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Effect of heat stress in synthesis of heat shock proteins and antioxidative enzyme response in *Trigonella foenum-graceum* L

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Abstract: Plants exposed to high temperature causes inhibition of photosynthesis, cell membrane damage and cell death. Plants have a number of protective systems against deleterious stress effects through the synthesis of proteins and enzymes. This study was performed to investigate the effects of heat stress on synthesis of proteins and reactive oxygen species scavenging enzymes in fenugreek (*Trigonella foenum-graceum*) plants. The plants were exposed to 30 ºC, 35 ºC and 40 ºC for 4 hrs and 6 hrs to induce heat stress. Catalase and peroxidase assays were performed to determine the defense mechanisms by *T. foenum-graceum* plants against reactive oxygen species produced due to heat stress. There was an increased enzymatic activity in response to exposure time and temperature. Proteins were extracted from heat stressed plants and SDS-PAGE analysis revealed the variation among the protein bands and high level of gene expression results in synthesis of proteins having molecular weight of 30 - 60 kDa. MALDI-TOF-MS was used to compare the peptide mass fingerprinting (PMF) data with SWISS-PORT and NCBI database for the identification of expressed proteins in response to heat stress.

Keywords: Heat Shock Proteins, Heat Stress, Reactive Oxygen Species, *Trigonella*

1. Introduction

Interaction of plants with biotic and abiotic factors put them under stresses which immediately block important metabolic processes [1]. Abiotic stresses adversely affect plant growth and productivity worldwide by more than 50% [2, 3]. Active plant growth in mesophiles is in between 10-40 °C and temperature which is under and below this range creates heat stress on metabolic activities of plants [4]. Heat stress affects photosynthesis, respiration and water relations in plants [5]. Heat stress damages plant membrane systems and can lead to loss of electrolytes [6]. Plants have developed different mechanisms for heat stress by capturing reactive oxygen species, maintenance of cell membrane stability, synthesis of antioxidants, osmoregulation and enhancing the transcription and signal transfer of chaperones [5].

Many endogenous protection systems are involved to tolerate the stresses by triggering defense mechanisms that leads to obvious gene expression [7, 8]. The changes in genotypic expression which is absent under normal conditions resulting in the synthesis of group of proteins called heat shock proteins (Hsps) or stress proteins [9-11]. Heat-shock proteins ranging from 10 to 200 KD are characterized as chaperones and participate in the induction of the signal during heat stress [12]. Heat shock proteins have been known to protect plant cells against deleterious stress effects.

Abiotic stresses can aggravate reactive oxygen species (ROS) [13] which destroy cell membranes and ultimately results in cell death [14]. Temperature stress produce devastating effect on plant metabolism leads to accumulation of toxic compounds that include reactive oxygen species [15]. Plants control the production of ROS is controlled by various enzymatic and non-enzymatic anti-oxidative systems. Enzymatic antioxidants include catalase (CAT), peroxidase (POX) and superoxide dismutase (SOD). This investigation was done to find out the synthesis of heat shock proteins and reactive oxygen species (ROS) scavenging enzymes in *Trigonella foenum-graceum* with response to temperature variations.

2. Materials and Methods

2.1. Growth Conditions

Fenugreek (*Trigonella foenum-graceum*) seeds were surface sterilized with $HgCl_2$ (1:500) and then germinated on sterile water agar (15 g l^{-1}) for 3 days. The seedlings were transplanted into pots containing soil:FYM:sand (3:1:1) and grown under controlled conditions (12 hrs photoperiod; 70% relative humidity). Watering was done daily up to the second leaf stage and the fourteen day old plants were divided into control and experimental groups.

2.2. Heat Shock Treatment

The experimental groups were exposed to 30 ºC, 35 ºC and 40 ºC for 4 hrs and 6 hrs in a growth chamber. Control group was maintained at ambient temperature (29 ± 2 ºC). Leaves were collected at the end of heat shock treatment (4 hrs and 6 hrs) and immediately frozen in liquid nitrogen and stored at -70°C.

2.3. Enzymatic Assay

2.3.1. Enzyme Extract

A 20% leaf homogenate was prepared in 0.1M phosphate buffer (pH 6.5), centrifuged and the supernatant was used for the peroxidase and catalase assays.

2.3.2. Catalase Assay

Catalase assay was adopted from Luck [16]. In an experimental cuvette, 0.3 ml of H_2O_2-phosphate buffer was taken, followed by the rapid addition of 40µl of enzyme extract. The time required for a decrease in absorbance by 0.05 units was recorded at 240 nm in a spectrophotometer. The enzyme solution containing H_2O_2-free phosphate buffer served as control. One enzyme unit was calculated as the amount of enzyme required to decrease the absorbance at 240 nm by 0.05 units.

2.3.3. Peroxidase Assay

Peroxidase assay was performed by following the procedure described by Reddy et al., [17]. To 3.0ml of pyrogallol solution (0.05 M in 0.1 M phosphate buffer, pH-6.5), 0.1ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at 430 nm. To the test cuvette, 0.5ml of H_2O_2 (1% in 0.1 M phosphate buffer, pH-6.5) was added and mixed. The change in absorbance was recorded every 30 seconds up to 3 minutes in a spectrophotometer. One unit of peroxidase was defined as the change in absorbance per minute at 430 nm.

2.3.4. Protein Extraction and SDS PAGE

After heat shock treatment, total protein was estimated from experimental and control groups according to the method described by Lin et al., [18]. In brief, the leaves were homogenized in 10 ml of 50 mM Tris-HCl (pH 8.5), 2% SDS, 2% β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride at room temperature. The homogenate was centrifuged for 30 min at 10,000 rpm and filtered. The extracted protein content was estimated by following the method of Lowry et al., [19]. The proteins were separated by SDS PAGE method through a 3% stacking gel and 10% separating gel following standard protocols.

2.3.5. Protein Analysis by MALDI TOF-MS

The differential and over expressed proteins were cut, digested and peptides were eluted for the identification by MALDI-TOF MS analysis. The peptide mass fingerprinting (PMF) data were used to search against the SWISS-PORT and NCBI database with mascot software. The corresponding MS value and mascot score was considered for the protein identification.

2.3.6. Experimental Design

Treatments were arranged in randomized complete block design with three replicates and the protein content was measured for three sub samples taken from each extraction sample. The mean was used in the analysis of variance.

3. Results

Data on catalase and peroxidase assay showed that under heat stress, the seedlings exhibited an increased enzyme activity (Fig 1 and 2). The activity continued to increase in response to duration and temperature.

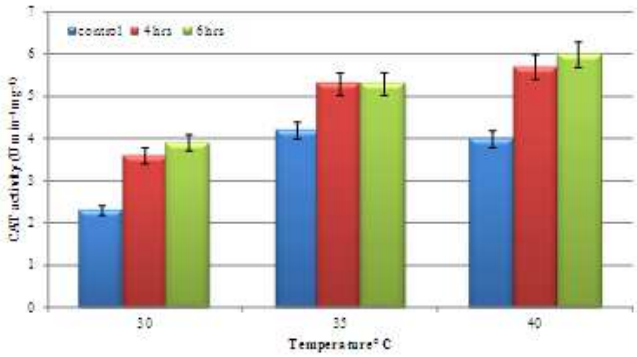

Fig 1. *Catalalse activity in response to heat stress*

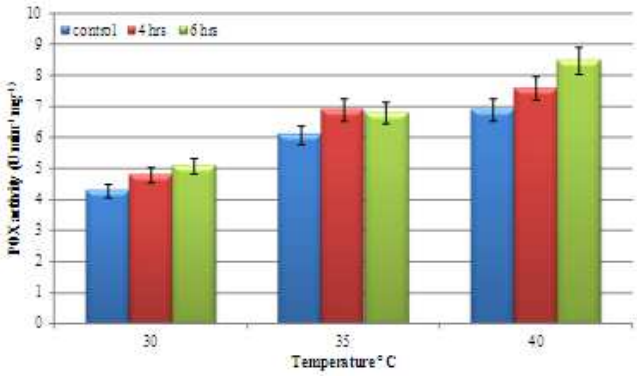

Fig 2. *Peroxidase activity in response to heat stress*

The effect of heat stress in protein synthesis at 30 ºC, 35 ºC and 40 ºC for 4hrs and 6 hrs on fenugreek plants is

shown in Fig-3. The heat stress resulted in the induction of heat shock protein bands with a molecular weight of ~16 kDa, ~50 kDa, ~54 kDa and ~60 kDa for 4 hrs and ~60 kDa, ~35 kDa and ~58 kDa for 6 hrs (Fig-4).

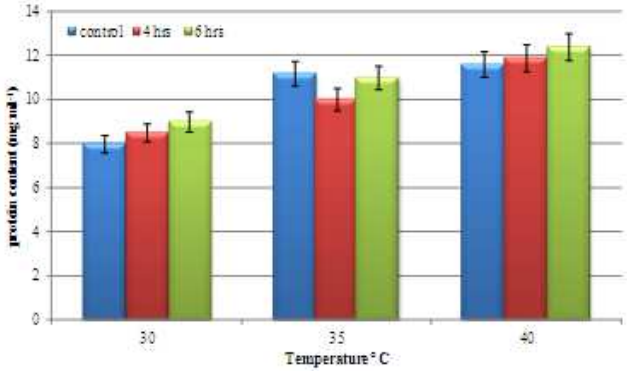

Fig 3. *Protein content in response to heat stress*

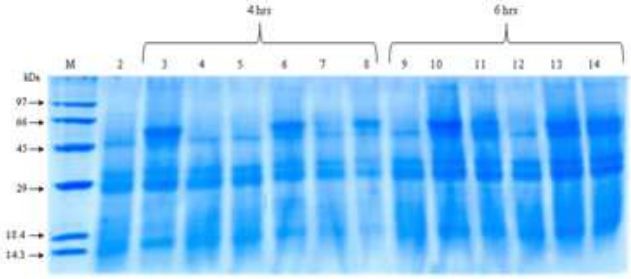

Fig 4. *1- Marker, 2- Control, 3- 30 ℃ (test); 4- 30 ℃(control); 5- 35 ℃ (control); 6- 35 ℃ (test); 7- 40 ℃ (control); 8- 40 ℃ (control); 9- 30 ℃ (control); 10- 30 ℃ (test); 11- 35 ℃ (test); 12- 35 ℃ (control); 13- 40 ℃(test); 14- 40 ℃ (control)*

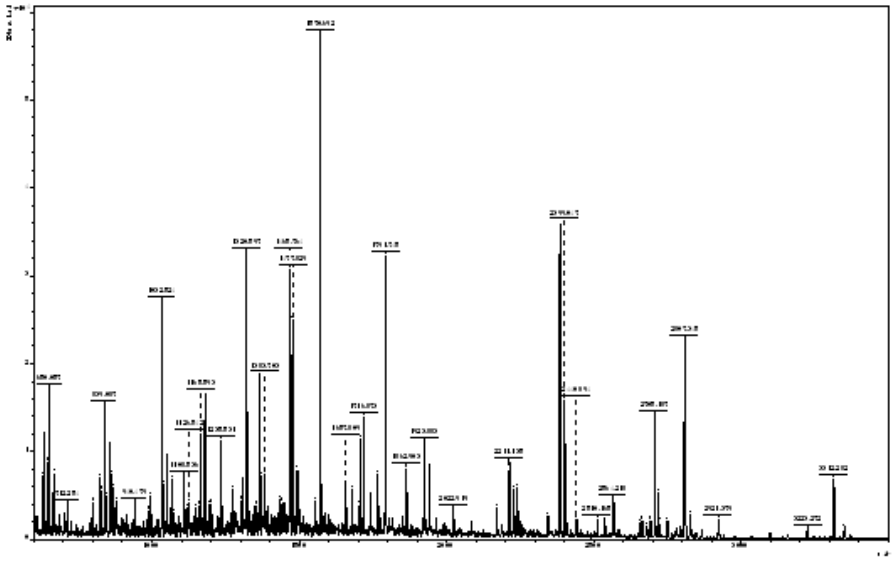

Fig 5. *MALDI-TOF-MS analysis of overexpressed protein1*

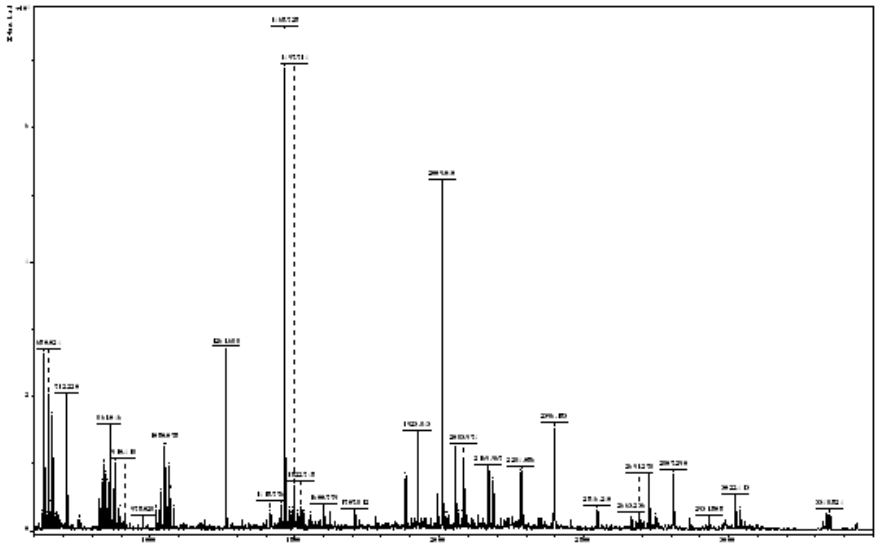

Fig 6. *MALDI-TOF-MS analysis of overexpressed protein2*

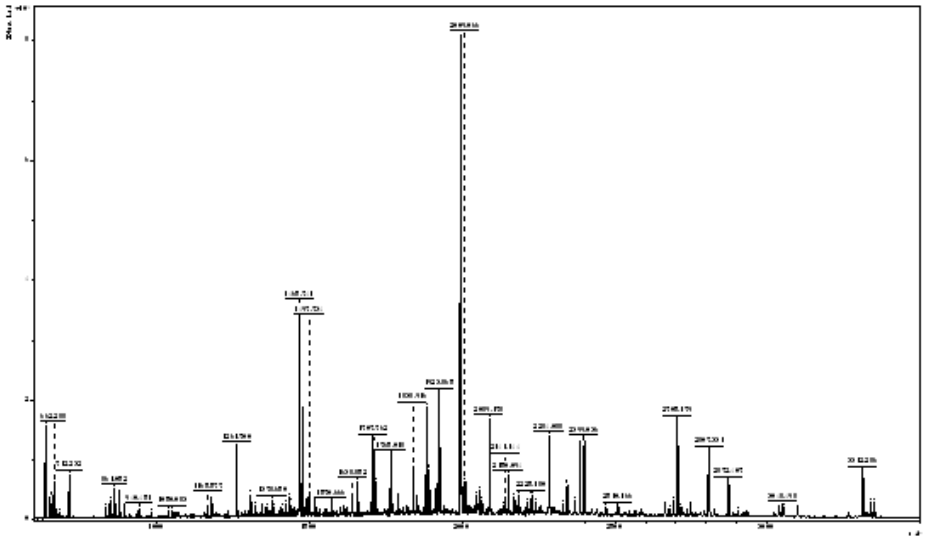

Fig 7. *MALDI-TOF-MS analysis of overexpressed protein3*

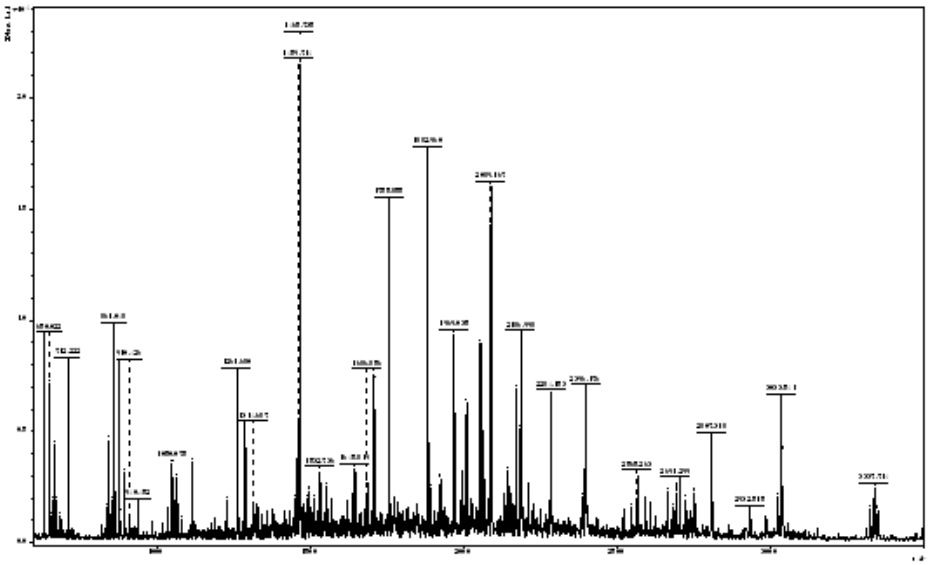

Fig 8. *MALDI-TOF-MS analysis of overexpressed protein4*

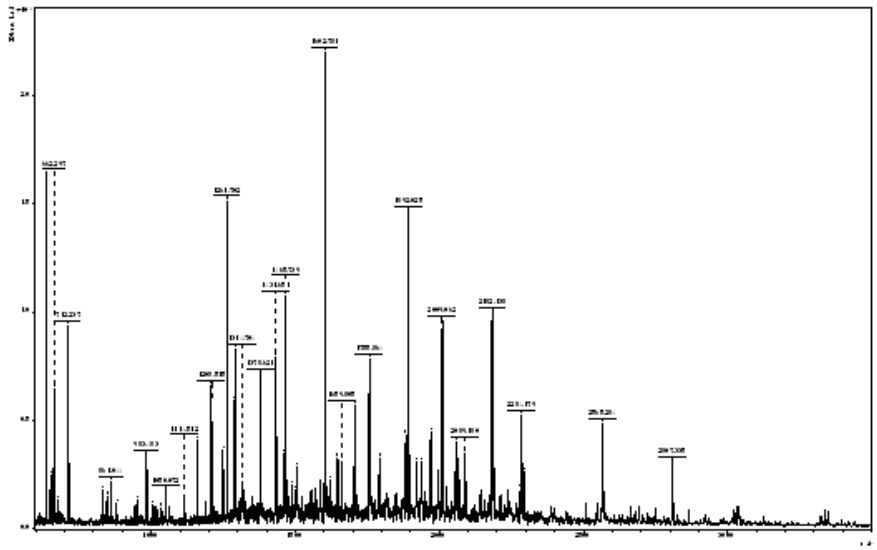

Fig 9. *MALDI-TOF-MS analysis of overexpressed protein5*

There was a high level expression of genes at ~30 kDa, ~60 kDa, ~58 kDa, ~35 kDa and ~50 kDa after exposure to variable temperatures which were further identified by MALDI-TOF-MS (Fig- 5, 6, 7, 8 and 9). Analysis of MS value and mascot score of highly expressed proteins revealed that the proteins belongs to gi|308805723 unnamed protein product, gi|297793845 expressed protein, gi|514716021 transmembrane protein 208 homolog, gi|297793845 expressed protein and gi|226532888 50S ribosomal protein L34.

4. Discussion

Stress conditions can cause increased production of reactive oxygen species (O_2 and H_2O_2) in plant tissues [20]. Plants posses a protective system composed of peroxidase and catalase to scavenge these reactive oxygen species [21]. The transcription and protein levels of many ROS scavenging enzymes were elevated by heat stress [22]. Catalase catalyzes the dismutation of H_2O_2 into H_2O and O_2 in peroxisomes. Ascorbate peroxidase breakdown H_2O_2 to form H_2O and monodehydroascorbate [23]. Reactive oxygen species (ROS) are formed during normal metabolism and cells must scavenge these toxic reactive species at the earliest to reach a homeostasis [24]. Failing to this, ROS production exceeds the antioxidant defense capability of the cell resulting in cellular damages. It is clear from our results that there was an increased activity of reactive oxygen species scavenging enzymes due to overproduced ROS in response to heat stress. High temperature stress tolerance in many crops has been associated with increased antioxidant enzymes activity.

Heat shock proteins serve important physiological functions which are related to heat and other stresses [25, 26]. Hsps protect plants against by reestablishing normal protein conformation for survival under stress [27]. Temperatures above 10-15 °C of optimal growth temperature induct heat shock protein response [27, 8 and 28]. *Trigonella foenum-graceum* has the optimal temperature of 8-27 °C and in this experiment the plants were exposed to 30 °C, 35 °C and 40 °C. Cooper et al., [30] has showed that optimal temperature for the synthesis of Hsps in maize was 45 °C and in this experiment, maximum temperature of 40 °C was chosen. In response to the increased temperatures, the plant was synthesizing a variety of heat shock proteins which was observed from SDS PAGE. Many heat shock proteins with molecular weights from 29-45 kDa were synthesized in acquisition of thermo tolerance [28] and in this study heat shock proteins with 30-60 kDa molecular weight were observed in *Trigonella* in response to heat stress.

5. Conclusion

During stress many structural proteins undergo deleterious structural and functional changes and the over expression of proteins after heat treatment were observed in the study. Variations in heat shock protein synthesis have been observed when the plants were grown under similar conditions but heat stresses for longer periods. Increased synthesis of reactive oxygen species scavenging enzymes indicated the defense mechanisms of the plant to heat stress. These findings suggest that fenugreek (*Trigonella foenum-graceum* L.) is heat tolerant and can be introduced in breeding programs to produce tolerant varieties.

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