ENHANCEMENT OF CYTOCHROME P-450 MEDIATED ARYL HYDROXYLATION OF BENTAZON IN RICE MICROSOMES

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Summary. Bentazon 6-hydroxylase (B6H) activity was determined in rice microsomes to study methods of enhancing cytochrome P-450 mediated aryl hydroxylation of bentazon by hydroxylase inducing compounds. Pretreating rice seeds with 1,8-naphthalic anhydride (0.5-2%) and fenclorim (8-12 uM) increased B6H activity. Treatments of rice seedlings with ethanol (2.5%) and phenobarbital (12 mM) enhanced B6H activity, also. Five-day-old rice seedlings showed higher B6H activity which decreased with seedling age.

INTRODUCTION

Cytochrome P-450 enzymes catalyze the oxygenation of many chemicals including herbicides. The most important metabolic reactions of herbicides that are mediated by cytochrome P-450 include hydroxylation and dealkylation.

Bentazon tolerance among crop species is due to detoxification of the herbicide via aryl hydroxylation and subsequent glycosyl conjugation (10). The aryl hydroxylation of bentazon that occurs in bentazon-tolerant species is thought to be catalyzed by a cytochrome P-450 monooxygenase. The NADPH dependence and the inhibitor sensitivity of the reaction suggested the involvement of a cytochrome P-450. McFadden *et al.*(9) suggested that in addition to its NADPH-dependence, the aryl hydroxylation of bentazon by corn microsomes is catalyzed by a cytochrome P-450 monooxygenase which requires oxygen and is strongly inhibited by pretreatment with carbon monoxide and tetcyclacis, a potent inhibitor of plant cytochrome P-450 enzymes.

Significant rates of bentazon hydroxylation has not been demonstrable in microsomal fractions from noninduced seedlings because of low levels of cytochrome P-450s in plants and the lability of this enzyme system during isolation (12). Gronwald (6) suggested that safeners confer crop protection by causing the induction of enzymes catalyzing herbicide detoxification. There is indirect evidence which suggests that pretreatment with the safener naphthalic anhydride increases the activity of monooxygenases catalyzing herbicide metabolism (2, 13). Microsomal fractions isolated from naphthalic anhydride-treated maize and sorghum shoots catalyzed the *in vitro* aryl hydroxylation of bentazon by a cytochrome P-450 (3,9).

Little work has been done concerning cytochrome P-450 responsible for aryl hydroxylation of bentazon in rice. Up to now, no demonstration of this type of reaction in an *in vitro* system of rice has been published.

We have tested several chemicals known to induce cytochrome P-450 monooxygenases in various crops to determine if they will induce B6H in rice.

METHODS

Rice (*Oryza sativa* L.) seeds were germinated and grown in rolled germination paper moistened with 1 mM Ca_2SO_4 solution for 6 days in the dark at 25°C.

1,8-naphthalic anhydride (NA) and fenclorim (CGA 123407; 4,6-dichloro-2-phenyl-pyrimidine) were applied directly as seed dressings. Ethanol and phenobarbital were applied by incubating 5-day-old seedlings in 0.5-liter Erlenmyer flasks in shaking water bath for 24 hr before shoot tissues were excised. B6H activity was also measured with microsomes from 4 - 14-day-old rice seedlings to test the influence of seedling age on B6H activity.

Microsome Isolation: Etiolated shoots of 6-day-old seedlings were excised and ground (using an ice-cold mortar and pestle) in 2 ml /g fresh weight of chilled 0.1 M sodium phosphate buffer (NaPi), pH 8, that contained 40 mM ascorbate, 14 mM 2-mercaptoethanol, and 10 mM EDTA. The homogenate was filtered through cheese cloth and centrifuged at 20,000*g* for 20 min. The supernatant was then centrifuged at 100,000*g* for 90 min. The microsomal pellet was resuspended in 0.1 M NaPi, pH 8.

Hydroxylase assay. Assays contained 0.1 M NaPi, pH 8, 1 mM NADPH, 1 mg microsomal protein, 25 uM ^{14}C-bentazon (13.9 uCi/umol) in 500 ul total volume and were conducted for 45 min at 30 C. Assays were initiated by addition of NADPH and terminated by addition of 50 ul 4N HCL, and 25 ul MeOH. The reaction was terminated by adding 75 ul of cold stop solution which was a mixture of 4N HCL and methanol (2:1).

Extraction and analysis. The terminated assays were extracted twice with 1 ml ethyl acetate; fractions were combined, dried under N_2 gas, and redissolved in 100% MeOH. Products were separated using HPLC (C_{18} column, 35% CH_3CHN/65% H_2O, 1 ml/min flow rate) and quantified with a radioactivity flow detector.

Each treatment was replicated three times and all experiments were conducted twice.

RESULTS AND DISCUSSION

Pretreatment of rice seeds with NA at 0.5 to 2.0% greatly increased microsomal B6H activity as compared to untreated seedling microsomes, in which the activity was barely detectable (Fig. 1). Pretreating rice seeds with NA at 2% caused a 19-fold increase in the *in vitro* activity of the P-450 catalyzing the aryl hydroxylation of bentazon. At a concentration of 0.5% (w/w), NA did not inhibit growth of rice seedling but higher concentrations greatly inhibited seedling growth. McFadden et al.(9) reported that there was a significant increase in metabolism of bentazon in naphthalic anhydride-treated corn tissue with only a small increase in total P-450 content. They suggested that naphthalic anhydride may act by increasing the level of a specific isozyme(s) in corn shoots responsible for bentazon metabolism and may also have other effects in vivo which serve to stabilize enzyme activity during isolation. Burton and Manness (3) suggested that NA treatment induces isozymes with a higher affinity for bentazon, as evidenced in the lower Km from NA-treated microsomal preparations. Alternatively, NA might stabilize or activate the constitutive P-450 during the extraction and preparation of microsomes.

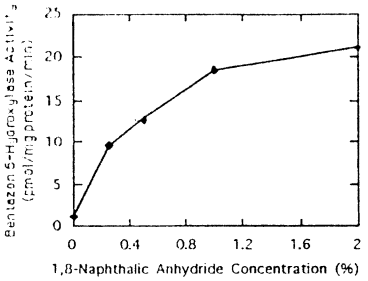

Fig. 1. Effect of 1,8-naphthalic anhydride on bentazon 6-hydroxylase activity in rice shoot microsomes.

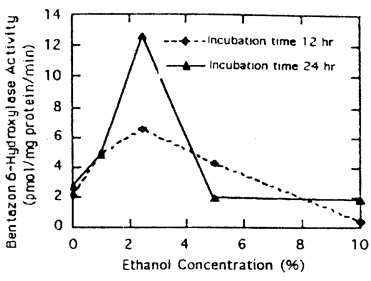

Fig. 2. Effect of ethanol on bentazon 6-hydroxylase activity in rice shoot microsomes.

As shown in Fig. 2, treatment of rice seedlings with ethanol 2.5% enhanced B6H activity (6.0-fold). Diclofop hydroxylase activity was increased 16-fold when wheat seedling tissues were treated with 10% ethanol (5). Hendry and Jones (8) also reported that 10% ethanol cause a 3-fold rise in cytochrome P-450 in intact mungbean.

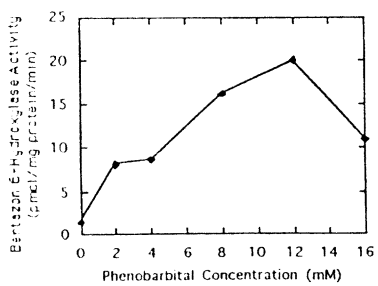

Fig. 3. Effect of phenobarbital on bentazon 6-hydroxylase activity in rice shoot microsomes.

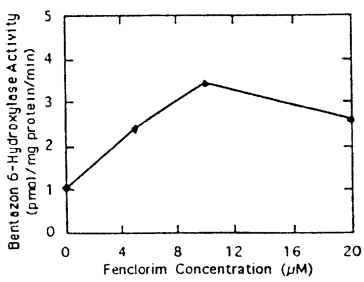

Fig. 4. Effect of fenclorim on bentazon 6-hydroxylase activity in rice shoot microsomes.

When rice seedlings were treated with phenobarbital at 12 mM, B6H activity was increased 13.1 times (Fig. 3). It is widely known that pretreating mammalian tissues with phenobarbital increases cytochrome P-450 levels and the rate of metabolism of selected xenobiotics because of the ability of phenobarbital to induce cytochrome P-450 isozymes (1, 11). Zimmerlin and Durst (14) reported that diclofop hydroxylase and cytochrome P-450 levels were increased 15.6- and 1.8-fold, respectively, when wheat seedlings were treated for 48 hr with 8 mM phenobarbital. Fonne-Pfister et al.(4) also reported monooxygenase induction in Jerusalem artichoke tissues treated with phenobarbital and clofibrate.

Induction of B6H activity by fenclorim at 8 to 12 uM was observed, but induction was not as effective as NA, ethanol, and phenobarbital (Fig. 4).

When microsomes were extracted from shoots of 4 to 14-day-old seedlings to test B6H activity at different seedling ages, B6H activity was highest in 5-day-old seedlings and then decreased as the age of the seedling tissues increased (Fig. 5). Hendry et al.(7) reported that constitutive cytochrome P-450 concentrations in mungbean microsomes decreased rapidly with age.

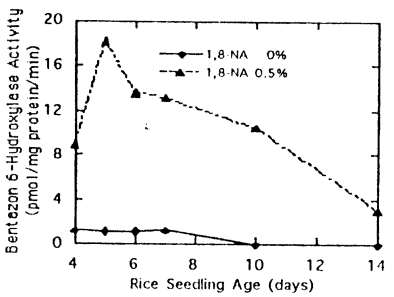

Fig. 5. Effect of seedling age on bentazon 6-hydroxylase activity in microsomes from 1,8-naphthalic anhydride-treated and untreated rice shoots.

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