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Effect of Salicylic Acid on Growth and Chilling Tolerance of Cucumber Seedlings

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The present study was undertaken to investigate the effect of low temperature and salicylic acid(SA) on the chilling tolerance of acclimated and nonacclimated cucumber(*Cucurmis sativus* L.) seedlings. The acclimation phenomenon was characterized in chilling-sensitive cucumber seedlings and found to have a significant effect on the survival and shoot dry weights. The injuries experienced by the acclimated seedlings in the third leaf stage were on average smaller by half than those experienced by the nonacclimated seedlings. Chilling also caused a large increase in the free proline levels, regardless of the acclimation status. Exogenous treatment with SA(0.5mM) resulted in improved growth and survival of the nonacclimated chilled seedlings, indicating that SA induced chilling tolerance and SA and acclimation had common effects. The application of cycloheximide in the presence of SA restored the acclimation-induced chilling tolerance. The elevated proline level observed in the cold-treated and SA-treated plants was more pronounced in the light than in the dark at a chilled temperature, indicating that endogenous proline may play a role in chilling tolerance by stabilizing the water status in response to chilling. From these results it is suggested that SA provided protection against low-temperature stress by increasing the proline accumulation, and pre-treatment with SA may induce antioxidant enzymes leading to increased chilling tolerance.

Key words : chilling, acclimation, proline accumulation, cycloheximide, *Cucurmis sativus*

1. Introduction

Plants native to warm regions are generally sensitive and injured by chilling temperatures. Recently, increasing attention has been given to growth substances as modulators of plant responses to stress conditions, including chilling[1,2]. Chilling injury is a physiological disorder that occurs in sensitive plants subjected to non-freezing temperatures below 12℃. However, the mechanism of chilling injuries in plants has not yet been fully explained. Considerable research has been conducted on the physiological basis of chilling injury in susceptible plant species using plant organs. The symptoms of chilling injury include stunted growth, reduced photosynthetic capacity, necrosis and discoloration, wilting, acceleration of senescence, and death. In addition, the effects of chilling on plant metabolism are numerous and have been reported in relation to respiration, photosynthesis, phenolic metabolism, sugar metabolism, and redox regulation. A number of tolerance mechanisms have been proposed on the physiological and biochemical changes associated with chilling injury[3,4]. Many approaches have been developed to reduce chilling injury in growing plants or harvested plant parts.

Many plants can be acclimated to low temperatures. Acclimation to chilling results in a lowering of the temperature at which the plant is damaged or killed by chilling. Exposure to noninjurious low temperatures enables plants to acquire the capacity to survive damaging temperatures. Chilling imposes severe oxidative stress, which may be responsible for chilling-associated damage

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in nonacclimated seedlings[5]. Yet, mild oxidative stress induced by either chemical treatment or chilling acclimation appears to be beneficial to subsequently chilled seedlings. Although the mechanisms of acclimation have already been previously documented, much remains to be discovered about how these metabolic changes coordinate to produce a level of low temperature tolerance during acclimation.

Low temperatures can cause various types of physiological damage and induce oxidative stress in the cells[6]. It has been found that the levels of certain amino acids increase under stress conditions, such as salt, water, and cold stress[7,8]. Although there is no definitive evidence on the adaptive value of proline under stress conditions, the involvement of proline accumulation in stress tolerance under adverse conditions is well established[9]. Kishor et al.[10] observed a proline- related increased resistance to water deficiency and salinity stress in transgenic tobacco plants, while Dörffling *et al.*[11] provided strong evidence on the inheritability of traits like 'increased frost tolerance' and 'increased proline content' in winter wheat. Osmotic stress has also been found to induce a strong accumulation of proline in canola leaf discs[8]. However, the authors were unable to determine whether the increase in frost tolerance was due to the proline over-accumulation or metabolic disturbances induced by this accumulation.

Salicylic acid(SA), an ubiquitous plant phenolic, plays an important role in the defense response of many plant species to different pathogen attacks[12]. SA is known to be a signal molecule for the development of systemic-acquired resistance, and mediates the oxidative burst that leads to cell death in a hypersensitive response[13]. Several studies have also found that SA plays a major role in modulating plant responses to various abiotic stresses[14]. As such, there has been considerable interest in the role of SA as regards inducing tolerance to low temperatures. Treating mustard seedlings with exogenous SA was found to improve their thermotolerance and heat acclimation[15]. In maize plants, pre-treatment with SA provided protection against low-temperature stress and induced antioxidant enzymes that led to an increased chilling tolerance[16]. However, despite strong evidence that SA can induce low-temperature tolerance, the means by which SA acts to produce such tolerance is still poorly understood.

The present paper reports on the effects of SA on seedling growth and chilling tolerance, and describes chilling acclimation in seedlings of chilling-sensitive cucumbers. In addition, the role of SA, with special regard to the changes in the proline content in the young cucumber plants, is discussed as a possible factor responsible for increasing chilling tolerance.

2. Materials and Methods

2.1 Plant material and growth conditions

Cucumber(*Cucurmis sativus* L.) seeds were planted in vermiculite and grown at 25 ℃ for 3 d in the dark. The plants were cultivated based on a photoperiod of 12 h at 300 μmol $m^{-2}sec^{-1}$, 25 ℃, and 60 % relative humidity in a growth chamber. The seedlings were then pre-exposed at either 14 ℃ for 3 d in the dark (acclimation period) followed by 7 d in the dark at 4 ℃ or directly transferred to 4 ℃ for 7 d in the dark(chilling period). The control seedlings did not receive the 14 ℃ treatment. Thereafter, the acclimated and control seedlings were exposed to chilling treatments at 4, 5, or 7 ℃ for 2, 4, 7, or 10 d. The final growth analysis was performed after the acclimated and nonacclimated seedlings were transferred to the initial growth conditions(25 ℃, 12 h photoperiod) and grown for 10 d. The surviving seedlings were counted and their dry weights measured before and after the 10-d grow-out period. The unchilled controls were also included. When calculating the survival percentage, the actively growing seedlings were determined to be survivors, while the non-growing and wilted seedlings were determined to be non-survivors. Only the survivors were included in the final weight analyses.

2.2 Chilling treatment

The experimental procedure used to determine the chilling tolerance consisted of pre-cultivation for 3 d at 25 ℃, subsequent chilling at 4 ℃ for 7 d, and a recovery phase at 25 ℃ for an additional 7 d. To investigate whether the exogenous application of SA affected the chilling tolerance, the seedlings were exposed to 0, 100, 500, or 1000

μM SA, then germinated in moist paper at 25 ℃ for 3 d in darkness. Thereafter, the imbibing solution was replaced and growth allowed for an additional 24 h. Next, the seedlings were transplanted into pots filled with a Hoagland solution, left at 25 ℃ for 12 h as a recovery period from oxidative shock, then transferred to 4 ℃ for 7 d. The culture medium was renewed at the beginning of the recovery phase. The controls were cultivated at 25 ℃ for the whole experiment with or without additions. When prechilled seedlings were used for the chemical treatments, 3-d-old seedlings were treated with 100 μM H_2O_2, a redox-cycling quinone that generates superoxide, for 4 h at 25 ℃ before being transferred to 4 ℃.

To further investigate the effect of SA on the chilling tolerance, cycloheximide(CH) was used to inhibit protein synthesis in an attempt to abolish the acclimation-induced chilling tolerance and determine whether SA was required. The CH was administered by allowing the seeds to imbibe in 0.1 mM CH at 25 ℃ in darkness. The CH-treated and untreated seeds(soaked in water) were then planted in pots filled with a Hoagland solution and watered with the same solution they had been soaked in.

Various cold treatments and preparatory treatments were applied to study the effect of SA on the chilling symptoms. No CH-treated seeds were used in the SA @4(25 ℃ for 2 d and 4 ℃ for 2 h in the presence of 500 μM SA). Following the various treatments, the seedlings were transferred to a growth chamber for a 7-d grow-out period. In another set of experiments on the long-term effect of SA, 3-d-old seedlings were pre-cultivated for 4 d at 25 ℃, subsequently chilled at 4℃ for 7 d, and allowed to recover at 25 ℃ for an additional 7 d. SA was added at the beginning of the pre-cultivation. Long-term experiments were also carried out with two-week-old seedlings to examine the necrotic injuries, survival rates, and proline contents in the entire seedling and third leaf. The time course of changes in the proline content and extent of necrotic damage were determined in acclimated and non-acclimated seedlings. After two weeks of growth(3-4 leaf stage) and 7 h after the beginning of the light period, the plants were acclimated or directly chilled. The acclimation lasted 4 d, followed by chilling at 5 ℃ for up to 5 d. After the chilling, the plants were transferred back to the initial growth conditions, then ten days later the necrotic damage suffered by the entire seedling and third leaf(in which the proline content was also measured) was estimated visually. The percentage of damaged area and necrotic spots was determined for all the leaves(whole seedling) and the third leaf. For the dead seedlings, 100 % damage was adopted. The third leaves of the young cucumber plants were also used to study the role of light in proline accumulation in cold-treated seedlings. The cold treatment of the two-week-old plants was carried out in the same type of chamber at 5 ℃ in continuous light or dark. The cold treatment started in the middle of the light period. The proline content in the third leaf was then analyzed after a 1-, 2-, or 3-d chilling period.

2.3 Proline determination

The proline content was determined according to the method of Bates *et al.*[16]. Approximately 0.5 g of the plant material was homogenized in 10 mL of 3 % aqueous sulphosalicylic acid and the homogenate centrifuged. Two mL of the extract was then treated with 2 mL of acid-ninhydrin and 2 mL of glacial acetic acid in a test tube for 1 h at 100 ℃ and the reaction terminated in an ice bath. Next, the reaction mixture was extracted with 4 mL and mixed vigorously for 15-20 sec. The chromphore containing toluene was separated and the absorbance read at 520 nm. The proline concentration was then calculated on a fresh weight basis.

All the experiments were conducted at least twice, with triplicate measurements for each treatment. All the values were means of at least 4 replications.

3. Results and Discussion

The visual symptoms of chilling damage ranged from a slight reduction in growth to a complete browning and decay of the entire seedling, depending on the severity of the chilling treatment. The chilling damage in the cucumber seedlings was found to be dependent on both the temperature and the duration of the exposure. Survival was unaffected by chilling stress less severe than 5 ℃ for 7 d, and the shoot dry weights decreased with

an increase in degree of chilling stress(Table 1). Acclimation (Ac) significantly affected the survival and shoot dry weights. The effect on survival was most evident with the 5 ℃ for 7 d treatment where survival with and without Ac was 78 % and 20 %, respectively. In terms of the dry weight, the Ac seedlings grew better than the control seedlings when the treatment involved sufficient stress to significantly affect growth. Treatment with more than 500 μM SA had a significant effect on the growth and survival of the seedlings exposed to 4 ℃ for 7 d(Table 2).

Table 1. Effects of acclimination, chilling temperature and chilling duration on survival and shoot dry weight of chilled cucumber seedlings

Temperature	Duration (d)	Percentage survival		Dry wt(mg)/ seedling	
		Ac	Non-ac	Ac	Non-ac
4℃	2	97	95	99	102
	4	97	93	70	44
	7	67	21	28	19
	10	12	3	25	13
5℃	2	96	97	120	84
	4	95	90	117	61
	7	78	20	47	28
	10	15	3	29	14
7℃	2	96	98	103	93
	4	97	97	78	62
	7	97	88	56	26
	10	90	52	38	31
Unchilled control		98	98	125	112

Table 2. Effect of exogenous SA treatment at different concentrations on survival and shoot dry weight of chilled seedlings

Treatment	Percentage survival	Dry wt(mg)/ seedling
0	1	22
100 μm SA	28	33
500 μm SA	45	57
1000 μm SA	66	80

The data in Table 3 illustrate the acclimation phenomenon experienced by the cucumber seedlings. Only 3 % of the nonacclimated seedlings survived chilling at 4 ℃ for 7 d compared to 68 % of the acclimated seedlings exposed to 14 ℃ for 3 d before chilling. The nonacclimated seedlings did not grow during the 10 d in the growth chamber, whereas the fresh and dry weights for the acclimated shoots increased nearly threefold. As such, acclimation and SA treatment were found to provide some degree of protection from chilling, and the exposure of the seedlings to the 14℃ acclimation temperature induced numerous changes that acted in concert to increase the chilling tolerance.

Table 3. Effects of acclimation, nonacclimation, SA and H_2O_2 treatments on the growth of cucumber seedlings

Treatment [a]	Conditions [b]	Percentage survival	Shoot	
			Fresh wt(g)	Dry wt (g)
NAc	I		0.77	0.08
	F	2.5	0.56	0.13
Ac	I		1.11	0.10
	F	68.0	3.05	0.30
SA	I		0.73	0.07
	F	57.8	2.27	0.24
H_2O_2	I		0.75	0.07
	F	58.0	2.71	0.29

[a] Ac, acclimation ; NAc, nonacclimation
[b] I, at the end of 4 ℃ treatment ; F, at the end of 10 days

The addition of H_2O_2 to the young seedlings before the cold treatment produced relatively similar results to those with SA(Table 3), i.e. an increased chilling tolerance without cold acclimation, probably due to the increased ability of certain antioxidant enzymes[5,18]. H_2O_2 activates SA biosynthesis in a catalase-mediated reaction[19]. Antioxidant systems play an important role in protecting plants against stress-induced oxidative damage[20]. Therefore, from the current results it can be assumed that the increase in the H_2O_2 level occurred due to a decrease in the catalase activity after 1 d of SA pretreatment under normal growth conditions, thereby increasing the activity of other

antioxidant enzymes along with the chilling tolerance in the young cucumber plants.

The inhibition of seedling growth and survival by cycloheximide(CH), an inhibitor of protein synthesis, dramatically affected the ability of the Ac seedlings to tolerate chilling stress, as shown in Table 4. For the unchilled seedlings, the CH treatment had no effect on survival, although the seedling weights were significantly reduced. AcCh seedlings showed a significant reduction in survival when treated with CH. The exogenous application of SA to the CH-treated seedlings restored their ability to survive chilling stress. SA applied at 4℃ provided some chilling tolerance, yet less than when it was applied at the time of planting. Similar results have been reported for maize[17]. However, 1 d of 0.5 mM SA pre-treatment was found to decrease the net photosynthesis, stomatal conductivity, and transpiration at the growth temperatures. As such, these results indicate that SA is essential for survival in the face of chilling stress, and SA and acclimation have a common effect. Yet no conclusive evidence was found that the acclimation process was mediated by SA. Although SA improved the survival of the chilled seedlings, it was not as effective as acclimation in inducing chilling tolerance. Thus, it is possible that SA-independent events occurred during acclimation that contributed to the observed level of chilling tolerance. In addition, the fact that CH abolished the acclimation-induced chilling tolerance does not exclude the possibility that SA and acclimation operate through separate mechanisms, both of which are required for the survival of chilled seedlings.

Table 4. Effect of SA and cycloheximide on survival and shoot dry weight of chilled cucumber seedlings exposed to 4 ℃ for 7 days

Treatment [a]	Percentage survival		Dry wt(mg)/ seedling	
	No CH	CH	No CH	CH
Cont	98	98	124	48
AcCh	79	12	37	18
AcSA	87	68	42	24
SACh	58	52	25	20
SA@4	40		27	
Ch	15		22	

[a] Cont, control ; AcCh, acclimated and chilled ; AcSA, SA treated then acclimated and chilled ; SACh, SA treated and chilled ; SA@4, SA treated at 4 ℃ ; Ch, chilled.

The proline levels found in the control, Ac, Ch, and AcCh shoots and at various times during acclimation are given in Fig. 1. Chilling caused a large increase in the free proline levels, regardless of the acclimation status. However, very little proline accumulation occurred during acclimation. Marginal increases in proline occurred during the first hours of acclimation, followed by a decline to the control level, however, the differences among the proline levels during the acclimation process were not statistically significant. The involvement of a rapidly increased and high level of proline has been suggested as an important prerequisite for chilling tolerance. Therefore, to investigate the effect of SA via proline, the proline level was analyzed in the shoots and roots (Fig. 2). The proline content increased during the chilling period in both the shoots and roots, however, there was a significant difference between the SA-treated and control plants. During the recovery phase, the proline levels decreased in a similar way in the shoots and roots of the controls and the roots of the SA-treated seedlings.

The extent of the necrotic injuries experienced by the whole seedlings and third leaves estimated after chilling was very similar, thus data is only given for the whole seedlings(Fig. 3). The chilling of the non-acclimated seedlings caused injuries that increased linearly with the chilling duration. The injuries experienced by the acclimated seedlings

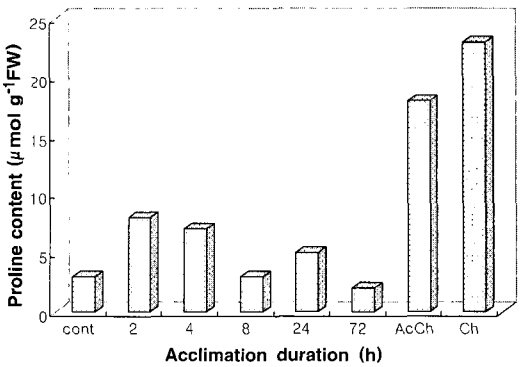

Fig. 1. Proline contents in shoot of cucumber seedlings exposed to various acclimation and chilling treatmetns.

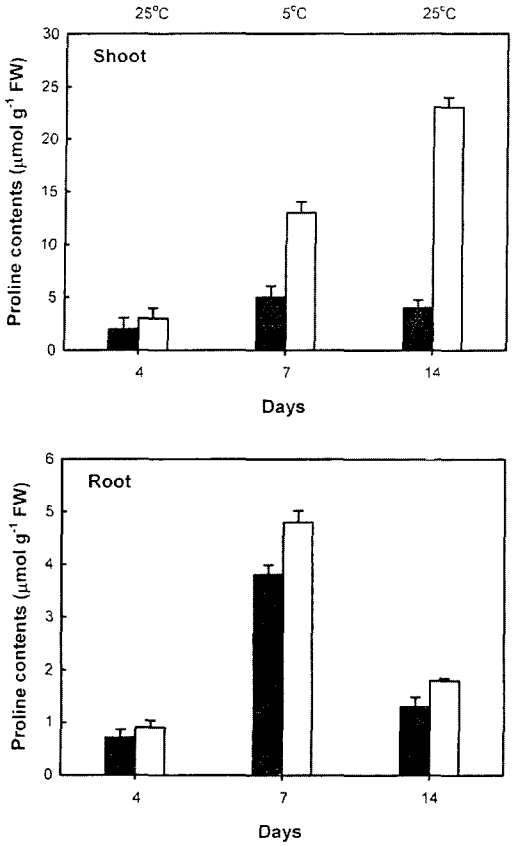

Fig. 2. Proline contents in shoot and root of cucumber seedlings cultivated with 0(open bars) or 500 μM SA(black bars) at 25 ℃ for 3 days after chilling for 7 days(14).

were on the average smaller by half than those experienced by the non-acclimated seedlings. During the chilling of the non-acclimated seedlings, the proline content increased in the third leaf, and continued to increase with further chilling(Fig. 4). Until the 2nd day of chilling, the increase in proline was more intensive in the nonacclimated seedlings compared to the acclimated seedlings. During the chilling of the acclimated seedlings, the proline content increased gradually with the duration of the chilling after a lag phase.

Acclimation only had a marginal influence on the proline content, yet markedly increased the chilling tolerance. The higher chilling tolerance of the acclimated seedlings was also accompanied by the ability for greater proline accumulation during chilling. This observation is consistent with previous findings that acclimation provides some degree of protection from chilling[2]. Although the action mechanisms of proline under stress conditions have not yet been fully explained, the current results indicate that endogenous proline clearly played a role in the chilling tolerance of the seedlings by stabilizing the water status, plus the higher chilling tolerance was related to the ability for greater and faster proline accumulation in response to chilling. To a certain degree the action of SA would seem to be similar to the effect of other regulatory molecules, e.g. jasmonic acid and ABA, on the processes of germination, growth, and ageing. The established effect of SA on the stomatal function, chlorophyll content, transpiration rate, and respiratory pathways raises the assumption that SA may posses another physiological function, most probably involve in the regulation of certain photosynthetic reactions. Thus, although the current results point to separate pathways of induction for SA-induced and

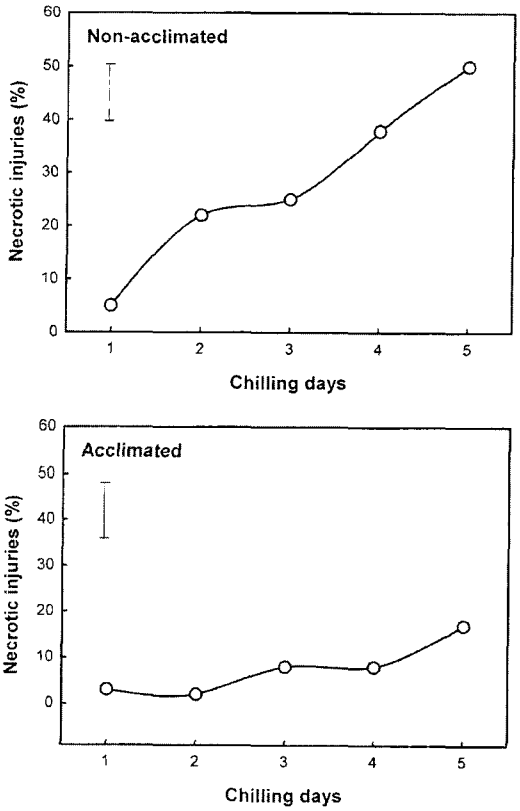

Fig. 3. Time course of necrotic injury development in the third leaf during chilling(5℃) in non-acclimated and acclimated seedlings.

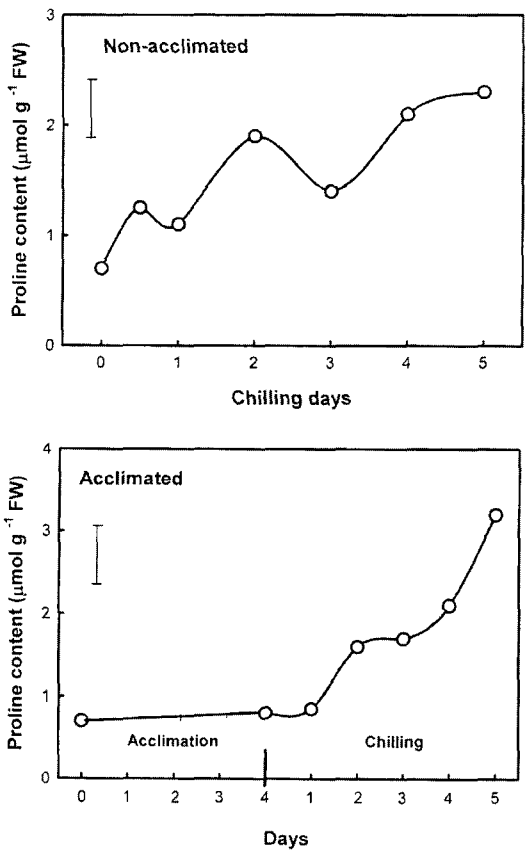

Fig. 4. Changes in proline contents in the two-week-old seedlings during chilling(5 ℃) in non-acclimated and acclimated seedlings.

acclimation-induced chilling tolerance, the possibility that SA plays a role during acclimation cannot be ruled out.

Two-week-old cucumber plants were chilled at 5 ℃ in the light or dark and the endogenous proline contents examined during a three-day chilling treatment(Fig. 5). There was a continuous increase in the proline level, which was more pronounced in the light than in the dark. When SA was added to the plants during the chilling treatment in the light and dark at a normal growth temperature, the proline content increased more in the light than in the dark. A low temperature and bright light can cause photoinhibition in the leaves of chilling-sensitive plants. When such plants are chilled in the light, the injury is either more severe or occurs sooner than if the same plant had been chilled in the dark. Chilling-sensitive plants can also be injury by dark chilling, yet in this case the damage is of a different nature. At low temperatures, both the cold and the light played a role in the chilling injury of the cucumber plants. Chilling stress in cold-sensitive maize plants was previously found lead to an increased susceptibility to photo-inhibition at low temperatures, and photoinhibition also had a role in the appearance of post-chilling symptoms[21]. However, the current study found that light had an influence on both the photosynthetic processes during chilling stress and other stress markers, such as free amino acids, determining the level of damage caused by chilling.

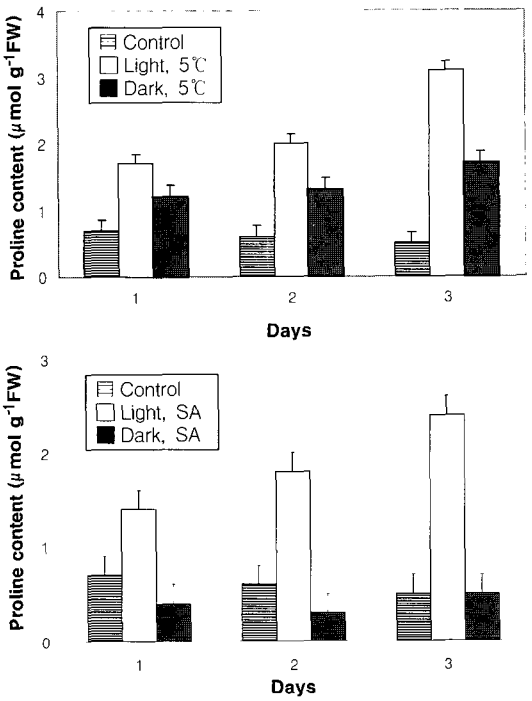

Fig. 5. Changes in proline contents in leaves of two-week-old cucumber seedlings grown at 25 ℃(control conditions) after 1,2 or 3 days of 5 ℃ cold treatment (A) or SA treatment during chilling(B) carried out in the light or in the dark.

The adaptive significance of higher proline accumulation in plants during environmental stress remains uncertain. Proline has already been shown to accumulate in the tissue/organs of plants subjected to water deficiency, high salinity, chilling, low and high temperature stress, heat, and heavy metal exposure[22]. Proline also plays a major role in osmoregulation and osmotolerance[23]. A recent

paper by Verslues and Sharp[24] attempted to explain apparently conflicting results by showing that, in stressed maized plants, the proline accumulated in the growing zone of the primary root was imported rather than synthesized in these tissues. This explanation is also in accordance with the fact that proline is not necessarily synthesized in tissues achieving osmotic adjustment[24]. Thus, it is questionable whether there is any adaptive value for proline synthesis and its accumulation in dehydrating tissues.

A dual role for SA has been proposed[25]. SA is necessary for the induction of antioxidant defences and essential for plant protection against the oxidative stress generated by O_3. SA accumulation can induce a programmed cell death pathway, leading to a hypersensitive reaction in response to O_3. The current authors previously showed that SA increases the oxidative damage generated by NaCl or osmotic stresses, which in turn is critical for seedling lethality[26]. Meanwhile the present study showed that SA is directly involved in the changes that take place in a plant under chilling and low temperature stress. Therefore, according to the current results, the apparent role of SA was potentiating the stress response of the cucumber seedlings during cold stress, which in turn resulted in a strong accumulation of proline.

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