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Conversion of ^{14}C-Glyphosate to Carbon Dioxide by Alligator Weed

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INTRODUCTION

Alligator weed (*Alternanthera philoxeroides* (Mart.) Griseb.) is a perennial plant native to South America. The terrestrial form of the plant, characterized by large aerial biomass and underground rhizomatous storage organs, has invaded several thousand hectares of low lying pasture land and small waterways in coastal south-eastern Australia. In these situations biological control is ineffective, and the weed is not controlled by glyphosate (N-phosphonomethyl-glycine) at rates up to 3.2 kg ha^{-1} (Bowmer et al. 1989).

Several processes may affect the tolerance of alligator weed to glyphosate. These include poor translocation to roots and rhizomes, the dilution of translocated herbicide by underground biomass, metabolism of glyphosate to non-toxic metabolites, and exudation of glyphosate from the roots.

In previous experiments (Bowmer et al. 1993, Bowmer and Eberbach 1993) plants of different phenology were produced by growth under short or long days, with the objectives of understanding processes underlying tolerance, and of optimizing timing of spray application in the field.

Plants grown under short days (SD) were relatively much smaller, more prostrate in growth and did not flower, in contrast to those grown under long days (LD). In SD plants a smaller proportion of radiolabel from foliar applied glyphosate was translocated to underground parts, but concentrations in roots and rhizomes were higher than in LD plants.

At least two factors contributed to these differences. The underground biomass was smaller in SD plants, so dilution of translocated label was reduced. Conversely more radioactivity was lost from SD plants in root exudation.

In current experiments the exudation losses were quantified, and the possible degradation of glyphosate to non-toxic metabolites was also investigated.

MATERIALS AND METHODS

Rhizome cuttings about 25 mm long were buried in river-washed sand in pots 98 mm upper diameter and 100 mm high, and were grown in a growth cabinet at 20C, 40-50% relative humidity, 203 μ mol photosynthetically active radiation m^{-2} s^{-1} and a 10 h (short-day) photoperiod. The pots were watered weekly with full-strength Hohenheim's solution and, if required, topped up daily with rain water. To obtain uniform plant sizes, plants were trimmed of excess shoot growth at weekly intervals until they were 9 weeks old and after 10 weeks were treated with glyphosate. The plants were actively growing at the time of treatment, were prostrate in form and about 20 cm tall. Further details of phenology and biomass are given in Bowmer and Eberbach (1993, Figure 1 and Table 3).

^{14}C-methyl labelled glyphosate was applied to a single leaf pair of each plant, 4 nodes from the apex of the longest stem. The application was similar to previous experiments (Bowmer et al. 1993) except that 26 droplets were used, each of 2 μL volume applied with a syringe. Each plant received 54.8 μg glyphosate per plant (calculated as glyphosate acid, but applied as glyphosate isopropylamine salt), together with additives in the commercial formulation, Roundup (Monsanto, Australia Pty Ltd).

Each pot was placed on a plastic tray and transferred to a sealed chamber. The chamber consisted of a closed cylinder of perspex 230 mm high and 120 mm diameter, sealed at its base with neoprene cemented to a sheet glass floor with Xantoprene Plus, (Neil Harcourt, Sydney), a non-toxic dental cement. Air was pumped through the chamber at 6.3 L h^{-1}, equivalent to 8 exchanges of air per hour over the plants. The chambers were placed in a growth cabinet and the treatment was replicated 3 times.

The air left each chamber through a condensation trap which collected water generated from evapotranspiration, then through a second trap containing a solution of 1 M NaOH to collect carbon dioxide. Checks were made on the trapping efficiency of $^{14}CO_2$ by the addition of acid to ^{14}C-labelled Na_2CO_3. Each day the NaOH was removed, a subsample assayed for radioactivity, and the trap refilled with fresh solution. Radioactivity was measured using liquid scintillation spectroscopy by adding 1 ml NaOH from the traps to 2 ml water and 2 ml of Ready-Gel (Beckman) scintillation cocktail.

Thin-layer chromatography (TLC) was used to separate glyphosate from possible metabolites, notably amino methyl phosphonic acid (AMPA), in sand and extracts of roots and rhizomes. Sand and plant tissues were washed with water. The water was filtered through coarse filter paper, the filtrate dried using a rotary evaporator at 40C and the residue resuspended in water (0.5 ml). Three plants were harvested 14 days after treatment with ^{14}C-glyphosate, separated into roots and rhizomes, and stored frozen. The tissues were later thawed, weighed, homogenized in water (30 ml) using an Ultra-Turrax rotary blade homogenizer and centrifuged at 3C for 10 minutes at 22700G to give a clear supernatant. This was decanted through a filter (Whatman No. 1) into a flask,

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and the extraction procedure repeated twice more. The three portions of filtered supernatant were combined, rotary evaporated to dryness at 40C and taken up in water (0.5 ml).

Commercial TLC plates of 0.1 mm thick DC cellulose (Merck, Cat. No. 5716) were dried at 70C before spotting with sample or standard using 2 µL spots. The mobile phase was ethanol (55 ml), water (35 ml), concentrated ammonium hydroxide (2.5 ml), trichloroacetic acid (3.5g) and glacial acetic acid (2 ml), as described by Sprankle et al. (1978). Standards used were sarcosine, glycine, AMPA, glyphosate acid and Roundup® (commercial formulation of glyphosate isopropylamine salt). The spots contained 2 µg of glyphosate or 1 µg of the other standards.

Plates were left in the mobile phase until a minimum run length of 13.5 cm was achieved, usually about 5 h. The plates were dried at 70C and sprayed with a solution of ninhydrin (1 g), absolute ethanol (700 ml), 2,4,6-collidine (29 ml) and glacial acetic acid (210 ml) as described by Pataki (1969).

Standards were separated by TLC and detected by the ninhydrin spray. Rf values were: glyphosate 0.19; AMPA 0.25; glycine 0.47; and sarcosine 0.62. Glyphosate acid and glyphosate isopropylamine salt as Roundup were not separated.

The plates were dried again and exposed to Kodak autoradiography film for 4 or 7 weeks. Spots in positions corresponding to glyphosate (Rf 0.19) and AMPA (Rf 0.25) were scraped off, eluted with the TLC mobile phase, and activities corrected by subtracting background activity. Minimum detectable radioactivity was estimated at 50 dpm above background, corresponding to about 0.3% of the activity applied to the plants.

RESULTS AND DISCUSSION

Root exudation

The efficiency of collection in the NaOH trap was 88% (range of 3 replicates was 86.3 - 88.9%). A further 12-14% of activity was collected in a second NaOH trap. In the final method one trap was used and results corrected for recovery.

Glyphosate applied to the plants was quickly converted to $^{14}CO_2$ (Figure 1). After 14 days, the trapped cumulated radioactivity generated from the plants represented about 29% of the total ^{14}C applied (after correction for incomplete recovery of $^{14}CO_2$ in the trap). Previous studies (Bowmer et al. 1993) showed negligible losses of glyphosate from a leaf surface of an excised shoot under similar ventilated conditions so we conclude that the observed $^{14}CO_2$ losses probably occurred by root exudation of glyphosate or its metabolites.

Root exudation has previously been proposed to account for a discrepancy between labelled glyphosate applied and recovered from various species. For example, Marshall et al. (1987) assumed root exudation losses though they did not make any direct measurements. Losses represented 17% of ^{14}C-glyphosate applied to field horsetail (*Equisetum arvense* (L.)) measured 8 weeks after treatment. Coupland and Caseley (1979) reported exudation of the intact glyphosate molecules from the roots of quackgrass (*Agropyron repens* (L.) Beauv.) and Rodrigues et al. (1982) found similar results for wheat.

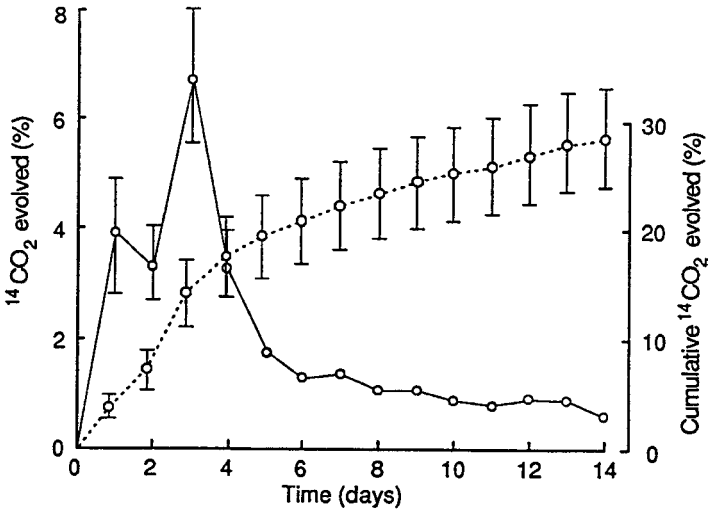

Figure 1. Evolution of $^{14}CO_2$ as a proportion of applied ^{14}C-activity after foliar application of ^{14}C-Glyphosate. Bars give standard errors for 3 replicates. $^{14}CO_2$ evolved (—); cumulative CO_2 evolved(·····).

Metabolism to non-toxic products

There was no evidence of glyphosate or metabolites in sand as determined by ninhydrin spraying, autoradiography or scintillation counting of plate eluates suggesting that any AMPA or other intermediates were rapidly degraded. Roots contained only small quantities of AMPA when measured 14 days after treatment. The proportion of radioactivity appearing as AMPA in root tissue was equivalent to about 0.4% of the foliar application. Rhizomes showed no AMPA in two extracts, and concentrations were similar to those observed in roots in a third extract.

These results contrast with those for field horsetail and quackgrass (*Agropyron repens* (L.) Beauv.) which metabolized glyphosate to AMPA (Coupland 1984, Marshall et al. 1987), but are similar to those obtained for many other species in which glyphosate in underground tissues remained unchanged (Wyrill and Burnside 1976, Gottrup et al. 1976, Zandstra and Nishimoto 1977, Schultz & Burnside 1980, Marquis et al. 1979) or was metabolized to only a limited extent (Putnam 1976, Sandberg et al. 1980).

Since there is little evidence of breakdown to AMPA or other non-toxic metabolites in below-ground tissues it is tempting to conclude that metabolism cannot account for the observed tolerance of alligator weed to glyphosate. However, it is possible that metabolism does occur with AMPA as a transitory intermediate which is rapidly degraded further, possibly to $^{14}CO_2$ either within the plant or after exudation into the soil. Observations that AMPA is microbially degraded in soil and water (Rueppell et al. 1977, Eberbach 1989), support this hypothesis. Further, Eberbach (1989) in other experiments, found that the decline of labelled glyphosate in soil was correlated to release of $^{14}CO_2$, suggesting that AMPA was in fact a transitory intermediate.

Mechanism of tolerance

Current experiments, taken together with previous results (Bowmer et al. 1993, Bowmer and Eberbach 1993) show that the most important mechanism of tolerance is poor translocation from the leaves. Loss of glyphosate as CO_2 from below ground tissues, possibly with AMPA as a transitory intermediate, can also be substantial, at least in plants growing in short day conditions.

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