

## Seasonal Changes in the Pattern of Assimilatory Enzymes and of Proteolytic Activities in Leaves of Juvenile Ivy

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Ivy growing under natural conditions is an interesting plant to study the influence of external (e.g. temperature, light) and internal (e.g. source/sink relations) factors on leaf metabolism. Leaves of this evergreen plant are subject several times to seasonal changes. The contents of selected assimilatory enzymes were well conserved throughout the winter indicating that ivy leaves are probably able to make use of short periods with higher temperatures and to immediately restart growth in spring. Total proteins and carbohydrates increased considerably between February and May before the emergence of the new leaf generation. The increase in the content of non-structural carbohydrates was due to the accumulation of starch, while soluble sugars peaked in winter and decreased in spring. From May onwards, the assimilates were retranslocated to the emerging young plant parts. Marked seasonal changes in the peptide hydrolase pattern were observed. All exo- and endopeptidases investigated were minimal during summer suggesting that the net protein remobilization from older leaves was not based on an increase in the level of these major peptide hydrolases. Source/sink interactions on a whole plant level seem to be decisive in the regulation of seasonal changes in the pattern of assimilatory enzymes and of proteolytic activities. Since ivy leaves remain active for several years, the changes must be reversible and occur repeatedly during the life-span of a particular leaf.

**Key words:** *Hedera helix* L., ivy, peptide hydrolase, assimilatory enzyme, low temperatures, retranslocation.

### INTRODUCTION

A leaf of an evergreen perennial plant is subject to seasonal changes several times during its life. It must therefore be able to adapt to the conditions in winter (characterized by low temperatures and low metabolic activities) and to re-adapt to warmer temperatures at the beginning of the following growth period (characterized by increasing metabolic activities).

Major changes in the levels of carbon and nitrogen compounds have been detected in juvenile ivy leaves in the course of a year (Parker, 1962). Soluble sugars peaked during the coldest phases of winter and rapidly decreased in spring. A cryoprotective role of sugars has been discussed in the past (Guy, 1990). Starch was not considered in the outdoor experiments mentioned (Parker, 1962), but a rapid increase of the starch content has been demonstrated in artificially cold-acclimated ivy plants after transition from 5 to 21 °C (Steponkus and Lanphear, 1968). Protein contents were high throughout the winter and reached maximal values in spring. However, enzymes from several plant species have been found to be cold-labile and to be inactivated by low temperatures, often because of a dissociation of the functional enzyme into its subunits (Guy, 1990). This may increase the accessibility for a proteolytic attack and might cause a temporal loss of metabolic capabilities during winter.

Changes in the levels of assimilatory enzymes and of peptide hydrolases have been investigated during leaf development in annual crop plants (Feller, 1990; Feller and

Fischer, 1994). The peptide hydrolase pattern in evergreen leaves of perennial plants, where the situation is more complex, have been less well investigated. In such leaves, enzyme levels should be interpreted with regard to (a) leaf age, (b) source/sink interactions on a whole plant level, and (c) metabolic adaptations to seasonal changes of external factors. The effects on selected enzymes of carbon and nitrogen assimilation as well as on major proteolytic activities in naturally growing ivy plants were the focus of our studies.

### MATERIALS AND METHODS

#### *Plant material*

Juvenile ivy (*Hedera helix* L.) leaves were collected between Nov. 1990 and Nov. 1991 from plants covering the soil in the Botanical Garden of the University of Bern, Switzerland. From spring to autumn 1991, young leaves (developed in 1991) and old leaves (developed in 1990 or earlier) were sampled separately.

#### *Extraction*

The fresh plant material was homogenized immediately after collection. Thirty-six leaf discs with a diameter of 1.4 cm (total area: 55.42 cm<sup>2</sup>) were extracted in 10 ml extraction medium (20 mM sodium phosphate buffer pH 7.5 containing 1% w/v polyvinylpyrrolidone and 0.1% (v/v)  $\beta$ -mercaptoethanol) with a Polytron mixer (Kinematica, Littau/Luzern) for 20 s at medium and for 5 s at full speed. The crude extracts were filtered through Miracloth (Calbiochem, San Diego).

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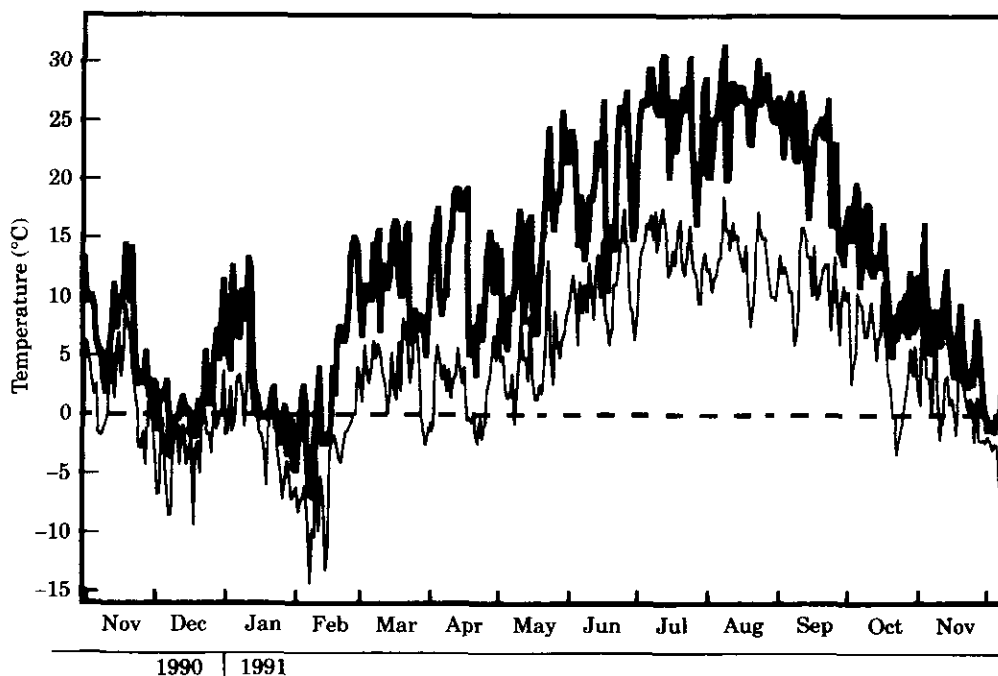


FIG. 1. Daily temperature maxima (bold line) and minima (thin line) in Bern between Nov. 1990 and Nov. 1991.

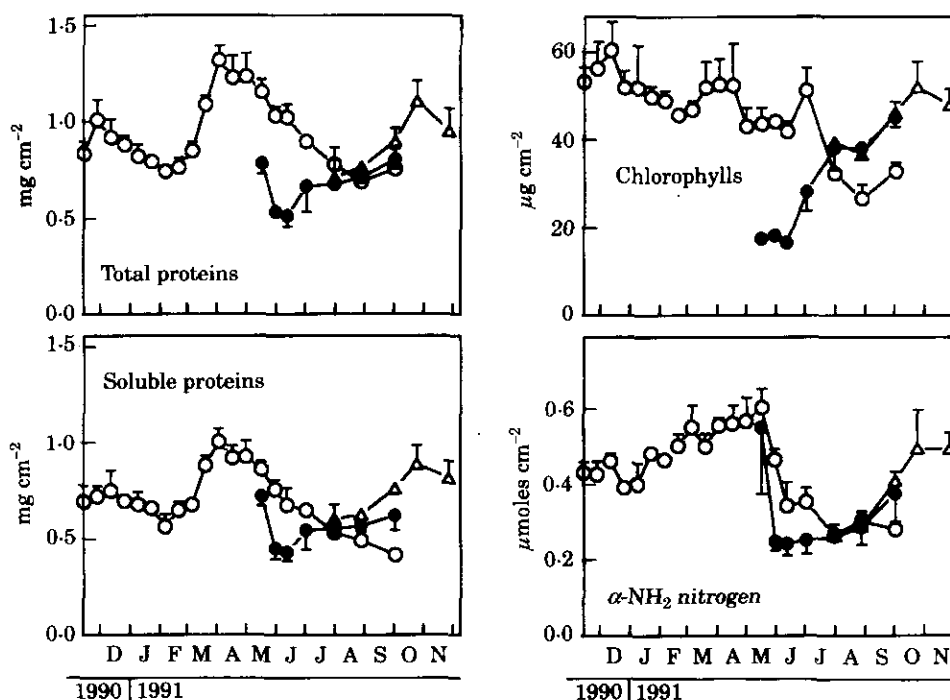


FIG. 2. Seasonal changes in nitrogen compounds. The sampling regime was as follows: leaves developed in 1990 or earlier (○) were collected at intervals from Nov. 1990 to Sep. 1991; the new leaves developed in 1991 (●) were collected separately from 17 May to Sep. 1991; a mixture of these leaf populations (△) was also analysed from Aug. 1991 to the end of Nov. 1991. Months from autumn 1990 to autumn 1991 are abbreviated by their first letter. Means and standard deviations were computed from four independent samples (each containing 55.4 cm<sup>2</sup> leaf area). The standard deviations are shown when exceeding the size of the symbol (on one side only for clarity).

#### Quantification of nitrogen compounds

The Miracloth filtrate was mixed with acetone (final concentration: 80%) and centrifuged for 5 min at 2300 g before photometry at 649 and 665 nm for the quantification

of chlorophylls. Contents were calculated according to Strain, Cope and Svec (1971). Total proteins (in the Miracloth filtrate) and soluble proteins (supernatants after centrifugation for 15 min at 12000 g) were quantified according to Bradford (1976) using bovine gamma globulin

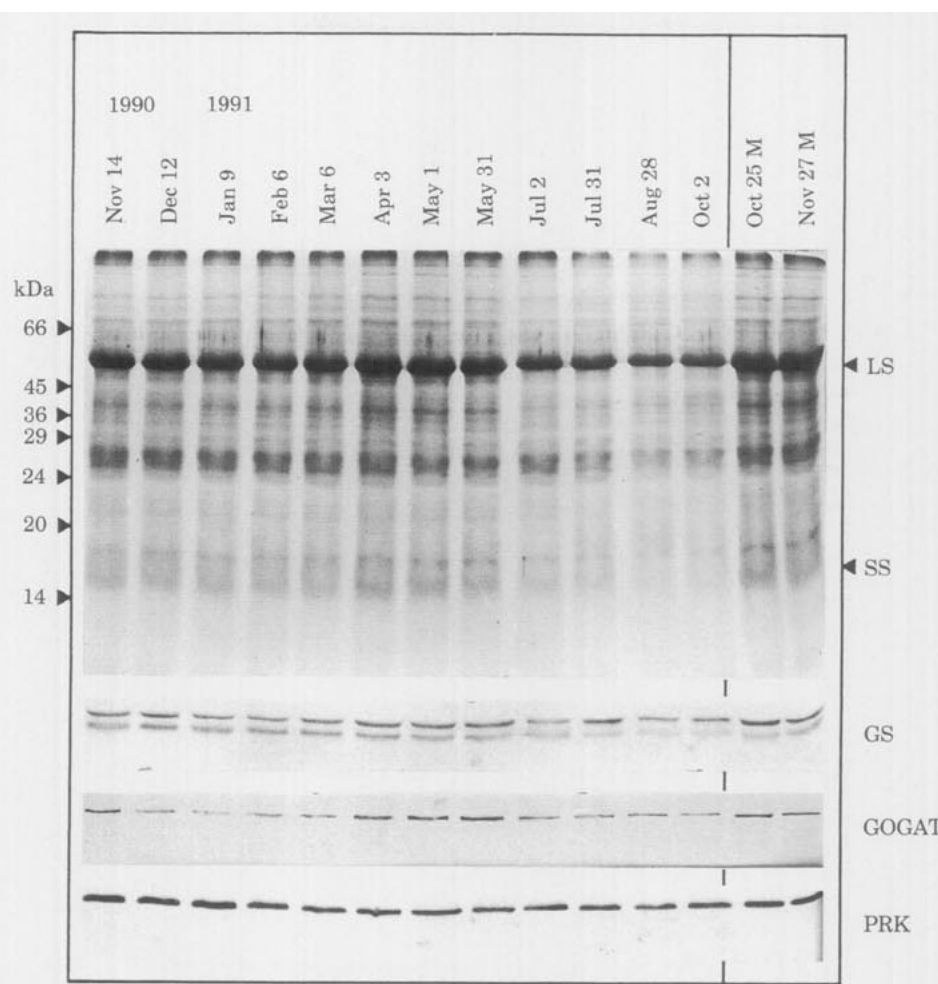


FIG. 3. Seasonal changes in the protein pattern. Old ivy leaves (developed in 1990 or earlier) were analysed by SDS-PAGE and stained with Coomassie Brilliant Blue R-250 for total proteins or by immunoblotting for selected enzymes between Nov. 1990 and Nov. 1991. Each lane was loaded with a sample equivalent to 2.0 mm<sup>2</sup> leaf area for SDS-PAGE or with a sample equivalent to 2.7 mm<sup>2</sup> leaf area for immunoblotting. The positions of the large (LS) and small (SS) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase are indicated on the right side of the Coomassie Blue-stained gel. Glutamine synthetase (GS), ferredoxin-dependent glutamate synthase (GOGAT) and phosphoribulokinase (PRK) were detected with specific antibodies. Samples containing both old leaves (developed in 1990 or earlier) and young leaves (developed in 1991) were analysed in Oct. (Oct 25 M) and Nov. (Nov 27 M) 1991 to allow a comparison with the results obtained in Nov. 1990.

as the standard. Samples were centrifuged for 10 min at 2300g and stored frozen (−20 °C) for the measurement of free amino acids with a ninhydrin reagent (Cramer, 1958).

#### *Soluble carbohydrates and starch*

Buffer-soluble carbohydrates were quantified with an anthron reagent (Fluri, 1959) as described by Fröhlich and Feller (1991). The supernatants of extracts centrifuged for 10 min at 2300 g were stored frozen (−20 °C) for these measurements. Starch was determined in the sediments (stored at −20 °C) of a 12000 g centrifugation. It was hydrolysed in 1.6 N perchloric acid for 80 min at 70 °C. The free glucose was quantified as described above.

#### *Potassium, magnesium and calcium*

Cations were quantified in the Miracloth filtrate (stored at −20 °C) according to Schenk and Feller (1990). The extracts

(200 µl) were mixed with 50 µl H<sub>2</sub>O<sub>2</sub> and heated to 110 °C until the samples were completely dry. The addition of H<sub>2</sub>O<sub>2</sub> and the heating were repeated once. After cooling 50 µl 10 N HCl and 1 ml H<sub>2</sub>O were added. K, Mg and Ca were measured by atomic absorption spectrometry after appropriate dilution with 1.267 g l<sup>−1</sup> CsCl suprapur in 0.1 N HCl (for K) or with 13.37 g l<sup>−1</sup> LaCl<sub>3</sub>·7H<sub>2</sub>O in 0.1 N HCl (for Ca and Mg).

#### *Activities of peptide hydrolases*

Aminopeptidase activities were determined in microtitration plates with a series of p-nitroanilides as substrates according to Blättler and Feller (1988) except that a buffer consisting of 100 mM Bis-Tris propane and 50 mM acetate (adjusted to the desired pH values with HCl) was used. The pH values for the various substrates were slightly different, according to the optima determined in a preliminary experiment. Carboxypeptidase activity was measured ac-

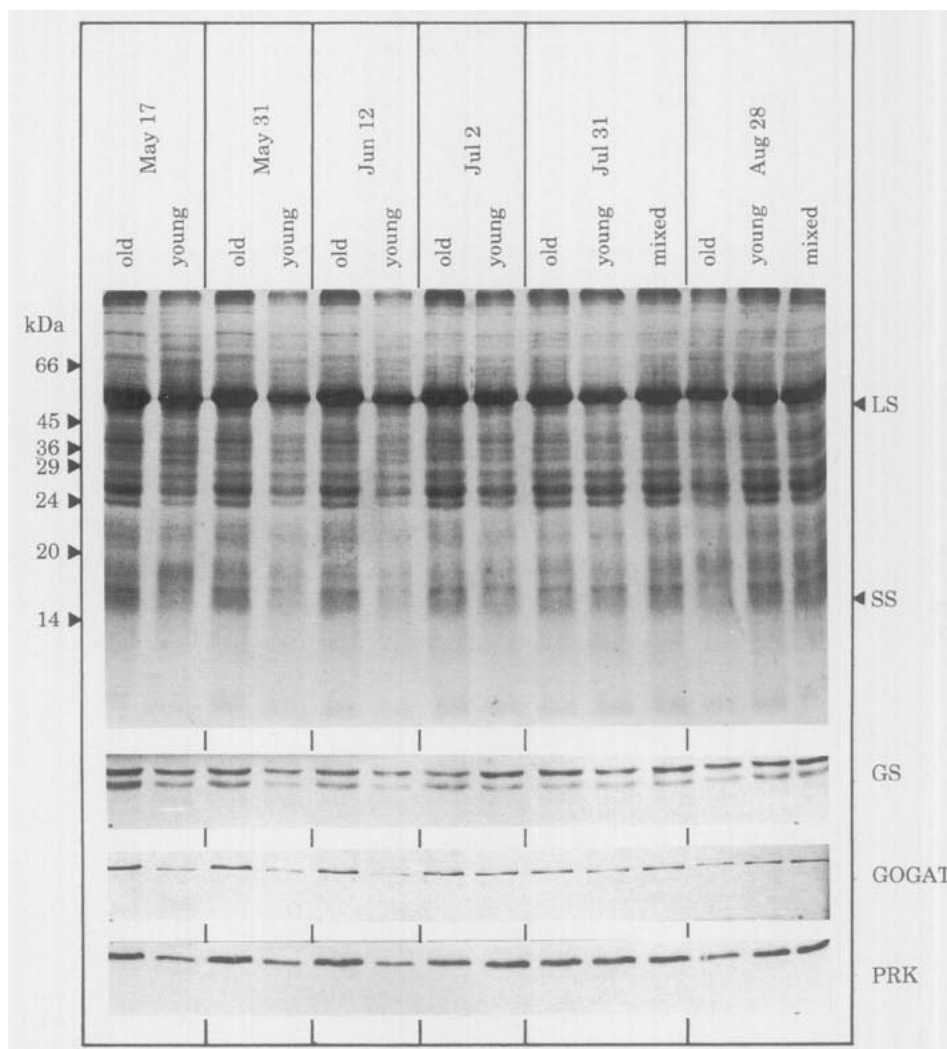


FIG. 4. Comparison of the protein patterns of old and young leaves. Old (developed in 1990 or earlier) and young ivy leaves (developed in 1991) were analysed separately by SDS-PAGE and staining with Coomassie Brilliant Blue R-250 for total proteins or by immunoblotting for selected enzymes between May and Aug. 1991. Samples containing both leaf types (mixed) were analysed additionally on 31 Jul. and 28 Aug. Each lane was loaded with a sample equivalent to 2.0 mm<sup>2</sup> leaf area for SDS-PAGE or with a sample equivalent to 2.7 mm<sup>2</sup> leaf area for immunoblotting. The positions of the large (LS) and small (SS) subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase are indicated on the right side of the Coomassie Blue-stained gel. Glutamine synthetase (GS), ferredoxin-dependent glutamate synthase (GOGAT) and phosphoribulokinase (PRK) were detected with specific antibodies.

cording to Salgó and Feller (1987) using the N-masked dipeptide N-carbobenzoxy-L-phenylalanine-L-alanine as substrate. Endopeptidase activities were determined by the formation of trichloroacetic acid-soluble products ( $\mu\text{g h}^{-1}$ ) from azocasein at three different pH values (5.0, 7.5 and 9.0) according to Fröhlich and Feller (1992). To eliminate interferences (probably with newly formed anthocyanins), all extracts prepared on 9 Jan. onwards were desalted by centrifugation through Sephadex G-25 (Feller, Soong and Hageman, 1977) equilibrated with 20 mM sodium phosphate buffer containing 0.1% (v/v)  $\beta$ -mercaptoethanol.

#### SDS-PAGE and immunoblotting

The samples were prepared from the Miracloth filtrate and stored at  $-20^{\circ}\text{C}$  prior to analysis. Polyacrylamide gel

electrophoresis (SDS-PAGE) using 0.75 mm thick slab gels (12%) was performed according to Laemmli (1970) in a Mini Protean II Dual Slab cell (Biorad, Richmond, UK). Western blotting (Tijssen, 1985) was performed as described by Fröhlich and Feller (1991). The blots were developed according to Mitsuhashi and Feller (1992). Plastidial glutamine synthetase (Höpfner, Reifferscheid and Wild, 1988), phosphoribulokinase (Crafts-Brandner, Salvucci and Egli, 1990) and ferredoxin-dependent glutamate synthase (Marquez *et al.*, 1988) were detected with specific antibodies raised in rabbits.

#### RESULTS

Climatic variations observed between Nov. 1990 and Nov. 1991 (daily minimal and maximal temperatures) are shown

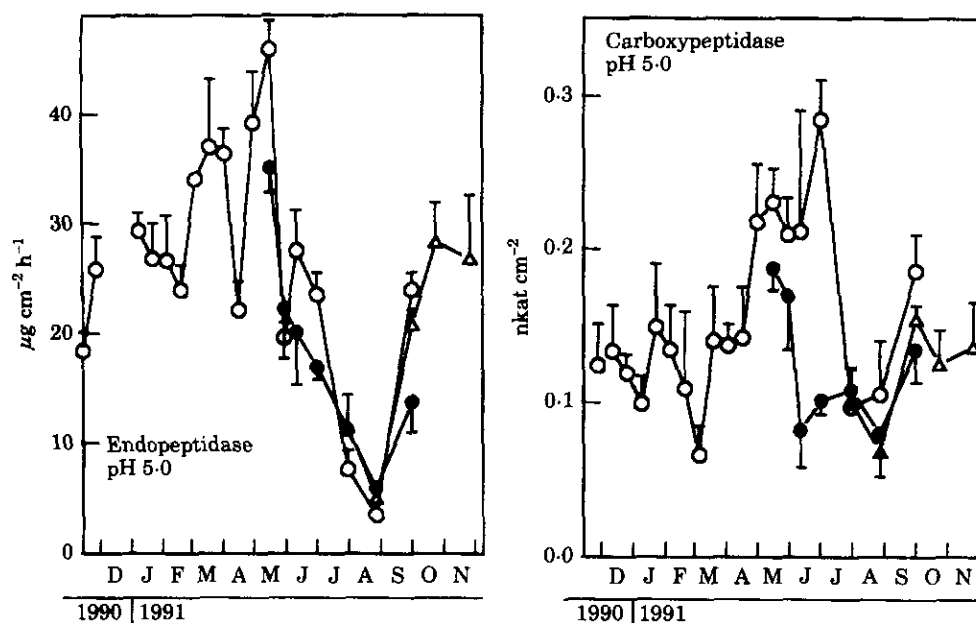


FIG. 5. Seasonal changes in endopeptidase and carboxypeptidase activities. The sampling regime was as follows: leaves developed in 1990 or earlier (○) were collected at intervals from Nov. 1990 to Sep. 1991; the new leaves developed in 1991 (●) were collected separately from 17 May to Sep. 1991; a mixture of these leaf populations (△) was also analysed from Aug. 1991 to the end of Nov. 1991. Analyses were carried out immediately after collection of the leaves. Months from autumn 1990 to autumn 1991 are abbreviated by their first letter. Means and standard deviations were computed from four independent samples (each containing 55.4 cm<sup>2</sup> leaf area). The standard deviations are shown when exceeding the size of the symbol (on one side only for clarity).

in Fig. 1. Two marked low-temperature phases were observed during the winter (in Dec. and Feb.), but the beginning of Jan. was rather warm.

Proteins and amino acids showed clear seasonal variations (Fig. 2). Protein levels peaked in Apr. and May just before the emergence of the first young leaves. A minor peak was observed in Oct./Nov. In spring and early summer protein contents per leaf area were considerably lower in the young leaves as compared to old leaves. Free amino acids also peaked before the development of the first young leaves, whereas no such peak was detected for chlorophylls (Fig. 2). The contents of some individual enzyme proteins were followed by SDS-PAGE and immunoblotting (Fig. 3). The seasonal changes visible for total proteins were also detected for ribulose-1,5-bisphosphate carboxylase/oxygenase, ferredoxin-dependent glutamate synthase and glutamine synthetase, but not (or much less pronounced) for phosphoribulokinase. Glutamine synthetase was detected with an antibody raised against the plastidial isoenzyme from mustard (Höpfner *et al.*, 1988). The double band (Fig. 3) may be due to a crossreactivity with the cytosolic isoform of the enzyme. Two different glutamine synthetase subunits have also been detected in pea leaves with an antibody raised against the plastidial isoenzyme from tobacco (Tingey, Walker and Coruzzi, 1987), and they have been identified as plastidial (upper band) and cytosolic glutamine synthetase (lower band).

The contents of all enzymes mentioned were lower in the young leaves just after their emergence as compared to mature leaves (Fig. 4). Low protein and chlorophyll contents were also detected in old leaves during late summer and

autumn, but the values obtained with the mixed leaf population in Nov. 1991 were comparable to Nov. 1990 (Fig. 2).

The main endopeptidase activity was found in the acidic pH range (5.0). Activities in the neutral and alkaline range were very low (data not presented). All investigated proteolytic enzymes showed major seasonal changes. Endopeptidase activity at pH 5.0 and most aminopeptidase activities peaked slightly before or during the development of the first young leaves in spring when protein contents were highest (Figs 5 and 6). The maximal carboxypeptidase activity was found about 2 months later (Fig. 5). Endopeptidase activity and some aminopeptidase activities were much lower in summer than during the winter. Considering old and young leaves, three different patterns of seasonal changes were found for aminopeptidase (Fig. 6). The first group consisted of activities hydrolysing alanine-, lysine- and arginine-p-nitroanilide; the second group of activities hydrolysing methionine-, proline-, phenylalanine- and leucine-p-nitroanilide; and a third pattern was found using glycine-p-nitroanilide as substrate. This may be a hint that different aminopeptidase forms are present in ivy leaves.

Buffer-soluble carbohydrates were high during the winter and peaked in Feb. during a marked low temperature phase (Figs 1 and 7). They started to decrease before the development of young leaves in spring. Starch content (Fig. 7) was marginal during the winter and increased sixty-fold between late Feb. (when temperatures started to rise) and May. The increase in starch by far over-compensated the decrease of soluble carbohydrates, leading to a maximum content of total carbohydrates during May when the first

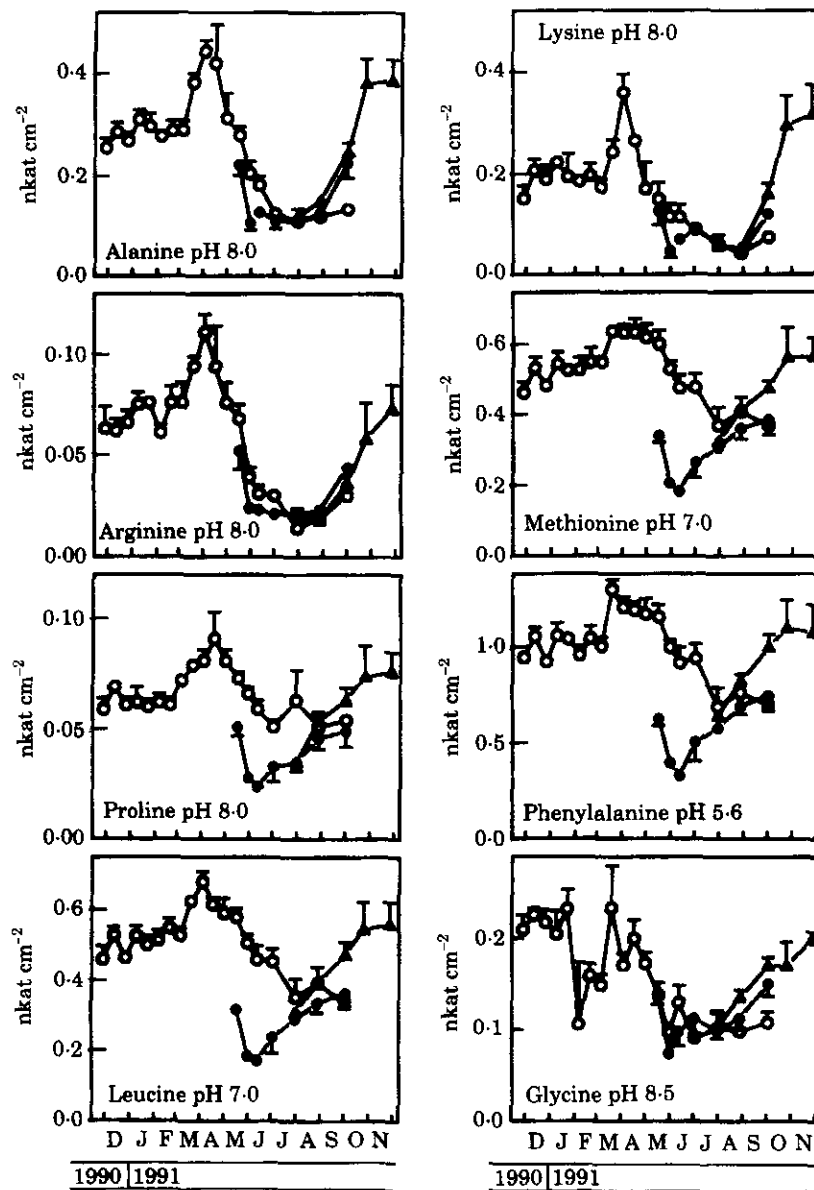


FIG. 6. Seasonal changes in aminopeptidase activities. The sampling regime was as follows: leaves developed in 1990 or earlier ( $\circ$ ) were collected at intervals from Nov. 1990 to Sep. 1991; the new leaves developed in 1991 ( $\bullet$ ) were collected separately from 17 May to Sep. 1991; a mixture of these leaf populations ( $\triangle$ ) was also analysed from Aug. 1991 to the end of Nov. 1991. Analyses were carried out immediately after collection of the leaves. The rate of hydrolysis was determined with p-nitroanilides of various amino acids at the pH indicated (according to the optima determined in a preliminary experiment). Months from autumn 1990 to autumn 1991 are abbreviated by their first letter. Means and standard deviations were computed from four independent samples (each containing 55.4 cm<sup>2</sup> leaf area). The standard deviations are shown when exceeding the size of the symbol (on one side only for clarity).

young leaves emerged. The following decrease in the old leaves may be explained by a redistribution of reduced carbon to the continuously developing young plant parts during the rest of the growth period. Although the seasonal variations of amino acids and proteins on the one hand and reduced carbon on the other were qualitatively comparable, they were more pronounced for the carbohydrates.

In contrast to the results obtained with reduced carbon and nitrogen, no seasonal changes were detected for the phloem-mobile elements potassium and magnesium (Fig.

8). Unlike potassium, magnesium was low in emerging young leaves. The phloem-immobile element calcium was even lower in the young leaves and did not reach the level detected in old leaves during the first vegetation period (Fig. 8).

## DISCUSSION

The investigated assimilatory enzyme proteins were essentially conserved throughout the winter. Ivy leaves may

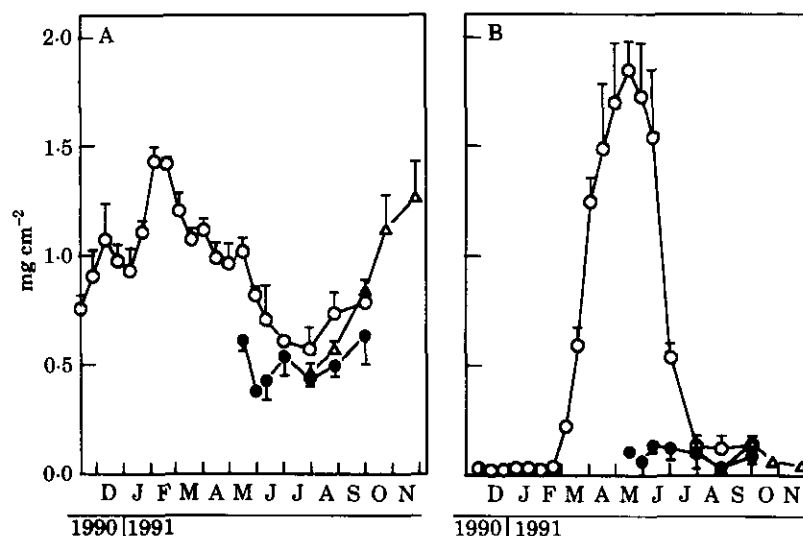


FIG. 7. Seasonal changes in the carbohydrate contents. A, soluble carbohydrates; B, starch. The sampling regime was as follows: leaves developed in 1990 or earlier ( $\circ$ ) were collected at intervals from Nov. 1990 to Sep. 1991; the new leaves developed in 1991 ( $\bullet$ ) were collected separately from 17 May to Sep. 1991; a mixture of these leaf populations ( $\triangle$ ) was also analysed from Aug. 1991 to the end of Nov. 1991. Months from autumn 1990 to autumn 1991 are abbreviated by their first letter. Means and standard deviations were computed from four independent samples (each containing  $55.4 \text{ cm}^2$  leaf area). The standard deviations are shown when exceeding the size of the symbol (on one side only for clarity).

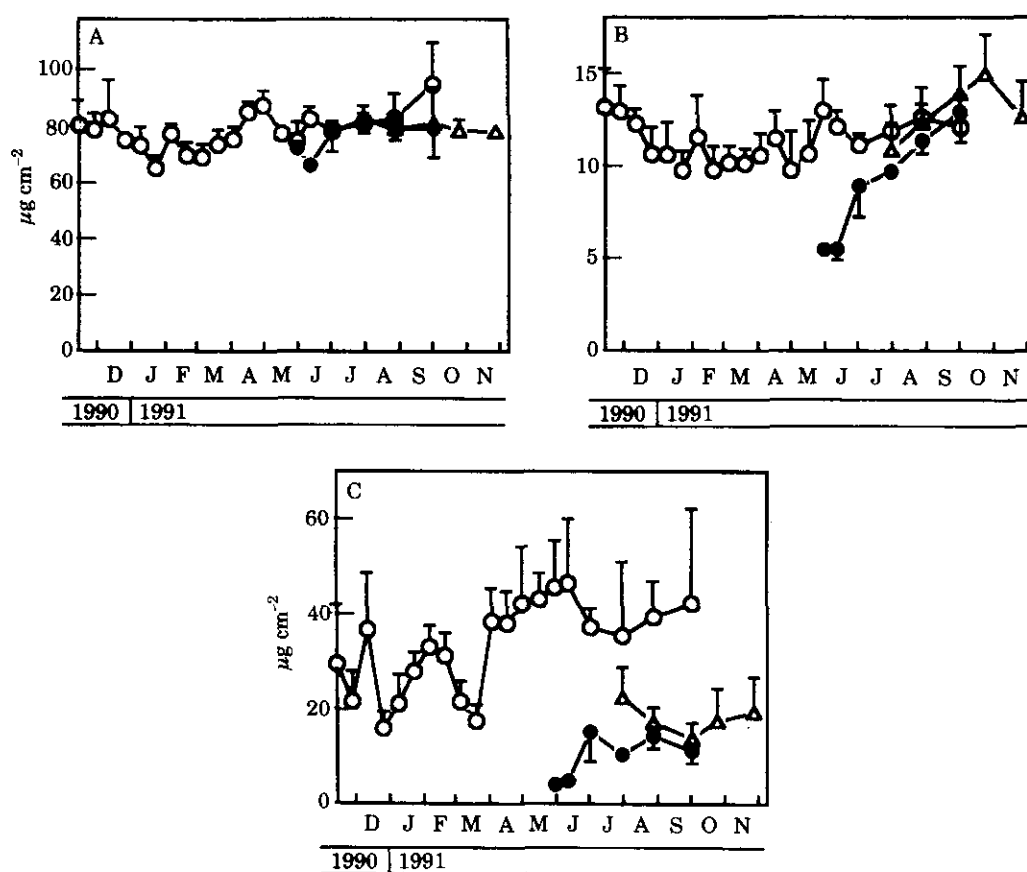


FIG. 8. Seasonal changes in the contents of cations. A, potassium; B, magnesium; C, calcium. The sampling regime was as follows: leaves developed in 1990 or earlier ( $\circ$ ) were collected at intervals from Nov. 1990 to Sep. 1991; the new leaves developed in 1991 ( $\bullet$ ) were collected separately from 17 May to Sep. 1991; a mixture of these leaf populations ( $\triangle$ ) was also analysed from Aug. 1991 to the end of Nov. 1991. Months from autumn 1990 to autumn 1991 are abbreviated by their first letter. Means and standard deviations were computed from four independent samples (each containing  $55.4 \text{ cm}^2$  leaf area). The standard deviations are shown when exceeding the size of the symbol (on one side only for clarity).

therefore be able to make use of short periods with higher temperatures during winter and to immediately start carbon and nitrogen assimilation with rising temperatures in spring. A comparable situation has been found in winter wheat leaves (Fischer and Feller, 1993).

Reduced nitrogen and carbon compounds synthesized between Feb. and May were not directly used for the growth of young plant parts. A 200% increase of non-structural carbohydrates (caused by a drastic increase in starch content) and a 50% increase of amino acids and proteins were detected in the existing leaves during this phase. The time courses between May and Jul. can only be interpreted as a net redistribution of carbon and nitrogen to emerging leaves.

Carboxypeptidase and a major endopeptidase have been localized in the vacuole of several plant species, while aminopeptidases have been found in the chloroplasts as well as outside of these organelles (Feller, 1986; Huffaker, 1990). It is noteworthy that marked seasonal changes were found in ivy leaves for all investigated proteolytic activities, irrespective of their subcellular localization. Three different types of time courses were detected for aminopeptidase activities. The substrate preferences associated with the different types are consistent with the substrate specificities reported for aminopeptidase forms from bean seeds (Blättler and Feller, 1988). At least three different aminopeptidase forms must therefore be present in ivy leaves. A strong increase of endopeptidase activity with a slightly basic pH optimum has been found during the phase of rapid protein remobilization from senescing wheat leaves (Feller, 1986; Fröhlich and Feller, 1992). No such increase was detected in ivy leaves during the phase of protein degradation in spring and early summer. However, it must be borne in mind that the situation in ivy leaves (repeated changes during several years) is different from that in senescing wheat leaves, where the remobilization process leads to the death of the organ. All peptide hydrolase activities investigated reached minimal activities earlier or later during summer.

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