Research Article

Protagonist Action of Plant Growth Regulators and Sublethal-Temperature in Inducing Thermotolerance in Green Gram (*Vigna radiata*) Seedlings

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Abstract | The specific proteins induced by sublethal-temperature are molecular chaperons that positively regulate plant growth and development that govern acclimation in plants but little has been known under lethal-temperature stress. Thus, the impact of induction of thermotolerance by sublethal-temperature (40 °C), 100 µM indoleacetic acid (IAA), and 100 µM gibberellic acid (GA_3) before lethal-temperature stress (50 °C) were assessed on green gram growth and protein profile. For this purpose, the distilled-water and phytohormones imbibed seeds of NM13-1 were pretreated with 40 °C (1 hour) before 50 °C (2 hours), after 24 hours seedling length, proteome profile by 1-D, and catalase (CAT), ascorbate (APX), and guaiacol peroxidase (GPX) activities were assessed. Study outcomes revealed that the combination of phytohormones and 40 °C before 50 °C exhibited improved growth and increased activity of APX, CAT, and GPX as to control and lethal-temperature treatments. The total soluble protein profile showed highly significant variations and was broadly divided into two major clusters. The 116, 113, 109, 106, 94, 86, 76, 62, and 61 kDa fractions were identified the first time, which may be involved in temperature stress acclimation. Conclusively, the sublethal-temperature along with phytohormones are involved in green gram growth, proteome expression, and thermotolerance via initiating antioxidant defense signaling.

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1. Introduction

Green gram is one of the most popular legumes in Asian countries including Pakistan, whose growth is negatively affected by lethal-temperature stress. Plants have a different optimum temperature for their best growth performance similarly green gram performs very well under 30 °C (Farheen *et al*., 2018). Furthermore, a temperature higher than optimum could lead to limited crop production and lethality. The lethal-temperature stress causes alterations in the expression of genes involved in direct protection from heat stress (Siddiqui *et al*., 2017; Khan *et al*., 2020). Furthermore, different plant species synthesize stress proteins to protect plants from the adverse effect of lethal-temperature stress (Siddiqui *et al*., 2017). Lethal-temperature may change membrane fluidity and osmotic effect (D'souza and Devaraj, 2013). Irreversible changes under lethal-temperature stress in cellular homeostasis may occur due to inadequate response during signaling and gene activation processes, responsible for the destruction

of structural activity ultimately leading to cell death (Siddiqui *et al.*, 2017; Wang *et al.*, 2018; Khan *et al.*, 2020). Another reason for cell death is the excessive production of reactive oxygen species (ROS) which is the outcome of lethal-temperature stress, interacts with many cellular components (Khan *et al.*, 2020).

The exposure of plants with moderately high-temperature before lethal-temperature causes thermotolerance (D'souza and Devaraj, 2013). Plants showing delayed heat-acclimation responses may be more sensitive to thermo-damage (D'souza and Devaraj, 2013). Other than exposure to moderately high-temperature, application of phytohormones is another effective way of inducing thermotolerance in plants, as these have growth-promoting and effective antioxidant capacity (D'souza and Devaraj, 2013). There is a number of antioxidant enzymes like catalase, guaiacol peroxidase, and ascorbate peroxidase which scavenge the increased levels of ROS (Khan *et al.*, 2020). Exogenous application of phytohormones like indoleacetic acid and gibberellic acid is an effective method in mitigating heat stress-induced damage in plants and has growth-promoting and antioxidant capacity (Siddiqui *et al.*, 2017; Farheen and Mansoor, 2020). The main objective of this study was to evaluate the damaging effect of heat stress on the growth, antioxidant enzymes, and protein profile of green gram. Also, the experiment was performed to find out the pretreatment effect of 40 ˚C and phytohormones on the acquisition of thermotolerance.

2. Materials and Methods

2.1. Plant material and experimental treatments

The seeds of green gram genotype NM 13-1 were obtained from the National Agricultural Research Center (NARC), Islamabad, Pakistan, and imbibed separately in distilled water (DW), 100 µM IAA and 100 µM GA_3 for 20 hours (h). Afterward, green gram 20 seeds treatment^{-1} replication^{-1} were allowed to germinate for 24 h at 30˚C (optimum temperature) in Petri dishes lined with two layers of filter paper moistened with DW. The two days old seedlings were divided into three groups. The first group labeled as control was kept at 30 ˚C for 3 h while the second group received sub-lethal temperature treatment as 40˚C for 1 h then subjected to 50˚C heat-shock for 2 h. The last group placed for 1 h under optimum temperature (30 ˚C) of green gram then exposed to lethal heat stress treatment (50 ˚C) for 2h. Subsequently, seedlings were allowed to grow at 30˚C for a further 24 h then harvested and saved for morphological and biochemical analysis.

2.2. Growth, biomolecules, and protein profiling

The total length of green gram seedling was recorded (Farheen *et al.*, 2018). The extraction of antioxidant enzymes was performed by the method of Mansoor and Naqvi (2013). Protein was estimated by the method of Lowry *et al.* (1951). GPX, APX, and CAT activity were measured by monitoring the decrease in absorbance due to hydrogen peroxide (H_2O_2) by the method of Mansoor and Naqvi (2013). SDS-PAGE was performed as previously described the method of Farheen and Mansoor (2020). Cluster analysis and genetic similarity indices were calculated by statistical software SPSS version 20 and dendrogram was constructed using presence or absence of protein bands in the treatments.

2.3. Statistical analysis

The experiment was conducted at the lab of the Department of Genetics, the University of Karachi in complete randomized design (CRD) with three replications (Steel and Torrie, 1997) and ANOVA was performed by statistical software SPSS version 20. Means were compared by using Duncan's multiple range test (DMRT) at $P \leq 0.05$ level of significance.

3. Results and Discussion

3.1 Growth and biomolecules

Plant growth is the most vital factor which irreversible increase size through cell division to form different parts of the plant (D'souza and Devaraj, 2013). It is essential to understand the complex mechanism of plant growth under lethal-temperature stress for the selection of competent genotypes of species. In this context, the protagonist action of various treatments such as sublethal-temperature along with two plant growth regulators was studied on green gram seedlings growth and biomolecules under lethal-temperature stress (Table 1). It was observed that the seedling length was significantly reduced to 61% at lethal-temperature stress (50 ˚C) that was improved 30% by the imbibition of 100 µM IAA before pretreatment of 40 ˚C for 1 h to 50 ˚C. Also, the pretreatment of IAA+50 ˚C and GA_3 +50 ˚C helped green gram seedlings to recover from lethal-temperature stress (Figure 1). Similar findings were reported earlier from lablab beans, where exposure

to extreme temperature produced ROS in excessive amount which ultimately harmed seedling length and fresh weight of seedlings (D'souza and Devaraj, 2013). Furthermore, an enhancement in the length of green gram seedling may have been due to the production of stress acclimation proteomes and the active synthesis of antioxidant enzymes. Moreover, an accumulation of heat-shock proteins and enzymes either in active or passive form is an imperative defense mechanism for thermotolerance in various plant species to defend cell organelles from severe injury due to dehydration (Wang *et al.*, 2018; Khan *et al.*, 2020).

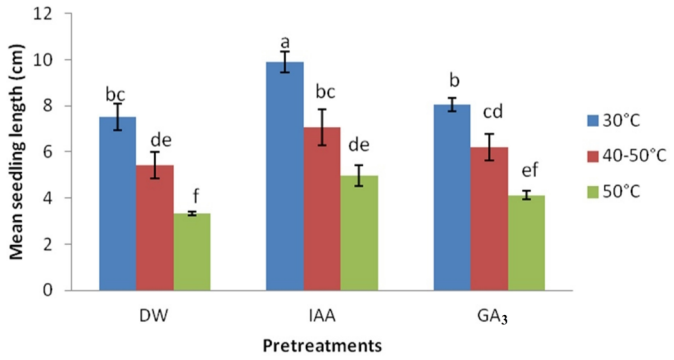

Figure 1: Effect of distilled water as control (DW for 20 h), gibberellic acid (GA3 for 20 h) and indoleacetic acid (IAA 20 h) imbibition, and pre-stress treatment of sub-lethal temperature (40°C for 1 h) on seedlings length of green gram under lethal-temperature stress. Vertical bars indicate standard error (n=3), and significant differences (P≤ 0.05) are marked with different letters.

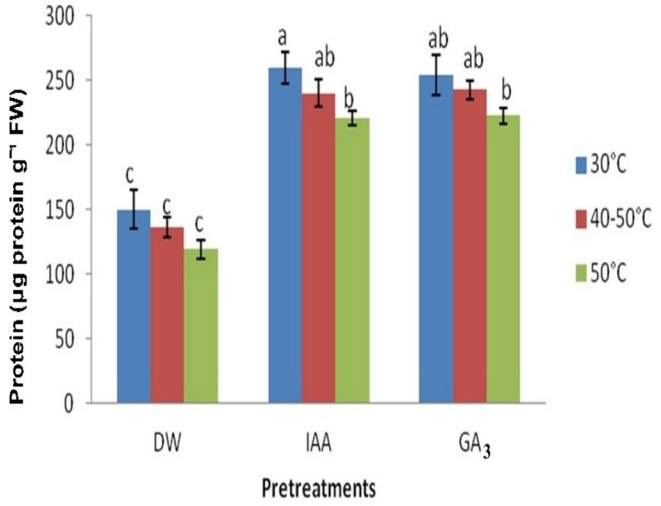

Figure 2: Effect of DW, GA3, IAA and 40°C on the total soluble protein of green gram under lethal-temperature stress. Vertical bars indicate standard error (n=3), and significant differences (P≤ 0.05) are marked with different letters.

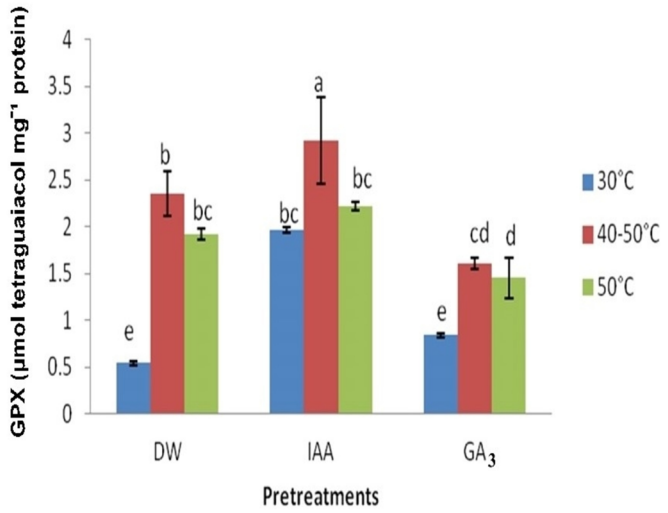

Figure 3: Effect of DW, GA3, IAA, and 40°C on ascorbate peroxidase (APX) activity in green gram under lethal-temperature stress. Vertical bars indicate standard error (n=3), and significant differences (P≤ 0.05) are marked with different letters.

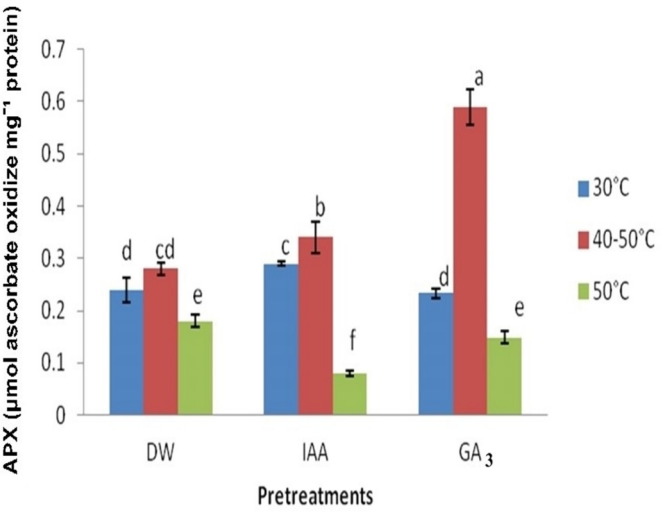

Figure 4: Effect of DW, GA3, IAA and 40°C on catalase (CAT) activity in green gram under lethal-temperature stress. Vertical bars indicate standard error (n=3), and significant differences (P≤ 0.05) are marked with different letters.

Under lethal-temperature stress, proteins, and antioxidants defense enzymes biosynthesis are alleviated in response to ROS (D'souza and Devaraj, 2013). The ROS mainly hydrogen peroxide and superoxide ion caused oxidative destruction in the plant cells as a result cell death occur. To rescue plant, cells scavenge superoxide ions through the production of GPX and SOD metalloprotein, and hydrogen peroxide degradation is accomplished via CAT and APX enzymes (Farheen *et al.*, 2018). To

recognize whether thermotolerance in green gram is related to the generation of proteins and antioxidant enzymes, 24 h phytohormones imbibed seedlings were analyzed for biomolecules level at lethal-temperature stress. In seedlings, both phytohormones together with 40 ˚C help to enhance 65% total soluble protein during lethal-temperature treatment in comparison to control (30 ˚C) (Figure 2). Treatment of green gram seedling that is, IAA+40 ˚C+50 ˚C resulted in outmost i.e. 500% GPX activity indicating that treatment-induced antioxidant enzyme synthesis most efficiently than other treatments (Figure 3). While APX and CAT were found to be 200% and 100% higher in GA3+40 ˚C+50 ˚C treatment, respectively (Figures 4-5). Therefore, the overproduction of antioxidants may responsible for further oxidative protection against H_2O_2. These observations are in consonance with GPX activity detected in high-temperature stress-tolerant lablab bean (D'souza and Devaraj, 2013). Likewise, numerous scientists have reported that elevated CAT and APX activities are related to the capability of species to recover from lethal-temperature stress when tolerant by using phytohormone and sublethal-temperature treatment (Siddiqui *et al* 2017; Khan *et al*., 2020). Also, APX and CAT are the most effective enzymes to prevent cellular damage by the detoxification of hydrogen peroxide into water molecules (Farheen *et al*., 2018). Additionally, the decrease in CAT and APX activity under lethal-temperature was due to the denaturation of proteins and antioxidant enzymes (Awasthi *et al*., 2015; Wang *et al*., 2018). Thus, an induction in antioxidant enzyme activity can be considered as an important mechanism to scavenge ROS and protect the cell from stress.

3.2 Protein profile

The response of lethal-temperature stress to green gram seedlings is also explained by the altered synthesis of proteins. It was observed that the different plant species show thermotolerance by synthesizing a group of proteins called stress proteins (Wang *et al*., 2018). In this context, the protein profile of green gram seedlings after different treatments was analyzed which revealed significant variations among control and treated samples (Table 2). The dendrogram was constructed among treatments based on the presence and absence of protein bands (Figure 6). The total of nine temperature treatments was broadly divided into two major clusters according to the percent homology. The largest cluster-I was composed of I-A and I-B. The sub-cluster I-A was minor, contained GA3 40-50 ˚C and GA3 50 ˚C which showed 99% similarity. While, sub-cluster I-B was the major one, consisted of four treatments in which, IAA 30 ˚C and GA3 30 ˚C were 93% similar. Further, the IAA 40-50 ˚C, and IAA 50 ˚C exhibited 92% and 88% similarity, respectively with IAA 30 ˚C and GA3 30 ˚C treatments. The cluster I-A was 84% similar to the cluster I-B. The cluster-II was also divided into II-A and II-B. Cluster II-A has two treatments namely 30 ˚C and 40-50 ˚C that were 93% similar to each other. Whereas, the sub-cluster II-B (50 ˚C treatment) was 89% like sub-cluster II-A.

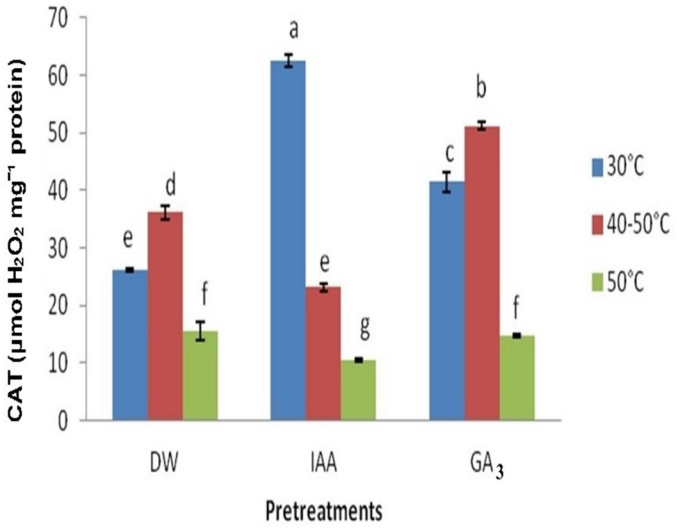

Figure 5: Effect of DW, GA3, IAA, and 40°C on guaiacol peroxidase (GPX) activity in green gram under lethal-temperature stress. Vertical bars indicate standard error (n=3), and significant differences (P≤ 0.05) are marked with different letters.

Table 1: Mean squares of seedling length, total soluble protein, GPX, APX, CAT between different treatments on NM13-1 genotype of green gram.

Sources of variations	Df	MS				
		Length	Protein	GPX	APX	CAT
B/w treatment	8	12.81*	8892.7**	1.953*	0.006*	953.19**
Within treatment	18	0.717	316.815	0.111	0.001	3.379
CV%		33.4	26.5	43.4	55.6	54.9

* as significant difference at P≤0.05 and ** as significant difference at P≤ 0.01 level of significance.

Table 2: Presence (+) and absence (-) of protein molecular weight (MW) bands based on relative mobility (RF) in green gram seedlings treated with two phytohormones and sublethal-temperature to acquire thermotolerance.

Bands	RF	MW (kDa)	30	40-50	50	IAA30	IAA 40-50	IAA50	GA330	GA3 40-50	GA350
1	0.17	116	–	–	–	+	+	+	+	+	+
2	0.19	113	–	+	+	–	–	–	–	+	+
3	0.20	109	–	–	–	+	+	+	+	–	–
4	0.22	106	+	–	+	–	–	–	+	–	+
5	0.23	103	–	+	+	+	+	+	–	+	+
6	0.25	100	–	–	–	+	–	+	+	–	+
7	0.28	94	–	–	–	–	–	–	–	+	–
8	0.29	91	+	+	–	+	+	–	+	–	+
9	0.30	86	–	–	+	–	–	+	–	+	+
10	0.32	80	–	–	–	+	+	–	+	+	–
11	0.33	76	+	+	+	–	–	–	–	–	–
12	0.35	71	–	–	–	+	+	–	+	+	+
13	0.36	67	+	+	+	–	–	+	–	–	–
14	0.38	62	+	–	–	–	+	+	+	–	–
15	0.39	61	–	–	+	+	–	–	–	+	–
16	0.41	60	+	+	–	–	–	–	–	–	–
17	0.42	59	–	–	–	–	–	–	–	+	–
18	0.43	58	–	–	–	–	–	–	–	+	–
19	0.45	57	+	+	+	+	–	–	–	–	–
20	0.46	56	–	–	–	–	+	+	+	–	–
22	0.49	55	+	+	+	+	–	–	–	–	–
23	0.51	54	–	–	–	+	+	+	+	–	–
24	0.52	53	+	+	+	–	–	–	–	–	–
25	0.54	52	–	–	–	–	+	–	–	+	–
27	0.57	51	+	+	+	+	–	+	+	–	–
28	0.59	49	–	–	–	–	–	+	+	+	+
29	0.61	48	–	+	–	–	–	–	–	–	–
30	0.64	47	–	–	+	–	+	–	–	–	–
31	0.65	46	+	–	–	+	–	+	+	+	+
32	0.67	43	–	+	+	–	–	–	–	+	+
33	0.68	41	–	–	–	–	+	–	–	–	–
34	0.70	39	–	–	–	–	–	+	–	–	–
35	0.71	37	–	+	+	+	–	–	+	–	–
36	0.72	35	–	+	–	–	+	+	+	+	+
37	0.74	33	+	–	+	+	–	–	–	–	–
38	0.75	31	–	+	–	–	–	–	–	–	–
39	0.77	29	–	–	–	–	–	–	+	–	–
40	0.78	28	–	–	–	–	–	+	–	–	–
41	0.80	27	+	–	–	+	+	–	+	+	+
42	0.81	24	–	–	+	–	–	–	–	–	–
43	0.83	22	+	+	–	+	+	+	–	–	–
44	0.84	20	–	–	–	–	–	–	+	–	–
45	0.86	18	–	–	–	–	–	–	–	+	+
46	0.87	16	–	–	+	+	+	+	+	+	+
47	0.88	15	–	+	–	–	–	–	–	–	–
	Total		14	17	17	18	16	17	19	18	15

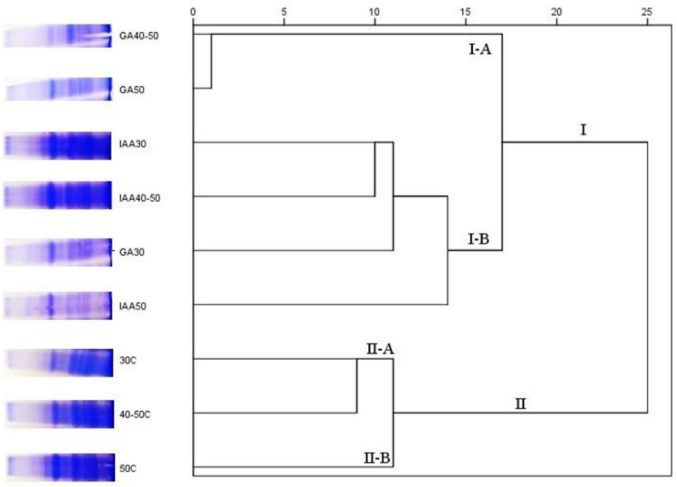

Figure 6: Dendrogram demonstrates the relationship between nine temperature and two phytohormone treatments on green gram seedlings, tested on the base of the protein profile.

The protein profiling revealed 116, 113, 109, 106, 94, 86, 76, 62 and 61 kDa fractions were unknown proteins that have first time identified in the current study. Other than these, 103, 100, 91, 80, 71, 67, 60, 59, 58, 57, 56, 55, 54, 53, 52, 51, 49, 48, 47, 46, 43, 41, 39, 37, 35, 33, 31, 29, 28, 27, 24, 22, 20, 18, 16, and 15 kDa were also found during the present investigation. Where 100 kDa proteome was coactivator-like protein (Sato *et al.*, 1998), 91 kDa act as sucrose synthase SS1 protein (Hameed *et al.*, 2012), 80 kDa was an outer envelope protein in *Arabidopsis thaliana* (Tabata *et al.*, 2000), 58 kDa was trans-cinnamate 4-monooxygenase (Mizutani *et al.*, 1993), 51 kDa, 35 kDa and 24 kDa were seed maturation proteins of *Glycine max* genome (Hameed *et al.*, 2012; Farheen and Mansoor, 2020), and 49 kDa was putative NADH-ubiquinone oxidoreductase subunit (Hameed *et al.*, 2012; Gostincar *et al.*, 2019; Farheen and Mansoor, 2020). While 47 kDa was chloroplastic photosystem II protein (Dempewolf *et al.*, 2010), 46 kDa was PP2A phosphatase-associated protein (Farheen and Mansoor, 2020), 39 kDa was NADH-ubiquinone oxidoreductase (Brumm *et al.*, 2015), 33 kDa was heat-shock protein 33 (Hamidian *et al.*, 2015; Farheen and Mansoor, 2020), 31 kDa was U11/U12 small nuclear ribonucleoprotein (Salanoubat *et al.*, 2000), 27 kDa was alpha-coixin or soybean toxin protein (Vasconcelos *et al.*, 2008) and 18 kDa was peptidyl-prolyl cis-trans isomerase (Kaga and Ishimoto, 1998).

Miclaus *et al.* (2011) identified that the 22 kDa protein related to the prolamin alpha zein z1C1_8 precursor and Guo *et al.* (2013) found that the 15 kDa has belonged to the prolamin beta zein precursor. Further, the 71, 67, 56, and 53 kDa peptide is vicilin 7S subunit and 60 kDa was 8S vicilin subunit that was reported earlier in the mung bean genotypes (Hameed *et al.*, 2012). While 52 kDa was 8S globulin alpha subunit (Ding *et al.*, 2008) and 16 kDa was 7S globulins (Mendoza *et al.*, 2001). Also, the serine/threonine-protein phosphatase associated 2A regulatory subunits B' were found in three isoforms like 59 kDa gamma isoform, 57 kDa kappa isoform, and 55 kDa beta isoform. Some other proteome including chloroplastic proteins such as 48 kDa dolichyl-diphosphooligosaccharide glycosyltransferase subunit, 41 kDa stem-loop binding protein, 37 kDa FKBP12-interacting proteins, 29 kDa thylakoid luminal protein, and signal recognition particles having 54 and 43 kDa molecular weight were reported earlier in *M. pruriens* (Nnadi *et al.*, 2018). Hameed *et al.* (2012) and Nnadi *et al.* (2018) were also found heat- and acid-stable phosphoprotein and chaperonin associated with 28 and 20 kDa molecular weights bands, respectively. Moreover, the variations in band number and intensity were also found in green gram genotypes under salt stress (Farheen and Mansoor, 2020). Hence, protein profiling of green gram revealed that the phytohormones such as IAA and GA3 could regulate the expression of heat shock proteins that are anticipated to play a vital part in the mechanism of heat tolerance.

Conclusions and Recommendations

The results presented in this study revealed that the imbibition of green gram seeds in plant growth regulator and treatment of sub-lethal temperature (40 °C) before lethal-temperature stress (50 °C) were easy and effective methods to induce thermotolerance at seedlings stage. This conclusion was drawn on the basis of improved growth, enhance the activity of antioxidant enzymes and significant variations in protein profile. Thus, it can be stated that lethal-temperature stress adversely affected the green gram physiology and biochemistry that was improved by the pretreatment of sublethal-temperature and phytohormones.

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Novelty Statement

Protein profiling via SDS-PAGE is the pivotal method in terms of discovering novel proteomes under various phytohormones together with sublethal-temperature treatment before lethal temperature. In this context, the study has found 116, 113, 109, 106, 94, 86, 76, 62, and 61 kDa proteome fractions for the first time in mungbean.

Author's Contribution

Simeen Mansoor has conceptualized the study, conduct the experiment, analysis the outcomes and initially draft the findings. Jabeen Farheen drafted whole research findings and done protein profiling by using Excel-2016 and SPSS v.20 software. Meher Hassan critically reviewed the study.

Conflict of interest

The authors have declared no conflict of interest.

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