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The influence of glyphosate on endogenous levels of free IAA and phenolic compounds in leafy spurge

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Introduction

Glyphosate (N-(phosphonomethyl) glycine) is a broad spectrum, foliar applied herbicide which is readily translocated to actively growing tissues in a plant. Its mode of action has been the subject of much research over the past several years, yet the exact means by which the plants are killed remains unclear. Initial research indicated that the shikimic acid pathway, a biochemical pathway unique to plants that is responsible for the biosynthesis of aromatic amino acids, was the site of inhibition (Jaworski 1972). This fact was demonstrated by the ability of the aromatic amino acids, phenylalanine, tyrosine and tryptophan to reverse the herbicidal effects of glyphosate. Subsequent studies have shown that glyphosate treated plant tissue accumulates shikimic acid, an intermediary compound in the pathway, and that glyphosate stops the conversion of shikimic acid to chorismic acid in cell free extracts (Amrhein *et al*. 1980). Recently it was shown that *Escherichia coli* cells could be made resistant to glyphosate by introducing a mutant gene into the bacterium for 3-enolpyruvylshikimic acid-5-phosphate (EPSP) synthase. EPSP synthase is the enzyme responsible for catalyzing the reaction of 5-phosphoshikimic acid with phosphoenolpyruvate to form 3-enolpyruvylshikimic acid-5-phosphate (Comai *et al*. 1983). Additional publications have supported the EPSP synthase inhibition theory, including one which offers evidence that glyphosate binds to the phosphoenolpyruvate binding site of EPSP synthase (Steinrucken *et al*. 1984), and another in which glyphosate resistant carrot cell cultures are shown to have increased EPSP synthase activity (Nafziger *et al*. 1984).

Although there is substantial evidence that EPSP synthase is the major site of glyphosate inhibition there is no proof that this interference directly causes the death of the plant. A deficiency of aromatic amino acids may lead to the disruption of protein synthesis but it has also been reported that after glyphosate treatment the concentration of phenylalanine in either the free state or the metabolic pool was not low enough to limit plant growth (Haderlie *et al*. 1977) Also, a growing body of evidence suggests that IAA levels are closely tied to glyphosate induced injury and this may provide a better explanation for the herbicidal effects. For example, sublethal quantities of glyphosate will produce multiple branches or “witches broom” effects in Bermuda grass (Fernandez *et al*.

1977) and will stimulate the release of lateral buds from apical dominance in soybean and pea seedlings, even though the apical bud is not dead (Lee 1984). Such responses appear similar to release from correlative inhibition in which IAA has traditionally been thought to play a major role. Furthermore, glyphosate also decreases IAA transport in corn and cotton tissue (Baur 1979) and Lee (1980b) found that IAA reversed glyphosate induced inhibition in soybean and tobacco tissue cultures. Likewise glyphosate and 2,4-D (a synthetic auxin), which both inhibit growth when applied separately, act antagonistically when applied to a plant at the same time (O'Sullivan *et al*. 1980). When glyphosate treated callus cultures are supplied with exogenous IAA, neither IAA nor the enzyme IAA oxidase appear to be affected by glyphosate directly, yet there is a decrease in the levels of free IAA while bound IAA and the products of IAA oxidation increase (Lee 1982a,b). These decreases in IAA levels may be due to an observed decrease in the level of phenolic compounds (Lee 1982b), which are known to influence the activity of IAA oxidase (Lee 1980a). It is reasonable to assume that phenolic concentrations are affected by glyphosate since the major precursors of these compounds are phenylalanine and tyrosine. Thus, a postulated site of glyphosate inhibition is linked with decreases in levels of the important phytohormone IAA that could further explain how plant senescence is induced. Since most of the experiments on this topic have been performed either on callus cultures or other isolated plant tissues, this paper presents data on the effects of glyphosate on endogenous levels of IAA and phenolics substances in whole plants.

Materials and methods

General

Leafy spurge was chosen for this experiment for two reasons. First of all, it is a very important weed and represents a potentially real target for glyphosate application. Secondly, leafy spurge possesses a highly unusual anatomy that makes it an interesting organism for the study of IAA, apical dominance, and systemically translocated herbicides. At the base of the stem and on the roots are adventitious buds which are normally held under correlative inhibition but which may, for reasons that are still not completely understood, be released to become new shoots.

The plant material was greenhouse grown leafy spurge, started from seed and used at 4 months of age. Only healthy, single stemmed, actively growing plants were used. The total numbers of plants were divided into 3 treatment groups: glyphosate treated, decapitated, and control. The glyphosate plants were given a foliar dose of 4 lb ae/acre glyphosate (as Roundup) using a field sprayer simulator. This dosage had been previously found to be lethal to similar plants. The decapitated group had the apical region (including the youngest leaves) removed at time zero. This group served as a control to simulate removal of an endogenous source of 1AA. The third group was left intact as a control. Plants were harvested at 0.5, 1, 3, 5 and 7 days after treatment. The soil was washed from the roots, they were lyophilized, and then divided into three parts: shoots (stems and leaves), hypocotylar region (containing most of the adventitious buds), and roots. The tissues were ground and passed through a fine mesh before chemical analysis.

Free IAA analysis

The plant tissue was extracted in 80% methanol at 4 C for a total of six hours, centrifuged, and the extract was evaporated to the aqueous phase under reduced pressure. A known amount of (2-^{14}C)IAA as an internal standard was added to the residue. The sample was then partitioned against ether using the method of Knegt and Bruinsma (1973) in which the IAA in a basic solution was shaken against ether. At a basic pH the IAA stayed in the aqueous phase and hydrophobic contaminants were removed. By changing the solution to an acidic pH the IAA became associated with the ether phase allowing for the removal of polar contaminants. The final ether phase was made basic and reduced to less than 0.5 ml volume. Additional purification was by HPLC using a Nucleosil C18 column and reversed phase chromatography with a 30-minute linear gradient from 0.lN acetic acid to 0.1N acetic acid in 50% ethanol. A U.V. detector set at 280 nm was used to monitor the runs and the IAA peak was collected according to the retention time of authentic IAA standards. The final analysis. was done by HPLC using an Adsorbophere HS C18 column and ion pair chromatography. The mobile phase consisted of 30% MeOH with 0.01 M $NaP0_4$ and 0.005 M tetrabutylammonium phosphate and was delivered isocratically. Identification and quantification was done using a sequential arrangement of fluorescence (excitation=254 nm, emission=340 nm) and electrochemical (potential= +0.8 volts) detectors in sequence. These are both very selective and sensitive detectors, such that IAA was the only compound to produce a simultaneous response. The IAA peak was collected and the internal standard recovery was measured by liquid scintillation spectrophotometer to determine the losses of IAA during the purification procedure. The identity of IAA was confirmed by methylation and mass spectrometer analysis of the putative IAA-containing fraction.

Total phenolics assay

The assay for total phenolic compounds was modified after that of Singleton *et al*. (1965). Phenolic compounds were extracted from plant tissue in boiling 80% methanol. After cooling and centrifugation, an aliquot of this solution was used in a reaction with the Folin-Ciocalteu reagent, forming a colored product that was spectrophotometrically quantified at 765 nm. The standard curve was prepared from an equimolar mixture of caffeic acid, chlorogenic acid, p-coumaric acid, and quercitin.

Results and discussion

Free IAA

The application of a lethal dose of glyphosate to leafy spurge plants caused a significant decrease in IAA concentration in all plant organs relative to the control. In shoots (fig. 1A) the glyphosate treated plants showed a 22% decrease in IAA concentration after only 12 hours indicating that glyphosate has a relatively rapid effect on the metabolism of IAA. Interestingly, the IAA concentration rises to near normal levels on the third day before dropping again to 33% less than the control on day 7. The decapitated plants showed initially normal levels of IAA but dropped on days 3 and 5 before rising substantially on

day 7. This could be explained by traditional apical dominance theory, which predicts that levels of IAA would decrease until lateral bud inhibition was removed whereupon the rapid growth of lateral buds would produce increased IAA concentrations. It should be pointed out that at 12 and 24 hours the IAA concentration in the glyphosate treated shoots are lower than that of the decapitated plants, implying that glyphosate may not only remove the source of IAA, but may cause an increase in IAA conjugation and/or degradation as shown in tissue culture (Lee 1982b).

The hypocotylar region shows higher variation in IAA concentrations (fig.1B), which may be due to the presence of adventitious buds in different states of metabolic activity, which commonly develop in this region of the plant axis. No noticeable change in the length of buds occurred after treatment. The glyphosate treated plants show lowered IAA concentrations with the exceptions of days 1 and 7. The decapitated plants display a steady increase until day 5, followed by a decrease. Again this could be explained as increasing IAA synthesis by the rapid growth of many buds newly released from the inhibition of apical dominance until one or two new shoots assert dominance over the others and the IAA levels return to normal.

Glyphosate treatment decreases the IAA concentrations in roots (fig. 1C) by 35% and 31% on days 3 and 5 respectively. However, this delayed response may represent the lag time between the time of herbicide application and when it reaches the roots. A (^{14}C)glyphosate study in leafy spurge demonstrated that two days were required for significant amounts of glyphosate to reach the root and maximum accumulation was not achieved until seven or more days (Gottrup *et al*. 1976). Thus the glyphposate induced decrease was not apparent until the third day. Decapitated plants display an opposite trend, rising steadily for the first three days rather than decreasing. It is possible that adventitious buds on the roots are being released from dormancy, becoming sites of IAA synthesis, and thus increasing the IAA concentration in the roots. Interestingly, decapitation seems to result in slightly elevated IAA concentrations by day 7 in all plant parts.

Finally, it is worthwhile to compare the relative concentrations of IAA among the plant parts under study. The mean values of the controls for the hypocotylar regions, shoots, and roots contained 195.3, 131.4, and 93.4 ng/g dry wt. IAA respectively. Of these, the roots showed the least amount of variation, followed by shoots and finally hypocotylar regions which showed the greatest fluctuations. We assume that this difference is related to the number and developmental stage of adventitious buds. A bud held under inhibition does not contain as much IAA as an actively growing bud (Hillman *et al*. 1977), yet they may appear similar under routine examination. Thus it appears that in young leafy spurge plants the adventitious buds of the hypocotylar region may contain considerably more IAA and so may be more likely to start growing than buds on either the roots or shoots.

Phenolic compounds

The application of glyphosate to intact leafy spurge plants produced a significant decrease in phenolic compound levels only in the hypocotylar region of the plant (fig. 1E). In roots (fig. 1F) there was no difference between the groups at any time, while in shoots (fig. 1D) at day 5 the glyphosate treated tissue contained significantly more phenolics

relative to the control but by day 7 the level had returned to normal. Decapitation resulted in increased phenolic levels in the shoots only. No other significant differences were found in the analysis of phenolic compounds.

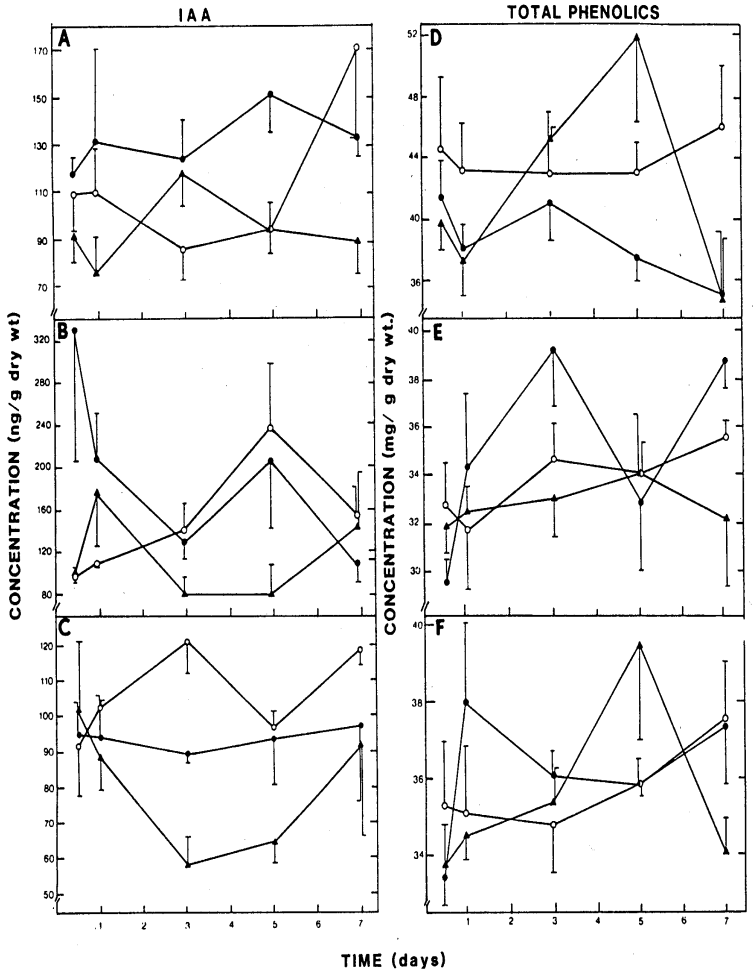

Figure 1. A) Effect of glyphosate and decapitation on free IAA levels in leafy spurge shoots; B) effect of glyphosate and decapitation on free IAA levels in leafy spurge hypocotylar regions; C) effect of glyphosate and decapitation on free IAA levels in leafy spurge roots; D) effect of glyphosate and decapitation on levels of total phenolics in leafy spurge shoots; E) effect of glyphosate and decapitation on levels of total phenolics in leafy spurge hypocotylar regions; F) effect of glyphosate and decapitation on levels of total plenolics in leafy spurge roots. ▲ -glyphosate treated, ○-decapitated, ●-control.

Conclusions

A lethal dose of glyphosate applied to intact, single stemmed leafy spurge plants produced a significant decrease in IAA concentrations in shoots, hypocotylar regions, and roots. Since this decrease did not resemble decapitation of the plants it serves as additional evidence that glyphosate in some way alters IAA metabolism. However, analysis

of phenolic compounds did not entirely support the previous reports of lowered phenolic compounds in glyphosate treated tissue. Only the hypocotylar region showed a significantly lower concentration of phenolic compounds. In summary, this experiment substantiates the hypothesis that glyphosate causes a reduction in endogenous levels of free IAA in whole plants but only partially supports a hypothesized decrease in total phenolics.

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