Naphthalic Anhydride Induces Imazethapyr Metabolism and Cytochrome P-450 Activity in Maize*

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Naphthalic Anhydride, Imazethapyr, Cytochrome P-450, Monooxygenase, Herbicide Metabolism, Imidazolinones

Naphthalic anhydride is a seed-applied herbicide safener which reduces the toxicity of imazethapyr, an imidazolinone herbicide, to maize (*Zea mays* L.). Protection of maize from imazethapyr was dependent on the amount of naphthalic anhydride applied to the seed. Metabolism of imazethapyr by maize roots and shoots was increased by exposure of the roots to a solution containing naphthalic anhydride. Increased imazethapyr metabolism due to naphthalic anhydride treatment of roots was observed within 4 h following safener exposure. Nitrogen, carbon monoxide and tetcyclacis inhibited imazethapyr metabolism in maize coleoptiles grown from naphthalic anhydride treated seed. This suggests that imazethapyr is metabolized by a cytochrome P-450 monooxygenase.

Naphthalic anhydride application to maize seed increased the level of cytochrome P-450 in the seedling shoots. Microsomes isolated from coleoptiles grown from naphthalic anhydride treated seed, but not untreated seed, converted bentazon to hydroxy-bentazon but did not metabolize imazethapyr. Protection of maize from imazethapyr damage by naphthalic anhydride is due to a safener-induced higher rate of imazethapyr metabolism associated with elevated cytochrome P-450 levels. However, this was not demonstrated *in vitro* for imazethapyr.

Introduction

Imazethapyr, (±)-2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1 H-imidazol-2-yl]-5-ethyl-3-pyridinecarboxylic acid, is an imidazolinone herbicide developed for weed control in soybeans. Imidazolinones are toxic to plants due to inhibition of the enzyme acetolactate synthase (ALS, EC 4.1.3.18) which catalyzes the production of the branched chain amino acids valine, leucine and isoleucine [1]. Selectivity for imidazolinones can be based on herbicide detoxification [2] or insensitive ALS [3] in tolerant plants.

Maize is sensitive to imazethapyr residues in soil [4]. However, treatment of maize seed with the herbicide safeners naphthalic anhydride, 1 H, 3 H-naphtho [1,8-*cd*]-pyran-1,3-dione, oxabetrinil, N-[1,3-dioxolan-2-yl-methoxy]-iminobenzeneacetonitrile, and flurazole, phenylmethyl-2-chloro-4-[trifluoromethyl]-5-thiazolecarboxylate, reduced maize injury from soil and foliar applied imazethapyr [5]. Naphthalic anhydride was also effective for reducing maize injury from imazaquin, 2-[4,5-dihydro-4-methyl-4-(1-methylethyl)-5-oxo-1 H-imidazol-2-yl]3-quinolinecarboxylic acid, another imidazolinone herbicide [6].

The primary mechanism of safener, particularly naphthalic anhydride, protection of maize from these imidazolinones, is an increased rate of conversion of the parent herbicide to metabolic products [5, 6]. In addition, radioactivity from root absorbed [^{14}C]imazethapyr is retained in the roots to a greater extent with naphthalic anhydride treatment [5]. This is likely a result of greater conversion of imazethapyr to less mobile metabolites with safener treatment.

This publication reports on further characterization of the imazethapyr metabolism response to naphthalic anhydride. The results support the hypothesis that naphthalic anhydride induces cytochrome P-450 monooxygenase activity responsible for imazethapyr metabolism.

Materials and Methods

The level of naphthalic anhydride needed to reduce maize (Pioneer 3369 A) injury from soil applied imazethapyr was studied by applying between 0 and 1% (w/w) safener to the seed. Seed was planted in soil and 0, 30 or 60 kg/ha imazetha-

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pyr was applied to the soil surface and watered into the soil. Weights of the maize shoots were determined after two weeks growth in a greenhouse.

The concentration of naphthalic anhydride in hydroponic solution needed to induce accelerated imazethapyr metabolism in maize was determined by placing the roots of one week old seedlings, grown from untreated seed in sand, into nutrient solution [7] containing 0 to 10 μM naphthalic anhydride. Seedlings remained in the naphthalic anhydride solution for 24 h after which they were transferred to a solution containing [^{14}C]imazethapyr (0.49 μCi or 18.1 kBq equivalent to 3.4 μM) for 24 h. Plants were extracted and imazethapyr quantified at the end of the 24 h period as previously described [5] except acetone replaced methanol in the extraction solution.

The time course of induction was determined by placing the roots of maize seedlings grown as above into a solution of 10 μM naphthalic anhydride for periods of 0 to 72 h. Plants were then transferred to a solution of [^{14}C]imazethapyr and the amount of imazethapyr metabolism over 24 h followed as above.

The effect of various inhibitors (CO, N_2 and 10 μM tetcyclacis) on *in vivo* metabolism of imazethapyr was studied by placing 1 g of 6 day old etiolated maize shoots grown from naphthalic anhydride treated (1% w/w) or untreated seed in 2 ml 25 mM MES (pH 6.2) containing 10 μM [^{14}C]imazethapyr (0.14 μCi or 5.3 kBq). Shoots were incubated in capped vials for 17 h and then imazethapyr and metabolites were extracted and quantified as described previously.

Cytochrome P-450 levels were determined in extracts of 6 day old etiolated maize shoots grown from naphthalic anhydride treated (1% w/w) and untreated seed. Shoots were homogenized in 2 × volume of homogenization buffer (0.1 M Na_3PO_4 [pH 7.5], 12 mM mercaptoethanol, 2 g/L PVP-40 and 0.25 M sucrose). The homogenate was filtered through cheesecloth and centrifuged 15 min at 10,000 × *g* and 20 min at 20,000 × *g* followed by centrifugation of the supernatant for 60 min at 100,000 × *g*. Pellets were resuspended to 2 mg protein/ml in 3 ml buffer (0.1 M Na_3PO_4 [pH 7.5], 300 g/L glycerol) for spectrophotometric assays. Cytochrome P-450 content was determined by the method of Omura and Sato [8] using an extinction coefficient of 91 cm^{-1} mm^{-1}.

In vitro metabolism of imazethapyr and bentazon, 3-(1-methylethyl)-(1 H)-2,1,3-benzothiadiazin-4-(3 H)-one 2,2-dioxide, was studied in microsomal suspensions isolated from 6 day old etiolated maize shoots grown from naphthalic anhydride treated (1% w/w) and untreated seed. Microsomes were isolated and bentazon metabolism quantified according to McFadden *et al.* [9]. [^{14}C]imazethapyr (0.025 μCi or 0.94 kBq) and unlabeled imazethapyr were included in the reaction mixture for a total concentration of 100 μM. Imazethapyr and metabolites were separated on an HPLC system equipped with an MCH-10 C-18 reversed phase column (Varian Instruments) and eluted at 1.0 ml/min with acetonitrile: 4% (v/v) acetic acid (40:60 v/v). Fractions were collected from the HPLC eluate and analyzed for associated radioactivity.

Results and Discussion

There was a dose response between the amount of naphthalic anhydride (NA) applied to maize seed and the level of protection from imazethapyr injury (Fig. 1). NA levels as low as 0.1% (w/w) overcame the injury. These levels are considerably lower than the 0.5% w/w rate of NA commonly used [10]. The higher NA rate may be needed to overcome inconsistent treatment at the farmer level. However, lower NA rates could overcome some of the phytotoxicity associated with NA [11, 12].

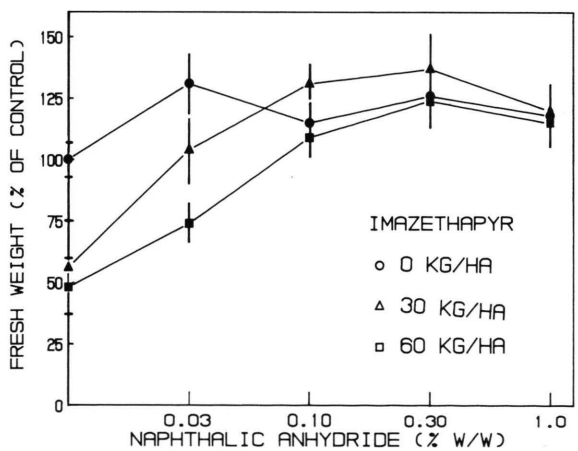

Fig. 1. Protection of maize from soil applied imazethapyr by seed treatment with naphthalic anhydride. Vertical bars represent the standard error of the individual means.

Concentrations of NA in the root bathing solution greater than 0.1 μM and 1.0 μM were effective for stimulating increased imazethapyr metabolism in roots and shoots, respectively (Fig. 2). Shoots metabolized more imazethapyr than roots without NA treatment. Earlier studies did not detect a large difference in imazethapyr metabolism between roots and shoots [5]. Maximum induction of imazethapyr metabolism by 10 μM NA occurred within 4 h of root tissue treatment (Fig. 3). However, there was a continued increase in imazethapyr metabolism in shoots with additional time of NA treatment. Both the higher NA concentration and time required by shoot tissue compared to roots for induction may simply reflect requirements for effective NA levels to be translocated from the roots to the shoots. Previous studies [5] demonstrated that the increase in imazethapyr metabolism due to NA treatment was greater in shoots (10 ×) than in roots (2 ×). This implies that shoots are as responsive as roots when exposed to NA through seed treatment. Sweetser [13] also found complete induction of chlorsulfuron, 2-chloro-N-[[(4-methoxy-6-methyl-1,3,5-triazin-2-yl)amino]carbonyl]benzenesulfonamide, metabolism within 4 h of treatment of maize shoots with 685 μM naphthalic acid. Further, it was shown cyclohexamide could block the induction, indicating that protein synthesis was required for increased chlorsulfuron metabolism.

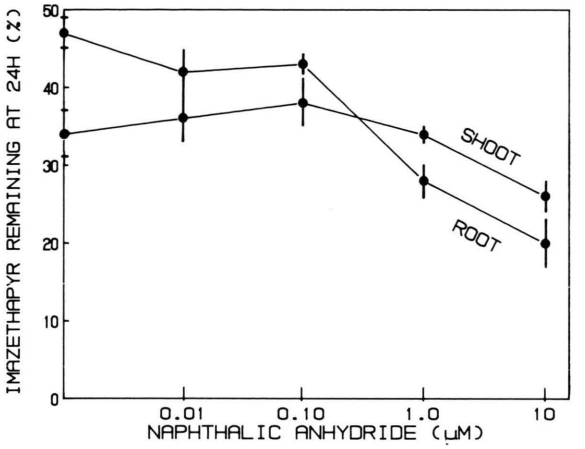

Fig. 2. Effect of root treatment with naphthalic anhydride concentrations for 24 h on imazethapyr metabolism in maize shoots and roots. Vertical bars represent the standard error of the individual means.

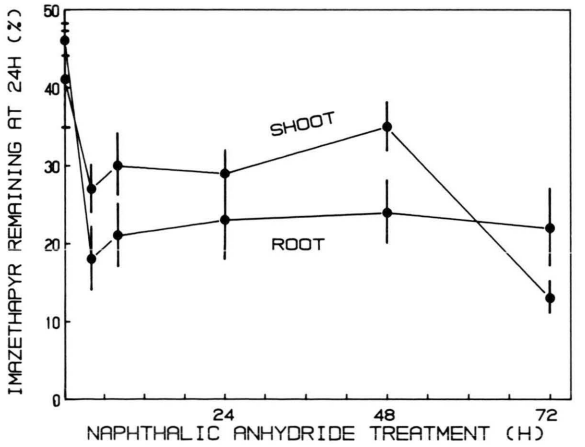

Fig. 3. Effect of time of root treatment with 10 μM naphthalic anhydride on imazethapyr metabolism in maize shoots and roots. Vertical bars represent the standard error of the individual means.

Nitrogen, carbon monoxide and tetcyclacis all reduced imazethapyr metabolism in shoots grown from NA-treated seed (Table I). While tetcyclacis was equally effective for reducing imazethapyr metabolism in shoots from safener treated and untreated seed, nitrogen and carbon monoxide were less inhibitory in the shoots from untreated seeds. This may reflect differences in the sensitivity of the enzyme systems to the gases in plants from NA-treated and untreated seed. Tetcyclacis and carbon monoxide are well known inhibitors of cytochrome P-450 monooxygenase activity in plants [9, 14–16]. Inhibition of imazethapyr metabolism by tetcyclacis in both shoots from treated und untreated seed suggests that both the constitutive and NA-induced imazethapyr metabolism is associated with cytochrome P-450 monooxygenase activity.

Etiolated maize shoots grown from NA-treated seeds contained more total cytochrome P-450 activity than shoots from untreated seeds. Cytochrome P-450 levels were 40.4 ± 12.7 and 26.1 ± 7.4 pmol P-450/mg protein for the treated and untreated shoots, respectively. This difference was significant by t-test. Although NA-treated shoots consistently contained more P-450 than untreated shoots, there was a large amount of variability in the total P-450 between separate groups of plants (extracted on separate days). Similar increases in P-450 levels were found due to treatment

Table I. Effect of nitrogen, carbon monoxide and tetcyclacis on *in vivo* imazethapyr metabolism in maize coleoptiles grown from naphthalic anhydride treated and untreated seed.

Inhibitor treatment	Naphthalic anhydride 0 Imazethapyr remaining* [% of total [^{14}C]recovered] ± standard error	Inhibition of metabolism [%]	Naphthalic anhydride 1% w.w. Imazethapyr remaining [% of total [^{14}C]recovered] ± standard error	Inhibition of metabolism [%]
None	21.5 ± 3.5	–	54.0 ± 12.7	–
N_2	19.0 ± 9.0	9	36.0 ± 8.5	33
CO	23.0 ± 0.0	0	34.0 ± 1.4	37
Tetcyclacis (10 μM)	9.5 ± 2.1	56	24.0 ± 15.5	56

* Percent imazethapyr of total [^{14}C]recovered in tissue 17 h after beginning incubation in [^{14}C]imazethapyr.

with the herbicide safeners cyometrinil, α-[(cyanomethoxy)imino] benzeneacetonitrile, and CGA 154281, 4-(dichloroacetyl)-3,4-dihydro-3-methyl-2H-1,4-benzoxazine, for wheat (*Triticum aestivum* L.) [17] and maize [14, 15], respectively. However, McFadden *et al.* [9] found no increase in total P-450 levels in maize due to seed treatment with NA.

Although total cytochrome P-450 levels were increased in the maize shoot tissue following NA treatment, attempts to demonstrate *in vitro* imazethapyr metabolism in microsomal preparations were unsuccessful. Preparations from NA-treated shoots, but not untreated shoots, were able to carry out NADPH dependent bentazon hydroxylation (138 ± 46 pmol/mg protein/h). McFadden *et al.* [9] also found hydroxybentazon formation in microsomes from NA-treated maize, but at a considerably higher level (930 pmol/mg protein/h).

Conclusion

NA decreases imazethapyr phytotoxicity to maize through an increased rate of herbicide metabolism in NA-treated plants. The indirect evidence suggests that both constitutive and NA-induced imazethapyr metabolism *in vivo* is associated with cytochrome P-450 monooxygenase activity. NA treatment increased total cytochrome P-450 levels in the maize shoots but *in vitro* imazethapyr metabolism in microsomal preparations was not demonstrated. Further characterization of imazethapyr metabolism and the NA induction will require development of an *in vitro* metabolism system for imazethapyr.

Acknowledgements

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[1] D. L. Shaner, P. C. Anderson, and M. A. Stidham, Plant Physiol. **76,** 545–546 (1984).
[2] D. L. Shaner and P. A. Robson, Weed Sci. **33,** 469–471 (1985).
[3] P. C. Anderson and M. Georgson, Abstracts VI Int. Cong. of Plant Tissue and Cell Culture, p. 437 (1986).
[4] K. A. Renner, W. F. Meggitt, and D. Penner, Weed Sci. **36,** 78–83 (1988).
[5] M. Barrett, Weed Sci. **37,** 296–301 (1989).
[6] M. Barrett, Weed Sci. **37,** 34–41 (1989).
[7] D. N. Maynard and A. V. Barker, Nutriculture: A guide to the soilless culture of plants, Publ. 41 Coop. Ext. Serv. Univ. Massachusetts 1970.
[8] T. Omura and R. Sato, J. Biol. Chem. **239,** 2370–2385 (1964).
[9] J. J. McFadden, J. W. Gronwald, and C. V. Eberlein, Biochem. Biophys. Res. Commun. **168,** 206–213 (1990).
[10] Anonymous, in: Herbicide Handbook of the Weed Science of America 5th Ed. (C. E. Beste, N. E. Humburg, H. M. Kemper, R. O. Radke, J. D. Riggleman, J. F. Stritzke, and G. R. Miller, eds.), p. 335, Champaign, Illinois 1983.
[11] E. Guneyli, Dissertation Abstracts **32B,** 1957–1958 (1971).
[12] J. S. Hickey and W. A. Krueger, Weed Sci. **22,** 86–90 (1974).
[13] P. B. Sweetser, Proc. 1985 Brit. Crop Prot. Conf.–Weeds, pp. 1147–1154 (1985).
[14] R. Fonné-Pfister and J. Gaudin, K. Kreuz, K. Ramsteiner, and E. Ebert, Pestic. Biochem. Physiol. **37,** 165–173 (1990).
[15] R. Fonné-Pfister and H. Kreuz, Phytochemistry **29,** 2793–2796 (1990).
[16] W. Rademacher, H. Fritsch, J. E. Grabe, H. Sauter, and J. Jung, Pestic. Sci. **21,** 241 (1987).
[17] C. Mougin, F. Cabanne, M. Canivenc, and R. Scalla, Plant Sci. **66,** 195–203 (1990).