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Evaluating the role of antioxidant defense system upon exposure to high temperature in different cultivars of *Curcuma longa* L

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Abstract
High temperature imposes adverse effects on plant's metabolic processes which influence its productivity and crop yield. *Curcuma longa* L is an economically important perennial medicinal crop plant cultivated globally. In general, *C. longa* L. grown in sub-tropical and tropical regions which encompasses temperatures around 40 °C. Any crop plants when subjected to high temperature will definitely prone to stress conditions ultimately affecting its productivity. Till date there are no reports on temperature stress studies in turmeric. Hence the present work is focused on monitoring antioxidant defense mechanisms by exposing potted plants of four different *Curcuma longa* L. cultivars aging 3 months to a temperature of 42 °C. Here we investigated levels of MDA and chlorophyll contents to assess the stress intensity. Further we examined the antioxidant enzyme activities (CAT, APX, GR) upon treatment with high temperature to understand the response of different cultivars. Our results revealed the alterations in antioxidant defense mechanism within cultivars conferring tolerance/sensitivity towards high temperature stress. Our given study would throw a light into key aspects of antioxidant defense systems in response to high temperature in *C. longa* L., this provides an platform for stress biology researchers to generate stress tolerant cultivars.

Keywords: Antioxidant defenses, *Curcuma longa* L, high temperature, oxidative stress, reactive oxygen species

1. Introduction
Turmeric (*Curcuma longa* L.) is one of the most important global spice crops generally termed as "golden spice" used in a variety of medicinal (germicidal, anti-bacterial, anti-tumour, anti-inflammatory, anti-helmenthic) [1, 2] and commercial purposes (food, pharmaceutical, confectionery, and cosmetic industries) [3]. It is a perennial rhizomatous herb belonging to Zingiberaceae family containing curcumoids which are important phenolic compounds chemically related to curcumin. About 50 different types of turmeric varieties are cultivated in India which is classified based on the duration of crop harvest as short, medium and long term cultivars. These cultivars vary in their curcuminoid content, essential oils (oleoresins) and other morphological attributes thereby altering in their medicinal value. The optimum temperature for turmeric cultivation with a vigorous vegetative growth in tropical countries like India is 20-35 °C which ensures maximal yield and productivity. Any further rise in temperatures due to seasonal variations may result in its decreased vigor causing a reduction in its qualitative attributes [4]. The reason behind its decreased productivity may be due to exposure of cellular and metabolic activities to stress.
As plants are sessile, dramatic changes in environment pose a serious threat to their normal growth which may be termed as stress caused by several biotic/abiotic factors. Extremes of temperature (both high and low), salinity, drought, floods, heavy metal accumulation etc., are different factors of abiotic stress. High temperature is considered to be as one of the major culprits which limit growth and yield of crop plants at varying periods of time exposure [5]. Extreme temperature changes leads to cellular distress causing disorganization of cell membrane, denaturation of proteins, destruction of photosynthetic apparatus and production of free oxygen radicals resulting in oxidative damage [6, 7, 8]. This type of damage in the

internal compartmentalization of cell organelles may lead to osmotic stress, nutritional imbalance and ionic toxicity. In consequence, these may result in severe morphological, biochemical and molecular associated physiological disturbances in plants [9]. Therefore, to capitalize agricultural productivity under altered climatic conditions, it has now become inevitable to study deep into the underlying mechanisms of plant heat stress responses [10]. In response, plants gain tolerance and/or adapt themselves against temperature stress by various mechanisms like detoxification of reactive oxygen species, adapt alternative metabolic pathways to defend themselves, accumulate compatible proteins, metabolites and alter the levels of phytohormones [11].

During hot summers, long term exposure of plants to high temperature (above 40 °C) will adversely affect their regular growth leading to damage in cellular and molecular interactions thereby resulting in decreased agricultural production [12, 13]. The mechanism of injury in plants involves the ROS (Reactive Oxygen Species) generation and reactions [14, 15, 16]. Development of detoxification systems take place to overcome stress conditions by breaking down ROS [17, 18]. The antioxidant defense system includes both enzymatic and non-enzymatic components. Many antioxidant enzymes like ascorbate peroxidase, superoxide dismutase, glutathione reductase, catalase protects plants from cytotoxicity of free radicals by neutralizing excess ROS produced during stress [19, 20]. Understanding the mechanism of temperature stress tolerance in Turmeric may further help to study in detail about its metabolic adjustments during stress. In the current study, we tried to assess the relationship between antioxidant defenses and high temperature tolerance in Turmeric. In general turmeric has a tendency to grow in varied climatic conditions such as temperature and humidity. This evoked us to investigate deep in to the physiological and metabolic adjustments taking place in different varieties of turmeric and their pattern of tolerance to high temperatures.

In this study we have chosen four cultivars of turmeric as our experimental model they are, Duggirala red, BSR, Pitamber and Salem. Reason behind selection of these varieties are as follows: Duggirala red, an indigenous; Salem, an high yielding; Pitamber and BSR are short term crops. The present investigation depicts the effect of high temperature on adjustments of antioxidant defense systems in *C. longa* plants. The oxidative damage caused due to high temperature results in elevated levels of reactive oxygen species (ROS), which need to be scavenged by antioxidant enzymes in order to maintain metabolomic equilibrium [17, 18]. Our study reveals differential thermo tolerance levels of four turmeric cultivars indicated by its alterations in biochemical activities. This study throws an insight into the precise understanding of antioxidant defense system behavior in response to high temperature which can be further deployed into crop improvement studies.

2. Material and Methods

2.1 Plant material

Rhizomes (seed material) of four different *C. longa* cultivars (Salem, Duggirala, BSR and Pitamber) were collected from local fields, India. For carrying out experiments these rhizomes were planted in the green house at Department of Biotechnology, Telangana University. Pots consisting soil and manure in 3:1 ratio was used to sow rhizomes in order to break their dormancy and help the rhizomes to germinate. The standard conditions of 27-35 °C day and 24-25 °C night temperatures with a relative humidity of 45-51% were maintained during the growth period of plantlets.

2.2 Temperature treatment

For performing effect of high temperature studies on four different cultivars of *C. longa* (Salem, Duggirala, BSR and Pitamber), plants aged three months were shifted from green house to the growth chamber. The experiments were carried out in a completely randomized block design consisting three replicates per each treatment. Treatment imposition was done by subjecting the plants to a temperature of 42 °C for 2 hours with an interval of 30 minutes in a growth chamber. Growth chamber was designed in the laboratory of Biotechnology, Telangana University using a wooden unit of size 4x8 feet fitted with high voltage lamps (four numbers) which emits light intensity approximately about 2000 LUX. Temperature was regularly monitored with a thermometer and any excess or deficit in the temperature was regulated by switching on/off one or more lights.

Sampling was done by taking leaf material at regular intervals of 30, 60, 90 and 120 minutes during exposure of plants to high temperature. Leaf samples collected prior treatment served as control. The harvested leaf samples were immediately frozen in liquid nitrogen and stored in -20 °C until experimentation.

2.3 Total chlorophyll content

The content of total chlorophyll was determined according to the protocol [21]. In brief: approximately 100 mg of sample was extracted using 5 ml of 80% (v/v) Acetone and absorbance was read at 646nm by subtracting the turbidity at 663 nm using UV-VIS spectrophotometer. The amount of total chlorophyll was calculated using the formula

Chl a (mg g^{-1} FW) = (12.27*A_{663})-(2.58*A_{645})

Chl b (mg g^{-1} FW) = (22.87*A_{645})-(4.67*A_{663})

The obtained results were expressed as mg/g FW.

2.4 Lipid peroxidation

Malondialdehyde (MDA) content was determined using the modified protocol [22]. In brief: one gram sample was homogenized using 1 ml 0.5% (w/v) TCA (Trichloroacetic acid) and centrifuged at 13000 g for 20 minutes. To 0.5ml of supernatant obtained, 2.5 ml of TBA (Thiobarbutiric acid) reagent was added (TBA reagent was prepared by adding 20% TCA (w/v) and 0.5% TBA (w/v) in 0.25 M HCl). Following addition, the sample was heated at 95 °C in a boiling water bath for 30 min and then cooled immediately on ice. The above mixture is centrifuged at 10000 xg for 10min. The absorbance of the supernatant is read at 532 nm by subtracting turbidity at 600 nm using a spectrophotometer. Calculation of the MDA content was done from the extinction coefficient 155 mM^{-1} cm^{-1} and expressed as µmol per g FW.

2.5 Determination of protein content

The amount of protein in the samples was estimated as per the of Lowry [23] using BSA as a standard. Approximately 100 mg of frozen leaf sample was homogenized in 1 ml of 100 mM extraction buffer (potassium phosphate buffer-pH

7.0 containing 1.0 mM EDTA). The obtained homogenate was subjected to centrifugation at 5,000 xg for 15 minutes. The resulting supernatant was henceforth referred to as leaf extract. 20 ml of leaf extract was made up to 1 ml using distilled water and 1 ml of solution D was added. Solution D was prepared using three different solutions a, b and c in 23:1:1 ratio. solution a is 2% sodium carbonate (Na_2CO_3) in 0.1 N NaOH, solution b is 2% sodium potassium tartrate ($C_4H_4O_6KNa.4H_2O$) Rochellis salt and solution c is 1% copper sulphate (CuSO4). Following incubation for 15 minutes, 200 ml of 1 N Folin-Coicalteau reagent was added and mixed thoroughly. Post mixing, it was incubated in dark for 30 minutes and absorbance was read at 750 nm using a spectrophotometer. The obtained values were used for performing enzyme assays.

2.6 Antioxidant enzyme assays

2.6.1 Catalase Activity

Measurement of Catalase activity was done as Aebi. H [24]. Briefly, 50 mg of leaf extract was incubated with 1 mM H_2O_2 and consumption of H_2O_2 was monitored at 240 nm using a UV-Vis spectrophotometer. The decrease in absorbance was quantified by the molar extinction coefficient of H_2O_2 (36 M^{-1} cm^{-1}) and the catalase activity was expressed as µmoles of H_2O_2 min^{-1} mg^{-1} protein.

2.6.2 Ascorbate Peroxidase

Ascorbate Peroxidase activity was monitored according to the standard Nacano and Asada [25]. In brief, 1ml reaction mixture contained 50 mM Potassium Phosphate buffer (7.0 pH), 0.1 mM AsA, 0.3 mM H_2O_2, 0.1 mM EDTA and protein extract. Reaction was initiated by adding H_2O_2 and absorbance was quantified at 290 nm using extinction coefficient (2.8 mM^{-1} cm^{-1}). The APX activity was expressed as decrease in µmoles of ascorbate min^{-1} mg^{-1} protein which was measured in UV–Vis spectrophotometer.

2.6.2 Glutathione Reductase

Glutathione Reductase (1.6.4.2) activity was measured as Foyer and Halliwell [26]. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.5), containing 0.5 mM DTNB in 0.01M potassium phosphate buffer (pH 7.5) (Ellmans reagent), 0.1 mM NADPH, enzyme extract. Reaction was started by adding 1 mM GSSG (oxidized glutathione). The rise in absorbance at 340 nm was recorded over a period of 3 min on a spectrophotometer using molar extinction coefficient of NADPH (6.2 mM^{-1} cm^{-1}). One unit GR is 1 µmol ml^{-1} GSSG (glutathione) reduced min^{-1}.

2.6.3 Statistical Analysis

Data resulted in the current study is the average of triplicate values (±SE) of leaf samples collected from four *C. longa* cultivars at different time points upon exposure to high temperature. Further statistical analysis was performed using SigmaPlot Version 12.0 by the method of one-way ANOVA (Holm-Sidak).

2.7 Result and Discussion

2.7.1 Effect on MDA content

In general, the accumulation of excess ROS was quantified as an equivalent value of MDA levels, MDA serves as a biomarker for lipid peroxidation [27, 28, 29] which is a decomposition product of polyunsaturated fatty acids. In this context, we exposed four cultivars of *C. longa* (BSR, Duggirala red, Pitamber and Salem) to high temperature (42 °C) and monitored MDA levels at 4 different time points (30, 60, 90 and 120 min). These time points hereafter called as Time of Exposure (TOE) with minutes as units, and the zero time point will be represented as respective control. Our results demonstrated increased MDA levels especially in BSR and Duggirala red with increased TOE of high temperature (Fig. 1a). Whereas, the pitamber cultivar has exhibited marginal increase initially (30 min) and later there observed a gradual drop in MDA levels when compared to control (Fig. 1a). In contrast to all the three cultivars, the salem cultivar shown considerably low MDA levels at all time periods (Fig. 1a). From this result it was quite apparent that the accumulation of MDA in Salem was low, moderate in Pitamber, elevated in both BSR and Duggirala red. These findings give us preliminary clue on thermo tolerance response of four *C. longa* cultivars. Our results are in corroboration with previous reported evidences which demonstrated the increased MDA levels under high temperature induced stress conditions [30, 31, 32].

2.7.2 Effect on total chlorophyll content

Elevated temperature was evident to cause an oxidative damage of chlorophyll complex which was due to damage of cellular and membrane components [3, 6]. So, keeping in view of this, we examined the chlorophyll content levels in four cultivars upon exposure to high temperature at different time points. The three cultivars BSR, Duggirala red and Pitamber have recorded low total chlorophyll contents with not much variation upon treatment with high temperature in comparison with control (Fig. 1b). While, the total chlorophyll contents in Salem cultivar remained high till 30 min and decrease significantly thereafter when compared to control (Fig. 1b). This stability of chlorophyll content in Salem variety even at high temperatures indicates that its thermo tolerance while the poor chlorophyll contents in Duggirala red, BSR and Pitamber shows the sensitivity nature towards high temperature. Our results are in agreement with the data reported on *Brassica juncea* [34].

2.7.3 Effect on antioxidant enzymes

Further, we evaluated the activities of ROS scavenging antioxidant enzymes (CAT, APX and GR) to predict the capability of different cultivars in combating the high temperature stress conditions. At first, we monitored the activity of CAT in four different cultivars of *C. longa* when treated with high temperature at varying time-points (Fig. 2a). The CAT activities in almost all 4 cultivars were similar with gradual rise till 60th min and exhibited downfall leaving the catalase activities in BSR, Duggirala red and Pitamber high. In contrast, the Salem cultivar has recorded low CAT activities with negligible variations at different time points (Fig. 2a). With this it was evident that BSR, Duggirala red and Pitamber were trying to overcome the oxidative stress by increasing their CAT activities. Our findings are in line with several reports [35, 36]. Further, we examined the APX activity in four different cultivars of *C. longa* up on exposure to high temperature at different time points (Fig. 2b). We observed a sharp increase in APX activities till 60th minute in Duggirala red, Pitamber and Salem varieties, further it decreased upon treatment with higher time points of HT. While BSR has initially exhibited rise in APX activity was significantly shown gradual decrease with increase in time of high temperature exposure (Fig.2b).

These findings are in correlation with wheat cultivars which exhibited tolerance towards high temperature due to enhanced activities of antioxidant enzymes [37, 38]. At last, we monitored the GR activity in four different cultivars of *C. longa* upon treatment with high temperature at varying time points. Dramatically, the activities of GR in BSR, Pitamber and Salem have exhibited low (Fig. 2c), while the GR activity in Duggirala red has been higher with gradual increase upon increase in TOE to high temperature (Fig. 2c). Previous reports revealed that there was pronounced role of ascorbate- glutathione cycle in maintenance of redox poise against abiotic stress in plant cells [39, 40, 17].

Illustrations of figures

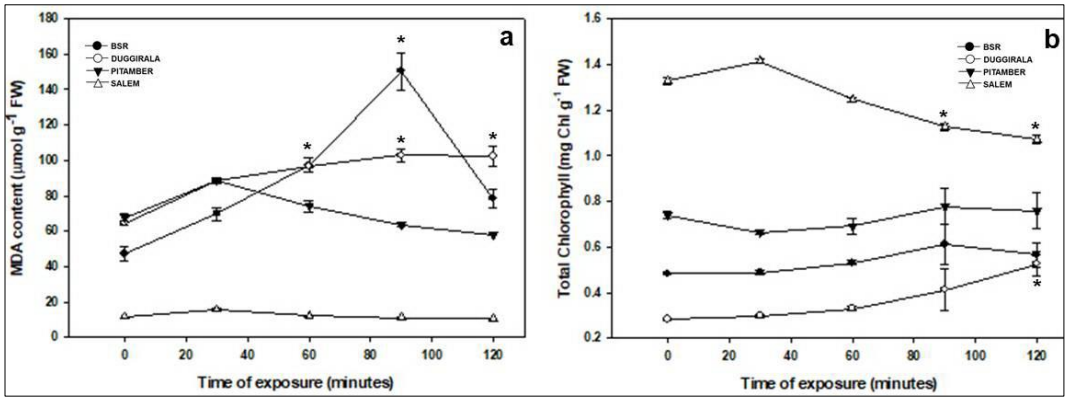

Fig 1: MDA content (a) and total chlorophyll content in different cultivars of *C. longa* upon exposure to high temperature at varying time points. Each bar represented as mean average ± standard deviation of three replicates per treatment performed randomly

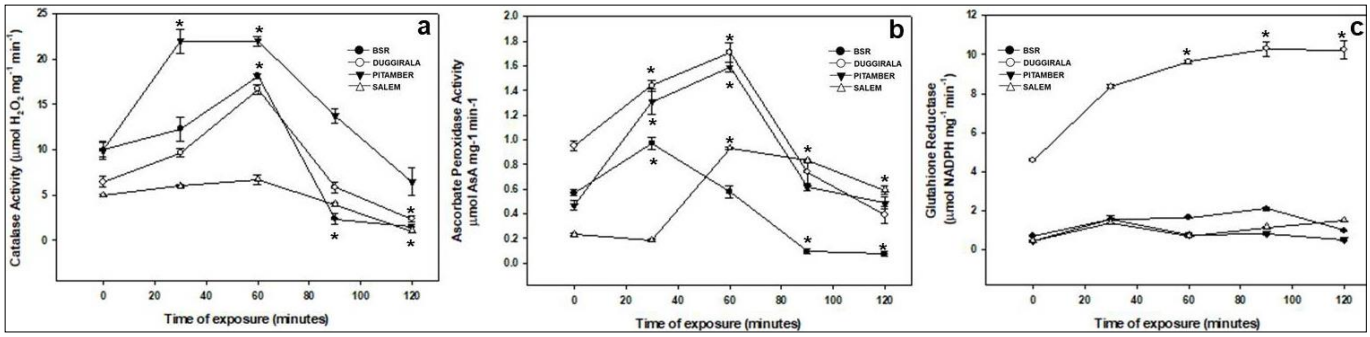

Fig 2: Effect of high temperature (42 °C) on catalase (a), ascorbate peroxidase (b) and glutathione reductase enzyme activities in different cultivars of *C. longa* upon exposure to high temperature at varying time points. Each bar represented as mean average ± standard deviation of three replicates per treatment performed randomly

Conclusions

The present study concludes that varying responses of different *C. longa* cultivars towards imposition of temperature stress. Out of four cultivars, Salem exhibited high tolerance while moderate by BSR, sensitive response by Pitamber and Duggirala red. This varied response would give a clue for researchers to improve the tolerance traits through modulation of antioxidant defenses.

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