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INTERACTIVE EFFECT OF INTEGRATED TEMPERATURE AND SALINITY STRESS ON EXPRESSION OF HEAT SHOCK PROTEINS (HSPS) AND PROTEIN CONTENTS OF *VIGNA MUNGO (L) HEPPER*

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ABSTRACT
The investigation is carried out to evaluate various Heat Shock Proteins produced by the plant during temperature and salinity stress as the plant can experience a wide fluctuation of temperature on a daily or seasonal basis. In the present study, the three day old seedlings of *Vigna mungo (L) Hepper* (Cultivar-AKU 15) are treated with various concentration of Nacl and LD-50 is recorded for the seeds presoaked in water for 12 hours and for dry seeds. The four different doses selected for final analysis for presoaked seeds viz-0.4%, 0.8%, 1.2%, and 1.6% and that for dry seeds were 0.2%, 0.4%, 0.6%, and 0.8%. The seeds were treated for 6 hours in the given concentration of Nacl, then sown in germination tray and maintained at 25^0C. The three day old seedling then suspended to temperature stress at different temperature viz-35^0C, 40^0C, and 45^0C for 3 hours in growth chamber. The seedling then crushed in mortal and pestle and proteins are isolated by centrifugation at 10000 rpm for 12 min. The separation of heat shock protein is carried out by Polyacrylamide Gel Electrophoresis (SDS-PAGE) and bands were evaluated on Ultra Info-tech gel documentation system (Genei) for molecular weight of proteins. The large numbers of small HSPs are found to be produced when the seeds presoaked in water for 12 hours treated with 0.8%, 1.2% and 1.6% Nacl solution are exposed to 45^0C. The most noticeable polypeptides were 7.2 kD, 6.5 kD, 5.0 kD, 3.6 kD and 1.3 kD. The 1.3 kD sHSP was found to be unique.

KEY WORDS: Heat Shock Proteins (HSPs), temperature, drought, salinity, LD-50, SDS-PAGE, AKU-15, protein quantification, Vigna mungo (L) Hepper etc.

INTRODUCTION
Abiotic stresses like temperature, salinity and drought are the primary causes of plant loss worldwide. Most eukaryotic cells respond to high temperature and certain other stresses with the production of HSPs (Lindquist *et al*., 1988). The plant can experience a wide fluctuation of temperature on daily or seasonal basis and therefore they are constantly exposed to changing environment. It was reported that heat stress disrupts water, ion and organic solute movement across plant membranes (Ibrahim *et al*., 2001), reduces chemical reactions, gas solubility, mineral absorption and water take up (Treshow M 1970), impairs photosynthetic electron transport system and increases oxidative degeneration of membrane lipids (Dash *et al*., 2002). Heat Shock Proteins are important in relation to stress tolerance and adaptation to environment. Heat shock proteins (HSPs) and other stress proteins have been known to protect cells against deleterious effects of stress (Blumenthal *et al*., 1990, Feder *et al*., 1999, Iba K 2002, Soransen *et al*., 2003 and Young *et al*., 2002).The HSPs buffers environmental variations and are therefore important factors for maintenance of homeostasis across environmental regimes (Banu *et al*., 2009). The 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 *et al*., 1992). The temperature of 30^0 to 40^0C combined with salt stress was introduced in Lentil seeds during germination. The expression of small HSPs declines at 30^0 to 40^0C, this declining was higher in 40^0C rather 30^0C (Dell A Quila *et al*., 2000). Heat stress is a major factor limiting the productivity and adaptation of crops, especially when temperature extreme coincides with critical stages of plant development. The rate of temperature change and duration and degree of high temperature all contributes to the intensity of heat stress (Chen *et al*., 1992). Presence of HSPs in higher plants were discovered in tobacco and soybean using cell culture technique (Barnett *et al*., 1980) when soybean was subjected to 40°C for four hours, ten new proteins were found, but disappeared after 3 h treatment at 28°C (Key *et al*., 1981). HSPs sometimes referred as stress proteins, also produced during the salt or saline stress .It may possible that combine effect of heat and salinity stress may induce expression of some novel HSPs. Therefore present study has been carried out to investigate the combined effect of temperature and salinity in *Vigna mungo (L) Hepper*.

MATERIALS AND METHODS
Plant Material
The genus *Vigna* contains several species that are economically important in world agriculture. The *Vigna mungo (L) Hepper* commonly known as black gram is grown in many tropical and subtropical countries mainly in India, Iran and Malaysia etc. It is rich in phosphoric acid, 5-10% more than other pulses. The pulse contains about 23.4% proteins, 1% fats and 57.3% carbohydrates. (Varshney *et al*., 2003). It prefers dry weather condition; optimum temperature is between 25^0C to 35^0C.

The germplasm of *Vigna mungo (L) Hepper* variety AKU-15 is procured from PKV Akola which is then treated with different concentration of Nacl and LD -50 was determined. The experiment was carried out in two sets one with dry 18 hours treatment and other with 12 hours presoaking in water plus 6 hours treatment of Nacl. The four different concentrations of Nacl were selected for dry seeds viz- 0.2%, 0.4%, 0.6%, 0.8% etc. and 0.4%, 0.8%, 1.2% and 1.6% for the presoaked seeds in water. The seeds were sown in germination tray where they allowed germinate at 25^0C and kept at same temperature up to the development of three day old seedlings. The germination trays then transferred to growth chamber where they incubated at 35^0C, 40^0c and 45^0C for three hours in different experimental set up. The protein estimation assay and Polyacrylamide Gel Electrophoresis is immediately carried out after temperature exposure.

Quantification of Protein

The extraction and isolation of proteins are carried out by crushing the three day old seedling in mortar and pestle followed by centrifugation at 10,000 rpm for 12 min, the supernatant was used as source of protein for protein estimation and SDS-PAGE assay. The protein estimation is done by Bradford method (Bradford 1976), for this Bradford reagent was prepared by mixing 100 mg Coomassie brilliant blue G-250, 50 ml of 95 % ethanol and 50 ml of ortho-phosphoric acid. The standard graph was plotted by using BSA protein solution. For this 100 mg of Bovine serum albumin was dissolved in 100 ml distilled water (Stock solution). The working standard is prepared by mixing 10 ml of stock solution with 90 ml of D. W. From this Working standard solution 0.2ml, 0.4ml, 0.6ml, 0.8ml and 1.0ml were pipetted out in five separate test tubes. The volume in each test tube is adjusted to 1 ml by adding D.W. The test tube containing 1 ml D. W. served as a blank. To each of this test tube 5 ml of diluted Bradford reagent was added and absorbance was recorded at 595 nm wavelength and standard graph was plotted. To estimate the protein in sample, 0.2 ml supernatant is collected in separate test tube and the volume is adjusted to 1 ml by adding 0.8 ml D. W. To this reaction mixture 5 ml of diluted Bradford reagent was added and absorbance was recorded at 595 nm wavelength. The quantity of protein was calculated from standard graph.

Analysis of HSPs by SDS-PAGE

The separation of Heat Shock Proteins is carried out by using Polyacrylamide Gel Electrophoresis i.e. SDS – PAGE (Laemmli 1970)) on vertical gel assembly (Genei-Midi). The 15 % polyacrylamide gel is prepared by using monomer acrylamide and cross linking agent bis-acrylamide. The tetramethylethylene diamine (TEMED) is used as chain initiator in the presence of ammonium persulphate as a catalyst. The isolated protein first treated with sodium dodecyl sulphate and mercaptoethanol which brings about denaturation of proteins. For this 50ul of protein extract is mixed with 100ul of sample buffer containing SDS and mercaptoethanol and mixture is incubated on boiling water bath for 10 min. The SDS is strong anionic detergent which binds to polypeptide and gives them net negative charge. These charged polypeptides migrate towards the positive electrode of applied electric field. The polypeptide with lowest molecular weight moves towards the bottom where as with that of heaviest molecular weight settled at top of gel. The protein sample requires 3 hours to electrophorized and then the gel is removed from the assembly and transferred to the staining jar. The protein stain solution is made by mixing 100 mg Coomassie brilliant blue R-250 in 40 ml methanol, 10 ml glacial acetic acid and 50 ml of distilled water. After keeping the gel in the stain solution overnight it is transferred to de-staining solution containing all ingredient of staining solution except stain. Where it is kept until clear bands of proteins is visible. This gel is then analyzed on Alpha-InfoTech gel documentation system to obtaining molecular weight of each separated protein. The molecular weight of proteins is determined by comparing them with standard proteins markers loaded at the time of electrophoresis.

RESULT AND DISCUSSION

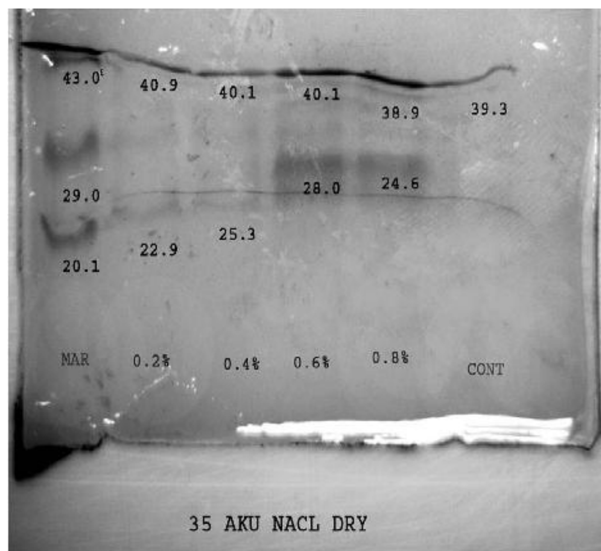

FIGURE 1:Protein profile of 35^0C+ Nacl dry treatment

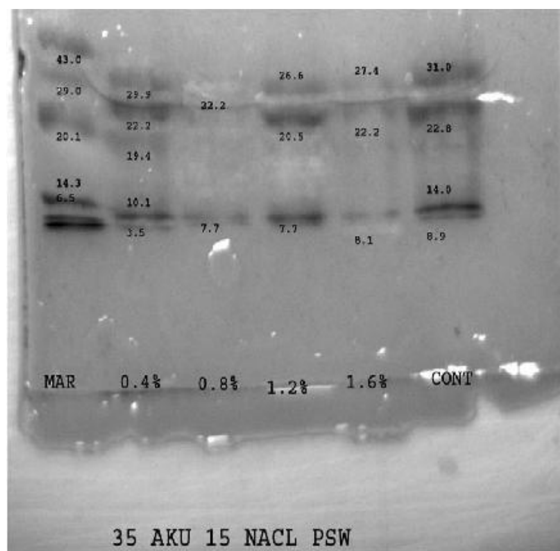

FIGURE 2: Protein profile-35^0C+ Nacl PSW treatment

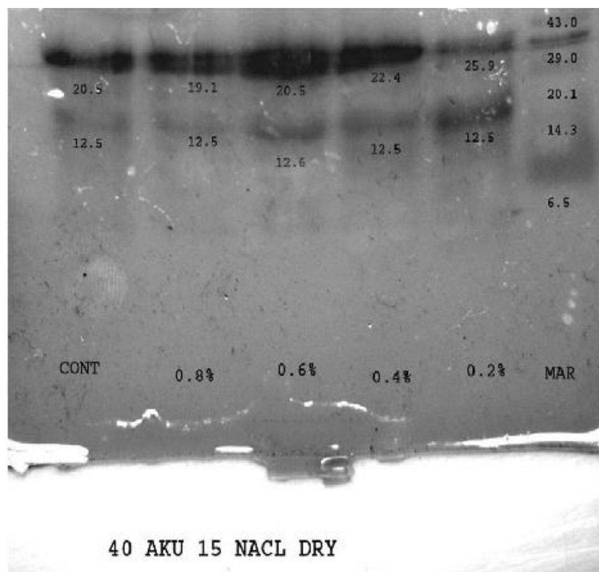

FIGURE 3: Protein profile- 40^0C+ Nacl dry treatment

FIGURE 4:Protein profile-40^0C+Nacl PSW treatment

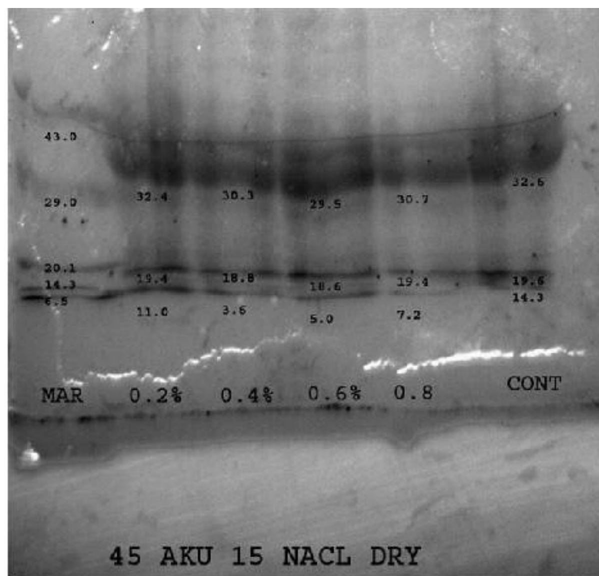

FIGURE 5: Protein profile- 45^0C+ Nacl dry treatment

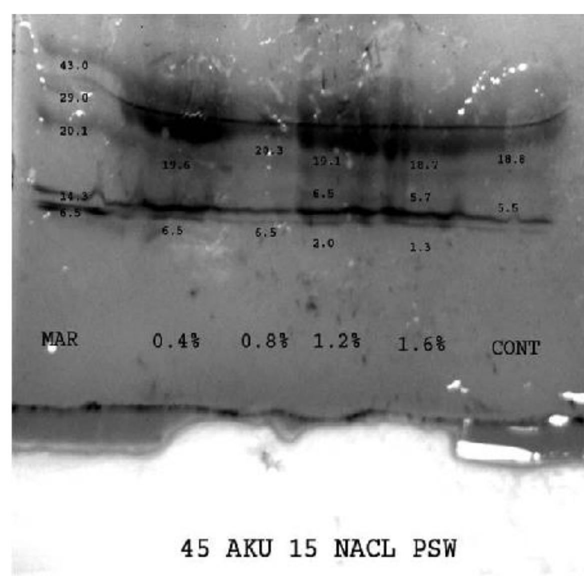

FIGURE 6: Protein profile- 45^0C+ Nacl PSW treatment

The electrophoresis pattern of three day old seedling of Vigna mungo (L) Hepper leads to some important observations. After the exposure to heat, plant synthesizes Heat Shock Proteins. In the given analysis the three day old seedlings exposed to 35^0C, 40^0C and 45^0C; all the three temperature was found to induce the expression of HSPs. The HSPs produced by heat treatment mostly ranges from 8- 40 kD. It can be observed from gel documentation analysis that temperature alone is responsible for induction of specific HSPs of average molecular weight (Fig-1, 3). The presoaking in water along with the combined temperature and salinity stress found to induce more varieties of HSPs (Fig-2, 4). The most noticeable polypeptides are 7.2 kD, 6.5 kD, 5.0 kD, 3.6 kD and 1.3 kD (Fig-5, 6). The large numbers of small HSPs are found to be produced when the seeds presoaked in water for 12 hours treated with 0.8%, 1.2% and 1.6% Nacl solution are exposed to 45^0C (Fig- 6). The 1.3 kD sHSP was found to be unique and 45^0C and 1.6% concentration of Nacl found to be effective in the expression of 1.3 kD polypeptides. When high temperature combines with salinity i.e. combination of 45^0c with 1.2% and 1.6% of Nacl , mostly expression of small HSPs takes place like polypeptides of 1.3 kD and 2.0 kD. It can be observed from table-1 that the protein expression pattern also varies with presoaking treatment of seed, where it is found to be decreased as compared to the dry treatment.

Plants in the field are often exposed to multiple stresses simultaneously. The soil which is more saline induces combine stress of temperature and salinity in the arid regions. The impact of this combined stress is analyzed in laboratory conditions. The result shows that as compared to the temperature alone, assisted salinity induces smaller HSPs. Plant small HSPs are controlled at transcriptional level at heat stress (Sun W, 2002). Small HSPs might protect and keep un-translated normal cellular m RNA

stable during heat stress. They have broad range function involving role in protein trafficking, prevention of protein aggregation and assistance in protein folding (Lan *et al.*, 2010) Many HSPs belongs to class ubiquitin. The ubiquitin is highly conserved low molecular weight proteins 75 – 76 amino acids residue and found in every eukaryotic cell (Vierstra, 2003). It is either found as a free or bound to various proteins by their terminal lysine amino acids. Heat stress induced synthesis of ubiquitin may have vital function for stress resistance. These proteins have a common alpha-crystalline domain containing 80–100 amino acid residues located in the C-terminal region (Seo *et al.*, 2006). One of the characteristic functions of this class is the degradation of the proteins that have unsuitable folding. The representative protein is the small HSPs ubiquitin (molecular weight is 8.5 KD) with its bounded enzymes (Ferguson *et al.*, 1990). The interaction of different biotic and abiotic stresses with heat stress was studied and analyzed, and the information in respect of transcription of Hsps in the plant *A. thaliana* was deposited in the database AtGenExpress Consortium (Schmid *et al.*, 2005). Results of the analysis indicated that all stresses interacted in the response pathways of heat-shock proteins and their factors, but the degree of interaction was different which suggested a cross-talk in the regulating net. Hu *et al.*, 2009 examined a global expression profiling with heat stressed rice seedling and then compared their own results with the previous rice data under cold, drought, and salt stresses. They concluded that Hsps might be important elements in cross-talk of different stress signal transduction networks.

Understanding the role of HSPs in relation to stress resistance in a more applied perspective as a potential indicator of stress is important and must be analyzed in detail in the area like Vidarbha(Maharashtra) as the soil in this region is very saline and climate is arid.

TABLE-1: Quantity (mg/ gm) of protein in various concentrations of Nacl

Temp.	Dry Treatment		PSW Treatment	
	Conc. of Nacl (%)	Quantity of Protein (mg/ gm)	Conc. of Nacl (%)	Quantity of Protein (mg/ gm)
	0.2	139	0.4	44.6
	0.4	146.6	0.8	52.2
35^0C	0.6	67.4	1.2	44.6
	0.8	103.2	1.6	27.6
	Control	59.8	Control	31.4
	0.2	14.6	0.4	37.2
	0.4	71.2	0.8	52.2
40^0C	0.6	8.8	1.2	31.4
	0.8	31.4	1.6	18.2
	Control	20.2	Control	25.8
	0.2	44.6	0.4	63.6
	0.4	80.6	0.8	52.2
45^0C	0.6	44.6	1.2	59.8
	0.8	5.0	1.6	25.8
	Control	29.2	Control	39.0

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