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Seasonal patterns of free proline and carbohydrate levels in cherry laurel (*Prunus laurocerasus*) and ivy (*Hedera 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

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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 (*Hedera 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 forma-
tion of ice in intracellular spaces, causing intracellular
water movement to extracellular spaces and dehydra-
tion of cells. In addition, cellular dehydration due to
low temperature may also be caused by reduced root
water uptake (Stéponkus et al. 1993, Webb and
Stéponkus 1993; Thomashow 1999; Xin and Browse
2000; Beck et al. 2004). Acclimation to cold (freez-
ing) temperature in woody plants occurs in early au-
tumn in response to shortening days and decreasing
temperature (slightly above zero), which induce
mechanisms responsible for increased freezing toler-
ance (Beck et al. 2004; Larcher 2005). These mecha-
nisms 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 oc-
currence of water deficit in cells (Nanjio 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 mem-
branes and proteins against detrimental effects of de-
hydration 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 af-
fter withdrawal of unfavourable conditions (Hare et al.

The objectives of the present study were (I) to ex-
amine seasonal changes in the levels of water-soluble
carbohydrates and free proline in leaves of cherry lau-
rel (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 Balka-
s. It was introduced to central Europe in 16th cen-
tury where is cultivated for ornamental purpose in
gardens and parks (Aniško 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,
latitude 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 de-
terminations 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 ninhydrin. Absorbance was read at 520 nm. The amount of
proline was calculated from a previously plotted stan-
dard curve and expressed in µg · g−1 of leaf dry matter
(DM). Water-soluble carbohydrate level was deter-
mined by the anthrone test according to Björnejso
(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 re-
action product of the anthrone reagent with carbohy-
drates soluble in the supernatant was read at 620 nm.
The amount of carbohydrates was calculated from a
standard curve previously plotted for glucose and ex-
pressed in mg · 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 cm³ of deionized water to remove surface ad-
hered electrolytes and put to 50 cm³ flask. One batch
of leaf samples was submerged in 10 cm³ 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 sur-
fase and kept for 24h at the temperature of −7°C. The
third batch of leaves was submerged in deionized wa-
ter (control) and kept for 24h at 10°C. After dehydra-
tion leaf pieces from the PEG-treated combination
were quickly washed three times with deionized wa-
ter and finally submerged in 10 cm³ of water. Leaf
pieces from the frost treated combination were sub-
merged in 10 cm³ of deionized water immediately af-
ter 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 in-
jury (%) was evaluated according to the formula:

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\text{Membrane injury index} = \frac{\text{WC before treatment} - \text{WC after treatment}}{\text{WC before treatment}} \times 100
\]
where \( C_1 \) and \( C_2 \) represent conductivity values of control samples before and after autoclaving, respectively; \( T_1 \) and \( T_2 \) 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-
cies the maximum proline level was recorded in April, amounting to 884 µg × g⁻¹ DM in laurel and to 182 µg × 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 µg × g⁻¹ DM), but in ivy from July to February (32–57 µg × g⁻¹ DM). An interesting fact is a high proline level in ivy leaves in June (110 µg × g⁻¹ DM), when laurel leaves exhibited the lowest proline level (51 µg × 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.

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

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


