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Effect of chilling on growth and nitrogen assimilation in *Azolla caroliniana*

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Abstract

Azolla caroliniana was exposed to 5 °C in darkness for 1, 2, 3, 5 or 7 d and then recovered for 7 d. Plants previously chilled for 2 or 3 d exhibited higher growth rates when transferred to normal temperature than either the control plants or those previously chilled for 5 or 7 d. Increased plant growth may be related to increased contents of chlorophyll, sucrose, and reducing sugars, due to increased photosynthetic capacity. In another experiment *Azolla* plants were chilled at 5 °C for 7 d and then transferred for 0, 4, 8, 12, or 16 d recovery to the N-free Hoagland solution or Hoagland solution containing 5 mM KNO_3. In previously chilled plants, the growth rate was decreased. In the medium supplemented with nitrogen, the growth rate was greater than in the N-free medium in both chilled and nonchilled plants. In chilled plants the decrease in growth rate may be related to the disturbance of *Anabaena azollae* cells where the protecting envelope of the heterocysts was deorganized. During the recovery the rate of N_2-fixation increased in both chilled and nonchilled plants up to 12 d after which both rates were similar. However, during the first 4 d the rate of the nonchilled plants was approximately 4-fold that of the previously chilled plants. Nitrate reductase and nitrite reductase activities in control plants were higher than in those previously chilled for 7 d. Both activities increased in nonchilled and previously chilled plants up to 12 d then decreased. The total protein content increased up to 12 d in chilled and nonchilled plants after which it decreased. Under all treatments, the values were higher in nonchilled plants than in those previously chilled ones and were also higher in presence of N than in its absence. Thus the presence of N-source in the medium counteracts the effect of chilling injury particularly during prolonged recovery.

Additional key words: nitrate reductase, nitrite reductase, rate of nitrogen fixation.

Introduction

Drought, salinity, and adverse temperatures are among the most encountered stresses which inhibit growth. Several tropical and subtropical higher plant species show characteristic damage symptoms when exposed to chilling temperatures, *i.e.* temperatures that are too low for normal growth but not low enough for ice to form (El-Saht 1998). Plants growing at suboptimal temperatures have a low chlorophyll (Chl) content, a low Chl *a/b* ratio, a high xanthophyll/β-carotene ratio, a reduced photosynthetic capacity, and damaged photosynthetic membranes (Hayden and Baker 1990, Haldimann *et al*. 1996). In addition, the effect of chilling injury included alteration in cell walls, nuclei, endoplasmatic reticulum, mitochondria, plastids, and ribosomes, and the extent of alterations varied greatly among cells (Lee *et al*. 2002).

Symptoms of chilling injury increase with the duration of exposure and with the reduction in temperature, and younger leaves are much more able to recover upon transfer to optimal temperature than the older ones (Jennings and Saltveit 1994). Jatimliansky *et al*. (2004) reported that maize and eastern gamagrass are known for their susceptibility to chilling injuries whereas their hybrid showed higher tolerance to low temperatures than its parents. Increased resistance to chilling temperatures is normally associated with content of saccharides, and exogenous saccharides impart chilling resistance to plants (King *et al*. 1988). Cyr *et al*. (1990) showed that contents of nitrate, amino acids, and total protein increase with chilling temperatures which may be an adaptive strategy for freezing tolerance. Furthermore, Huang and Guo (2005) reported that higher activities of defence enzymes and higher content of antioxidant in rice under stress were associated with tolerance to chilling.

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Abbreviations: Chl - chlorophyll; NR - nitrate reductase; NiR - nitrite reductase; RGR - relative growth rate; RS - reducing sugars; Su - sucrose; TAS - total available sugars.

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The aim of this work was to study the effect of chilling on growth, ultrastructure, and metabolic activities of an aquatic fern native of Asia and Africa. There is a symbiotic relationship between *Azolla* and the nitrogen fixer, blue green alga *Anabaena azollae* which invades certain cavities on the dorsal lobes of the leaves with true roots on the ventral surface. The genus *Azolla* is of agronomic importance as a potential N fertilizer in rice crops and also as fodder for livestock. Growth of *Azolla* plants was repeatedly observed to decrease markedly during winter.

Materials and methods

Plants and cultivation: *Azolla caroliniana* Wild. (known as water velvet) was provided by Prof. C. Van Hove, Catholic University of Louvain, Belgium. The plants were acclimated in the greenhouse of the Faculty of Science, Alexandria, in 2500 cm^3 polyethylene vessels which were filled with a nitrogen free, modified [KNO_3 and $Ca(NO_3)_2$ were replaced by KCl and $CaCl_2$, respectively] Hoagland solution (2/5 concentration, pH 5.1). About 5 g (fresh mass) of *Azolla* from the stock material were inoculated in each vessel. After 15 d an inoculum was taken from each vessel to make a new subculture, and so on.

The plants were freed from epiphytic microorganisms by thorough washing with water. The cultures were grown in a growth chamber under 16-h photoperiod at irradiance of 1200 μmol m^{-2} s^{-1} (cool white fluorescent tubes) and (light/dark) temperature of 28-30/20-25 °C for 7 d (stock culture). Before being used the plants were surface sterillized with 0.2 % *Clorax* (El-Aggan 1982), then thoroughly washed with water.

Treatments: Plants from the stock culture were kept at 5 °C in darkness in a controlled temperature-incubator. After 1, 2, 3, 5 and 7 d of chilling, 4.0 g samples were taken and cultured in the modified Hoagland solution at laboratory conditions for 7 d for recovery.

In further experiments, a sample of 7-d-old plants was chilled at 5 °C for 7 d, then half the lot was transferred to the N-free Hoagland solution to estimate the rate of N_2-fixation, and the other half to the Hoagland solution containing 5 mM KNO_3 to estimate nitrate reduction. A control experiment was set in which the chilling treatment was ommitted. After 0, 4, 8, 12 and 16 recovery days, samples were taken for chemical analyses and enzyme assays. Samples of 7-d chilled plants after 4 d recovery were taken for ultrastructure examination.

Methods: The number of generations and doubling time were determined from the fresh mass and duration of experiment employing the expression given by Peters *et al.* (1979): n (final mass) = $n_o \times 2G$, where G = number of generations, n_o = initial mass of *Azolla* plants (mass of inoculum). Doubling time (DT) = duration of experiment per one generation.

Chl *a* and *b* contents were determined using the spectrophotometric method (*Jenway*, UK, *6305 UV/Vis* spectrophotometer) according to Inskeep and Bloom (1985). The pigments were extracted with N,N-dimethyl-formamide. Reducing sugar (RS) content was determined by the anthrone/sulphuric acid method as described by Scott and Melvin (1956). Sucrose (Su) was determined enzymatically by hydro-lyzing the extract with invertase, then anthrone/sulphuric method followed, and sucrose was calculated by subtracting the reducing sugar content from the total sugar content after hydrolysis. Total available sugar (TAS) content was determined by hydrolyzing a known mass of the finely powdered dried tissue with 0.7 M HCl as described by Murata *et al.* (1968). Half a gram of the finely powdered plant material was digested with H_2SO_4/H_2O_2 and total nitrogen content was estimated by the spectrophotometric method of Solarzano (1969). Total protein content was estimated according to Hartree (1972). Nitrate reductase (NR) activity was assayed by the *in vivo* method described by Saber *et al.* (1989) which is based on the excretion of nitrite in the assay medium. The fresh plants were transferred to the assay medium (0.1 M KNO_3 and 0.5 M phosphate buffer, pH 7.5), the mixture was degassed at 3 °C under reduced light and then incubated at 30 °C for 1 h. The nitrite formed was then estimated by a modified method of Snell and Snell (1949) as follows: Two cm^3 of the assay medium were added to 1.0 cm^3 of 0.5 % sulphanillic acid in 20 % HCl, then 1.0 cm^3 of 0.01 % (m/v) N-1-naphthyl ethylene diamine dihydrochloride was added. After 30 min, the colour intensity was measured at 530 nm. Nitrite reductase (NiR) activity was assayed by the same technique as that for NR except the replacement of KNO_3 in the assay medium by $NaNO_2$. Activity of NiR was based upon the disappearance of nitrite from the medium. Nitrogen fixation rate was determined using the formula of Termaat and Munns (1986): $J = [(N_2 - N_1)/(M_2 - M_1)] \times RGR$, where J is the rate of nitrogen fixation, (N_2-N_1) is the change in nitrogen content of plants growing on N-free medium from time 1 to time 2, (M_2-M_1) is the change in fresh mass of the plant from time 1 to time 2, and RGR is the relative growth rate over that period. RGR was calculated according to the formula of Hunt (1990): $RGR = (\lg M_2 - \lg M_1)/(t_2 - t_1)$, where M_2 and M_1 are the plant masses at times t_2 and t_1, respectively.

Transverse sections of *Azolla* plants were examined by both light microscope (LM) and transmission electron

microscopy (TEM). Sectioning and staining were done as described in the pamphlet of EM unit, then examined by *Jeol* (Japan) *CX 100* electron microscope.

Statistics: The results from triplicate samples and triplicate determinations were statistically analysed using the least significant difference (LSD) at 5 % level and standard deviation.

Results and discussion

When *Azolla* plants were chilled for 3 d and then recovered under laboratory conditions for 7 d, increases in fresh and dry masses were recorded (Table 1). This was accompanied by an increase in Chl *a* and *b*, RS, Su and TAS contents. Longer chilling resulted in a progressive decrease in all parameters to reach values which were lower than the control ones, except for RS. Alonso *et al*. (1997) reported that tropical and subtropical higher plant species showed marked growth inhibition when chilled. Under the prevailing experimental conditions reduction of *Azolla* growth was markedly affected by increasing duration of chilling (Jennings and Saltveit 1994).

Increasing the chilling period to 7 d resulted in a significant decrease in Chl *a* and *b* contents in spite of the 7-d recovery (Table 1). Hayden and Baker (1990) reported that reduction in contents of photosynthetic pigments in response to low temperature treatment was related to disturbance of photosynthetic membranes. Haldimann *et al*. (1996) recorded a marked reduction in photosynthetic capacity in maize plants after transferring to low temperature. In contrast, the *Azolla* plants, previously chilled for 2 or 3 d exhibited greater growth rates when transferred to normal temperature than either the control plants or those previously chilled for 5 or 7 d. Therefore, the increased growth of *Azolla* chilled for 3 d and then recovered for 7 d may be related to the increase in Chl synthesis and contents of sucrose and reducing sugars, due to an increased photosynthetic capacity. Purvis and Grierson (1982) and King *et al*. (1988) reported that reducing sugars and sucrose may be involved in decreasing chilling sensitivity and preventing membrane dysfunctions in chilled grape fruit and tomato seedlings. Joly and Hahn (1991) showed that net CO_2 assimilation rate of chilled cacao seedlings was recovered within 7 d. Haldimann (1996) found that when temperature was increased from 14 to 24 °C, Chl synthesis in *Zea mays* was increased. According to Kudoh and Sonoike (2002) the amount of functional photosystem 1 in the leaves of a chilling-sensitive cucumber plant was recovered to 90 % of the original content 6 d after the chilling.

Table 1. Effect of exposure of *Azolla caroliniana* to 5 °C for 0 (control), 1, 2, 3, 5 and 7 d on fresh and dry masses and contents of Chl *a* and *b*, reducing sugars (RS), sucrose (Su), and total available saccharides (TAS). Values carrying different letters are significantly different at $P \leq 0.05$.

Chilling [d]	FM [g culture^{-1}]	DM [g culture^{-1}]	Chl *a* [mg kg^{-1}(f.m.)]	Chl *b* [mg kg^{-1}(f.m.)]	Chl *a*+*b* [mg kg^{-1}(f.m.)]	RS [g kg^{-1}(d.m.)]	Su [g kg^{-1}(d.m.)]	TAS [g kg^{-1}(d.m.)]
0	19.05 ^{b}	0.76 ^{b}	166.4 ^{b}	42.3 ^{bc}	208.7 ^{b}	23.1 ^{e}	20.8 ^{bc}	99.8 ^{bc}
1	19.13 ^{b}	0.78 ^{ab}	169.1 ^{ab}	46.0 ^{abe}	215.1^{ab}	32.9 ^{d}	25.9 ^{bc}	104.7 ^{b}
2	19.86 ^{ab}	0.84 ^{a}	175.3 ^{a}	46.2 ^{abd}	221.5 ^{ab}	65.3 ^{a}	42.2 ^{a}	166.1 ^{a}
3	22.31^{a}	0.88 ^{a}	184.3 ^{a}	51.1 ^{a}	235.4 ^{a}	68.6 ^{a}	41.3 ^{a}	158.2 ^{a}
5	14.38 ^{c}	0.59 ^{c}	139.7 ^{c}	41.2 ^{cde}	180.9 ^{c}	53.7 ^{b}	17.0 ^{c}	92.8 ^{cd}
7	12.75 ^{c}	0.52 ^{c}	136.1 ^{c}	40.5 ^{ce}	176.6 ^{c}	39.7 ^{c}	20.5 ^{bc}	87.2 ^{d}

Chilling for 14 d resulted in severe inhibition of growth and chilling for 21 d induced a death of plants. Therefore, in the following experiments, plants were chilled for 7 d and then grown under laboratory conditions in presence or absence of a N-source for 4, 8, 12, and 16 d. In N-free medium, the rate of growth of control plants decreased with age. In previously chilled plants, the doubling time (DT) increased compared to nonchilled plants, *i.e*. the rate of growth was decreased. In the medium supplemented with N, the rate of growth

Table 2. Growth, characterized by doubling time, DT [d], of unchilled and previously chilled *Azolla* plants during 16-d recovery in presence (+N) or absence (-N) of N-source. Means of three replicates ± SD.

Recovery [d]	-N unchilled	-N chilled	+N unchilled	+N chilled
4	3.20 ± 0.36	6.70 ± 0.26	2.80 ± 0.56	4.50 ± 0.36
8	3.70 ± 0.47	6.50 ± 0.26	2.50 ± 0.44	3.90 ± 0.61
12	4.40 ± 0.44	6.70 ± 0.56	3.70 ± 0.36	4.10 ± 0.36
16	5.90 ± 0.44	6.80 ± 0.61	3.70 ± 0.44	4.40 ± 0.44

was better than in the N-free medium in both chilled and nonchilled plants (Table 2). El-Aggan (1982) showed that low concentration of nitrate significantly increased the growth of *Azolla*, in comparison with growth in the nitrogen-free medium since nitrate is the most preferred N form for the growth of green plants and nitrate assimilation consumes relatively less energy than the N_2-fixation. Also when nitrate was absorbed from the external medium, the pH of the culture medium increased to the favourable value for *Azolla* growth. Recently, Cruz *et al*. (2004) reported that activities of nitrate reductase and other related enzymes in cassava were considerably lower in low nitrate supply than in higher one.

Under chilling treatment the increase in DT (decreased growth rates) may be related to the disturbance of *A. azollae* cells. Examination of chilled plants with LM and TEM showed that the cyanobacterial cavities of the plants became distorted with the cells around them being slightly plasmolysed (Fig. 1*B*,*E*). However, they recovered after 4 d where the vegetative cells and heterocysts were arranged in a regular pattern (Fig. 1*C*,*F*). In the plants previously chilled this arrangement was disturbed (Fig. 2*G*-*I*) and TEM showed dramatic changes in the morphology and ultrastructure of the heterocysts, where the protecting envelope was deorganized (Fig. 2*J*-*L*). This envelope acts as an O_2 diffusion barrier (Garcia-Gonzalez *et al*. 1988). Thus the decrease in growth may be related to a decrease in the rate of N_2-fixation and protein content. Chilling had a marked inhibitory effect on the rate of N_2-fixation in *Azolla* plants (Table 3).

During the first four days of recovery, the rate of N_2-fixation of unchilled plants was approximately 4-fold that of the plants previously chilled. However, during the recovery days, the rate increased in both plants up to 12 d then decreased and, in addition, NR and NiR activities increased up to 12 d and in the plants previously chilled the increase continued till the end of experiment. However, enzyme activities were higher in the control plants than in those previously chilled for 7 d (Fig. 3*A*).

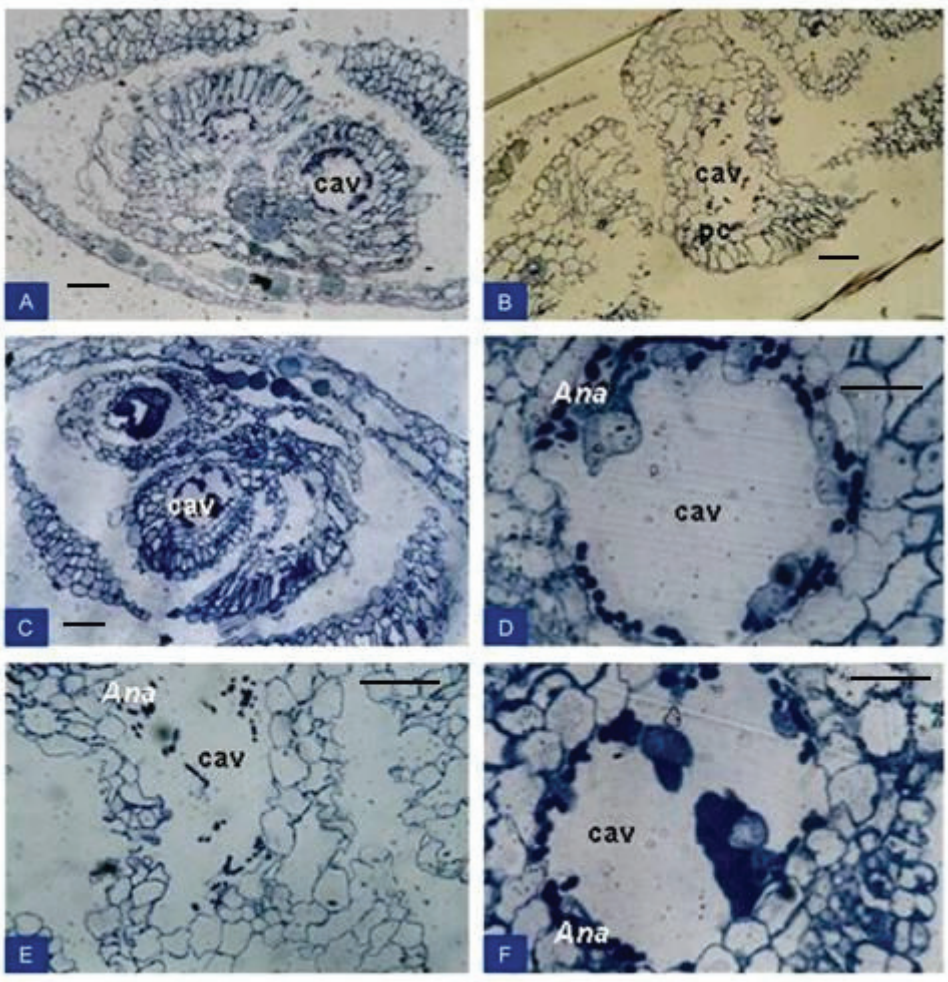

Fig. 1. *Azolla* shoot sections. Microscopic photographs of control 4-d-old plants (*A*), chilled plants (*B*), and plants after 4-d recovery (*C*). Cavities including *Anabaena azollae* (*D*, *E*. *F* corresponding to *A*, *B*, *C*, respectively). Ana - *Anabaena* cells, cav - cavity, pc - plasmolyzed cells; *bar* = 25 μM.

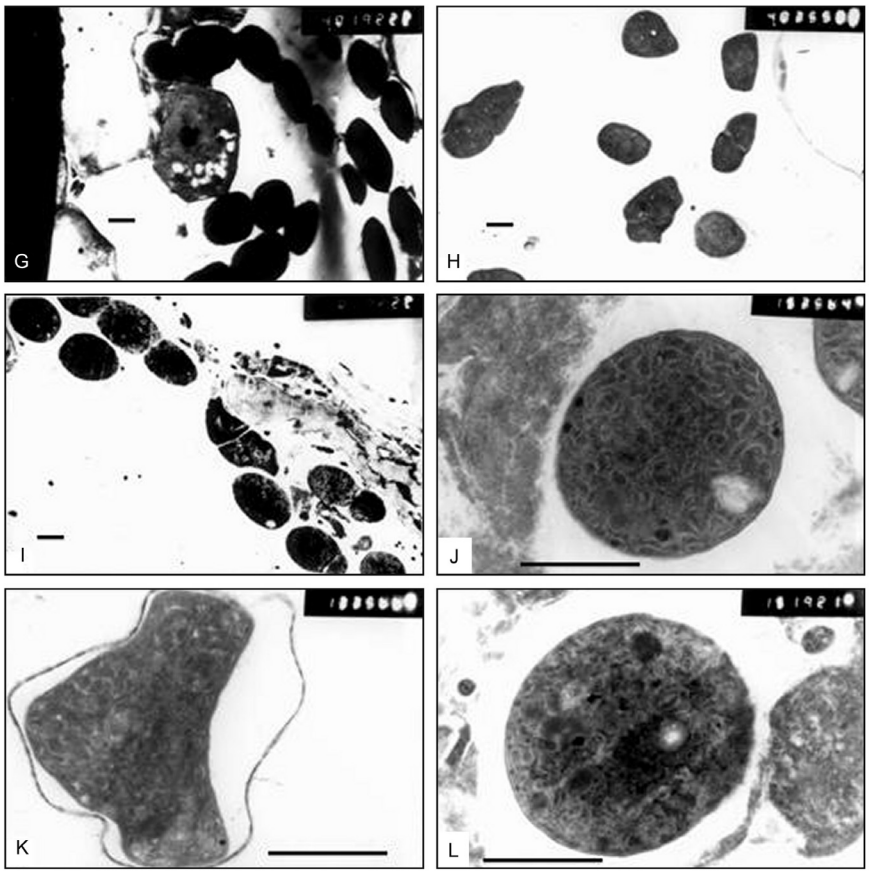

Fig. 2. Electron micrographs of cells (*G* - *I*) and heterocysts (*J* - *L*) corresponding to Fig. 1*A* - *C*, respectively. *Bar* = 1 μM.

Table 3. Rate of nitrogen fixation of unchilled and previously chilled *Azolla* plants during 16 recovery days in nitrogen-free Hoagland solution. Values carrying different letters are significantly different at $P \leq 0.05$.

Recovery [d]	Rate of nitrogen fixation [g(N) kg^{-1}(d.m.) d^{-1}] unchilled	chilled
0 - 4	2.19 ^{b}	0.51 ^{b}
4 - 8	4.85 ^{a}	0.81 ^{b}
8 - 12	4.69 ^{a}	2.17 ^{a}
12 - 16	1.87 ^{b}	1.71 ^{a}

Hence chilling may have an impact on nitrogenase and nitrate assimilating enzymes. The inhibitory effect on nitrogenase may be related to the increased diffusion of oxygen due to the disturbance of heterocyst envelopes thus increasing the competition between oxygen and nitrogen on hydrogen donor (El-Aggan 1982). Vogel and Dawson (1991) reported that recovery of nitrogenase activity of root nodules of black alder and *in vivo* nitrate reductase of leaves occurred within 7 d after chilling.

The total protein content increased up to 12 d in chilled and nonchilled plants, especially in those grown in presence of N-source, after which the protein content decreased. Under all treatments, the total protein content

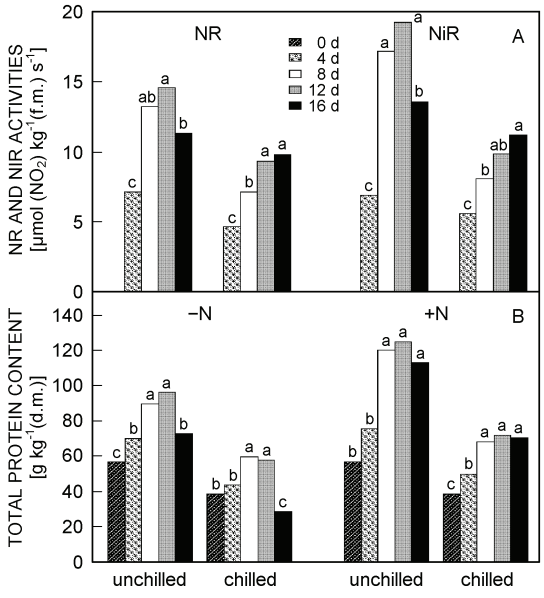

Fig. 3. Nitrate reductase (NR) and nitrite reductase (NiR) activities of unchilled (control) and previously chilled *Azolla* plants (*A*), and total protein content (*B*) after 16-d recovery in presence of 5 mM KNO_3 as a nitrogen source or absence of N source. Values carrying different letters are significantly different at $P \leq 0.05$.

was higher in nonchilled plants than in the previously chilled ones and was also higher in presence of nitrogen than in its absence (Fig. 3*B*). Thus the increase in growth during recovery may be due to an increase in activities of nitrogenase and nitrite and nitrate reductase resulting from increased photosynthetic capacity.

References

Alonso, A., Queiroz, C.S., Magalhaes, A.C.: Chilling stress leads to increased cell membrane rigidity in roots of coffee (*Coffea arabica* L.) seedlings. - Biochim. biophys. Acta **1323**: 75-84, 1997.

Cruz, J.L., Mosquim, P.R., Pelacani, C.R., Araújo, W.L., Damatta, F.M.: Effect of nitrate nutrition on nitrogen metabolism in cassava. - Biol. Plant. **48**: 67-72, 2004.

Cyr, D.R., Bewley, J.D., Dumbroff, E.B.: Seasonal dynamics of carbohydrates and nitrogenous compounds in the roots of perennial weeds. - Plant Cell Environ. **13**: 359-365, 1990.

El-Aggan, W.H.: A Comparative Study of the Growth and Nitrogenase Activity of five *Azolla* Species as Affected by Various Environmental Factors. - Ph.D. Thesis, Université Catholique De Louvain, Louvain 1982.

El-Saht, H.M.: Responses to chilling stress in French bean seedlings: antioxidant compounds. - Biol. Plant. **41**: 395-402, 1998.

Garcia-Gonzalez, M., Mateo, P., Bonilla, I.: Boron protection for O_2 diffusion in heterocysts of *Anabaena* sp. PCC 7119. - Plant Physiol. **87**: 785-789, 1988.

Haldimann, P.: Effects of changes in growth temperature on photosynthesis and carotenoid composition in *Zea mays* leaves. - Physiol. Plant. **97**: 554-562, 1996.

Haldimann, P., Fracheboud, Y., Stamp, P.: Photosynthetic performance and resistance to photoinhibition of *Zea mays* L. leaves grown at suboptimal temperature. - Plant Cell Environ. **19**: 85-92, 1996.

Hartree, E.F.: A modification of the lowry method that gives a linear photometric response. - Anal. Biochem. **48**: 422-425, 1972.

Hayden, D.B., Baker, N.R.: Damage to photosynthetic membranes in chillings sensitive plants: maize, a case study. - CRC Rev. Biotechnol. **9**: 321-341, 1990.

Huang, M., Guo, Z.: Responses of antioxidative system to chilling stress in two rice cultivars differing in sensitivity. - Biol. Plant. **49**: 81-84, 2005.

Hunt, R.: Basic Growth Analysis. - Unwin Hyman, London 1990.

Inskeep, W.P., Bloom, P.R.: Extinction coefficients of chlorophyll *a* and *b* in N,N-dimethylformamide and 80 % acetone. - Plant Physiol. **77**: 483-485, 1985.

Jatimliansky, J.R., Garcia, M.D., Moliana, M.C.: Response to chilling of *Zea mays*, *Tripsacum dactyloides* and their hybrid. - Biol. Plant. **48**: 561-567, 2004.

Jennings, P., Saltveit, M.E.: Temperature and chemical shocks induce chilling tolerance of germinating *Cucumis sativus* (cv. Poinselt 76) seeds. - Physiol. Plant. **91**: 703-707, 1994.

Joly, R.J., Hahn, D.T.: Net CO_2 assimilation of cacao seedlings following dark chilling. - Tree Physiol. **9**: 415-424, 1991.

King, A.I., Joyce, D.C., Reid, M.S.: Role of carbohydrates in diurnal chilling sensitivity of tomato seedlings. - Plant Physiol. **86**: 764-768, 1988.

Kudoh, H., Sonoike, K: Irreversible damage to photosystem I by chilling in the light: cause of the degradation of chlorophyll after returning to normal growth temperature. - Planta **215**: 541-548, 2002.

Lee, S.H., Singh, A.P., Chung, G.C., Kim, Y.S., Kong, I.B.: Chilling root temperature causes rapid ultrastructural changes in cortical cells of cucumber (*Cucumis sativus* L.) root tips. - J. exp. Bot. **53**: 2225-2237, 2002.

Murata, T., Akazawa, T., Shikiko, F.: Enzymic mechanisms of starch breakdown in germinating rice seeds. - Plant Physiol. **43**: 1899-1905, 1968.

Peters, G.A., Mayne, B.C., Ray, T.B., Toia, R.E., Jr.: Physiology and Biochemistry of the *Azolla-Anabaena* symbiosis. - In: Nitrogen and Rice. Pp. 325-344. International Research Institute, Los Baños 1979.

Purvis, A.C., Grierson, W.: Accumulation of reducing sugar and resistance of grapefruit peel to chilling injury as related to winter temperatures. - J. amer. Soc. hort. Sci. **107**: 139-142, 1982.

Saber, N.E., El-Aggan, W.H., Barakat, S.Y., Dabash, E.M.: Nitrate uptake and nitrate reductase in the water-fern *Azolla caroliniana*. - Commun. Sci. Res. **26**: 111-125, 1989.

Scott, T.A., Melvin, E.H.: Anthrone colorimetric method. - In: Whistler, R.L., Walfrom M.L. (ed.): Methods in Carbohydrate Chemistry. Vol. I. P. 384. Academic Press, New York - London 1956.

Snell, F.D., Snell, C.T.: Colorimetric Method of Analysis. 3rd Ed. - Van Nostrand and Co., New York 1949.

Solarzano, L.: Determination of ammonia in natural waters by the phenolhypochlorite method. - Limnol. Oceanogr. **14**: 799-807, 1969.

Termaat, A., Munns, R.: Use of concentrated macro-nutrients solution to separate osmotic from NaCl specific effects on plant growth. - Aust. J. Plant Physiol. **13**: 509-514,1986.

Vogel, C.S., Dawson, J.O.: Nitrate reductase activity, nitrogenase activity and photosynthesis of black alder exposed to chilling temperatures. - Physiol. Plant. **82**: 551-558, 1991.