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Full Length Research Paper

Physiological responses of two rice (*Oryza sativa* L.) genotypes to chilling stress at seedling stage

A. Aghaee[1], F. Moradi[2], H. Zare-Maivan[1]*, F. Zarinkamar[1], H. Pour Irandoost[2] and P. Sharifi[3]

[1]Department of Plant Biology, Faculty of Biological Sciences, Tarbiat Modares University, P. O. Box 14115-154, Tehran, Iran.
[2]Division of Molecular Physiology, Agricultural Biotechnology Research Institute of Iran (ABRII), P. O. Box 31535-1897, Karaj, Iran.
[3]Department of Agronomy and Plant Breeding, Faculty of Agriculture, Urmia University, P. O. Box 57153-165, Urmia, Iran.

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In this study, quantitative changes of dry matter, proline and total soluble protein in shoot and root, stomatal conductance (g_s), total chlorophyll, chlorophyll stability index (CSI) and soil and plant analyzer development (SPAD) number of latest fully-expanded leaves were determined in an Iranian cold-sensitive rice genotype (Hoveizeh) in comparison to an international check genotype (IRCTN34, cold-tolerant). The hydroponic experiment was arranged in a completely randomized design with three replications under the growth chamber condition under a controlled environment of 29/22°C (day/night) and 12 h light photoperiod. Then, the treatment plants were exposed to 15/10°C (day/night) cold stress for two weeks and control plants were kept at 29/22°C (day/night). Dry matter accumulation decreased with chilling stress in the two genotypes, with decreases been more pronounced in Hoveizeh genotype. Our results showed that cold treatment increased accumulation of total soluble protein (only in cold-tolerant genotype) and proline in rice seedlings, while it decreased the content of chlorophyll, stomatal conductance, total soluble protein (only in cold-sensitive genotype) and dry matter. The results indicated that higher contents of protein and chlorophyll under stress were associated with tolerance to chilling.

Key words: Abiotic stress, cold, total soluble protein, proline, total chlorophyll, stomatal conductance.

INTRODUCTION

The temperatures on the Earth's surface are very different, changing during the seasons as well as during the day and night. Low temperatures act as an abiotic stress factor that has a strong impact on the survival, growth, reproduction and distribution of plants. Each plant is characterized by a certain genetically fixed level of resistance to low temperatures, which reduces its metabolic activity. This level of resistance can vary among individual plants and species. Chilling damage can be observed on many plants of tropical and subtropical origin when they are exposed to low positive temperatures.

*Corresponding author. E-mail: zaremaih@modares.ac.ir. Tel: +98 9123831060. Fax: +98 2182884717.

For plants of temperate origin, the chilling temperatures usually range from 0 to 15°C. The chilling effect is manifested by physiological perturbations, generally called low-temperature injury (Lyons,1973; Hudak and Salaj, 1999; Yan et al., 2010 and Zhang et al., 2010).

Rice is a temperature-sensitive crop; low temperatures dramatically reduce its production. During the early growth stages of rice, the occurrence of low temperature stress inhibits seedling establishment and eventually leads to nonuniform crop maturation. Good cold tolerance at the seedling stage is an important character for stable rice production. Developing cold-tolerant genotype is one of the most effective ways to avoid the low-temperature damage (Lou et al., 2007).

The chilling stress below 15°C often happens in the rice-growing regions in the north of Iran in April, which

makes early-season rice seedlings rotten, causing heavy seed loss and a delayed growth period (Sharifi, 2010).

In this study, we examined whether cold-sensitive Hoveizeh and cold-tolerant IRCTN34 respond differently to cold treatment in terms of differential total soluble protein, proline in shoots and roots and changes in total chlorophyll and stomatal conductance in leaves of rice in a treatment comprising shifts from 29/22 to 15/10°C.

MATERIALS AND METHODS

Plant material and growth condition

Oryza sativa cold-sensitive Hoveizeh (from ABRII (Agricultural Biotechnology Institute of Iran), Iran) and cold-tolerant IRCTN34 (a germplasm of International Rice Cold Tolerant Nursery 2005 (IRCTN 2005) from IRRI (International Rice Research Institute), Philippines) genotypes were used in the experiments. The surface of the seeds was sterilized by sodium hypochlorite (2.5% v/v) for 2 min and thoroughly washed with sterile distilled water. Seeds were germinated on Whatman no. 1 filter paper moistened with de-ionized water in Petri dishes at 28°C in the dark. Two days after sowing (DAS), uniformly germinated seeds were selected and transferred to trays containing 18 L of de-ionized water. The mean air temperature in the growth room was 29 and 22°C during the day and the night, respectively. Plants were illuminated for 12 h, keeping the light intensity at approximately 400±50 µmol m^{-2} s^{-1} at leaf level. Relative air humidity varied between 70 and 75%. At 6 DAS, plants were provided with full-strength Yoshida solution (Yoshida et al., 1976). Nutrient solutions were renewed every 3 days. On the 18th DAS, tri-leaf plants were divided into two groups. One group was maintained in the same chamber with 12 h light photoperiod at 29/22°C (day/night) as the control. The other group was transferred to a chamber for cold treatment with 12 h light photoperiod and at 15/10°C (day/night) for two weeks. Then, on the 14th day after treatment, randomly selected control and cold-treated seedlings were each divided into shoots and roots. Samples were transferred to liquid nitrogen (within 2 min of harvesting) and kept at -80°C for further analyses.

Shoot and root dry weights

Dry weights of roots and shoots were determined after drying in a forced draft oven at 65°C for 72 h and the shoot/root ratio and root/shoot ratio were calculated.

Leaf SPAD value

Chlorophyll or SPAD meter (SPAD-502, 1989 Minolta Co., Ltd) was used to measure the greenness or relative chlorophyll content of leaves (Inada, 1985). The youngest fully expanded leaf of a plant was used for SPAD measurement. A mean of 10 values per plant was taken as the measured SPAD value.

Chlorophyll (Chl) assay

A ground fresh sample of 0.5 g was extracted with 80% (v/v) aqueous acetone. The absorbance of the resulting supernatant was recorded at 664 and 647 nm using an UV-visible spectrophotometer (Cary300, Varian, Inc.) (Arnon, 1949). The chlorophyll stability index (CSI) was determined according to Sairam et al. (1997) and calculated as follows:

CSI = (Total Chl under stress/Total Chl under control) × 100

Leaf stomatal conductance

Stomatal conductance (g_s) of leaves was determined using a portable automatic diffusion porometer (Delta-T AP_4, Delta-T Devices, Cambridge, UK). The measurements were taken on the youngest fully expanded leaves of ten replicate plants of each treatment in the morning between 8.00 and 9.00 h. The readings were accomplished during 1 h to avoid the diurnal pattern of variation of the leaves. The porometer was calibrated before measurement using a set of known resistances on a calibration plate.

Proline assay

Free proline content was estimated using the acid ninhydrin method described by Bates et al. (1973). Fresh shoot and root samples (0.5 g) were ground in a mortar and pestle with 10 ml of 3% (w/v) sulfosalicylic acid aqueous solutions and the homogenate was filtered through Whatman no. 1 filter paper, then 2 ml of filtered extract was taken for the analysis to which 2 ml acid ninhydrin (1.25 g ninhydrin was warmed in a mixture of 30 ml glacial acetic acid and 20 ml of 6 M phosphoric acid until dissolved) and 2 ml glacial acetic acid were added. The reaction mixture was incubated in a boiling water bath (100°C) for 1 h and the reaction was then terminated in an ice bath. 4 ml of toluene was added to the reaction mixture and the organic phase (chromophore containing toluene) was warmed to room temperature and its optical density was measured at 520 nm using toluene as blank by UV-visible spectrophotometer (Cary300, Varian, Inc.). The amount of proline was determined from a standard curve.

Protein determination

Protein concentrations were measured using a modified Bradford procedure with bovine serum albumin (BSA) as standard protein (Bradford, 1976). Protein extracts were thawed and their concentration determined by a colorimetric method. The absorbance at the wavelength of 595 nm was determined against the blank and the standard curve of absorbance versus protein concentration plotted. Reactions containing dilutions of the soluble protein extracts (unknown concentrations) were set up as mentioned earlier and the absorbance at 595 nm determined using a spectrophotometer (Cary300, Varian, Inc.). Protein contents of the extracts were determined from the standard curve.

Statistical analysis

Data obtained were subjected to analysis of variance (ANOVA). Mean comparison was conducted using Duncan's multiple range tests ($P < 0.05$ and 0.01).

RESULTS

It was observed during the two-week growth period after the exposure to chilling that the morphological differences such as reduction in plant height, leaf area and

Table 1. Shoot and Root Dry Weights (DW), Shoot /Root and Root/Shoot Ratio of Hoveizeh (cold-sensitive) and IRCTN34 (cold-tolerant) seedlings subjected to 29/22°C and 15/10°C.

Genotypes	Temperature Treatment (°C)	Shoot DW (g plant^{-1})	Decrease (%)	Root DW (g plant^{-1})	Decrease (%)	Shoot/Root Ratio	Root/Shoot Ratio
Hoveizeh	29/22	0.846a	72.58	0.141b	78.01	6b	0.167c
	15/10	0.232d		0.031d		7.48a	0.137d
IRCTN34	29/22	0.730b	46.71	0.173a	36.74	4.21c	0.237b
	15/10	0.389c		0.112c		3.47d	0.289a
Mean	-	0.549	59.645	0.11	57.38	5.29	0.208
Genotype	-	**	-	**	-	**	**
Temperature	-	**	-	**	-	**	**
G × T	-	**	-	**	-	**	**

Duncan's multiple range test: Mean values sharing the same letter are not significantly different (P < 0.05). ns = not significant. * P < 0.05, ** P < 0.01.

Table 2. Total Chlorophyll content, chlorophyll stability index (CSI), SPAD number and stomatal conductance (gs) in youngest fully-expanded leaves of Hoveizeh (cold-sensitive) and IRCTN34 (cold-tolerant) seedlings subjected to 29/22°C and 15/10°C.

Genotypes	Temperature Treatment (°C)	Total Chlorophyll (mg g^{-1} FW)	Chlorophyll Stability Index (CSI) (%)	SPAD Number	g_s (mmol m^{-2} s^{-1})
Hoveizeh	29/22	3.74b	49.47	36.7b	442b
	15/10	1.85d		24.83c	43d
IRCTN34	29/22	4.076a	74.29	42.5a	473a
	15/10	3.028c		37.27b	180c
Mean	-	3.17	61.88	35.33	284.5
Genotype	-	**	-	**	**
Temperature	-	**	-	**	**
G × T	-	**	-	**	**

Duncan's multiple range test: Mean values sharing the same letter are not significantly different (P < 0.05). ns = not significant. * P < 0.05, ** P < 0.01.

appearance of necrotic spots between cold-sensitive and cold-tolerant plants became larger (data not shown). Thus, chilling may have damaged the growing points of rice seedlings. The analysis of variance revealed highly significant differences between the genotypes for all the characters studied.

Shoot and root dry weights

Dry weight results indicated that growth was negatively correlated with the cold treatment (Table 1). Rice plants grown at normal temperature (29/22°C) had significantly higher dry weights in contrast to plants grown at low temperature (15/10°C) which showed growth depression as indicated by reduced dry weights.

Chlorophyll content

Under chilling conditions, tolerant cultivar IRCTN34 showed little change of SPAD value compared with its control plants, while the SPAD value of Hoveizeh decreased significantly (Table 2). In this study, the amount of total chlorophyll was decreased in chilling phase (Table 2). Under chilling stress, Hoveizeh showed a drastic decrease in chlorophyll content and higher chlorophyll content was observed in IRCTN34. Chlorophyll stability index (CSI) under chilling stress was higher in IRCTN34. Hoveizeh, however, showed significantly lower CSI under chilling stress.

Table 3. Proline and total soluble protein contents in shoots and roots of Hoveizeh (cold-sensitive) and IRCTN34 (cold-tolerant) seedlings subjected to 29/22°C and 15/10°C.

Genotypes	Temperature Treatment (°C)	Shoot Proline (µmol g^{-1} FW)	Root Proline (µmol g^{-1} FW)	Shoot Total Soluble Protein (mg g^{-1} FW)	Root Total Soluble Protein (mg g^{-1} FW)
Hoveizeh	29/22	0.36b	0.24c	25.4a	8.38b
	15/10	0.77a	0.39a	12.51d	5.27d
IRCTN34	29/22	0.22c	0.18d	16.53c	7.50c
	15/10	0.37b	0.26b	22.97b	11.43a
Mean	-	0.43	0.27	19.35	8.15
Genotype	-	**	**	ns	**
Temperature	-	**	**	**	ns
G × T	-	**	**	**	**

Duncan's multiple range test: Mean values sharing the same letter are not significantly different ($P < 0.05$). ns = not significant. * $P < 0.05$, ** $P < 0.01$.

Stomatal conductance

Stomatal conductance of the rice plants was significantly influenced by chilling stress (Table 2).

Proline content

IRCTN34 genotype showed lower root proline contents than that of Hoveizeh genotype (Table 3).

Total soluble protein content

In this study, the transfer of seedlings from 29/22 to 15/10°C (day/night) for two weeks resulted in increase in the total soluble protein amounts in IRCTN34 genotype during low temperature period compared with the seedlings maintained at a control temperature of 29/22°C. Also, it was observed that the total soluble protein content in the shoot and roots of the cold-treated seedlings of Hoveizeh were lower than those of the control seedlings (Table 3).

DISCUSSION

We compared different aspects of the physiology of two genotypes of rice differing in chilling-sensitivity (Hoveizeh and IRCTN34), in respect to seedling growth at low temperature. To develop cold tolerant rice cultivars (for example through genetically modified genotypes), it is essential to understand how chilling induces its injurious effects on plants. Although Iran has high rice yield potential, the rice production areas are rather limited. In order to expand rice production areas, the development of cold tolerant varieties is required.

The results of the biomass indicated that cold treatment inhibited the growth of rice plant and led to a decrease in biomass. This might be related to the effect of cold stress which resulted in limited water uptake and nutrient supply by roots, biochemical processes and also declines of the rates of net photosynthesis due to adverse effect on CO_2 assimilation. This, in turn, lowered plant growth through lowering of the rates of both cell division and elongation. When seedlings were transferred to the low temperature conditions, the shoot/root ratio decreased in cold-tolerant genotypes, mainly due to a higher root dry matter. High root biomass in IRCTN34 seemed to be correlated with chilling tolerance and reduction in shoot biomass and height could be the avoidance mechanism by adjusting the growth rate in this genotype. In addition, clear chilling-caused spots were found in Hoveizeh but not in IRCTN34 during the 14 days treatment.

Low temperature is one of the most important factors that limit photosynthetic activity. It has been reported that chlorophyll *a* and *b* content was decreased in plants when plants were subjected to cold treatment (Wise and Naylor, 1987). Results of this research confirmed that the total chlorophyll concentration of rice leaves was reduced by the cold treatment. This reduction could be a typical symptom of oxidative stress. These results also indicated that the photosynthetic pigments in the tolerant genotype IRCTN34 were better protected against injury of chilling damage than the susceptible genotype Hoveizeh In this investigation, the high values of chlorophyll satiability index (CSI%) verified the tolerance of IRCTN34 plants to chilling stress. However, chilling temperature around the leaves could disrupt key processes in photosynthesis, including thylakoid electron transport, carbon assimilation and stomatal control (Allen and Ort, 2001).

Decrease of g_s in the chilling-stressed seedlings was due to the stomatal closure. Closure of stomata may result

from hydropassive or hydroactive closures (Mahajan and Tuteja, 2005). These processes reduced water loss by stomatal resistance.

Many plants accumulate high levels of free proline in response to a wide range of biotic and abiotic stresses and proline is considered as a signal/regulatory compound able to activate multiple physiological or molecular mechanisms. The role of endogenous proline under oxidative stress includes stabilization of protein complexes, regulation of cytosolic pH, regulation of NAD/NADH ratio or as a scavenger of oxygen free radicals. There are conflicting reports concerning the function of proline in the chilling resistance of plants. The higher level of free proline in cold-stressed plants has been suggested as a factor conferring chilling tolerance. In contrast, proline accumulation has also been considered as a symptom of injury rather than an indicator of low temperature tolerance (Hawrylak-Nowak et al., 2010). It is clear from the presented results that chilling effectively enhanced the proline accumulation under chilling stress conditions.

Our results showed that total protein has protective role in cold-tolerant genotype subject to low temperature. Synthesis of specific proteins is an important mechanism involved in increasing cold tolerance (Koc et al., 2010).

In conclusion, the results of this study showed that chilling sensitivity of Iranian genotype is related to the rapid reduction in shoot and root proteins and leaf chlorophyll content.

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REFERENCES

Allen DJ, Ort DR (2001). Impacts of chilling temperatures on photosynthesis in warm-climate plants. Trends Plant Sci. 6: 36-42.

Arnon DI (1949). Copper enzymes in isolated chloroplasts, polyphenol oxidase in *Beta vulgaris* L. Plant Physiol. 24: 1-15.

Bates LS, Waldren RP, Teare ID (1973). Rapid determination of free proline for water stress studies. Plant Soil, 39: 205-208.

Bradford MM (1976). A rapid and sensitive for the quantitation of microgram quantitites of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Hawrylak-Nowak B, Matraszek R, Szymanska M (2010). Selenium modifies the effect of short-term chilling stress on cucumber plants. Biol. Trace Elem. Res. 138: 307-315.

Hudak J, Salaj J (1999). Effect of low temperatures on the structure of plant cells. In: Handbook of Plant and Crop Stress, 2nd ed. (Ed. Pessarakli M). Marcel Dekker Press, New York, pp. 441-464.

Inada K (1985). Spectral ratio of reflectance for estimating chlorophyll content of leaf, Jpn. J. Crop Sci. 54: 261-265.

Koc E, Islek C, Ustun AS (2010). Effect of Cold on Protein, Proline, Phenolic Compounds and Chlorophyll Content of Two Pepper (*Capsicum annuum* L.) Varieties. GU J. Sci. 23(1): 1-6.

Lou Q, Chen L, Sun Z, Xing Y, Li J, Xu X, Mei H, Luo L (2007). A major QTL associated with cold tolerance at seedling stage in rice (*Oryza sativa* L.). Euphytica, 158: 87-94.

Lyons JM (1973). Chilling injury in plants. Ann. Rev. Plant Physiol. 24: 445-466.

Mahajan S, Tuteja N (2005). Cold, Salinity and Drought Stresses. An Overview. Arch. Biochem. Biophys. 444: 139-158.

Sairam RK, Deshmuhu PS and Shukla DS (1997). Tolerance of drought and temperature stress in relation to increased antioxidant enzyme activity in wheat. J. Agron. Crop Sci. 178: 171-178.

Sharifi P (2010). Evaluation on Sixty-eight Rice Germplasms in Cold Tolerance at Germination Stage. Rice Sci. 17(1): 77-81.

Wise RR, Naylor AW (1987). Chilling-enhanced photooxidation. Evidence for the role of singlet oxygen and superoxide in the breakdown of pigments and endogenous antioxidants. Plant Physiol. 83: 278-282.

Yan WW, Bai LP, Zhang L, Chen G, Fan JG, Gu XH, Cui WS, Guo ZF (2010). Comparative study for cold acclimation physiological indicators of *Forsythia mandshurica* Uyeki and *Forsythia viridissima* Ind. Middle-East J. Sci. Res 6(6): 556-562.

Yoshida S, Forno DA, Cock JH (1976). Routine procedure for growing rice plants in culture solution. IRRI Laboratory Manual for Physiol. Studies of Rice, IRRI, LosBanos, Philippines, (eds), pp. 61-66.

Zhang S, Jiang H, Peng S, Korpelainen H, Li C (2010). Sex-related differences in morphological, physiological, and ultrastructural responses of *Populus cathayana* to chilling. J. Exp. Bot. pp. 1-12.