Indian J. Hort. 64(4), December 2007: 373-380

Stress responsive proteins in coconut seedlings subjected to water, high-light, flooding and high-temperature stresses

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ABSTRACT

Coconut seedlings of different cultivars and cross combinations were subjected to stresses like water, high temperature and flooding stresses independently. Results indicated increase in the concentration of heat stable protein fraction (HSPF) in leaf tissue even when the total protein concentration reduced due to stress. The percentage of HSPF in leaf tissue increased with decrease in leaf water potentials. Quantitative changes in proteins also were observed in leaf and root tissue due to temperature and flooding. In HSPF, proteins of ~66KDa are found in root and leaf tissue. Leaflet tissues have specific proteins in the range of ~10 and ~14 KDa which are not present in the root tissues. Two extra proteins 66 KDa and ~ 76 KDa appeared in water stressed WCT seedlings. They were also present in seedlings exposed to high light intensity (~1,500 mmol/m^2/s) apart from an extra LMW HSP of 14.4 KDa. Among the MMW- proteins, protein of 53 KDa is present in all WCT samples. The LMW protein of 20.1 KDa, present in non-stress seedlings disappeared during water stress period. In temperature induced and flooded leaflet tissue, new proteins (LMW range ~17 KDa) were observed. In temperature induced root tissue, a protein band in the range of 30 KDa was observed but not in flooded root or in control root. Results indicate quantitative and qualitative variations in stress proteins in coconut seedlings subjected to different abiotic stresses.

Key words: Stress proteins, abiotic stress, coconut, water stress, flooding, temperature, water potential.

INTRODUCTION

The rate of protein biosynthesis declines during stress conditions. However, despite reduction in overall biosynthesis of proteins, cells under stress preferentially synthesize a few specific proteins (Pareek *et al.,* 10). The new or existing proteins which are preferentially synthesized in plant tissue in repose to stress are called stress responsive proteins. These proteins are known to be synthesized under the abiotic stresses like desiccation (Bray, 3); anoxia (Czarnecka *et al.,* 5), high temperature (Lindquist and Craig, 8), etc. The role of these proteins is to protect the cells from the adverse effects of stress (Pareek *et al.,* 11). The proteins are synthesized when the system is exposed to non-lethal or induction stress. The stress protein synthesis is related to the turgor-dependent gene expression (Bray, 4).

Studies on stress responsive proteins assume importance for several reasons. Firstly, the information on sum total of alterations of cellular transcripts provides measure of complexity of stress response. Secondly, it reveals the possible number of genes with altered action. Thirdly, it serves as one of the tools for screening germplasm for stress tolerance. Fourthly, possibility of finding common stress responsive proteins providing tolerance across the stresses and finally provides information on their role in imparting tolerance to specific stress.

Using the criteria that stress responsive proteins are having characteristics of (i) stable at high temperatures, (ii) inducible by non-lethal stress (induced stress) conditions (Bray, 4; Pareek *et al.,* 10), the heat stable protein fractions were estimated which were synthesized in coconut seedlings under different stress conditions. Analysis of protein profiles before and after stress treatments is an important approach for identification of stress-responsive genes (Pareek *et al.,* 11). Apart from studies scavenging enzymes during water stress (Shivashankar, 16) these there has been no information available on the stress responsive proteins or on heat stable proteins. In the current study, coconut seedlings were exposed to various stresses like water stress, flooding, high temperature and high light intensities in the nursery to assess the response of coconut seedlings to the above stresses in terms of quantitative and qualitative alterations in proteins.

MATERIALS AND METHODS

Four experiments were conducted using one year old uniform coconut seedlings of different cross combinations and their parents which were maintained in polybag nursery in a net house. The seedlings were provided irrigation once in four days. The polybags contained the potting mixture of uniformly fertile soil, sand and farm yard manure. For each cross combination and cultivar 10 seedlings were maintained for experimental purpose. The seedlings were grown continuously in net house at low light intensities ~350

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*Corresponding author's E-mail: nareshkumar.soora@gmail.com; snk_66@yahoo.com µmol/m²/s. The seedlings were subjected to various treatments. All experimental observations were made during summer months of March to May for two consecutive years.

In experiment I, the seedlings of seven cross combinations *viz.*, COD x WCT, WCT x COD, LCT x COD, ADOT x GBGD, WCT x GBGD, LCT x GBGD, WCT x MYD and their parents *viz.*, COD, MYD, WCT were maintained in polybag nursery under uniform levels of light at ~350 µmol/m²/s to rule out the influence of high light intensity. The seedlings were maintained under well irrigated conditions to get the pre-stress observations and then were subjected to the water stress for 25 days and after taking the observations during stress period they were again watered to get the data during recovery. The recordings on leaf water potentials (Skye, USA) and leaflet protein estimations were done during pre-water stress, stress and recovery periods.

In experiment II, one-year-old uniform coconut seedlings of cv. West Coast Tall, were maintained in polybag nursery. A total of two sets of seedlings were maintained for experimental purpose. One set of seedlings was grown continuously in net house at low light intensities (~350 µmol/m²/s). The second set was given high light intensity (1500 µmol/m²/s) treatment. Both the sets were subjected to water stress for 20 days. Estimation of protein and HSPF concentrations were done in experimental seedlings.

In experiment III, one-year-old uniform coconut seedlings of cv. West Coast Tall were maintained in polybag nursery in a net house as indicated above. A total of two sets of seedlings were maintained. One set of seedlings was grown continuously under flooding for 20 days before protein estimation is made. Another set without flooding treatment served as control. The leaf and root tissue protein concentrations and HSPF were estimated.

In experiment IV, in order to study the impact of high temperature on coconut proteins, the temperature induction technique was used on the excised leaflet of WCT cultivar. Since in natural conditions the changes occur gradually, the changes in temperature also occur in a gradual manner. Hence the plants were generally exposed to sub-lethal stress before they face severe stress. The plants which acquire tolerance during sub-lethal stress are more capable of withstanding severe stress condition. During the sub-lethal stress, plant cells respond by changes in physiological and biochemical events. Based on this principle, the temperature induction experiment was conducted on excise leaf tissue of WCT seedlings. The leaflets were excised from the third leaf from top of well watered WCT seedlings and the leaf pieces were given four types of treatments. First two sets were exposed to (i) temperature induction cycle of 35-40-45-50-55°C in high humid (RH~80%) conditions and (ii) under dry low humid (~20% RH) conditions in an incubator. The time maintained at each temperature step induction was 1 hour. Another two sets were exposed directly to (iii) high temperature of 55 °C in high humid (RH~80%) conditions and (iv) under dry low humid (~20% RH) conditions in an incubator for five hours. Freshly excised roots also were subjected to similar treatments. The leaf tissue protein concentrations and HSPF were estimated.

For estimation of proteins and HSPFs, leaflet tissue (500 mg) from the third leaf from top was frozen in liquid nitrogen immediately after excise and homogenized in 1:4 volumes of tris-extraction buffer (Tris 50 mM; NaCl 50 mM; EDTA 2 mM, α-mercaptoethanol 5 mM, PMSF 1 mM) pH 8.0 in the presence of insoluble PVPP. The extract was centrifuged at 12,000 rpm for 10 min. at 4 °C in refrigerated centrifuge (Hareus, Germany). The protein in the extract was estimated as per the method of Bradford (2) using CBB G250 dye. For separation of heat stable proteins soluble protein extract was incubated at 70°C for 10 min. in water bath and quickly chilled denatured proteins were removed by centrifugation at 12,000 rpm for 10 min. The HSPF content in supernatant was estimated as per Bradford (2). To the above fraction TCA was added and chilled in ice for one hour and then centrifuged at 10,000 rpm for 15 min. The pellet was incubated in chilled acetone at -20 °C for 10 min. The acetone was removed by centrifugation at 10,000 rpm for 15 min. The vacuum dried pellet was dissolved in Laemmli–SDS sample buffer (pH 6.8), consisting tris-HCl 0.0625 M, SDS 5%; α-mercaptoethanol 2%; sucrose 10%; bromophenol blue 0.002%.

For SDS-PAGE, the gel recipe consisted of 1M tris-HCl (pH 6.8) (stacking gel); 1.5 M tris-HCl (pH 8.8) (resolving gel); acrylamide mix 30%; SDS 10%; APS 1.5%; TEMED. The reservoir buffer was tris-glycine (pH 8.3). The markers of HMW and LMW were also placed on the gel. The gel was run at 50 v (stacking) and 100 v (resolving) at 15 °C. The gels were stained in CBB staining solution (CBB R-250, methanol and GAA) to spot the specific proteins. The samples from non-stressed seedlings formed the control. The data were analysed for statistical significance in GLM using SPSS software (SPSS ver. 10.0).

RESULTS AND DISCUSSION

The results obtained in the experiment on water stress indicate clear patterns in changes in proteins, HSPF and in qualitative changes in proteins (Fig. 1). Leaflet tissue protein concentrations varied from 20 to 35 mg/g fresh tissue in different coconut cultivars during

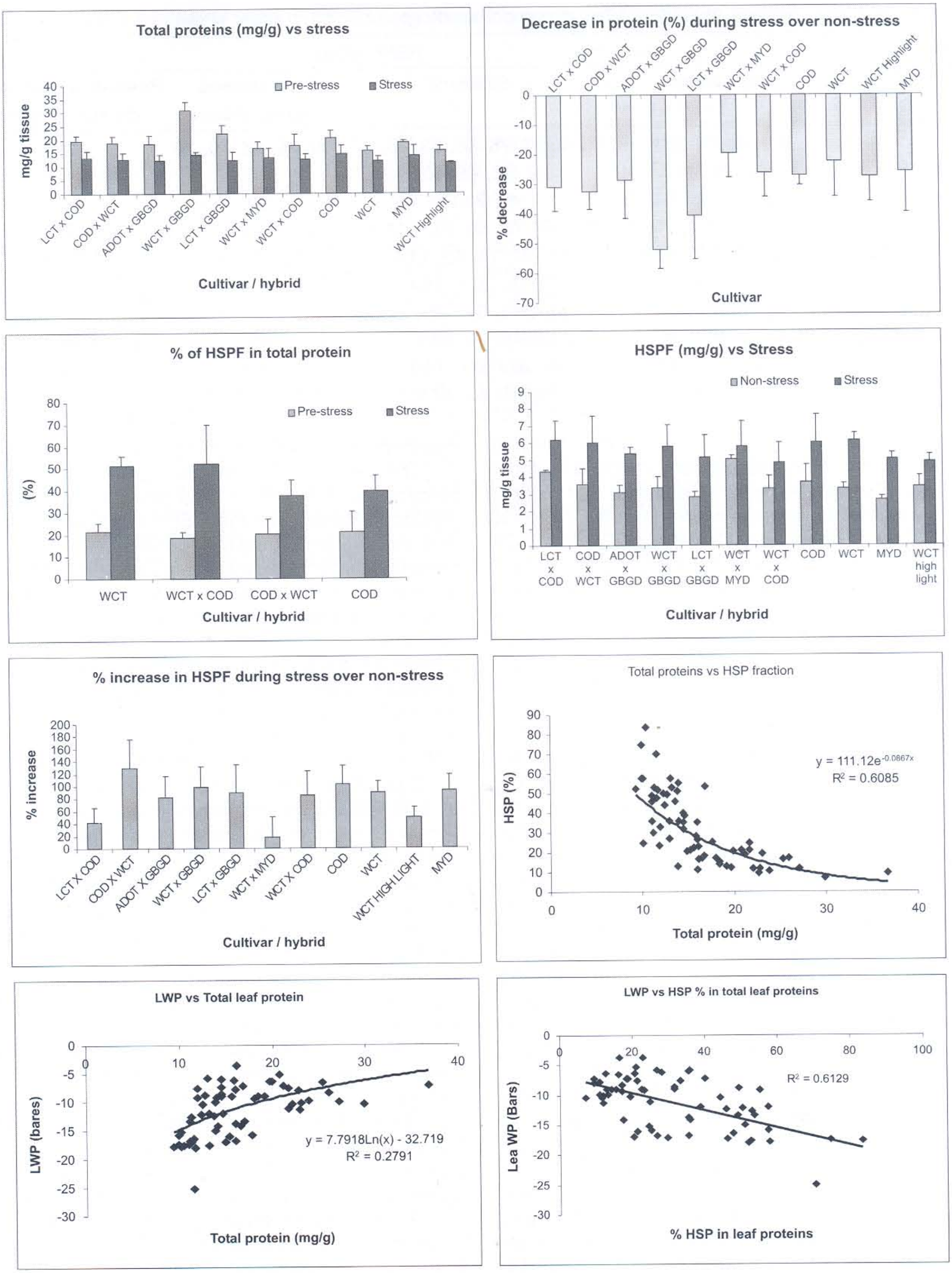

Fig. 1. Changes in leaf proteins, HSPFs and their relationship with leaf water potentials in seedlings of coconut cultivars subjected to water stress.

Table 1. Qualitative analysis of HSPFs (kDa) in coconut seedlings subjected to water stress.

Cultivar	HSPF (kDa)			
	Non-stress	Stress	Extra proteins during stress	Proteins not detected/ low during stress
WCT	97, 53, 45, 20.1	116, 97, 76, 66, 53, 30,	116, 76, 66, 53, 30	45
COD	97, 76, 30	97, 76, 45, 30	45	-
MYD	53, 30, 20.1	45, 20.1	30	53
COD x WCT	170, 116, 97, 66, 20.1	116, 97, 76, 66, 20.1, 14.4	14.4	170
WCT x COD	170, 97, 76, 66	116, 97, 76, 45, 14.4	116, 45, 14.4	170, 66
WCT x MYD	97, 53, 45, 30, 20.1	97, 45, 20.1, 14.4	14.4	30
WCT x GBGD	97, 45, 14.4	97, 45, 30, 14.4	30	-
LCT x GBGD	45, 30, 20.1, 14.4	53, 45, 30, 20.1	53	45
LCT x COD	53, 45, 30, 20.1,	45, 30, 20.1, 14.4	14.4	30
ADOT x GBGD	97, 53, 20.1	97, 45, 30, 14.4	45, 30, 14.4	53

non-stress periods. However, the protein concentrations during stress were reduced to 12-15 mg/g fresh tissue. Per cent reduction in leaf proteins due to water stress were the maximum in WCT x GBGD and LCT x GBGD and least in WCT x COD and WCT. Variation among cultivars for reduction in protein concentration was noted. Heat stable protein fraction during non-stress was present in leaf tissue at concentrations lower than 5 mg/g fresh tissue. But during the stress period the concentrations increased above 6 mg/g fresh tissue. Per cent increase in HSPF fraction during stress over non-stress period was to the tune of 140% in COD x WCT and 20% in WCT x MYD. Variation in relative accumulation of HSPF and relative decrease in protein concentration indicates differential response of cultivars and hybrids to water stress. In reciprocal crosses of WCT x COD and COD x WCT and their parental lines, the per cent HSPF in total protein was more in WCT and WCT x COD than COD and COD x WCT indicating the predominant influence of female parent on this character. The cross combinations with GBGD and MYD seems to have more per cent reduction in protein concentration. The per cent HSPF in leaf tissue increased with decrease in leaf water potentials. However, these combinations also have higher per cent increase in HSPF during stress over non-stress periods. It is known that coconut cultivars and hybrids varied for their tolerance to drought stress (Rajagopal *et al.,* 12). Variations in tolerance levels were linked to the anatomical, physiological and biochemical characters like epiculticular wax, paranchyma cell size, lipid peroxidation, stomatal resistance, scavenging enzymes, etc. (Naresh Kumar *et al.*, 9; Rajagopal and Kasturi Bai, 13; Rajagopal and Naresh Kumar, 14). These results indicate that apart from the above factors, heat stable proteins play a major role in subsequent metabolic responses imparting tolerance to coconut.

The relationship between leaf proteins and percentage of HSPF in total proteins had inverse relation indicating that the HSPF is limited to a level and under unfavourable conditions HSPs are preferentially synthesized even as some of the thermo-labile proteins are destroyed. There existed a weak positive relationship between leaf water potential and leaf protein concentration. It is apparent that normal protein synthesis is hampered at low leaf water potentials. However, the results clearly indicate that at low leaf water potentials, the cells shifted their preference to HSP synthesis, thus the percentage of HSPs had negative relationship with water potentials.

Qualitative analysis of HSPF was done by resolving on SDS-PAGE. The fractionation indicated presence of new proteins due to water stress (Table 2). The results indicated that the HMW protein at 97 KDa is present in all the samples of WCT, whereas two extra proteins (66 KDa and ~76 KDa) appeared in water stressed WCT seedlings. They were also present in seedlings exposed to high light intensity (~1500 mmol/ m^2/s) apart from an extra LMW HSP of 14.4 KDa. In COD, the 97 KDa and ~76 KDa proteins are present in both non-stressed and water stressed seedlings.

In WCT X COD, the 97, 76 and 66 kDa were present during non-stress but 76 kDa disappeared during stress while in COD X WCT the reverse trend was observed. Among the MMW- proteins, a 53 KDa MW protein is present in all WCT samples. The LMW protein with 20.1 KDa MW, present in non-stress seedlings, disappeared during water stress period. The results indicate that cultivars and hybrids not only varied for quantity of proteins but also for quality. Differential expression of two stress proteins in cultivars with varying response to salt stress was reported earlier also (Pareek *et al.,* 11).

Table 2. Qualitative analysis of HSPs (kDa) in coconut seedlings subjected to high light intensity.

Cultivar	HSP (kDa)			
	Non-stress	Stress	Extra proteins during stress	Proteins not detected/ low during stress
WCT	97, 53, 45, 20.1	116, 97, 76, 66, 53, 30,	116, 76, 66, 53, 30	45
WCT (High light)		116, 97, 76, 66, 53, 20.1, 14.4	116, 76, 66, 20.1, 14.4	45

In the second experiment, one set of seedlings were grown continuously in net house at low light intensities (~350 µmol/m²/s). The second set was given high light intensity (1,500 µmol/m²/s) treatment (Fig. 2). High intensity of light enhanced reduction in protein concentration under water stress conditions. More deleteriously, under high light, increase in HSPF percentage was half of the increase in HSPF under shaded conditions. This indicates why water stress coupled with high light intensity is more damaging to the cells. The response of coconut seedlings to high light intensities was almost similar as in case of water stress. However, LMW polypeptide of 14.4 kDa seems to be extra protein expressing under high light intensities (Table 2). Comparison with results on water stressed seedlings indicates that this protein seemed to be expressed in seedlings under severe stress.

The leaflet protein concentration in WCT seedlings, grown continuously under flooding conditions for 20 days, was increased slightly over control seedlings (Table 3). However, there was a significant increase in HSPF in leaf tissue due to flooding. In contrast, the protein concentration and HSPF were reduced in root tissue. Anoxia caused at root zone due to flooding seems to have direct impact on the protein concentrations of root. The HSP fraction was high in the stressed seedling tissue. Flooding causes anoxia at the root zone thus affecting the metabolism in root

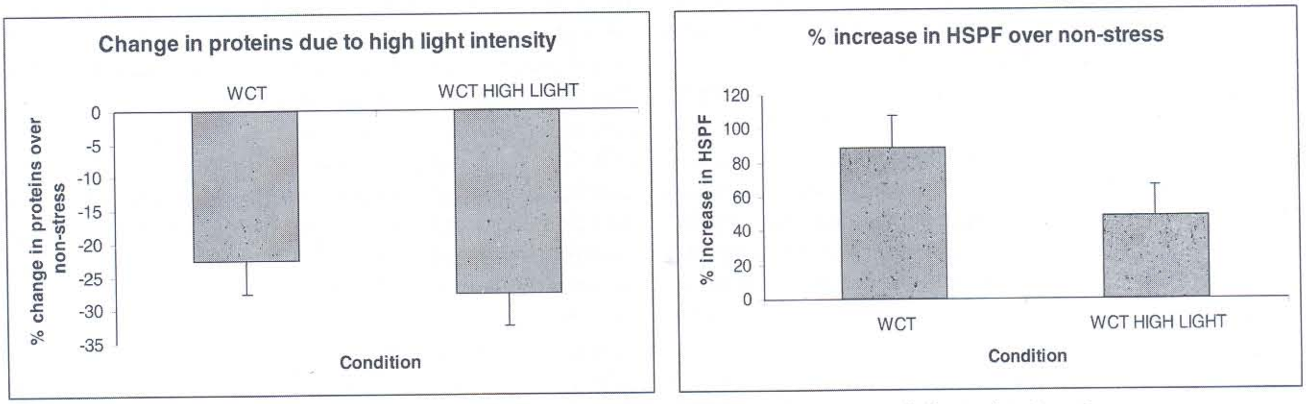

Fig. 2. Changes in leaflet proteins and HSPF in coconut seedlings exposed to high light and water stress.

Table 3. Changes in tissue proteins and HSPF due to flooding for 20 days under shade (500 µmol/m²/s) in WCT seedlings.

Treatment and tissue	Proteins (µg/mg of leaf tissue on FW basis)	Per cent change over stress	HSPF (µg/mg of leaf on FW basis)	Per cent change over non-stress	Per cent of HSPF in protein
Flooding					
Leaflet	19.94	9.50	6.01	66.48	30.09
Root	5.43	-37.66	1.73	-24.12	31.91
Control					
Leaflet	18.21		3.61		19.77
Root	8.71		2.28		26.25
CD (P = 0.05)	1.62		0.63		

cells. The results on root proteins indicated that the flooding caused severe damage to the root proteins. Changes in leaf proteins indicate a plant response possibly triggered by root signal with possible involvement of ABA and ethylene signals which are produced in response to stress (Shinozaki and Shinozaki, 15).

Studies on the impact of high temperature on coconut proteins, using temperature induction technique on the excised leaflet and roots of WCT cultivar, indicated that the temperature cycle with high RH is suitable for studying the impact of temperature on tissue proteins. Maintenance of RH was crucial in these experiments.

Since under the natural conditions the changes occur gradually, the changes in temperature also occur in a gradual manner. Hence, plants are generally exposed to sub-lethal stress before they face severe stress. Thus, the plants which acquire tolerance during sub-lethal stress are more capable of withstanding severe stress condition. During the sub-lethal stress, plant cells respond by changes in physiological and biochemical events. The genotypes with enhanced synthesis of stress responsive proteins or synthesis of new proteins under induced stress condition will survive better under severe stress conditions. Thus, the induction protocols are useful for assessing the genetic variability in stress response and to study the expression pattern of stress responsive proteins.

The results indicated that the protein concentration in leaf tissue under temperature induction at high RH has increased over the control tissue indicating the active synthesis of proteins under induction (Table 4). On the other hand, concentration of proteins in tissue treated with temperature induction under dry (RH at 30%) conditions was estimated at higher concentrations than in tissue at high RH. Under low RH conditions at high temperatures tissue dehydrates and this may be prime reason for estimation of increased concentration even though presumably protein degradation takes place in dehydrating tissue. This can be supported from the data that in tissue exposed to high temperature at high RH, protein concentration was very low indicating rapid protein degradation. In tissue exposed to high temperature under dry conditions gain showed increased concentration of proteins due to rapid dehydration as indicated above. In case of HSPF, the temperature induced tissue under high RH conditions could synthesize the HSPFs as indicated by increase in per cent concentration over control. In other cass, the concentrations of HSPFs either increased or decreased mainly due to the reasons mentioned above. As regards the temperature induced root tissue, the protein concentration decreased over control (Table 5). In other treatments, the results were in similar trends as in case of leaf tissue because of the reasons mentioned earlier. Trends in HSPF also were similar to that in leaf tissue.

The results indicate that for temperature induction treatment on excised leaf or root tissue, maintenance of high RH is crucial to rule out the interference of desiccation on protein concentration estimations. Gradual increase in temperature from sub-lethal levels caused cells to survive even at lethal temperature (55°C). This temperature induction promoted the synthesis of HSPs as reported in other plant species and possibly importing thermo-tolerance (Howarth, 6). Sudden exposure of tissue to high temperature (55°C) proved lethal and resulted in rapid dehydration of tissue and death as reported earlier (Lin *et al.*, 7). However, under natural conditions, temperature buildup is gradual thus the temperature induction technique provides possible means for studying the seedling

Table 4. Changes in leaflet tissue proteins due to temperature induction in WCT seedlings.

Treatment	Protein (µg/mg of fresh leaf tissue)	Per cent change over non-stress	HSPF (µg/mg of fresh leaf tissue)	Per cent change over non-stress	Per cent of HSPF in protein
Temperature cycle (35-40-45-50-55 °C)					
With high RH (over 80%)	22.12	21.47	5.20	44.04	23.51
Dry	23.36	28.28	6.57	81.99	28.13
At 55 °C					
With high RH (over 80%)	9.2	-49.48	1.81	-49.86	19.57
Dry	25.42	39.59	6.51	80.33	25.55
Control	18.21		3.61		19.77
CD (P = 0.05)	3.46		0.81		

Table 5. Changes in root tissue proteins due to temperature induction in WCT seedlings.

Treatment	Protein (µg/mg) of fresh leaf tissue)	Per cent change over stress	HSPF (µg/mg of fresh leaf tissue)	Per cent change over non-stress	Per cent of HSPF in protein
Temperature cycle (35-40-45-50-55 °C)					
With high RH (over 80%)	6.88	-20.92	2.37	3.95	34.48
Dry	9.00	3.43	2.40	5.26	26.67
At 55 °C					
With high RH (over 80%)	5.30	-39.08	1.17	-48.68	22.11
Dry	21.37	145.63	5.98	162.28	27.96
Control	8.71		2.28		26.25
CD (P = 0.05)	1.86		0.57		

tolerance to high temperature. However, more studies are needed before adopting this method as a screening technique.

HSPFs in root and leaf tissue of WCT coconut seedlings subjected to flooding and temperature and drought stress were resolved on SDS-PAGE. The results indicated leaflet tissue have specific proteins in the range of ~10 and ~14 KDa, which are not present in the root tissue. In HSPF, proteins of ~66 KDa are found in root and leaf tissue. In temperature induced and flooded leaflet tissue, new proteins (LMW range ~17 KDa) were observed. In temperature induced root tissue, a protein band in the range of 30 KDa was observed but not in flooded root or in control root. The results are in conformity with the unique feature of the heat-shock response in higher plants in which small HSPs, ranging in size from 15 to 30 kDa, dominate the protein synthesis during temperature stress and accumulate in abundance (Vierling, 17; Waters *et al.*, 18; Boston *et al.*, 1). These proteins act as molecular chaperones (Vierling, 17) thus imparting the tolerance to plant cell. Set of proteins in HMW range showed to accumulate in response to high temperature stress as well as other stress conditions apart from the accumulation of proteins specific to stress (Pareek *et al.*, 11).

It can be concluded from the above findings that coconut seedlings respond to the abiotic stresses by preferentially synthesizing stress responsive proteins as indicated by increase in the HSP fraction of proteins even though over all protein synthesis is decreased during stress. Synthesis of stress responsive proteins is related to leaf water potentials. These proteins are in LMW (14.4 and 17 kDa), MMW (30, 66 and 76 kDa) and HMW (116 kDa) range specific to type of stress.

Table 6. Quantitative and qualitative changes of proteins in coconut seedlings subjected to different stresses.

Type of stress on seedlings/ tissue	Quantitative and qualitative changes in proteins		
	Protein concentration	HSPFs	Extra proteins during stress (kDa)
Water stress (leaflet)	Decrease	Increase	116, 76, 30, 14.4
Water stress + High light intensity (leaflet)	Decrease	Increase	116, 76, 66, 20.1, 14.4
Temperature induction			
Leaflet	Increase	Increase	17
Root	Decrease	Increase	30
High temperature			
Leaflet	Decrease	Decrease	-
Root	Decrease	Decrease	-
Flooding			
Leaflet	Increase	Increase	17
Root	Decrease	Decrease	17

ACKNOWLEDGEMENTS

The authors are thankful to Dr K.U.K. Nampoothiri and Dr V. Rajagopal, former Directors of CPCRI, Kasaragod for their encouragement and support for the study. The help rendered by Mr. Keeran (JTO), Late Mrs. Mohinibai, Mr. Pramod and Vinu K. Cherian (RA) are gratefully acknowledged.

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(Received: November, 2006; Revised: June, 2007;
Accepted: September, 2007)