DENDROBIOLOGY
2009, vol. 62, 3–9

Hanna Bandurska, Marlena Płachta, Marta Woszczyk

Seasonal patterns of free proline and carbohydrate levels in cherry laurel (*Prunus laurocerasus*) and ivy (*Hederea helix*) leaves and resistance to freezing and water deficit

Received: 26 February 2009; Accepted: 25 May 2009

Abstract: Seasonal changes in the levels of water-soluble carbohydrates, free proline and tolerance to freezing and water deficit were studied in leaves of cherry laurel (*Prunus laurocerasus*) and ivy (*Hedera helix*). Frost and water deficit tolerance was estimated based on the measurement of electrical conductivity of aqueous media containing leaf discs that were previously treated with frost (–7°C) or polyethylene glycol solution (PEG 600), respectively. Carbohydrate content in leaves of examined species changed differently throughout the measurement period. In laurel leaves the highest carbohydrate level was found in February, while it was lowest in June. In contrast, in ivy leaves the highest carbohydrate content was recorded during summer (June, July), while the lowest in February. However, a lack of correlation between soluble carbohydrate level and membrane injury index was shown in this study. Free proline content in leaves of investigated species was the highest in early spring, i.e. April. The lowest level of free proline in laurel leaves was found in July, September and October, but in ivy from July to February. A negative correlation between proline content and membrane injury index observed in frost and PEG treated leaves of both species suggest that this amino acid may have resulted on membrane protection.

Additional key words: evergreen shrub, membrane injury, PEG test

Address: Hanna Bandurska, Department of Plant Physiology, Poznań University of Life Sciences, Wołyńska 35, 60-637 Poznań, Poland, e-mail: bandur@jay.au.poznan.pl

Introduction

Woody plants grown in temperate climate must survive in an environment where temperatures fluctuate drastically with the change of seasons. The ability of woody plants to survive freezing temperatures is based on different evolutionary driven strategies (Larcher 2005). Deciduous woody plants preparing to low winter temperature shed their leaves. This genetically controlled process, known as autumn leaf senescence, is preceded by remobilization of nutrients and carbohydrates, which are transported from leaves into the stem (Keskitalo et al. 2005). Both cherry laurel (*Prunus laurocerasus*) and ivy (*Hederea helix*) are overwintering evergreen shrubs, with leaves that remain green and vital throughout several seasons. Foliage is gradually replaced and shedding of old leaves is accompanied by the formation of new leaves, thus these plants are never leafless. These plants survive seasonal drops of temperature below zero thanks to appropriate anatomical, biochemical and physiological adaptations. Freezing damage may arise from the

destabilization of cell membranes as well as formation of ice in intracellular spaces, causing intracellular water movement to extracellular spaces and dehydration of cells. In addition, cellular dehydration due to low temperature may also be caused by reduced root water uptake (Steponkus et al. 1993, Webb and Steponkus 1993; Thomashow 1999; Xin and Browse 2000; Beck et al. 2004). Acclimation to cold (freezing) temperature in woody plants occurs in early autumn in response to shortening days and decreasing temperature (slightly above zero), which induce mechanisms responsible for increased freezing tolerance (Beck et al. 2004; Larcher 2005). These mechanisms include modifications of cell membranes (lipid composition) and the accumulation of water-soluble carbohydrates and an amino acid, proline (Pukacki 1995, Sauter 1988; Stitt and Hurry 2002; Hare et al. 1998; Frankow-Lindberg 2001, Mahajan and Tuteja 2005). Increased concentrations of these compounds lead to a reduction of osmotic potential of cell sap and a lowering of freezing point, thus preventing the occurrence of water deficit in cells (Nanjo et al. 1999, Xin and Browse 2000, Kishor et al. 2005, Qureshi et al. 2007). These compounds, as non-toxic compatible solutes, also protect structural integrity of cell membranes and proteins against detrimental effects of dehydration and freezing temperature (Thomashow 1999, Smallwood and Bowles 2002; Öztürk and Demir 2002; Ashraf and Foolad 2007). In addition, both carbohydrates and proline when accumulated may serve as a source of carbon, nitrogen and energy during the first stage of restored metabolic activity after withdrawal of unfavourable conditions (Hare et al. 1998; Frankow-Lindberg 2001).

The objectives of the present study were (I) to examine seasonal changes in the levels of water-soluble carbohydrates and free proline in leaves of cherry laurel (*Prunus laurocerasus*) and ivy (*Hedera helix*), and (II) to relate these changes to freezing temperature (–7°C) and water deficit tolerance. Ivy (*Hedera helix*) is native to most of Europe and western Asia. It is widely cultivated as ornamental landscaping plant growing as a ground cover and a climbing vine (Swearingen and Dietrich 2006). Cherry laurel has a natural range stretching from south of Caspian Sea through the western Caucasus Mountain and Trnscaucasia, south of Black Sea and the eastern Balkans. It was introduced to central Europe in 16th century where is cultivated for ornamental purpose in gardens and parks (Aniśko 2002).

Material and methods

The experiment was carried out on leaves of cherry laurel (*Prunus laurocerasus*) and ivy (*Hedera helix*) plants, growing in the Dendrological Garden of the Poznań University of Life Sciences (latitude 16°52'E, longitiude 52°25'N, altitude about 50 m a.s.l.). Young, fully developed leaves of each species were collected from March 2006 to February 2007 during eight successive sampling procedures. Harvested leaves were used to estimate water content, proline and carbohydrate levels as well as the effect of frost and water deficit, applied *in vitro*, on cell membrane injury index. For each estimated parameter, five determinations were made.

Water content (*WC*) in leaves was estimated by measuring leaf fresh weight and dry weight following oven drying of fresh leaf samples at 70°C.

Determination of proline and carbohydrates. 0.3 g samples of fresh leaf tissues were freeze-dried in liquid nitrogen and stored at –20°C until analysis. Proline content was determined according to Bates et al. (1973) by measuring the quantity of the coloured reaction product of proline with ninhydric acid. Absorbance was read at 520 nm. The amount of proline was calculated from a previously plotted standard curve and expressed in $\mu g \cdot g^{-1}$ of leaf dry matter (DM). Water-soluble carbohydrate level was determined by the anthrone test according to Björnejsö (1955). Frozen leaf samples were homogenized in a chilled mortar with water and centrifuged at 10 000 *g* for 15 min at 4°C. The absorbance of the coloured reaction product of the anthrone reagent with carbohydrates soluble in the supernatant was read at 620 nm. The amount of carbohydrates was calculated from a standard curve previously plotted for glucose and expressed in $mg \cdot g^{-1}$ of leaf DM.

Membrane injury index. The effect of frost (–7°C) and water deficit (PEG test) on cell membrane injury was determined *in vitro* (Sullivan 1971) according to Premachendra and Shimada (1987). Leaf pieces (five pieces of 1.5 cm diameter) were washed three times in 10 cm^3 of deionized water to remove surface adhered electrolytes and put to 50 cm^3 flask. One batch of leaf samples was submerged in 10 cm^3 of 25% PEG 600 solution of osmotic potential –1.8 MPa (water deficit) and kept at 10°C for 24h. The second leaf batch was lightly dried to remove water from leaf surface and kept for 24h at the temperature of –7°C. The third batch of leaves was submerged in deionized water (control) and kept for 24h at 10°C. After dehydration leaf pieces from the PEG-treated combination were quickly washed three times with deionized water and finally submerged in 10 cm^3 of water. Leaf pieces from the frost treated combination were submerged in 10 cm^3 of deionized water immediately after treatment. Samples from all combinations were kept for 24h at 10°C, warmed to 25°C, shaken, and the electrical conductivity of effusate was measured. Next tissues were killed by autoclaving for 15 min, cooled down to 25°C, and electrical conductivity of the effusate was measured once again. Membrane injury (%) was evaluated according to the formula:

$$MI = 1 - \frac{1-(T1/T2)}{1-(C1/C2)} \times 100,$$

where $C1$ and $C2$ represent conductivity values of control samples before and after autoclaving, respectively; $T1$ and $T2$ represent conductivity values for PEG or frost temperature treated samples before and after autoclaving, respectively.

Statistical analysis. Tukey's test was applied to determine the significances between the mean values. Pearson's correlation coefficients were calculated for the relationships between estimated parameters. All numerical analyses were performed using the STATISTICA 8.0 software package.

Results

Water content (*WC*) in leaves of examined species varied slightly from March 2006 to February 2007 (Fig. 1). Leaves of laurel were characterized by the lowest hydration in June (45%) and the highest in September (66%). The fluctuation in *WC* of ivy leaves was different throughout the experimental period. The maximum *WC* was recorded in June (77%) and the minimum in March (57%).

Carbohydrate level throughout the experimental period was lower in laurel (av. 0.64 mg × g^{-1} DM) when compared to ivy (av. 2.8 mg × g^{-1} DM) leaves ($p < 0.0001$, Tukey's test). Seasonal changes of carbohydrate level proceeded differently in examined species (Fig. 2). The maximum carbohydrate level in leaves of laurel was found in February (0.99 mg × g^{-1} DM) and minimum in June (0.32 mg × g^{-1} DM). In contrast, the maximum carbohydrate level in ivy leaves was recorded in June (4.2 mg × g^{-1} DM) and minimum in February (1.8 mg × g^{-1} DM).

Significant differences in leaf free proline content between laurel (av. 192 μg × g^{-1} DM) and ivy (av. 77 μg × g^{-1} DM) were observed during the measurement period ($p < 0.01$, Tukey's test). In leaves of both spe-

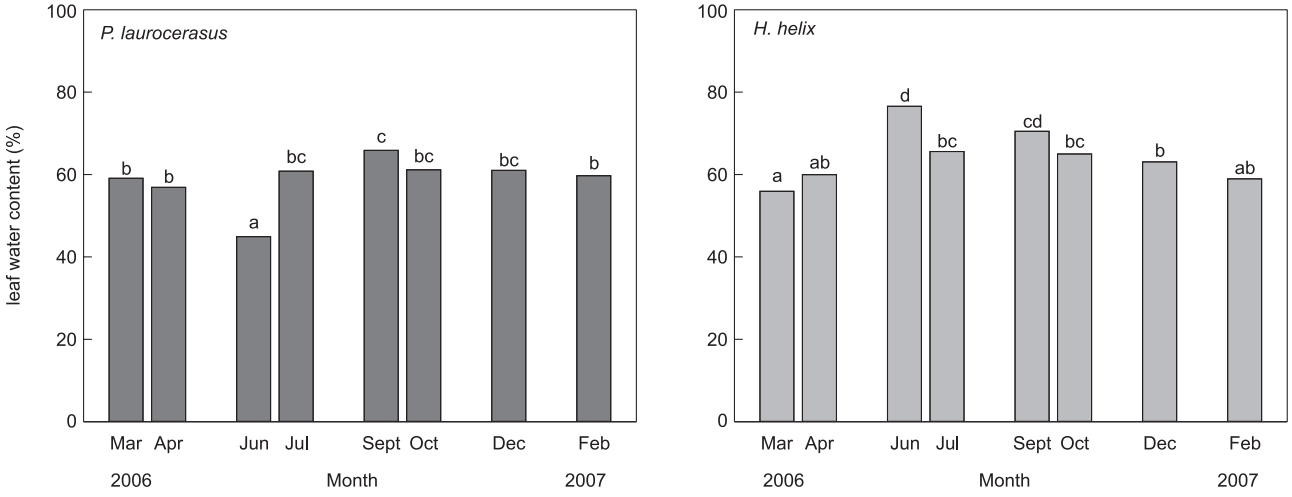

Fig. 1. Seasonal patterns of leaf water content (% of fresh weight) in laurel (*Prunus laurocerasus*) and ivy ((*Hederea helix*). Different letters indicate significant differences between means ($p < 0.05$)

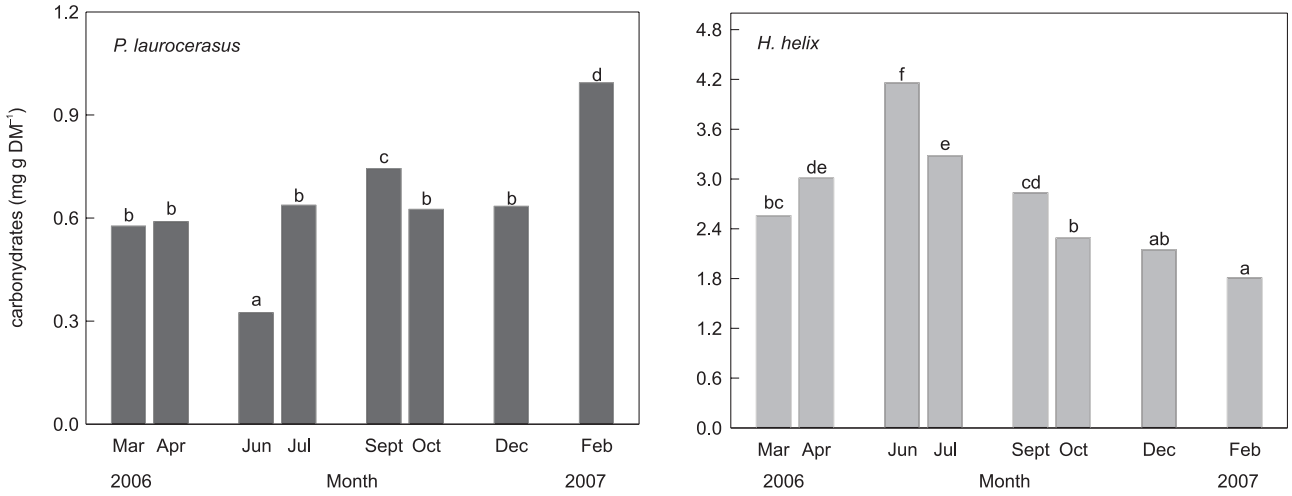

Fig. 2. Seasonal patterns of leaf carbohydrates content in laurel (*Prunus laurocerasus*) and ivy ((*Hederea helix*). Different letters indicate significant differences between means ($p < 0.05$)

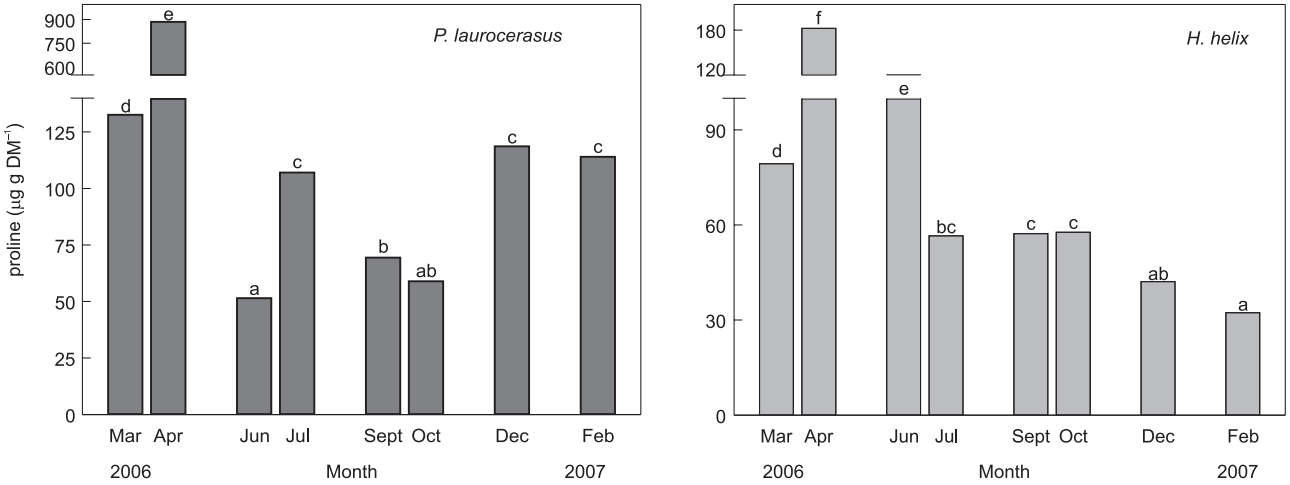

Fig. 3. Seasonal patterns of leaf free proline content in laurel (*Prunus laurocerasus*) and ivy ((*Hederea helix*). Different letters indicate significant differences between means ($p < 0.05$)

cies the maximum proline level was recorded in April, amounting to 884 $\mu g \times g^{-1}$ DM in laurel and to 182 $\mu g \times g^{-1}$ DM) in ivy (Fig. 3). A high proline level in both species was also observed in March. The lowest level of free proline in laurel leaves was found in July, September and October (51–69 $\mu g \times g^{-1}$ DM), but in ivy from July to February (32–57 $\mu g \times g^{-1}$ DM). An interesting fact is a high proline level in ivy leaves in June (110 $\mu g \times g^{-1}$ DM), when laurel leaves exhibited the lowest proline level (51 $\mu g \times g^{-1}$ DM).

No correlation was observed between *WC* and proline content as well as soluble carbohydrate content in leaves of both species.

The frost (–7°C) induced cell membrane damage in leaves of both species changed considerably during the measurement period. Membrane injury index ranged from 5.3% to 97% in laurel and from 3.3% to 98% in ivy (Fig. 4). The highest percentage of membrane damage, from 79% to 97% in laurel and from 87% to 98% in ivy, was recorded during summer (June to September). In contrast, the lowest membrane injury was recorded in laurel leaves from February to April (5.5–11%), while in ivy from March to April (3.3–5.4%).

PEG-induced cell membrane damage changed during the experimental period from 29% to 97% in laurel leaves and from 22% to 89% in ivy leaves (Fig. 5). The biggest damage rates both in laurel (97–86%) and in ivy (89–83%) was recorded from July to September. In contrast, the lowest damage rates were observed in laurel from March to April (29–33%), but in ivy from February to April (22–41%).

A negative correlation was shown between leaf proline content and membrane injury index in frost- ($r = -0.4325$; $p < 0.05$) and PEG-treated ($r = -0.5507$; $p < 0.05$) laurel leaves. Weaker correlations between proline and membrane injury indices ($r = -0.1064$; $p<0.05$ for frost, $r = -0.3318$; $p < 0.05$ for PEG) were recorded in ivy. However, no such correlation was observed between soluble carbohydrate content and membrane damage affected by frost and PEG.

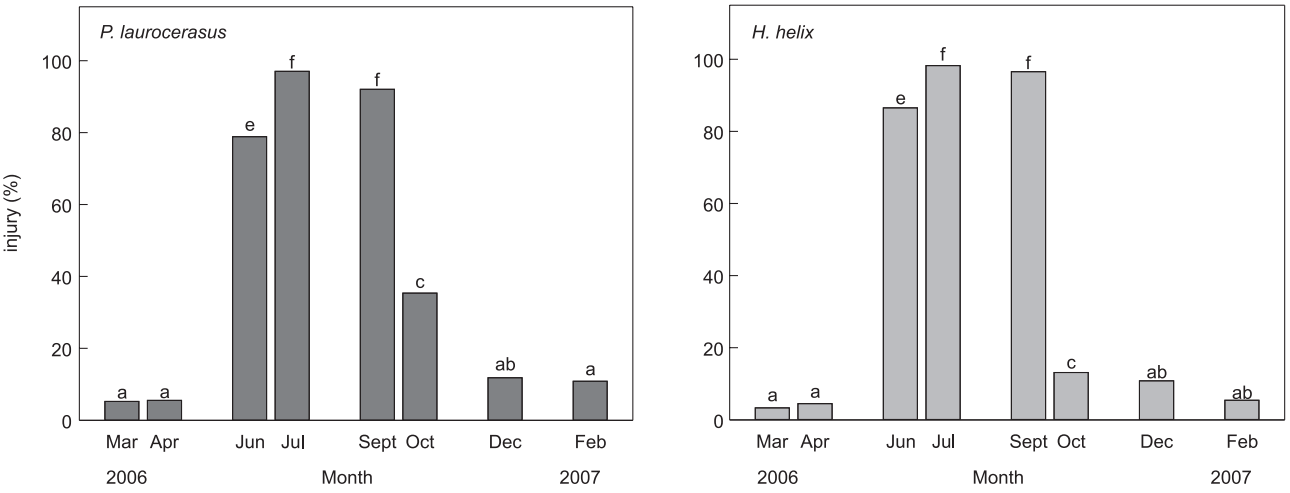

Fig. 4. Seasonal patterns of membrane injury in freeze treated (–7°C) leaves of laurel (*Prunus laurocerasus*) and ivy (*Hederea helix*). Different letters indicate significant differences between means ($p < 0.05$)

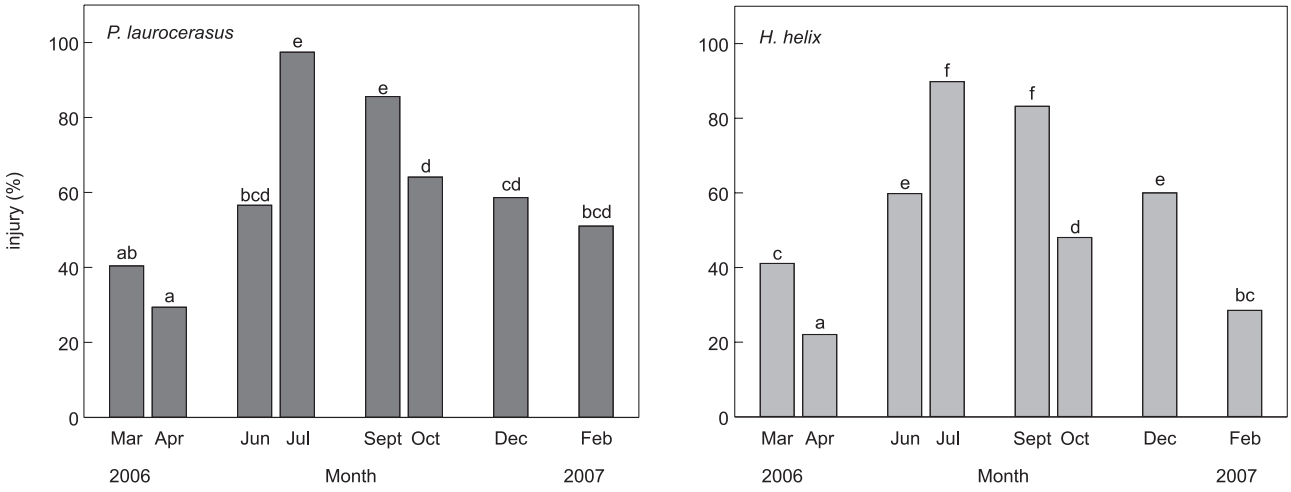

Fig. 5. Seasonal patterns of membrane injury index measured by PEG test in leaves of laurel (*Prunus laurocerasus*) and ivy ((*Hederea helix*). Different letters indicate significant differences between means ($p < 0.05$)

Discussion

Seasonal changes in sensitivity to frost (–7°C) and PEG-induced water deficit were comparable in both species. The biggest and parallel sensitivity to the action of both factors was observed in summer (Jun to Sept). A higher sensitivity to frost than to water deficit in both species was observed in June. At the other dates a considerable reduction in sensitivity to both factors was observed, as well as a generally higher sensitivity to water deficit than to frost. The lowest frost sensitivity was found in both species in early spring and winter. Needles of pine *(Pinus sylvestris*) were characterized by the lowest frost sensitivity in winter months, which increased in spring (Beck et al. 2004). It needs to be stressed that pine needles were resistant to the temperature of –40°C, while for laurel and ivy the temperature of –20°C was harmful (unpublished data). The lowest sensitivity to water deficit throughout the examined period was observed in both species in early spring and in ivy also in winter. In contrast, Premachandra and Shimada (1987) showed that in leaves of orchardgrass (*Dactylis gromerata* L.) cell membrane damage caused by the PEG-induced water deficit was higher in the spring-summer period than in the autumn-winter season.

Improved tolerance to water deficit and frost tolerance in orchardgrass (*Dactylis glomerata* L.), detected as a decrease of cell membrane injury, was associated with increased soluble carbohydrate concentrations (Premachandra et al. 1993). A considerable increase in the level of soluble carbohydrates and high stability of cell membranes during frost were observed in leaves of saltgrass (*Distichlis spicata* L. Greene) as well as rhizomes and stolons of zoysiagrass (*Zyosia* spp.) (Shahba et al. 2003; Patton et al. 2007). An increased concentration of soluble carbohydrates with a possible cryoprotective function made it possible to survive freezing temperatures during winter for overwintering cereals and evergreen plants (Hurry et al. 1995; Miyazawa and Kikuzawa 2005). Oleksyn et al. (2000) reported increased soluble carbohydrate concentrations during autumn and winter in needles of Scots pine (*Pinus silvestris* L.). Results presented in this study have shown that the highest carbohydrates level in laurel leaves was recorded in February, while the lowest in June. In contrast, in leaves of ivy the highest carbohydrate content was found during summer (Jun and Jul), whereas the lowest in winter (Dec to Feb). A high carbohydrates accumulation and at the same time small damage to cell membranes under the influence of both factors was observed only in laurel leaves harvested in February. However, a lack of correlation between soluble carbohydrates level and membrane injury index shown in this study indicates that these compounds did not play a significant role in membrane protection in stressed leaves of examined species.

Many authors revealed the positive role of proline in plant resistance to water deficit (Bandurska 2000; Ashraf, Foolad 2007; Moussa and Abdel-Azis 2008). Antisense transgenic *Arabidopsis thaliana* lines with suppression of the proline degradation enzyme accumulated higher proline levels than the wild type under either stressed or unstressed conditions and showed an enhanced tolerance to osmotic stress and freezing stress (Nanjo et al. 1999). The beneficial role of proline accumulation in tolerance to low temperature was found both in annuals (Swaaij et al. 1985; Patton et al. 2007) and in perennial woody plants (Karolewski and Pukacki 1995; Molinari et al 2004). Free proline content in leaves of investigated species was highest in early spring, i.e. March and April. Moreover, in laurel leaves an increased proline level was also recorded in summer (July) and in winter (December, February), while in ivy also in June.

Diamantoglou and Rhizolopoulou (1992) showed that the lowest proline level in leaves of three evergreen species from the East Mediterranean region (*Ceratonia siliqua* L., *Laurus nobilis* L., *Myrtus communis* L.) was found in spring and the highest in summer. In the opinion of those authors increased proline content in summer resulted from water deficit in leaves, caused by a lack of rainfall. However, no correlation was observed between proline level and leaf water content either in laurel or ivy, which indicates that leaf hydration did not affect free proline level in these evergreen species. Results recorded in this study point to a potential role of proline in the seasonal increase of resistance to frost and water deficit in both species. A negative correlation between proline content and membrane injury index observed in frost and PEG treated leaves of both species suggest that this amino acid may have resulted on membrane protection. Under natural conditions, frost hardening starts when day length falls below 12 h, which takes place at the end of summer. Woody plants maintain extreme frost hardiness during the entire winter (Thomashow 1999; Beck et al. 2004), which was also confirmed by our results. Leaves of both laurel and ivy were characterized by very small damage of cell membranes in the winter season as indicated by the freezing test. Our results proved that proline might play a role in membrane protection during winter in laurel leaves, since its level was increased in that time. Results of our study show also that sensitivity of cell membranes to water deficit and frost in leaves of both evergreen shrubs was lowest from March to April. In that period leaves of both species characterized by a high proline level. These results may indicate a possible involvement of proline in the protection of cell structures and cell proteins during the potential occurrence of frost and drought in early spring both in laurel and ivy leaves.

References

Ashraf M., Foolad M.R. 2007. Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany 59: 206–216.

Aniśko T. 2002. Cold hardiness of evergreen cherries. American Nurseryman 195(6): 42–48.

Bandurska H. 2000. Does proline accumulated in leaves of water deficit stressed barley plants confine cell membrane injury? I. Free proline accumulation and membrane injury index in drought and osmotically stressed plants. Acta Physiologiae Plantarum 22: 409–415.

Bates L.S., Waldren R.P., Teare J.D. 1973. Rapid determination of proline for water stress studies. Plant and Soil 39: 205–207.

Beck E.H., Heim R., Hansen J. 2004. Plant resistance to cold stress: Mechanisms and environmental signals triggering frost hardening and dehardening. Journal of Biosciences 29: 449–459.

Björnesjö K.B. 1955. Analysis of protein-bound serum polysaccharides with anthrone reagent. Scandinavian Journal of Clinical and Laboratory Investigation 7: 147–152.

Diamantoglou S., Rhizopoulou S. 1992. Free proline accumulation in sapwood, bark and leaves of three evergreen sclerophylls and comparison with an evergreen conifer. Journal of Plant Physiology 140: 361–365.

Frankow-Lindberg B.E. 2001. Adaptation to winter stress in nine white clover populations: Changes in non-structural carbohydrates during exposure to simulated winter conditions and spring re-growth potential. Annals of Botany 88: 745–751.

Hare P.D., Cress W.A., Van Staden J. 1998. Dissecting the roles of osmolyte accumulation during stress. Plant Cell and Environment 21: 535–553.

Hurry V.M., Strand Å. Tobićson M., Gardeström P., Öquist G. 1995. Cold hardening of spring and winter wheat and rape results in differential effects on growth, carbon metabolism, and carbohydrate content. Plant Physiology 109: 697–706.

Karolewski P., Pukacki P.M. 1995. Free proline as an indicator of sensitivity of Norway spruce (*Picea abies*) to low temperature. Arboretum Kórnickie 40: 159–167.

Kishor P.B.K., Sangam S., Amrutha R.N., Laxmi P.S., Naidu K.R., Rao K.R.S.S., Rao S., Reddy K.J., Theriappan P., Sreenivasulu N. 2005. Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: Its implications in plants growth and abiotic stress tolerance. Current Science 88: 424–438.

Keskitalo J., Bergquist G., Gardeström P., Jansson S. 2005. A cellular timetable of autumn senescence. Plant Physiology 139: 1635–1648.

Larcher W. 2005. Climatic constraints drive the evolution of low temperature resistance in woody plants. Journal of Agricultural Meteorology 61: 189–202.

Mahajan S., Tuteja N. 2005. Cold, salinity and drought stresses: An overview. Archives of Biochemistry and Biophysics 444: 139–158.

Molinari H.B.C., Marur C.J., Bespalhok J.C., Kobayashi A.K., Pileggi M., Leite R.P., Pereira L.F.P., Vieira L.G.E. 2004. Osmotic adjustment in transgenic citrus rootstock Carrizo citrange (*Citrus sinensis* OSB. X *Poncirus trifoliata* L. Raf.) overproducing proline. Plant Science 167: 1375–1381.

Moussa H.R., Abel-Azis S.M. 2008. Comparative response of drought tolerant and drought sensitive

maize genotypes to water stress. Australian Journal of Crop Science 1: 31–36.

Miyazawa Y., Kikuzawa K. 2005. Winter photosynthesis by saplings of evergreen broad leaved trees in deciduous temperate forest. New Phytologist 165: 857–866.

Nanjo T., Kobayashi M., Yoshiba Y., Kakubari Y., Yamaguchi-Shinozaki K., Shinozaki K. 1999. Antisense suppression of proline degradation improves tolerance to freezing and salinity in *Arabidopsis thaliana*. FEBS Letters 461: 205–210.

Öztürk L., Demir Y. 2002. I *vivo* and *in vitro* protective role of proline. Plant Growth Regulation 38: 259–264.

Oleksyn J., Zytkowiak P., Karolewski P., Reich P.B., Tjoelker M.G. 2000. Genetic and environmental control of seasonal carbohydrate dynamics in trees of diverse *Pinus sylvestris* populations. Tree Physiology 20: 837–847.

Patton A.J., Cunningham S.M., Volenec J.J., Reicher Z.J. 2007. Differences in freeze tolerance of zoysiagrasses: II. Carbohydrate and proline accumulation. Crop Science 47: 2170–2181.

Premachandra G.S., Saneoka H., Fujita K., Ogata S. 1993. Seasonal changes in leaf water relations and cell membrane stability in Orchardgrass (*Dactylis glomerata*). Journal of Agricultural Science 121: 169–175.

Premachandra G.S., Shimada T. 1987. Measurement of drought tolerance in orchardgrass (*Dactylis glomerata* L.). I. Polyethylene glycol test of measuring cell membrane stability. Journal of Japanese Society of Grassland Science 33: 140–148.

Pukacki P.M. 1995. Membrane properties of Norway spruce cell during long-term freezing stress. Arboretum Kórnickie 40: 125–133.

Qureshi M.I., Qaidr S., Zolla L. 2007 Proteomic-based dissection of stress-responsive pathways in plants. Journal of Plant Physiology 164: 1239–1260.

Sauter J.J. 1988. Temperature-induced changes in starch and sugars in steam of *Populus* × *canadiensis* "Robusta". Journal of Plant Physiology 132: 608–612.

Shahba M.A., Qian Y.L., Hughes H.G., Koski A.J., Christensen D. 2003. Relationships of soluble carbohydrates and freeze tolerance in saltgrass. Crop Science 43: 2148–2153.

Smallwood M., Bowles D.J. 2002. Plants in a cold climate. Philosophical Transactions of Royal Society (B) 357: 831–847.

Steponkus P.L., Uemura M., Webb M.S. 1993. Membrane destabilization during freeze-induced dehydration. Current Topics in Plant Physiology 10: 37–47.

Sullivan C.Y. 1971. Techniques for measuring plant drought stress. In: Drought injury and resistance in crops. Larson K.I., Eastin J.D. (eds.). Crop Science Society of America, Madison, Wisconsin, pp. 1–18.

Stitt M., Hurry V. 2002. A plant of all seasons: alteration in photosynthetic carbon metabolism during cold acclimation in *Arabidopsis*. Current Opinion in Plant Biology 5: 199–206.

Swaaij A.C., Jacobsen E., Feenstra W.J. 1985. Effect of cold hardening, wilting and exogenously applied proline on leaf proline content and frost tolerance of several genotypes of *Solanum*. Physiologia Plantarum 64: 230–236.

Swearingen J.M., Dietrich S. 2006. English ivy. http://www.invasive.org/weedcd/pdfs/wgw/englishivy.pdf

Thomashow M.F. 1999. Plant cold acclimation: Freezing tolerance genes and regulatory mechanisms. Annual Review of Plant Physiology and Plant Molecular Biology 50: 571–599.

Webb M.S., Steponkus P.L. 1993. Freeze-induced membrane ultrastructural alterations in Rye (*Secale cereale*) leaves. Plant Physiology 101: 955–963.

Xin Z., Browse J. 2000. Cold comfort farm: The acclimation of plant to freezing temperatures. Plant Cell and Environment 23: 893–902.