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# Phloem Transport of D,L-Glufosinate and Acetyl-L-Glufosinate in Glufosinate-Resistant and -Susceptible *Brassica napus*[1]

**Jennifer N. Beriault[2], Geoff P. Horsman, and Malcolm D. Devine[3]***

Department of Plant Sciences, University of Saskatchewan, 51 Campus Drive, Saskatoon, Saskatchewan, Canada S7N 5A8

**Phloem transport of D,L-[$^{14}$C]glufosinate, D-[$^{14}$C]glufosinate, and acetyl-L-[$^{14}$C]glufosinate was examined in the susceptible *Brassica napus* cv Excel and a glufosinate-resistant genotype (HCN27) derived by transformation of cv Excel with the phosphinothricin-*N*-acetyltransferase (*pat*) gene. Considerably more $^{14}$C was exported from an expanded leaf in HCN27 than in cv Excel following application of D,L-[$^{14}$C]glufosinate (25% versus 6.3% of applied, respectively, 72 h after treatment). The inactive isomer, D-glufosinate, was much more phloem mobile in cv Excel than racemic D,L-glufosinate. Foliar or root supplementation with 1 mM glutamine increased D,L-[$^{14}$C]glufosinate translocation in cv Excel but only transiently, suggesting that glutamine depletion is not the major cause of the limited phloem transport. Acetyl-L-[$^{14}$C]glufosinate (applied as such or derived from L-glufosinate in *pat* transformants) was translocated extensively in the phloem of both genotypes. Acetyl-L-[$^{14}$C]glufosinate was readily transported into the floral buds and flowers, and accumulated in the anthers in both genotypes. These results suggest that phloem transport of D,L-glufosinate is limited by rapid physiological effects of the L-isomer in source leaf tissue. The accumulation of acetyl-L-glufosinate in the anthers indicates that it is sufficiently phloem mobile to act as a foliar-applied chemical inducer of male sterility in plants expressing a deacetylase gene in the tapetum, generating toxic concentrations of L-glufosinate in pollen-producing tissues.**

The herbicide glufosinate inhibits Gln synthetase (GS), a key enzyme in the assimilation of inorganic nitrogen into organic compounds. Inhibition of GS by L-glufosinate, the active isomer, leads to depletion of the amino acid Gln, a concomitant accumulation of ammonia in treated tissues (Köcher, 1983; Wild and Manderscheid, 1984; Tachibana et al., 1986b), and glyoxylate accumulation, which inhibits Rubisco and carbon fixation (Wendler et al., 1992).

Resistance to glufosinate has been created through the insertion of the phosphinothricin-*N*-acetyltransferase (*pat*) gene, derived from the homologous gene from *Streptomyces viridochromogenes*. This gene codes for L-phosphinothricin (= glufosinate) acetyl-transferase (PAT), which catalyzes the acetylation of L-glufosinate to *N*-acetyl-L-glufosinate (De Block et al., 1987; Wohlleben et al., 1988; Broer et al., 1989; Dröge et al., 1992) (see Fig. 1 for structures). Acetyl-L-glufosinate is not phytotoxic, and constitutive expression of the *pat* gene confers glufosinate resistance in transformed plants. PAT is stereospecific for the L-isomer of glufosinate; D-glufosinate in the racemic D,L mixture is not acetylated and is not phytotoxic to plants. Glufosinate-resistant *Brassica napus* (canola) cultivars have been available to farmers in western Canada since 1995.

Glufosinate is not translocated extensively from the site of application in susceptible plants (Bromilow et al., 1993; Steckel et al., 1997), but the reason for this limited transport is unknown. Based on its physicochemical properties ($pK_a$ = <2, 2.9, and 9.8; log $K_{ow}$ estimated at −3.9), glufosinate has the requisite characteristics for phloem mobility (Kleier, 1988; Hsu and Kleier, 1990). Localized phytotoxicity is apparent relatively soon after glufosinate application to the leaves. Such rapid action at the site of application is known to limit phloem transport of other herbicides, including chlorsulfuron and glyphosate. It is possible that glufosinate may also limit its own translocation through rapid action in source leaf tissue.

Little information is available on the translocation of glufosinate in resistant plants or the translocation of acetyl-L-glufosinate in susceptible or resistant plants. Dröge-Laser et al. (1994) reported that, based on autoradiographs, acetyl-L-glufosinate was distributed in the same way as D,L-glufosinate; i.e. most of it remained in the treated leaf, with a small amount transported to the stem and upper leaves. However, no quantitative data on acetyl-L-glufosinate translocation are available from either resistant or susceptible plants.

A chemical male sterility system based on the tissue-specific conversion of acetyl-L-glufosinate to L-glufosinate has been described recently. An Arg deacetylase from *Escherichia coli* expressed in tobacco (*Nicotiana tabacum*) plants under the control of a tapetum-specific promoter catalyzed the conversion of acetyl-L-glufosinate to L-glufosinate (Kriete et al., 1996). In some of the transformants, complete male sterility was induced following application of acetyl-L-glufosinate to the flowers. However, it is not known if acetyl-L-glufosinate is sufficiently phloem mobile to be translocated from the leaves to the flowers following foliar application, the most convenient method for the induction of male sterility in plants on a large scale.

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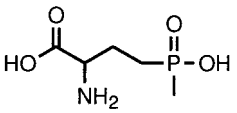
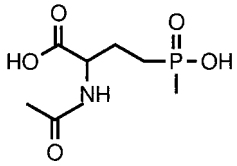
[2] Present address: AgrEvo Canada, 295 Henderson Drive, Regina, SK, Canada S4N 6C2.

[3] Present address: AgrEvo Canada, 203–407 Downey Road, Saskatoon, SK, Canada S7N 4L8.

* Corresponding author; e-mail malcolm.devine@agrevo.com; fax 306–934–8337.



Glufosinate



*N*-Acetyl-glufosinate

**Figure 1.** Structures of glufosinate and *N*-acetyl-glufosinate.

The objectives of this research were to quantify the phloem transport of D,L-glufosinate and acetyl-L-glufosinate in resistant and susceptible *B. napus* genotypes, and in particular the translocation of acetyl-L-glufosinate into the floral tissues, as part of an assessment of its potential as a chemical inducer of male sterility. A further objective was to determine if phloem transport of D,L-glufosinate is limited by phytotoxic effects of the herbicide in source tissues.

## MATERIALS AND METHODS

### Plant Material and Growth Conditions

Seeds of the *Brassica napus* cvs Excel (susceptible) and HCN27 (resistant) were provided by AgrEvo Canada (Saskatoon, Saskatchewan, Canada). Glufosinate resistance in HCN27 was generated by *Agrobacterium tumefaciens*-mediated transformation of cv Excel protoplasts with the *pat* gene under the control of a constitutive promoter (R. MacDonald, AgrEvo Canada, personal communication). The original transformant was selfed to create the homozygous line HCN27.

Seeds of the resistant and susceptible genotypes were grown in perlite at 22°C/18°C day/night temperatures with a 16-h photoperiod at 350 $\mu$E $m^{-2}$ $s^{-1}$ and 70% RH. Two seeds were planted in each 195-mL styrofoam cup and were later thinned to one plant per cup. The plants received equal amounts of Hoagland's nutrient solution (Hoagland and Arnon, 1950). Plants were grown until they reached the appropriate growth stages for application of glufosinate or acetyl-glufosinate.

### Herbicide Application

D,L-[3,4-$^{14}$C]Glufosinate (specific activity 1,040 MBq $g^{-1}$) was prepared in the "150 SN" formulation (proprietary formulation from AgrEvo Canada, Saskatoon, SK, Canada), which included surfactant, at a concentration of $1.67 \times 10^3$ Bq/10 $\mu$L. Acetyl-L-[3,4-$^{14}$C]glufosinate (specific activity 32.2 MBq $g^{-1}$) was also prepared in the "150 SN" formulation at a concentration of $2.67 \times 10^3$ Bq/10 $\mu$L. Ten microliters of D,L-[$^{14}$C]glufosinate or acetyl-L-[$^{14}$C]glufosinate solution were applied using a Wiretrol (Drummond Scientific, Broomall, PA) pipette on either side of the midrib halfway between the base and the tip of the leaf. The $^{14}$C compounds were applied to the first fully expanded leaf (two-leaf stage treatments), the third fully expanded leaf (four-leaf and green-bud-stage treatments), or a mature fully expanded upper leaf (early-flowering-stage treatments). The D,L-[$^{14}$C]glufosinate or acetyl-L-[$^{14}$C]glufosinate was applied shortly after the start of the photoperiod. The total quantity of D,L-glufosinate applied was approximately three times the amount that would be applied per plant in a typical field application. D-[3,4-$^{14}$C]Glufosinate (specific activity 2,080 MBq $g^{-1}$) was prepared in the "150 SN" formulation at a concentration of $1.67 \times 10^3$ Bq/10 $\mu$L.

The following experiments were conducted: (a) comparison of D,L-[$^{14}$C]glufosinate and acetyl-L-[$^{14}$C]glufosinate absorption and translocation in plants at the four-leaf stage; (b) translocation of D,L-[$^{14}$C]glufosinate and acetyl-L-[$^{14}$C]glufosinate to the upper shoot and flower buds in plants at the green-bud stage; (c) translocation of D,L-[$^{14}$C]glufosinate and acetyl-L-[$^{14}$C]glufosinate to the upper shoot, flowers, and anthers of plants at the early-flowering stage; (d) translocation of D,L-[$^{14}$C]glufosinate and D-[$^{14}$C]glufosinate in plants at the two-leaf stage; and (e) effect of Gln supplementation on translocation of D,L-[$^{14}$C]glufosinate in plants at the two-leaf stage. Two methods of Gln supplementation were used: Gln (1 mM) was supplied either in the nutrient solution from 48 h before treatment with D,L-[$^{14}$C]glufosinate until harvest or was included in the D,L-[$^{14}$C]glufosinate treatment solution.

### Measurement of $^{14}$C Absorption and Translocation

Foliar absorption of all compounds was determined by washing the treated area of the treated leaf three times with 5 mL of 50% (v/v) ethanol/water at various time intervals after treatment. The rinsates were collected and the $^{14}$C content measured by liquid scintillation spectroscopy. A separate experiment showed that this solution rinsed over 99% of the applied D,L-[$^{14}$C]glufosinate from a glass slide. Absorption was calculated by subtracting the radioactivity in the leaf washes from the total applied radioactivity (determined by liquid scintillation spectroscopy of an aliquot of the treatment solution).

Following the leaf wash, the plants were divided into various parts depending on the experiment. Parts harvested included the treated leaf, tissue above the treated leaf, flower buds, flowers, anthers, tissue below the treated leaf, and roots. The treated leaf was further divided into three portions: the treated (mid) portion, the basal portion, and the portion near the tip. All plant parts were air-dried for 72 h, combusted in a biological oxidizer, and the $^{14}$C content determined by liquid scintillation spectroscopy. Translocation was determined by summing the $^{14}$C recovered in the tissue above and below the treated leaf, including the roots, and is expressed as a percentage of the applied $^{14}$C.

All treatments were replicated four times per experiment, and all experiments were conducted twice. The re-

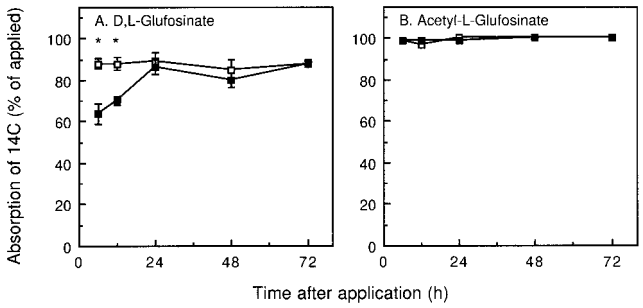



**Figure 2.** Foliar absorption of D,L-[$^{14}$C]glufosinate (A) and acetyl-L-[$^{14}$C]glufosinate (B) in cv Excel (■) and HCN27 (□) plants at the four-leaf stage. Absorption was determined by rinsing unabsorbed material from the leaf surface at the times indicated. Vertical bars represent SE; asterisks indicate a significant difference between cv Excel and HCN27 at that harvest time.

sults from duplicate experiments were generally consistent, and combined data from the duplicate experiments are presented. Means and SEs were calculated for absorption and translocation in each genotype at each harvest time. Comparisons between genotypes at individual harvest times were compared using a one-way ANOVA ($P < 0.05$). Reference to differences between treatments implies that the data were significantly different according to this test.

### Identification of Translocated $^{14}$C

D,L-[$^{14}$C]Glufosinate or acetyl-L-[$^{14}$C]glufosinate was applied to cv Excel and HCN27 plants at the four-leaf, green-bud, and the early-flowering stages at three times the dose previously described, and the following tissue samples were collected for HPLC analysis: the treated leaf and the remainder of the plant (combined upper shoot, lower shoot, and roots), both at the four-leaf stage, and the floral buds and flowers at the green-bud and early-flowering stages. All plants were harvested 72 h after treatment, and the treated leaves were washed to remove unabsorbed $^{14}$C, as previously described. The plant tissue samples were wrapped in aluminum foil and frozen at −20°C until extraction.

The samples were homogenized with a mortar and pestle in liquid nitrogen. Thirty milliliters of water was then added to the tissue sample in a 125-mL Erlenmeyer flask. The extract was stirred for 30 min, and then 30 mL of chilled acetone was added and the mixture was centrifuged (model J2-21 centrifuge, Beckman Instruments, Fullerton, CA) at 12,100*g* for 15 min. The extract was transferred to a 250-mL round-bottom flask and concentrated in a rotovaporator at 50°C to a volume of 0.5 mL. The extracts were then cleaned on a $C_{18}$ solid-phase extraction column (Fisher Scientific, Loughborough, Leicestershire, UK). The $^{14}$C content was determined in three 1-mL subsamples before and after centrifugation, and in three 50-$\mu$L subsamples before and after cleaning on the $C_{18}$ column.

D,L-[$^{14}$C]Glufosinate, acetyl-L-[$^{14}$C]glufosinate, and other minor unidentified metabolites were separated by HPLC using a ZORBAX SAX (Hewlett-Packard, Palo Alto, CA) column (4.6 mm × 25 cm) at ambient temperature. Forty-five microliters of clean extract was injected per analysis. The HPLC mobile phases were 0.05 M $KH_2PO_4$ adjusted to pH 2.1 with $H_3PO_4$ (A) and methanol (B). The isocratic elution was set at an A to B ratio of 90:10 and a flow rate of 0.6 mL min$^{-1}$. The elution times were compared with those of D,L-[$^{14}$C]glufosinate and acetyl-L-[$^{14}$C]glufosinate standards injected directly into the HPLC. Identification was replicated twice with two different tissue samples. This HPLC method did not distinguish between the D- and L-isomers of glufosinate.

## RESULTS

### Foliar Absorption of D,L-[$^{14}$C]Glufosinate and Acetyl-L-[$^{14}$C]Glufosinate

Absorption of D,L-[$^{14}$C]glufosinate and acetyl-L-[$^{14}$C]glufosinate at the four-leaf stage was consistently high in HCN27 and in cv Excel, reaching >85% after 24 h (Fig. 2, A and B). Similar results were obtained with both compounds at all other growth stages, and with $^{14}$C-D-glufosinate at the two-leaf stage (data not shown).

### Translocation of [$^{14}$C]Glufosinate and Acetyl-L-[$^{14}$C]Glufosinate

*Four-Leaf Stage*

Significantly more $^{14}$C was translocated out of the treated leaf of HCN27 plants than out of that of cv Excel plants following application of D,L-[$^{14}$C]glufosinate (Fig. 3A). Approximately 2% and 14% of the applied $^{14}$C was translocated out of the treated leaf 24 h after treatment, and 6% and 25% 72 h after treatment in cv Excel and HCN27, respectively. Approximately 80% (cv Excel) and 47% (HCN27) of the applied $^{14}$C remained in the treated leaves after 72 h (Table I). Eleven percent of the applied $^{14}$C was recovered in the roots of the HCN27 plants after 72 h and 9% in the tissue above the treated leaf; much less $^{14}$C was recovered in the roots and upper shoot of cv Excel (Table I).

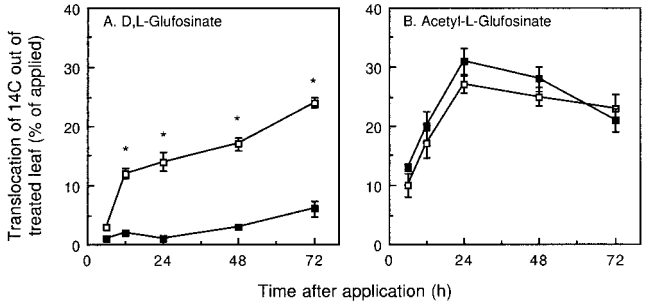



**Figure 3.** Translocation of $^{14}$C out of the treated leaf of cv Excel (■) and HCN27 (□) plants following application of D,L-[$^{14}$C]glufosinate and acetyl-L-[$^{14}$C]glufosinate at the four-leaf stage. Translocation was determined by summation of the $^{14}$C recovered in all plant parts other than the treated leaf. Vertical bars represent SE; asterisks indicate a significant difference between cv Excel and HCN27 at that harvest time.

**Table I.** *Distribution of $^{14}C$ in cv Excel and HCN27 72 h after application of D,L-[$^{14}C$]glufosinate or acetyl-L-[$^{14}C$]glufosinate at the four-leaf stage*

Values are means, with SE shown in parentheses.

| Plant Part | D,L-[$^{14}C$]Glufosinate[a] | | Acetyl-L-[$^{14}C$]Glufosinate[a] | |
|---|---|---|---|---|
| | cv Excel | HCN27 | cv Excel | HCN27 |
| | *% of applied* | | | |
| Leaf wash | 12.0 (4.8) | 12.1 (1.8) | 0.3 (0.1) | 0.3 (0.1) |
| Treated leaf | | | | |
| Leaf tip | 15.0 (1.4) | 8.8 (0.8) | 2.7 (0.6) | 3.1 (0.6) |
| Treated area | 51.3 (2.1) | 34.3 (1.1) | 40.0 (3.5) | 49.2 (2.1) |
| Basal leaf | 14.2 (1.1) | 4.1 (0.1) | 4.4 (1.0) | 4.4 (0.8) |
| Shoot above treated leaf | 1.8 (0.5) | 9.1 (1.0) | 5.3 (0.4) | 8.4 (1.4) |
| Shoot below treated leaf | 3.3 (1.0) | 4.4 (0.4) | 2.8 (0.3) | 3.3 (0.3) |
| Roots | 1.2 (0.1) | 11.4 (0.8) | 12.4 (1.8) | 11.1 (0.9) |
| Total recovery | 98.8 (3.8) | 84.2 (1.5) | 67.9 (2.8) | 79.8 (3.3) |

Acetyl-L-[$^{14}C$]glufosinate was much more phloem mobile than D,L-[$^{14}C$]glufosinate, with approximately 31% and 27% of the applied $^{14}C$ exported from the treated leaf 24 h after application to cv Excel and HCN27 plants, respectively (Fig. 3B). Recovery of $^{14}C$ in plant parts other than the treated leaf did not change beyond 24 h after treatment, although the total recovery of $^{14}C$ declined (Table I). The distribution of acetyl-L-[$^{14}C$]glufosinate in both genotypes 72 h after treatment was similar to that following application of D,L-[$^{14}C$]glufosinate to HCN27 (Table I).

### *Green-Bud Stage*

More $^{14}C$ was translocated into the tissue above the treated leaf in HCN27 than in cv Excel following application of D,L-[$^{14}C$]glufosinate at the green-bud stage (Fig. 4A). Conversely, more $^{14}C$ remained in the treated leaf of cv Excel (58% of applied) than in that of HCN27 (38% of applied) 72 h after treatment (Table II). More $^{14}C$ was recovered in the upper stem of HCN27 than cv Excel at all sampling times, and more $^{14}C$ was translocated into the floral buds and flowers in HCN27 than in cv Excel 72 h after application of D,L-[$^{14}C$]glufosinate (approximately 3.0% versus 1.2% of the applied $^{14}C$, respectively; Table II).

Transport of $^{14}C$ into the tissue above the treated leaf following application of acetyl-L-[$^{14}C$]glufosinate was similar in HCN27 and cv Excel, with 21% to 22% translocated after 72 h (Fig. 4B). Translocation into the floral buds and flowers was also similar following application of acetyl-L-[$^{14}C$]glufosinate, with approximately 3% of the applied $^{14}C$ recovered in the floral buds and flowers after 72 h (Table II).

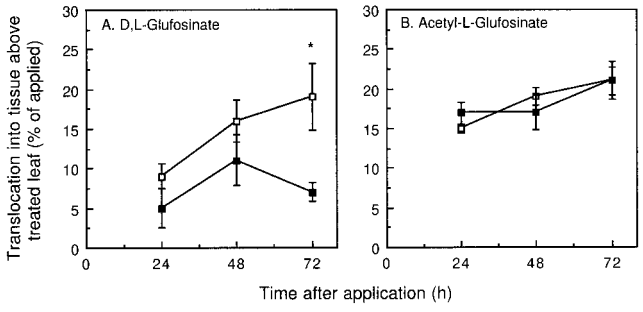



**Figure 4.** Translocation of $^{14}C$ into the tissue above the treated leaf of cv Excel (■) and HCN27 (□) plants following application of D,L-[$^{14}C$]glufosinate (A) and acetyl-L-[$^{14}C$]glufosinate (B) at the green-bud stage. Vertical bars represent SE; asterisks indicate a significant difference between cv Excel and HCN27 at that harvest time.

### *Early-Flowering Stage*

Fourteen percent of the applied $^{14}C$ was recovered in the floral tissues of flowering HCN27 plants 96 h after application of D,L-[$^{14}C$]glufosinate (Fig. 5A). This was much higher than in cv Excel (2%–5% of the applied $^{14}C$ recovered in the floral tissues). Translocation of $^{14}C$ into the floral tissue, expressed as disintegrations per minute per milligram of dry tissue, was also higher in HCN27 than in cv Excel 96 h after application of D,L-[$^{14}C$]glufosinate (14 versus 3 dpm $mg^{-1}$, respectively). More $^{14}C$ was transported into the floral tissues of HCN27 than into those of cv Excel following application of acetyl-L-[$^{14}C$]glufosinate (Fig. 5B). Translocation of $^{14}C$ into the floral tissue of HCN27 increased up to 48 h after application of acetyl-L-[$^{14}C$]glufosinate, then remained stable at about 10% of the applied dose.

The flowers of these plants were subdivided so that the amount in the anthers could be determined. More $^{14}C$ was translocated into the anthers of HCN27 than into those of cv Excel 96 h after application of D,L-[$^{14}C$]glufosinate (approximately 1.0% versus 0.2% of applied; Fig. 6A). Translocation of $^{14}C$ into the anthers following application of acetyl-L-[$^{14}C$]glufosinate increased over time in both genotypes, reaching 0.6% to 0.8% after 96 h (Fig. 6B).

## Comparative Translocation of D-[$^{14}C$]Glufosinate and D,L-[$^{14}C$]Glufosinate

D-[$^{14}C$]Glufosinate, the inactive isomer, was much more phloem mobile than D,L-[$^{14}C$]glufosinate in the susceptible genotype, cv Excel. Significantly more $^{14}C$ was exported out of the treated leaf after application of D-[$^{14}C$]-glufosinate than D,L-[$^{14}C$]glufosinate 24 or 72 h after application (Table III).

**Table II.** *Distribution of $^{14}C$ in the treated leaf and tissues above the treated leaf of cv Excel and HCN27 plants 72 h after application of D,L-[$^{14}C$]glufosinate or acetyl-L-[$^{14}C$]glufosinate*

The plants were treated at the green-bud stage, i.e. when flower buds were first apparent at the apex of the main flowering stem. Foliar absorption ranged from 87% to 97% of the applied $^{14}C$. Not all plant parts were harvested; therefore, total recovery of $^{14}C$ was not determined. Values are means, with SE shown in parentheses.

| Plant Part | D,L-[$^{14}C$]Glufosinate[a] | | Acetyl-L-[$^{14}C$]Glufosinate[a] | |
|---|---|---|---|---|
| | cv Excel | HCN27 | cv Excel | HCN27 |
| | *% of applied* | | | |
| Leaf wash | 13.0 (3.0) | 13.6 (2.5) | 2.8 (1.6) | 3.7 (1.5) |
| Treated leaf | 58.4 (2.4) | 37.5 (1.3) | 54.2 (3.5) | 43.9 (2.4) |
| Leaves above the treated leaf | 2.5 (0.5) | 5.4 (0.4) | 7.3 (0.6) | 5.9 (0.5) |
| Stem above treated leaf | 3.1 (0.6) | 10.9 (1.7) | 10.7 (1.1) | 12.3 (1.0) |
| Floral buds and flowers | 1.2 (0.2) | 3.0 (0.4) | 3.1 (0.5) | 3.3 (0.6) |

### Effect of Gln Supplementation on Translocation of D,L-[$^{14}C$]Glufosinate

Gln supplementation (either to the root or the leaves) stimulated export of D,L-[$^{14}C$]glufosinate 24 h after application, but not beyond that (Table IV). By 72 h after application, equal quantities of D,L-[$^{14}C$]glufosinate had been exported from the treated leaf in all treatments.

### Identification of Translocated $^{14}C$

In general, the quantities of $^{14}C$ recovered in various tissues in the metabolism experiments were similar to those found in the previous experiments (data not shown). Therefore, the [$^{14}C$]glufosinate and acetyl-L-[$^{14}C$]glufosinate metabolism data are presented as a percentage of recovered $^{14}C$ in the selected tissues (Table V). When the total recovery did not equal 100%, the balance was comprised of minor, unidentified $^{14}C$ compounds. The stereochemistry of the compounds detected was not determined.

Most of the D,L-[$^{14}C$]glufosinate applied to the susceptible genotype, cv Excel, was recovered as [$^{14}C$]glufosinate (Table V). Approximately 30% of the radiolabel recovered in the rest of the plant was in the form of unidentified compounds, but no acetyl-[$^{14}C$]glufosinate was recovered in these plants. Similarly, all of the acetyl-L-[$^{14}C$]glufosinate applied to cv Excel plants was recovered as acetyl-[$^{14}C$]glufosinate (Table V).

[$^{14}C$]Glufosinate and acetyl-[$^{14}C$]glufosinate were identified in approximately equal quantities in all HCN27 tissues following application of D,L-[$^{14}C$]glufosinate (Table V). Only acetyl-[$^{14}C$]glufosinate was identified in HCN27 tissue following application of acetyl-L-[$^{14}C$]glufosinate.

## DISCUSSION

Phloem mobility is an important component of the biological activity of many xenobiotics, particularly when accumulation in specific sink tissues is critical to that biological activity. Our objectives in this study were to develop a more complete understanding of the phloem mobility of D,L-glufosinate and its major metabolite, acetyl-L-glufosinate, in resistant and susceptible plants.

Phloem transport of D,L-glufosinate in the susceptible genotype was limited (Tables I–IV), which is in agreement with previous results (Mersey et al., 1990; Steckel et al., 1997). Contrary to the qualitative results reported by Dröge-Laser et al. (1994), the majority of the D,L-glufosinate exported from the treated leaf in the susceptible *B. napus* genotype was transported to the lower portion of the plants. Although Dröge-Laser et al. (1994) concluded that glufosinate transport likely occurred in the xylem, our

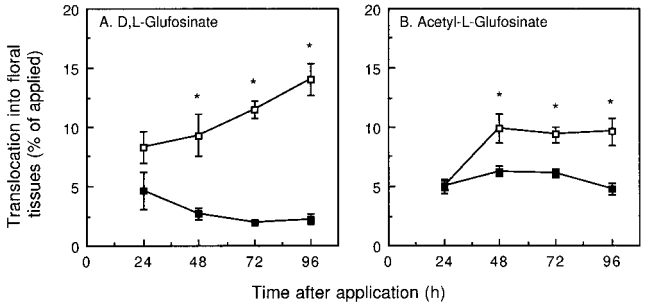



**Figure 5.** Translocation of $^{14}C$ into the floral tissues of cv Excel (■) and HCN27 (□) plants following application of D,L-[$^{14}C$]glufosinate (A) and acetyl-L-[$^{14}C$]glufosinate (B) at early flowering. Floral tissues included unopened flower buds and all parts of open flowers. Vertical bars represent SE; asterisks indicate a significant difference between cv Excel and HCN27 at that harvest time.

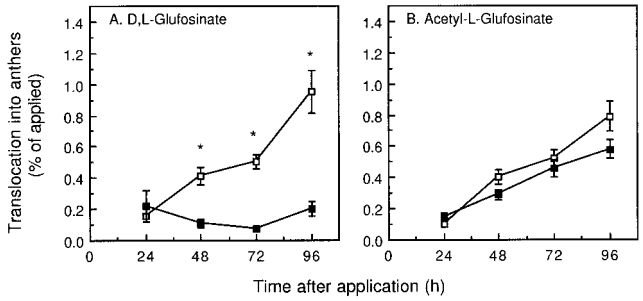



**Figure 6.** Translocation of $^{14}C$ into the anthers of cv Excel (■) and HCN27 (□) plants following application of D,L-[$^{14}C$]glufosinate (A) and acetyl-L-[$^{14}C$]glufosinate (B) at early flowering. Flowers were removed from the plants and the anthers excised by hand. Vertical bars represent SE; asterisks indicate a significant difference between cv Excel and HCN27 at that harvest time.

**Table III.** *Translocation of D-[$^{14}$C]glufosinate and D,L-[$^{14}$C]glufosinate out of the treated leaf of cv Excel plants*

D-[$^{14}$C]Glufosinate and D,L-[$^{14}$C]glufosinate were applied to the second leaf of plants at the two- to three-leaf stage. Translocation was determined by summing the $^{14}$C content of all tissues other than the treated leaf. Approximately 95% of the applied D-[$^{14}$C]glufosinate and D,L-[$^{14}$C]glufosinate was absorbed by the treated leaves 72 HAT. Values are means, with SE values shown in parentheses.

| Hours after Treatment | Translocation out of the Treated Leaf[a] | |
|---|---|---|
| | D-[$^{14}$C]Glufosinate | D,L-[$^{14}$C]Glufosinate |
| | *% of applied* | |
| 24 | 25.5 (2.4) | 2.3 (0.4) |
| 72 | 35.1 (4.8) | 8.4 (1.8) |

[a] Mean (SE).

results indicate that racemic D,L-glufosinate is phloem mobile, although only to a limited extent. In contrast, acetyl-L-[$^{14}$C]glufosinate was very phloem mobile, with substantial quantities exported to the upper leaves, flowers, anthers, and roots.

Based on the physicochemical properties required for phloem mobility of herbicides (intermediate membrane permeability and/or a functional weak acid group; Tyree et al., 1979; Kleier, 1988; Bromilow et al., 1990), glufosinate would be predicted to move readily in the phloem. However, the phloem mobility of D,L-glufosinate was low compared with that of other compounds with similar properties. For example, glyphosate ($pK_a$ values of 2.6, 5.6, and 10.3; log $K_{ow}$ = −2.7 to −3.2) is very phloem mobile in most species (Devine, 1989; Bromilow et al., 1993). Since D-glufosinate was much more phloem mobile than D,L-glufosinate (Table IV), and only the L-isomer of glufosinate is herbicidal (Manderscheid and Wild, 1986), we conclude that the phytotoxic effect of L-glufosinate on the plant limits its own translocation. The transient reversal of this effect by Gln supplementation (Table IV) supports this conclusion.

The phenomenon of "self-limitation" of phloem translocation has been documented previously for several herbicides, including glyphosate and chlorsulfuron. Glyphosate reduces carbon fixation in some species due to the rapid depletion of ribulose bisphosphate (Servaites et al., 1987; Shieh et al., 1991). This occurs as a consequence of the deregulation of carbon metabolism and increased carbon flow into the shikimate pathway following the inhibition of 5-*enol*pyruvylshikimate-3-phosphate synthase, the herbicide target site (Steinrücken and Amrhein, 1980; Gougler and Geiger, 1984; Geiger et al., 1986, 1987). Chlorsulfuron, on the other hand, does not reduce carbon fixation or hexose/Suc synthesis, but prevents Suc loading in source leaf tissue (Bestman et al., 1990; Devine et al., 1990; Hall and Devine, 1993). Therefore, the phloem mobility of chlorsulfuron was considerably greater in an Arabidopsis mutant with a resistant form of acetolactate synthase (the herbicide target site [Ray, 1984]) than in a susceptible biotype (Hall and Devine, 1993). The end result of these effects is similar: less herbicide is exported from leaves than would be predicted based on their physicochemical properties.

There are several possible explanations for the limited phloem transport of D,L-glufosinate in the susceptible genotype. The immediate consequences of GS inhibition by L-glufosinate are ammonia accumulation in the tissue and depletion of Gln. The accumulation of ammonia disrupts cell membranes, leading to the death of the treated tissues (Köcher, 1983; Wild and Manderscheid, 1984; Tachibana et al., 1986a, 1986b). L-Glufosinate also induces rapid depolarization of the plasma membrane electrogenic potential, although at relatively high concentrations (0.1–1.0 mM) (Ullrich et al., 1990), and reduces membrane protein content in sensitive species (Senaratna et al., 1997). These effects may disrupt other membrane transport processes, including Suc loading and export from leaves.

Gln depletion may play a minor role in the limited export of glufosinate. Root-applied Gln reversed the glufosinate-induced suppression of hairy root growth in *B. napus* (Downs et al., 1994). In our experiments, exogenously supplied Gln temporarily enhanced D,L-[$^{14}$C]glufosinate translocation, but did not maintain it at the same level as that of D-[$^{14}$C]glufosinate (Table IV). Injury symptoms (yellow lesions at the site of application) were evident in cv Excel plants 12 h after application of D,L-glufosinate. Over time, these lesions spread across the treated leaf. Plants supplied with exogenous Gln did not show these symptoms initially, but did by 72 h after glufosinate application, indicating that Gln supplementation only temporarily reversed the toxic effects of the herbicide.

Finally, accumulation of glyoxylate and the resulting reduction in photosynthetic carbon fixation may also contribute to the limited translocation of D,L-glufosinate (Sauer et al., 1987; Wild et al., 1987; Zeigler and Wild, 1989; Wendler and Wild, 1990; Lacuesta et al., 1992; Wendler et al., 1992). This would represent a similar effect to that reported for glyphosate (Geiger et al., 1986, 1987) but via a different mechanism. The limited export of D,L-glufosinate from source leaves is likely a result of a combination of the mechanisms described here.

As expected, approximately one-half of the applied D,L-[$^{14}$C]glufosinate was metabolized to acetyl-[$^{14}$C]glufosinate in HCN27 (Table V). Since PAT is specific for the L-isomer and does not acetylate D-glufosinate (Dröge et al., 1992), the two compounds exported from the treated leaf in

**Table IV.** *Effect of Gln supplementation on the export of D,L-[$^{14}$C]glufosinate from the treated leaf of cv Excel plants*

D,L-[$^{14}$C]Glufosinate was applied to the second leaf of plants at the two- to three-leaf stage. Gln (1 mM) was supplied either in the nutrient solution (root) or as part of the herbicide application solution (foliar). Control plants did not receive supplemental Gln. Values are means, with SE values shown in parentheses.

| Hours after Treatment | Translocation out of the Treated Leaf[a] | | |
|---|---|---|---|
| | Control | Gln supplementation | |
| | | Root | Foliar |
| | *% of applied* | | |
| 24 | 2.3 (0.4) | 9.5 (1.3) | 8.6 (1.0) |
| 72 | 8.4 (1.8) | 7.9 (0.3) | 8.7 (0.5) |

**Table V.** *Identity of $^{14}C$ metabolites in the treated leaf and the rest of the plant (combined upper shoot, lower shoot, and roots) at the four-leaf stage, and in floral tissues (flowers and buds) at the early-flowering stage, 72 h after application of D,L-[$^{14}C$]glufosinate or acetyl-L-[$^{14}C$]glufosinate*

Plants at the two growth stages were treated with D,L-[$^{14}C$]glufosinate or acetyl-L-[$^{14}C$]glufosinate, and $^{14}C$ metabolites extracted and identified as described in "Materials and Methods." The data are means of two samples from two separate tissue extractions. Values are means, with SE shown in parentheses.

| Genotype Tissue | $^{14}C$ Compound Applied | $^{14}C$ Compound Identified[a] | |
|---|---|---|---|
| | | [$^{14}C$]Glufosinate | Acetyl-[$^{14}C$]glufosinate |
| | | *% of $^{14}C$ recovered in tissue* | |
| cv Excel | | | |
| Treated leaf | D,L-[$^{14}C$]Glufosinate | 90.6 (5.7) | 0 |
| Rest of plant | D,L-[$^{14}C$]Glufosinate | 67.2 (2.9) | 0 |
| Flowers | D,L-[$^{14}C$]Glufosinate | 96.0 (2.2) | 0 |
| Treated leaf | Acetyl-L-[$^{14}C$]glufosinate | 0 | 97.7 (0.6) |
| Rest of plant | Acetyl-L-[$^{14}C$]glufosinate | 0 | 98.6 (1.1) |
| Flowers | Acetyl-L-[$^{14}C$]glufosinate | 0 | 98.6 (1.0) |
| HCN27 | | | |
| Treated leaf | D,L-[$^{14}C$]Glufosinate | 55.5 (1.5) | 43.0 (1.9) |
| Rest of plant | D,L-[$^{14}C$]Glufosinate | 37.0 (0.4) | 58.6 (2.3) |
| Flowers | D,L-[$^{14}C$]Glufosinate | 46.8 (0.7) | 52.6 (1.1) |
| Treated leaf | Acetyl-L-[$^{14}C$]glufosinate | 0 | 98.4 (1.1) |
| Rest of plant | Acetyl-L-[$^{14}C$]glufosinate | 0 | 97.0 (1.9) |
| Flowers | Acetyl-L-[$^{14}C$]glufosinate | 0 | 97.0 (0.2) |

HCN27 are assumed to be acetyl-L-[$^{14}C$]glufosinate and D-[$^{14}C$]glufosinate.

Recovery of acetyl-L-[$^{14}C$]glufosinate in both genotypes decreased over time (72-h data shown in Table I). This was evident in all plants treated with acetyl-L-[$^{14}C$]glufosinate and in HCN27 plants treated with D,L-[$^{14}C$]glufosinate. Possible reasons for the reduced recovery include loss of $^{14}CO_2$ as a product of metabolism, loss of root tissue when the roots were extracted from the growth medium, and loss of acetyl-L-[$^{14}C$]glufosinate and/or D-[$^{14}C$]glufosinate through root exudation into the surrounding growth medium.

Both D,L-glufosinate and acetyl-L-glufosinate are relatively stable in non-transformed plants. For example, no $^{14}CO_2$ was released from the susceptible tobacco and carrot plants when they were treated with [3,4-$^{14}C$]-L-glufosinate (Dröge et al., 1992). D,L-Glufosinate and acetyl-L-glufosinate are non-volatile and are not subject to photodecomposition, so it is unlikely that $^{14}C$ was lost from the leaf surface prior to absorption. In a separate experiment, 5% to 10% of the absorbed acetyl-L-[$^{14}C$]glufosinate was exuded from the roots of hydroponically grown plants treated at the two-leaf stage. Previous research has demonstrated root exudation of phloem-mobile herbicides, including glyphosate, 2,4-D, and picloram (Sharma et al., 1971; Coupland and Caseley, 1979; Devine, 1989). Root exudation may have been enhanced under these hydroponic conditions compared with typical plants growing in soil; it is unlikely that significant root exudation of acetyl-L-glufosinate occurs in plants growing in soil under conditions representative of field conditions.

Kriete et al. (1996) have described a male sterility system involving the conversion of acetyl-L-glufosinate to L-glufosinate through the expression of a tapetum-specific Arg deacetylase from *Escherichia coli*. Based on the results of this study, foliar application of acetyl-L-glufosinate and its anther-specific conversion to L-glufosinate may generate sufficient concentrations of L-glufosinate to ablate the developing pollen. An early-flowering *B. napus* plant has a leaf-spray target area (from directly above) of approximately 150 $cm^2$. Assuming that acetyl-L-glufosinate was applied to early-flowering *B. napus* plants at a rate of 1.0 kg $ha^{-1}$, each plant would receive approximately 1.5 mg of acetyl-L-glufosinate. If 1% of the applied acetyl-L-glufosinate were translocated into the anthers, they would contain 15 $\mu$g of acetyl-L-glufosinate. In this study, the fresh weight of the anthers of an early-flowering plant was approximately 850 mg. Assuming the density of the anthers to be 1.0 g $mL^{-1}$, this would yield an acetyl-L-glufosinate concentration of 18 $\mu$g $mL^{-1}$, which would be converted to 14 $\mu$g $mL^{-1}$ glufosinate (equivalent to 79 $\mu$M) in the anthers of deacetylase transformants. A concentration of 2 to 10 $\mu$M of glufosinate is required to inhibit GS activity by 50%, depending on the source species (Wild and Manderscheid 1984; Ericson, 1985; Lea and Ridely, 1989; Walker et al., 1990). Based on these assumptions, therefore, the concentration of L-glufosinate generated in the anthers in deacetylase transformants should be sufficient to substantially inhibit GS and induce male sterility in plants treated with this rate of acetyl-L-glufosinate.

One unresolved question arising from this work is the mechanism of membrane transport of L-glufosinate and acetyl-L-glufosinate. L-Glufosinate activity depends on transport into the plastids, where the major sensitive form of GS is localized (Ericson, 1985; Ridley and McNally, 1985). Although most membrane transport of herbicides is by simple diffusion (Devine, 1989), carrier-mediated transport has been demonstrated for certain herbicides that mimic endogenous substrates (e.g. Minocha and Nissen, 1985; Denis and Delrot, 1993). The structural similarity

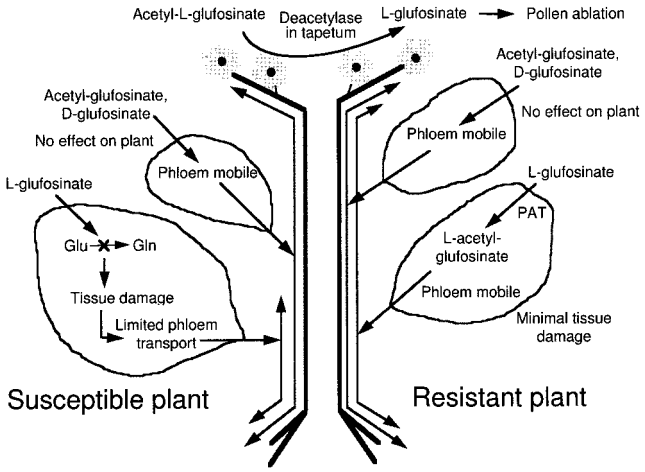



**Figure 7.** A descriptive model of phloem transport of D,L-glufosinate and acetyl-L-glufosinate in susceptible and resistant plants. D-glufosinate and acetyl-L-glufosinate are phloem mobile, are readily exported from source leaves, and accumulate in sink tissues. L-Glufosinate causes localized toxicity at the site of entry into the leaves, limiting its own transport to the rest of the plant.

between L-glufosinate and L-glutamate suggests that the former may be transported by a glutamate carrier, although no evidence has been produced for this. Regardless of the mechanism of membrane transport, the phytotoxicity of L-glufosinate and acetyl-L-glufosinate in deacetylase-transformed tobacco (Kriete et al., 1996) indicate that both compounds are permeable to the plasma membrane and that L-glufosinate is also permeable to the plastid envelope.

It remains to be determined if complete pollen ablation can be induced through the conversion of acetyl-L-glufosinate to L-glufosinate in the anthers following foliar application of acetyl-L-glufosinate. Complete male sterility may require repeat applications of acetyl-L-glufosinate, given the extended flowering period of *B. napus* (ranging from 3–4 weeks, depending on the growing conditions), coupled with high expression of the deacetylase gene. Further research on dosage, frequency of application, and spatial arrangement of male sterile and pollen donor plants will be needed to optimize the use of acetyl-L-glufosinate as a chemical inducer of male sterility in a hybrid production system.

## CONCLUSIONS

Based on the results reported here, the transport behavior of D,L-glufosinate and acetyl-L-glufosinate in resistant and susceptible plants can be described as shown in Figure 7. In susceptible plants, L-glufosinate causes tissue damage to the cells at the site of application, limiting its export from the leaves. The low phloem mobility of D,L-glufosinate is likely due to the combined effects of ammonia accumulation and the associated effects on membrane structure and function, glyoxylate accumulation and reduced carbon fixation, and depletion of Gln in the source tissue. D-Glufosinate and acetyl-L-glufosinate are phloem mobile but not phytotoxic, and are readily exported from the leaves to sink tissues. However, the transport of D-glufosinate in the racemic D,L mixture is limited by the effect of L-glufosinate. Resistant plants expressing the *pat* gene selectively metabolize L-glufosinate to acetyl-L-glufosinate, while the non-phytotoxic D-glufosinate remains unacetylated; both compounds are exported in the phloem. Acetyl-L-glufosinate is translocated readily into floral tissues, including the anthers, in amounts likely to be sufficient to induce male sterility in transformants expressing a tapetum-specific deacetylase gene.

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