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HARDENING PRETREATMENT BY DROUGHT AND LOW TEMPERATURE ENHANCED CHILLING STRESS TOLERANCE OF CUCUMBER SEEDLINGS

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ABSTRACT

Chilling stress is of major limiting factors influencing the growth and development of warm-season crops like cucumber. In this research, the possibility of chilling tolerance of cucumber seedlings was investigated through employing the drought and low-temperature pretreatments. The factorial experiment consisted of two factors including cucumber cultivars (i.e. 'Super Dominos' and 'Super Star') and hardening treatments (control, low temperatures at 10°C, and 15°C and drought simulated by 10% and 20% PEG) based on completely randomized design (CRD) in 3 replications. After applying treatments and providing them 48 h opportunity to be recovered, the seedlings were subjected to 3°C for a six-day period and 6 h for each day. All hardening treatments improved seedlings' growth, chlorophyll content, total phenol (TP) and antioxidant enzyme activities, while reducing chilling injury index and malondialdehyde (MDA) content. Comparing to temperature hardening, the drought pretreatment showed to have a better effect on inducing the chilling tolerance into cultivars. Overall, the results of this experiment showed that employing drought and low-temperature pretreatments enabled cucumber seedlings to mitigate the harmful effects of chilling.

Key words: antioxidant, catalase, chilling injury, chlorophyll, hardening

INTRODUCTION

Plants, due to their static nature, are continuously exposed to various environmental stresses including drought, salinity and extreme light and temperature. During germination and early seedling growth, chilling stress is one of major limiting factors affecting plant growth and productivity [Saltveitt 2001, Baninasab 2009] and inducing considerable changes in development and geographical distribution of many plant species around the world [Allen and Ort 2001]. Similar to other warm-season crops, cucumber is sensitive to chilling and easily injured by low temperatures (<10°C) [Helmy et al. 1997, Dong et al. 2013]. Although some parts of Iran, in terms of environmental conditions, are suitable for planting cucumber, chilling stress, due to occurring at early stage of growth season, acts as a limiting factor in growing plant in these regions. In this regard, the seedlings planted in the field may be exposed to chilling for few hours before stabilizing temperature of environment around them [Baninasab 2009]; and this causes to decrease plants' growth and yield as well as delays their harvesting, and even in case of severe chilling, it leads to kill the plants [Korkmaz and Dufault 2001]. Applying different techniques such as breeding, genetic engineering, chemical usage and changing in cultivation practices, can mitigate damage caused by chilling [Hossain et al. 2015]. In this regard, an increasing number of studies

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showed that exposing plants to one stress could stimulate tolerance to others [Pardossi et al. 1978, Ao et al. 2013, Dong et al. 2013, Hura et al. 2015]; this phenomenon is so-called cross-resistance or cross-adaptation. For example, drought pretreatment induced cold tolerance [Pardossi et al. 1987, Dong et al. 2013], low-temperature pretreatment induced chilling resistance [Helmy et al. 1997, Li et al. 2011, Ao et al. 2013], heat shock increased resistance to heat and chilling [Gong et al. 2001], and cold acclimation increased resistance to heat [Mei and Song 2010] and heavy metals [Streb et al. 2008]. So far, various mechanisms justifying the role of cross-adaptation phenomenon on inducing plants' tolerance or resistance to stresses have been proposed [Hossain et al. 2015]. Such a simple and inexpensive technique is so reliable and safe that it can be taken into account as a suitable replacement for current-using techniques. In some cases, some physiological and biochemical mechanisms involving the cross-resistance have not been well known yet. Thus, the goal of this research was to investigate the physiological and biochemical effects of drought and low-temperature pretreatments on improving cucumber's tolerance to chilling stress at early stage of growth.

MATERIALS AND METHODS

Planting conditions and treatments. This experiment was carried out in a form of factorial based on CRD with three replicates in 2017. At first, the seeds of two well-known Iranian cucumber cultivars i.e. 'Super Dominos' and 'Super Star' were planted in plastic pots filled with perlite and vermiculite (2 : 1) and kept in greenhouse exploiting sun light directly. By a pad-and-fan cooling system, the temperature of greenhouse was maintained at 25/18°C (day/night) during the experiment. In order to grow seedlings normally, they were fertigated with Phosamco Bio fertilizer. After germinating, the thinning practice was accomplished and the extreme seedlings were eliminated and finally just one seedling was remained per each pot. At two-leaf stage, seedlings have been subjected to the pretreatments (drought and low-temperature) for two days. The pretreatments consisted of drought pretreatment simulated by adding 10% and 20% PEG-6000 into nutrient solution and temperature pretreatment resulted by a growth chamber adjusted at 10°C and 15°C and 100 µmol m^{-2} s^{-1} light intensity, 70–80% RH, and 12-h photoperiod.

After receiving the treatments, seedlings have been kept in the greenhouse for 48 h (without applying any treatment). Then, seedlings were transferred into growth chamber in order to experience chilling condition (3°C) for 6 days and 6 h each day. After completing the chilling period, all seedlings were transferred into the greenhouse and 72 h later, the traits were measured [Baninasab 2009].

Measurements. To determine shoot dry weight (SDW) and root dry weight (RDW), plants were gently removed from culture medium and roots were washed with distilled water. Then, they were placed on paper bags and transferred into an electric oven and dried at 75°C for 72 h, and finally dry weight of samples was recoded.

In order to measure the rate of chlorophyll, 0.1 g of fresh leaf tissue was grinded with 5 mL acetone 80% until producing homogenized product. After centrifuging the solution at 5000 rpm for 10 min, the supernatant was taken and its absorbance was read at 663 nm and 645 nm by spectrophotometer. According to the following formula, the rate of chlorophyll was determined and reported as mg g^{-1} FW [Arnon 1949].
Total chlorophyll:

$$[20.21\ (A_{645}) + 8.02(A_{663})] \times V/1000 \times W$$

A – absorbance of specific wavelength,
V – volume of chlorophyll extract,
W – fresh weight of tissue.

For TP measurement, the lyophilized material was homogenized in 80% ethanol. The TP content was determined using the Folin-Ciocalteau method of Singleton and Rossi [1965]. The absorbance was measured at 760 nm and gallic acid was used as a standard.

The MDA content was measured by Stewart and Bewley [1980] method with some modification. 0.3 gram of fresh leaf was homogenized by addition of 5 ml phosphate buffer (50 mM) in a bath. Then,

the homogenate was transferred into a tube and centrifuged at 12,000 rpm for 30 min at 4°C. 1 ml of supernatant and 1 ml of 0.5% thiobarbituric acid (Merck, Darmstadt, Germany) in 20% trichloroacetic acid (Merck, Darmstadt, Germany) solution were added into a new tube. This mixture was incubated at 98°C for 30 min, then cooled and centrifuged at 10,000 rpm for 10 min. The supernatant was subjected to analysis with the spectrophotometer. The MDA content was calculated from the subtracted absorbance (A_{532}–A_{600}) using the extinction coefficient of 155 m m^{-1} cm^{-1}.

Fresh leaf samples (0.2 gram) were homogenized with 2 ml of 50 mM phosphate buffer (pH 7.5) containing 1 mM EDTA (Merck, Darmstadt, Germany), 1 mM phenylmethylsulfonyl fluoride (PMSF) (Sigma-aldrich, USA) and 2% poly vinyl pyrrolidone (PVP) (Sigma-aldrich, USA) in ice water-bath and then centrifuged at 12000 rpm for 20 min at 4°C. The supernatant was collected and used for enzyme assay with a UV-Visible spectrophotometer (Cary 100, Varian, USA). Catalase (CAT, EC 1.11.1.6) activity was measured by estimating the breakdown of H_2O_2, which was determined at 240 nm as described by Dhindsa et al. [1981]. The activity of guaiacol peroxidase (GPX) (EC 1.11.1.7) was determined at 470 nm due to guaiacol oxidation and the reaction solution contained of 100 µl guaiacol, 100 µl H_2O_2, 50 µl of enzyme extract and 2.5 ml phosphate buffer [Plewa et al. 1991].

Wilting and necrosis of shoots were considered as the indictors of chilling injury index and classified by using the following scale: normal, no visible symptoms; trace, small necrotic areas on shoots but without growth restrictions (less than 5% of leaf area necrotic); slight, small necrotic areas on shoots (less than 15% of leaf area necrotic); moderate, well-defined necrotic areas on shoots (less than 30% of leaf area necrotic); and severe, extensive necrotic areas and severe growth restrictions (more than 50% of leaf area necrotic but plant still alive). By assigning values of 1, 2, 3, 4 and 5 respectively to each group, the average injury for each treatment was calculated [Baninasab 2009].

Statistical analysis. Data from the analytical determinations were subjected to analysis of variance (ANOVA). Sources of variation were cultivars and hardening treatments. Means comparisons were assessed by Duncan's test with differences being considered significant at $p < 0.05$. All analyses were performed with SAS software (Version 9.1).

RESULTS AND DISCUSSIONS

Growth parameters. The results of variance analysis showed that the main effect of cultivar on RDW was significant ($p < 0.01$). The main effect of treatment on height, RDW and SDW was significant ($p < 0.01$), but on the stem diameter, it was significant at $p < 0.05$. The interaction of cultivar × treatment on growth parameters was not significant (Tab. 1). Comparison of means of main effects showed that 'Super Star' in comparison to 'Super Dominos' produced higher RDW (Tab. 2). The results showed that different hardening treatments had different effects on cucumber's growth parameters. As compared to control, the treatments did not have significant effects on stem diameters, although 20% PEG reduced stem' diameters in comparison to 10% PEG and 15°C treatments. Comparing to control, 20% PEG and 10°C significantly reduced the height of seedlings. Also, the results showed that 10% PEG and 15°C increased the growth of seedlings compared to control (Tab. 2).

Berova et al. [2002] pointed out that chilling stress reduced growth of the plants subjected to chilling through reduction in chlorophyll content, photosynthesis enzyme activities, stomata conductance, CO_2 absorption, electron transporting in photosynthesis. The results of this research showed that drought and low-temperature hardening improved growth of seedlings after experiencing chilling stress. The result of this research is in agreement with that of Paradossi et al. [1988], who stated that drought pretreatment induced tomato's tolerance to chilling and consequently increased its growth and yield under chilling condition. Cayuela et al. [2007] reported that applying PEG increased tomato growth under salinity stress, which confirms our findings. Also, Nayyar et al. [2005] sho-wed that low-temperature pretreatment at 10/7°C (day/ night) for 6 days increased plant growth under chilling condition. Also, it has been reported that applying low-temperature

Table 1. Analysis of variance (ANOVA) for cultivar, hardening pretreatment and their interactions effects on evaluated dependent variables in cucumber seedlings

Dependent variable	Cultivar	Treatment	Cultivar × treatment
Plant height	ns	**	ns
Stem diameter	ns	*	ns
Shoot dry weight	ns	**	ns
Root dry weight	**	**	ns
Chlorophyll	**	**	*
Phenol	ns	**	ns
Malondialdehyde	ns	**	ns
CAT activity	*	**	**
GPX activity	**	**	**
Chilling index	ns	**	ns

** and * represent significance at the 0.01 and 0.05 levels, respectively, and ns represents non-significance at $p < 0.05$

Table 2. Comparison of the main effects cultivar and hardening treatments on studied traits

Treatments	Level of treatments	Plant height (cm)	Stem diameter (cm)	Shoot dry weight (gram)	Root dry weight (gram)	Phenol (mg 100 g^{-1} FW)	Malondi-aldehyde (nmol g^{-1} FW)	Chilling index (Score)
Cultivars	'Super Dominos'	11.97a	0.56a	0.59a	0.29b	7.04a	1.17a	2.01a
	'Super Star'	12.43a	0.57a	0.60a	0.32a	7.80a	1.20a	2.04a
Hardening	Control	13.21a	0.56abc	0.55b	0.29b	5.95d	1.45a	3.31a
	10% PEG	12.69a	0.60ab	0.65a	0.33a	7.37b	1.05c	1.61b
	20% PEG	11.18a	0.53c	0.59b	0.28b	7.72a	1.14b	1.55b
	10°C	11.33b	0.54bc	0.56b	0.29b	7.30b	1.16b	1.81b
	15°C	12.59a	0.61a	0.63a	0.33a	6.96c	1.13bc	1.85b

Values followed by different letters were significant difference according to Duncan's Multiple Range Test at $p < 0.05$

pretreatment induced cucumber tolerance to chilling and reinforced its survival and growth by alteration in antioxidant enzyme activity [Kang and Saltveit 2001]. The merits of hardening effects on escalating plant growth and ameliorating adverse effects of chilling can be served as suitable tools to minimize problems resulted by low temperature.

Chlorophyll. The results of this experiment showed that the main effects of cultivar, treatments, and their interaction effect had a significant effect on chlorophyll content (Tab. 1). Comparison of means of cultivar × treatment in both cultivars showed that the pretreatments of 10% and 20% PEG and 10°C significantly increased chlorophyll content compared to controls. In 'Super Dominos', PEG pretreatment, in comparison to temperature one, had higher effect on chlorophyll content (Fig. 1).

It has been reported that chilling stress caused a disturbance in chlorophyll production due to its dependency on temperature [Mahajan and Tuteja 2005]. By measuring chlorophyll content, it will be clear whether a plant is sensitive or tolerant to chilling conditions [Tewari and Tripathy 1998]. Under chilling condition, reduction in chlorophyll content may be due to chlorophyll disintegration as a result of reactive oxygen species (ROS) activities [Schütz and Fangmeier 2001]. The results of this research showed that drought and low-temperature

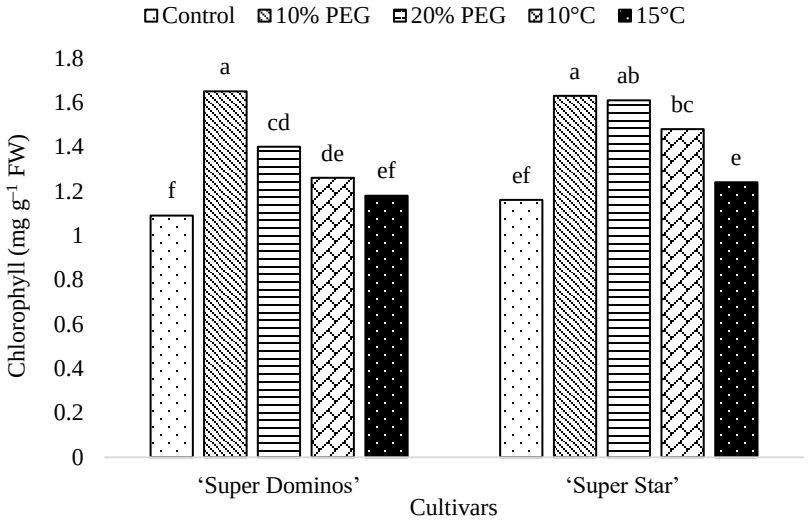

Fig. 1. The effect of hardening pretreatment on chlorophyll content in cucumber cultivars

pretreatment increased chlorophyll content in cucumber seedlings subjected to chilling stress. Li et al. [2014] reported that mild drought maintained wheat's chlorophyll integrity under low temperature. Also, Helmy et al. [1999] reported that the hardening of cucumber seedlings by low-temperature pretreatment increased chlorophyll content under chilling stress, which is in agreement with our findings. Therefore, the impact of hardening treatments on chlorophyll may be contributed to its influence on some physiological changes such as antioxidant enzyme activity.

Total phenol. Analysis of variance showed that the main effect of treatments on total phenol (TP) was significant ($p < 0.01$) (Tab. 1). All hardening treatments increased the TP as compared to control, although a significant difference between treatments was observed and highest TP was observed by 20% PEG (Tab. 2).

TP content in stressed plants generally goes up and this has been reported by a great number of studies and amount of TP in such plants will be considered as cell defensive mechanisms against unfavorable condition [Dat et al. 2000]. Antioxidant properties of phenolic compounds are due to their reductive activities and unique chemical structures effective on neutralizing ROS, creating a metal-ion complex, and silencing molecules of singlet and triplet oxygen. Phenolic compounds, as donors of electrons to free radicals, neutralize the reactions of fatty oxidation [Rice-Evans et al. 1997]. In a similar way, Hura et al. [2016] showed that the rate of phenolic compounds remarkably increased in rapeseed hardened to tolerate chilling stress and these compounds efficiently improved plants' tolerance to chilling. As mentioned above, an increase in the rate of cucumber seedlings' phenol compounds under drought stress can be considered as one of effective mechanisms involving in improving plants tolerance to chilling.

Malondialdehyde. The results of variance analysis showed that the main effect of treatment on the malondialdehyde (MDA) content of seedlings was significant ($p < 0.01$). The main effects of cultivar and interaction of cultivar × treatments on MDA content were not significant (Tab. 1). Comparison of means of main effects showed that they totally had a significant effect on reducing MDA content as compared to control (Tab. 2). By investigating effect of low-temperature pretreatment on inducing barley's tolerance to high temperature stress, Mei and Song [2010] reported that low-temperature hardening prevented an increase in MDA accumulation under high temperature. Also, Dong et al. [2013] showed that drought pretreatment simulated by PEG significantly reduced MDA accumulation in cucumber seedlings

subjected to chilling condition and this is in agreement with ours. It was previously shown that a reduction in MDA content resulted in high stability of unsaturated fatty acids located in cellular membrane and inducing more tolerance to stressful conditions [Maali-Amiri et al. 2007]. It seems that hardening plants with drought and low-temperature pretreatments, through activating defensive mechanisms, reduces the magnitude of damage to cellular membrane.

Antioxidant enzyme activities. Analysis of variance showed that the interaction of cultivar × treatment had a significant effect on catalase (CAT) and GPX activities (Tab. 1). In both cultivars, all hardening treatments significantly increased CAT activities and it was higher in 'Super Star' relative to 'Super Dominos'. The highest CAT activities on both cultivars were observed by applying 15°C pretreatment (Fig. 2). Different hardening treatments had different effects on GPX activities in cucumber seedlings. In 'Super Dominos', GPX activity was increased by 10% and 20% PEG as well as 15°C pretreatments, but 10°C did not have a significant effect on GPX activity (Fig. 3).

Exposing plants to low temperature leads to generate more ROS resulted in oxidative stress [Song et al. 2014]. Antioxidant enzymes such as CAT and GPX disturb hydrogen peroxide (H_2O_2) activity. H_2O_2 is one of major members of ROS inflicting damage to plants during oxidative stress [Szollosi 2014]. Thus, an increase in antioxidant activities is of underlying mechanisms in order to assist plants to cope with stressful conditions [Anjum 2015]. In this research, drought and low-temperature pretreatments significantly increased CAT and GPX activities in both chilling-stressed cultivars. Our findings are in agreement with those by Dong et al. [2013], in which drought pretreatment improved cucumber seedlings' tolerance to chilling through changing in activities of antioxidant enzymes. In order to investigate the importance of glutathione antioxidant in favor of stimulating bean's tolerance to chilling, Yu et al. [2002] maintained bean seedlings for 36 h at 8°C; he found approximately a six-fold increase in glutathione content under this condition. Also, Ao et al. [2013] showed that low-temperature hardening increased the activities of CAT, GPX, superoxide dismutase (SOD), ascorbate peroxidase (APX), and glutathione reeducates (GR) as well as the contents of glutathione and ascorbate; and thereby inducing plants' tolerance to chilling. With respect to obtained results, hardening may increase activities of antioxidant enzymes by which defensive mechanisms in plants will be activated against stressful conditions.

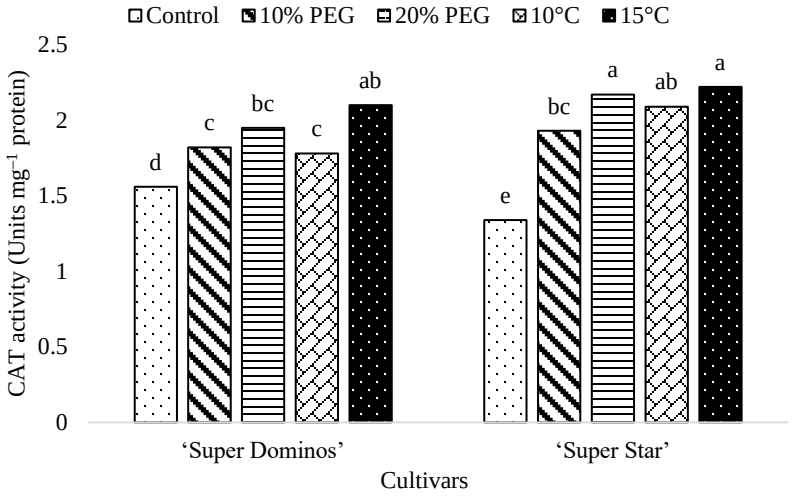

Fig. 2. The effect of hardening pretreatment on catalase (CAT) activity in cucumber cultivars

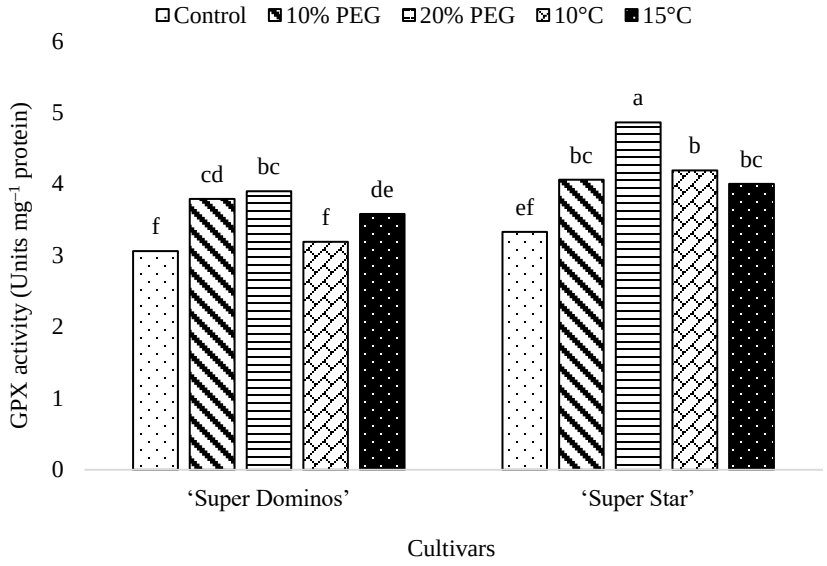

Fig. 3. The effect of hardening pretreatment on guaiacol peroxidase (GPX) activity in cucumber cultivars

Chilling injury index. Analysis of variance showed that the treatment had a significant effect on chilling index of cucumber seedlings ($p < 0.01$), while the main effect of cultivar as well as interaction of cultivar × treatment on chilling index were not significant (Tab. 1). The results showed that all hardening treatments reduced the amount of chilling injury as compared to control (Tab. 2). The visual symptoms of chilling injury in sensitive plants usually appear 48 to 72 h after having subjected to stress condition. Leaf chlorosis and necrosis, wilting, and growth reduction are of main plants' visual symptoms after experiencing chilling injury [Mahajan and Tuteja 2005]. In this research, to determine the indices of chilling in cucumber seedlings, some phenomena such as necrotic spots and chlorosis on leaves as well as the extent of damage inflicted to leaf and shoots of stressed plants were measured. The results showed that applying 10% and 20% PEG and low-temperature pretreatments at 10°C and 15°C reduced the symptoms of chilling in cucumber seedlings subjected to low temperature stress. Similarly, Dong et al. [2013] reported that drought pretreatment reduced chilling indices through an increase in antioxidant enzyme activities. Reduction in deleterious effect of chilling injury to maize by cold pretreatment [Li et al. 2011], to *Jatropha* by low-temperature hardening [Ao et al. 2013], and to cucumber by heat shock [Kang and Saltveit 2001] have been previously reported, which all are fully in agreement with our results. Applying drought and low-temperature hardening likely prevents occurring chilling injury through impacting on chlorophyll content, antioxidant enzyme activities and phenol accumulation.

CONCLUSION

In conclusion, results of this research revealed that drought and low-temperature pretreatments not only improved growth parameters, but also protected plants from chilling stress, as compared to controls. The efficacy of hardening is mainly contributed to its ability to stimulate antioxidant activities and enhance chlorophyll content and phenol metabolism. In general, employing drought pretreatment simulated by 20% PEG was more effective relative to other treatments. In a nutshell, regarding the detrimental effects of chilling stress on sensitive plant at early of growth season, applying drought pretreatment can efficiently mitigate the harmful effects of chilling stress.

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