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Effects of Low Temperature on Plant Growth and Their Protein Patterns in Phloem Exudates of *Luffa cylindrica*

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ABSTRACT: One-week-old seedlings of *Luffa cylindrica* were grown in phytotron at 15, 20, 25 and 30 ℃ for four weeks. Growth curves of the plant organs were compared with their fresh weight and size during the cultured period. Isozymes of defensive enzymes and phloem proteins were measured on elctrophorogram of native gel and 2-dimensional gel, respectively. Low temperature not only retards plant growth, but also increases the ratio of root/shoot. Leaf blades of plants grown at 25 and 30 ℃ have typical sigmoid growth curve but with different slope factors. Plants grown at 15 ℃ do not show the normal sigmoid curves in leaf growth. Isozyme patterns in plant grown at 15 ℃ are changed as follows: two minor bands in group II isozyme of acid phosphatase were induced in green leaf blades and cotyledons; group II isozymes of peroxidase were enhanced in hypocotyls and cotyledons; group III isozyme of acid phosphatase was induced in leaf. All isozyme patterns show the specificity in tissues and developmental stages. One of phloem proteins with molecular weight of 31.5 kDa and pI at 4.9 was induced in plants grown at 15 ℃.

KEY WORD: Acid phosphatase, Peroxidase, Proteins, SOD, *Luffa cylindrica*.

INTRODUCTION

Low but non-freezing temperature has a severe and ubiquitous effect on the growth and metabolism of tropical and subtropical plants (Levitt, 1980). In low temperature, less available metabolic energy, restriction of water and nutrient uptake, less biosynthetic assimilation and retardation of growth were observed (Farrar, 1988). The major physiology processes influenced by low temperature were reported in areas of growth and developmental stage, root structure and function, photosynthesis, respiration, partitioning, and reproduction (Haellgen and Oequist, 1990). Short term effects often result in the imbalances of partial process of metabolic pathways. The longer the period of low temperature, the more severe the consequence is (Larcher, 1995). In Taiwan, chilling injury is a major limiting factor for the growth of annual plants.

The effect of low temperature on partitioning of source/sink is related to its effect on root/shoot relationship (Clarkson *et al.*, 1988). The accumulation of carbohydrates in both sources and sinks at low temperature is due partially to the greater sensitivity of growth in comparison to photosynthesis at low temperature. Differential sensitivity of enzymes and

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reduction in phloem transport are of primary importance for partitioning. Within sources and sinks, enzymatic processes must dominate the response to altered temperature, but very little is known about the temperature sensitivity of individual enzyme. Meanwhile, reduced glutathione (GSH) has a role in resistance to low-temperature stress. The presence of several isozymes of glutathione reductase in spinach and two isozymes in pea emphasizes that low temperature stress affects on particular isozyme (Bielawski and Joy, 1986).

Ethylene production is stimulated in the tissues of plants with subtropical or tropical origin when exposed to temperature below 10-13℃ (Wang *et al.*, 1990). Early studies show that translatable factors promote or inhibite the development of plants (Carter and Brenner, 1985). Low temperature enhances the ABA and ethylene production, whereas reduces the biosynthesis of gibberellic acids (Morgan, 1990). Enzyme changes during cold treatment are comprehensively different in isozymes, secondary modification, electrophoretic mobility and kinetic parameters. (Tseng and Li, 1987).

In *Luffa cylindrica* phloem exudate contains higher protein content than that the homogenate of non-vascular tissues. Besides seasonal change of protein content in phloem exudate was found (Wang and Chen, 1997). In this study, patterns of phloem proteins and isozymes of acid phosphatase, peroxidase and superoxide dismutase in plants grown under low temperature were examined.

MATERIALS AND METHODS

Plant materials

Seeds of *Luffa cylindrica* var. Chung-chang were sowed and germinated in a vermiculite-containing pot. After germination, young seedlings were transferred to different culture rooms of a phytotron with temperatures at 30, 25, 20, and 15℃ for four weeks. The growth of leaf blades, stems and roots was measured. At the same times, plant materials were collected for further experiments.

Protein content of phloem exudate

Different plant organs were transversely cut off with a razor blade. Phloem exudates were collected with pipetman and mixed well with same volume of collection buffer containing 10 mM Tris and 0.13 M β-mercaptoethanol. Meanwhile, collecting buffer was also applied on surface of wounding cut for further bleeding of phloem exudates.

The protein contents of phloem exudate were determined with Coomassie Brilliant Blue G 250-binding method (Bradford, 1976), using bovine serum albumin as a standard.

Isozyme analysis

Crude extracts of plant tissues were electrophoretically separated with 7.5% polyacrylamide gel and 0.38 M glycine buffer (pH 8.3). Gel thickness was 0.75 mm and voltage was at 200 V. For detection of acid phosphatase, the stainer contained 0.2 M sodium acetate buffer (pH 5.0), 0.1% fast red TR salt, 5 mM $MgCl_2$ and 0.1% α-naphthyl phosphate (Hooley, 1984); for peroxidase, the reaction mixture contained 0.1% benzidine, 0.1% H_2O_2 and 0.2 M phosphate buffer at pH 7 (Clare *et al.*, 1984); and for superoxide dismutase (SOD),

the method of Fridovish's group (Clare *et al.*, 1984) was followed. After staining, gels were destained, photographed, and dried.

Two-dimensional gel electrophoresis

The separation of phloem proteins followed the method of O'Farrell (1975) with some modification. Brief descriptions of first dimensional separation were as follows: glass tubes (0.5 x 11 cm) pre-casted with isoelectrofocusing (IEF) mixture were added 20 μl of 0.1% Pyronin Y on top and were pre-run in disc IEF apparatus at 100 V for 15min, 300 V for 30 min and 400 V for 30 min; after the formation of pH gradient in tube, protein samples were applied on top of each tube and further separated in IEF apparatus at 250 V for 17 h and 800 V for 1 h; then, gel columns were forced out with hydraulic water and equilibrated twice with SDS-denatured buffer for 45 min and 15 min, respectively. For second dimensional separation, equilibrated gels were sealed with 15 % SDS gel by using 0.75% agarose gel and run in a electrophoretic apparatus at 100 V for 9 h. Protein bands on gel were shown by silver staining after proper washing (Oakley *et al.*, 1980).

RESULTS

Effect of low temperature on growth

L. cylindrica is the tropical and creepy annual vines with alternate leaves in the open field. The one-week-old seedlings with epigeous, oval and persistent green cotyledons are germinated from submerged seeds. Later, the epicotyl apex produces leaves, nodes and internodes (Figs. 1A-D). The growth of seedlings was influenced by low temperature. The one-week-seedlings grown at 30 and 25℃ had the first new leaf rising from shoot apex. Leaf numbers on each seedling were increased with the rising of the test temperature. The elongation of hypocotyl was also inhibited by low temperature. In five-week-old seedlings, the lengths of hypocotyl at 30, 25 and 20℃ are 4.5, 3.1 and 1.8 times longer than that of 15 ºC, respectively (data not shown). Trichomes covered the whole plant body of seedlings. The lower the tested temperature is, the higher the density of trichomes on leaves and shoots is (Figs. 1A-D).

Low temperature affected the growth of roots and shoots. However, the affected patterns of low temperature on root and shoot growth were quite different (Table 1). in the test period the best growing temperature for shoot was 30℃, whereas for roots was 25℃. However in the tested temperatures, 15℃ was the poorest for both root and shoot growths. Whole plants of seedlings grown for four weeks at 15 and 20℃ were only one tenth and one third of the plants grown at 30℃ in fresh weight, respectively. Moreover, the ratio of root/shoot was increased as seedling-age extended (data not shown). Meanwhile, low temperatures (15 and 20℃) affect on ratios of root/shoot was more prominent than that of higher temperature (30 ℃). It indicated that low temperatures inhibited shoot growth more prominently than root growth.

The leaves of *L. cylindrica* are penta-lobed palmate. Low temperatures influenced the growth and external feature of leaf blade. Seedling leaves grown at 15℃ was characteristic with the densely trichomes on leaf surface, the short and stunt petiole, and the dark green blades with curving and folding from their margin (Fig. 1D). The growth patterns of leaves

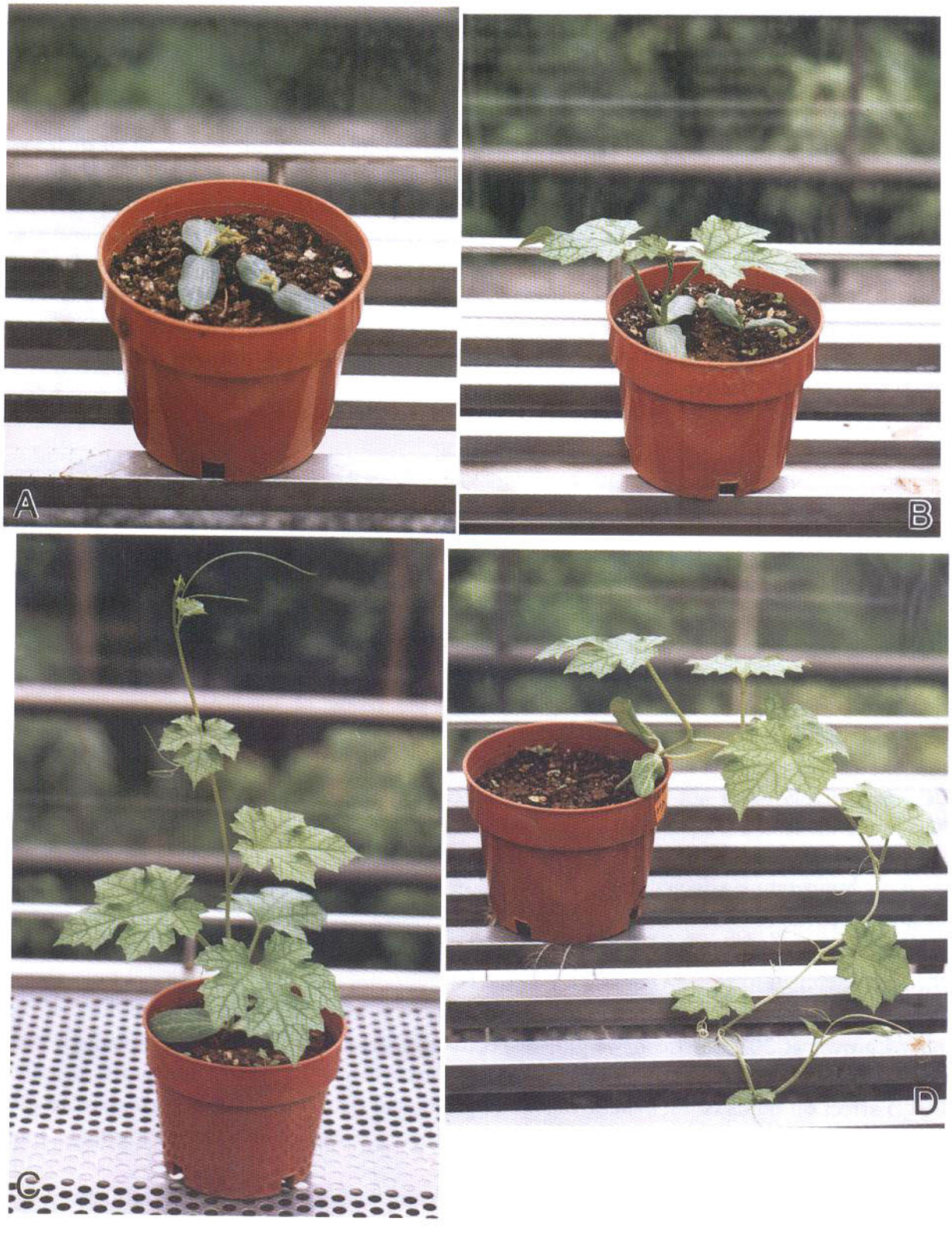

Fig. 1. The external feature of 5-week-old seedlings grown at 15℃(A), 20℃(B), 25℃(C) and 30℃(D).

Table 1. The effect of temperatures on the growth of *Luffa cylindrica* seedlings* in-5-week-old.

Seedlings grown at (℃)	Shoot length (cm)	Fresh weight			
		Shoot (g)	Root (g)	whole plant (g)	Root/Shoot
15	8.3	1.4	0.7	2.1	0.56
20	34.1	6.4	3.4	9.8	0.53
25	102.8	24.4	7.9	32.3	0.32
30	133.3	26.4	5.8	32.3	0.22

*All test samples were triplicate.

at different temperatures were shown in Figs. 2A-D. All leaf blades of young seedlings grown at 30 and 25℃ did show sigmoid curve in their growth patterns. Leaf blades of 20℃ grown seedlings with parts of non-regular sigmoid curve and parts of non sigmoid curve were observed. Meanwhile, leaf blades of 15℃ grown seedlings were all non sigmoid-shaped curve and had much factors in their growth pattern. All the observed phenomena showed that the analysis of leaf growth was tremendously hindered by low temperature.

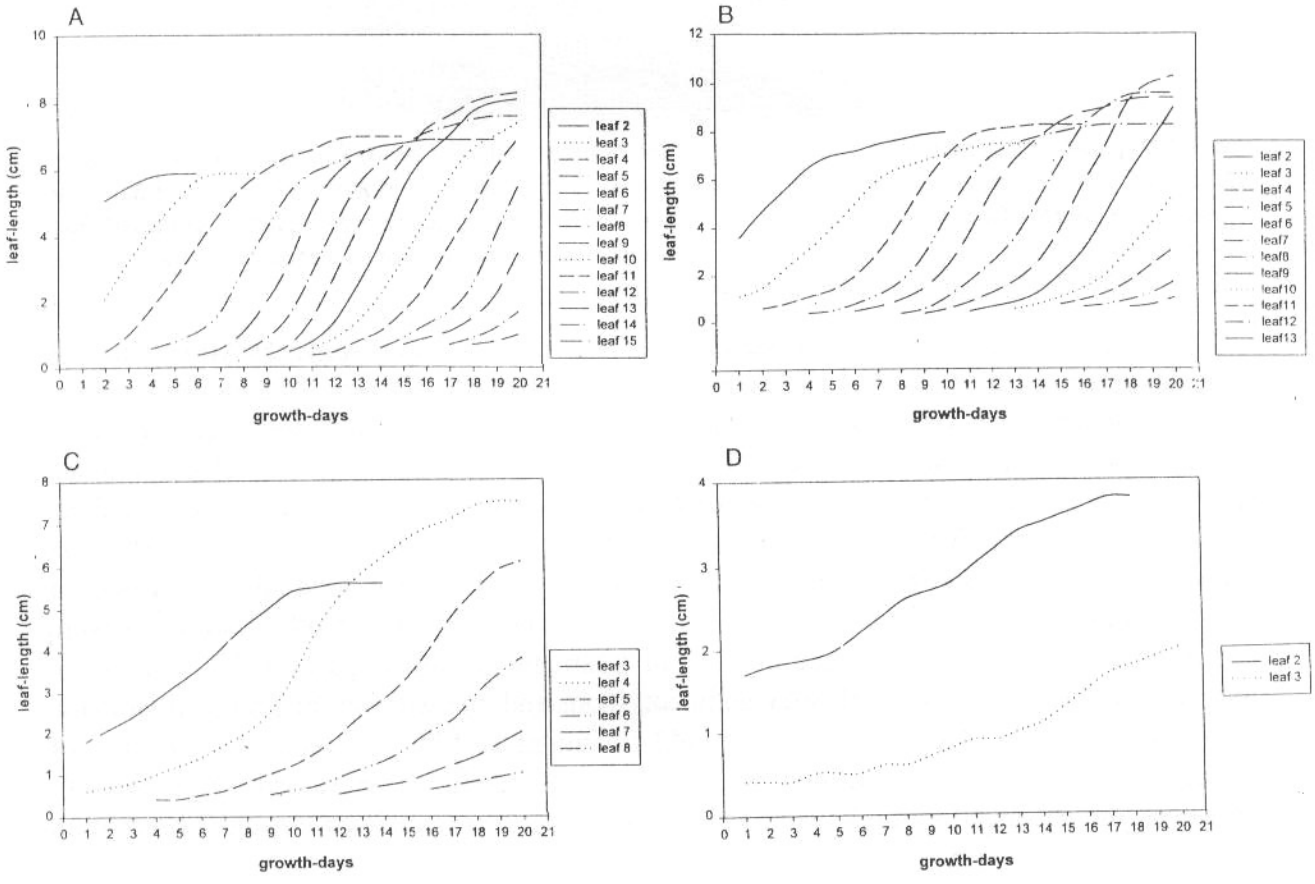

Fig. 2. The growth patterns of leaf blades on seedlings grown at different temperatures. 30℃(A), 25℃(B), 20℃(C) and 15℃(D).

Effect of low temperature on isozymes and phloem proteins

Four groups of peroxidase isozymes identified in electrophorogram were as follows: group I isozyme with single band; group II isozyme with two bands; groups III isozyme with 3 to 5 bands depending on sources of plants tissues; group IV isozyme with 1-2 minor bands. Root peroxidases with strong activity containing most of isozymes (Fig. 3). Group II and group IV isozymes were root-specific specificity. There was no much difference in isozyme patterns of leaf blades, cotyledons and hypocotyles. Only cotyledon contained one extra band in group III isozyme. Low temperature (15℃) only impressed the activities of group III isozymes in hypocotyls and cotyledons. However, a fast moving band and a slow moving band of group III isozymes in cotyledons were great enhanced by cold treatment.

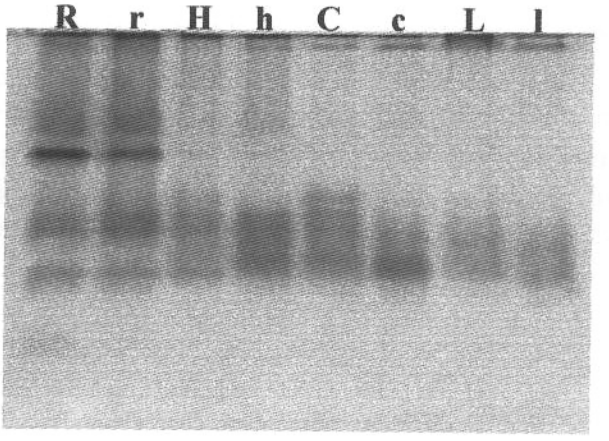

Fig. 3. Comparison of isoperioxidase in phloem exudate of different organs in seedlings grown at 25℃ and 15 ℃. Lanes R and r are roots collected from seedlings grown at 25℃ and 15℃ respectively. Lanes H and h are hypocotyles collected from seedlings grown at 25 ℃and 15 ℃. Lanes C and c are cotyledons collected from seedlings grown at 25℃ and 15℃. Lanes L and l are leaves blades collected from seedlings grown at 25℃ and 15℃. About 10 μg of protein have been applied on each well.

There were three groups of acid phosphatase isozymes. Group I isozyme (AP I) with 1 to 3 bands, group II (AP II) with 1 to 2 bands and group III (AP III) with 2 bands were shown on electrophorogram (Figs. 4A-D). As shown in Fig. 4A, organ-specificity of acid phosphatase isozymes was prominent. The leaves and cotyledons in green color contained only group I and II isozymes, whereas roots and hypocotyles in white color had all three groups of isozymes. Meanwhile, both cotyledons and leaves in one-week-old seedlings grown at 25℃ were deficient in AP II-1. Group III isozyme was gradually decreased in enzyme activity of growing seedlings (Figs. 4A-D). The activities of other isozymes were not influenced by the age of seedlings. Cold treatment of seedlings at 15℃ resulted in inducing production of group III isozyme in cotyldons and intensifying the group III isozyme in hypocotyles (Fig. 4A). The effect of cold treatment at 15℃ on group III isozyme of cotyledons was gradually declined with the age of seedlings increased (Figs. 4A-D). However, the enhanced group III isozymes in hypocotyl were persisted through observation period.

Three groups of SOD isozymes were shown on electrophorogram (Fig. 5). Group I isozyme with one major band and two minor bands, group II isozyme with two major bands and two to four minor bands, and group III isozyme with one major band and one minor

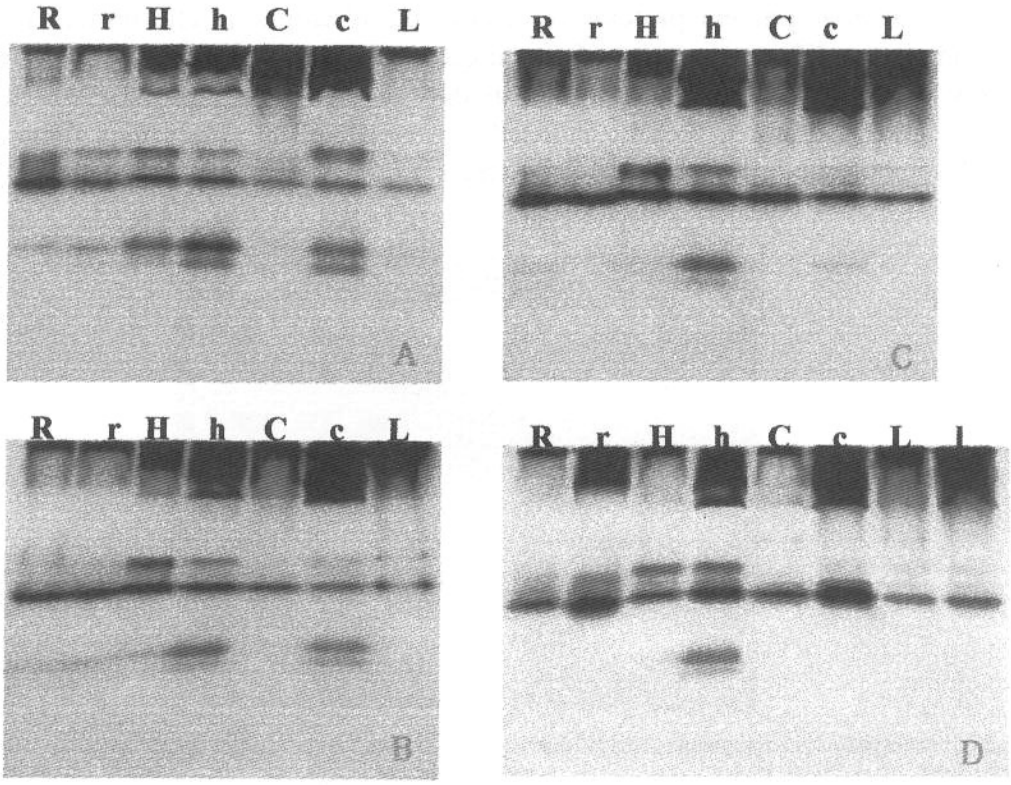

Fig. 4. Effect of low temperature and developmental stage on acid phosphatase isozyme in phloem exudate of different organs in seedlings grown at different temperatures. A, phloem exudate from 2-week-old seedlings. B, phloem exudate from 3-week-old seedlings. C, phloem exudate from 4-week-old seedlings. D, phloem exudate from 5-week-old seedlings. Lanes R and r are roots collected from seedlings grown at 25℃ and 15℃ respectively. Lanes H and h are hypocotyles collected from seedlings grown at 25℃ and 15℃. Lanes C and c are cotyledons collected from seedlings grown at 25℃ and 15℃. Lanes L and l are leaves blades collected from seedlings grown at 25℃ and 15℃. About 10 μg of protein had been applied on each well.

band were found in tissue extracts of seedlings. All organs contained three groups of isozymes. However, root SOD had two additional tissue-specific bands appeared in group I isozyme and two minor bands in group II isozyme too. Meanwhile, leaf extracts had only one major band in group I and two major bands in group II isozymes. Seedlings grown at 15 ^{o}C enhanced two slow-moving minor bands in group II isozyme in cotyledons and it also induced the formation of two fast-moving minor bands in leaves. However, the isozyme pattern of SOD was not affected by the developmental stage of seedlings during culture period (data not shown).

Two-dimensional electrophorogram of proteins in stem phloem exudate showed that there were three major groups of proteins (Fig. 6A). Group I proteins have 7-10 proteins with the molecular weight between 36 and 55 kDa and pI between 4.5 and 6.1. Group II proteins aggregated together into a mixture group with the molecular weight between 25 and 31 kDa and pI between 3.9 and 4.4. Group III proteins with the molecular weight smaller than 24 kDa could be further divided into two sub-groups. Group III-1 proteins had the molecular weight between 17 and 24 kDa and pI between 3.9 and 4.2. Group III-2 proteins contained five proteins with their molecular weight at 19 kDa and pI above 5.8. Seedlings grown at 15

and 25℃ had similar phloem proteins on 2-D electrophorogram. The protein patterns in group II and groups III proteins were changed qualitatively rather than quantitatively, even though low temperature treatment resulted in the decrease of protein content in phloem extracts. However, a low temperature induced a protein with the molecular weight of 31.5 kDa and pI 4.9 in group I protein of phloem extract (Fig. 6B, arrowed).

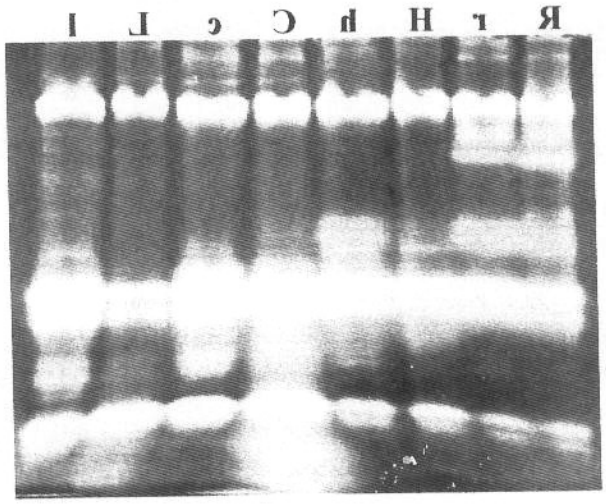

Fig. 5. The isozyme patterns of superoxide dismutase in phloem exudate of different organs in seedlings grown at 25℃ and 15℃. Lanes R and r are roots collected from seedlings grown at 25℃ and 15℃ respectively. Lanes H and h are hypocotyles collected from seedlings grown at 25℃ and 15℃. Lanes C and c are cotyledons collected from seedlings grown at 25℃ and 15℃. Lanes L and l are leaves blades collected from seedlings grown at 25℃ and 15℃. About 10 μg of protein have been applied on each well.

DISCUSSION

Tropical and subtropical plants have less capacity to tolerate low temperature. Once plants exposed external temperature down to 10-12℃ for a period, it results in the metabolic disfunction and the plant even dies later (Haellgen and Oequist, 1990). Young seedlings of *L. cylindrica* showed a typical growth retardation at 15℃. However, for the growth of this tropical plant 15℃ is the sub-lethal temperature. Mature plant grown at 25℃ for 110 day is flowering, whereas plants grown at 15℃ for the same days still keep in stunted and upright vegetative body with short interned and curving leaves (data not shown). The elongation of stem and the retardation of the growth of leaf retarded by low temperature were often reported (Simillie, 1976; Haellgen and Oequist, 1990). However, the mechanism of low temperature affecting on flowering is still unknown.

Different parts of plant growth inhibited by sub-lethal low temperature in various ways were observed in tropical and subtropical plants. Low temperature and low nutrient availability gave a higher root/shoot ratio in weight (Osmond *et al.*, 1980). The inhibition of nutrient uptake at low temperature was partially accountable for increasing the root/shoot ratio (Chapin, 1980). The increasing size of root is partially compensated for the decreasing nutrient uptake rate at low temperature. Seedlings of *L. cylindrica* with higher root/shoot ratios at 15, 20 and 25℃ than that at 30℃ was observed in this study. It might provide a model system for studying annual plants in response to low temperature.

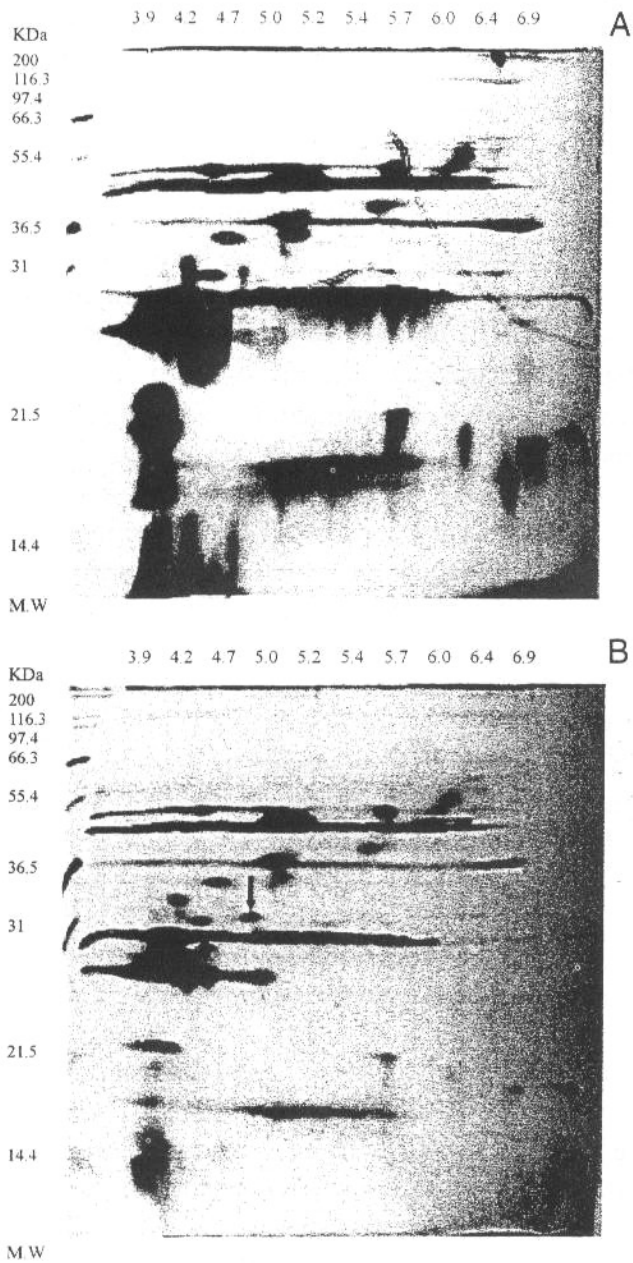

Fig. 6. Electrophorogram of two-dimensional gel for separation of protein patterns in phloem exudate of seedlings grown at 25 ℃ (A) and 15 ℃ (B). The low temperature induced protien is arrowed in B. About 150 μg of protein have been applied in each electrophorogram.

Seedlings were more sensitive to low temperature than the adult plants. The effects of low temperature on vegetative organs are easily observed in the field and in the laboratory (Haellgen and Oequist, 1990). Leaf development in seedlings is one of the best marker for tracing the effect of low temperature (Simillie, 1976). In *L. cylindrica*, 20℃ was the turning-point temperature for the normal growth of leaf blades with sigmoid curve. Seedlings grown above 20℃ showed the typical sigmoid curves with different slope factors. At 15℃, the growth curve of leaf blade was non sigmoid one. Choose right temperature is very important for growth analysis of plant vegetative growth.

Low temperature affects plants on the decrease in root hydraulic conductivity and stomatal closure and thus results in the decrease in transpiration (Running and Read, 1980). As the temperature decreases, water viscosity increased and permeability of root cells to water reduced. In chilling sensitive plants, an abrupt decrease in water conductivity at low temperature was observed (Berry and Raison, 1981). All the above facts indicate that the reduction of water flow through the roots has the consequence of the decrease of stomatal conductance in the short-term observation. For long-term adaptation, the formation of folding leaves and dense trichomes on the surface of *L. cylindrica* might also provide good way for preventing excessive water loss from plant body.

The functions of peroxidases related to IAA oxidation, lignification, defensive enzymes and differentiation had been documented (Scandalios, 1974). The biosynthesis of peroxidase is under the internal hormones and phytochrome control (Lee, 1972; Penel *et al.*, 1976) and

outside stress regulation (Tao and Khan, 1976). However, only some isozymes are functional for cell wall lignification (Gaspar *et al.*, 1991; Siegel, 1993). Group III isozymes in cotyledons and hypocotyl induced by low temperature may relate to strengthen framework of plant body (Anderson *et al.*, 1995). In *L. cylindrica* group III isozymes also related to the formation of stunt stem, thick hypocotyl and short petioles. Further studies on cytochemical localization of these isozymes are needed.

Changes in isozyme pattern of acid phosphatase were found in hormonal treatment (Gabard and Jones, 1986) and environmental stress (Gabbreilli *et al.*, 1989). Inactivation of extra-cytoplasmic acid phosphatase treated at low concentration of NaF was found in yeast-like cell of *Sporothrix schenckii.* Halperin (1969) demonstrated that only in senescent cell of carrot acid phosphatase was cytochemically localized in the nucleus. In ripening tomato grown at 33℃, enzyme activity of acid phosphatase decreased sharply, whereas declined slowly at 22℃ (Shirai *et al.*, 1984). In present study, different isozymes of acid phosphatase in *L. cylindrica* seedlings had different properties in response to low temperature. The sensitivities of different plant parts in response to low temperature were as follows: cotyledons > hypocotyls > roots > leaf blades. Group III isozymes in seedlings were induced by low temperature. Inducible isozymes of acid phosphatase were also reported in germinating seedling of maize (Teno *et al.*, 1987) and triticale (Ching *et al.*, 1984).

In the present study, low temperature (15℃) induced two minor bands in group II isozyme of SOD in cotyledons and hypocotyls of *L. cylindrica.* It might result from the interaction of cold temperature and light and was correlated with the increasing scavenger activity of ascobate-glutathione cycle. The SOD expression in response to environmental changes and oxidative stress was well documented (Hassan and Scandalios, 1990). Leaves of tobacco albinos under light stress had higher acitvity of Mn-SOD and higher rate of aerobic repiration than that of green leaves under dim-light (Bowler *et al.*, 1989); Under strong light, the activity of Fe-SOD in plastid of tomato was greatly enhanced (Tsang *et al.*, 1991); Under $CuSO_4$ treatment, the activity of Cu, Zn-SOD in soybean was increased (Praphasri *et al.*, 1992). The expression pattern of isozymes in SOD could provide a good indicator for oxidative stresses.

The protein contents of phloem exudate obtained from pepo of *Cucurbita* spp were higher than that from stem (Alosi *et al.*, 1989). Similar results found in *L. cylindrica* were as following order: pepo > petiole > stem (Wang and Chen, 1997). There were four groups of proteins on one-dimensional SDS-gel (Chang, 1995). Two-dimensional electrophorogram stained with silver-staining provided a better resolution for phloem protein separation (Lin, 1997). Most of investigated phloem proteins were lectins (Behnke and Sjolund, 1990). However, recently α-amylase (Wang *et al.*, 1995), thioredoxin h (Ishiwatari *et al*, 1995), trypsin inhibitors (Murrary and Christeller, 1995) and transduction factors (Kessmann and Ryals, 1993) were identified in phloem tissues. In the present study, the well-resolved phloem proteins on 2-D electrophorogram may provide a source for determining N-terminal amino acid sequence and molecular cloning.

The enzyme changes and soluble protein synthesis under cold treatment were observed in many species. Differences in isozymes, secondary modification, and cold stability have also been studied (Tseng and Li, 1987). Under low temperature, not only the amount of Rubisco was significantly increased, but also structural change with improved catalytic activity was

established in some cold acclimated species (Berry and Bjoerkman, 1980). Further studies on long-term effects of low temperature are needed.

ACKNOWLEDGMENT

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低溫逆境下絲瓜的生長及其韌皮部蛋白質類型的的變化

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摘　　要

一週大的絲瓜 (*Luffa cylindrica*) 幼苗置於人工氣候室內，分別在 30, 25, 20 及 15℃下處理四週後，以鮮重量及大小為生長指標，同時以單向電泳活性檢定法及雙維電泳分析法，調查植株體上抗性同功酵素與韌皮部蛋白質的類型變化。低溫處理不但抑制植物的生長，同時也增高根/莖比值。在 30, 25℃下植株的葉片生長曲線都呈 s 形，然而 15℃下則為非 s 形。植物長在 15℃下其抗性同功酵素呈出：綠色的葉片及子葉的第二群同功超氧歧化酶有增強的現象；下胚軸及子葉上的第二群同功過氧化酶有增強現象；葉子上的第三群同功酸性磷酸酶被誘導生成。以上同功酵素圖譜也顯示其類型具組織和發生時期的特異性。此外，植株長在 15℃下也誘導一新合成的韌皮部蛋白質，其分子量為 36.5 kDa 而等電點為 pH 4.9。

關鍵詞：酸性磷酸酶，過氧化酶，蛋白質，超氧歧化酶，絲瓜。

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