Carbohydrate Accumulation in Leaves of Plants Treated with the Herbicide Chlorsulfuron or Imazethapyr Is Due to a Decrease in Sink Strength

ANA ZABALZA, LUIS ORCARAY, SUSANA GASTON, AND MERCEDES ROYUELA*

Departamento Ciencias del Medio Natural, Universidad Pública de Navarra, Campus Arrosadia,
E-31006 Pamplona, Spain

Herbicides that inhibit branched chain amino acid biosynthesis produce a rapid carbohydrate increase in leaves of treated plants. The relationship between these processes is not known nor is the importance of carbohydrate accumulation in the growth inhibition caused by these herbicides. This work analyzes carbohydrate concentration in sources and sinks after herbicide treatments in pea (*Pisum sativum* L.), as well as photosynthetic carbon assimilation, using two classes of chemicals, chlorsulfuron and imazethapyr, applied to roots or leaves. The most remarkable result was that, in addition to carbohydrate accumulation in leaves, accumulation of sucrose and/or starch in roots was detected. This pattern of carbohydrate accumulation was similar for both herbicides and independent of whether the herbicides were applied to leaves or roots. This indicates that root growth inhibition was not caused by sugar starvation in sinks. Nevertheless, the results are consistent with a decrease in sink strength, leading to the inhibition of photoassimilate translocation.

KEYWORDS: Imazethapyr; chlorsulfuron; carbohydrates; *Pisum sativum*

INTRODUCTION

Herbicides that block essential amino acid biosynthesis are limited in activity to organisms possessing the target biochemical pathways, namely, plants and microorganisms. Valine, leucine, and isoleucine are essential amino acids for animals, and the inhibition of their biosynthetic pathway is the mechanism of action of four main classes of herbicides: imidazolinones, sulfonylureas, triazolopyrimidines, and pyrimidinylsalicilyc acids (*1*). All of these herbicides inhibit the first common enzyme in this pathway [acetohydroxyacid synthase (EC 4.1.3.18) or acetolactate synthase (ALS)] and together account for ∼20% of the herbicide market (*2*). ALS inhibition as the mechanism of action of these herbicides was unequivocally described using physiological and enzymatic approaches and also genetically, after generation of herbicide-resistant plants by modification with genes coding a resistant ALS (*3*, *4*).

Although the primary site of action has been ascertained, the sequence of changes leading to plant death is unclear. Because meristematic tissues are more susceptible to ALS inhibitors (*5*), one of the first physiological effects is growth arrest. ALS inhibitors act slowly, so the death of the whole plant can take several weeks (*5*). Several biochemical and physiological effects have been described as a secondary consequence of the primary action of ALS inhibitors: accumulation of 2-ketobutyric acid (*6*, *7*), accumulation of 2-aminobutyric acid (the transamination product of 2-ketobutyric acid) (*8*), increase in free amino acid

pool, and decrease in protein levels (*9*–*12*). A rapid carbohydrate increase in leaves of plants treated with this type of herbicide has been widely reported (*9*, *13*, *14*), but the relationship with ALS activity or branched chain amino acid biosynthesis inhibition remains unclear. In short-term studies, photosynthesis is not affected by ALS inhibition, so the observed carbohydrate accumulation in treated leaves was suggested to be related to a decreased photoassimilate translocation to sink tissues (*14*, *15*).

On the basis of carbohydrate studies in treated leaves and exudates from excised leaves (*9*, *13*, *16*), it was suggested that carbohydrate accumulation in leaves might be caused by an impairment of phloem loading. This would explain the inhibition of photoassimilate translocation and may be part of the plant death mechanism, because meristematic tissues would be carbon starved (*5*, *14*, *17*). Although Hall and Devine (*18*) did not detect any effect of chlorsulfuron on H^+-ATPase activity, Kim and Vanden Born (*17*, *19*) suggested that the inhibition of photoassimilate transport was due to a reduced ability of leaf tissue to load sucrose into the phloem, based on structural alterations of mesophyll cells.

Alternatively, carbohydrate accumulation in leaves might be caused by a decrease in sink strength. However, carbohydrate concentration in sinks after herbicide supply has not been thoroughly addressed. Sugar accumulation in pea leaves and roots after a long-term sublethal imazethapyr (IM) treatment (4 weeks) has been shown (*20*), although this observation does not preclude the possibility of a shortage of carbon skeletons in the roots at the beginning of the treatment. However, it has

been reported (*21*) that total soluble sugar accumulation in roots occurs before starch accumulation in leaves of IM-treated plants, indicating that carbohydrate accumulation in leaves is the consequence of a decrease in sink strength. However, these studies (*20, 21*) were performed with herbicides being applied to the root system. The different method of herbicide application (to leaves or to roots) may influence the carbohydrate accumulation pattern and may explain the different results found in different studies.

The aim of this work was to ascertain whether there is a general pattern of carbon allocation following ALS-inhibiting herbicide supply, independent of herbicide chemical class and site of application (spray to leaves or supply to the roots via the nutrient solution). For this purpose, carbon fixation in leaves and carbohydrate content in leaves and roots were studied in pea plants treated with two classes of ALS inhibitors: the sulfonylurea chlorsulfuron (CS) and the imidazolinone IM.

MATERIALS AND METHODS

Chemicals and Apparatus. Commercial IM (Pursuit 10) was supplied by BASF Española SA (Barcelona, Spain), and commercial CS (Glean) was supplied by DuPont (DuPont Ibérica SA, Barcelona, Spain). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

A portable infrared gas analyzer (IRGA) (system Li-6200, Li-Cor, Lincoln, NE) was used to determine CO_2 assimilation rate and internal CO_2 concentration. Leaf area was determined using an Li-3000 system (Li-Cor). Stomatal conductance was determined by a porometer (Delta T Device Ltd., Cambridge, U.K.). Capillary electrophoresis was carried out using a P/ACE system 5500 (Beckman Instruments, Fullerton, CA).

Plant Material and Herbicide Treatment. Seeds of pea (*Pisum sativum* L. cv. Snap Sugar Boys) were surface sterilized as described in ref *22* prior to germination. For germination, seeds were grown in vermiculite for 96 h at 26 °C in darkness and then were transferred to hydroponic tanks of 2.7 L and placed in a growth chamber. The nutrient solution (*23*) was supplemented with 10 mM KNO_3, aerated continuously (700 mL tank^{-1} min^{-1}), and refreshed every 3 days. Growth conditions were 300 μmol m^{-2} s^{-1} (PPF; 14 h photoperiod) and 25/18 °C and 60/70% relative humidity day/night. When plants were 12 days old, the tanks were divided into groups: one group was left as the control, and the other groups were treated with herbicides. The four herbicide treatments were IM or CS applied to the nutrient solution and IM or CS applied to the leaves. There were eight plants per tank and three replicates of tanks per treatment.

Herbicide (IM and CS) concentrations necessary to induce similar effects on pea after application to roots or to leaves were determined in preliminary studies. The herbicide concentrations in the nutrient solution were maintained constant throughout the experiment: IM, 20 mg of active ingredient IM L^{-1} (69 μM); and CS, 10 μg of active ingredient CS L^{-1} (28 nM). These concentrations corresponded to 100 times the recommended field application rate for IM (IM can be used on legume crops) and 0.2 times for CS (pea is very susceptible to CS). The herbicides applied to leaves were sprayed with a mechanical sprayer. IM was sprayed to plants at a concentration of 1.5 g of active ingredient IM L^{-1} (5.2 mM), and CS was sprayed at 10 mg of active ingredient CS L^{-1} (28 μM).

For carbohydrate analysis, leaf and root samples were taken 5 h after the beginning of the photoperiod at 0, 1, 3, and 7 days after treatment. Plant material was immediately frozen in liquid nitrogen and stored at −80 °C. Some material was dried for 48 h at 75−80 °C to obtain the fresh weight/dry weight ratio. Root and shoot lengths were measured at 0, 1, 3, and 7 days after treatment. Gas exchange measurements were conducted daily for 7 days after the onset of treatment. Results given in this paper are the mean of two independent experiments with three replicates in each experiment.

Gas Exchange Measurements. Net CO_2 assimilation rate, internal CO_2 concentration, and leaf area were measured 3 h after the beginning of the photoperiod in intact plants in the youngest expanded leaf with
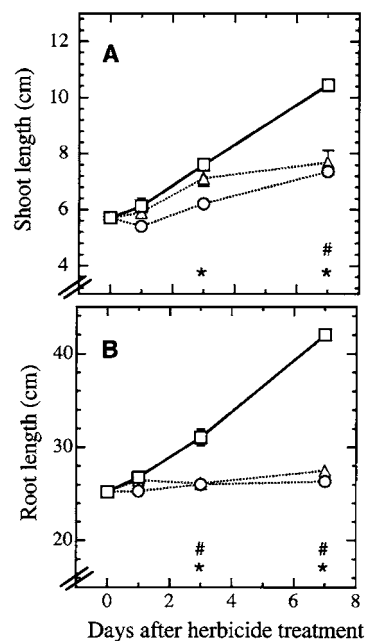

Figure 1. Shoot (**A**) and root (**B**) lengths of control pea (□) or plants treated with imazethapyr (○) or chlorsulfuron (△) applied to the nutrient solution. Each value is the mean ± standard error (*n* = 10). * indicates significant difference between control and imazethapyr and #, between control and chlorsulfuron (*p* ≤ 0.05) at a given day.

a portable IRGA. Measurements were made in growth conditions using an initial concentration of 450 ppm of CO_2 and a flow rate of ∼230 μmol s^{-1}. Stomatal conductance was measured in intact plants in the youngest expanded leaf with a portable porometer.

Carbohydrate Extraction and Determination. Roots and leaves (0.3 g of fresh weight) were exhaustively extracted in boiling 80% (v/v) ethanol (12 mL for roots; 16 mL for leaves). Ethanol-soluble extracts were dried in a Turbovap (Zymark, Hopkinton, MA), and soluble compounds were redissolved with 4 mL of distilled water, mixed, and centrifuged at 2300*g* for 10 min. The supernatant was immediately frozen and stored at −20 °C until its utilization. The ethanol-insoluble residue was extracted for starch, and the glucose produced by amyloglucosidase enzyme (*24*) was analyzed as for soluble compounds.

The contents of fructose, glucose, and sucrose were analyzed by high-performance capillary electrophoresis as described in ref *25*. The background buffer was 10 mM benzoate (pH 12) containing 0.5 mM myristyltrimethylammonium bromide (MTAB). The applied potential was −15 kV, and the capillary tubing was 50 μm i.d. and 31.4/38.4 cm long. The indirect UV detection wavelength was set at 225 nm. Soluble sugars were expressed as milligrams per gram of dry weight (DW), and starch was expressed as milligrams of glucose per gram of DW. In roots, no detectable levels of glucose and fructose were found.

Statistical Analysis. Results shown in this paper were examined by one-way analysis of variance. Replications refer to single plants. In the figures, * and # indicate significant difference between control and herbicide-treated plants (*p* ≤ 0.05) at a given day of treatment using Fisher's test.

RESULTS

Effects of Herbicide Treatments on Growth. Preliminary studies were conducted to determine herbicide concentrations with effects on growth similar to those described after the supply of 20 mg L^{-1} IM to the nutrient solution (*21*). The four treatments studied caused an arrest of root elongation after the onset of treatment, and this growth inhibition was significant within the third day. Shoot growth inhibition caused by the four treatments was smaller than root growth inhibition (**Figures 1** and **2**). In all cases net photosynthesis was almost zero after 20
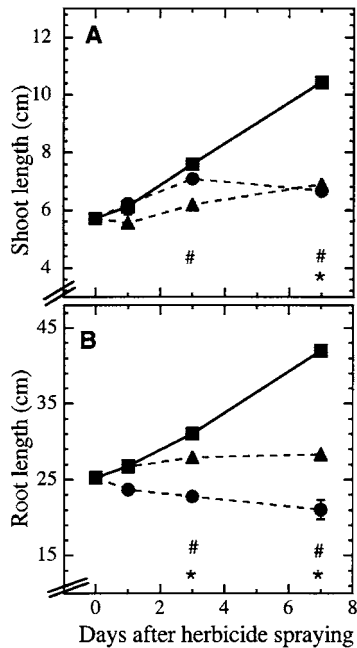
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Figure 2. Shoot (**A**) and root (**B**) lengths of control pea (■) or plants sprayed with imazethapyr (●) or chlorsulfuron (▲) to the foliage. Each value is the mean ± standard error (*n* = 10). * and # indicate as in **Figure 1**.

Figure 3. Sucrose (**A**), glucose (**B**), fructose (**C**), and starch (**D**) contents in leaves of control pea plants (□) or plants treated with imazethapyr (○) or chlorsulfuron (△) applied to the nutrient solution. Each value is the mean ± standard error (*n* = 6). * and # indicate as in **Figure 1**.

Figure 4. Sucrose (**A**) and starch (**B**) contents in roots of control pea plants (□) or plants treated with imazethapyr (○) or chlorsulfuron (△) applied to the nutrient solution. Each value is the mean ± standard error (*n* = 6). * and # indicate as in **Figure 1**.

days from the onset of herbicide treatment, and plant death took ~22−23 days. The concentrations used were suitable for analysis of the physiological effects after ALS inhibition because, although these concentrations caused a lethal phenotype, results presented in this study were carried out for 7 days at the initial phase of toxicity, in which plant viability was not compromised.

Carbohydrate Accumulation in Leaves and Roots. IM and CS supply to the nutrient solution led to a carbohydrate accumulation in leaves both as soluble carbohydrates (glucose and sucrose) and as starch from the onset of treatment. Sucrose content was immediately increased under IM treatment (**Figure 3A**), reaching up to twice the content of nontreated leaves by day 1. Glucose and fructose (**Figure 3B,C**) increased up to 9.5 and 23 times, respectively, by day 7, and starch accumulation was observed within 3 days and was twice the control values by 7 days (**Figure 3D**).

CS supplied to the nutrient solution did not lead to such a drastic carbohydrate accumulation, but there was an increase in sucrose content after 3 days (**Figure 3A**) and in glucose content from 3 days of treatment (**Figure 3B**). Leaves of CS-treated plants showed an increase in starch content by day 1, but then decreased to nearly zero at day 7 (**Figure 3D**).

Despite carbohydrate accumulation in leaves, there was no carbohydrate shortage in roots. Indeed, carbohydrates were also accumulated in roots when herbicides were added to the nutrient solution (**Figure 4**). IM caused a significant increase in sucrose and starch contents in roots from day 1 throughout the treatment, whereas CS led to only sucrose accumulation.

ALS-inhibiting herbicides applied by spraying caused an increase in carbohydrate concentration of leaves. With IM, there was a significant increase in sucrose and glucose at day 3 (**Figure 5A,B**). Although no variation in fructose content was detected in IM-sprayed plants, the starch content increased throughout the course of the experiment (**Figure 5C,D**). In CS-sprayed leaves, fructose accumulation was significant within 1 day from the onset of treatment (**Figure 5C**), and sucrose and
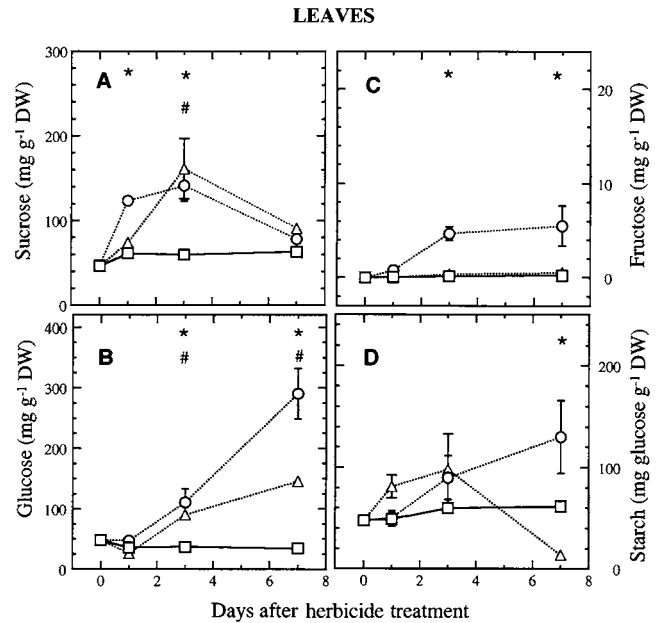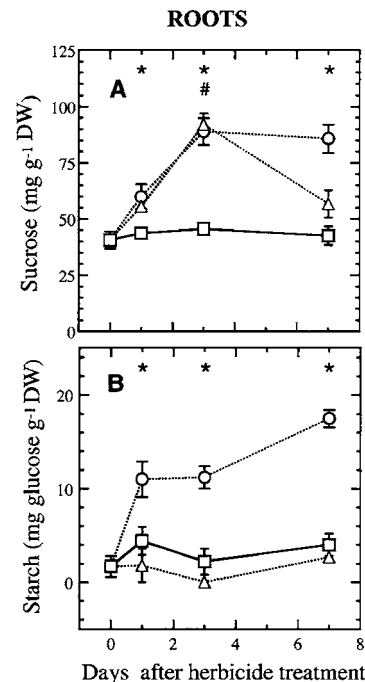
starch accumulations were significant from day 3 (**Figure 5A,D**).

Similarly, root carbohydrate content was also affected when herbicides were sprayed to the foliage, although with different patterns. With IM, a nonsignificant increase of starch mobilization at day 1 and a significant increase in sucrose content from day 3 were detected, whereas with CS, there was a significant increase in starch at day 3 (**Figure 6**).

Photosynthetic Parameters under ALS Inhibition. Supplying IM to the nutrient solution led to an inhibition of photosynthetic parameters in leaves (**Figure 7**). The inhibition
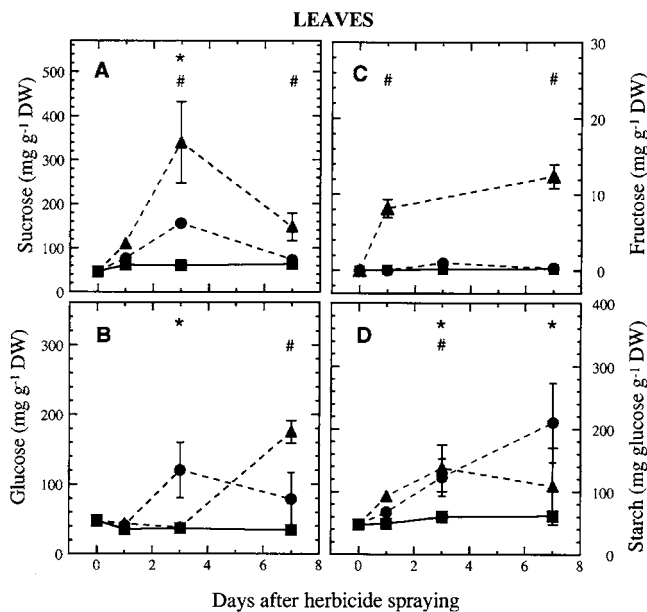
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LEAVES

Figure 5. Sucrose (**A**), glucose (**B**), fructose (**C**), and starch (**D**) contents in leaves of control pea plants (■) or plants sprayed with imazethapyr (●) or chlorsulfuron (▲) to the foliage. Each value is the mean ± standard error (*n* = 6). * and # indicate as in **Figure 1**.

ROOTS

Figure 6. Sucrose (**A**) and starch (**B**) contents in roots of control pea plants (■) or plants sprayed with imazethapyr (●) or chlorsulfuron (▲) to the foliage. Each value is the mean ± standard error (*n* = 6). * and # indicate as in **Figure 1**.
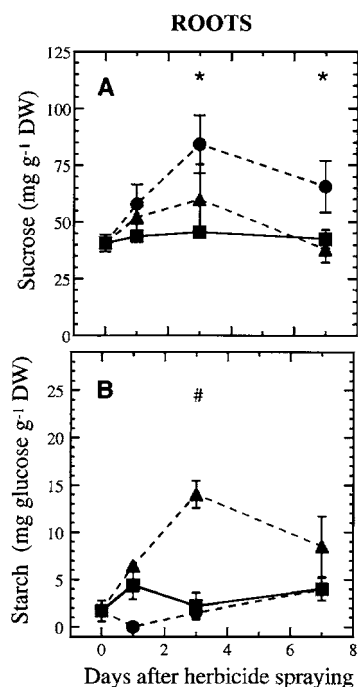
of stomatal conductance, internal CO_2 concentration, and photosynthesis rate were significant from days 2, 3, and 4, respectively. Although CS supply to the nutrient solution did not cause a similar decline in conductance, net photosynthesis declined from day 5, being 50% inhibited after 7 days of treatment (**Figure 7A,C**). Thus, at the end of the study period (7 days) net photosynthesis was similarly affected by CS and by IM.

When herbicides were sprayed onto the foliage, only CS caused a slight decline in net photosynthesis after a slight decline of stomatal conductance (**Figure 8A,C**). Internal CO_2 concen-
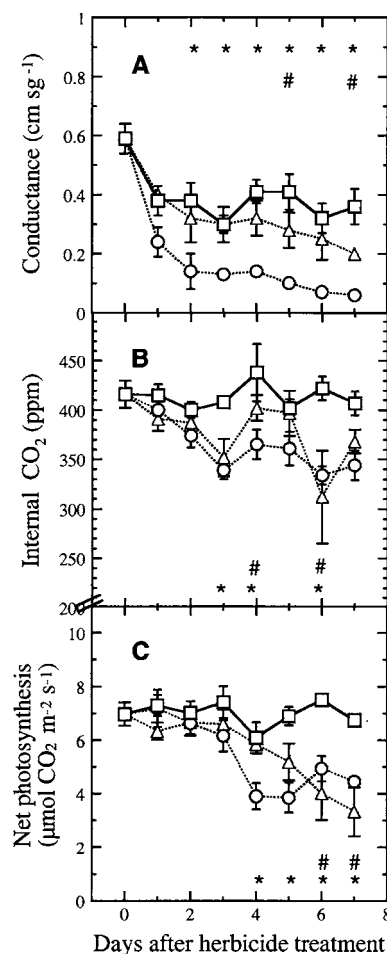

Figure 7. Stomatal conductance (**A**), internal CO_2 concentration (**B**), and net photosynthesis (**C**) in leaves of nontreated pea plants (□) or plants treated with imazethapyr (○) or chlorsulfuron (△) applied to the nutrient solution. Measurements were made daily on the youngest fully expanded leaf. Each value is the mean ± standard error (*n* = 8). * and # indicate as in **Figure 1**.

tration was not significantly altered by IM spray to the foliage. Stomatal conductance and net photosynthesis in IM-sprayed leaves differed only slightly from those of untreated leaves (**Figure 8A,C**). None of the four treatments caused any effect in the water content of leaves, so it could not be related to changes in stomatal conductance.

DISCUSSION

Carbohydrate accumulation in leaves has been repeatedly reported after treatment with ALS inhibitors (*26*). On the contrary, a decrease in glucose and starch in IM-treated soybean (*27*) and no effect on total carbohydrate content in maize (*28*) have been described. Nevertheless, these contradictory effects may be due to differences in the dosages of herbicides used, as the latter results were found at a sublethal level, with dosages that did not produce any effect on growth (*28*).

In this paper, carbohydrate accumulation in leaves after 1 and 3 days from the onset of the treatment was clearly detected after either leaf or root application, by supplying lethal concentrations of ALS inhibitors (causing plant death in ~20 days). It was suggested that growth inhibition and plant death induced by ALS inhibitors could be explained by a possible carbon starvation in meristematic tissues caused by inhibition of assimilate translocation (*5, 14, 17*). Nevertheless, our results
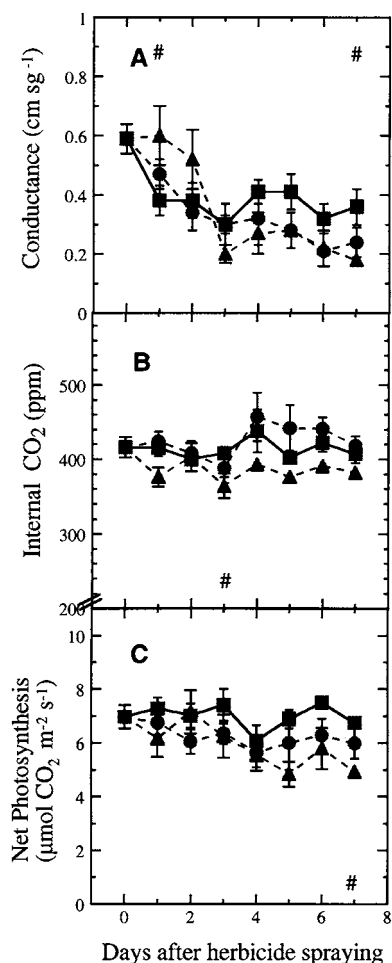
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Figure 8. Stomatal conductance (**A**), internal CO_2 concentration (**B**), and net photosynthesis (**C**) in leaves of control pea plants (■) or plants sprayed with imazethapyr (●) or chlorsulfuron (▲) to the foliage. Measurements were made daily on the youngest fully expanded leaf. Each value is the mean ± standard error ($n = 8$). * and # indicate as in **Figure 1**.

show that sugar accumulation occurs not only in leaves but also in roots. This supports the suggestion that root growth inhibition of treated plants is not due to a shortage of carbon skeletons. It seems that metabolic alteration induced by ALS inhibitors (as described in refs *20* and *21*) impairs carbohydrate utilization, without much effect on photosynthesis, leading to carbohydrate accumulation in roots. The increase in sucrose and starch content in sinks suggests that sucrose is transported from leaves to the roots at a higher rate than sinks are able to use it. Under these conditions, the gradient of sugars required for long-distance transport is abolished, and phloem loading would be impaired. This decrease in sink strength seems to cause the carbohydrate accumulation in leaves.

Although previous studies (*20, 21*) supported the hypothesis that carbohydrate accumulation in leaves is the consequence of decreased sink strength, it remained to be determined if this was a general effect of ALS inhibitors, independent of the site of application and the chemical class of herbicide. Our results show that the carbohydrate accumulation pattern in leaves and roots, evaluated with two different classes of herbicides and two different applications, is a general physiological effect of ALS inhibitors. There are few studies considering the role of carbohydrate content in sinks of plants treated with ALS inhibitors. Although phloem transport depends on the intensity of sink strength, most studies dealing with carbohydrate accumulation caused by ALS inhibitors in leaves have been performed in leaves or even excised leaves without considering root performance. The results shown in this paper assess the time course of the pattern of carbohydrate content not only in leaves but also in roots, discerning the role of root carbohydrate content under in vivo conditions.

The inhibition of cellular division by ALS-inhibiting herbicides (*29*) may cause a decrease in root growth, although carbohydrates are not limiting in that organ, as we have detected. If cellular division is inhibited, carbohydrates may not be metabolized to obtain energy or to produce structural molecules, and that is why carbohydrates may accumulate in the roots. It remains unclear why the inhibition of the biosynthesis of branched-chain amino acids leads to the inhibition of cellular division. An alteration of intermediate metabolite pools (*21*) or the ATP levels after ALS inhibition could cause the inhibition of cellular division.

It is noticeable that plants treated with this class of herbicides maintain enough photosynthetic carbon assimilation rates to accumulate carbohydrates in sources and sinks for a long time in a situation when growth is arrested and, ultimately, leads to plant death. Sugar accumulation showed a trend to decline to control values after 7 days of treatment, but it does not mean a recovery, because it matches up with the decline in carbon assimilation, which makes it difficult to maintain the same carbohydrate accumulation throughout the treatment period. In contrast, no effect on carbon fixation rates was reported shortly after application (within hours) of ALS inhibitors (*9, 14, 30*) and after long-time applications of sublethal concentrations of IM (*20*).

The time courses of conductance, internal CO_2 concentration, and photosynthesis decline in leaves of IM-treated plants strongly support the assumption that the decline in conductance within 1 day of IM supply elicits the decline in net photosynthesis after 4 days. Another possibility is that the effect on assimilation rates may be associated with the high carbohydrate availability of the treated leaves. This has been described before under ALS inhibition (*27*) and other physiological situations (*31–33*). It is proposed that the inhibition of photosynthesis detected after ALS inhibition is mediated by a stomatal closure and is a response to the increased carbohydrate content of the treated leaves. In contrast, when herbicides were sprayed to the foliage, photosynthesis slightly declined only in response to the high carbohydrate content, because no changes in stomatal conductance could be detected. This difference can explain the more noticeable decline in CO_2 fixation rates when herbicides were supplied to the nutrient solution. It is difficult to explain the difference in stomatal conductance response between the two types of applications. It might be due to the fact that when ALS inhibitors were sprayed to the foliage, it was only a sole application, whereas in the case of herbicides supplied to the nutrient solution, they remained in the container during the experimental time course.

The results presented in this paper indicate the existence of a close relationship between ALS inhibition and carbohydrate accumulation. The lack of use of the available carbohydrates by the root can be related to a slowing of metabolism in plants treated by ALS inhibitors (*20, 21, 34*).

We conclude that carbohydrate accumulation in leaves of treated plants is attributed to a decrease in sink strength and is not due to an inhibition of sucrose loading, because roots of treated plants also accumulated carbohydrates. The validity of this hypothesis has been demonstrated after herbicide supply to the nutrient solution and after herbicide spray to the foliage. The inhibition of carbon fixation several days after treatment

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with a lethal dose of IM or CS, which was not detected in shorter or sublethal treatments, is a secondary response of ALS inhibition, and it can be explained by two phenomena: reduced stomatal conductance and high carbohydrate accumulation.

ABBREVIATIONS USED

ALS, acetolactate synthase; IM, imazethapyr; CS, chlorsulfuron; DW, dry weight.

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