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Research article

IMPROVING RICE SEEDLING PHYSIOLOGICAL AND BIOCHEMICAL PROCESSES UNDER LOW TEMPERATURE BY SEED PRIMING WITH SALICYLIC ACID

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ABSTRACT: Temperature is one of the major environmental limiting factors that affect rice (*Oryza sativa* L.) production. This study was carried out to induce chilling tolerance in rice (cv. Hashemi) by seed priming with salicylic acid and to unravel the physiological and biochemical processes at early growth stage. Seeds were soaked in 0, 20, 50 and 100 mg.l^{-1} salicylic acid solutions for 24 hours and then dried back to the original moisture content and were sown in three temperatures (28°C as normal, 12°C and 8°C as chilling stress). Sharp decrease in photosynthesis pigments was observed under low temperature in rice seedling leaves in compared to control though chilling stress effects on photosynthetic pigments was mitigated by seed priming with salicylic acid. Maximum decrease in protein contents (35%) was observed in 8°C though it was 4% for 12°C. seed priming with salicylic acid improved protein contents in all concentrations. Soluble sugars content increased with decreasing in temperature as it was 33% and 41% in 12 and 8°C, respectively. Maximum soluble sugars accumulation (34.41 mg.g-1 f_w) was recorded in seedling raised from primed seeds with 100 mg.l-1 SA at 12°C. Indeed, seed priming with salicylic acid via increasing in photosynthesis pigments, proline, soluble sugars and protein content could decrease low temperature harmful effects and therefore increase rice seedling root and shoot growth.
Keywords: Carotenoid, Photosynthesis Pigment, Proline, Protein, Soluble sugars

INTRODUCTION

Temperature is one of the major environmental limiting factors that affect plants distribution. Many plants growth in subtropical regions for instance rice, tomato and maize are affected by low temperature every year in the world wide. As a matter of fact, we are able to maintain production at low temperature conditions by improving chilling tolerance in these crops. Rice (*Oryza sativa* L.) is a temperature-sensitive crop. The range of air temperature below 15 to 20° C results in chilling damage in rice though the level of damage is related to period of chilling stress, growth stage and genotypes [21]. In northern regions of Iran, rice is sown from early April to middle of May, when mean temperature is around 15° C which leads to rotten rice seedlings, causing heavy seed loss and a delay in growth period. Low vigor and growth rates are the symptoms of the crops exposed to chilling stress conditions without other visual symptoms of damage. Decrease of growth rates at low temperature conditions is because of photosynthesis inhibition which may be a consequence of photooxidative injury to the photosystems in the membranes of chloroplasts. Photooxidative damage causes lipid peroxidation and degradation of chlorophyll and carotene. Plants undergo physiological and biochemical changes under low temperature conditions that enable them to survive under these conditions [35]. In general, physiological and biochemical dysfunction induced by low temperature in plants are not equal and most of them can be converted if plant returns to optimum condition before appearance of damage. Therefore, disturbances of physiological and biochemical dysfunctions induced by low temperature do not lead to visible manifestations of damage to reduce the rate of plant photosynthesis and growth, since disturbances of physiological and biochemical functions are reversible. Some studies on different plants have shown that seed priming treatments have been effectively increase germination, early seedling growth especially at abnormal conditions and also improve seedlings resistance to stresses conditions [3]. Seed priming in order to improve germination, increases seedling stand in field and stress tolerance.

Salicylic acid (SA) is a growth regulator which influences a range of processes in plants including germination, photosynthesis and growth rate, ion uptake and transport, membrane permeability, and is also known to provide protection against several abiotic stresses [16]. El- Tayeb [6] revealed that barely seed priming by 1 mM SA solution caused an increase in seedling salt tolerance. Moreover, seed priming by SA solution improved wheat seedling growth under salt stress condition [24]. Horvath et al. [13] reported that SA improved drought and chilling stress tolerance in wheat (*Triticum aestivum* L.) via protecting plant cell structure under low temperature. In another study, exogenous SA application decreased the rate of relative electrolyte leakage in grape (*Vitis vinifera* L.) leaves under chilling condition [31]. The authors concluded that exogenous application of SA caused chilling resistance via increase in antioxidant activity and also improved physiological and biochemical processes. Farooq et al. [7] showed that maize seed priming by SA solution improved germination characteristics and seedling root and shoot growth under both normal and low temperature conditions. They mentioned that main reason of increase in maize seedling growth under chilling stress condition is activation of antioxidant enzymes system, including catalase (CAT), superoxide dismutase (SOD) and ascourbate peroxides (APX).

In this study, we exposed the rice seedling raised from seed primed by SA solution to short period chilling temperatures in order to investigate chilling negative effect on physiological and biochemical processes and SA mitigating effect on them.

MATERIALS AND METHODS

Seed

Rice genotype seeds (cv. Hashemi) were obtained from the Rice Research Institute of Iran (RRII). Healthy seeds of similar size lacking any sign of disease were selected. The seeds had been sent by RRII research farms in October 2010. Germination rate as 91 % was recorded in the first germination test via between paper methods. The seeds were kept in air-tight container and placed in 4° C in refrigerator for further experiments. The experiments were conducted in the Seed Research Laboratory of the Department of Crop Science, Ferdowsi University of Mashhad in summer 2011.

Seed priming

To perform seed priming treatments they were soaked in SA solutions concentrations (0, 20, 50 and 100 $mg.l^{-1}$) for 24 h at 28 ± 2° C. then they were washed six times with distilled water and dried back to primary moisture level (8%) at 27 ± 3° C temperature condition [18]. Unprimed seeds were used as control

Emergence in sand

Trays (45 ×25 ×10 cm) contain moist sand were used for seed cultivation. Seeds were sown in 1 cm depth and then trays were put in a growth chamber with 15/9h light/dark for 19 days at 28° C as optimum temperature condition. To induce low temperature conditions another two sets were put in the same above conditions for 15 days, before moving to 8° C and 12° C for four days, respectively. The experiment was conducted in a completely randomized design with four replications of 25 seeds.

Biochemical analysis

For biochemical analysis, samples (second top leaf from main tiller) were taken from control and chilling stressed plants on 19th day after sowing.

Determination of photosynthesis pigment content

To determine photosynthesis pigments, the method proposed by Dere et al. [5] with some modifications was used. Briefly, 100 mg fresh leaf samples were used for preparation of solution to be used for spectrophotometer (Jenway Model 6305) measurement. Absorbance in three wave length (470, 653 and 666 nm) were recorded and then to estimate the amount of chlorophyll a, b and carotenoid the equation 1 was used.

$$Ca = 15.65\,A666 \quad 7.340\,A653$$

$$Cb = 27.05\,A653 - 11.21\,A666$$

$$Cx+c = \frac{(1000\,A470 - 2.860Ca - 129.2\,Cb)}{245}$$

where, Ca is the chlorophyll *a* content; Cb is the chlorophyll *b* content and Cx+c is the carotenoid content. A470 is the absorbance at 470 nm, A653 is the absorbance at 653 nm and A666 is the absorbance at 666 nm.

Determination of malondialdehyde (MDA) accumulation and POX activity

Method of Heath and Packer [11] was used for determination of MDA. In order to estimate the activity of POX the method proposed by Srinivas et al. [27] was used.

Determination of proline content

To estimate proline content, 100 mg fresh leaf samples were pulverized in liquid nitrogen and homogenized in 5 ml of 3% sulfosalicylic acid. The homogenate (1 ml) was mixed with 1 ml of acid ninhydrin for 60 min at 100 °C in oven. The reaction mixture was thoroughly mixed with 2 ml of toluene and placed at room temperature for 30 minutes. Absorbance of the solution was measured spectrophotometrically at 520 nm using toluene as a blank.

Determination of protein and sugars content

Protein concentration was evaluated using a modified Bradford procedure with bovine serum albumin (BSA) as standard protein [3]. Soluble sugars were analysed after sequential extraction with an ethanol/water mixture (80/20) and a boiling 3% HCl solution, respectively, using a modification of the phenol sulphuric acid method as described by Buysee and Merckx [4].

Shoot and root length and dry weights

Ten seedlings for each replicate were carefully removed from seed bed and washed before measuring the root (RL) and shoot (SL) length. Then all seedlings were dried in an oven at 65° C for 72 hours to estimate root (RDW) and shoot (SDW) dry weight.

RESULT

Temperature had significant effect on chlorophyll content (Table 1). The highest decrease in chlorophyll a due to chilling stress as 17 percent was observed at 8° C and it was 14 percent for 12° C. Seed priming with SA had a positive effect on seedlings leaves chlorophyll in every temperature conditions (Table 1). As in 12° C, the sharp increase (12%) in chlorophyll a content was occurred at 50 mg.l-1 SA solution and was 9 and 6 percent for 28° C and 8° C, respectively. Chlorophyll b was lowered by decrease in temperature condition as it was 21 and 25 percent decrease in 12° C and 8° C, respectively. Seedling raised from seed primed with 50 mg.l-1 SA solution, showed the highest content (493 micro g.g-1 fw) of chlorophyll b than control.

Table 1. The effects of chilling stress and seed priming with salicylic acid on rice seedling chlorophyll a, chlorophyll b, carotenoid, proline, protein and soluble sugars.

Treatments		Chl.a (µ $g.g^{-1}$ fw)	Chl.b (µ $g.g^{-1}$ fw)	Carotenoid (µ $g.g^{-1}$ fw)	Proline (µ $g.g^{-1}$ fw)	Protein ($mg.g^{-1}$ fw)	Sugar ($mg.g^{-1}$ fw)
T1	P1	697	529	434	137	14.01	21.14
	P2	703	532	432	138	14.64	21.95
	P3	746	581	456	149	16.59	24.39
	P4	758	583	469	142	15.69	26.57
	P5	739	572	439	143	14.54	23.77
T2	P1	598	419	475	171	13.38	28.09
	P2	584	422	479	174	14.02	29.91
	P3	637	461	510	186	15.07	30.34
	P4	668	493	526	194	16.97	33.53
	P5	656	482	525	187	16.58	31.72
T3	P1	577	396	482	168	9.05	29.78
	P2	576	404	486	175	9.19	30.59
	P3	596	422	489	180	10.74	31.03
	P4	612	429	497	190	11.64	32.22
	P5	608	433	495	191	11.58	34.41
LSD		42	31	28	14	2.81	3.12

P1, untreated seeds (control); P2, distilled water; P3, 20 $mg.l^{-1}$ salicylic acid; P4, 50 $mg.l^{-1}$ salicylic acid; P5, 100 $mg.l^{-1}$ salicylic acid; T1, Temperature of the whole period was 28°C; T2, fifteen days at 28°C and then four days at 12° C; T3, fifteen days at 28° C and then four days at 8°C.

Table 2. The effects of chilling stress and seed priming with salicylic acid on rice seedling malondfialdehyde (MDA), peroxides activity (POX), root dry weight (RDW), shoot dry weight (SDW), root length (RL) and shoot length (SL).

Treatments		MDA (mmol.g^{-1} fw)	POX (unit.mg^{-1} protein)	RDW (mg.plant^{-1})	SDW (mg.plant^{-1})	RL (mm)	SL (mm)
T1	P1	19.94	15.34	126	840	75	124
	P2	19.18	16.21	131	835	77	122
	P3	16.91	19.61	146	903	86	144
	P4	16.07	18.24	140	934	84	151
	P5	16.67	16.72	138	882	80	139
T2	P1	32.61	25.46	98	788	54	96
	P2	30.84	26.62	103	784	55	95
	P3	25.58	29.73	118	830	70	110
	P4	24.74	38.81	127	843	75	116
	P5	27.33	36.56	123	836	72	104
T3	P1	40.21	27.67	95	705	51	85
	P2	38.44	27.76	96	698	53	84
	P3	33.18	32.16	103	730	54	90
	P4	32.35	39.78	109	745	57	98
	P5	33.93	38.67	112	753	61	99
LSD		4.1	2.25	8.5	45	5.2	12

P1, untreated seeds (control); P2, distilled water; P3, 20 mg.l^{-1} salicylic acid; P4, 50 mg.l^{-1} salicylic acid; P5, 100 mg.l^{-1} salicylic acid; T1, Temperature of the whole period was 28°C; T2, fifteen days at 28°C and then four days at 12° C; T3, fifteen days at 28° C and then four days at 8°C.

Temperature, seed priming and the interaction of temperature*priming had a significant effect on carotenoid content (Table 1). 8°C and 28°C with 482 and 434 micro g.g^{-1} f_w had the highest and the lowest content of carotenoid content, respectively. Among priming treatments, 50 mg l^{-1} SA solution in 12° C and unprimed seed in 28° C with 526 and 434 micro g.g^{-1} f_w had the highest and the lowest amount of carotenoid content, respectively. At all temperatures, seedling raised from primed seeds showed the highest amount of carotenoid pigments in compared with control (Table 1).

The amount of proline in seedling leaves significantly increased in both low temperatures. This increase was 25 and 23 percent in 12 and 8°C in compared to normal condition. Furthermore, rice seed priming by SA solutions increased the proline concentration in all temperature conditions (Table 1). For instance in 8°C, seed priming with SA 100 mg.l^{-1} increased (14%) the proline from 168 μ g.g^{-1} f_w in untreated seeds to 191 μ g.g^{-1} f_w in treated seeds.

Data presented in Table 1 showed that different temperature conditions and treatments had significant effect on protein content of seedling leaves. Maximum decrease in protein content (35%) was observed in 8° C though it was 4% for 12°C. seed priming with SA solutions improved protein contents in all concentrations. For instance in 8°C, seed priming with SA 50 mg.l^{-1} increased (29%) the protein content from 9.05 in untreated seeds to 11.64 in treated seeds (Table 1).

Analysis of variance revealed a significant effect of temperature and priming on soluble sugars content of seedling leaves (Table 1). Soluble sugars content increased with decrease in temperature as it was 33% and 41% in 12 and 8°C, respectively. Maximum soluble sugars accumulation (34.41 mg.g^{-1} f_w) was recorded in seedling raised from primed seeds with 100 mg.l^{-1} SA concentration solution at 12°C condition. As a matter of fact, soluble sugars content was increased by seed priming in all three temperatures. Although, the more SA solutions effect (26%) was observed in normal temperature. Peroxides (POX) activity increased significantly in rice seedling leaves as temperature decreased (Table 2), So that it was 66% and 80% for 12° C and 8°C, respectively. At normal and low temperatures, seed priming with SA improved the POX activity compared to control. However, the maximum POX activity was recorded from100 mg.l^{-1} SA under 8° C temperature (Table 2). The MAD of the seedling leaves significantly increased in low temperatures. This increase was 102 percent in 8° C in compared with normal condition. However, rice seed priming by SA solutions decreased the MAD in every temperature conditions (Table 2). As in 12° C, seed priming with SA 50 mg.l^{-1} decreased (24%) the MDA from 32.61 in untreated seeds to 24.74 in treated seeds.

Although chilling temperatures considerably decreased the RDW than optimal temperature; seed priming with SA solutions improved it at temperature conditions (Table 2). The highest increase in RDW (30%) was obtained by 20 $mg.l^{-1}$ SA solution treatment in 12°C chilling conditions. Between two chilling temperatures, 8°C had the highest decrease (25%) in RDW though it was 22% for 12°C.

Although chilling stress considerably decreased the SDW (16%), seed priming with SA improved the SDW in both chilling stress conditions and normal temperature. Under optimum temperature conditions seedlings raised from primed seeds with 50 $mg.l^{-1}$ SA solution had the highest SDW (934 mg/plant) though in 12°C and 8°C chilling conditions the highest SDW (843 and 753 mg/plant) was obtain from primed seeds with 50 $mg.l^{-1}$ and 10050 $mg.l^{-1}$ SA solution (Table 2). Chilling stress significantly reduced root length (32%) compared with control (Table 2). Although, Seed priming improved the root growth in all temperatures particularly at 12°C where root length increased (39%) from 54 mm in untreated seeds to 75 mm in primed seeds with 50 $mg.l^{-1}$ SA solution (Table 2). Seedling shoot length was decreased significantly in low temperatures. This decrease was 31 percent in 8°C compared to normal condition. However, rice seed priming by SA solutions increased the shoot length in all temperature conditions (Table 2). For instance in 28° C, seed priming with 50 $mg.l^{-1}$ SA solution increased (22%) the shoot length from 124 mm in untreated seeds to 151 mm in treated seeds.

DISCUSSION

In current study, sharp decrease in chlorophyll a and b was observed under chilling temperature in rice seedling leaves compared to control though chilling stress effect on photosynthetic pigments was mitigated by seed priming with SA solution.

Biotic and abiotic stresses can decrease the plants photosynthesis rate via damage in photosynthesis pigments. Low temperature stress is one of abiotic stresses that can induce photosynthesis pigments injury in crops and lead to decrease in photosynthesis rate. Wise and Naylor [34] showed that low temperature stress damaged the photosynthesis pigments. They mentioned that low temperature via produce ROS in plant lead to damage in pigments and therefore reduce in photosynthesis rate. Aghaee et al. [1] demonstrated that the amount of photosynthesis pigment (chlorophyll a and b) in rice seedling leaves was decreased as temperature was lowered. Decrease in photosynthesis rate caused by chilling stress via inducing damage in pigments is a famous response of chilling sensitive plants. In another study conducted by Yadegari et al. [36] on soybean (*Glycine max* L.), chilling stress decreased pigment concentration in seedling leaves as compared with control.

In our study, High amount of carotenoid (482 micro $g.g^{-1}$ f_w) was observed in the leaves of the rice seedlings under chilling stress (8° C) and seed priming with SA enhanced the responses that can be related to SA protective effect on cell membrane and organs.

In our study, SA solution treatments were able to mitigate chilling stress negative effect on photosynthesis pigments. It can be concluded that SA, via improving seedling antioxidant system, decreased the ROS negative effect on photosynthesis pigments. Salicylic acid (SA) is a growth regulator that affects several processes in plants including germination, ion uptake, membrane permeability, photosynthesis, antioxidant activity, signal transduction and also protective effects for many environmental stresses [22]. Kaydan et al. [14] in a study showed that wheat seed priming with SA solution led to increase photosynthesis pigments (chlorophylls and carotenoids) content and improved root and shoot growth under stress condition. Similarly, Türkyılmaz et al. [29] mentioned that in the field condition SA solution spray improved photosynthesis pigments (chlorophyll a, b and carotenoid) concentration in bean (*Phaseolus vulgaris* L.).

The results indicated that the proline synthesis could be induced by decreasing in temperature and seed priming with SA solution could intensify it. Nowak et al. [20] mentioned that accumulation of proline in plant cells is a protective mechanism which plants use to protect themselves against abiotic stresses such as low temperature. They also reported that proline acts as a signal/regulatory compound affecting several physiological and biochemical processes in plants under normal and stresses conditions. Aghaee et al. [1] demonstrated that with decrease in temperature, proline concentration increased. They concluded that increase in proline content is a proper response of plant to increase in ROS level in cells caused by chilling stress. Similarly, Yadegari et al. [36] revealed that proline concentration in soybean seedling leaves increased as temperature decreased.

Chilling stress (8° C) reduced the protein content (35%) in untreated seeds while the reduction of 6% in protein content was observed when the seeds were primed with SA 50 $mg.l^{-1}$ (Table 1). Therefore, seed priming with SA solution was able to mitigate (29%) the negative effect of chilling stress on protein content of seedling leaves. Mercado et al. [19] demonstrated that low temperature causes decrease in protein content of chilling sensitive plants. They concluded that this decrease in protein content is because of chilling stress negative effect on protein synthesis.

Koc et al. [17] concluded that cold tolerance is enhanced in some plants via synthesis of specific proteins at low temperature. They found a change in proteins content of plant leaves though the deference was not significant. Kester et al. [15] reported that seed priming is able to increase in plant tissue protein via conferring protection to the cellular proteins damaged, improving the performance of the protein synthesis system and also increase in L-isoaspartyl protein methyltransferase enzyme that repairs plant tissue protein. In current study, probably, seed priming with SA solution by the above reasons was the reason of improvement in protein content of rice seedling in low temperature as compared with control. Soluble sugar content of rice seedling leaves considerably increased as temperature decreased and seed priming with SA enhanced the responses. In fact, some plants improve their chilling tolerance via change in soluble sugar content. Wanner and Junttila [32] reported that increase in soluble sugar content is a rapid response of Arabidopsis plant when they were exposed to chilling stress condition. Uemura and Steponkus [30] mentioned that soluble sugar (sucrose) in low concentration acts as a substrate for metabolic processes induced by chilling stress though at higher amount it has a protective effect on plant cell membranes. Basra et al. [2] demonstrated that rice seed priming with KNO_3 increased the amount of soluble sugar content in rice seedling compared to unprimed seeds. Similarly, in another study Lee and Kim [18] showed that seed priming improved concentration of soluble sugar content in both aged and normal seeds. The MAD was increased in rice seedling leaves (102%) by chilling stress in untreated seeds while the reduction of 20% MDA was observed when the seeds were primed with SA solution at 50 $mg.l^{-1}$ concentration. Low temperature stress via induce ROS conducts many malfunctions include membrane injury in plant cells therefore, increasing their permeability. In fact, Cell membranes are the major targets of environmental stresses. ROS produced by chilling stress via peroxidation of cell membrane lipids causes to accumulation of MDA. Therefore, accumulation of MDA was considered to be an indicator of membrane damage induced by low temperature. In our study, the amount of MDA in seedling raised from primed seeds was lower than that of unprimed seeds which can be a sign of SA protection role in low temperature condition. In our opinion, SA decreased the peroxidation of membrane lipids and the amount of MDA via increasing the activity of antioxidants (SOD, CAT. APX, glutathione reductase (GR) and POX) under low temperature. Pouramir et al. [22] reported that rice seed priming with SA solution decreased the amount of ROS in rice seedling under low temperature by increasing the antioxidant activity. POX activity was increased by decrease in temperature and seed priming with SA enhanced the responses compared to unprimed seeds. It is probable that high level of POX activity in low temperature conditions is a response to ROS production in this condition. Farooq et al. [7] reported that antioxidant activity plays an important role in maize chilling tolerance at early growth stage. Similarly, Saruyama and Tanida [25] revealed that activity of some antioxidants including CAT and POX under low temperature conditions are the main reason for chilling tolerance in rice cultivars. Hodges et al. [12] also displayed that probably high activity of CAT, MDHAR (monodehydroascorbate reductase) and POX under chilling stress are the main reason of chilling tolerance in maize seedling. Chilling stress reduced root and shoot length and also RDM and SDM but this reduction was lower in seedling raised from primed seeds. Granier et al. [10] claimed that low temperature can influence root and shoot growth by preventing both cell division and expansion. Rymen et al. [23] demonstrated that low temperature can affect plant growth via influencing enzymes activity in several biochemical reactions. They mentioned that chilling stress by lengthened cell cycle duration and therefore decrease in cell production leads to inhibition in leaf growth. Granier et al. [10] reported that abiotic stresses result in reduction of growth rate by decreasing in both cell production and cell size. West et al. [33] showed that main reason for reduction of Arabidopsis growth under low temperature was reducing in cell production and smaller length of mature cell though average duration of cell cycle was not affected.

In our study, harmful effects of chilling stress on rice seedling root and shoot growth and their dry matter was mitigated by seed priming with SA solutions. It is probable that, SA enhanced root and shoot growth via improvement of cell cycle, cell division in germination stage and also improve the amount of photosynthesis pigments, soluble sugars and antioxidant in rice seedling. Promotion of seedling meristems cells into the S and G2 stage of cell cycle, enhancement the percentage of nuclei having 4 C DNA content has been reported to occur during seed priming. Sunitha et al. [28] mentioned that tomato (*Solanum lycopersicum* L.) seed priming result in increase in replication of DNAsynthesis in meristem nuclei though an increase in the percentage of 4 C nuclei doesn't seem to be essential for germination advancement. Pouramir et al. [22] reported that rice seed priming with SA solution led to increase in root and soot length. Similarly, Farooq et al. [7] showed that maize seed priming with SA solution resulting an increase in root and shoot length and also seedling fresh and dry weights under low temperature condition. They concluded that increase in seedling growth rate might be as a result of increase in cell division in maize seedling meristem.

CONCLUSION

This study revealed that seed priming with SA solution improved rice seedling growth (cv. Hashemi) under low temperature condition. Indeed SA could decrease low temperature harmful effects and therefore increase seedling root and shoot growth by increasing proline, soluble sugars and protein content.

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