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벼에서 Bentazon 히드록시화반응에 관련된 Cytochrome P-450 活性 增進에 관한 硏究*

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The Enhancement of Cytochrome P-450 Mediated Aryl Hydroxylation of Bentazon in Rice Microsomes*

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ABSTRACT

Bentazon 6-hydroxylase (B6H) and cinnamic acid 4-hydroxylase (CA4H) activities were determined in rice (*Oryza sativa* L.) microsomes to study methods of enhancing cytochrome P-450 mediated aryl hydroxylation of bentazon by hydoxylase inducing compounds. Pretreating rice seeds with 1,8-naphthalic anhydride at 0.5-2% and fenclorim at 5 and 10 μM increased B6H and CA4H activities. Treatments of rice seedling with ethanol 2.5% enhanced B6H and CA4H activities, and with phenobarbital at 12 mM enhanced B6H activity, and CA4H activity was enhanced at 2 mM. B6H activity was synergistically enhanced by combined treatments of ethanol 2.5 or 5% and phenobarbital 8 or 12mM and also that of 1,8-naphthalic anhydride 0.5 or 1% and phenobarbital 8 or 12 mM, but CA4H activity was decreased by combined treatment. Five-day-old rice seedlings showed higher B6H and CA4H activities which decreased with seedling age.

Key word : bentazon, cytochrome P-450, bentazon 6-hydroxylase, cinnamic acid 4-hydroxylase, rice

INTRODUCTION

Higher plants are frequently able to metabolize the xenobiotics that they absorb. 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 oxidative dealkylation in plants.

Cytochrome P-450 plays a major role in the detoxification of herbicides by plants and is important in regulating herbicide selectivity.[9] Aryl hydroxylation is an important method of herbicidal detoxification which is carried out by cytochrome P-450 dependent monooxygenases(E.C.1.14.14.1). Microsomal enzyme activity would be higher in more tolerant species or after treatment with saf-

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eners.[14] Bentazon tolerance among crop species is due to detoxification of the herbicide via aryl hydroxylation and subsequent glycosyl conjugation.[13] Indirect evidence suggests that the aryl hydroxylation of bentazon in corn shoots is thought to be catalyzed by a cytochrome P-450 monooxygenase.[12] The NADPH dependence and the inhibitor sensitivity of the reaction suggested the involvement of a cytochrome P-450. McFadden *et al.*[12] suggest 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 have 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.[18]

Cytochrome P-450 and associated oxygenase activity is inducible. Gronwald[8] suggests 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.[1,19] Microsomal fractions isolated from naphthalic anhydride-treated maize and sorghum shoots catalyzed the in vitro aryl hydroxylation of bentazon cytochrome P-450.[4,12] Treatments causing increasing cytochrome P-450 are varied : slicing or damaging tubers caused substantial increases in cytochrome P-450 concentration as did treatment of tubers with manganese and phenobarbital, a potent inducer of mammalian P-450.[16]

Cinnamic 4-hydroxylase(EC 1.14.13.11), a cytochrome P-450 monooxygenase found in only in plants, which catalyses hydroxylation of trans-cinnamic acid into p-hydroxy-cinnamic acid. CA4H catalyzes the first hydroxylation reaction in the phenylpropanoid pathway, leading to the biosynthesis of important compounds like lignins, tannins, anthocyanins, and several classes of phytoalexins. Involvement of a microsomal cytochrome P-450 in this hydroxylation has been demonstrated.[15,5] Cinnamic acid 4-hydroxylase was purified from microsomes of manganese-induced Jerusalem artichoke(*Helianthus tuberousus* L.) tuber tissues.[7]

Little work has been done concerning cytochrome P-450 responsible for aryl hydroxylation of bentazon in rice microsomal fractions. Up to now, no demonstration of this type of reaction in an *in vitro* system of rice has been published. Activity of microsomal bentazon 6-hydroxylase (B6H) in comparison with cinnamic 4-hydroxylase (CA4H) were determined if safeners and other chemicals would enhance aryl hydroxylation of bentazon in rice shoot microsomes.

MATERIALS AND METHODS

Rice seeds were germinated and grown on folded sheets of germination paper and placed upright in 2000ml plastic containers that contained 1mM Ca_2SO_4 solution for 6 days in the dark at 25 C.

1, 8-naphthalic anhydride at 0.25, 0.5, 1.0, and 2.0% and fenclorim (CGA 123407; 4,6-dichloro-2-phenyl-pyrimidine) at 5, 10, and 20 μM were applied directly as seed dressings. Ethanol at 2.5, 5.0, and 10.0%, and phenobarbital at 2, 4, 8, 12 and 16mM were applied by incubating 5-day-old rice seedlings in 0.5-liter Erlenmyer flasks in shaking water bath for 24 hr before shoot tissues were excised. B6H and CA4H activities were also measured with microsomes from 4 ~ 14-day-old rice seedlings to test the influence of seedling age on B6H and CA4H activities.

Microsome Isolation : Microsomal preparations

from treated and untreated rice shoots were used to study hydroxylation of bentazon and cinnamic acid *in vitro*. Etiolated shoots of 6-day-old seedling were excised and ground(using an ice-cold mortar and pestle) in 2ml/g fresh weight of chilled 0.1 M sodium phosphate buffer(NaPi), pH 8, that contained 40mM ascorbate, 14mM 2-mercaptoethanol, and 10mM EDTA. The homogenate was filtered through cheese cloth and centrifuged at 20,000g for 20 min. The supernatant was then centrifuged at 100,000g for 90min. The microsomal pellet was resuspended in 0.1M NaPi, pH 8.

Hydroxylase Assay : Microsomal protein was determined spectrophotomtically by the method of Bradford[3] with crystalline bovine serum albumin as a standard. Assays contained 0.1M NaPi, pH 8, 1mM NADPH, 1mg and 0.1mg microsomal protein for B6H and Ca4H, respectively, 25 μM 14C-bentazon(13.9 μCi/umol) or 25 μM 14C-cinnamic acid(50.8 μCi/umol) in 500 μl total volume and were conducted for 45min for B6H and 15min for CA4H at 30C. Assays were initiated by addition of NADPH and terminated by addition of 50 μl 4N HCL, and 25 μl MeOH. The reaction was terminated by adding 75 μl 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 1ml 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 radiocativity flow detector(Packard Flo-One/Beta A-500).

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

RESULTS AND DISCUSSION

Pretreatment of rice seeds with 1,8-naphthalic anhydride 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 1,8-naphthalic anhydride at 2% caused a 19 -fold increase in the *in vitro* activity of the cytochrome P-450 catalyzing the aryl hydroxylation of bentazon. Cinnamic acid 4-hydroxylase activity was also greatly increased at 1,8-naphthalic anhydride-treated rice seedlings with the increase of concentrations from 0.5 to 2%. At a concentration of 0.5%(w/w), 1,8-naphthalic anhydride did not inhibit growth of rice seedling but higher concentrations greatly inhibited seedling growth (Data not shown). McFadden et al.[12] reported that there was a significant increase in metabolism of bentazon in naphthalic anhydride-treated corn tissue with only a small increase in total cytochrome P-450 content. They suggested that naphthalic anhydride may act by increasing the level of 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[4] suggests that 1,8-naphthlic anhydride treatment induces isozymes with a higher affinity

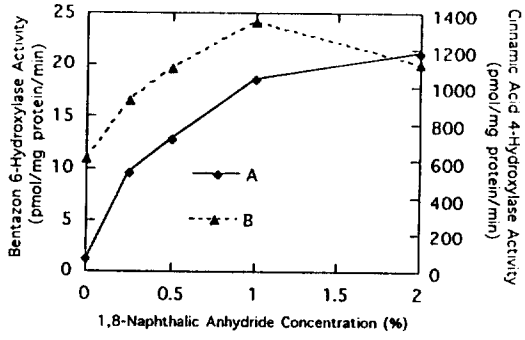

Fig. 1. Effect of 1,8-naphthalic anhydride on bentazon 6-hydroxylase (A) and cinnamic acid 4-hdroxylase (B) in rice shoot microsomes.

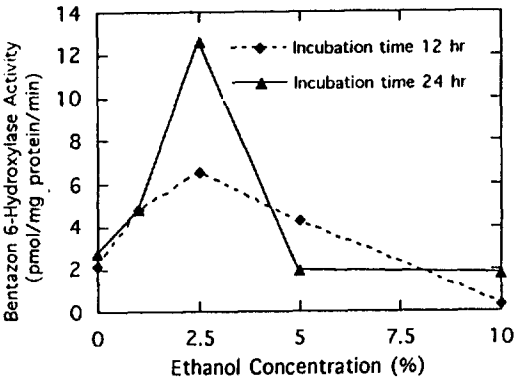

Fig. 2. Effect of ethanol on bentazon 6-hydroxylase activity in rice shoot microsomes.

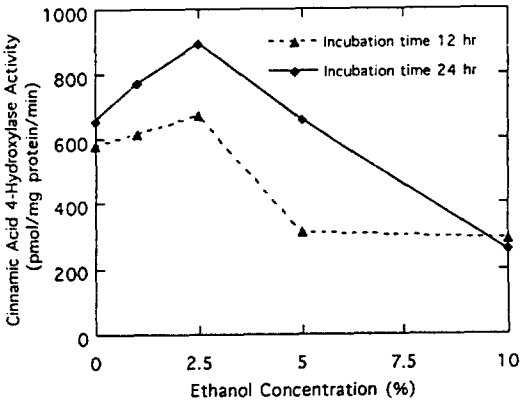

Fig. 3. Effect of ethanol on cinnamic acid 4-hydroxylase activity in rice shoot microsomes.

for bentazon, as evidenced in the lower Km from 1,8-naphthalic anhydride-treated microsomal preparations. Alternatively, 1,8-naphthalic anhydride might stabilize or activate the constitutive P-450 during the extraction and preparation of microsomes.

As shown in Fig. 2 and 3, treatment of rice seedling with ethanol 2.5% for 12 and 24 hr enhanced B6H(6.0-fold) and CA4H activities, and activities were higher at 24 hr incubation. However, CA4H activity was decreased at ethanol 5 and 10%. Previously it was found that various alcohols increased the microsomal hydroxylasing system from Jerusalem artichoke tissue.[16] Diclofop hydroxylase activity was increased 16-fold when wheat seedling tissues were treated with 10%

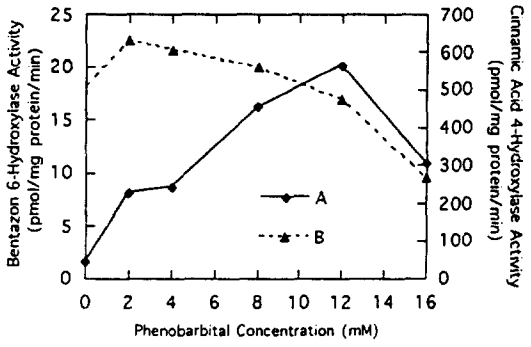

Fig. 4. Effect of phenobarbital on bentazon 6-hydroxylase (A) and cinnamic 4-hydroxylase (B) activities in rice shoot microsomes.

ethanol.[6] Hendry and Jones[11] also reported that 10% ethanol caused a 3-fold rise in cytochrome P-450 in intact mung bean.

When rice seedlings were treated with phenobaribital, a typical inducer of cytochrome P-450 enzyme in mammalian liver, at 12mM, B6H activity was increased 13.1 times(Fig. 4) and CA4H activity was highest at 2mM and decreased with the increase of concentrations. 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.[2,17] Zimmerlin and Durst[20] 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 8mM phenobarbital. Fonne -Pfister *et al.*[5] also reported monooxygenase induction in Jerusalem artichoke tissues treated with phenobarbital and clofibrate.

Induction of B6H activity by fenclorim at 8 to 12 μM was observed, but induction was not as effective as 1,8-NA, ethanol, and phenobarbital (Fig. 5). However, CA4H activity was greatly enhanced by fenclorim at 5, 10 and 20uM.

B6H activity was synergistically increased when rice seedlings treated with 1,8-naphthalic anhydride 0.5 or 1% were incubated at phenobarbital 8 or

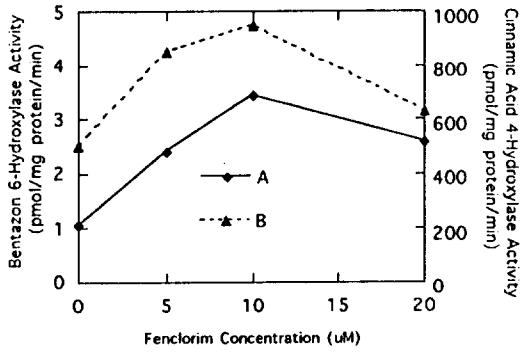

Fig. 5. Effect of fenclorim on bentazon 6-hydroxylase (A) and cinnamic acid 4-hydroxylase (B) activities in rice shoot microsomes.

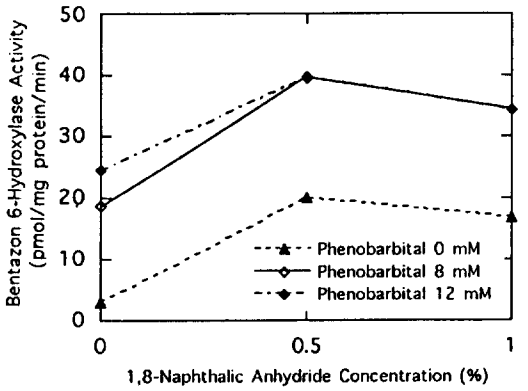

Fig. 6. Effect of 1,8-naphthalic anhydride plus phenobarbital on bentazon 6-hydroxylase activity in rice shoot microsomes.

12mM solution(Fig. 6), but CA4H activity was decreased by combined treatment(Date not shown). Coating of seeds with 1,8-NA and subsequently aging on phenobarbital strongly depressed CA4H activity.[21]

When rice seedlings were incubated at ethanol 2.5 or 5% + phenobarbital 8 or 12mM solution, B6H activity was synergistically enhanced(Fig. 7), but induction of CA4H activity was not observed by combined treatments of ethanol and phenobarbital(Date not shown). These results suggest that at least two different cytochrome P-450 monooxygenase are involved in the metabolism of bentazon. Zimmerlin *et al.*[21] suggest that in plants exposed to xenobiotics, some isozyme

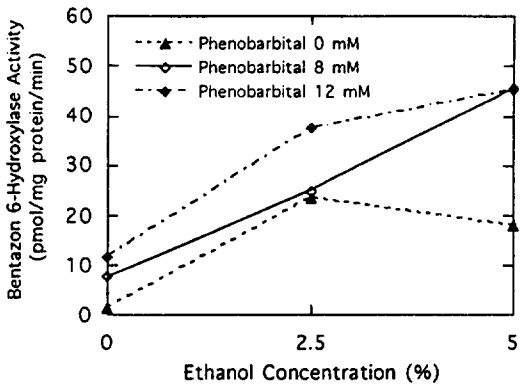

Fig. 7. Effect of ethanol plus phenobarbital on bentazon 6-hydroxylase activity in rice shoot microsomes.

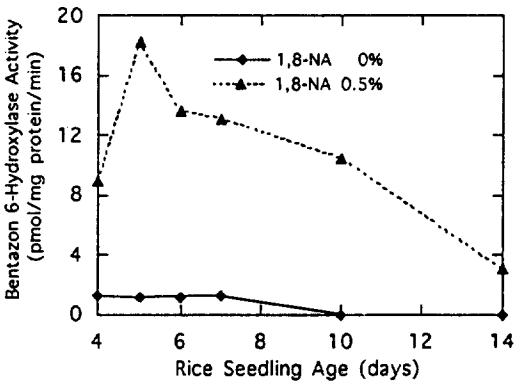

Fig. 8. Effect of seedling age on bentazon 6-hydroxylase activity in microsomes from 1,8-naphthalic anhydride-treated and untreated rice shoots.

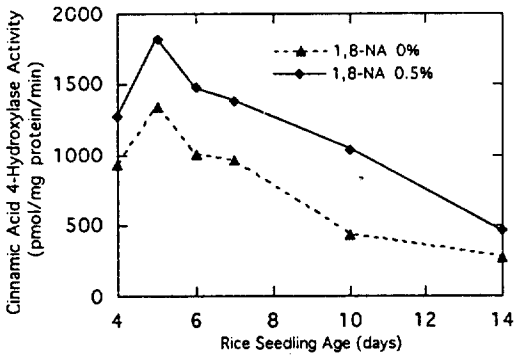

Fig. 9. Effect of seedling age on cinnamic acid 4-hydroxylase activity in microsomes from 1,8-naphthalic anhydride-treated and untreated rice shoots.

activities may be selectively stimulated, whereas others are unchanged or decreased.

When microsomes were extracted from shoots of 4 to 14-day-old rice seedlings to test B6H and CA4H activities at different seedling ages, B6H and CA4H activities were highest in 5-day -old seedlings and then decreased as the age of the seedling tissues increased(Fig. 8, 9). Hendry *et al.*[10] reported that constitutive cytochrome P-450 concentrations in mung bean microsomes decreased rapidly with age.

적 요

Bentazon이 첫 분해대사물질인 6-hydroxy bentazon으로 변화하는데 관련하는 cytochrome P-450 활성을 증진시키는 방법을 모색하기 위하여 벼 microsome에서 hydroxylase 효소 유기물질을 처리하여 bentazon 6-hydroxylase(B6H)와 식물에서 흔히 볼 수 있는 cinnamic acid 4-hydroxylase(CA4H) 효소를 대조하여 효소활성을 검정하였다.

1,8-naphthalic anhydride 0.5-2% 농도를 벼 종자에 분의처리하거나 fenclorim 5, 10 μM을 벼 종자에 침지처리함에 따라 B6H와 CA4H 효소 활성이 증대되었다. Ethanol 2.5%를 벼 유묘에 처리함으로써 B6H와 CA4H 활성이 증대되었으며, phenobarbital 12mM 처리에서 B6H 활성이 증대되었고 phenobarbital 2mM 처리에서는 CA4H 활성이 증대되었다. B6H 효소활성은 ethanol 2.5, 5%와 phenobarbital 8, 12mM 혼합처리 또는 1,8-naphthalic anhydride 0.5, 1%와 phenobarbital 8, 12mM 혼합처리에서 상승적으로 증가하였으며, CA4H 효소는 혼합처리에 의하여 활성이 저하되었다. 한편 벼 5일묘에서는 B6H와 CA4H 활성이 가장 높았으며 묘령이 진전될수록 효소활성은 현저히 감소되는 경향을 나타냈다.

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