Assimilate partitioning in leaves of the raffinose-storing herb *Lamium album* L.: the effects of altering source-sink balance

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ABSTRACT – (Assimilate partitioning in leaves of the raffinose-storing herb *Lamium album* L.: the effects of altering source-sink balance). In *Lamium album*, sucrose and raffinose-family oligosaccharides are the major products of photosynthesis that are stored in leaves. Using gas analysis and $^{14}CO_2$ feeding, we compared photosynthesis and the partitioning of recently-fixed carbon in plants where sink activity was lowered by excision of flowers and chilling of roots with those where sink activity was not modified. Reduction in sink activity led to a reduction in the maximum rate of photosynthesis, to retention of fixed carbon in source leaves and to the progressive accumulation of raffinose-family oligosaccharides. This ultimately affected the extractable activities of invertase and sucrose phosphate synthase. At the end of the light period, invertase activity was significantly higher in treated plants. By contrast sucrose phosphate synthase activity was significantly lower in treated plants. We propose that reducing sink activity in L. album is associated with a shift in metabolism away from starch and sucrose synthesis and towards sucrose catabolism, galactinol utilisation and the synthesis of raffinose-family oligosaccharides.

Key words - *Lamium album*, oligosaccharides, source-sink interactions, starch, sucrose

RESUMO – (Partição de assimilados em folhas de *Lamium album* L., uma planta herbácea que acumula rafinose: os efeitos da alteração do balanço fonte-dreno). Em *Lamium album*, sacarose e oligossacarídeos da família rafinósica (OFR) são os principais produtos da fotossíntese que são armazenados nas folhas. Utilizando análise de trocas gasosas e fornecimento de $^{14}CO_2$ foram comparadas a fotossíntese e a partição de carbono recentemente fixado, em plantas nas quais a atividade dreno foi reduzida através da excisão das flores e resfriamento das raízes, com plantas nas quais a intensidade de dreno não foi modificada. A redução da atividade de dreno levou a uma diminuição na taxa máxima de fotossíntese, à retenção de carbono fixado nas folhas fonte e a um acúmulo progressivo dos OFR. Isso afetou as atividades extraíveis de invertase e sacarose fosfato sintase. Ao final do período de luz, a atividade de invertase foi significativamente maior nas plantas tratadas. Em contraste, a atividade de sacarose fosfato sintase foi significativamente menor nas plantas tratadas. Nós propomos que a redução da atividade de dreno em *L. album* esteja associada a uma alteração metabólica não relacionada ao metabolismo de síntese de amido e sacarose, mas sim ao catabolismo da sacarose, utilização de galactinol e síntese de OFR.

Palavras-chave - amido, interações fonte-dreno, *Lamium album*, oligosacarídeos, sacarose

Introduction

Long-distance interactions between sources and sinks in plants affect both photosynthesis and carbon partitioning (Farrar *et al*. 2000). Both increased photosynthesis and reduction of sink activity may result in the accumulation of carbohydrates in leaves. Sucrose and starch are the major immediate products of photosynthesis although some plant species accumulate a range of other carbohydrates (Pollock *et al.* 1999). Accumulation of sugars in leaves can be associated with a decline in the activities of enzymes involved in carbohydrate synthesis and ultimately result in feed back inhibition of both sucrose synthesis and photosynthesis (Stitt 1996, Farrar *et al.* 2000). The extent of feed back inhibition varies from plant to plant. In plants such as soybean, cotton and cucumber, which store starch in their leaves, feed back inhibition is observed, while there is less evidence of inhibition in plants like spinach which store sucrose in their leaves (Goldschmidt & Huber 1992). Studies on fructan-accumulating monocots (which store very little starch in the leaves) indicate that photosynthetic down-regulation is minimal, perhaps due to the vacuolar sequestration of carbohydrate (Farrar *et al.* 2000). In a previous paper, we described the partitioning of carbon in leaves of *Lamium album* L. (Kochhar *et al.* 2003). *L. album* was used because raffinose family

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oligosaccharides (RFOs) are major products of photosynthesis along with sucrose and starch, and this species therefore provides a contrast between high starch leaves and those of low-starch fructan-accumulators. Measurement of carbohydrate content showed that sucrose and starch were more abundant than RFOs in leaves of *L. album* grown under moderate irradiance with high sink activity (Kochhar *et al.* 2003). We suggested that only sucrose was synthesized at the beginning of the light period, with RFO and/or starch synthesis becoming significant later in the photoperiod.

In the study reported here, the activities of the major sinks (roots and flowers) were reduced in order to decrease sink demand. The effects of the resulting accumulation of photosynthetic products on carbon partitioning, photosynthesis and enzyme activities were determined. It is generally accepted that the activities of enzymes of both sucrose synthesis (e.g. sucrose phosphate synthase (SPS; Kerr & Huber 1987, Gerhardt *et al.* 1987) and sucrose degradation (e.g. invertase; Huber 1989) are regulated by the concentration of sucrose inside the cytosol. Sucrose accumulation may, therefore, be associated with the inhibition of SPS and the stimulation of the activities of invertase isoforms (Walker *et al.* 1997, Kingston-Smith *et al.* 1998).

Material and methods

Plant cultivation and physiological treatments - Plants of *Lamium album* L. (white dead nettle) were grown hydroponically from cuttings in controlled environment conditions as described previously (Kochhar *et al.* 2003). Growth temperature was 20 °C, photoperiod 12 h, irradiance 500 - 550 $\mu Em^{-2}s^{-1}$. Carbon partitioning, photosynthesis and enzyme activities were measured under two contrasting conditions. In sink–limited (treated) plants, roots were chilled by circulating ethylene glycol at 4 °C through the nutrient solution and developing flowers were pinched off. Experiments were begun thirty-six h after sink manipulation was imposed. This delay overcame the direct effects of treatment on stomatal closure. Control plants were grown without any sink manipulation.

Gas exchange measurements - Measurements were made on fully-mature leaves at a range of times during the photoperiod on a number of occasions following sink manipulation. For comparative purposes, only the results for the third photoperiod after sink manipulation are presented. Net CO_2 exchange, transpiration, stomatal conductance and intercellular CO_2 concentrations were measured using a PP Systems CIRAS-1 gas analyser with a clip-on leaf cuvette (Lewis *et al.* 1999). A_{max} values were measured using a Hansatech leaf disk oxygen electrode under saturating

irradiance and CO_2 concentrations (Housley & Pollock 1985).

Feeding $^{14}CO_2$ to intact plants of *L. album* - Replicate groups of ten hydroponically-grown plants were taken for $^{14}CO_2$ feeding. Because of the opposed pattern of leaf development in *L. album*, two feeding chambers were constructed, each of which held ten source leaves, each from a different plant. These chambers were arranged in parallel, with the plants in between them, inside a larger perspex chamber (123 × 29 × 41 cm). Connectors were sealed into the large chamber to allow coolant to be circulated through the root nutrient solution and there were fans on the upper surface to promote air movement. The two feeding chambers (112 × 7.5 × 2.5 cm) had removable lids and the inner walls had ten equally distant notches (4 × 3 mm) milled into the upper surface. The roots were kept in aerated nutrient solution that, for treated plants, could be cooled via a heat exchanger linked to an external constant temperature water bath. The plants were moved into the outer chamber two nights before the experiment, and were kept at 500 - 550 $\mu Em^{-2}s^{-1}$. On the day of the experiment, prior to illumination, the petioles of fully-mature attached leaves were sealed into the slots with Blu-Tak and the lids replaced.

Both control and treated plants were illuminated for twenty minutes with CO_2-free air being passed through the feeding chambers for the last 10 minutes. $^{14}CO_2$ was then administered to 20 mature leaves from 10 plants (10 leaves in each chamber). Sodium [^{14}C] bicarbonate (1.2 mL; 2.4 mci) was injected into each feeding chamber onto excess lactic acid. The feeding period was 30 min, after which the chamber was flushed with ambient air for 10 min. KOH (10% v/v) was used to trap the unfixed $^{14}CO_2$. During the subsequent chase period, ambient air was passed through the chambers. Duplicate plants were harvested after 1h (immediately after feeding), 6 h (after 6 h illumination), 12 h (after 12 h illumination), 18 h (12 h light, 6 h of the subsequent dark period) and 24 h (12 h light, 12 h of the subsequent dark period). Extraction, fractionation and measurement of radioactivity was carried out as described previously (Kochhar *et al.* 2003). Soluble material was fractionated by ion exchange chromatography into an acidic fraction (organic acids plus acid-stable phosphorylated intermediates); a basic fraction (mainly amino acids) and a neutral fraction (carbohydrates). The neutral fraction was separated further by TLC, sugars were identified by co-chromatography with markers and radioactivity quantified by autoradiography/densitometry (Kochhar *et al.* 2003). Insoluble material was counted directly following solubilisation and aliquots were also digested with amyloglucosidase/ á-amylase and the glucose released from starch separated by TLC (Kochhar *et al.* 2003). During the chase period, respired $^{14}CO_2$ was trapped by passage through KOH (10% v/v) and aliquots counted by liquid scintillation spectrometry.

Enzyme extraction and assay - SPS and invertase activities were measured in extracts from the middle leaves of *L. album* harvested 0, 4, 8, 12, 18 and 24 h after the beginning of the photoperiod commencing thirty-six h after sink manipulation

for treated plants. The light period was 12 h. Concentrations of substrates as well as incubation time, temperature and pH, were optimized to obtain V_{max} estimates. For the extraction of enzymes, leaf samples were harvested and ground in liquid N_2. Weighed samples (200-300 mg) of the powdered leaves were placed in a 2 mL screw-capped microtube and extracted according to Huber *et al.* (1989). The extracts were desalted prior to assay.

Assay of SPS - The activity of SPS was determined under V_{max} conditions by measuring fructose-6-phosphate-dependent formation of sucrose and sucrose-P from uridine diphosphoglucose. Substrate concentrations were as described in Huber *et al.* (1989) except that fructose-6-phosphate was at 5 mM.

Assay of invertase - Invertase was assayed by measuring the release of glucose and fructose from sucrose under the conditions described by Lyne & ap Rees (1971) except that the assay pH was 4.5. Released glucose and fructose were determined using a microtitre plate reader. In each well 10-100 μL of the assay mix was added together with 200 μL of buffer containing 50 mM HEPES (pH 7.5), 5 mM $MgCl_2$, 10 mM NAD^+, 10 mM ATP. The reaction was started by the addition of 5 μL of enzyme cocktail containing 1 U mL^{-1} of phosphoglucose isomerase, 1 U mL^{-1} of hexokinase and 1 U mL^{-1} of glucose-6-phosphate dehydrogenase. The overall increase in absorbance at 340 nm was measured for each well.

Results

Photosynthetic parameters - These are summarized in figure 1 for mature leaves in control and sink-manipulated (treated) plants measured at various times during the photoperiod three days after sink manipulation. Similar results were obtained from other measurements made over a seven-day period following sink manipulation. In all cases, photosynthesis was reduced in treated plants, as was stomatal conductance. This was accompanied by a reduction in transpirational water loss but no significant increase in intracellular CO_2 content. A_{max} values over the first three days following sink manipulation showed a significant and persistent reduction in treated plants (figure 2). These results suggest that photosynthesis declined due both to stomatal regulation (Setter & Brun 1980) and to down-regulation of photosynthesis (Suwignyo *et al.* 1995).

Total radioactivity - The distribution of radioactivity in middle leaves of control and treated plants is shown in figure 3. Immediately after the feeding period, the total amount of radioactivity was lower in treated than in control plants. This is probably due to the lower rate of photosynthesis and decreased stomatal conductance observed in treated plants (figure 1). During the dark period, total radioactivity increased in the middle leaves

of treated plants. It is possible that, in the absence of other sinks, the younger leaves of treated plants acted as temporary sinks and that secondary transfer of radioactivity was responsible for this increase. Autoradiographs of $^{14}CO_2$-fed plants (results not shown) indicated much greater retention of radioactivity in the leaves of treated plants throughout the 24 h chase period. Immediately after the feeding period, more radioactivity
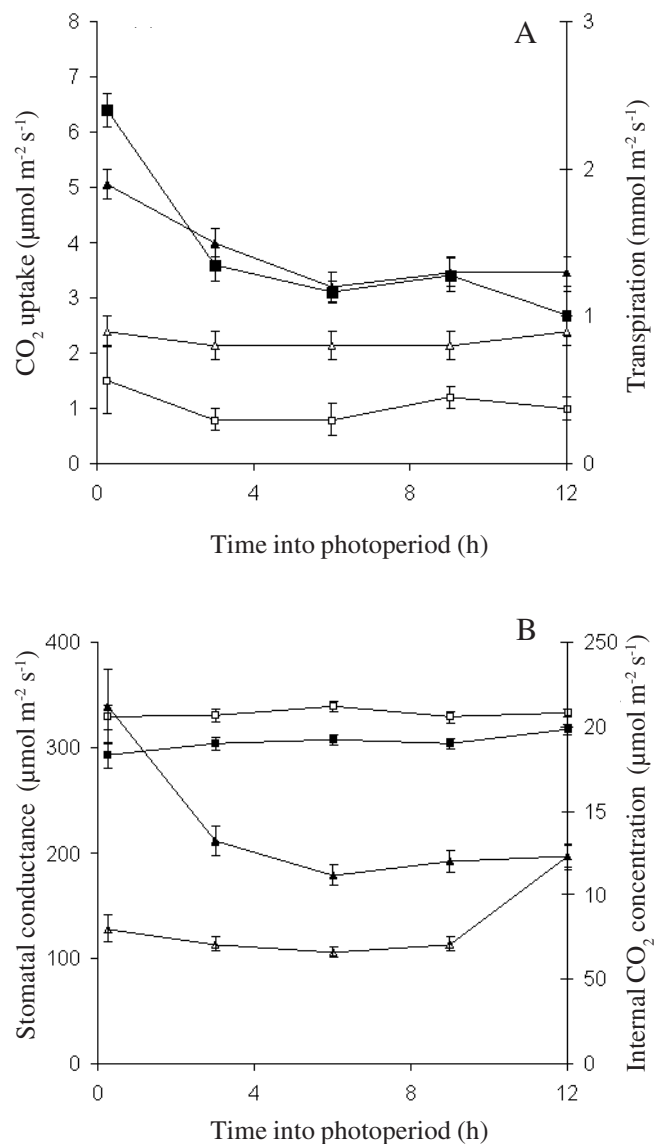

Figure 1. Photosynthetic paprameters measured in mature leaves of *Lamium album* at various times during the photoperiod. Measurements were made three days after the imposition of root cooling and flower removal in treated plants. Values are means ± sem of at least eight determinations. Closed symbols control plants; open symbols treated plants. A: ■ = CO_2 uptake; ▲ = transpiration. B: ■ = internal CO_2 concentration; ▲ = stomatal conductance.
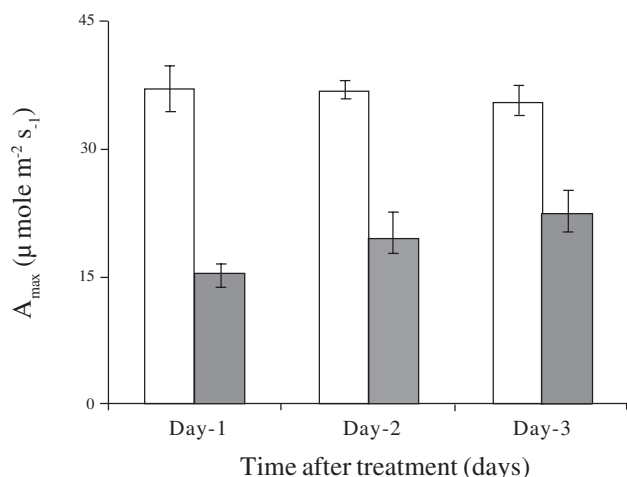
Figure 2. A_{max} values for mature leaves of control (□) and treated (■) plants harvested at various times after cooling of roots and removal of flowers. Values are means ± sem of five individual samples, measured mid-way through the light period.

was released as $^{14}CO_2$ from treated plants than from control plants. The amount of $^{14}CO_2$ released from both types of plant fell steadily during the chase period (figure 3).

Distribution of radioactivity within mature leaves - Treated plants had more radioactivity in the soluble fraction than did control plants (figure 4A, B). In both cases, radioactivity decreased in the soluble fraction with time and increased in the insoluble fraction until the end of the chase period. Generally, treated plants accumulated less radioactivity in the insoluble fraction and in starch than did control plants.

Distribution of radioactivity within soluble components - The middle leaves from treated plants had a greater proportion of the retained radioactivity in neutral components throughout the chase period (figure 4C, D). In the basic fraction, radioactivity in control leaves was almost double than in treated leaves between 6 and 24 h. We suggest that the high proportion of radioactivity in the neutral components of extracts from mature leaves of treated plants was due to reduction in export associated with sink manipulation. The pattern of changes in radioactivity over time differed between control and treated leaves. The highest percentage of radioactivity in the neutral fraction from control plants was observed immediately after the feeding period. It was then relatively stable till 18 h, whilst the opposite was observed in treated plants. The subsequent decrease in neutral sugars in control plants could be due to transport into sink tissues or repartitioning into the chloroplast as starch.

Further analysis of the neutral fractions from middle leaves of both control and treated plants indicated that substantial amounts of radioactivity were present in stachyose, raffinose and galactinol as well as in sucrose (figure 4E, F). In control plants, the radioactivity in sucrose fluctuated throughout the chase period, whereas it was more stable in treated plants. At 12 h (the end of the light period) treated leaves had more radioactivity in stachyose than in any other neutral sugar, whereas the opposite was true for control leaves. By contrast, the partitioning of radioactivity into galactinol was greater in the control leaves.
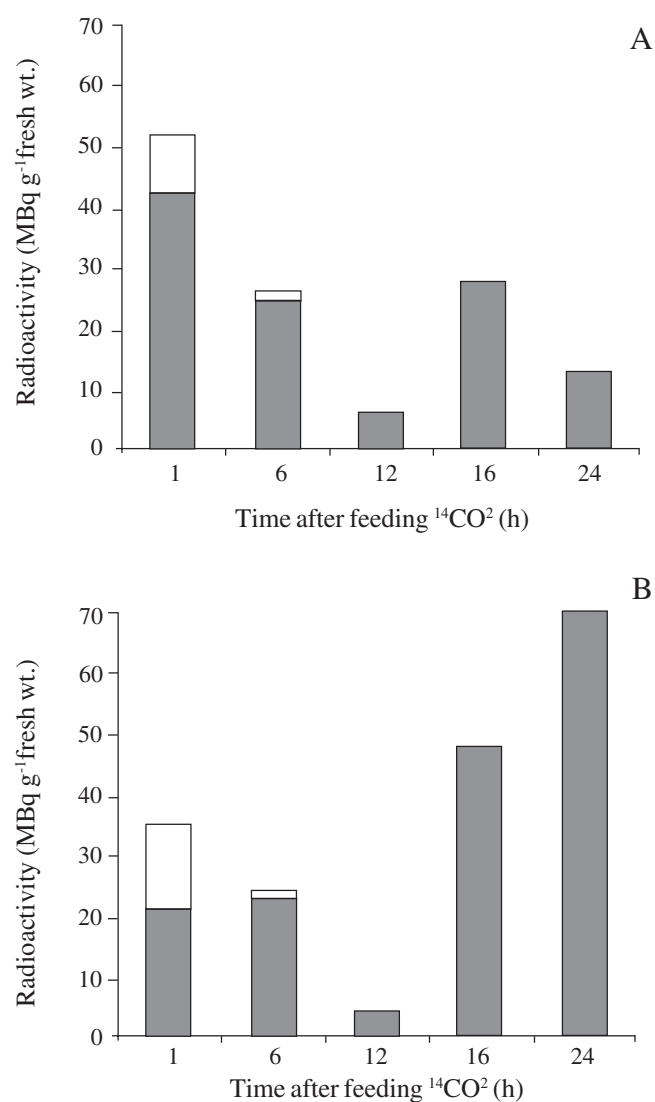

Figure 3. Total radioactivity accumulated in the middle leaves of (A) control and (B) treated plants of *L. album* at different times after feeding $^{14}CO_2$. ■ = radioactivity in the leaf; □ = radioactivity released as $^{14}CO_2$ by respiration following feeding. Values are means of duplicates.
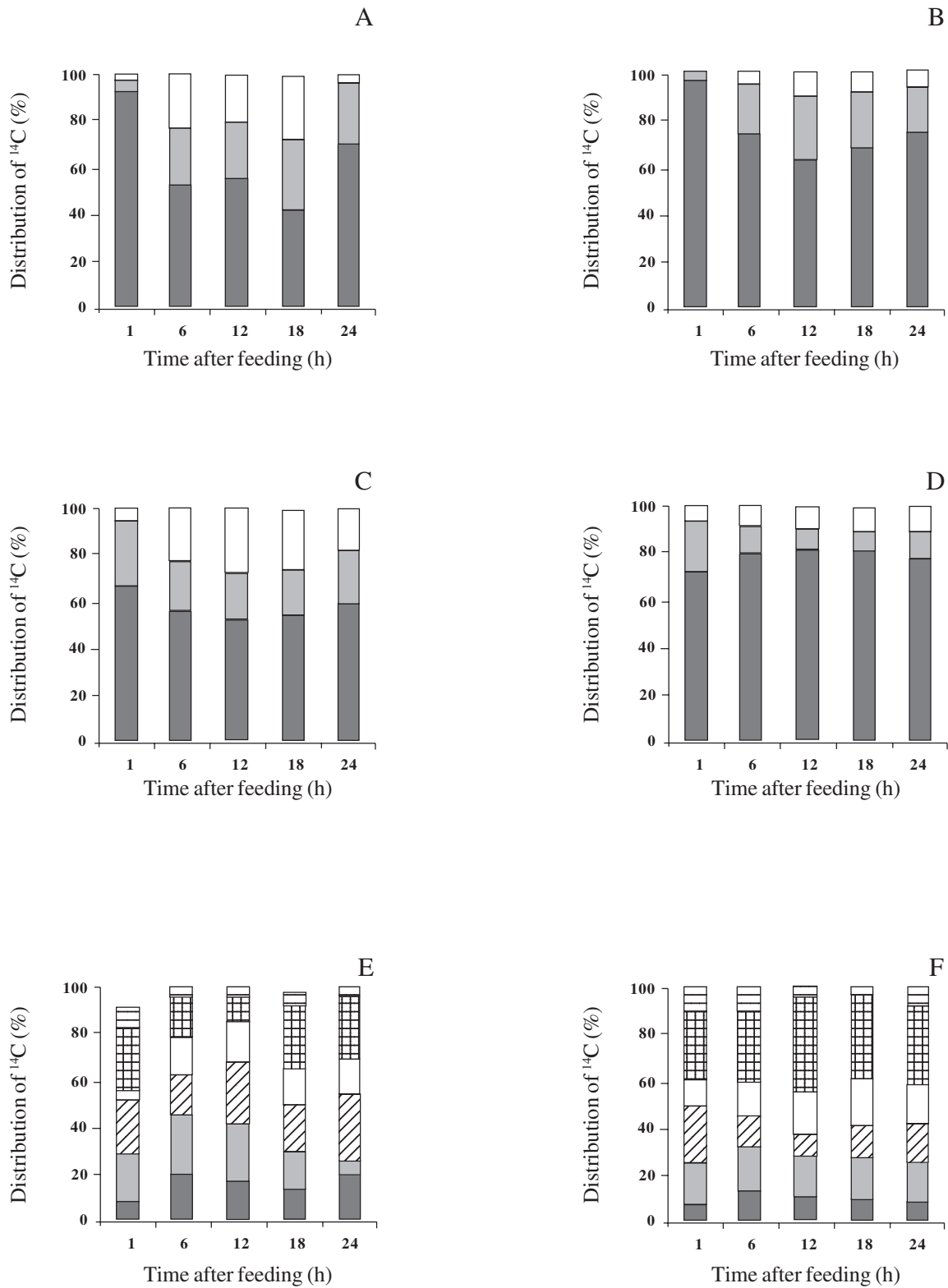
Figure 4. Distribution of radioactivity between various components harvested from control (A, C, E) and treated (B, D, F) leaves of *Lamium album* at various times after feeding $^{14}CO_2$. 4A, B. Distribution between water-solubles (■), water-insolubles (■), and starch (□). 4C, D. Distribution between neutral (■), basic (■), and acidic (□) components of the water-soluble fraction. 4E, F. Distribution between individual sugars within the neutral components. (■ = hexose; ■ = sucrose; ▨ = galactinol; □ = raffinose; ⊞ = stachyose; ▤ = verbascose). Values are means of duplicates.

Enzyme activity - There was a small increase in extractable SPS activity during the day in extracts from leaves of both control and treated plants of *L. album* (figure 5A) despite the increase in sucrose content that accompanies photosynthesis in this species (Kochhar *et al.* 2002). The increase was greater in extracts from control plants. The apparent sucrose-insensitivity of SPS may be a result of vacuolar sequestration of sucrose. There was a marked decrease in SPS activity in both control and treated plants by 18 h (6 h into the dark period) with a more marked decrease in activity in extracts from treated plants. Activities recovered to control values by 24 h.

A small but statistically significant increase in total soluble acid invertase activity from extracts of both treated and control leaves was observed during illumination (figure 5B). This increase was more marked in extracts from treated leaves, although this difference declined during the subsequent dark period.

Discussion

Photosynthesis and uptake of $^{14}CO_2$ - Restriction of sink activity was accompanied by a decline in both CO_2 uptake and A_{max} (figures 1, 2). In cucumber leaves, inhibition of photosynthesis was observed four days after removal of aerial sinks (Mayoral *et al.* 1985) whereas in temperate gramineae net photosynthesis appears to remain substantially independent of sink activity for some time after experimental manipulation (Natr 1969, Pollock & Housley 1985). Reductions in net photosynthesis can be caused by stomatal closure (Setter & Brun 1980, and in this study stomatal conductance and transpirational loss were reduced as a result of sink manipulation. However, intercellular CO_2 contents remained similar, suggesting that photosynthetic processes were also inhibited in treated leaves, and this is supported by the decline in A_{max} (figure 2). Goldschmidt & Huber (1992) observed declines in net photosynthesis and A_{max} in a range of plants that store both starch and sucrose, suggesting that the ability to accumulate RFOs does not lead to the insensitivity of photosynthesis to assimilate abundance observed during fructan accumulation in temperate gramineae (Pollock & Housley 1985).

Partitioning of fixed $^{14}CO_2$ between mature leaves and the remainder of the plant was also altered markedly by sink manipulation (figure 3). In control leaves, decline in radioactivity following feeding was rapid, although there was some apparent re-allocation of ^{14}C back into the fed leaves during the first part of the dark period. In treated leaves, radioactivity was also lost during the light period but there was extensive re-allocation during the dark period, leading to a considerable excess of radioactivity in treated leaves after 24 h. Autoradiography of the plants (data not shown) confirmed this excess of radioactivity within fed leaves on treated plants. Reduction in sink activity frequently alters carbon partitioning. In sorghum leaves where translocation was blocked completely by steam girdling, an increase in radioactivity in starch was observed (Watson & Wardlaw 1981). In maize source leaves, increasing sink demand led to an increase in the radioactive sucrose pool (Jeannette *et al.* 1995). Chilling sinks in vegetative plants of *L. temulentum* (Simpson *et al.* 1991) also led to a significant accumulation of soluble material in the source leaves. It appears that the treatments imposed on *L. album* plants in this study produced changes in photosynthesis and spatial partitioning that are similar to those observed for other species

Metabolic partitioning of $^{14}CO_2$ within mature source leaves - There were significant and progressive differences in the partitioning of ^{14}C within the fed leaves (figure 4). In leaves from treated plants, partitioning of carbon into insoluble material (including starch) was reduced, and there was little evidence of starch mobilisation during the dark period. This contrasted with the greater accumulation of starch in control leaves during the light period and its rapid turnover in darkness (figure 4A, B).

Within the soluble components, partitioning of ^{14}C into acidic and basic compounds was significantly inhibited in leaves from treated plants (figure 4C, D), and this was associated with increased accumulation of ^{14}C in raffinose, stachyose and verbascose. Stachyose was the major soluble sugar in which ^{14}C accumulated in leaves from treated plants, whereas in leaves from control plants, radioactivity was more evenly distributed (figure 4E, F). In leaves from control plants, the accumulation of ^{14}C in sucrose increased during the light period and fell again in darkness. By contrast, the ^{14}C content of sucrose remained similar in leaves from treated plants, with stachyose exhibiting a rise in the light period, followed by a decline in the subsequent dark period. The reduced radioactivity in sucrose and galactinol in leaves from treated plants suggested that there was transfer of radioactivity from these sugars to RFOs. Galactinol is proposed to function as a galactosyl donor in the biosynthesis of raffinose series oligosaccharides (Senser & Kandler 1967, Kandler 1967).

These results, taken with the shift away from starch accumulation, indicate that RFO accumulation in *L. album* is sensitive to the balance between supply of and demand for fixed carbon, and to that extent functions as an analog to fructan accumulation in leaves of temperate gramineae (Pollock *et al.* 1999). Significant amounts of radioactivity also accumulated in RFOs when *L. album* plants were exposed to very high irradiance. Under these experimental conditions no radioactivity was present in starch (Kochhar *et al.* 2003).

Enzymes of sucrose metabolism - Although the changes were not large, mature leaves from treated plants showed significant differences in the extractable activity of both SPS and soluble acid invertase (figure 5). Both of these enzymes are thought to play a key role in regulating sucrose content (Stitt 1996). SPS activity showed a decline in the early part of the dark period,

which was more marked in leaf extracts from treated plants. This decline has also been observed in other species (Pollock & Housley 1985). In this study, it is not possible to ascribe regulatory significance to these differences, which may merely reflect differences in substrate or product abundance. We also did not measure the distribution of invertase between vacuole and apoplast or between photosynthetic and heterotrophic tissues. However, Suwignyo *et al.* (1995) observed a direct correlation between the activity of SPS, sucrose content and carbon dioxide exchange rate in soybean leaves. In turn, vacuolar invertase activity has been proposed to be involved in the regulation of the amount of sucrose in leaves (Huber 1989). A similar decrease in SPS activity and an increase in invertase activity were observed in water-stressed mature maize leaves (Pelleschi *et al.* 1997). These workers suggested that this was due to a combination of low rate of photosynthesis, lower stomatal conductance and accumulation of sugars.

In *L. album*, carbon partitioning during sink limitation was shifted away from starch and sucrose towards the synthesis of RFOs. We suggest that this may be linked to a reduction in photosynthetic capacity. This was associated with a reduction in the capacity to synthesise sucrose and an increase in saccharolytic activity.
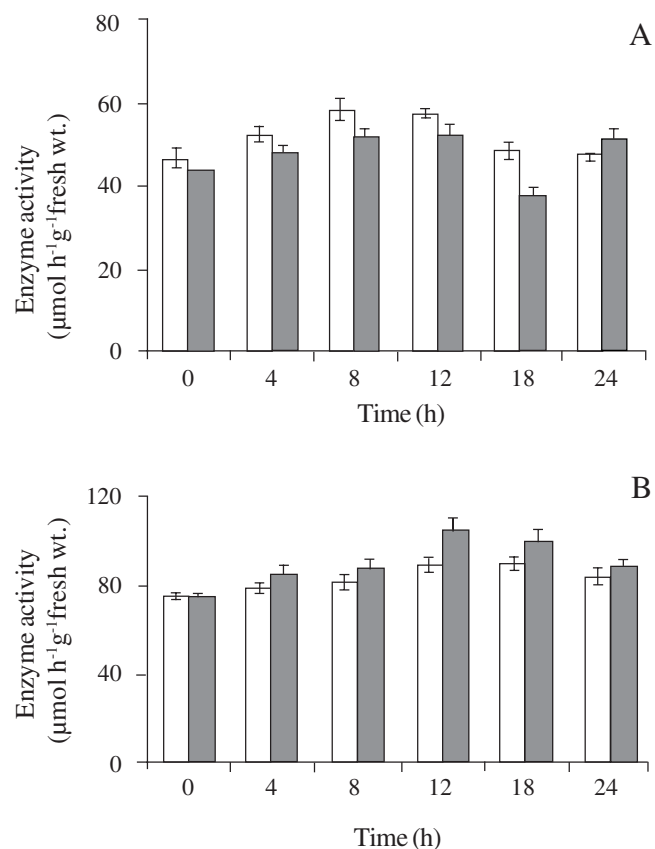
Figure 5. Sucrose phosphate synthase (A) and invertase (B) activities measured in extracts from mature leaves of control (%) and treated (%) plants of *L. album* over a 24 h period. The thick line on the horizontal axis represents the duration of the dark period. Values are mean ± sem of five individual samples. Between-treatement differences were significant at the 5% level (Student's t test) for sucrose phosphate synthase at the 18 h harvest and for invertase at the 12 h harvest.

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