

# Reversal of Glyphosate Inhibition of Carrot Cell Culture Growth by Glycolytic Intermediates and Organic and Amino Acids<sup>1</sup>

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JOHN KILLMER<sup>2</sup>, JACK WIDHOLM, AND FRED SLIFE  
Department of Agronomy, University of Illinois, Urbana, Illinois 61801

## ABSTRACT

Various cytokinins and purines were ineffective in reversing glyphosate (0.25 millimolar)-induced growth inhibition of carrot (*Daucus carota* L.) cell suspension cultures. Aspartate was particularly effective in reversing glyphosate inhibition, but asparagine and various combinations of lysine, methionine, threonine, and homoserine (eventual products of aspartate metabolism) were not effective. When organic acids of the tricarboxylic acid cycle were added to the medium, particularly good reversal of inhibition could be obtained with  $\alpha$ -ketoglutarate, succinate, and malate. Citrate provided only moderate reversal but the reversal given by glutamate was comparable to that of aspartate and the more effective tricarboxylic acid cycle intermediates. Pyruvate was somewhat toxic to cells when added early in the cell cycle but was most effective at reversing glyphosate inhibition when added at this time. If pyruvate addition was delayed, it was less toxic but was also a less effective reversing agent for glyphosate inhibition.

All of the effective reversing agents for glyphosate inhibition found in this study can serve either directly or indirectly as carbon skeletons for respiration and ammonia assimilation and have previously been shown to be effective detoxifying agents for ammonia in cell culture systems. The results of this study suggest that glyphosate inhibition of growth in this system may be due to depletion of respiratory substrate which may eventually result in ammonia accumulation.

Previous work on the mode of action of the nonselective herbicide glyphosate (*N*-[phosphonomethyl] glycine) indicates some relationship with the biosynthesis or metabolism of aromatic amino acids since these can reverse glyphosate toxicity in duckweed (18), *Rhizobium japonicum* (18), *Escherichia coli* (12, 24), *Chlamydomonas reinhardtii* (12), and carrot (12, 13), tobacco (13), and soybean (12) cell cultures. Gresshoff (12) reported reversal of glyphosate inhibition of *Arabidopsis thaliana* seedlings with a combination of phenylalanine and tyrosine. In contrast, Duke and Hoagland (6) observed increased inhibition by glyphosate of corn seedling growth when phenylalanine and tyrosine were added.

Several studies (7, 8, 16) show that glyphosate treatment can decrease free phenylalanine and tyrosine levels. Recent studies have indicated that glyphosate inhibits the synthesis of flavonoids and chlorogenic acid in buckwheat hypocotyls (17) and causes an accumulation of shikimate that is significantly correlated with a

reduction of anthocyanin formation (1). Extension of these studies using a cell-free bacterial system has shown that glyphosate inhibits the conversion of shikimate to anthranilate due to inhibition of 5-enolpyruvylshikimate acid-3-P synthase (25). Others (6, 15) have shown that glyphosate induces PAL<sup>3</sup> activity and it was suggested that this may in turn cause growth inhibition by decreasing phenylalanine levels and/or by increasing the level of inhibitory phenolics or intracellular ammonia. Glyphosate-induced increases in levels of ammonia (7, 13, 23), glutamate, and glutamine (7, 13, 15) have been reported in several different plant test systems. More recent work (8) has shown that *L*- $\alpha$ -aminoxy- $\beta$ -phenylpropionic acid, a specific inhibitor of PAL, provides only a slight reversal of glyphosate inhibition of soybean seedling growth.

Hadacidin (*N*-formyl-*N*-hydroxyglycine) is an amino acid analog which, like glyphosate, does not have a free amino group. Hadacidin inhibits adenylosuccinate synthetase (14) and causes an inhibition of apical dominance (20) similar to sublethal doses of glyphosate (2, 4), effects which can be reversed by cytokinins and other metabolites (2, 20).

The studies reported here were attempts to reverse glyphosate inhibition of cultured carrot cell growth by adding various compounds to the culture medium.

## MATERIALS AND METHODS

**Plant Material.** Garden carrot (*Daucus carota* L. cv. Danvers) cells were cultured in a defined medium which was slightly modified from that reported elsewhere (22). Modifications included substitution of 2,4-D (0.4 mg/l) for IAA and the elimination of kinetin, Edamin, and agar as components in the media. Growth experiments were initiated by inoculating duplicate flasks with 0.5 g fresh weight of 11- to 13-day-old cells from a single or several combined flasks into 100 ml liquid medium. Cells were grown until the early stationary phase (11-12 days), harvested on Miracloth via suction filtration, and the fresh weight determined. Data are presented as per cent increase in fresh weight of a control and were calculated for each treatment as (+glyphosate) + (-glyphosate).

**Additions to Media.** Glyphosate (0.25 mM, 94% technical grade, or 98% analytical grade, Monsanto Co.) was used in all growth studies and was routinely autoclaved (13). All other additions to the media were as 0.5-ml or 1.0-ml solutions that had been brought to a pH of 5.7 to 5.9 (KOH or HCl) and filter sterilized. Both 10 mM KH<sub>2</sub>PO<sub>4</sub> and 20 mM KCl did not affect cell growth or glyphosate inhibition.

## RESULTS

**Aspartate Reversal of Glyphosate Inhibition.** Inasmuch as hadacidin and glyphosate have structural and activity characteristics

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<sup>2</sup> Present address: Monsanto Co., 800 N. Lindbergh Blvd., St. Louis, MO 63166.

<sup>3</sup> Abbreviation: PAL, phenylalanine ammonia lyase.

in common, the initial studies carried out were to determine whether reversing agents for hadacidin inhibition would effectively reverse glyphosate inhibition. Attempts to reverse glyphosate inhibition with the cytokinins isopentenyladenine (0.1–1.0  $\mu\text{M}$ ) and BA (0.89–4.44  $\mu\text{M}$ ) were unsuccessful (19). Combinations of the purines adenine, guanine, inosine, and xanthine ( $0.125\text{--}1 \times 10^{-4}$  M) were also ineffective reversing agents (19).

Inhibition of adenylosuccinate synthetase by hadacidin could be reversed with aspartate (14), as could inhibition of bud growth in peas (20). Likewise, 2.5 mM aspartate was moderately effective in reversing glyphosate inhibition of growth in these carrot suspension cultures (Table I). Reversal increased from 8 to 27% of the control as the aspartate concentration increased from 0.05 to 2.5 mM. Previous work using duckweed (18) had shown no reversal of glyphosate inhibition with this amino acid.

**Attempted Reversal with Products of Aspartate Metabolism.** Since aspartate reversed glyphosate inhibition and various purines and cytokinins did not, the possible role of glyphosate as an inhibitory aspartate analog in the synthesis of asparagine and the various aspartate family amino acids were investigated. Asparagine supplied in the media inhibited cell growth at 6.6 mM, while lower concentrations (0.007–0.606 mM) neither inhibited growth nor reversed glyphosate toxicity (19).

Regulation of the aspartate family amino acid pathway is complex (5), so components of the pathway (lysine, methionine, threonine, and homoserine) were added in various concentrations (0.10–0.30 mM) and combinations in an effort to overcome the possible inhibitory feedback effects of single components. Inhibition of cell growth by these amino acids alone or in combination was always less than 20%. In no instance did any of the supplements provide convincing reversal of glyphosate inhibition (19).

**Reversal with Tricarboxylic Acid Cycle Intermediates and Glutamate.** The lack of reversal by asparagine and aspartate family amino acids suggests that aspartate reversal of glyphosate inhibition may occur through deamination or transamination of aspartate resulting in a donation of carbon skeletons to the tricarboxylic acid cycle via oxaloacetate. Subsequent experiments (Table II) indicated that high concentrations of tricarboxylic acid cycle

Table I. Concentration-Dependent Reversal by Aspartate of Glyphosate Inhibition of Carrot Cell Growth

| Addition to Culture Medium | Increase in Fresh Weight |                            | Per cent Control |
|----------------------------|--------------------------|----------------------------|------------------|
|                            | –Glyphosate              | +0.25 mM Gly-<br>phosphate |                  |
|                            | <i>g</i>                 |                            |                  |
| None                       | 38.33 $\pm$ 1.10         | 0.97 $\pm$ 0.18            | 2.53             |
| 0.05 mM Asp                | 35.89 $\pm$ 1.33         | 3.00 $\pm$ 0.27            | 8.35             |
| 0.25 mM Asp                | 34.30 $\pm$ 9.16         | 3.69 $\pm$ 0.54            | 10.75            |
| 0.50 mM Asp                | 37.49 $\pm$ 0.10         | 5.71 $\pm$ 0.79            | 15.23            |
| 2.50 mM Asp                | 35.24 $\pm$ 1.61         | 9.65 $\pm$ 1.75            | 27.38            |

Table II. Reversal of Glyphosate Inhibition by Glutamate and Intermediates of the Tricarboxylic Acid Cycle

| Addition (10 mM) to Culture Medium | Increase in Fresh Weight |                            | Per cent Control |
|------------------------------------|--------------------------|----------------------------|------------------|
|                                    | –Glyphosate              | +0.25 mM Gly-<br>phosphate |                  |
|                                    | <i>g</i>                 |                            |                  |
| None                               | 34.81 $\pm$ 2.84         | 6.21 $\pm$ 0.83            | 17.83            |
| Citrate                            | 27.96 $\pm$ 2.24         | 9.33 $\pm$ 0.30            | 33.36            |
| $\alpha$ -Ketoglutarate            | 37.57 $\pm$ 0.82         | 34.07 $\pm$ 2.02           | 90.68            |
| Succinate                          | 33.12 $\pm$ 5.42         | 26.30 $\pm$ 0.35           | 79.40            |
| DL-Malate                          | 31.06 $\pm$ 1.38         | 26.02 $\pm$ 2.04           | 83.77            |
| Glu                                | 32.00 $\pm$ 2.54         | 30.40 $\pm$ 1.20           | 95.00            |

Table III. Comparison of the Relative Effectiveness of Reversal of Glyphosate Inhibition by Glutamate, Aspartate, and  $\alpha$ -Ketoglutarate

| Addition (1 mM) Culture Medium | Increase in Fresh Weight |                             | Per cent Control |
|--------------------------------|--------------------------|-----------------------------|------------------|
|                                | –Glyphosate              | + 0.25 mM Gly-<br>phosphate |                  |
|                                | <i>g</i>                 |                             |                  |
| None                           | 35.88 $\pm$ 4.82         | 1.34 $\pm$ 4.82             | 3.73             |
| 1 Glu                          | 34.93 $\pm$ 0.27         | 7.76 $\pm$ 1.02             | 22.21            |
| 1 Asp                          | 33.61 $\pm$ 0.11         | 11.09 $\pm$ 1.17            | 32.99            |
| 1 $\alpha$ -Ketoglutarate      | 37.21 $\pm$ 0.46         | 3.28 $\pm$ 0.06             | 8.81             |

Table IV. Effect of Time of Addition on Pyruvate Reversal of Glyphosate Inhibition of Carrot Cell Growth

| Addition (20 mM) to Culture Medium | Increase in Fresh Weight |                             | Per cent Control |
|------------------------------------|--------------------------|-----------------------------|------------------|
|                                    | –Glyphosate              | + 0.25 mM Gly-<br>phosphate |                  |
|                                    | <i>g</i>                 |                             |                  |
| None                               | 38.76 $\pm$ 2.93         | 1.47 $\pm$ 0.32             | 3.79             |
| Pyr (day 0)                        | 19.05 $\pm$ 0.43         | 18.13 $\pm$ 1.41            | 95.17            |
| Pyr (day 1)                        | 26.27 $\pm$ 1.89         | 13.78 $\pm$ 0.30            | 52.45            |
| Pyr (day 2)                        | 29.30 $\pm$ 3.28         | 11.30 $\pm$ 0.95            | 38.56            |
| Pyr (day 3)                        | 30.43 $\pm$ 1.99         | 9.67 $\pm$ 0.76             | 31.77            |

intermediates (citrate,  $\alpha$ -ketoglutarate, succinate, and malate) or glutamate could effectively reverse glyphosate inhibition. Citrate was the least effective of all the tricarboxylic acid cycle intermediates tested. Comparisons of 1 mM concentrations of  $\alpha$ -ketoglutarate and glutamate with aspartate (Table III) suggest that aspartate is the better reversing agent.

Inasmuch as glutamate reduced glyphosate inhibition and arginine, citrulline, and ornithine are derived from glutamate, they too were used to attempt to reverse the inhibition. However, even high concentrations of 10 mM citrulline, 10 mM ornithine, or 20 mM arginine, while relatively nontoxic to these cultures, provided no reversal of glyphosate inhibition (19).

Thus, it seems that the action of aspartate may be due to its ability to provide additional carbon to the tricarboxylic acid cycle.

**Reversal of Inhibition with Pyruvate.** Results reported above suggest that glyphosate inhibition may be reversed by provision of additional carbon in the form of tricarboxylic acid cycle intermediates or the closely related amino acids aspartate and glutamate. To determine whether the apparent lack of respiratory substrate resulted from a lack of carbon flow from glycolysis, subsequent experiments were done to determine whether the glycolytic intermediate pyruvate could reverse glyphosate inhibition.

Pyruvate (20 mM) was slightly toxic if provided to freshly subcultured cells (Table IV). Lactic acid (2.5, 5.0, and 10 mM) was considerably more toxic than equimolar levels of pyruvate (19) suggesting that pyruvate toxicity may be due to lactic acid formation. Pyruvate, however, proved to be an excellent reversing agent for glyphosate inhibition (Table IV).

If pyruvate was added to cultures at 0, 1, 2, and 3 days after inoculation, there was a decrease in toxicity with time (Table IV). Yet when 20 mM pyruvate was present at inoculation, the growth of glyphosate-treated cells was greater than 95% of the pyruvate control. There was a decreasing reversal if pyruvate addition was delayed for 1 to 3 days after inoculation. These results might suggest that the toxic action of glyphosate takes place very early in the cell cycle. However, reversal of glyphosate inhibition by aromatic amino acids could occur after 8 days (13) and the decreasing effectiveness of pyruvate with time may be related to factors such as lower uptake or utilization which may limit pyru-

vate inhibition later in the cell cycle.

**Reversal of Inhibition by Other Glycolytic Intermediates.** The reversal of glyphosate inhibition with pyruvate prompted attempts to reverse inhibition with other glycolytic intermediates. The cyclohexylammonium salt of P-enolpyruvate proved to be quite toxic to cells at concentrations greater than 2.5 mM and was an ineffective reversing agent at this concentration. The toxicity of cyclohexylamine to cultured carrot cells was not tested. Phosphoglyceric acid at 10 mM inhibited cell growth about 50% but was a moderately effective (40%) reversing agent. Various other glycolytic intermediates were provided in an effort to determine whether some blockage in this pathway was responsible for an apparent inadequacy of respiratory substrate. Studies with 10 mM additions of glucose-1-P, glucose-6-P, fructose-6-P, and 20 mM DL-glycerate were all ineffective in reversing glyphosate inhibition. Perhaps uptake of these highly polar molecules was not great enough to provide adequate supplementary carbon or breakdown in the medium may have occurred before uptake. However, the same may be said for 3-P-glycerate which was a moderately effective reversing agent.

The addition of  $\alpha$ -glycerol phosphate to the medium was inhibitory to cell growth and was only slightly effective in reversing glyphosate toxicity (Table V). If a low (5 mM) concentration of pyruvate was combined with  $\alpha$ -glycerol phosphate, inhibition of cell growth was greater than when either component was added alone, but reversal of glyphosate inhibition was 2 times greater than when pyruvate was added alone. Citrate was mildly inhibitory and only slightly effective in reversing glyphosate toxicity. However, combinations of citrate with  $\alpha$ -glycerol phosphate or pyruvate provided reversal of glyphosate inhibition that was greater than pyruvate alone.

## DISCUSSION

These studies provide evidence that glyphosate inhibition in this system may be due to insufficient respiratory substrate. Decreases in carbon substrate available for respiration would also limit substrate for ammonia assimilation and might result in a buildup of ammonia and glutamine as has been reported for several tissues treated with glyphosate (7, 13, 16, 23). Amide accumulation is reported to occur in plants in the presence of high ammonia levels (11). Here, glyphosate inhibition was shown to be reversed by succinate, malate,  $\alpha$ -ketoglutarate, glutamate, pyruvate, and aspartate. Citrate reversal of glyphosate inhibition was slight. Behrend and Mateles (3) showed that while succinate, fumarate, malate,  $\alpha$ -keto-glutarate, glutamate, pyruvate, and to a lesser extent, citrate could reverse ammonia toxicity in tobacco cell cultures, acetate, tartrate, lactate, and glycolate were ineffective. Others (9) have reported growth of soybean cell suspension cultures with ammonium salts as the sole nitrogen source if tricarboxylic acid cycle acids were added. Matsumoto *et al.* (21)

Table V. Effect of Pyruvate, Citrate, and  $\alpha$ -Glycerol Phosphate ( $\alpha$ GP) and Combinations of These Metabolites on Glyphosate Inhibition of Carrot Cell Growth

| Addition (5 mM) to Culture Medium | Increase in Fresh Weight |                        | Per cent Control |
|-----------------------------------|--------------------------|------------------------|------------------|
|                                   | -Glyphosate              | +0.25 mM Gly-phosphate |                  |
|                                   | $\bar{x}$                |                        |                  |
| None                              | 25.16 $\pm$ 9.15         | 0.20 $\pm$ 0.20        | 0.79             |
| Pyr                               | 20.73 $\pm$ 0.12         | 3.96 $\pm$ 0.11        | 19.10            |
| Cit                               | 20.70 $\pm$ 3.25         | 1.36 $\pm$ 0.23        | 6.57             |
| $\alpha$ GP                       | 20.99 $\pm$ 3.90         | 1.95 $\pm$ 0.40        | 9.29             |
| Pyr + Cit                         | 12.54 $\pm$ 2.13         | 4.47 $\pm$ 0.01        | 35.64            |
| Pyr + $\alpha$ GP                 | 15.07 $\pm$ 0.40         | 8.15 $\pm$ 0.39        | 54.08            |
| Cit + $\alpha$ GP                 | 18.30 $\pm$ 3.19         | 4.94 $\pm$ 0.91        | 26.99            |

showed suppression of ammonia toxicity and lower levels of free ammonia in leaves of hydroponically grown cucumber plants when organic acids were present in the nutrient solution.

The effective reversal of glyphosate toxicity with combinations of aromatic amino acids (12, 13, 18) seems to argue against ammonia arising from PAL. However, aromatic amino acids increased glyphosate inhibition in whole plants and caused a decrease in PAL activity (6). Another possible explanation for amide and ammonia accumulation is the inhibition of transamination reactions as proposed by Nilsson (23). This might result in lower aromatic amino acid and higher ammonia levels.

These studies, however, indicate that a lack of respiratory substrate may be the cause of the observed ammonia accumulation in this (13) and other systems (7, 16, 23). Reversal of glyphosate inhibition by glycolytic intermediates such as pyruvate and to a lesser extent 3-P-glycerate indicate that flow of carbon from these points in glycolysis may be unhindered. The additive reversal by low concentrations of pyruvate and  $\alpha$ -glycerol phosphate may indicate free carbon flow throughout much of the glycolytic pathway.

Recently, it has been shown that glyphosate inhibits bacterial 5-enolpyruvylshikimic acid-3-P synthase (25) and causes an accumulation of shikimate in tissues of many plant species (1). This enzymic site is compatible with data showing reversal of glyphosate inhibition by aromatic amino acids. The regulation of biosynthesis of products of the shikimic acid pathway in higher plants is currently thought to be limited to feedback inhibition of chorismate mutase and anthranilate synthase (10). Accumulation of shikimate in glyphosate-treated tissues further suggests the lack of feedback inhibition within this section of the pathway. One result of the unregulated accumulation of shikimic acid may be the depletion of respiratory substrate in the form of P-enolpyruvate and erythrose-4-P, because 1 mol each is required for each mol shikimate. If this is the mechanism of glyphosate inhibition in these studies, then the many metabolites shown to reverse inhibition might do so by their ultimate utilization as substrates for respiration and ammonia assimilation.

Thus, it is possible that in the carrot culture system the growth inhibition occurs because of the depletion of respiratory substrate and not aromatic amino acids. Aromatic amino acids may reverse glyphosate growth inhibition by feedback inhibiting the early steps in the shikimate pathway which would prevent the large accumulation of shikimate and in turn the drain of respiratory substrate.

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