

Characterization of Chilling-Sensitivity of Three Submersed Aquatic Angiosperms

SASADHAR JANA AND MONOJIT A. CHOUDHURI

Senior Research Fellow, and Reader in Botany,
Plant Physiology and Biochemistry Laboratory,
Department of Botany, University of Burdwan,
Burdwan 713 104, India

ABSTRACT

Chilling-sensitivity of the submersed aquatic angiosperms, sago pondweed (*Potamogeton pectinatus* L.), vallisneria (*Vallisneria spiralis* L.) and hydrilla (*Hydrilla verticillata* (L.f.) Royle), was characterized on the basis of the changes in Hill activity and protein level of chilled leaves (0 C in the dark) in comparison with that of a terrestrial chilling-resistant plant spinach (*Spinacia oleracea* L.). Concomitant with the loss of Hill activity, the protein content gradually decreased in chilled mature leaves of both vallisneria and hydrilla. In sago pondweed and spinach the Hill activity and protein level were not affected. The rate of protein loss at 0 C was faster than that observed at 22 C in detached leaves of vallisneria and hydrilla. It was concluded that vallisneria and hydrilla are chilling-sensitive while sago pondweed is a chilling-resistant species. The restoration of Hill activity and protein level of chilled leaves occurred upon irradiance for 150 min in vallisneria and 120 min in hydrilla but it did not occur in leaves of either species kept at 22 C. Incubation at 0 C for 2 days in the dark produced maximum restoration. Young leaves of vallisneria and hydrilla were affected most during chilling and mature leaves the least. Pretreatment of mature leaves of the two chilling-sensitive plants with 1 mM each of MgCl₂, MnCl₂ and CaCl₂ and 0.23 mM kinetin solution enhanced the rate of restoration upon irradiance indicating lowering of chilling-sensitivity.

INTRODUCTION

Submersed aquatic angiosperms have attracted the attention of biologists in recent time possibly due to their unique habitational characteristics (3, 7, 9, 10, 11, 21). Of the three submersed macrophytes, sago pondweed (*Potamogeton pectinatus* L.), vallisneria (*Vallisneria spiralis* L.) and hydrilla (*Hydrilla verticillata* (L.f.) Royle), the first one grows only during winter months, while the growth of the other two is more vigorous in summer months in the tropics and subtropics (8). It has been stressed by some scientists that the measurement of Hill activity of isolated chloroplasts (17) and the faster degradation of protein (19) of chilled leaves, which is associated with senescence, can serve as indices of chilling-sensitivity of a plant. The present study, therefore, aims to (1) characterize the chilling-sensitivity of the three submersed plants on the basis of changes in Hill activity and protein level of chilled leaves in com-

parison with that of a terrestrial chilling-resistant plant, (2) study the effect of aging on chilling-sensitivity, and (3) determine the effect of pretreatment of isolated mature leaves with Mg²⁺, Mn²⁺, Ca²⁺ and kinetin on Hill activity and protein level of chilled leaves with a view to imposing the induction of any chilling resistance property in such pretreated leaves.

METHODS AND MATERIALS

Sago pondweed, vallisneria and hydrilla from the ponds and lakes around the University of Burdwan, India, and spinach (12) from the University Crop Research Farm, were collected during the winter season.

Isolated leaves were sterilized by dipping them in 95% ethanol for 10 seconds and they were immediately washed with sterile distilled water. The leaves were kept in sterile distilled water for 1 h to allow them to recover the dehydrating effect of ethanol before being used in experiments. Isolated leaves or chloroplast suspensions were placed in an opaque container and stored for different durations (1, 2 and 3 days) at 0 C or 22 C in the dark. Cold-stored leaves refer to those stored in the dark at 0 C. Reactivation of the cold-stored leaves was carried out at 25 C by the irradiance of leaves with various quantum flux densities for different periods.

The effect of aging on chilling-sensitivity was determined using either three different age groups (young, mature and old) or isolated mature leaves incubated in the dark (inducing aging). The isolated mature leaves of sago pondweed (leaf age: 40 to 50 days), vallisneria (leaf age: 60 to 70 days), hydrilla (leaf age: 30 to 40 days) and spinach (leaf age: 40 to 50 days) were used in induced aging experiments. The leaf age of sago pondweed, vallisneria and spinach was determined on the basis of leaf area and dry weight, while in the case of hydrilla, twig length and dry weight were considered for this purpose (8).

Methods of isolation of chloroplasts from leaves, the measurement of Hill activity of isolated chloroplasts, and effects of different environmental factors on the Hill activity are described elsewhere (10). The Hill activity of sago pondweed (quantum flux density 60 $\mu\text{E}/\text{m}^2\cdot\text{sec}$, temperature 20 C, pH 7.5, and Mg²⁺ concentration of 3 mM during chloroplast isolation and assay), vallisneria (100 $\mu\text{E}/\text{m}^2\cdot\text{sec}$, 20 C, pH 6.4, and Mg²⁺ 5 mM during chloroplast isolation and 2 mM during assay), hydrilla (100 $\mu\text{E}/\text{m}^2\cdot\text{sec}$, 30 C, pH 6.0, and Mg²⁺ 4 mM during chloroplast isolation

and 3 mM during assay) and spinach (240 $\mu\text{E}/\text{m}^2\cdot\text{sec}$, 20 C pH 7.5, and Mg^{2+} 3 mM during chloroplast isolation and assay) was measured under defined conditions (8). Chlorophyll content of the chloroplast suspension and leaves was measured according to Arnon (2). Protein was extracted from the leaves after removal of chlorophyll by digesting with 0.5 N NaOH at 80 C for 1 h and estimated by Folin-phenol reagent (14).

Sterilized mature leaves were floated in Petri dishes containing solutions of previously determined optimum effective doses of 1 mM MgCl_2 , 1 mM MnCl_2 , 1 mM CaCl_2 and 0.23 mM kinetin for 3 days in the dark at 0 C or 22 C. For further precautionary measure against epiphytic microorganisms, streptomycin sulphate and sodium penicillate (25 $\mu\text{g}/\text{ml}$) were added to the experimental and control sets.

Each experiment was replicated ten times and the data included in the tables were statistically analyzed by Duncan's Multiple Range Test (5) at 95% confidence limits. Standard errors (SE) around the mean were also calculated (5) and plotted in the figures.

RESULTS AND DISCUSSION

As indicated in Figure 1, the Hill activity was almost completely lost after 4 days of storage of mature leaves

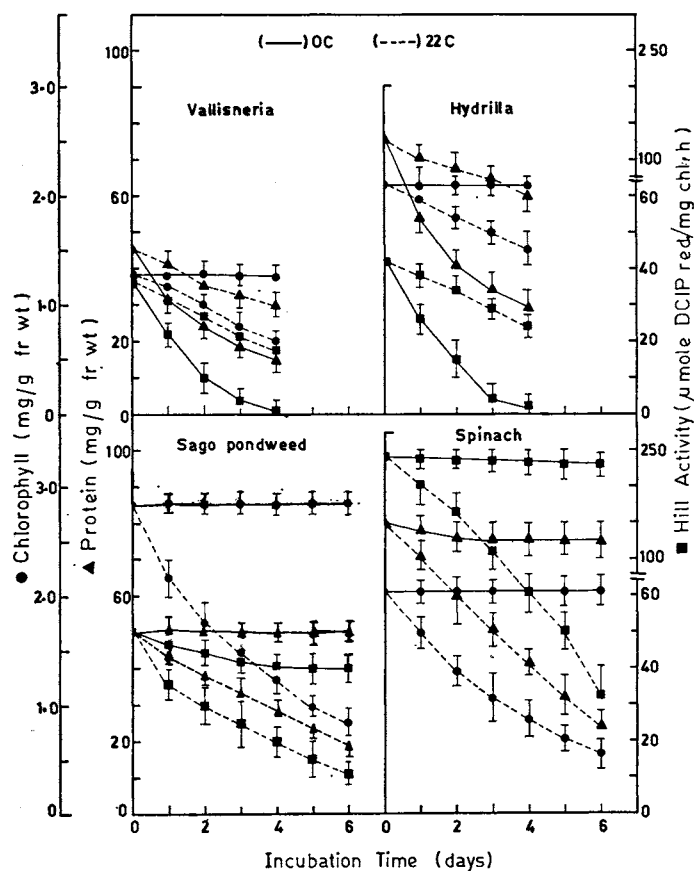


Figure 1. Changes in Hill activity and the contents of chlorophyll and protein during aging of isolated mature leaves of three submersed aquatic angiosperms and a terrestrial chilling-resistant plant spinach. Leaves were stored at 0 C or 22 C in the dark. The vertical lines indicate the standard errors of the means. Each point is the mean of ten replications.

of vallisneria and hydrilla at 0 C in the dark. In contrast to these plants, cold storage of sago pondweed and spinach leaves for up to 6 days did not affect their Hill activity. The chlorophyll level in the leaves of these aquatic plants and of spinach was not affected by such a cold treatment. However, the protein content gradually decreased concomitant with the loss of Hill activity in the leaves of vallisneria and hydrilla. In sago pondweed and spinach the protein content was not affected. The Hill activity and the contents of both chlorophyll and protein decreased during aging of isolated mature leaves of these plants kept at 22 C in the dark. The rate of protein loss at 0 C was faster than that observed at 22 C in detached leaves of vallisneria and hydrilla. The present study suggests that chlorophyll cannot be taken as a reliable parameter for determining chilling-sensitivity of a plant. Detached leaves of chilling-sensitive plants subjected to cold and dark storage exhibit several metabolic changes that are characteristic of senescence at room temperature, of which degradation of protein and chlorophyll is commonly studied (18). A more rapid rate of protein loss at chilling temperature in several chilling-sensitive plants than that at room temperature has been reported by several workers (6, 20). The main protein fraction which degraded following cold and dark storage of leaves of chilling-sensitive plants was the fraction of cytoplasmic protein of about 45,000 molecular weight (18). According to Peterson and Huffacker (15), the chloroplast is the main site of proteolysis during senescence and ribulose-1,5-bisphosphate carboxylase is the major protein degraded. Thus from the above observations as well as from the studies reported here, it can be concluded that vallisneria and hydrilla are chilling-sensitive while sago pondweed is a chilling-resistant species.

Figure 2 shows the effect of irradiance time on Hill activity and protein level of mature leaves stored at 0 C and 22 C for 3 days in the dark. The restoration of Hill activity, and an increase in protein level occurred upon irradiance for 150 min for vallisneria and 120 min for hydrilla. Increase in irradiance time beyond that produced no further improvement in restoration of the above two parameters. In the case of sago pondweed and spinach, restoration of Hill activity and protein level was not observed, instead there was a slight increase in Hill activity and protein level. This is the result of these plants being chilling-resistant species. Chlorophyll level remained unaffected in the leaves of all species subjected to chilling. No restoration of Hill activity and changes in the levels of chlorophyll and protein over control were observed in leaves kept at 22 C. This lack of restoration might be due to irreversible changes of the subcellular particles of the leaves, phenomena generally associated with senescence development. It is noteworthy that the changes in cellular contents occurring as a result of chilling treatment are reversible upon irradiance, while those occurring at higher temperature (22 C) are irreversible. This suggests that the mechanism of cellular deterioration in chilled and senescing leaves is not identical.

The effect of incubation for different durations at 0 C in the dark on the restoration of Hill activity and protein level of intact leaves and the effect of irradiance of chloro-

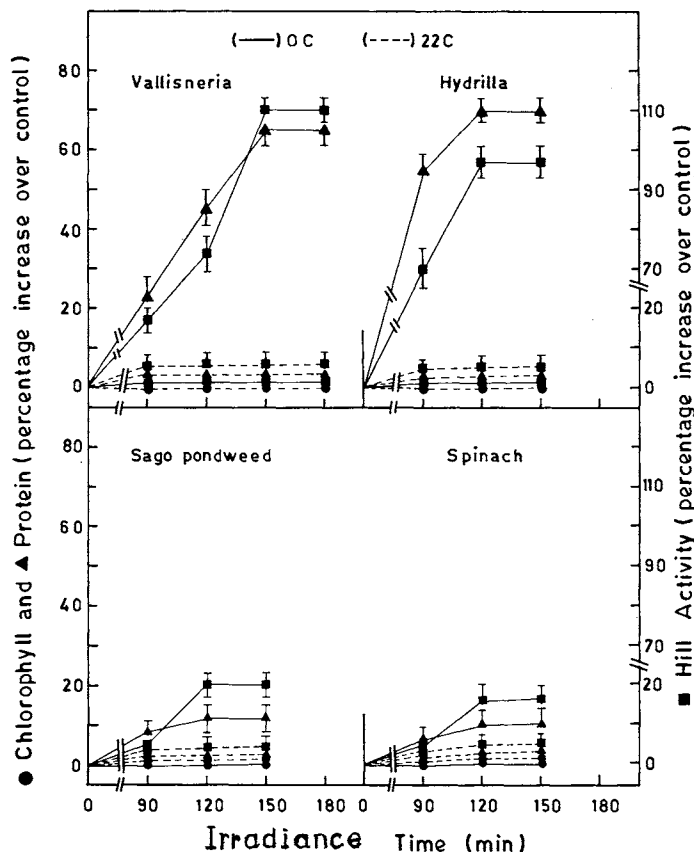


Figure 2. Effect of irradiance time on Hill activity and the contents of chlorophyll and protein of isolated mature leaves of three submersed aquatic angiosperms and a chilling-resistant plant spinach. Leaves were stored at 0 C or 22 C in the dark for 3 days. Leaves were then irradiated at the quantum flux density of 100 for vallisneria and hydrilla, 60 for sago pondweed and 240 $\mu\text{E}/\text{m}^2\cdot\text{sec}$ for spinach at 25 C and Hill activity, chlorophyll and protein were measured. The vertical lines indicate the standard errors of the means. Each point is the mean of ten replications.

plast suspension on Hill activity and protein level following the cold and dark storage of leaves can be observed from Table 1. The restoration of Hill activity and an increase

in protein level were achieved to the greatest extent after two days of cold storage of leaves in the dark in chilling-sensitive plants, viz., vallisneria and hydrilla. On the other hand, no increase in Hill activity and protein level of chloroplast suspension upon irradiance could be observed in these two species. This confirms the observations of Kaniuga *et al.* (12) who have demonstrated that intact cell structure is necessary for restoration of Hill activity in several chilling-sensitive land plants.

The effect of leaf age on the Hill activity and protein level in the cold and dark stored (3 days) leaves of vallisneria and hydrilla, and such effect on the restoration upon irradiance were studied in the two chilling-sensitive plants (Table 2). Young leaves were affected most during chilling treatment and the mature leaves the least. This is evident from the reactivation experiments which showed that the maximum amount of restoration of Hill activity and increase in protein level were achieved in mature and the minimum in young leaves. This observation indicates that a certain degree of membrane integrity of tissue is essential for offering resistance against the deleterious effects caused by chilling treatment (1).

Table 3 indicates the effect of pretreatment of leaves with 1 mM each of MgCl_2 , MnCl_2 , and CaCl_2 and 0.23 mM kinetin on restoration of Hill activity and protein content in the cold and dark stored (3 days) mature leaves of chilling-sensitive vallisneria and hydrilla plants. All the above chemicals arrested the loss of Hill activity and protein level of chilled leaves and enhanced the rate of restoration upon irradiance. Among the three cations used, Mn^{2+} was most effective in this respect. A report (13) concerning the role of Mn^{2+} in chilling-sensitive plants points out that loosely bound Mn^{2+} is lost from chloroplast and Mn-containing superoxide dismutase activity in chloroplast diminishes during chilling in the dark. It has been further suggested that during irradiance of chilled leaves, the restoration of Hill activity could be achieved by re-incorporation of Mn^{2+} into the thylakoid membrane and partial restoration of superoxide dismutase activity in the chloroplast (13). In

TABLE 1. EFFECT OF INCUBATION FOR DIFFERENT DURATIONS AT 0 C IN DARKNESS OF INTACT MATURE LEAVES OF THE RESTORATION OF HILL ACTIVITY AND PROTEIN LEVEL AND THE EFFECT OF IRRADIANCE OF CHLOROPLAST SUSPENSION ON HILL ACTIVITY AND PROTEIN LEVEL FOLLOWING THE COLD AND DARK STORAGE OF LEAVES.^a

Species and treatment	Percentage increase (+) or decrease (-) over control					
	Days of incubation					
	1		2		3	
	Hill activity	Protein	Hill activity	Protein	Hill activity	Protein
Vallisneria						
Intact leaves	+95 c	+58 c	+125 d	+71 c	+112 d	+65 c
Chloroplast suspension	0 a	0 a	0 a	0 a	-2 a	-1 a
Hydrilla						
Intact leaves	+88 c	+65 d	+100 c	+79 d	+97 c	+74 d
Chloroplast suspension	0 a	0 a	0 a	0 a	-1 a	-1 a
Sago pondweed						
Intact leaves	+10 b	+7 b	+22 b	+13 b	+20 b	+12 b
Chloroplast suspension	0 a	0 a	0 a	0 a	-1 a	-1 a
Spinach						
Intact leaves	+9 b	+5 b	+18 b	+14 b	+15 b	+11 b
Chloroplast suspension	0 a	0 a	0 a	0 a	-2 a	-1 a

^aValues in a column followed by the same letter are not significantly different at the 5% level as determined by Duncan's Multiple Range Test. Each value is the mean of ten replications.

TABLE 2. EFFECT OF LEAF AGE ON HILL ACTIVITY (μ MOLE DCIP RED./MG CHL•H) AND PROTEIN CONTENT (MG/G FR WT) IN THE COLD AND DARK STORED LEAVES (3 DAYS) OF VALLISNERIA AND HYDRILLA.^a

Species and stage of leaf	Initial		0 C		Stored at 0 C followed by irradiance of leaves	
	Hill activity	Protein	Hill activity	Protein	Hill activity	Protein
Vallisneria						
Young (40 to 50 days old)	42.1 c	40.1 d	1.7 a	16.3 a	28.3 b	30.0 b
Mature (60 to 70 days old)	39.3 b	46.3 c	3.3 b	22.5 b	35.2 c	41.6 c
Old (75 to 90 days old)	19.0 a	34.2 a	1.0 a	13.2 a	14.3 a	25.6 a
Hydrilla						
Young Twig (10 to 15 days old)	48.2 d	67.3 e	2.1 a	30.0 cd	30.4 b	49.5 d
Mature Twig (30 to 40 days old)	42.7 c	76.5 f	4.2 b	35.2 d	38.0 c	65.2 e
Old Twig (55 to 65 days old)	18.3 a	16.1 d	1.0 a	27.9 bc	12.7 a	42.3 c

^aValues in a column followed by the same letter are not significantly different at the 5% level as determined by Duncan's Multiple Range Test. Each value is the mean of ten replications.

TABLE 3. EFFECT OF DIFFERENT CATIONS AND KINETIN ON RESTORATION OF HILL ACTIVITY AND PROTEIN IN COLD AND DARK STORED (3 DAYS) MATURE LEAVES OF CHILLING-SENSITIVE VALLISNERIA AND HYDRILLA PLANTS.^a

Species and pretreatment	Percentage increase (+) over control			
	0 C		Stored at 0 C followed by irradiance of leaves	
	Hill activity	Protein	Hill activity	Protein
Vallisneria				
MgCl ₂ (1 mM)	+ 95 d	+20 b	+ 7 ab	+ 5 ab
MnCl ₂ (1 mM)	+112 e	+28 cd	+19 c	+14 c
CaCl ₂ (1 mM)	+ 85 cd	+14 a	+ 3 a	+ 3 a
Kinetin (0.23 mM)	+ 62 a	+12 a	+ 2 a	+ 9 b
Hydrilla				
MgCl ₂ (1 mM)	+ 92 d	+21 b	+10 b	+ 1 a
MnCl ₂ (1 mM)	+127 f	+33 d	+34 d	+10 bc
CaCl ₂ (1 mM)	+ 81 bc	+20 b	+ 5 a	+ 2 a
Kinetin (0.23 mM)	+ 73 b	+25 bc	+ 3 a	+ 8 b

^aValues in a column followed by the same letter are not significantly different at the 5% level as determined by Duncan's Multiple Range Test. Each value is the mean of ten replications.

the present study, Mn²⁺ may exert its influence in the above way to reduce the loss of Hill activity and protein level of chilled leaves of vallisneria and hydrilla. The effect of Mg²⁺ might be explained in the same way as that of Mn²⁺, but there is no available experimental data to support this view. The effect of Ca²⁺ and kinetin might be mediated possibly by maintaining the membrane integrity of the tissue (4, 16). Thus from the experimental results reported here, it appears that the mechanism of chilling-sensitivity of submersed aquatic angiosperms is essentially the same as that of land plants.

LITERATURE CITED

- Anderson, J. M. 1975. The molecular organization of chloroplast thylakoids. *Biochem. Biophys. Acta* 416:191-235.
- Arnon, D. I. 1949. Copper enzymes in isolated chloroplasts: polyphenoloxidase in *Beta vulgaris*. *Plant Physiol.* 24:1-15.
- Benedict, C. R. 1978. Nature of obligate photoautotrophy. *Annu. Rev. Plant Physiol.* 29:67-93.
- Biswas, A. K. and M. A. Choudhuri. 1978. Regulatory role of some hormones and nutrients on senescence development of soybean leaf discs. *Indian J. Exp. Biol.* 16:1313-1314.
- Clarke, G. M. 1969. *Statistics and Experimental Design* (1st Edn.). Edward Arnold (Publishers) Ltd., London, pp. 91-100.
- Goldthwaite, L. and W. M. Laetch. 1967. Regulation of senescence in bean leaf discs by light and chemical growth regulators. *Plant Physiol.* 42:1757-1762.
- Holaday, A. S. and G. Bowes. 1980. C₄ acid metabolism and dark CO₂ fixation in a submersed aquatic macrophyte (*Hydrilla verticillata*). *Plant Physiol.* 65:331-335.
- Jana, S. 1982. Studies on the Physiology of a few Submersed Aquatic Angiosperms. Ph.D. Thesis, University of Burdwan, Burdwan, India, pp. 63-104.
- Jana, S. and M. A. Choudhuri. 1979. Photosynthetic, photo-respiratory and respiratory behaviour of three submersed aquatic angiosperms. *Aquat. Bot.* 7:13-19.
- Jana, S. and M. A. Choudhuri. 1980. Characterization of Hill activity of a submersed aquatic angiosperm (sago pondweed). *J. Aquat. Plant Manage.* 18:30-34.
- Jana, S. and M. A. Choudhuri. 1980. Senescence in submersed aquatic angiosperms: Changes in intact and isolated leaves during aging. *New Phytol.* 86:191-198.
- Kaniuga, Z., B. Sochanowicz, J. Zabek and K. Krzystyniak. 1978. Photosynthetic apparatus in chilling-sensitive plants. I. Reactivation of Hill reaction activity inhibited on the cold and dark storage of detached leaves and intact plants. *Planta* 140:121-128.
- Kaniuga, Z., J. Zabek and B. Sochanowicz. 1978. Photosynthetic apparatus in chilling-sensitive plants. III. Contribution of loosely-bound manganese to the mechanism of reversible inactivation of Hill reaction activity following cold and dark storage and illumination of leaves. *Planta* 144:49-56.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with Folin-phenol reagent. *J. Biol. Chem.* 193:265-275.
- Peterson, W. and R. C. Huffacker. 1975. Loss of ribulose-1, 5-diphosphate carboxylase and increase in proteolytic activity during senescence of detached primary barley leaves. *Plant Physiol.* 55:1009-1015.
- Poovaiah, W. B. and A. C. Leopold. 1973. Deferral of leaf senescence with calcium. *Plant Physiol.* 52:236-239.
- Smillie, R. M. and R. Nott. 1979. Assay of chilling injury in wild and domestic tomatoes based on photosystem activity of the chilled leaves. *Plant Physiol.* 63:799-801.
- Sochanowicz, B. and Z. Kaniuga. 1979. Photosynthetic apparatus in chilling-sensitive plants. IV. Changes in ATP and protein levels in cold and dark stored and illuminated tomato leaves in relation to Hill reaction activity. *Planta* 144:153-159.
- Sochanowicz, B. and Z. Kaniuga. 1979. Photosynthetic apparatus in chilling-sensitive plants. V. Changes in protein fractions of leaves and isolated chloroplasts following cold and dark storage and illumination of tomato leaves. *Planta* 145:137-143.
- Takegami, T. 1975. A study on senescence in tobacco leaf discs. I. Inhibition by benzylaminopurine of decrease in protein level. *Plant Cell Physiol.* 16:407-416.
- Winter, K. 1978. Short-term fixation of ¹⁴Carbon by the submersed aquatic angiosperm *Potamogeton pectinatus*. *J. Exp. Bot.* 29:1169-1172.