Vierling, 1991).

The heat stress response is a highly conserved response involving multiple pathways, regulatory networks, and cellular compartments (Kotak *and al.*, 2007). Changes in ambient temperature occur within hours, unlike drought and salinity stresses. Therefore, plants need to suppress and respond to the adverse effects of heat in a very short time. Gradual temperature increase in a day could cause some alterations in antioxidant metabolism or in other physiological responses. Improving tolerance to heat stress is a major challenge in many C_3 crops given the threat of global warming. Most of the earlier studies on the effects of multitude of abiotic stresses showed changes in the level of several physiological parameters including lipid peroxidation, H_2O_2 production and proline accumulation (Georgieva, 1999).

Reactive oxygen species (ROS) control in-plant processes such as growth, development, stomata signaling, and biotic and abiotic stress responses (Zaninotto *and al.*, 2006). The steady-state level of ROS in cells, as well as the duration, subcellular localization, and intensity of ROS signals, are thought to be controlled in cells by the ROS gene network (Bailey-Serres and Mittler, 2006). Complex antioxidant systems are very important for protecting cellular membranes and organelles from the damaging effects of active oxygen species. These include both enzymatic like GPX and CAT and non enzymatic antioxidants.

The change of peroxydase and specific peroxydase activities are represented in Table 2 and 3. In the present investigation, a significant increase in peroxydase activity was observed in the three plants under differential heat shock (HS) treatment. The

highest activity of peroxydase was observed in response to HS of 42°C for 3 h in three plants. Maximum high peroxydase (211 ± 2.696 U/g) and specific peroxydase (23.28 ± 1.248 U/mg protein) activities were detected in *C.equisetifolia* as compared to *A. retinodes* (66.68 ± 1.483 U/g, 7.02 ± 0.846 U/mg protein (40°C) peroxydase and specific peroxydase activities respectively) and *B. orientalis* (56.80 ± 1.238 U/g, 7.06±0.701 U/mg protein (40°C) peroxydase and specific peroxydase activities respectively).

Peroxidases are enzymes that oxidize various hydrogen donors in the presence of H_2O_2 or organic hydroperoxides. They catalyse many different and important biochemical and physiological reactions in most living organisms. Plant peroxidases are involved in diverse physiological functions such as lignin biosynthesis (Gross, 2008), suberization (Bernards *and a*l., 1999), wound healing (Kumar *and al.*, 2007), fruit ripening (Huang *and al.*, 2007), auxin metabolism and disease resistance (Veitch, 2004).

Even under normal growth conditions, many metabolic processes produce ROS in plants, such as superoxide (O_2-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH^-) (Sudhakar, 2001). Meanwhile, plants possess efficient antioxidant defense systems for scavenging ROS (Xu and Huang, 2004). CAT and GPX are the major antioxidant enzymes. Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage. Increase in CAT and GPX activity is supposed to be an adaptive trait possibly helping to overcome the damage to the tissue metabolism by reducing toxic levels of H_2O_2 produced during cell metabolism and protection against oxidative stress (Zhu *and al.*, 2004). A change in the level of cell membrane stability, H_2O_2 production, proline accumulation and antioxidant isoenzymes activities in plant cells is an indicator of oxidative stress. Hydrogen peroxide acts as a signaling molecule inside the plant system and it is considered as the second line of defense in response to heat stress.

Table 2. Comparison of total peroxydase activity (U/g)

	Temperature					
Name of plant	Control	36°C	38°C	40°C	42°C	44°C
C. equisetifolia	117.36c	183.36b	186.00b	202.40a	211.20a	191.80b
A. retinodes	50.00c	60.68b	60.23b	63.51ab	66.68a	64.51ab
B. orientalis	13.52c	36.40b	39.04b	40.64b	56.80a	51.36a

Table 3. Comparison of specific peroxydase activity (U/mg protein)

	Temperature					
Name of plant	Control	36°C	38°C	40°C	42°C	44°C
C. equisetifolia	12.53e	18.20c	15.66a	19.39d	23.28a	21.17
A. retinodes	6.16b	6.27b	6.10b	7.02a	6.78a	6.19b
B. orientalis	1.57d	3.15c	3.48c	3.84c	5.43b	7.06a

In the present investigation, a significant increase in catalase and specific catalase activities was observed in *C. equisetifolia, A. retinodes* and *B. orientalis* under differential heat shock (HS) treatment (Table 4 and 5). The highest of catalase was observed in response to heat shock treatment of 38°C for 3 h in *C. equisetifolia* (292.68 ± 3.246 U/g) as compared at *B. orientalis* (18.13 ± 0.946 U/g) at the same treatment, *which* showed significantly lower activity than the other two species. Catalase activity in *C. equisetifolia* was 16 fold higher than that of *B. orientalis* and 4 fold higher than those of *A. retinodes*, respectively. The results of catalase specific activities from *C. equisetifolia,*

B. orientalis and *A. retinodes* leaves are summarized in Table 5. These tree plants display different levels of specific activities under heat stress. Catalase specific activities among these plants ranged from 0.68 to 24.64 U/mg proteins. The highest specific activity was found in plant leaves from *C. equisetifolia* at 38°C, followed by *A. retinodes* at 36°C and *B.orientalis* at 38°C.

Table 4. Comparison of total catalase activity (U/g)

	Temperature					
Name of plant	Control	36°C	38°C	40°C	42°C	44°C
C. equisetifolia	51.83e	95.04cd	292.68a	187.92b	131.28c	69.13d
A. retinodes	13.44d	80.44a	62.40b	67.20b	61.44b	39.36c
B. orientalis	7.45d	14.36b	18.13a	14.58b	12.64c	11.02c

Table 5. Comparison of specific catalase activity (U/mg protein)

	Temperature					
Name of plant	Control	36°C	38°C	40°C	42°C	44°C
C. equisetifolia	5.43e	9.44cd	24.64a	18.0b	14.51c	7.65d
A. retinodes	1.66d	8.90a	6.32b	5.77b	5.61b	3.78c
B. orientalis	0.68c	1.28b	1.57a	1.38b	1.34b	1.37b

The results of this study experiments demonstrate that heat stress induce an accumulation of CAT that plays a role in the photosystem II (PSII) operation. It is probable that seedlings increased CAT activity in order to neutralize H_2O_2 and thus avoid cellular damage caused by accumulation of the al., substrate. This result converges with previous researches where stress tolerance were correlated with a higher concentration of CAT and the ability to remove AOS (Xu and Huang, 2004), but, it is in contradiction with others (Jiang and Huang, 2001).

CAT scavenges H_2O_2 to nontoxic levels or catalyzes the formation of water and oxygen and hence, an increase in CAT activity could play a role in the protection of the plants from damaging effect of H_2O_2 (in higher concentration in response to heat shock.

CAT and GPX are the major antioxidant enzymes. Plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to this oxidative damage (Zhu *and al.,* 2004). Increase in CAT and GPX activity is supposed to be an adaptive trait possibly helping to overcome the damage to the tissue metabolism by reducing toxic levels of H_2O_2 produced during cell metabolism and protection against oxidative stress. Species difference (*C. equisetifolia*, *A. retinodes* and *B. orientalis*) in heat tolerance is associated with tolerance to oxidative stress and the difference in sensitivity is due to the accumulation of H_2O_2 rather than tolerance to H_2O_2.

CONCLUSION:

The evaluation of the antioxidant- enzyme (catalase and peroxydase activities) measured in leaves of various species (*C. equisetifolia*, *A. retinodes* and *B. orientalis*) seedlings showed different values of specific activity under heat stress. All young plants noted an increase of CAT and GPX activities when heat stress increase. The increase of antioxidant activity values could deduce a tolerance/adaptation. It appeared those *C. equisetifolia*, *A. retinodes* and *B. orientalis* have different level of CAT and GPX activities. CAT and GPX from those *C. equisetifolia* exhibited the highest specific activities. The present contribution shows that those *C. equisetifolia* cultivated in Mediterranean climate such as east Algeria may be an alternative source to horseradish for peroxidases and catalase. It may also display interesting catalytic properties as well as thermal resistance.

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