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Glyphosate Is an Inhibitor of Plant Cytochrome P450: Functional Expression of *Thlaspi arvensae* Cytochrome P45071B1/Reductase Fusion Protein in *Escherichia coli*

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Glyphosate (Roundup) is an herbicide used extensively worldwide which acts as an inhibitor of 5′*enol*-pyruvylshikimate-3-phosphate synthase and for which transgenic herbicide resistant plants have been developed. Here we report for the first time that glyphosate is an inhibitor of cytochrome P450 using a functional expression system for *Thlaspi arvensae* CYP71B1 in *Escherichia coli*. CYP71B1 was fused to the soluble domain of a plant cytochrome P450 reductase (CPR) from *Catharanthus roseus*. CYP71B1 could obtain reducing equivalents in this fusion construct and metabolised the polycyclic aromatic hydrocarbon, benzo(a)pyrene. The fusion protein retained normal spectral characteristics having a Soret peak at 448 nm in the reduced carbon monoxide difference spectrum. Addition of the herbicide resulted in a Type II spectrum indicative of binding via the nitrogen group to haem as a sixth ligand. A K_s of 60 μM was observed and an IC_{50} of 12 μM was observed for glyphosate inhibition of CYP71B1 activity. The implications of these results are discussed.

Cytochrome P450 enzymes represent an extensive superfamily undertaking an unprecedented array of endogenous reactions in primary and secondary metabolism as well as chemical detoxification functions of importance for determining sensitivity of plants to herbicides (1). Individual plants contain numerous CYP proteins belonging to different families and the function of many proteins revealed in genome projects and by PCR techniques remain to be determined (2). One such protein is CYP71B1 from *Thlaspi arvensae* which was cloned using redundant primers corresponding to CYP conserved domains (3).

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The role of CYP71 enzymes remains unclear, but activity in geraniol epoxidation has been observed for CYP71A1 (4). Heterologous expression represents an obvious route towards characterisation of the spectrum of individual CYP activities and a productive system for this is *Escherichia coli*. General routes to successful expression have been determined previously which include alteration of the N-terminus to that used for CYP17 (5). *E. coli* does not possess an effective electron donor system to support CYP activity and addition of appropriate purified NADPH cytochrome P450 reductase (CPR) is essential to reconstitute normal activity. An alternative approach has involved coexpression of CPR [6] or fusion of the soluble domain (lacking the N-terminal membrane anchor) to the C-terminus of CYP [7].

We have been interested to develop heterologous expression systems to investigate plant cytochrome P450 (CYP) activity in endogenous and xenobiotic metabolism as well as for biophysical studies. Here we present the successful expression of a fusion protein of CYP71B1 to CPR from *Catharanthus roseus*. The protein is demonstrated to be active for xenobiotic metabolism. In the course of these investigations we discovered for the first time that glyphosate (figure 1) is an inhibitor of cytochrome P450, a phenomenon which may have implications and application to future herbicide development.

2. MATERIALS AND METHODS

2.1 Construction of expression vectors. Plasmid pSP19g10L, which is derived from pSPORT, was used for the expression of the *CYP71B1* and plant P450 reductase. This plasmid contains the inducible *tac* promoter [8]. The yeast expression plasmid Th_i was used as the source of the cDNA for CYP71B1 and a plant λ library was used as DNA source for isolation of plant P450 reductase. All manipulations were checked by diagnostic restriction analysis and the successful construct (CYP71B1:CPR) was sequenced to confirm the integrity of the clone, using T7 polymerase (Sequenase, USB). Using the polymerase chain reaction (PCR) the N-terminus of CYP71B1

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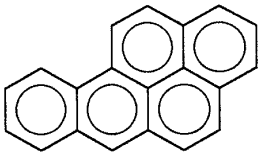
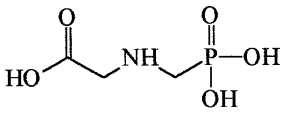


Benzo(a)pyrene



Glyphosate

FIG. 1. The chemical structures of (a) glyphosate and (b) benzo(a)-pyrene.

was modified to encode the codons representing the initial eight amino acids of CYP17A. Modifications to create CYP71B1 were introduced using the following 5′ PCR primer: 5′-GCGCATATGGCTGCT-AAGAAAAGCTCTAAAGCT-3′. Mutagenesis was performed to modify the coding sequence for the carboxyl terminus of CYP71B1 and the coding sequence for the amino terminus of plant NADPH-cytochrome P450 [9] reductase in accordance with previous studies in order to allow fusion of these sequences with one encoding a dipeptide linker, Ser-Thr. Plant CYP71B1 was amplified by PCR using a 3′ primer deleting the TGA stop codon and incorporating a *Sal* I site encoding Ser-Thr: 5′-AGATCTGATCAGTCTTGCAATA GCTGCG-3′. Plant NADPH cytochrome P450 reductase was amplified by PCR using primers deleting the amino terminal membrane-anchoring region and incorporating a *Sal* I site encoding Ser-Thr as a linker; 5′ primer, 5′-AGACCTG GTACGTGCAATGAGGTCCCATGC-3′ and 3′ primer, 5′AACTGTGCCTGA TGCTAACCGTTGCAGGTC-3′. The 3′ reductase primer incorporated a *Bam* HI site after the TAG stop codon. The CYP71B1 PCR fragment was digested with *Nde* I/*Sal* I and the plant NADPH cytochrome P450 reductase PCR fragment was digested with *Sal* I/*Bam* HI. Both fragments were gel isolated and ligated into pSP19g10L previously digested with *Nde* I/*Bam* HI (figure 1a) using T4 DNA Ligase (Promega). Positive colonies were identified by restriction digestion analysis.

2.2 Expression of CYP71B1:CPR fusion protein. Expression conditions were a modification of those described elsewhere for other P450 proteins [5]. DH5α cells were transformed with CYP71B1:CPR and transformants isolated on LB agar plates containing 100μg/ml ampicillin. A single transformed colony was inoculated into 5 ml LB medium with ampicillin (100μg/ml) and grown overnight at 37°C. Then, 0.5 l of Terrific Broth modified and supplemented with ampicillin (100 mg/litre) as described [10] and contained in a 2 litre flask was inoculated with 1/10 dilution of the overnight culture and the culture was incubated at 37°C, 240 rpm, until A_{600} reached 0.4, then δ-aminoevulinic acid (ALA) was added to a final concentration of 80 mg/l. Following an incubation for 15 min at 25°C with shaking at 200 rpm, the expression of the fusion protein was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were harvested 48h later by centrifugation at 5000 $\times$ *g* for 10 min at 4°C.

2.3 Membrane fractionation. E. coli suspensions from 500 ml culture were resuspended in 25 ml 10 mM potassium phosphate buffer, pH 7.4, containing 1 mM EDTA and 20 % (v/v) glycerol. Lysozyme (0.5 mg/ml) was added and the suspension left on ice for 30 min. Phenyl methyl sulphonyl fluoride (PMSF) was added to a final concentration of 1 mM and the mixture was sonicated on ice with a Soniprep cell disruptor using six 20-s pulses with 20-s intervals at 80 % of the maximum power. Cell debris was removed by centrifugation at 5000 $\times$ *g* for 10 min and the membranes pelleted by centrifugation at 100000 $\times$ *g* for 60 min. The pellet was resuspended in 2 ml 10 mM potassium phosphate buffer, pH 7.4 and stored at −80°C until use.

2.4 Spectral studies. Light absorption spectra were measured using a Phillips PU8800/02 scanning spectrophotometer. For estimation of P450 concentration, membranes were diluted in 10 mM potassium phosphate buffer, pH 7.4, and the CO reduced difference spectra according to Omura and Sato, [11], using an extinction coefficient of 91 mM^{-1} cm^{-1}. Binding spectra with glyphosate were recording according to established procedures [12]. Briefly membranes were diluted to a P450 concentration of 0.2 nmol/ ml and placed in both sample and reference cuvettes (1 cm path length). Following the recording of a baseline, glyphosate, dissolved in water was added direct to the sample cuvette, the contents mixed and the spectrum was recorded between 500 and 350 nm. By adding glyphosate, the change in absorbance between the type II peak (420 nm) and the corresponding trough (390 nm) was related to the concentration of glyphosate added. Data were analysed by linear regression of substrate concentration vs the ratio of the substrate concentration to the proportion of the maximal spectral shift from 420 to 390 nm.

2.5 Reconstitution of catalytic activity. Bacterial membrane fractions containing 500 pmol of CYP71B1 fusion protein were incubated with varying concentrations of benzo(a)pyrene (10 mg/ml) in 50 mM potassium phosphate buffer, pH 7.4. NADPH was added to a final concentration of 1 mM, in a total volume of 1 ml, to start the reaction, and reactions were incubated at 30°C with shaking for 20 min. Following incubation, reactions were stopped by the addition of 1 ml ice-cold acetone, 3.25 ml of hexane were added and the samples were incubated with shaking at 37°C for 10 min. A 1 ml sample of the organic phase was extracted with 3 ml of 1N NaOH. Samples were then measured for fluorescence using a Perkin Elmer 3000 fluorescence spectrophotometer. Benzo(a)pyrene hydroxylase activity was measured according to the method of Nebert and Gelboin, [13].

2.6 Glyphosate inhibition studies. CYP71B1:CPR activity was assayed as described above using a concentration of benzo(a)pyrene at 25μM. Glyphosate was added to the reaction mixtures at varying concentrations for assessment of its inhibitory activity towards CYP71B1.

3. RESULTS AND DISCUSSION

CYP71B1:CPR was constructed as shown in figure 2a and expressed in *E. coli* as a modified protein where the native N-terminal sequence was replaced with that employed for the heterologous expression of CYP17A in *E. coli* [5], thus incorporating codons that facilitate expression in *E. coli*. CYP71B1:CPR levels were low (29 nmol/l) compared with those found for other animal CYP/CPR fusion proteins such as CYP3A4 (200 nmol/ l, [144]) and CYP4A1 (700 nmol/l, [15]). Supplementation of the growth medium with the haem precursor δ-aminoeluvinic acid increased the expression levels approximately twofold. Typical spectra for bacterial membranes containing CYP71B1:CPR and transformants containing the control plasmid pSP19g10L are shown in figure 2b. The CPR activity of the fusion protein was calculated to be 650 nmol cytochrome *c* reduced/min per mg membrane protein reflecting an equivalence of CYP and CPR levels.

The enzymatic activity of CYP71B1 fused with its electron donor, cytochrome P450 reductase, was analysed using benzo(a)pyrene as a substrate. No substrate interaction with geraniol was observed as occurred for CYP71A1 and the enzyme was not geraniol 10-hydroxylase as had been thought possible. However metabolism of the polycyclic aromatic hydrocarbon benzo(a)pyrene was observed. A K_M of 23 μM was calcu-

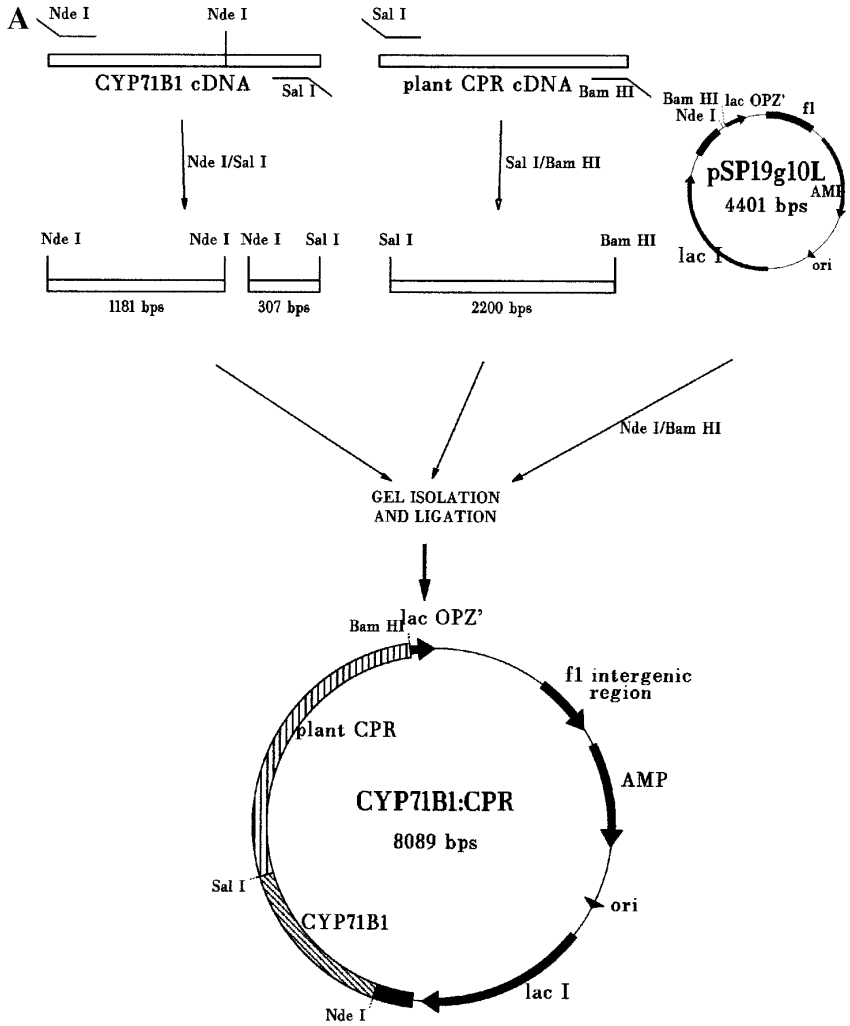


FIG. 2. (a) Construction of the expression construct for the fusion protein of CYP71B with CPR. Both coding sequences were amplified by PCR to contain the desired alterations to the N-terminus of CYP71B1 and for the production of a N-terminal truncated CPR. The fusion was cloned behind the *tac* promotor of pSP19g10L. (b) Reduced carbon monoxide difference spectrum of CYP71B1:CPR expressed in membrane fractions of *E. coli*. (—) denotes the spectrum for CYP71B1:CPR and (- - -) denotes the spectrum for control membranes from transformants containing pSP19g10L alone.

lated and a V_{MAX} of 0.56 nmol product formed/min/nmol CYP71B1:CPR. Benzo(a)pyrene metabolism was not detected in membranes prepared from *E. coli* transformed with the control plasmid pSP19g10L alone.

Addition of glyphosate to *E. coli* membranes expressing CYP71B1:CPR gave rise to a typical type II difference spectrum (figure 3a). Type II spectra are indicative of an interaction of a free electron pair located within an atom of the binding molecule (usually nitrogen) with the iron atom located in the centre of the haem in the P450 molecule. The type II spectrum obtained with glyphosate was characterised by a peak at 420 nm and a trough at 390 nm. The spectral dissociation constant measured by titration of CYP71B1:CPR with ligand indicated that glyphosate had reasonable affinity for the enzyme ($K_S = 60\mu M$). The mode of binding is via the nitrogen of glyphosate the same as that of azole fungicides and drugs where they also bind as a sixth ligand via an azole moiety. Specificity of azole fungicide inhibition is determined by interaction with of the N-1 substituent group to the CYP apoprotein and similar structure/activity studies could be addressed using analogues of glyphosate.

The catalytic activity of CYP71B1:CPR in a reconstituted system containing bacterial membranes expressing the fusion protein was assayed by measuring the conversion of benzo(a)pyrene to 3-hydroxybenzo(a)pyrene. An IC_{50} of 12μM was observed. Figure 3b shows the results of an experiment in which 0.5 nmol of CYP71B1:CPR was titrated with increasing amounts of glyphosate. The complete inhibition of enzyme activity occurred at a concentration equal to 30μM, indicating

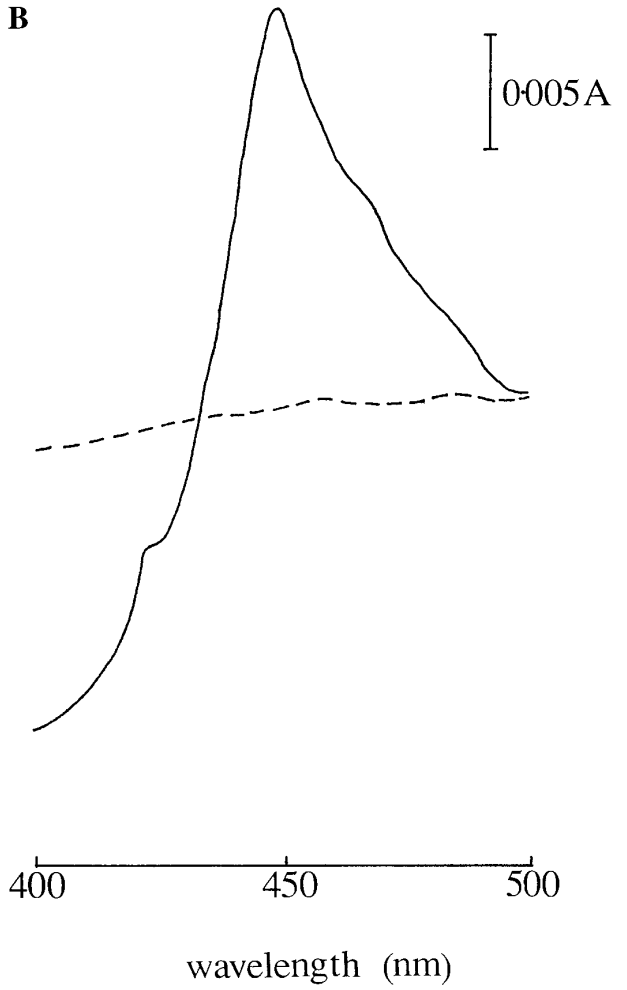


FIG. 2—*Continued*

that a 60-fold excess of glyphosate to CYP71B1:CPR was required to abolish enzymatic activity.

Glyphosate is used extensively as a herbicide, but to our knowledge, no information on interaction with CYP enzymes has been reported. The molecular structure and Type II spectra observed on binding of glyphosate to CYP71B1:CPR show the single nitrogen of the molecule binds to haem as a sixth ligand. The activity of the molecule as an inhibitor of CYP enzymes could be significant, especially if other plant CYP enzymes have significantly higher affinity and are involved in important endogenous or xenobiotic metabolism. We have also examined CYP71B1 expression in yeast and shown this enzyme has activity in chlortoluron metabolism [16] and together with the benzo(a)pyrene metabolism presented here suggests a general detoxification role for CYP71s which should extend to crop species.

The observation of glyphosate as an inhibitor might be useful for future development of CYP inhibitors which are very important as fungicides (inhibitors of CYP51), potential herbicides (inhibitors of plant CYP51), plant growth regulators (inhibitors of giberellin biosynthesis) [17]. Compounds might be used as adjuncts inhibiting herbicide detoxification by CYP enzymes while still acting on it's own molecular target, 5′*enol*pyruvylshikamate-3-phosphate synthase or single compounds may be developed with dual modes action. Some reports of glyphosate resistant weeds [18, 19] have appeared and CYPs could be glyphosate-binding proteins altering sensitivity to the herbicide in weeds or transgenic plants.

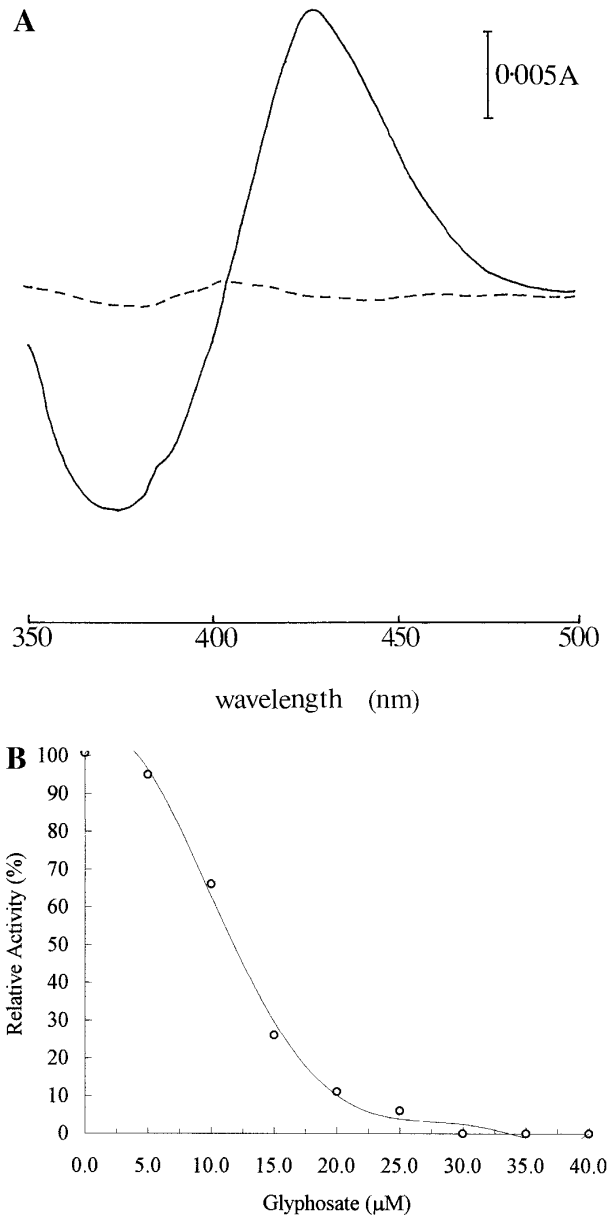


FIG. 3. (a) Type II binding spectrum on addition of glyphosate to membrane fractions of *E. coli* expressing CYP71B1:CPR (—). (- - -) denotes the result obtained using control membranes from *E. coli* transformants containing pSP19g10L alone. The Type II spectrum indicates coordination of glyphosate to haem as a sixth ligand via the nitrogen atom. (b) Inhibition by varying concentrations of glyphosate of CYP71B1:CPR mediated metabolism of benzo(a)pyrene.

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REFERENCES

1. Mougin, C., Cabanne, F., Canivenc, M-C., and Scalla, R. (1990) *Plant Science* **66,** 195–203.
2. Durst, F. (1991) *in* Frontiers in Biotransformation (Ruckpaul, K., and Rein, H., Eds.) Vol. 4, pp. 191–232.
3. Udvardi, M. K., Metzger, J. D., Krishnapillai, V., Peacock, W. J., and Dennis, J. N. (1994) *Plant Physiol.* **105,** 755–756
4. Hallahan, D. L., Lau, S-M. C., Harder, P. A., Smiley, D. W. M., Dawson, G. W., Pickett, J. A., Christoffersen, R. E., and O'Keefe, D. P. (1991) *Biochem. Biophys. Acta* **272,** 115–124.
5. Barnes, H. J., Arlotto, M. P., and Waterman, M. R. (1991) *Proc. Natl. Acad. Sci.* **88,** 5597–5601.
6. Blake, J. A. R., Pritchard, M., Ding, S., Smith, G. C. M., Burchell, B., Wolf, C. R., and Friedberg, T. (1996) *FEBS Lett.* **397,** 210–214.
7. Murakami, H., Yabusaki, Y., Sakaki, T., Shibata, M., and Ohkawa, H. (1987) *DNA* **6,** 189–197.
8. Bak, S., Kahn, R. H., Olsen, C-E., and Halkier, B. A. (1997) *Plant J.*, in press.
9. Meijer, A. H., Cardoso, M. I. L., Voskuilen, J. T., De Waal, A., Verpoorte, R., and Hogge, J. H. C. (1993) *Plant J.* **4,** 47–60.
10. Gillam, E. M. J., Baba, T., Kim, B-R., Ohmori, S., and Guengerich, F. P. B (1993) *Arch. Biochem. Biophys.* **305,** 123–131.
11. Omura, T., and Sato, R. (1964) *J. Biol. Chem.* **239,** 2370–2378.
12. Jefcoate, C. R. (1978) *in* Methods in Enzymology (Fleisher, S., and Parker, L., Eds.), Vol. 52, pp. 258–279.
13. Nebert, D. W., and Gelboin, H. V. (1968) *J. Biol. Chem.* **243,** 6242–6249.
14. Shet, S. S., Fisher, C. W., Holmans, P. L., and Estabrook, R. W. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 11748–11752.
15. Fisher, C. W., Shet, M. S., Caudle, D. L., Martin-Wixtrom, C. A., and Estabrook, R. W. (1992) *Proc. Natl. Acad. Sci.* **89,** 10817–10821.
16. Kelly, D. E., Hanley, S. Z., Mehmood, Z., Hallahan, D., Udvardi, M., and Kelly, S. L. (1995) *in* Third International Symposium on Cytochrome P450 Biodiversity, Woods Hole, p. II.16.
17. Buchenauer, H. (1995) *in* Modern Selective Fungicides (Lyr, H., Ed.), Vol. 2, pp. 259–290.
18. Racchi, M. L., Rebecchi, M., Todesco, G., Nielsen, E., and Forlani, G. (1995) *Euphytica* **82,** 165–173.
19. Escorial, M. C., Sixto, H., Garcia-Baudin, J. M., and Chueca, M. C. (1996) *Plant Cell. Tiss. Organ Cult.* **46,** 179–186.