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Enzymes of ammonia assimilation, photosynthesis, and respiration in alfalfa leaves of different ages

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Abstract

The activities of enzymes involved in ammonia metabolism ferredoxin-dependent glutamate synthase (Fd-GOGAT), glutamine synthetase (GS) and glutamate dehydrogenase (GDH), the rates of photosynthetic oxygen evolution, dark respiration, and the activity of RuBP carboxylase (RuBPC) were determined in alfalfa (*Medicago sativa* L.) leaves taken from the apex (apical leaves), from the second to the fourth internode (mature leaves) and from the bottom of the canopy (basal leaves). Photosynthetic rate and the activities of RuBPC, GS and Fd-GOGAT showed their maximum in the mature leaves. The respiration rate together with amino acid and ammonium contents decreased with leaf age, whereas the opposite was true for GDH activity. Basal leaves still maintained substantial levels of chlorophylls, GS and Fd-GOGAT activities and oxygen evolution rate, thus suggesting that photosynthesis has some role in the reassimilation of the nitrogen liberated during protein degradation.

Additional key words: glutamate dehydrogenase, glutamate synthase, glutamine synthetase, leaf development, *Medicago sativa*, nitrogen metabolism, RuBP carboxylase.

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Abbreviations: DTT - dithiothreitol; EDTA - ethylendiaminetetraacetic acid; Fd-GOGAT - ferredoxin-dependent glutamate synthase; GS - glutamine synthetase; GDH - glutamate dehydrogenase; HEPES - N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid); PAGE - polyacrylamide gel electrophoresis; PMSF - phenylmethylsulphonylfluoride; PVPP - polyvinylpolypyrrolidone; RuBPC - ribulose 1,6 bisphosphate carboxylase; TRIS - Tris(hydroxymethyl)aminomethane.

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Introduction

During development and ageing, leaves undergo many anatomical, physiological and metabolic changes: in the transition from heterotrophy to autotrophy the leaf reaches photosynthetic competence and the rate of respiration and synthesis of proteins and lipids declines owing to the decreased need of growth (Nakamura and Hashimoto 1988, Turgeon 1989, Klerk *et al.* 1992).

Nitrogen is an essential element for plant growth, but despite its high abundance in the atmosphere, the availability of nitrogen is often limited. Differently from cereal plants, leguminous plants have evolved the capability to co-operate symbiotically with *Rhizobium* bacteria so to obtain reduced nitrogen for their needs. Nevertheless, with the onset of senescence, nitrogen released from protein degradation is reassimilated and exported to growing leaves, through the involvement of proteases and other enzymes (Peoples and Dalling 1988, Feller 1990). Nitrogen metabolism during leaf development has been intensively studied, both in cereal and leguminous plants (Peoples and Dalling 1988). However, there are few data on enzymes of ammonia assimilation in leaves of alfalfa. Furthermore, attempts to relate developmental changes in enzyme activities of nitrogen metabolism with both photosynthesis and respiration are scanty. Therefore, the aim of this work was to determine the activities of important enzymes involved in ammonia metabolism and to compare them with photosynthetic capabilities and respiration rates, in leaves of different age of *Medicago sativa* L.

Materials and methods

Plants: Leaves were collected in August 1996, in the morning, from 70 - 80 cm tall, 1-year-old alfalfa plants (*Medicago sativa* L., cv. Leonicena) grown in the field. Apical leaves were light green, partially unfolded, at about 70 % of full size; mature leaves were dark green, fully expanded and were collected from the second to the fourth internode; the basal leaves, starting to yellow, were taken from the bottom of plant canopy. Except for O_2 exchange measurements, leaves of the same kind were pooled, rinsed with distilled water and blotted dry with paper. Sub-samples (0.5 g) were frozen and stored in liquid N_2 for later analysis. The results reported are the mean of three separate harvests.

Oxygen exchange measurements: Evolution and uptake of O_2 were measured in a gas-phase leaf-disc O_2-electrode (*LD2, Hansatech*, Kings Lynn, UK). Measurements were performed on individual leaf blades which were weighed and immediately placed in the measurement chamber. Respiration was measured at 25 °C in water-saturated air after 10 min of equilibration in darkness (Delieu and Walker 1981). Photosynthetic O_2 evolution was measured in saturating CO_2, supplied from 0.4 cm^3 of a 1 M $KHCO_3/K_2CO_3$ mixture (pH 9.1). Irradiance (1050 μmol m^{-2} s^{-1} PAR at the top of the chamber) was delivered from a light source *LS2* (*Hansatech*, Kings Lynn, UK) fitted with a heat filter. Oxygen evolution was monitored for 20 min, and was

constant during the last 10 - 15 min. Controls were carried out using leaf tissue killed by the freezing and thawing.

Enzyme extraction and assays: Frozen leaf tissue (0.5 g fresh mass) was ground to a powder in a chilled mortar with liquid N_2. Five cm^3 extraction buffer containing 50 mM HEPES-KOH (pH 7.4), 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 5 % (v/v) glycerol, 0.1 % (v/v) Triton X-100, 0.5 mM PMSF and 2 % (m/v) PVPP. The mixture was ground until complete thawing, filtered through muslin cloth and centrifuged (30 000 *g* for 5 min at 2 °C). A 2.5-cm^3 aliquot of the resulting supernatant was desalted at 4 °C on a *Sephadex G-25* column (*PD-25 Pharmacia*, Uppsala, Sweden) equilibrated with 30 cm^3 of the above extraction buffer minus Triton X-100 and PVPP. The activity of RuBP carboxylase (RuBPC; EC 4.1.1.39) was determined spectrophotometrically in a coupled assay by measuring the 3-phosphoglycerate produced in the reaction after a 5-min period of incubation with 10 mM $MgCl_2$ and 20 mM $NaHCO_3$ (Di Marco and Tricoli 1983). The activity of glutamine synthetase (GS; EC 6.3.1.2.) was measured by the synthetase assay as described by Lea *et al.* (1990). Ferredoxin-dependent glutamate synthase (Fd-GOGAT; EC 1.4.7.1) was determined according to Lea *et al.* (1990), through the separation of glutamate from the reaction mixture by anion-exchange chromatography and its quantitation with ninhydrin. Glutamate dehydrogenase (GDH; EC 1.4.1.2) was assayed spectrophotometrically at 340 nm in a final volume of 1 cm^3 containing 70 mM TRIS-HCl (pH 8.1), 1 mM $CaCl_2$, 0.1 mM NADH, 150 mM NH_4^+-acetate, starting the reaction with 20 mM 2-oxoglutarate. All reported activities were measured in triplicate on each sample and were linear with time and proportional to the amount of extract added. Leaf protein content was determined on desalted extracts according to Bradford (1976), using bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis was performed on 5 % acrylamide gels according to Davis (1964). GS activity on native gels was detected by the transferase assay as described by Barrat (1980). To distinguish between GS1 and GS2 isoforms, the extracts were incubated for 30 min at 45 °C as described by Hirel and Gadal (1980) prior to PAGE.

Other measurements: Chlorophylls were extracted and assayed according to Moran (1982). Total amino acids and ammonia were extracted with 1 M perchloric acid. After neutralization with 3 M KOH, amino acids were determined by the ninhydrin method (Moore and Stein 1948) using glutamate as a standard. Ammonia was determined in a final volume of 1 cm^3 containing 55 mM TRIS-HCl (pH 8.1), 10 mM α-keto-glutarate, 0.1 mM NADH and measuring the absorbance change at 340 nm 30 min after the addition of 12U GDH (*Boehringer*, Mannheim, Germany). A proper blank was prepared to substract ammonia contaminations of the solutions.

Results

Protein and chlorophyll content increased with leaf expansion, reaching the highest values in fully developed leaves, and decreased in basal samples to about 70 % and 80 % of the values in mature leaves, respectively (Fig. 1*A,B*).

Photosynthetic rate of apical leaves, measured as oxygen evolution, was about 60 % of that obtained with fully expanded leaves (Fig. 1*C*). Basal samples still

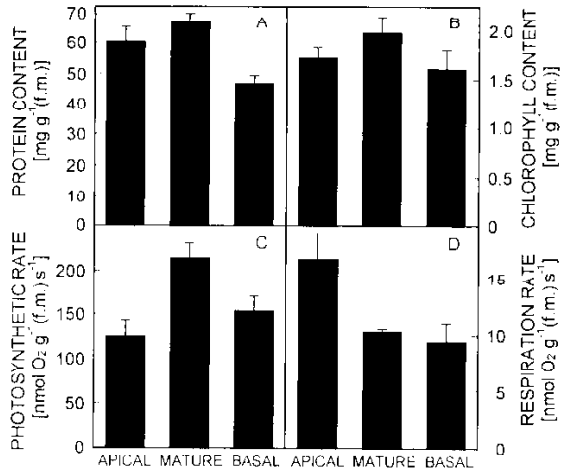

Fig. 1. Protein (*A*) and chlorophyll (*B*) contents, photosynthetic O_2 evolution (*C*) and dark respiration (*D*) of alfalfa leaves of different ages. Data are the mean ± SE of three separate experiments, each one with three replicate samples.

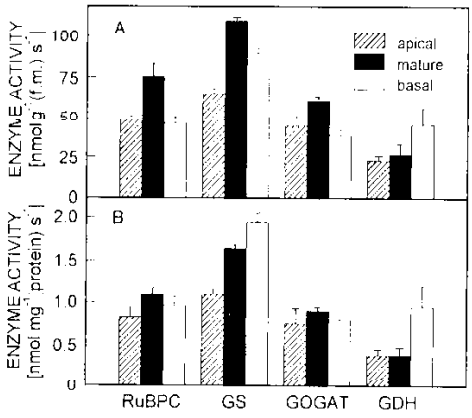

Fig. 2. RuBPC, GS, GOGAT and GDH in alfalfa leaves of different ages. *A* - activity expressed on a fresh mass basis; *B* - specific activity expressed on a protein basis. Data are the mean ± SE of three separate experiments each with three replicate samples.

exhibited a high rate of oxygen evolution (about 70 % of mature leaves). Dark respiration rates (Fig. 1*D*) were highest in expanding leaves, whereas in mature and basal leaves exhibited similar values (about 60 % of apical samples).

RuBPC, GS and Fd-GOGAT activities exhibited a bell-shaped profile along leaf development, mature tissues showing the maximum level, whereas GDH activity remarkably increased with leaf age (Fig. 2*A*). GDH and GS extracted from basal leaves had higher specific activity than fully expanded ones (Fig. 2*B*), whereas Fd-GOGAT and RuBPC specific activities continued to show the highest activity in mature leaves, even if to a less pronounced extent.

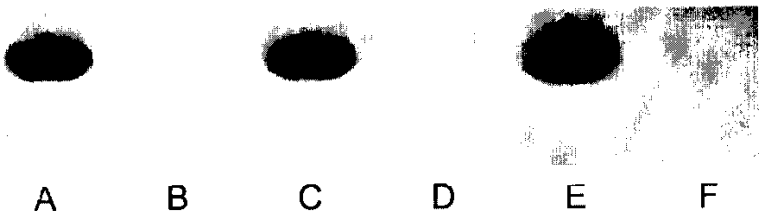

Fig. 3. GS bands after native PAGE of extracts of the three kinds of alfalfa leaves. *Lane A,C,E*: apical, mature and basal leaf samples; *Lane B,D,F*: apical, mature and basal samples subjected to thermal treatment (45 °C for 30 min) before PAGE.

The three kinds of alfalfa leaves show one band positive for GS presence (Fig. 3*A,C,E*), which disappears under thermal treatment (Fig. 3*B,D,F*).

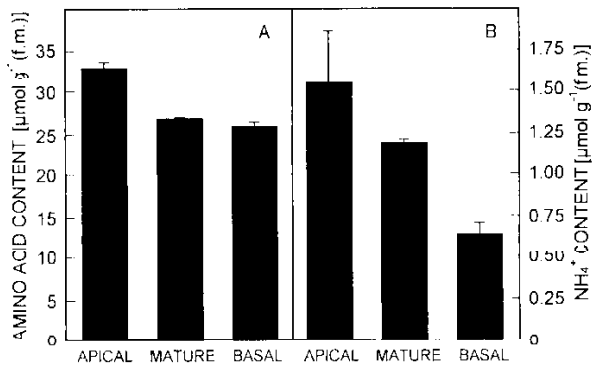

Fig. 4. Total amino acids (*A*) and ammonium (*B*) contents of alfalfa leaves of different ages. Data are the mean ± SE of three separate experiments each with three replicate samples.

The amino acid pool was highest in apical leaves, mature and basal samples showing similar levels (Fig. 4*A*). Ammonium content too was highest in apical leaves, and it declined in mature and basal ones (Fig. 4*B*).

Discussion

In alfalfa leaves, activities of GOGAT, GS and GDH were comparable to those measured in leaves of cereals (Peeters and Van Laere 1992, Schjoerring *et al.* 1993) and other leguminous plants (Storey and Beevers 1978, Streit and Feller 1982). RuBPC accounts for about 50 % of soluble protein content of C_3 plants and its activity increases during leaf development up to full expansion, when it begins to decline with senescence (Zima and Šesták 1985). In accordance with these results, in alfalfa plants, RuBPC activity, chlorophyll and protein contents and photosynthetic oxygen evolution increased from apical leaves towards the mature ones, and decreased in the samples collected at the bottom of plant canopy. Conversely, the rate of dark respiration was highest in young tissues, whereas mature and basal leaves exhibited similar values. This is in accordance with the general statement that the respiration rate is retained longer than photosynthesis during leaf ontogeny (Peoples and Dalling 1988).

It is well known that the nitrogen released from chlorophyll and proteins during senescence is used for the growth of developing organs (Feller 1990). Accordingly, in our experiments, amino acid and ammonia contents were highest in the still developing apical leaves and decreased with leaf age. Conversely, GDH activity was at its minimum in apical tissues and increased with leaf age, so resembling the developmental pattern of the enzyme extracted from cereal and other leguminous plants (Streit and Feller 1982, Laurière 1983, Peoples and Dalling 1988). The role of GDH in plant metabolism is still a matter of debate, as it catalyze a reversible reaction of amination of oxoglutarate and deamination of glutamate with contrasting efficiencies (Loulakakis and Roubelakis-Angelakis 1996, Turano *et al.* 1996). Furthermore it exists in multiple isoforms, which are affected by factors such as environment, growth stage and nutrition (Srivastava and Singh 1987). It has been proposed that *in vivo* GDH acts as a deaminating enzyme providing senescing (Thomas 1978), or C-deprived cells (Robinson *et al.* 1991) with carbon skeletons for the functioning of Krebs cycle. In our experiments the basal leaves still showed a significant photosynthetic oxygen evolution rate, therefore, a role for the GDH extracted from these leaves, of more replenishment of Krebs cycle, seems limiting. Rather, from the opposite developmental pattern of ammonium content and GDH activity, we might suggest that the enzyme has an anabolic, aminating role rather than a deaminating one. Recently, Oliveira *et al.* (1997) suggested that GDH functions in the direction of glutamate biosynthesis under conditions of carbon and ammonium excess, whereas it performs a catabolic role under carbon limitation. Therefore, in the still photosynthetizing basal leaves an anabolic GDH coupled to GS, whose specific activity increases with leaf age, may be important in the utilization of ammonia derived from protein degradation to the purpose of producing N-exporting amides.

In plants there are at least two isoforms of GS, the main enzyme in ammonia assimilation (Miflin and Lea 1976): the GS2, which is found in leaf plastids, and which seems important to support photorespiration, and the GS1 which is localized in the cytosol and which is reported to be stable during leaf senescence (Oak 1994). Considerable species-specific differences in the ratio of these two isoforms are

reported (McNally *et al.* 1983). From PAGE experiments it appears that the GS extracted from the three kinds of leaves shows one band which responds to thermal treatments like the chloroplastic GS2 isoform (Hirel and Gadal 1980). Therefore, in alfalfa leaves the GS2 isoform seems to be the most important ammonia assimilating enzyme in all the three kinds of leaf. This is in accordance with McNally *et al.* (1983), who found that the examined members of leguminous plants show exclusive, or prevalent (more than 70 %) GS2 activity. However, more recently, with immunocytochemical methods it was possible to detect the presence of a cytosolic GS isoform in the vascular tissues of plants originally classified as having only GS2 activity (Carvalho *et al.* 1992).

Similarly to GS activity and to oxygen evolution rates, the chloroplastic Fd dependent GOGAT was highest in the fully expanded leaves but continued to show an appreciable activity in basal leaves.

In conclusion our results show that, in alfalfa, the leaves taken from the bottom of the plant canopy are able to maintain a relatively high photosynthetic competence, even if coupled to senescence symptoms (decreased protein and chlorophyll content, enhanced GDH activity). These data, together with results on activities and localization of GS and GOGAT, suggest that in these leaves the photosynthetic process may be important in the reassimilation of the nitrogen liberated during protein degradation. However, the existence of multiple forms of the enzymes of ammonia assimilation, which are differently expressed in different tissues or growth stage, stresses the importance of the study of enzyme isoforms and localization during leaf development.

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