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# INVOLVEMENT OF HEMOPROTEINS FROM BLACK LOCUST ROOTS IN PHYTOREMEDIATION

Kenneth J. Zanon

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By

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## ABSTRACT

## INVOLVEMENT OF HEMOPROTEINS FROM BLACK LOCUST ROOTS IN PHYTOREMEDIATION

By

Kenneth J. Zanon

The ability of black locust (*Robinia pseudoacacia*) to survive in contaminated soil may be attributed to enzymes such as cytochrome P450 and peroxidase, which are capable of metabolizing contaminants. These enzymes were produced by the roots of black locust seedlings. Cytochrome P450 was found in its P420 state using carbon monoxide difference spectrometry. Peroxidases from the roots of black locust seedlings catalyzed the oxidation of both hydroquinone and 4-chloro-1-naphthol, and may be involved in phytoremediation. The role of cytochromes P450 in black locust has not been characterized, and more work is needed to determine their function and possible participation in phytoremediation.

## DEDICATION

This work is dedicated to my wife Rachel, my parents Ken and Deb and my sister Lisa. Thank you for always being there for me.

## ACKNOWLEDGMENTS

As I was writing this section I realized there are too many people who I need to thank. I would like to thank all of those professors and staff in the Chemistry Department at NMU. You teach, inspire and actually care about the education a student in your classes receives. I truly do not know how all of you find the time to accomplish all that you do. I would especially like to than those on my Graduate Chair Committee for helping me in making my thesis what it is today. Thanks to Pauline Gould and Dave Erickson, they have always been there for all of the students, if we needed something they would help. Most of all, I would have to thank Dr. Lesley Putman, for guiding my research and thesis, traveling with me to a P450 symposium at Purdue, and for driving almost 500 miles to my wedding. Also, thanks to Jen Lyons for her help in the lab.

I would also like to thank all my family members for your support throughout the years, especially those who I dedicated this thesis to. I would also like to thank Roger Zanon, who was always there for support, to add great insight into overcoming challenges and for all the great times. There are also several other friends and family who I would like to thank, and they know who they are. I feel blessed knowing I am surrounded by such wonderful people.

This thesis follows the format prescribed by the ACS Style Guide and the Department of Chemistry.

## PREFACE

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| BSA | Bovine Serum Albumin |
| CO | Carbon Monoxide |
| DEET | N,N-diethyl-3-methylbenzamide |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediamine Tetraacetic Acid |
| EGTA | [Ethylenebis(oxyethylenenitrilo)]tetraacetic acid |
| ER | Endoplasmic Reticulum |
| EST | Expression Sequence Tag |
| kDA | kilo Daltons |
| 3MC | 3-methylcholanthrene |
| mRNA | messenger Ribonucleic Acid |
| Monuron | 3-(4-chlorophenyl)-1, 1-dimethylurea |
| PCR | Polymerase Chain Reaction |
| PMSF | Phenylmethylsulfonyl fluoride |
| PVPP | Polyvinylpolypyrolidine |
| RT-PCR | Reverse Transcriptase Polymerase Chain Reaction |
| TCE | Trichloroethylen |

# INTRODUCTION

Throughout the years many organic chemicals have been used in the manufacturing industry to produce useful, everyday products and in agriculture as insecticides and herbicides to protect crops. Though these chemicals are very useful some have been found to contaminate soil and water. Many of these compounds have also been found to be harmful to either the environment or mammals, or both, and need to be removed. Current processes for the remediation of these chemicals are either not viable, not efficient or not complete. Therefore research into the use of plants for either the uptake, degradation or simply the immobilization of these harmful contaminants is gaining momentum, and has been named phytoremediation. The ideal route of contaminant removal by phytoremediation would be phytodegradation.

In phytodegradation enzymes produced by the plants metabolize these harmful chemicals. Cytochromes P450 and peroxidases are two large enzyme families found in plants that are known to oxidize a large amount and variety of organic compounds. Being found in plants and known to oxidize many organic compounds, these two enzyme families have great potential to be involved in phytodegradation and are of importance in phytoremediation research.

In this research, black locust, a tree known to participate in phytoremediation, will be studied to determine if cytochromes P450 and peroxidase are present and involved in the phytoremediation process.

*Cytochrome P450*

Cytochrome P450 (P450) has been a mysterious enzyme since its discovery in 1958 by Klingenberg (*1*) in liver microsomes of rats. Since then P450s have been found in all eukaryotic organisms, animals, plants and fungi. In addition some prokaryotic organisms produce an isoform of this enzyme (*2*). In fact, much research performed has been on the soluble bacterial P450, P450cam, produced by *Pseudomonas putida*, first purified in 1970 (*3*). The research performed on the P450 enzyme superfamily is enormous in itself, generating thousands of papers each year (*4*).

P450s make up an enormous superfamily of hemoproteins that outrank any other family of enzymes by the shear "number of substrates recognized, the number of reactions catalyzed and the number of inducers" (*4*). Recently the name "diversozyme" has been proposed for P450 due to the variation of reactions and substrates being so diverse and numerous (*4*, *5*). All P450s appear to have evolved from a single ancestor, and were originally identified by a carbon monoxide difference spectrum, where solutions in both sample and reference cuvettes are reduced, then the sample is bubbled with CO, and a discernable peak at 450 nm is observed. However, this characteristic alone will not classify a protein as a P450. The P450 Nomenclature Committee only "accepts as P450 those proteins that can be satisfactorily aligned with and show discernible homology to, other P450 sequences" (*4*). An example from Kahn and Durst (*4*) is the nitric oxide synthase. This enzyme is a hemoprotein oxygenase that displays the CO difference spectrum as other P450s, however the phylogenetic criteria is not met and this enzyme is therefore excluded.

In the 1980's there was an increasing amount of P450s discovered, this made naming them based on their function confusing (*2*), since more than one isozyme may have the same function. Therefore a scheme was proposed for giving systematic names based on amino acid sequence. P450 genes with at least 40% amino acid positional identity are grouped in a family, and 55% identity for placement in a subfamily. This phylogenetic make up is then used to name the protein. For example, CYP2C5 is the fifth member of subfamily C of family 2 (*4*). The majority of the 2500 P450 genes cloned to date (*6*) have not had their function determined, so classification by phylogenetics is very useful.

Four main molecular procedures exist for cloning and isolation of P450 genes (*4*). The first is the polymerase chain reaction (PCR). This procedure works best when isolation of a specific P450 gene is not as much of a priority as finding any P450 gene. Isolation is complicated with this procedure, but still is possible. The second is the reverse transcriptase-polymerase chain reaction (RT-PCR). This procedure is used to reverse transcribe mRNA and then amplify the product using PCR. The third is differential display of mRNA (DD-RT-PCR) (*4*). Differentially expressed genes may be identified by this procedure (*7*). The fourth is the use of the expressed sequence tags (EST) databases for the identity and isolation of specific genes (*8*). Conserved regions (to be discussed later) are also used to classify a hemoprotein as a P450 (*4*).

*Cytochrome P450 Structure*

The elucidation of a P450 tertiary structure occurred more than two decades after its purification. According to Omura (2), in 1970 the water soluble $P450_{CAM}$ became the

first P450 enzyme purified (*3*), it was crystallized in 1974 (*9*), its amino acid sequence was determined in 1982 (*10*), and its tertiary structure observed in 1985 following X-ray crystallographic analysis (*11*, *12*). *Pseudomonas putida*'s P450cam has an overall "asymmetrical triangular prism shape" (*2*). Making up this structure are 12 α-helices and 5 anti-parallel β-sheets. The iron heme lies below the active site, which consists of hydrophobic amino acid residues, and is between the α-helices I and L. Other bacterial P450s have been crystallized and examined using X-ray crystallography and their structures have been found to have a similar fold (*13*).

Sequence similarity between P450s can be very low; however according to Kahn and Durst (*4*) four conserved domains of proteins in this superfamily exist and denote some proteins of low identity as P450. Therefore, even if a protein does not contain the requisite positional identity, it still can be classified as a P450. The first is the well known domain, which contains the cysteine that binds the apoprotein to the iron of the heme (*2*, *4*), in the sequence of FXXGXXX**C**XG. Here "X" means any amino acid residue. According to Kahn and Durst (*4*) the only two exceptions are CYP74A and CYP74B that contain only **C**XG and **C** respectively. This cysteine, in thiolate anion form, is the fifth ligand of the heme iron, and was found by a model compound experiment (*14*) and later confirmed by X-ray crystallographic analysis of $P450_{CAM}$ (*11*). This thiolate anion ligand was displaced when the P450 was in the presence of detergents; also the carbon monoxide binding spectrum no longer had a peak at 450 nm, but at 420 nm. This form of the enzyme is called P420 (*2*). The shift in the carbon monoxide binding spectrum is caused from a realignment of the protein's conformation, and a change in the fifth ligand of the iron heme, from cysteine to histidine. The EXXR

motif in Helix K, which contains two charged residues (*4*), is the second conserved domain and is thought to bind to the 'meander' region. The third lies between the heme-binding region and Helix K known as the 'meander' region. This conserved region contains many prolines and aromatic residues. In the rest of known P450s the fourth and final conserved region is a motif that is part of the oxygen binding pocket, in the form of AGX[E/D]T, and located in Helix I. However, in the CYP74 family, this region is not conserved (*3*).

The eukaryotic forms of P450 have always been found to be membrane bound according to Omura (*2*), which makes purification and crystallization challenging due to the instability of these enzymes. The first X-ray crystallographic analysis of a membrane bound P450 was that of CYP2C5 (*15*), and was very similar to that of the soluble bacterial P450s. Microsomal P450s have a 20-25 amino acid residue "signal anchor sequence" of highly hydrophobic residues (*16*). This anchor sequence is also responsible for targeting the protein to the ER membrane. Mitochondrial P450s differ slightly: they have a 20-40 amino acid sequence that is removed once in the mitochondria (*17*). Additionally, these enzymes are initially water soluble and developed in the cytoplasm. However, the means of anchoring mitochondrial P450s to the inner membrane of the mitochondria is not known (*2*).

Information on the structure of cytochromes P450 is extensive; much of this information was obtained from review written by Kahn and Durst (*4*) as well as Omura (*2*). However, several additional review papers are available.

*P450 Catalyzed Reactions*

Many reactions are catalyzed by P450s in eukaryotic organisms, from specific cellular metabolic reactions to the more intense metabolism of xenobiotics. The P450 monooxygenase follows a cyclic reaction scheme where it receives electrons from the flavoprotein NADPH-P450 reductase, which utilizes NADPH for the generation of electrons (*18*). The reaction follows the cycle in **Figure 1** and was inspired by Kahn and Durst (*4*). The stoichiometry of a P450 monooxygenase reaction is $RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+$, where RH is the substrate being oxidized (*4*). Many substrates are known so far to be oxidized from this reaction. In microsomes it was found that P450 and NADPH-cytochrome P450 reductase make up a P450 system (*19*, *20*). A difference was found between mammals and plants in that mammals use only one reductase and plants utilize two to three (*21-24*).

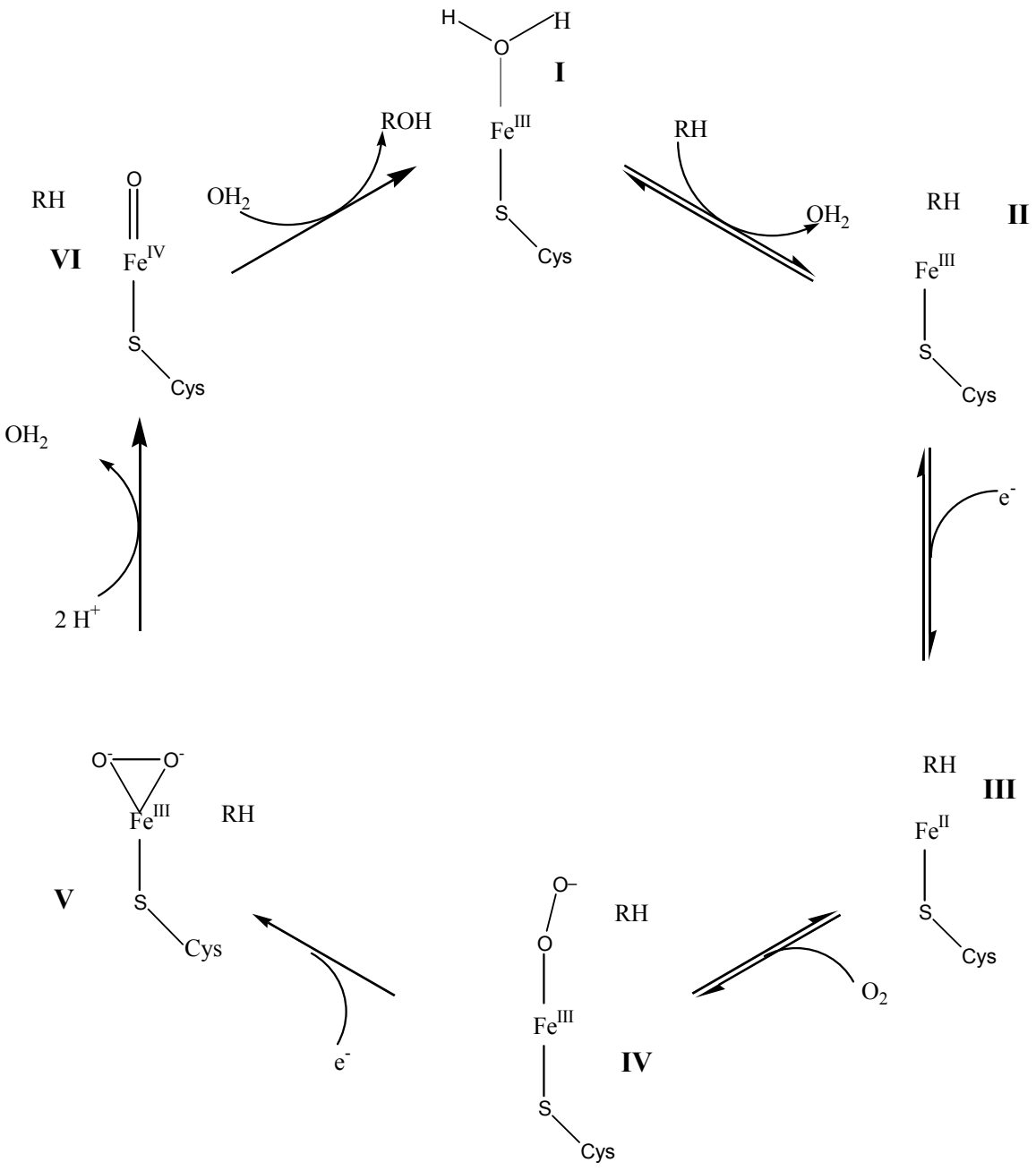



**Figure 1: General P450 Reaction Scheme**. The substrate enters the active site with the enzyme in its resting state (I) and displaces the water molecule bound to the iron (II), causing a transfer of an electron from the reductase to reduce the iron to Fe II (III). Molecular oxygen will then bind the iron (IV) and become activated with the next electron transfer from the reductase (V). Two protons allow the removal of one iron-bound-oxygen to produce a water molecule (VI). The remaining iron-bound-oxygen is then transferred to the substrate, while water binds the iron and the enzyme-heme is left in its resting state (I). Figure has been modified from Kahn and Durst (*4*).

Synthesis of many natural compounds within plants can only occur with the catalyzing potential of P450s. Alkaloids are one type of compound synthesized with the help of P450s. Alkaloids are organic nitrogenous bases, which are found primarily in plants but also in some micro-organisms and animals. Some plant produced alkaloids such as strychnine, function as a defense mechanism against other organisms; other alkaloids, such as morphine and codeine, are used in medicinal applications. While many enzymes are involved in the biosynthesis of alkaloids, P450s are the regulators of the pathway (*4*). Additionally, the reactions needed to produce these intermediates and products are very difficult to repeat with traditional, non-enzymatic organic synthesis, as opposed to being catalyzed by P450 enzymes.

Three functional types of P450 enzymes are involved in alkaloid biosynthesis as are stated by Kahn and Durst (*4*): hydroxylases, methylenedioxy bridge enzymes and C-O and C-C coupling enzymes. *Eschscholzia californica* contains the enzyme protopine-6-hydroxylase, a P450 enzyme involved in the hydroxylation of protopine, which will then spontaneously rearrange to dihydrosanquinarine. Plant species with this enzyme produce benzophenanthrine alkaloids (*25*, *26*). Two reactions are catalyzed by P450 in the conversion of stylopine from reticuline. Each reaction will produce a separate methylenedioxy bridge during the pathway from the hydroxylation of an *ortho*-methoxyphenol (*27*, *28*). Each reaction is catalyzed via a separate enzyme. The P450 (S)-canadine synthase will form a methylenedioxy bridge in berberine (*29*). Isoquinoline alkaloids are known to also be synthesized by P450s. Salutaridine, an isoquinoline, is biosynthesized from reticuline in *Papaver somniferum* and is catalyzed by a highly specific P450 C-C coupling reaction (*30*, *31*). The synthesis of colchicines in *Colchicum*

*autumnale* requires a C-C coupling reaction in the conversion of utumnaline from isoandrocymbine, catalyzed by a P450 (*32*). The bisbenzylisoquinoline alkaloid berbamunine is the major product of oxidative phenol coupling, C-O coupling, the reaction catalyzed by the P450 berbamunine synthase (*31*, *33*).

The participation of P450 in the biosynthesis of phenylpropanoids is very significant. These compounds are only found in plants and micro-organisms and are derived from the Shikimate (phenylpropanoid) pathway. This pathway is used to develop many compounds including "cinnamic acids, lignin monomers, coumarins, flavonoids, isoflavonoids and pterocurans" (*4*). Many of these compounds have "evolved for the protection" of the plants from "biotic and abiotic factors". These compounds allow the plant to survive in a varying environment and in the presence of microorganisms, such as plant parasites and diseases. According to Kahn and Durst (*4*) the sequence of this pathway is the production of *trans*-cinnamic acid from phenylalanine, which is then metabolized to *p*-coumaric acid. The final step utilizes cinnamate-4-hydroxylase (C4H), an enzyme that is prevalent within the plant kingdom (*34*). The substrate and product are both used as regulators of the pathway (35 and 36). *p*-Coumaric acid is the point where lignin biosynthesis branches off of the phenylpropanoid pathway, and after several steps is converted to ferulic acid (*4*). Ferulate is then hydroxylated to form 5-hydroxyferulate, a reaction catalyzed by the ferulate-5-hydroxylase (F5H), a cytochrome P450 (*37*). However, ferulate has come under investigation as not being as important to lignin biosynthesis, since two other compounds, coniferaldehyde and coniferyl alcohol, are better substrates for F5H (*38*). In chlorogenic acid biosynthesis the p-coumaroylshikimate-3'-hydroxylation reaction shows evidence for the requirement of

P450 catalysis (*39*, *40*). The involvement of P450 in the "phenylpropanoid metabolism" can also be found in Bolwell *et al.* (41).

Many P450 catalyzed reactions have been found in the biosynthesis of flavonoids and coumarins, which are compounds used for defense in plants, according to Bolwell *et al.* (*41*). Licodione synthase hydroxylates (2S)-liquiritigenin to produce 2-hydroxyliquiritigenin, which spontaneously converts to licodione (*42*). Naringenin and eriodictyol have also been shown to be metabolized by this P450 (*43*). Additionally, the intramolecular rearrangement of an aromatic ring to an adjacent carbon is used to produce isoflavanoids, and is a P450 catalyzed reaction (*44*). The hydroxylations of dihydrokaempferol, apigenin and kaempferol have been found to be catalyzed by P450 (*45*).

Terpenoid biosynthesis is very dependent on P450s in plants, in fact "monoterpenes, sequiterpenes and diterpenes are substrates for cytochromes P450s", as stated by Bolwell *et al.* (*41*). Plants produce compounds such as hormones (gibberellic acids, abscisic acid), carotenoids, phytosterols, volatile oils and phytoalexins that interact with their environment (*46*, *47*). One of the first plant P450s to be characterized was the hydroxylation nerol and geraniol (*48*, *49*). Many cyclic monoterpenes are known to be hydroxylated by cytochromes P450 (*41*). For example, limonene is hydroxylated at three separate carbons via P450s (*50*). Terpenoid derived phytohormones are biosynthesized with the help of P450s, and are required for shoot elongation according to Kahn and Durst (*4*). At the very least the initial and final oxidative reactions for the conversion of kaurene to gibberellins are all catalyzed by P450s (*51*, *46*). The degradation of abscisic acid is started with a P450 catalyzed hydroxylation (*52*). Brassinosteroids are

polyhydroxysteroids which participate in the development of plants (*4*). In their production is the demethylation of obtusifoliol that is catalyzed by P450 (*53*, *54*). Other steroids in plants are formed with the catalyzing effect of P450s, including the 12β-hydroxylation of digitoxin (*55*) and the hydroxylation of 18β-glycyrrhetinic acid (*56*).

Cyanogenic glucosides are released during tissue disruption, when a plant is wounded or infected, and then are metabolized into hydrogen cyanide to protect the plant (*41*). Cyanogenic glucosides are biosynthesized from amino acids via P450s (*57*, *58*).

The metabolism of many fatty acids occurs with the help of P450, with the examples that follow given by Kahn and Durst (*4*) and Bolwell *et al.* (*41*). Some highly specific P450s are known to hydroxylate fatty acids at several positions, including the ω (methyl terminus), ω-1 and other in-chain carbons (*59-63*). Cytochromes P450 are also known to be involved in the epoxygenation of several fatty acids. The first epoxy reaction described is of 18-hydroxyoleic (*64*), others include lauric acid unsaturated analogues (*65*, *66*), 11-dodecenoic acid (*67*) and lineolate (*68*). An unusual P450 is the allene oxide synthase. This enzyme uses the 13-hydroperoxide of linoleic acid instead of molecular oxygen (*69*).

The reported substrates and products of reactions catalyzed by members of the cytochromes P450 family are extensive with only a brief description of these enzymes listed here. However, several review papers that contributed to the information here in are available including those by Omura (*2*), Kahn and Durst (*4*) and Bolwell *et al.* (*41*).

*Peroxidase*

Peroxidases are hemoproteins found in a wide variety of organisms, including microorganisms, plants and mammals (*70*). All plant peroxidases found are between 40 and 50 kDa. The structure of plant peroxidases varies between the different families, however, nearly all of the known plant peroxidases contain a ferriprotoporphyrin IX, with Fe (III), and have 13 helices. Additionally, all of the hemes are located between helices B and F (*71*). Helix B contains the catalytic residues Arg48 and His52, whereas helix F contains the fifth ligand for the heme iron, which is an imidazole (His175), coincidentally the same as with cytochrome P420. Peroxidases can also be subjected to a CO difference spectrum as with cytochrome P450/P420, and will have a peak at 420 nm as with P420 due to the imidazole fifth ligand. Additionally, a distinct dip is seen at 440 nm of the peroxidase CO difference spectrum. These enzymes have the ability to catalyze the oxidation of many organic compounds with the addition of $H_2O_2$, generally following the reaction scheme depicted in **Figure 2**. Peroxidases and cytochromes P450 are both involved in the oxidation of organic compounds; however, while P450 inserts an oxygen, peroxidase will create a radical, following the removal of a proton. Peroxidases are also involved in xenobiotic metabolism, as will be discussed later.

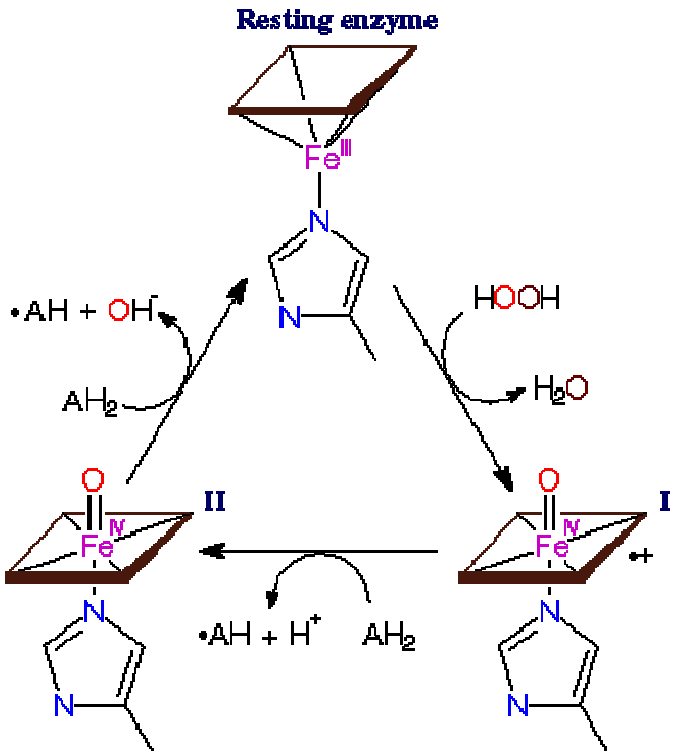



**Figure 2: General Peroxidase Reaction Scheme**. The general reaction scheme of oxidation of an organic compound catalyzed by peroxidase. The resting enzyme reacts with hydrogen peroxide to give the porphyrin cation radical and oxidized iron of compound I. Next a generic organic compound is oxidized to a one-electron radical and compound II. Following another one electron oxidation of the substrate the iron heme is reduced back to its native resting state (72).

*Xenobiotic Metabolism*

Xenobiotic metabolism in animals is an important subject due to its relation to pharmaceutical drug discovery and effectiveness. P450s catalyze many reactions involved in the metabolism of foreign compounds according to Omura (*2*). Cytochrome P450 activity was first discovered in liver microsomes with the metabolism of xenobiotics, with characteristics of the xenobiotic metabolism activity being discovered in 1949 (*73*). However, at this time the enzyme responsible for the hydroxylations observed was unknown. The enzyme responsible for the oxidation reactions was determined to be P450 in 1965, using carbon monoxide inhibition (*74*). The metabolism

of foreign compounds in plants has also become an interesting subject over the past 20 years. Xenobiotics can be toxic in all living organisms if they accumulate, and the most effective way to combat this is to metabolize them to either harmless or useful compounds or to ones that can easily be removed by the system to prevent accumulation. According to Kahn and Durst (*4*), the products of P450 catalyzed reactions can be further modified for their storage in places such as the cell wall and vacuoles.

P450s have been found to perform several types of reactions on xenobiotics. The first reaction performed on a xenobiotic compound by a purified plant P450 was the demethylation of *p*-chloro-N-methylaniline (*75*). According to Bolwell *et al.* (*41*), reactions found to be catalyzed by P450s to date are N- and O- dealkylations, ring hydroxylations, ring-methyl hydroxylations and S-oxidation. Monuron is demethylated twice (*76*) and O-dealkylation occurred in ethoxycoumarin and ethoxyresorufin (*77*). Ring hydroxylations are the most reported xenobiotic reaction in plants. Some of the examples are the hydroxylation of bentazon (*78*), primisulfuron (*79*), flumetsulam (*80*), diclofop (*81*, *82*) and triasulfuron (*83*). Also, N-nitrosodimethylamine and N-nitrosomethylaniline both appear to be ring-hydroxylated by P450 (*84*). Methyl groups on rings have also been found to be hydroxylated by P450, including chlorotoluron (*85*) and flumetsulam (*80*). An S-oxidation occurs on diazinon (*86*). The detoxification of these foreign compounds has been found to be very important in plants, and as within nearly all other living organisms P450 plays a significant role in this process. In fact, a P450 from Jerusalem artichoke has been cloned in plants to increase their resistance to and increase their ability to metabolize herbicides (*87*).

Individual members of the P450 superfamily have been found to have very specific functions in secondary metabolism in both animals and plants as stated previously. However, the reactions carried out on xenobiotics catalyzed by P450s appear to be due to the evolution and work as a defense mechanism. These xenobiotics that are metabolized by P450s are not a natural substrate of the enzyme, but a compound that is able to fit in the active site and be oxidized by the enzyme. For example the P450 that catalyzes the fatty acid (ω-1)-hydroxylation has also been found to hydroxylate the herbicide diclofop (*88*).

Plant peroxidases have also been shown to metabolize xenobiotics. For example, the xenobiotics *N*-nitrosodimethylamine (NDMA), *N*-nitroso-*N*-methylaniline (NMA), aminopyrine and 1-phenylazo-2-naphthol (Sudan I) were oxidized by plant peroxidases (*84*). Interestingly, these xenobiotics were also oxidized by P450, and provide an insight into the diversity of these two enzyme families. Peroxidases can metabolize a large number of compounds (*70*, *89*), however not to the extent and diversity of P450.

A more in-depth review of xenobiotic metabolism by cytochromes P450 is given by Bolwell *et al.* (*41*) and Durst *et al.* (*90*), from where most of this information was obtained.

*Induction of P450*

Induction of P450 has also been seen in all organisms studied and has been useful for several reasons. Induction studies of liver microsomes with phenobarbitol and 3MC in 1966 provided evidence of multiple forms of P450 in rats (*91*). Many other compounds are able to induce the production of P450s in animals, and have been greatly

studied since the 1980s (*2*). Plant P450s have also been shown to be induced several ways, which may be split into three categories: physical, chemical and challenge by pathogens (*90*). The physical factors that induce P450s include light, wounding and oxygenation. Pathogens include fungi, viruses and elicitors such as fungal membrane extracts. Many chemicals have been found to induce the production of P450s, such as manganese, cadmium, mercury, clofibrate**,** phenobarbital, aminopyrine, ethanol, arochlor, β-naphthoflavone, biphenyl, many herbicides and safeners. The herbicides monuron and dichlobenyl were also found to induce the production of P450 in higher plants (*92*).

*Phytoremediation*

Phytoremediation is the use of plants to decontaminate soil or water, by the means of phytoaccumulation, enhanced rhizosphere biodegradation, phytostabilization, rhizofiltration and phytodegradation (*93*). In phytoaccumulation the plant takes up the contaminant and stores it somewhere within its tissue. This is very useful for the uptake of metals because the plants may then be removed and disposed of and the metals may be recycled. The use of plants to stimulate the growth of microorganisms that degrade contaminants is known as enhanced rhizosphere biodegradation. Plants provide an optimized environment for microorganisms to grow, most notably around the roots. Metabolites from the roots will stimulate bacterial growth while enhancing the microorganisms' xenobiotic metabolism activity. Microorganisms have long been known to biodegrade contaminants in soil and water, known as bioremediation. The roots of vegetation are able to stop the migration of contaminants in the soil as they accumulate on the roots, a condition known as phytostabilization. This will stop the contaminants

from reaching ground water. Rhizofiltration is the use of plants to accumulate contaminants from water. And finally, phytodegradation is the use of plants to breakdown contaminants into harmless and possibly useful metabolites.

As stated earlier, plants contain P450s that have been shown to metabolize xenobiotics: this is an example of phytodegradation. Another important aspect is that many of these xenobiotics have been reported to be inducers of P450s. With the possible exception of enhanced rhizosphere biodegradation, phytodegradation is definitely the most efficient use of phytoremediation. This is due to all other mechanisms needing removal and incineration of the vegetation. Phytodegradation on the other hand will produce either metabolites which the plant can use or safe compounds that may be released. Induction of the xenobiotic-metabolizing enzymes, most notably P450s, is a phenomenon which has not gone unnoticed. This has proved that plants have evolved to clean up soil and water and that P450s play a significant natural-role in phytoremediation.

*Black Locust*

*Robinia pseudoacacia*, the black locust tree, has become the most widely distributed fast-growing hardwood in temperate climate zones around the world (94). Black locust trees have been planted in many places including abandoned farmland for erosion control and in other poor soils to reclaim the area for reforestation. Other areas of poor soil where black locust is able to grow and in some cases has been used to reclaim the soil include strip mines (*95*) and areas contaminated with industrial polyaromatic pollutants (*96*, *97*, *98*). In one study, black locust was found to have a high concentration of soil bacteria that could degrade polychlorinated biphenyls and

chlorobenzoic acids (*98*). The black locust is also good for use as a nitrogen-fixing plant, which will help fertilize the soil, and is thus helpful for agriculture and forests (*99*). Although no evidence exists for the presence of P450 in black locust, peroxidases have been found within the leaf tissue of this tree (*100*). These attributes of black locust make it a prime candidate for phytoremediation.

*Objectives*

There are three major objectives of this project. The first objective is to determine if the roots of black locust seedlings contain any P450s by the use of CO difference spectrometry. The second is to determine if seedlings exposed to a xenobiotic are induced to produce a large amount of P450. The final objective is to determine if black locust seedlings contain peroxidase that will catalyze the oxidation of hydroquinone and 4-chloro-1-naphthol.

## MATERIALS & METHODS

*Reagents and Equipment*

Several of the reagents used for this project were purchased from Sigma, including EGTA, PMSF, Amberlite XAD-4, PVPP, Caffeic acid, bovine serum albumin (BSA) and 4-chloro-1-naphthol. Bradford reagent was from Bio-Rad. Monuron (3-(4-chlorophenyl)-1, 1-dimethylurea) and hydroquinone were from Aldrich. DEET (N,N-diethyl-3-methylbenzamide) was from The Supply Sergeant in Escanaba, MI. All other reagents were of research grade.

The majority of centrifugations were performed on the Beckman L-60 Ultracentrifuge. Protein assays were performed using the Shimadzu UV-160 Spectrophotometer, whereas all other spectra were obtained using the Shimadzu UV3101PC Spectrophotometer.

*Plant Tissue*

Black locust seeds (*Robinia pseudoacacia*) were obtained from Mistletoe Quality seeds in Goleta, CA. Black locust seedlings were grown in four inch pots in the greenhouse at Northern Michigan University. They were harvested at various times between the ages of 45-400 days. Other specimens include mature black locust trees, mature *Populus tremuloides* (poplar), and *Vigna radiate* mung bean sprouts. Etiolated mung bean sprouts were grown from seeds obtained from the Marquette Food Co-op. The mung bean seeds were soaked in water for 1 day, and then grown up in a completely dark environment for approximately 3 weeks with proper hydrating.

*Xenobiotics*

The xenobiotics used were 32 ppm naphthalene, 800 or 1000 ppm DEET, 100 ppm trichloroethylene (TCE), 2 mM Monuron, 1.01 mM Monuron in 20 or 15% ethanol, 0.25 mM Monuron in 4% ethanol, 0.25 mM Monuron (dissolved in ethanol, which was left to evaporate), and 25 mM $MnCl_2$. All solutions were prepared in tap water. Volumes of xenobiotic used for exposure of seedlings varied with the amount of water the seedling needed. Groups of seedlings were watered with tap water instead of xenobiotic solution in order to give a control. Wounding of mung bean sprouts was performed by producing a 1 cm slice up the stalk just prior to exposure to the xenobiotic.

*Extraction Buffers*

The extraction of proteins from black locust seedlings and poplar stems was performed with a buffer containing components that would stabilize cytochromes P450. Only one buffer was used in a given extraction. The first buffer employed for the extraction of P450 from black locust seedling roots was prepared according to Werck-Reichhart and co-workers (*101*) with some changes. This was **Buffer A**, and contained 100 mM sodium phosphate, at pH 7.4, 25 mM sucrose, 0.1% (v/v) 2-mercaptoethanol, 1 mM EDTA, 40 mM ascorbic acid and 0.5 mM PMSF. **Buffer B** contained 100 mM sodium phosphate, at pH 7.4, 250 mM sucrose, 0.1% (v/v) 2-mercaptoethanol, 1 mM EDTA, 40 mM ascorbic acid and 0.5 mM PMSF. **Buffer C** contained 100 mM sodium phosphate, at pH 7.4, 250 mM sucrose, 0.15% (v/v) 2-mercaptoethanol, 1 mM EDTA, 40 mM ascorbic acid and 1 mM PMSF. **Buffer D** was prepared accordingly to Hefner and Croteau (*102*) with a few modifications and contained 100 mM sodium phosphate, 0.12%

(v/v) 2-mercaptoethanol, 250 mM sucrose, 2 mM EGTA, 40 mM ascorbic acid, 1 mM PMSF, and 1 mM p-bromoacetophenone at pH 7.4. **Buffer E** contained 200 mM sodium phosphate, 0.12% (v/v) 2-mercaptoethanol, 250 mM sucrose, 2 mM EGTA, 40 mM ascorbic acid, 1 mM PMSF, and 1 mM p-bromoacetophenone at pH 6.95. **Buffer F** contained 200 mM sodium phosphate, 0.12% (v/v) 2-mercaptoethanol, 250 mM sucrose, 2 mM EGTA, 40 mM ascorbic acid, 1 mM PMSF, 25 mM $Na_2S_2O_5$ and 1 mM p-bromoacetophenone at pH 6.70. **Buffer G** contained 200 mM sodium phosphate, 0.12% (v/v) 2-mercaptoethanol, 250 mM sucrose, 4 mM EGTA, 40 mM ascorbic acid, 1 mM PMSF, 25 mM $Na_2S_2O_5$, 4 mM EDTA and 1 mM p-bromoacetophenone at pH 6.70. **Buffer H** contained 200 mM sodium phosphate, 0.12% (v/v) 2-mercaptoethanol, 250 mM sucrose, 4 mM EGTA, 40 mM ascorbic acid, 1 mM PMSF, 25 mM $Na_2S_2O_5$, 10 mM EDTA and 1 mM p-bromoacetophenone at pH 6.70. **Buffer I** contained 200 mM sodium phosphate, 0.5% (v/v) 2-mercaptoethanol, 250 mM sucrose, 4 mM EGTA, 40 mM ascorbic acid, 1 mM PMSF, 25 mM $Na_2S_2O_5$, 10 mM EDTA and 1 mM p-bromoacetophenone at pH 6.70. **Buffer J** contained 200 mM sodium phosphate, 0.5% (v/v) 2-mercaptoethanol, 250 mM sucrose, 4 mM EGTA, 40 mM ascorbic acid, 1 mM PMSF, 25 mM $Na_2S_2O_5$, 10 mM EDTA, 1% (m/v) BSA and 1 mM p-bromoacetophenone at pH 6.70. **Buffer K** contained the same components as **Buffer J**, however the BSA was defatted according to Chen (*103*). Commercially available BSA will contain fatty acids that act as a detergent and disrupt microsomes.

The extraction buffers employed in the extraction of peroxidase from black locust seedling roots varied only slightly from those used when P450 was the enzyme of interest. **Buffer L** was the extraction buffer used for the homogenization and protection

of peroxidase, containing 200 mM dibasic potassium phosphate, 250 mM sucrose, 2 mM EDTA, 1 mM PMSF, 2 mM L-ascorbate and 0.2% (v/v) 2-mercaptoethanol, at pH 7.0.

The extraction buffers used for homogenizing etiolated mung bean sprout tissues differed slightly from that used for the seedlings, and was prepared according to Mizutani and co-workers (*104*). **Buffer M** contained 200 mM sodium phosphate, 300 mM sucrose 2 mM EDTA, 0.4 mM PMSF 0.156% (v/v) 2-mercaptoethanol, 2 mM *trans*-cinnamic acid and 10% (m/v) PVPP at pH 7.25. The addition of *trans*-cinnamic acid was used in an attempt to stabilize the cinnamate-4-hydroxylase isoform of P450.

*Extraction Procedures*

All extractions were carried out on ice and with ice cold equipment and solutions, or at 4°C. For a stem extraction, the phloem (inner bark) and cambium layer were used. When extractions were performed on root tissue, the cortex (living outer tissue surrounding the endodermis) was removed from the stele and homogenized. Homogenizations were performed in one of the several extraction buffers. The extractions of proteins from black locust roots involved homogenization with a mortar and pestle, using approximately 5-7 mL of extraction buffer per gram of tissue. This value is larger than what Hefner and Croteau recommend (4-5 mL) (*102*). The increase in volume is due to the root tissue used for the sample being relatively dry. Even though the soft tissue of the roots was used for the extractions of P450, this is hard, fibrous tissue (*105*) and difficult to completely homogenize with conventional methods. To overcome the problem of homogenizing a hard tissue, liquid nitrogen was tested for its efficiency in grinding the samples (*102*). Using a mortar and pestle, this procedure would give a near-

powder-like substance. However, it was clear that conventional homogenization in buffer using a mortar and pestle should also be used, after the use of liquid nitrogen. Washed sea sand (approximately 0.6 grams per gram of tissue) was used in this final homogenization step in order to grind up the cells closer to homogenization, as used by Yoshida (*105*). Amberlite XAD-4 (10% w/v) and PVPP (polyvinylpolypyrolidine) (0.3 grams per gram of sample) were also used on occasion in the extraction buffer. The advantages of these two substances will be discussed later. Mung bean sprouts were homogenized with a kitchen blender. In all cases the homogenates were shaken for approximately 20 minutes and filtered through cheese cloth.

*Recovery of Microsomes and Soluble Proteins*

The filtrates recovered from filtering the homogenates through cheese cloth were then fractionated by centrifugation, or homogenized further with a teflon homogenizer in a glass tube and then centrifuged. All centrifugations were carried out at 4°C for a specified time with the g forces being given as the maximum. The maximum force is used because the volumes of the solutions varied between samples being centrifuged. Several centrifugation methods were used in order to obtain more concentrated and/or purer fractions. These can be expressed in four basic categories. **Fractionation A** was performed at approximately 10,000 x g for 20 minutes. **Fractionation B** involved one centrifugation at 15,000 x g for 15 minutes and a microsomal fractionation (pellet) on the supernatant recovered at 180,000 x g for 60 minutes. **Fractionation C** involved three centrifugations, the first two to remove unwanted components from the supernatant, and the third to obtain a microsomal fraction. The first was at 4,000 x g for 25 minutes, the

second at 25,000 x g for 25 minutes and the final was at 186,000 x g for 90 minutes. **Fractionation D** was used in the recovery of microsomes from mung bean sprouts. The filtrate was centrifuged at 22,500 x g for 17 minutes, the supernatant was collected and centrifuged again at 160,000 x g for 60 minutes to obtain the microsomal pellet. A total protein extract, used for analysis of peroxidases, was obtained by centrifuging the filtrate at 15,500 x g for 20 minutes, with the supernatant (total protein extract) being collected for assays.

*Suspension of microsomes*

Microsome pellets were placed in buffer and homogenized using a glass homogenizer. The first suspension buffer used for this procedure was prepared according to Werck-Reichhart *et. al.* (*101*). This was **Buffer N**, containing 10 mM sodium phosphate (pH 7.4), 0.01% (v/v) 2-mercaptoethanol and 30% (v/v) glycerol. The next was **Buffer O** that contained 10 mM sodium phosphate (pH 7.4), 0.015% (v/v) 2-mercaptoethanol and 10% (v/v) glycerol. The next was **Buffer P**, prepared according to Hefner and Croteau (*102*), containing 25 mM sodium phosphate, 1 mM EGTA, 20% glycerol, 0.012% 2-mercaptoethanol, 10 mM $MgCl_2$ and 25 mM KCl at pH 7.4. **Buffer Q** contained 25 mM sodium phosphate, 1 mM EGTA, 20% glycerol, 0.055% 2-mercaptoethanol, 10 mM $MgCl_2$ and 25 mM KCl at pH 7.4. **Buffer R** contained 25 mM sodium phosphate, 1 mM EGTA, 15% glycerol, 0.055% 2-mercaptoethanol, 10 mM $MgCl_2$ and 25 mM KCl at pH 7. **Buffer S**, used for the suspension of microsomes from mung bean sprouts contained 100 mM sodium phosphate, 1 mM EDTA, 0.0156% 2-mercaptoethanol and 30% glycerol at pH 7.25.

*Difference Spectrometry*

The microsome suspensions and microsomal supernatant fractions were analyzed for P450 using carbon monoxide difference spectrometry. Three variations of this method were employed. **CO A**, involved bubbling the sample with helium for 30 seconds prior to reduction of the sample solution with a few milligrams of sodium dithionite (*106*). The sample was then split in half, the baseline taken between the sample and reference cells, and then the sample cell was bubbled in carbon monoxide for 30 seconds. The difference spectrum was then taken. The second method, **CO B**, was performed similarly, but without He bubbling (*107*). **CO C** was performed by first taking the baseline of the sample and reference cells, bubbling the sample cell with CO for 30 seconds and finally reducing both cells (*108*) with the sodium dithionite. **CO D** was performed as the previous but with reducing the two solutions with 50 μL of a 1% sodium dithionite and 2% sodium carbonate solution (*109*), instead of only the solid sodium dithionite.

*Peroxidase Assays*

Peroxidases can also be detected using CO difference spectroscopy, following the same procedure used for the assay of P450. Peroxidases were also measured by observing UV/VIS spectra during and following the metabolism of caffeic acid, hydroquinone and 4-chloro-1-naphthol. The metabolism of caffeic acid was observed spectrophotometrically at 315 nm, in 0.1 M acetate at pH 4.4 with 0.1 mM caffeic acid and 0.01% $H_2O_2$ (*110*). Microsomal supernatant or microsomal fraction suspension (10 μL) was added to start the reaction. The conversion of hydroquinone to benzoquinone

was seen spectrophotometrically by observing the formation of benzoquinone at around 250 nm, and was performed according to Zapata and co-workers (*111*). The molar absorptivity is 1.9 $M^{-1}cm^{-1}$ for benzoquinone at the specified wavelength. The reaction mixture contained 30 mM potassium phosphate pH 7.0, 0.5 mM hydroquinone and 0.01% $H_2O_2$, with 10 μL of total protein extract (in **Buffer L**) added to initialize the reaction. The formation of a product from 4-chloro-1-naphthol was observed spectrophotometrically, and the procedure was performed according to Adler and co-workers (*112*) with slight modification. The reaction mixture contained 20 mM Tris-HCl pH 7.0, 500 mM NaCl, 0.5 mM 4-chloro-1-naphthol, 0.01 % $H_2O_2$, and was initiated with the addition of 10 μL of total protein extract (in **Buffer L**). The 4-chloro-1-naphthol was a solution of 17.70 mM 4-chloro-1-naphthol in 20 mL of ice cold methanol.

*Bradford Protein Assay*

Protein content was measured using the "Bradford Protein Assay" (*113*). BSA was the protein used for the standard curve, with a range of 6-90 μg in 60 μL, (0.100-1.500 μg/μL) for the protein sample and diluted to 3 mL with Bradford reagent, with the absorbance at 595 nm observed 5 minutes after the dilution. All BSA standard solutions were prepared with the respective buffer that the sample being analyzed was in.

Samples that were tested for protein concentration were diluted the appropriate amount to be measured within the standard curve.

## RESULTS & DISCUSSION

*Extraction Development*

The extraction of P450s from plant species requires an extraction buffer with an environment of certain conditions and compounds for the protection of this family of enzymes. This is due to this membrane bound enzyme being in a weakened state and susceptible to many potentially harmful cellular components. Among the compounds used in the extraction buffer are: sucrose, 2-mercaptoethanol, EDTA, EGTA, PMSF, *p*-bromoacetophenone, sodium metabisulfite, and BSA. Either sucrose or glycerol can be used to stabilize the enzyme in its active form (*102*). EDTA is commonly used to chelate calcium and magnesium during the extraction of enzymes. Phospholipase $A_2$ activity will produce detergents that will disrupt microsomal membranes which in turn will solubilize P450. The activity of this enzyme is dependent on free calcium. EGTA chelates only calcium and can be substituted for EDTA to decrease the activity of phospholipase $A_2$. In addition *p*-bromoacetophenone is used as an inhibitor of this enzyme. PMSF is used as a protease inhibitor and ascorbic acid is used as an antioxidant to keep the enzyme in its active form (*102*). Sodium metabisulfate was added following a personal communication with Rodney Croteau, in order to possibly prevent the denaturing of P450. Sodium metabisulfite is an antioxidant used to maintain the activity of cytochrome P450s. BSA has been found to bind many plant cellular components, as summarized by Loomis (*114*), which would otherwise interfere with the extraction of intact and undisturbed organelles. Characteristically BSA will bind to both anions and hydrophobic compounds including but not limited to phenols, quinones and lipids. The

ability of BSA to perform this function is due to its high content of both hydrophobic and cationic amino acids. Commercially available BSA contains fatty acids unless specified. These fatty acids will act as a detergent and disrupt the microsomes, and were later removed according to Chen (*103*). Amberlite XAD-4 and PVPP were used to extract the enzyme in active form, and were added to the extraction buffer immediately before the extraction. Amberlite XAD-4 is a very hydrophobic polystyrene bead which will remove hydrophobic compounds. PVPP is capable of binding phenolics and quinones in plant extracts. Phenolics are natural plant compounds that will form hydrogen bonds between their hydroxyl group and oxygen atoms of the polypeptide backbone (*114*). The lowering of the pH was recommended by Hefner and Croteau (*102*), as well as Loomis (*114*), to help eliminate phenolates by protonating them and thereby increasing the effectiveness of polymeric adsorbents.

The extraction of peroxidases from black locust roots was not successful until **Buffer C** was employed in the extraction, since the characteristic peak at 420 nm and dip at 440 nm were not observed until certain components in this buffer were used. One major component of this solution was L-ascorbate. When this anti-oxidant was not present nearly all peroxidase activity for catalyzing the oxidation of hydroquinone was lost shortly after recovery of the microsomal fraction supernatant. However, when this component was present there was no significant loss of activity, even with storage of the protein extract at 4°C, for two weeks.

The soft tissue of the roots of black locust seedlings was used as the sample for the extractions. This tissue is the cortex and the site of living cells, and where phytoremediation will occur. However, as stated by Yoshida the black locust has “thick

and hard cell walls" (*105*). Sea sand was then incorporated into the extraction procedure and used when homogenizing with a mortar and pestle to facilitate a greater degree of homogenization. Grinding the tissues in liquid nitrogen prior to addition of the extraction buffer was also incorporated into the procedure to attempt greater disruption of the cell walls and mitochondria (*102*). This was attempted when no active P450 was detected, possibly due to insufficient homogenization and the majority of the P450 enzymes remaining in the mitochondria.

*Microsome Purification*

Initially, all extraction samples were centrifuged only once after filtration through cheese cloth. This centrifugation step was carried out at a maximum of approximately 10,000 x g. The purpose of this was to pellet cellular debris from the root extracts in order to remove it from the sample. However, P450 in plant tissue (eukaryote) only exists in a membrane bound form. When a root cell is broken up by homogenization, membrane bound enzymes will be in fragments of the lipid bilayer, and microsomes will form from these fragments. Cytochromes P450 usually exist in low concentrations, but may be concentrated by using a high centrifugal g force to precipitate the microsomes out of solution. Centrifugation of greater than 100,000 x g was used for one hour to precipitate these microsomes. Later on, the initial centrifugation to remove cellular debris was reduced to an approximate maximum of 3,000 x g. Another centrifugation was then used to remove dense organelles, at an approximate maximum of 20,000 x g. This centrifugation would promote further purification of microsomes, by removing these dense organelles that would otherwise pellet with the microsomes. These first two

centrifugations were performed for 20 minutes, whereas the 100,000 x g centrifuge was performed for 90 min.

*CO Difference Spectrometry Development*

The hemoprotein cytochrome P450 was analyzed by a CO difference spectrum. The first technique, used for the initial extractions from black locust roots, followed the procedure reported by O'Keeffe and co-authors (*106*) with work on *P. putida* cells. This is described in the methods section as **CO A**. The difference spectrum was later obtained by the methods developed by Omura and Sato (*107*), and is referred to in the methods section as **CO B**. This procedure was the same as the previous technique but excluded bubbling with helium (argon). This procedure was used to test for P450 in all extracts until it was noticed that Omura and Sato (*108*) suggested that aerobic sample cells need to be bubbled with CO first, and then reduced with sodium dithionite, and is mentioned in the methods section as **CO C**. This was performed to prevent the reaction of the reduced iron heme with oxygen in the solution, which would decrease the amount of CO complex formed. This procedure produced the same results as the previous. With the proper buffer components, as in **Buffer C**, a large peak at 420 nm and a large dip at 440 nm were observed. These characteristics in the spectrum may be attributed to peroxidases, and will be discussed later. This procedure was used until a personal communication with Omura (*109*) in which he suggested using a solution containing 1% sodium dithionite and 2% sodium carbonate, in place of solid sodium dithionite. The procedure followed the previous one exactly, except instead of using solid sodium dithionite, 50 μL

of the 1% sodium dithionite and 2% sodium carbonate was used to reduce the heme, and is referred to in the methods section as **CO D**.

Throughout the majority of the extractions Amberlite XAD-4 was incorporated during the homogenizations in order to remove harmful hydrophobic metabolites, possibly produced from the degradation of xenobiotics. Many compounds within the homogenate, including xenobiotics and their metabolites have been shown to alter the CO difference spectrum. Another component, defatted BSA, was added to the extraction buffer in order to remove even more of these possible cellular and xenobiotic metabolites. The results did not vary, no peak was found at 450 nm within the microsomal fractions from black locust seedling roots.

*Protein Concentration*

When plants or animals are exposed to xenobiotics, the organism will produce more of the enzymes responsible for the metabolism of these compounds. This will cause an increase in protein concentration within the organism. The concentration of protein within the black locust seedling root was assayed to determine if the xenobiotic(s) induced protein production within the organism. Since the organism will be induced to produce more of these enzymes the total concentration of protein within the organism should increase as a direct effect. Additionally, if the production of cytochrome P450s is increased the concentration of protein within the microsomal fraction will be greater.

When seedlings were exposed to either DEET, TCE or a combination of Monuron and TCE, they produced a marked increase in concentration of protein per gram of root. Seedlings (75 days old) were exposed to concentrations of 1000 ppm DEET, 100 ppm

TCE or tap water for three days prior to homogenization of roots, and showed significant increases in protein concentration per gram of root. For reference these will be called **Seedling Group A**. The roots of this group were homogenized in **Buffer C** using 6 mL of buffer and 0.2 g of PVPP per gram of sample. The microsomes and soluble proteins were separated using **Fractionation B**. The microsomes were then suspended in **Buffer P**. Soluble protein per gram of root for DEET and TCE exposed seedlings was found to be 35.8 and 33.5% greater than the control's value, respectively, as seen in **Figure 3**. The concentrations of microsomal protein per gram of root for the DEET and TCE exposed seedlings were both 4.5 times that of the control's as seen in **Figure 4**.

When seedlings (70 days old) were exposed to 0.25 mM Monuron (initially dissolved in ethanol that was evaporated off) and 100 ppm TCE for 3 days prior to a two day rest before the homogenization of the roots, the amount of protein per gram of root was also greatly increased. These seedlings will be referred to as **Seedling Group B**. The roots of the seedlings were homogenized in 6 mL of **Buffer I** containing 10% (w/v) Amberlite XAD-4, 0.3 g of PVPP, and 0.6 g of washed sea sand per gram of sample. Cell debris and dense organelles were removed and the soluble proteins and microsomes were separated using **Fractionation C**. The microsomes were suspended in **Buffer S** and homogenized. These exposed subjects showed an increase of 79.3% in soluble protein per gram of root above that of the control, as shown in **Figure 3**. Microsomal protein concentration in per gram of root can be seen as being more than double the concentration of the control in **Figure 4**.

Seedlings (85 days old) were exposed to 0.25 mM Monuron (initially dissolved in ethanol that was evaporated off) two days prior to homogenization of the roots. These

subjects will be referred to as **Seedling Group C**. The roots were homogenized in 6 mL of **Buffer K** containing 10 % (w/v) Amberlite XAD-4, 0.6 g washed sea sand and 0.3 g PVPP per gram of root. The filtrates were subjected to **Fractionation C** in order to purify and separate the soluble proteins and microsomes. The microsomes were then suspended in **Buffer S** and homogenized. These seedlings showed an increase of 16.2% of total microsomal protein per gram of root over that of the control as seen in **Figure 4**. Soluble protein could not be accurately analyzed due to the addition of BSA to the extraction buffer; therefore total protein could not be calculated.

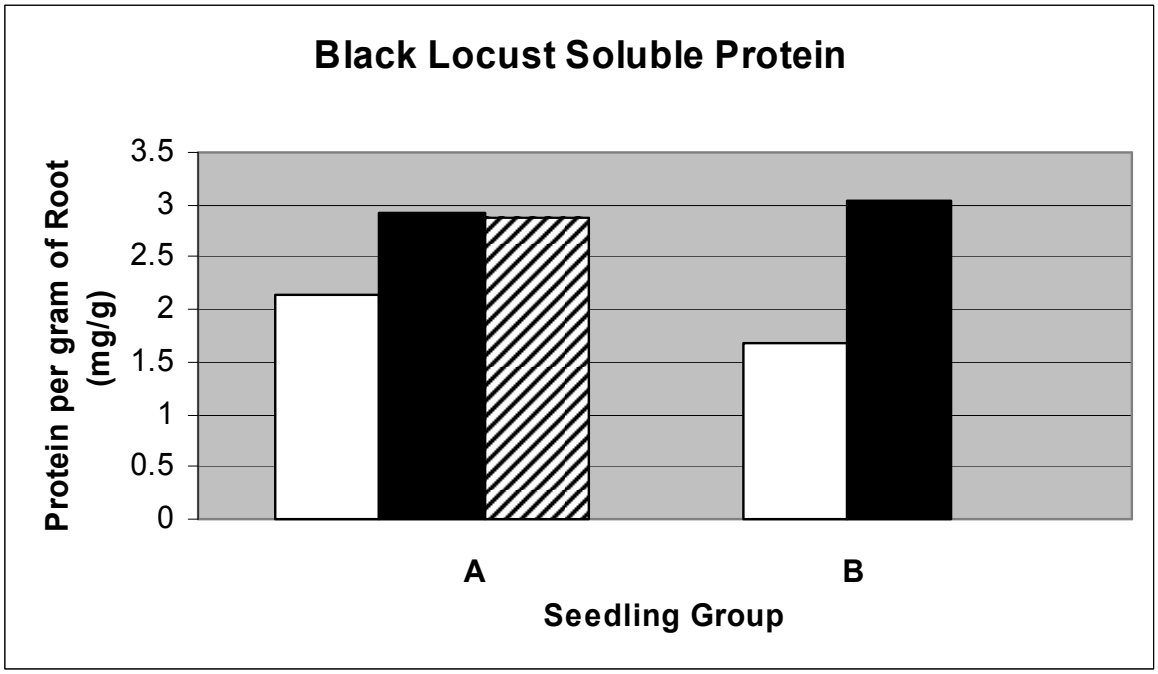



**Figure 3**: **Black Locust Soluble Protein**. The soluble protein of black locust seedling roots from **Seedling Groups A** and **B**, determined using the Bradford Protein Assay. **Seedling Group A** contains values for soluble protein in the control (open), DEET (filled) and TCE (hashed) exposed seedlings, respectively. The standard curve for this group was $Y=0.0102(X) + 0.568$ with $R^2=0.978$. **Seedling Group B** illustrates values for the control (open) and monuron and TCE exposed seedlings (filled). The standard curve for this group was $Y=0.0118(X) + 0.0915$ with an $R^2$ of 0.944.

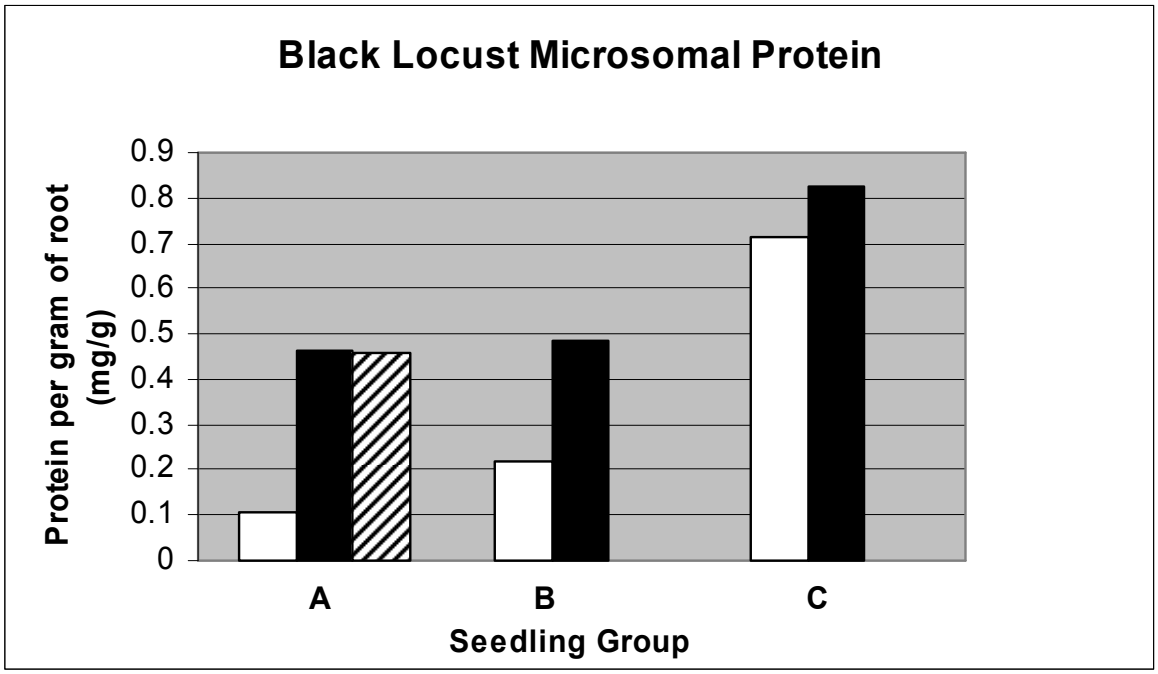
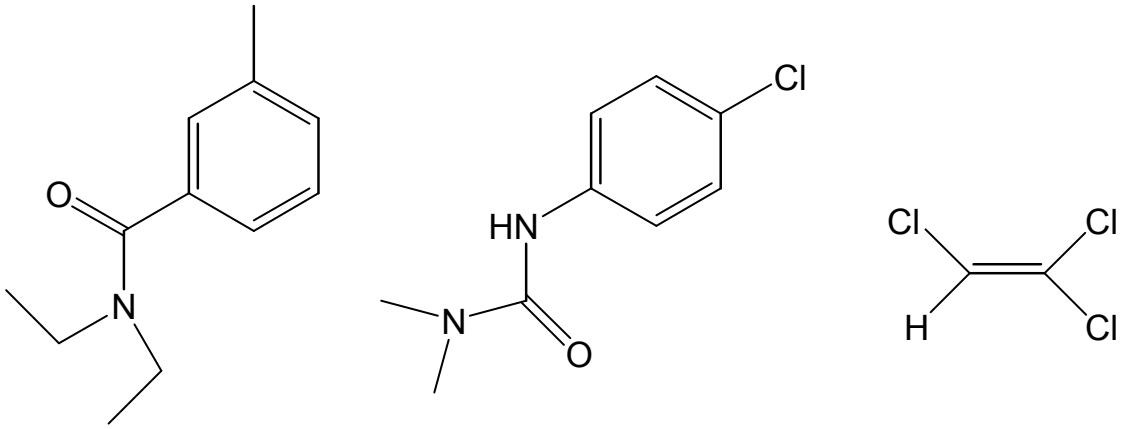



**Figure 4**: **Black Locust Microsomal Protein**. The microsomal protein of black locust seedling roots from **Seedling Groups A**, **B** and **C**. **Seedling Group A** depicts the microsomal protein obtained from control (open), DEET (filled) and TCE (hashed) exposed seedlings. The standard curve for this group was Y=0.0110(X) + 0.581 with $R^2$=0.990. **Seedling Group B** shows the microsomal protein of the control (open) and monuron and TCE (filled) exposed seedlings. The standard curve for this group was Y=0.0117(X) + 0.0574 with an $R^2$ of 0.981. **Seedling Group C** contains the values of the control (open) and monuron (filled) exposed seedlings. The standard curve for this group was Y=0.0102(X) + 0.0477 with an $R^2$ of 0.972.

In all instances the concentration of protein in the root sample increased from the control to the exposed seedlings, regardless of the age of the plants analyzed and the xenobiotic used. The lesser change in this final group may be due to the BSA used in the extraction buffer, and will be discussed later.




DEET Monuron TCE

**Figure 5: Xenobiotics Affecting Protein Regulation in Black Locust.** The compounds DEET, Monuron and TCE were used to induce both membrane bound and cytosolic proteins in black locust seedling roots.

*CO Difference Spectrometry*

The microsomal fraction supernatant (soluble proteins) and the microsome suspensions (membrane bound proteins) were assayed for cytochrome P450 and P420 using CO difference spectrometry. Cytochrome P450 has a very distinct carbon monoxide difference spectrum. The spectrum is produced by taking the difference of a sample bubbled in CO and reduced with sodium dithionite, versus that of just a reduced sample. A baseline of the two cuvettes containing an equal volume of the same sample added to them was produced in order to remove any unequal absorbance between them. The molar concentration of the microsomal and solubilized forms of the enzyme may also be determined by taking A450–490 and A420-490 (1). The absorbance at 490 nm is used as the baseline value. A peak in the spectrum at 450 nm has been observed with microsomes of several plants (*4*, *41*) and animals (*2*) and is the basis of P450 discovery and purification. However, peaks are also found at 420 nm, which would be the

solubilized, partially denatured and inactive form of P450 (*2*). This solubilized form should not be confused with the P450s of most bacteria. This is because P450 found in bacteria is naturally cytosolic, not membrane bound. P450s in eukaryotes have always been found in microsomes, which are sections of a lipid bilayer reformed after the cell and organelles have been broken up, as opposed to being soluble and cytosolic.

Throughout most of the project a large peak was found at 420 nm within the CO difference spectrum of the microsomal fraction supernatant, collected following a high g force centrifuge. Initially this peak was believed to be that of the solubilized form of P450, P420. A smaller peak was found at 420 nm in the microsomal pellet samples, also believed to be P420. The peak of P420 results from the membrane bound form being removed from the membrane or when the membrane is removed by the use of reagents which will disrupt the membrane, and therefore destabilize the protein. Following destabilization, the link between the iron heme and the cysteine (the fifth ligand) will be broken, and will be displaced by histidine, specifically its imidazole group (*8*). **Figure 2** shows an iron heme of peroxidase that is much like that of P450, with the difference being that peroxidase contains histidine as the fifth ligand and P450 has cysteine as the fifth ligand. For nearly all of the extractions the reagents chosen for both the extraction and suspension buffers (those in which the microsomal pellet is homogenized) were those needed to both protect the P450 enzyme against interfering materials and to stabilize it in the membrane during the extraction. In essence these compounds were employed to keep P450 active and in its natural state. Ascorbate was used as an antioxidant, sucrose and glycerol were used to stabilize the protein within the membrane, PMSF was included as a protease inhibitor, 2-mercaptoethanol was used as a reducing agent to keep the enzyme

active, and EDTA was used to chelate metal ions (*102*). These were the components initially added to the buffer used in the extraction of black locust roots which produced the peak at 420 nm in the CO difference spectrum of the microsomal fraction supernatant shown in **Figure 6**.

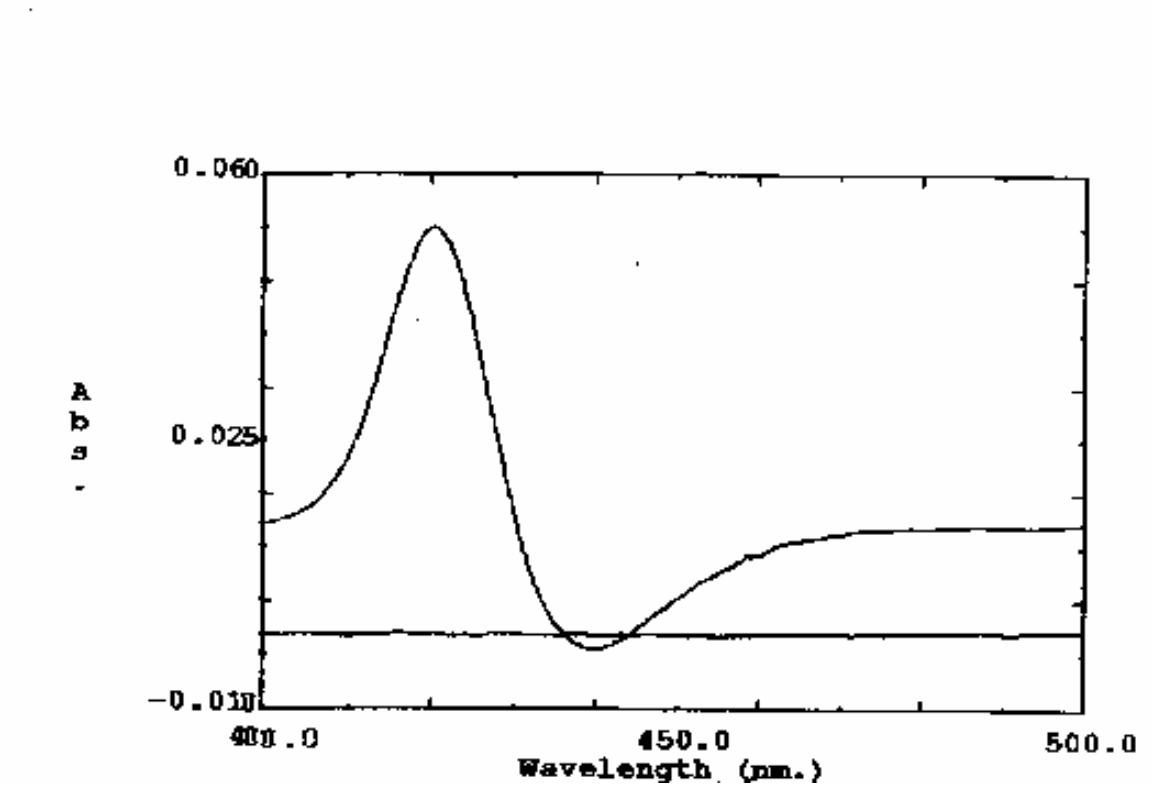



**Figure 6**: **Black Locust Soluble Protein CO Difference Spectrum.** This CO difference spectrum was performed with the supernatant collected following the microsomal fractionation (**Fractionation B**) on the filtrate from the black locust seedling roots extracted with **Buffer C**. Both the sample and reference cells contained the supernatant, and followed the **CO D** method. The large peak obtained is at 420 nm, with the dip being at 440 nm.

Measures were taken to inhibit cellular components that would break up the microsomes and prevent recovery of active cytochrome P450. The most likely component in woody tissue was suggested to be phospholipase $A_2$ (*102*). This enzyme is

able to produce fatty acids that are natural detergents from the phospholipids that make up the membrane. However, the activity of this enzyme is dependent on free calcium. The use of EGTA was then incorporated because it will chelate calcium ions. An inhibitor of the phospholipase $A_2$, *p*-bromoacetophenone, was also incorporated into the extraction buffer. However, the major peak at 420 nm found in the CO difference spectrum of the supernatant remained, as well as the occasional and lesser peak at the same wavelength for the microsomes as shown in **Figure 7**.

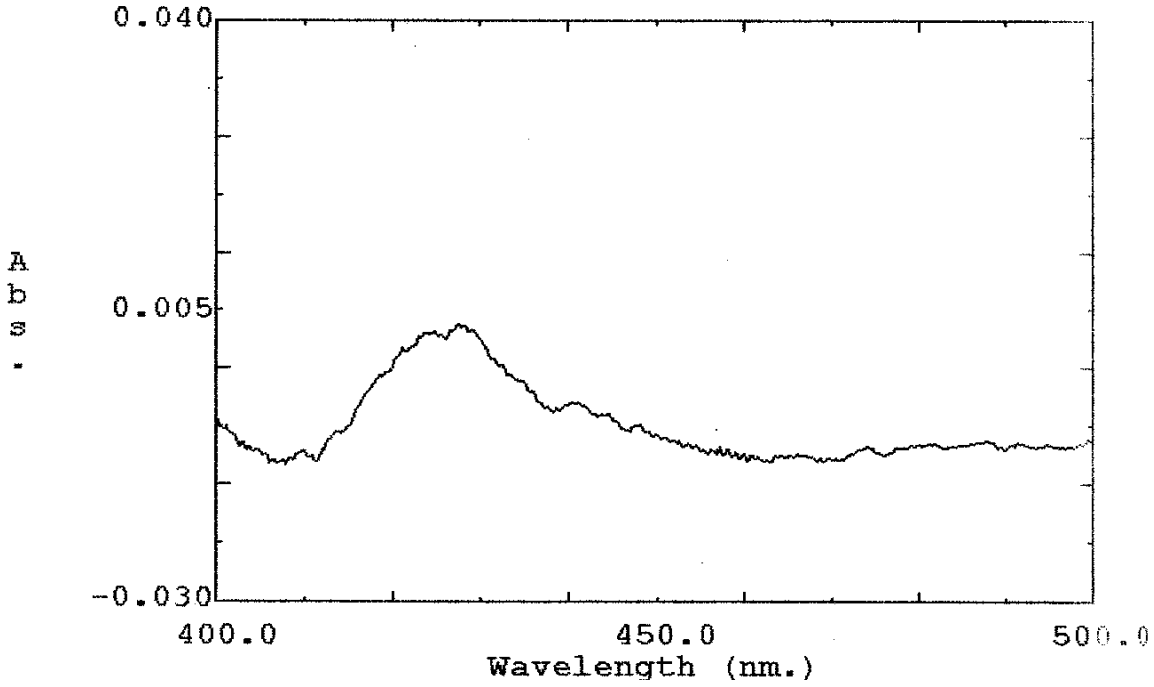



**Figure 7**: **Black Locust Microsomal Protein CO Difference Spectrum.** CO difference spectrum of microsomal suspension collected from black locust roots, at 120 days old, treated with 0.1% DEET for 3 days then homogenized in **Buffer C**. Microsomes were recovered using **Fractionation B** and suspended in **Buffer O**. Absorbance values for 420, 450 and 490 nm are 0.001, -0.010 and -0.012, respectively. These values will correspond to 1.2E-4 mM of P420 and 2E-5 mM of P450, using molar coefficients of 110 $cm^{-1}mM^{-1}$ and 91$cm^{-1}mM^{-1}$, respectively (101). The method **CO D** was used in obtaining this spectrum.

*Peroxidase*

Peroxidases are known to have a maximum absorbance at 420 nm, accompanied by a large dip at 440 nm in a CO difference spectrum. The information provided by the work of Rodgers and co-workers (*110*), where peroxidase was found in their microsomes, actually indicates that the peak found in the microsomal fraction supernatant from black locust roots (**Figure 6)**, was produced from the presence of peroxidases. Rogers *et. al.* showed that the presence of peroxidase eliminated nearly all absorbance by P450 at 450 nm within the microsome suspension.

In this project, peaks produced from the various microsomal fraction suspensions thus far were very small at 420 nm and no dip was observed at 440 nm, therefore this would indicate that no peroxidase was present. To verify that this was not peroxidase, the high-salt-concentration washings of the microsomal fraction were performed, as suggested by Rodgers *et. al*., followed by a high g force centrifugation to re-pellet the microsomes. This procedure would eliminate any peroxidase contamination. When this procedure was performed on microsomes from black locust seedlings, however, there was no decrease in the absorbance at 420 nm, nor was there an increase in the absorbance at 450 nm. This suggests that the 420 nm peak in the microsomal suspension from black locust roots is produced by P420, and is not due to peroxidase.

The use of caffeic acid, hydroquinone and 4-chloro-1-naphthol for detection and characterization of peroxidase from the roots of black locust seedlings was employed. In the microsomal fraction supernatant of black locust roots caffeic acid was determined to be metabolized by peroxidase. No metabolism of this substrate was seen without the addition of the supernatant, or $H_2O_2$, nor did the microsomal fraction metabolize this

substrate. Therefore, no peroxidase was contained within the microsomal fraction; all peroxidase activity was found within the microsomal fraction supernatant. Furthermore, the peak found at 420 nm within the CO difference spectrum of the microsomal fraction was not generated by peroxidase.

Hydroquinone and 4-chloro-1-naphthol were also used as substrates for peroxidases, and both were quickly metabolized by peroxidases. The conversion of hydroquinone to benzoquinone was very rapid when the total protein extract (low g force supernatant collected following centrifugation) from the black locust roots was added along with hydrogen peroxide, as seen in **Figure 8**. The spectrum with the lowest absorbance at 250 nm would be that of the substrate hydroquinone, which has peaks at 280 and 225 nm. A spectrum was taken every minute following addition of the protein extract, the first having the lowest absorbance at 250 nm, the last with the greatest at this wavelength. The enzymatic oxidation of hydroquinone to benzoquinone was further studied by determining the kinetics of the reaction. At a protein concentration of 3.6 μg/mL (10.8 μg of protein), 0.01% $H_2O_2$, and 3.3 μM of ascorbate, the velocities of the production of benzoquinone were determined at hydroquinone concentrations of 0.1404 to 0.7020 mM. The $K_m$ was determined to be 1.2 mM and the $V_{max}$ was $7.7x10^{-4}$ mmole/min, using the Lineweaver-Burk plot in **Figure 9**. All hydroquinone and 4-chloro-1-naphthol assays were performed using a total protein extract obtained by homogenization of the roots in **Buffer L**.

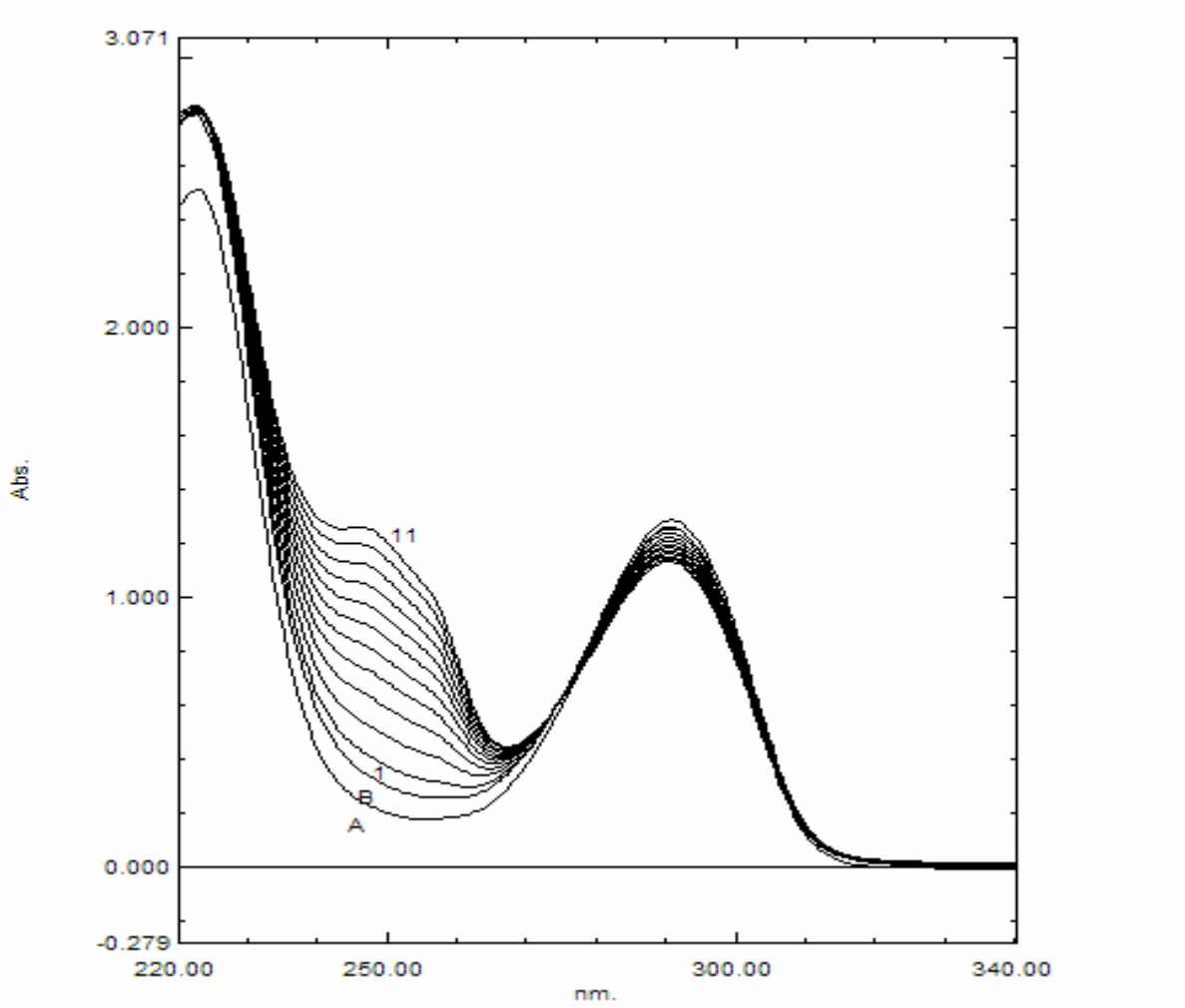



**Figure 8**: **Peroxidase Oxidation of Hydroquinone to Benzoquinone**. Reaction mixture was 50 mM potassium phosphate (pH 7.0), 0.5 mM hydroquinone, 0.01% $H_2O_2$, and is labeled **A**. The reaction was initiated by the addition of 10 µL of total protein extract (13.6 µg of protein). A spectrum was taken after addition of the protein extract and is labeled **B**. The substrate has a peak at 225 and 280 nm, with a large dip around 250 nm. When hydrogen peroxide and protein extract were added, the product benzoquinone produced an increase in absorbance around 250 nm. Every spectrum above the spectrum labeled **B** was taken 1 minute apart. Spectrums labeled **1** and **11** were taken at 1 and 11 minutes following the addition of the protein extract, respectively.

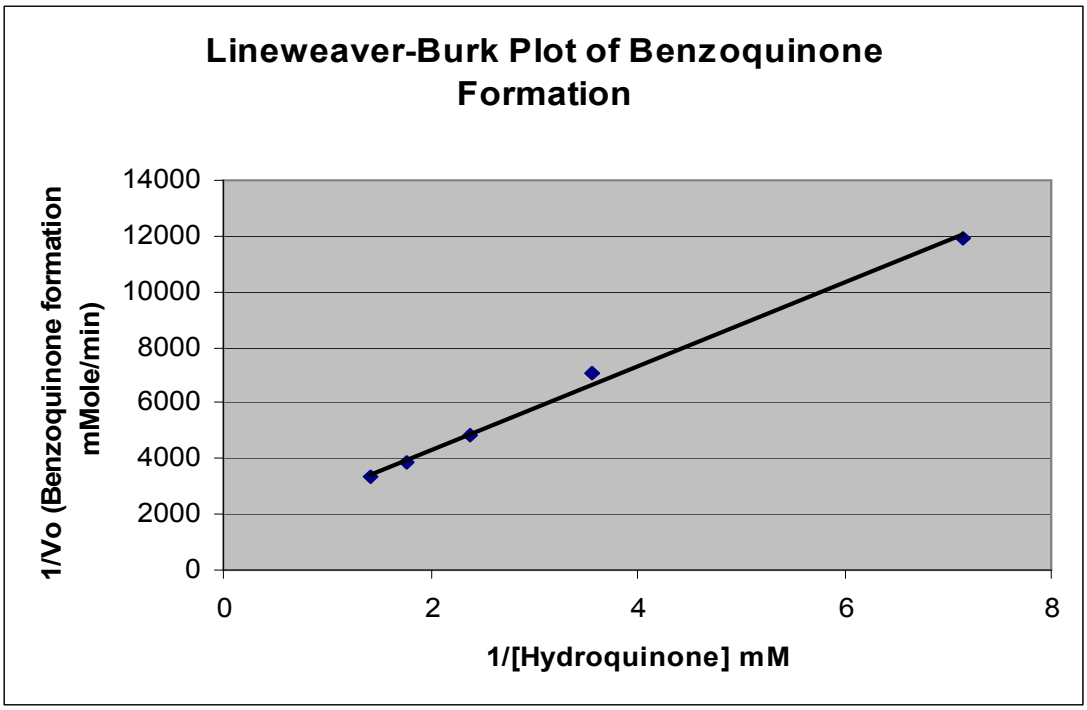



**Figure 9: Lineweaver-Burk Plot of Benzoquinone Formation**. The reaction kinetics of the catalysis of hydroquinone by peroxidase was determined to have a Km of 1.2 mM and a Vmax of $7.7x10^{-4}$ mmole/min. Reaction mixture was 50 mM potassium phosphate (pH 7.0) and 0.01% $H_2O_2$ and hydroquinone. Concentrations of hydroquinone were 0.140, 0.281, 0.421, 0.562 and 0.702 mM. The reaction was initiated by the addition of 10 µL of total protein extract (13.6 µg of protein). The equation for the linear plot is $Y=1500(X) + 1300$ with an $R^2$ of 0.9952.

The metabolism of 4-chloro-1-naphthol can also be seen by taking the spectrum of the sample at different times, as seen in **Figure 10**. The substrate has no absorbance at the wavelengths in this spectrum. Every minute following addition of the protein extract another spectrum was taken, and product formation is shown by the increases in absorbance at all wavelengths.

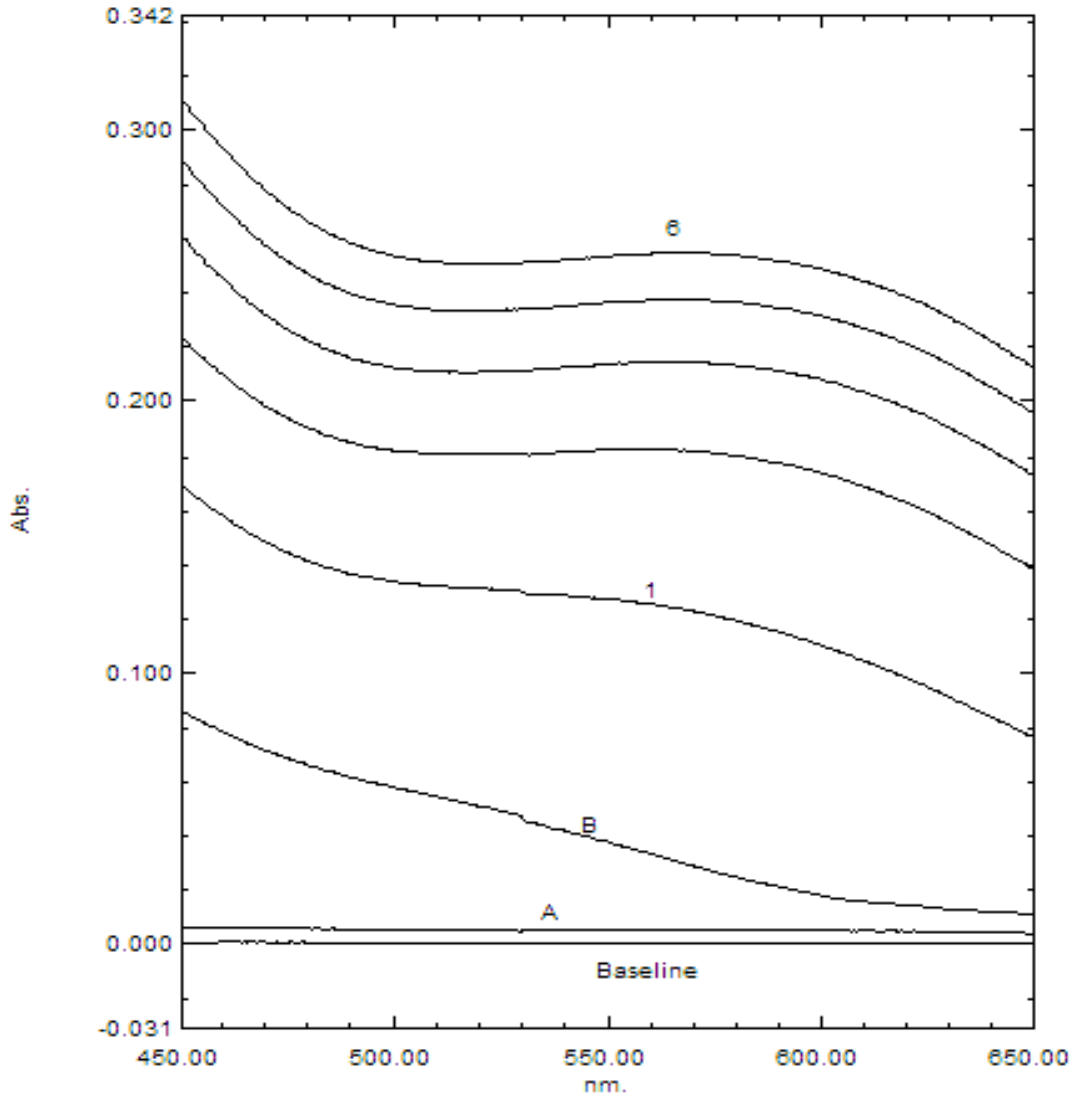
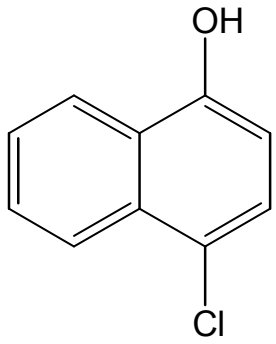
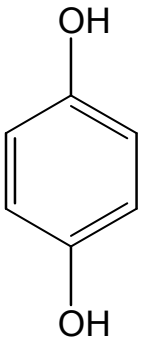


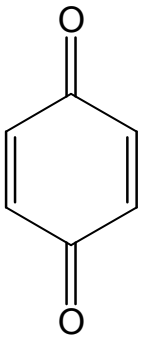

**Figure 10: Peroxidase Oxidation of 4-chloro-1-naphthol**. Formation of a product from the catalysis of 4-chloro-1-naphthol by peroxidase. Reaction mixture contained 20 mM Tris-HCl (pH 7.0), 500 mM NaCl, 0.826 mM 4-chloro-1-naphthol, 4.67% methanol and 0.005% $H_2O_2$. The spectrum of the reaction mixture is labeled **A**. Reaction was initiated by the addition of 10 µL of total protein extract (13.6 µg of protein), and the spectrum after its addition is labeled **B**. The substrate does not absorb light at any of these wavelengths in this spectrum. When either protein extract or hydrogen peroxide was not added to the substrate, no product was formed. Spectra were taken at one minute intervals after protein extract addition. Spectra labeled **1** and **6** were taken at the time intervals one and six minutes, respectively.



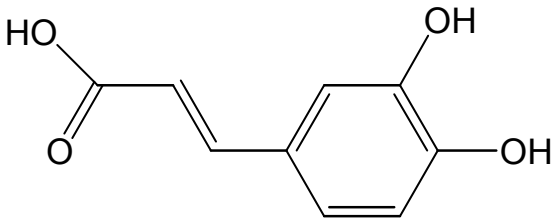

4-Chloro-1-naphthol




Hydroquinone




Benzoquinone




Caffeic Acid

**Figure 11: Substrates and Products of Peroxidase Catalyzed Reactions.** Caffeic Acid, 4-chloro-1-naphthol and hydroquinone are all oxidized by peroxidase extracted from the roots of black locust seedlings. Benzoquinone is formed from the oxidation of hydroquinone by peroxidase.

*Poplar & Mung Bean Analysis*

Extractions were performed on other plant species including a poplar branch and mung bean sprouts. Mung bean sprouts have been shown to contain cytochromes P450 (*104*), the poplar tree also seemed to be a good candidate from its ability to grow quickly, in a variety of environments and its use in current phytoremediation projects (*115*). The poplar tree branch was shown to have a very large peak within the CO difference spectrum of its microsomal supernatant. Interestingly, no dip at 440 nm was observed, suggesting very low or zero concentration of peroxidases. The microsome suspension was very concentrated with chlorophylls that made obtaining a clear difference spectrum

difficult. Even with this interference a peak at 420 nm, with a significant A420-490, was observed, as well as a broad peak at 450 nm. These peaks may be seen in **Figure 12**. This broad peak at 450 nm is likely the result of P450s.

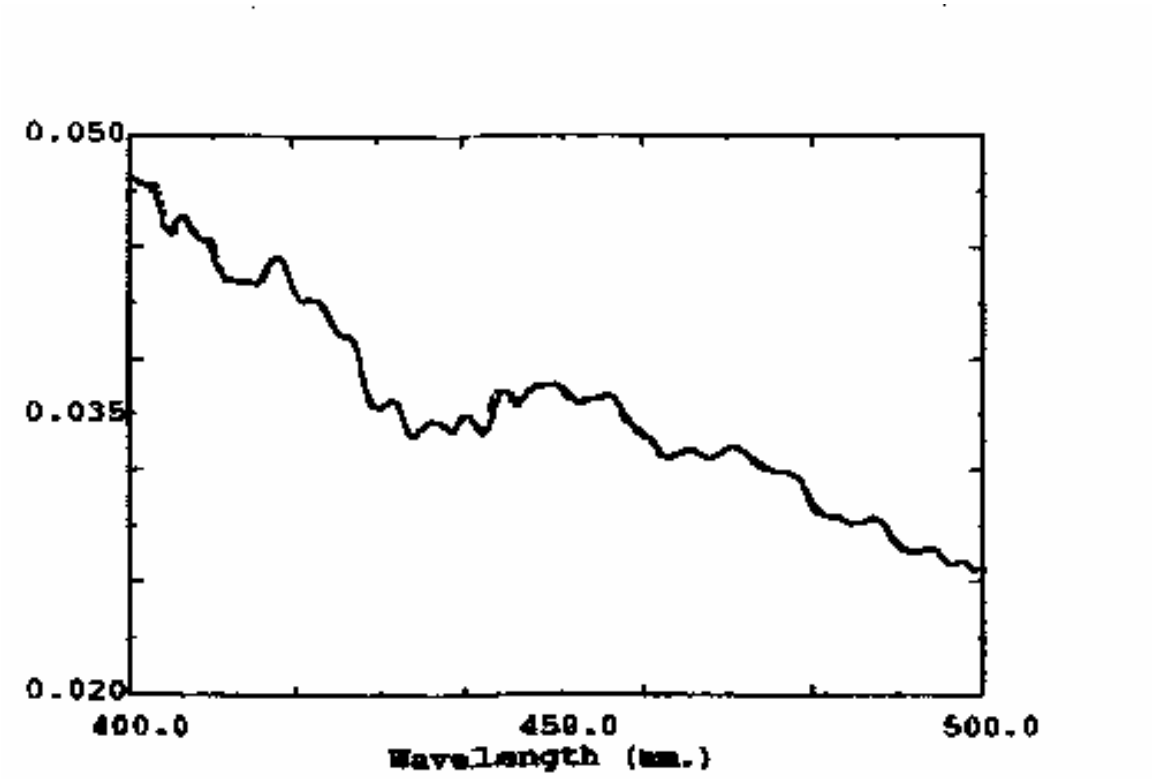



**Figure 12: Poplar Microsomal CO Difference Spectrum**. CO difference spectrum obtained from microsomes homogenized and suspended in **Buffer Q** and obtained from the living tissue of branches from *Populus tremuloides* from Marquette, MI. The cambium and phloem layers were homogenized in **Buffer F** at pH 6.7, and followed **Centrifuge Fractionation C**. With the visibly high amount of chlorophylls within the microsomes, the spectrum seen above is cloudy. However, a discernable broad peak at 450 nm is visible. Absorbance values at 420, 450 and 490 nm are 0.042, 0.036 and 0.028, respectively. Concentrations were found using the same molar coefficients as before (107), and were 1.3E-4 mM and 9E-5 for P420 and P450, respectively.

Mung bean seedlings were grown without light so they were etiolated so as not to have any interference in the CO difference spectrum by chlorophylls. Exposed sprouts from the first set of seedlings, **Mung Group A** were wounded at an age of 17 days and treated with 25 mM $MnCl_2$ in tap water. Manganese had been previously found to be an inducer of P450 in plants (*92*). The control group of **Mung Group A** was not wounded and watered with tap water with no xenobiotic. Four days later the seedlings of **Mung Group A** were homogenized in 1 ml of **Buffer M** (containing 10 % PVPP) per gram of sample, using a kitchen blender. Microsomes from the filtrate were collected using **Fractionation D**. Microsomes were suspended and homogenized in **Buffer S**. Sprouts from **Mung Group B** were grown for 9 days, wounded and exposed to 25 mM $MnCl_2$. The sprouts were homogenized, centrifuged and the microsomes suspended with the same procedure as **Mung Group A** three days later. Sprouts from **Mung Group C** were grown as before for 11 days, wounded and exposed to 25 mM $MnCl_2$ for 2.5 days. The homogenization, centrifugation and suspension of microsomes were performed as before.

Microsomal protein concentration was assayed using the Bradford Assay. The concentration of microsomal protein within the microsomes per gram of tissue for **Mung Group A** was 1.07 mg/g for the exposed and 0.685 for the control. **Mung Group B** had concentrations for the exposed and control at 0.488 and 0.322 mg/g, respectively. **Mung Group C** consisted of three groups of exposed sprouts and three groups for the control. The exposed had an average of 0.304 and the control an average of 0.169 mg/g. **Figure 13** shows the values and standard deviation within the exposed and control groups of **Mung Group C.** All of the groups of exposed sprouts showed a major increase in microsomal protein concentration over that of their respective control group. In values of

percentages this corresponds to an increase of 56.2, 51.6 and 82.7% for **Mung Group A**, **B** and **C** respectively. This shows that mung bean seedlings respond to changes in their environment, most likely by producing enzymes to counter the change.

Cytochrome P450 concentration in the microsomal suspension was measured in both control and exposed groups of seedlings using the CO difference spectrometry. The microsomes of exposed sprouts in **Mung Group A** showed a presence of P450, whereas the control sprouts showed no evidence of P450. The CO difference spectrum produced by the microsomes of the exposed sprouts of **Mung Group A** may be seen in **Figure 14**, which was nearly identical to the CO difference spectrum produced from the microsomes of **Mung Group B**. Control sprouts showed no peaks at 420 nor 450 nm, and no changes in absorbance when compared to A490. These results show that the concentration of P450s in mung bean seedlings is increased when the plant is under stress and needs to defend itself, either from a wound or xenobiotics.

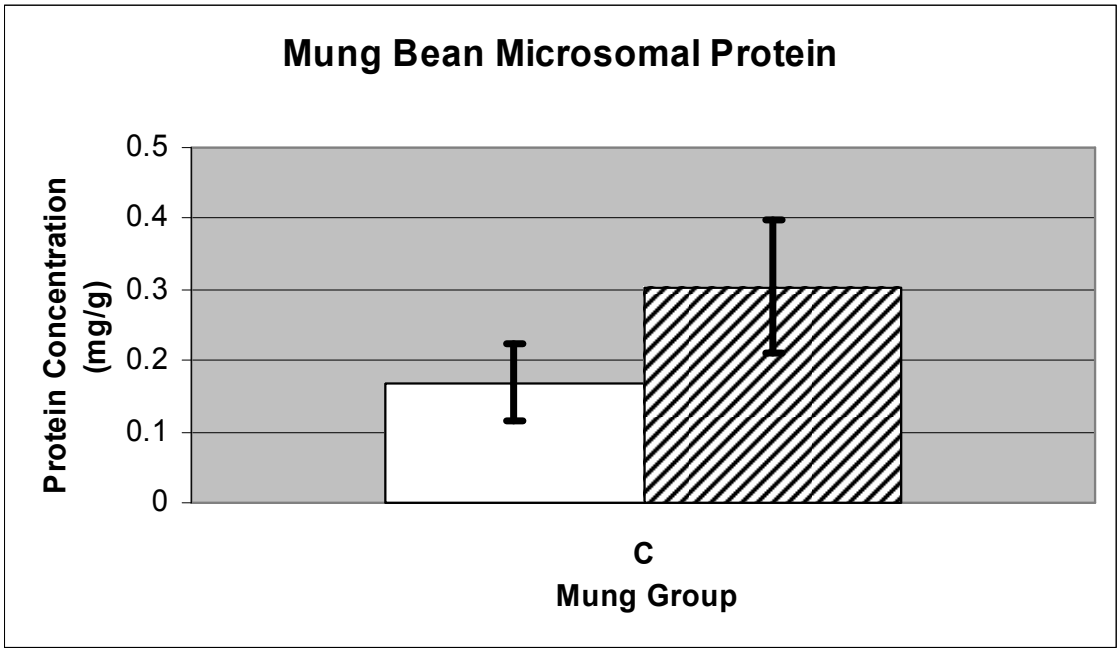



**Figure 13: Mung Bean Sprout Microsomal Protein**. **Mung Group C** was comprised of six groups of sprouts, three were controls (open) three were wounded and exposed to 25 mM $MnCl_2$ (hashed). Standard deviations were +/-0.056 and 0.093 for the control and exposed, respectively. The equation of the standard curve was Y=0.0121(X) + 0.0767 with an $R^2$ of 0.964.

Cytochrome P450s exist in nearly all forms of life, normally in very small amounts, especially in respect to plants. This problem is complicated by two additional anomalies, according to Daisuku Ohta in a personal communication (*114*). One is the fact that many pigments will be extracted from plant cells and be contained within the microsomes. These pigments turn the homogenized microsomal suspension very cloudy, evidently interfering with the P450 peak to be observed. The second is that when P450s are in the reduced CO-complex, the enzyme will turn into P420 rather rapidly, due to the enzyme being unstable in this form.

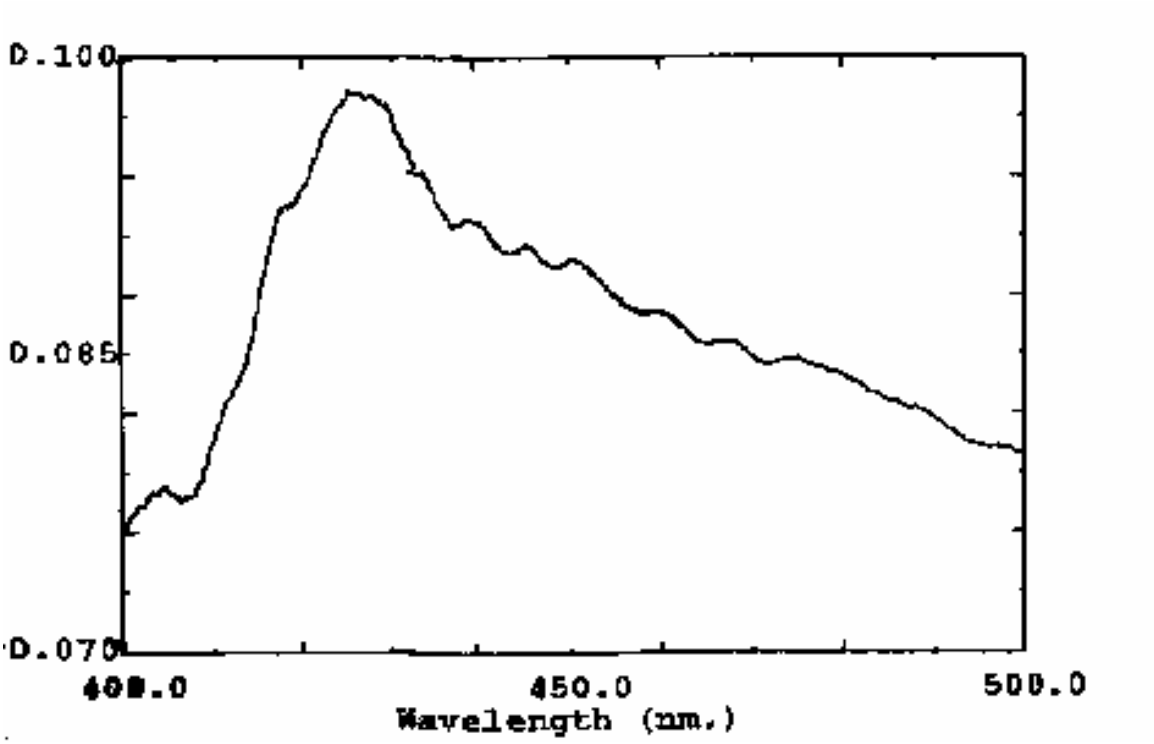



**Figure 14: Mung Bean Microsomal Protein CO Difference Spectrum**. Carbon Monoxide Difference Spectrum of the microsomal fraction obtained from sprouts **Mung Group A** that were exposed and wounded. The final method for the CO difference spectrum was used to generate this spectrum. Absorbance values are 0.093, 0.090 and 0.082 for 420, 450 and 490 nm, respectively. These correspond to P420 and P450 concentrations of 10E-5 and 9E-5 mM, respectively.

# CONCLUSIONS

The goal of this research was to determine if black locust seedlings produced an enzyme of the cytochrome P450 family and/or a peroxidase to be used in the process of phytoremediation, more specifically phytodegradation. As stated before, other plant species have been induced to produce higher quantities of the P450 enzyme in response to the presence of xenobiotics (*92*). In this project, black locust seedlings were exposed to individual or multiple xenobiotics to determine if they would respond in a way that would prove they participate in phytodegradation. This was measured by comparing both the solubilized and microsomal protein concentrations in xenobiotic-exposed and control seedlings, assaying P450 concentration by CO difference spectrometry, and qualitatively determining the presence of peroxidase.

As seen in **Figure 3** and **4** black locust seedlings exposed to DEET, TCE and a combination of Monuron and TCE had an increased concentration of soluble and microsomal protein within their roots compared to that of their respective controls. The majority of all protein extracted from black locust roots was soluble, only a small amount came from the microsomes. Both the microsomal and soluble protein concentrations of exposed roots were always greater than the control. The increase in microsomal protein does not imply that the seedlings are producing additional amounts of P450 to degrade the added xenobiotics, as will be discussed later. These results indicate that production of cytosolic and membrane bound proteins is increased in black locust seedlings as a response to xenobiotic exposure.

To determine if any cytochromes P450 were present and if so, their subsequent concentration, the CO difference spectrometry assay was employed. A major advantage of this assay is that it is very non-specific in respect to the many different types of P450. This was important for this project, since P450 in general was being analyzed, not a specific enzyme of this superfamily. The microsomal supernatant fractions could not be analyzed for P450, due to the presence of peroxidase, therefore, no conclusions could be made on whether the conditions of the extraction were effective in preserving P450. Only the microsome suspensions could be analyzed for any presence of P450. Many of the microsome suspensions produced from black locust roots produced a peak around 420 nm, as seen in **Figure 7**, and had a positive A420-490. However, few of the microsome suspensions would have a positive A450-490, and none of these CO difference spectra contained a peak at 450 nm. These results indicate that P450 was seen, in the form of P420. Since these spectra were produced from the microsomal fraction suspensions, P450 was denaturing to P420 following collection of the microsomes. This could be due to P450 denaturing to P420 following addition of the sodium dithionite (*116*).

However, other conditions may also cause the P450 identifying peak not to be seen. For example, pigments exist within the microsomes and will cover the peak (*116*). Also, it is a possibility that other cellular components could exist which may interfere with obtaining a proper P450 peak. As stated before, hydrophobic cellular compounds interfere with the extraction of active P450 enzymes, and some of these may also interfere with the CO difference spectrum (*117*).

There are two known characteristics that a compound must have to be bound to the active site of P450. One is that the molecule must be small enough to fit in the active

site; second is that said molecule must be hydrophobic. Compounds containing amines or hydroxyl groups which are hydrophobic enough will interfere with the CO difference spectrum of P450 (*117*). Also, the products of metabolism of xenobiotics may interfere with the spectrum. It is still believed, however, that the two main reasons for not obtaining a P450 peak are pigments interfering with the spectrum and the denaturing of P450 to P420.

The CO difference spectrum is not only useful in quantifying and detecting P450, but in the detection of peroxidase. When a solution containing active peroxidase is subjected to the same CO difference spectrum procedure as that of P450, a highly distinct peak at around 420 nm and a large dip at 440 nm is visible. This characteristic spectrum was also seen by Rodgers *et al.* (*110*), but in a suspension of microsomes. This is a major disadvantage for determining if P450 is denaturing to P420, more specifically being removed from the membrane, during extraction or recovering the microsomes. This is due to the peroxidase being soluble, and therefore contained within the microsomal fraction supernatant. However, the fact that peroxidase and P420 are seen in the CO difference spectra (**Figures 6** and **7**), proves the efficacy of the reagents and procedure. In doing so, the fact that a spectra showing native P450 was not seen is in no way due to any part of the procedure or reagents, but more indicative of interfering compounds and/or denaturing to P420.

Cytochromes P450 have been extracted from many other plant species and were assayed with CO difference spectrometry. Other than black locust, mung bean sprouts and native poplar were assayed for microsomal protein concentration and P450 as stated before. In all of the mung bean sprout groups, the exposed/wounded groups contained a

much larger concentration of microsomal protein than the control groups. For **Mung Group A**, **B** and **C** the increase in microsomal protein per gram of sample found in the exposed/wounded sprouts were much greater than their control. This indicates that the mung bean sprouts respond to the xenobiotic and wounding by producing additional membrane bound proteins. When a CO difference spectrum was performed on microsome suspensions from exposed/wounded mung bean sprouts a spectrum was obtained that was reminiscent of the spectrum seen in **Figure 12**. No peak was ever seen in these spectra at 450 nm, but peaks at around 420 nm were always seen. This would indicate that P450 is denaturing to P420, as with the black locust samples. The peaks at 420 nm were very large and had a high A420-490. The spectra also had a positive A450-490. The reason for the large peak at 420 nm and no peak at 450 nm would be due to the same reasons as with the black locust samples as stated previously, due to pigments and denaturing to P420.

The quaking aspen (poplar) was also analyzed as stated before. However, unlike the black locust and mung bean, the microsomal fraction suspension did show a discernable peak at 450 nm, seen in **Figure 10**, and is believed to be a form of P450. The extraction was performed on the living tissue of a branch, and therefore contained a visible amount of chlorophyll. This fact combined with the other cellular pigments would cause the spectrum to not be smooth.

Cytochromes P450 were only one family of enzymes assayed for. As stated before, peroxidases were found to exist in the black locust seedling roots by observance of their characteristic CO difference spectrum within the microsomal supernatant fraction. Peroxidase has also been found to metabolize xenobiotics (*84*), and was found

in this paper to oxidize the same xenobiotics as P450. In this study as well as with Rodgers *et. al.* (*110*), peroxidases were found to be contained within the microsomes. No indication of peroxidase being in the microsomes of black locust roots was seen in the CO difference spectra, since no dip at 440 nm accompanied the peak at 420 nm. The caffeic acid assay was then employed to determine the fractions that contained peroxidase. The microsomal supernatant fraction was found to metabolize the caffeic acid, however, no activity was found in the microsome suspension. This indicates that the 420 nm peak found in CO difference spectra from the microsome suspensions of black locust roots was due to P420. The total protein extract was then assayed for peroxidase activity using hydroquinone and 4-chloro-1-naphthol as substrates. Both of these substrates were readily oxidized by peroxidase, as stated and shown before (**Figures 8** and **9**). Kinetics of the production of the metabolites were only found with hydroquinone as the substrate. The $K_m$ was determined to be 1.2 mM and the $V_{max}$ was $7.7x10^{-4}$ mmole/min, for the formation of benzoquinone.

In summary, the black locust seedlings responded to the presence of the xenobiotics by producing additional protein, both cytosolic and membrane bound. These could be enzymes, possibly P450 or peroxidase that have the ability to be involved in phytodegradation, and as stated before were determined to metabolize xenobiotics. No peak at 450 nm was found in any CO difference spectrum from the black locust seedling's microsomes, however, a peak at 420 nm indicates that P420 was present. The reagents and procedure for the CO difference spectrum are reliable due to the appearance of peroxidases in the spectra. The work done on mung bean seedlings indicates a marked

increase in P450s when exposed to a xenobiotic and wounded, where P450s are involved in a response.

Further research including additional purification with detergents and affinity column chromatography (*101*, *104*, *116*, *118*) would determine the involvement of P450 when black locust seedlings are exposed to a xenobiotic. When looking at the response of an organism to exposure to a xenobiotic, enzymes are not the only assayable macromolecule. The mRNA expressed in this response can also be detected and quantified. DNA microarrays have been used to determine the genes expressed and the amount of their expression in response to xenobiotics (*119*), by analyzing the amount of mRNA transcribed. This would indicate the amount of a protein that will be produced. Also, the use of the reverse-transcriptase polymerase chain reaction (RT-PCR) (4) could be used to monitor the production of mRNA when the organism is exposed to the xenobiotic(s). The use of real-time RT-PCR is highly useful in quantifying the amount of mRNA produced from the exposure to xenobiotics (*120*, *121*), and therefore the amount of enzyme. Real-time RT-PCR is also used to validate results from DNA microarrays, and is much more sensitive than all other methods of mRNA quantification (*122*). The aforementioned methods are very useful in quantifying the expression of genes: they will greatly define the response on a transcriptional level and would compliment the analysis of an enzyme's function and metabolism of xenobiotics.

## REFERENCES


1. Klingenberg, M. Pigments Of Rat Liver Microsomes. *Arch. Biochem. Biophys*. **1958**, *75*, 376-386.

2. Omura, T. Forty Years Of Cytochrome P450. *Biochem. Biophys. Res. Comm*. **1999**, *266*, 690-698.

3. Dus, K.; Kafagiri, M.; Yu, C. A.; Erbes, D. L.; Gunsalus, I. C. Chemical Characterization Of Cytochrome $P450_{CAM}$. *Biochem. Biophys. Res. Commun*. **1970**, *40*, 1423-1430.

4. Kahn, R.; Durst, F. Function And Evolution Of Plant Cytochrome P450. *Recent Adv. Phytochem.* **2000**, *34*, 151-189

5. Coon, M. J.; Vaz, A .D. N.; Bestervelt, L .L. Cytochrome P450. Two Peroxidative Reactions Of Diversozymes. *FASEB. J.* **1996**, *10*, 428-434.

6. Nelson, D. Cytochrome P450 Homepage. http://drnelson.utmem.edu/cytochromeP450.html (accessed 5/31/2006). The Dupont Co.

7. Liang, P.; Pardee, A. B. Differential Display Of Eukaryotic Messenger Rna By Means Of The Polymerase Chain Reaction. *Science* **1992**, *257*, 967-971.

8. Newman, T.; DeBruijn, F. J.; Green, P.; Keegstroy, K.; Kende, H.; Mcintosh, L.; Ohlrogge, J.; Raikhel, N.; Somerville, S.; Thomashow, M. Genes Galore: A Summary Of Methods For Accessing Results From Large-Scale Partial Sequencing Of Anonymous Arabidopsis cDNA Clones. *Plant Phys*. **1994**, *106*, 1241-1255.

9. Yu, C. A.; Gunsalus, I. C. Cytochrome $P450_{CAM}$ I. Crystallization And Properties. *J. Biol. Chem.* **1974**, *249*, 94-101.

10. Hanieu, M.; Armes, L. G.; Tanaka, M.; Yasunobu, K. T.; Shastry, R. S.; Wagner, G. C.; Gunsalus, I. C.; The Primary Structure Of The Monooxygenase Cytochrome $P450_{CAM}$. *Biochem. Biophys. Res. Commun.* **1982**, *105*, 889-894.

11. Poulos, T. L.; Finzel, B. C.; Gunsalus, I. C.; Wagner, G. C.; Kraut, J. The 2.6-A Structure Of *Pseudomonas putida* Cytochrome P450. *J. Biol. Chem.* **1985**, *260*, 16122-16130.

12. Poulos, T. L.; Finzel, B. C.; Howard, A. J. High Resolution Crystal Structure Of Cytochrome $P450_{CAM}$. *J. Mol. Biol.* **1987**, *195*, 687-700.

13. Graham, S. E.; Peterson, J. A. How Similar Are P450s And What Can Their Differences Teach Us?. *Arch. Biochem. Biophys.* **1999**, *369*, 24-29.

14. Stern, J. O.; Peisach, J. A Model Compound Study Of The Co-Adduct Of Cytochrome P-450. *J. Biol. Chem*. **1974**, *249*, 7495-7498.

15. Williams, P. A.; Cosme, J.; Sridhar, V.; Johnson, E. F.; McRee, D. E. Mammalian Microsomal Cytochrome P450 Monooxygenase: Structural Adaptations For Membrane Binding And Functional Diversity. *Mol. Cell*. **1999**, *5*, 121-131

16. Sakaguchi, M.; Mihara, K.; Sato, R. A Short Amino-Terminal Segment Of Microsomal Cytochrome P-450 Functions Both As An Insertion Signal And As A Stop-Transfer Sequence. *EMBO J*. **1987**, *6*, 2425-2431.

17. Omura, T. In *Handbook of Experimental Pharmacology*. Schenkman, J. B.; Greim, H.; Eds.; Springer-Verlag: Berlin, 1993; Vol. 105, pp 61-69.

18. Omura, T.; Sanders, E.; Estabrook, R. W.; Cooper, D. Y.; Rosenthal, O. Isolation From Adrenal Cortex Of A Non-Heme Iron Protein And A Flavoprotein Functional As A Reduced Triphosphopyridine Nucleotide-Cytochrome P-450 Reductase. *Arch. Biochem. Biophys*. **1966**, *117*, 660-673.

19. Lu, A. Y. H.; Coon, M. J. Role Of Hemoprotein Cytochrome P450 In Fatty Acid Ω-Hydroxylation In A Soluble Enzyme System From Liver Microsomes. *J. Biol. Chem*. **1968**, *243*, 1331-1332.

20. Masters, B. S. S.; Baron, J.; Taylor, W. E.; Isaacson, E. L.; Lospallutol, J. Immunological Studies On The Electron Transport Chains Involving Cytochrome P450. I. Effects Of Antibodies To Pig Liver Microsomal Reduced Triphosphopyridine Nucleotide-Cytochrome C Reductase And The Non-Heme Iron Protein From Bovine Adrenocortical Mitochondria. *J. Biol. Chem*. **1971**, *246*, 4143-4150.

21. Beveniste, I.; Lesof, A.; Hasenfratz, M. P.; Kochs, G.; Durst, F. Multiple Forms Of NADPH-Cytochrome P450 Reductase In Higher Plants. *Biochem. Biophys. Res. Commun*. **1991**, *177*, 105-112.

22. Shet, M. S.; Sathasivan, K.; Arlotto, M. A.; Mehdy, M. C.; Estabrook, R. W. Purification, Characterization And cDNA Cloning Of An NADPH-Cytochrome P450 Reductase From Mung Bean. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 2890-2894.

23. Louerat-Oriou, B.; Perret, A.; Pompon, D. Differential Redox And Electron-Transfer Properties Of Purified Yeast, Plant And Human NADPH-Cytochrome P450 Reductases Highly Modulate Cytochrome P450 Activities. *Euro. J. Biochem*. **1998**, *258*, 1040-1049.

24. Rosco, A.; Pauli, H. H.; Priesner, W.; Kutchan, T. M. Cloning And Heterologus Expression Of NADPH-Cytochrome P450 Reductases From The Papaveraceae. *Arch. Biochem. Biophys*. **1997**, *348*, 369-377.

25. Tanahashi, T.; Zenk, M. H. One Step Enzymatic Synthesis Of Dihydrosanquinarine From Protopine. *Tetrahedron Lett*. **1988**, *29*, 5625-5628.

26. Tanahashi, T.; Zenk, M. H. Elicitor Induction And Characterization Of Microsomal Protopine-6-Hydroxylase, The Central Enzyme In Benzophenathridine Alkaloid Biosynthesis. *Phytochemistry* **1990**, *29*, 1113-1122.

27. Bauer, W.; Zenk, M. H. Formation Of Both Methylenedioxy Groups In The Alkaloid (S)-Stylopine Is Catalyzed By Cytochrome P-450 Enzymes. *Tetrahedron Lett*. **1989**, *39*, 5257-5260.

28. Bauer, W.; Zenk, M. H. Two Methylenedioxy Bridge Forming Cytochrome P-450 Dependent Enzymes Are Involved In (S)-Stylopine Biosynthesis. *Phytochemistry* **1991**, *30*, 2953-2961.

29. Rueffer, M.; Zenk, M. Canadine Synthase From *Thalictrum tuberosum* Cell Cultures Catalyzes The Formation Of The Methylenedioxy Bridge In Berberine Synthesis. *Phytochemistry* **1994**, *36*, 1219-1223.

30. Gerardy, R.; Zenk, M. H. Formation Of Salutaridine From (R)-Reticuline By A Membrane-Bound Cytochrome P450 Enzyme From *Papaver somniferum*. *Phytochemistry* **1992**, *32*, 79-86.

31. Zenk, M.; Gerardy, R.; Stadler, R. Phenol Oxidative Coupling Of Benzylisoquinoline Alkaloids Is Catalyzed By Regio- And Stereo-Selective Cytochrome P-450 Linked Plant Enzymes: Salutaridine And Berbamunine. *J. Chem. Soc. Shem. Commun*. **1989**, *22*, 1725-1727.

32. Nasreen, A.; Rueffer, M.; Zenk, M. H. Cytochrome P-450-Dependent Formation Of Isoandrocymbine From Autumnaline In Colchicine Biosynthesis. *Tetrahedron Lett.* **1996**, *37*, 8161-8164.

33. Stadler, R.; Zenk, M. H. The Purification And Characterization Of A Unique Cytochrome P450 Enzyme From *Berberis stolonifera* Plant Cell Cultures. *J. Biol. Chem.* **1993**, *268*, 823-831.

34. Potts, J. M.; Weklych, R.; Conu, E. C. The 4-Hydroxylation Of Cinnamic Acid By Sorghum Microsomes And The Requirement For Cytochrome P-450. *J. Biol. Chem.* **1974**, *249*, 5019-5026.

35. Werck-Reichhart, D. Cytochrome P450s In Phenylpropanoid Metabolism. *Drug Metab. Drug Interact.* **1995**, *12*, 221-243.

36. Dixon, R. A.; Paiva, N. L. Stress-Induced Phenylpropanoid Metabolism. *The Plant Cell* **1995**, 7, 1085-1097.

37. Grand, C. Ferulic Acid 5-Hydroxylase: A New Cytochrome P-450-Dependent Enzyme From Higher Plant Microsomes Involved In Lignin Synthesis. *FEBS Lett*. **1984**, *169*, 7-11.

38. Humphreys, J. M.; Hemm, M. R.; Chapple, C. New Routes For Lignin Biosynthesis Defined By Biochemical Characterization Of Recombinant Ferulate 5-Hydroxylase, A Multifunctional Cytochrome P450-Dependent Monooxygenase. *Proc. Natl. Sci. USA* 1999, *96*, 10045-10050.

39. Heller, W.; Kuhnl, T. Elicitor Induction Of A Microsomal 5-O-(4-Coumaroyl)Shikimate 3'-Hydroxylase In Parsley Cell Suspension Cultures. *Arch. Biochem. Biophys*. **1985**, *241*, 453-460.

40. Kuhnl, T.; Koch, U.; Heller, W.; Wellman, E. Chlorogenic Acid Biosynthesis: Characterization Of A Light-Induced Microsomal 5-O-(4-Coumaroyl)-D-Quinate/Shikimate 3'-Hydroxylase From Carrot (*Daucus carota* L.) Cell Suspenstion Cultures. *Arch. Biochem. Biophys.* **1987**, *258*, 226-232.

41. Bolwell, G. P.; Bozak, K.; Zimmerlin, A. Plant Cytochrome P450. *Phytochemistry* **1994**, *37*, 1491-1506.

42. Otani, K.; Takahashi, T.; Furuya, T.; Ayabe, S. Licodione Synthase, A Cytochrome P450 Monooxygenase Catalyzing 2-Hydroxylation Of 5-Deoxyflavanone, In Cultured *Glycyrrhiza echinata L*. Cells. *Plant Phys.* **1994**, *105*, 1427-1432.

43. Akashi, T.; Aoki, T.; Ayabe, S. Idenitfication Of A Cytochrome P450 Cdna Encoding (2s)-Flavanone 2-Hydroxylase Of Licorice (*Glycyrrhiza echinata* L.*; Fabaceae*) Which Represents Licodione Syntahase And Flavone Synthase Ii. *FEBS Lett.* **1998**, *431*, 287-290.

44. Dewick, P. M. In *Medicinal Natural Products: A Biosynthetic Approach*; John Wiley & Sons: New York, 1997; p 466.

45. Hagmann, M. L.; Heller, W.; Grisebach, H. Induction And Characterization Of A Microsomal Flavonoid 3'-Hydroxylase From Parsley Cell Cultures. *Eur. J. Biochem.* **1983**, *134*, 547-554.

46. Coolbaugh, R. C.; Moore, T. C. Localization Of Enzymes Catalysing Kaurene Biosynthesis In Immature Pea Seeds. *Phytochemistry* **1971,** *10*, 2395-2400.

47. McGarvey, D.; Croteau, R. Terpenoid Metabolism. *The Plant Cell* **1995**, *7*, 1015-1026.

48. Meehan, T. D.; Coscia, C. J. Hydroxylations Of Geraniol And Nerol By A Monoxygenase From *Vinca rosea*. *Biochem. Biophys. Res. Commun.* **1973**, *53*, 1043-1048.

49. Madyastha, K. M.; Meehan, T. D.; Cocia, C. J. Characterization Of A Cytochrome P450 Dependent Monoterpene Hydroxylase From The Higher Plant *Vinca rosea*. *Biochemistry* **1976**, *15*, 1097-1102.

50. Karp, F.; Mihaliak, C. A.; Harris, J. L.; Croteau, R. Monoterpene Biosynthesis: Specificity Of The Hydroxylations Of (-)-Limonene By Enzyme Preparations From Peppermint (*Mentha piperita*), Spearmint (*Mentha spicata*), And Perilla (*Perilla frutescens*) Leaves. *Arch. Biochem. Biophys.* **1990**, *276*, 219-226.

51. Murphy, P. J.; West, C. A. Role Of Mixed Function Oxidases In Kaurene Metabolism *In Echinocystis macrocarpa* Greene Endosperm. *Arch. Biochem. Biophys.* **1969**, *133*, 395-407.

52. Gillard, D. F.; Walton, D. C. Abscisic Acid Metabolism By A Cell-Free Preparation From *Echinocystis lobata* Liquid Endosperm. *Plant Phys*. **1976**, *58*, 790-795.

53. Rahier, A.; Taton, M. The 14 Alpha-Demethylation Of Obtusifoliol By A Cytochrome P-450 Monooxygenase From Higher Plants' Microsomes**.** *Biochem. Biophys. Res. Commun.* **1986**, *140*, 1064-1072.

54. Kahn, R. A.; Bak, S.; Olsen, C. E.; Svendsen, I.; Moller, B. L. Isolation and Reconstitution of the Heme-Thiolate Protein Obtusifoliol 14α-Demethylase from *Sorghum bicolor* (*L*.) Moench. *J. Biol. Chem*. **1996**, *271*, 32944-32950.

55. Peterson, M. A.; Alfermann, A. W.; Reinhard, E.; Seitz, H. U. Immobilization Of Digitoxin 12β-Hydroxylase, A Cytochrome P450-Dependent Enzyme From Cell Cultures Of *Digitalis lanata* EHRH. *Plant Cell Rep.* **1987**, *6*, 200-203.

56. Hayashi, H.; Henaoka, S.; Tanaka, S.; Fukui, H.; Tabutu, M. Glycyrrhetinic Acid 24-Hydroxylase Activity In Microsomes Of Cultured Licorice Cells. *Phytochemistry* **1993**, *34*, 1303-1307.

57. Bak, S.; Kahn, R. A.; Nielsen, H. L.; Moller, B. L.; Halkier, B. A. Cloning Of Three A-Type Cytochromes P450, CYP71E1, CYP98, And CYP99 From *Sorghum bicolor* (L.) Moench By A PCR Approach And Identification By Expression In *Escherichia coli* Of CYP71E1 As A Multifunctional Cytochrome P450 In The Biosynthesis Of The Cyanogenic Glucoside Dhurrin. *Plant Mol. Biol*. **1998**, *36*, 393-405.

58. Koch, B. M.; Sibbesen, O.; Halkier, B. A.; Svendsen, I.; Moller, B. L. The Primary Sequence Of Cytochrome P450tyr, The Multifunctional N-Hydroxylase Catalyzing The Conversion Of L-Tyrosine To *P*-Hydroxyphenylacetaldehyde Oxime In The Biosynthesis Of The Cyanogenic Ggucoside Dhurrin In *Sorghum bicolor* (L.) Moench. *Arch. Biochem. Biophys.* **1995**, *323*, 177-186.

59. Salaun, J.–P.; Helvig, C. Cytochrome P450-Dependent Oxidation Of Fatty Acids. *Drug Metab. Drug Interact.* **1995**, *12*, 262-284.

60. Salaun, J.–P.; Benveniste, I.; Reichhart, D.; Durst, F. Induction And Specificity Of A Cytochrome P-450 Dependent Laurate In-Chain Hydroxylase From Higher Plant Microsomes. *Eur. J. Biochem.* **1981**, *119*, 651-655.

61. Salaun, J.–P.; Simon, A.; Durst, F. Specific Induction Of Lauric Acid Ω-Hydroxylast By Clofibrate, Diethylhexyl-Phtalate And 2,4-Dichlorophenoxyacetic Acid In Higher Plants. *Lipids* **1986**, *21*, 776-779.

62. Soliday, C. L.; Kolattukudy, P. E. Midchain Hydroxylation Of 16-Hydroxypalmitic Acid By The Endoplansmic Reticulum Fraction From Germinating *Vicia faba*. *Arch. Biochem. Biophys*. **1978**, *188*, 338-347.

63. Pinot, F.; Alayrac, C.; Mioskowski, C.; Durst, F.; Salaun, J.–P. New Cytochrome P450-Dependent Reactions From Wheat: Terminal And Sub-Terminal Hydroxylation Of Oleic Acid By Microsomes From Naphthalic Acid Anhydride And Phenobarbital Induced Wheat Seedlings. *Biochem. Biophys. Res. Comm*. **1994**, *198*, 795-803.

64. Croteau, R.; Kolattukudy, P. E. Biosynthesis Of Hydroxyfatty Acid Polymers. Enzymatic Epoxidation Of 18-Hydroxyoleic Acid To 18-Hydroxy-Cis-9,10-Epoxystearic Acid By A Particulate Preparation From Spinach (*Spinacia Oleracea*). *Arch. Biochem. Biophys*. **1975**, *170*, 61-72.

65. Salaun, J.–P.; Weissbart, D.; Helvig, C.; Durst, F.; Mioskowski, C. Regioselective Hydroxylation And Epoxidation Of Lauric Acid And Unsaturated Analogues By Cytochrome P450 In Jerusalem Artichoke Microsomes. *Plant Physiol. Biochem.* **1993**, *31*, 285-293.

66. Salaun, J. –P.; Weisbart, D.; Durst, F.; Pflieger, P.; Mioskowski, C. Epoxidation Of *Cis* And *Trans* $\Delta^9$-Unsaturated Lauic Acids By Cytochrome P450-Dependent System From Higher Plant Microsomes. *FEBS Lett*. **1989***, 246*, 120-126.

67. Weissbart, D.; Salaun, J. –P.; Durst, F.; Pflieger, P.; Mioskowski, C. Regioselectivity Of A Plant Lauric Acid Omega Hydroxylase. Omega Hydroxylation Of *Cis* And *Trans* Unsaturated Lauric Acid Analogs And Epoxygenation Of The Terminal Olefin By Plant Cytochrome P-450. *Biochem. Biophys, Acta*. **1992**, *1124*, 135-142.

68. Bafor, M.; Smith, M. A.; Jonsson, K.; Stobart, K.; Stymne, S.; Biosynthesis Of Vernoleate (*Cis*-12-Epoxyoctadeca-*Cis*-9-Enoate) In Microsomal Preparations From Developing Endosperm Of *Euphorbia Lagascae. Arch. Biochem. Biophys.* **1993**, *303*, 145-151.

69. Lau, M. C.; Harder, P. A.; O'Keefe, D. Low Carbon Monoxide Affinity Allene Oxide Synthase Is The Predominant Cytochrome P450 In Many Plant Tissues. *Biochemistry* **1993**, *32*, 1945-1950.

70. *Plant Peroxidases: 1970-1980*; Gaspar, T.; Penel, C.; Thorpe, T.; Greppin, H. Eds.; Universite De Geneve-Centre De Botanique: Geneve, 1982; 9-13.

71. Welinder, K. G. Plant peroxidases: Structure-Function Relationships. In *Plant Peroxidases: 1970-1980. Topics and Detailed Literature on Molecular, Biochemical, and Physiological Aspects*; University of Geneva: Geneve, 1992; 1-17.

72. Dawson, J. H. Probing Structure-Function Relations In Heme Containing Oxygenases And Peroxidases. *Science* **1988**, *240*, 433-439.

73. Mueller, G. C.; Miller, J. A. The Reductive Cleavage Of 4-Dimethylamino-azobenzene By Rat Liver: The Intracellular Distribution Of The Enzyme System And Its Requirement For Triphosphopyridine Nucleotide. *J. Biol. Chem*. **1949**, *180*, 1125-1136.

74. Cooper, D. Y.; Levin, S. S.; Narasimhulu, S.; Rosenthal, O.; Estabrook, R. W. Photochemical Action Spectrum Of Terminal Xxidase Of Mixed Function Oxidase Systems. *Science* **1965**, *147*, 400-402.

75. O'Keefe, D.; Leto, K. J. Cytochrome P-450 From The Mesocarp Of Avocado (*Persea Americana*). *Plant Phys.* **1989**, *89*, 1141-1149.

76. Frear, D. S.; Swanson, H. R.; Tanaka, F. S. N-Demethylation Of Substituted 3-(Phenyl)-1-Methylureas; Isolation And Characterization Of A Microsomal Mixed Function Oxidase From Cotton. *Phytochemistry* **1969**, *8*, 2157-2169.

77. Werck-Reichhart, D.; Gabriac, B.; Teutsch, H.; Durst, F. Two Cytochrome P-450 Isoforms Catalyzing O-De-Ethylation Of Ethoxycoumarin And Ethoxyresorufin In Higher Plants. *Biochem. J*. **1990**, *270*, 729-735.

78. McFadden, J. J.; Gronwald, J. W.; Ebelein, C. V. In Vitro Hydroxylation Of Bentazon By Microsomes From Naphthalic Anhydride-Treated Corn Shoots. *Biochem. Biophys. Res. Comm*. **1990**, *168*, 206-213.

79. Fonne-Pfister, R.; Gaudin, J.; Kreuz, K.; Ramsteiner, K.; Ebert, E. Hydroxylation Of Primisulfuron By An Inducible Cytochrome P450-Dependent Monooxygenase System From Maize. *Pestc. Biochem. Physiol*. **1990**, *37*, 165-173.

80. Frear, D. S.; Swanson, H. R.; Tanaka, F. S. Metabolism Of Flumetsulam (DE-498) In Wheat, Corn And Barley. *Pestic. Biochem. Physiol*. **1993**, *45*, 178-192.

81. McFadden, J. J.; Frear, D. S.; Mansager, E. R. Aryl Hydroxylation Of Diclofop By A Cytochrome P450 Dependent Monooxygenase From Wheat. *Pestic. Biochem. Physiol*. **1989**, *34*, 92-100.

82. Zimmerlin, A.; Durst, F. Xenobiotic Metabolism In Plants: Aryl Hydroxylation Of Diclofop By A Cytochrome P-450 Enzyme From Wheat. *Phytochemistry* **1990**, *29*, 1729-1732.

83. Frear, D. S.; Swanson, H. R.; Thalacker, F. W. Induced Microsomal Oxidation Of Diclofop, Triasulfuron, Chlorsulfuron, And Linuron In Wheat. *Pestic. Biochem. Physiol.* **1991**, *41*, 274-287.

84. Stiborova, M.; Schmeiser, H. H.; Frei, E. Oxidation Of Xenobiotics By Plant Microsomes, A Reconstituted Cytochrome P450 System And Peroxidases: A Comparative Study. *Phytochemistry* **2000**, *54*, 353-362.

85. Fonne-Pfister, R.; Kreuz, K. Ring-Methyl Hydroxylation Of Chlortoluron By An Inducible Cytochrome P450-Dependent Enzyme From Maize. *Phytochemistry* **1990**, *29*, 2793-2796.

86. Moreland, D. E.; Corbin, F. T.; McFarland, J. E. Oxidation Of Multiple Substrates By Corn Shoot Microsomes. *Pestic. Biochem. Physiol*. **1993**, *47*, 206-214.

87. Didierjean, L.; Gonet, L.; Perkins, R.; Lau, S-M. C.; Schaller, H.; O'Keefe, D. P.; Werck-Reichhart, D. Engineering Herbicide Metabolism In Tobacco And Arabidopsis With CYP76B1, A Cytochrome P450 Enzyme From Jerusalem Artichoke. *Plant Phys*. **2002**, *130*, 179-189.

88. Zimmerlin, A.; Durst, F. Aryl Hydroxylation Of The Herbicide Diclofop By A Wheat Cytochrome P-450 Monooxygenase: Substrate Specificity And Physiological Activity. *Plant Phys*. **1992**, *100*, 874-881.

89. Brenda: The Comprehensive Enzyme Information System. http://www.brenda.uni.koeln.de (accessed 8/10/2006), Cologne University Bioinformatics Center.

90. Durst, F.; Benveniste, I.; Salaun, J.-P.; Werck, D. Function And Diversity Of Plant Cytochrome P450. In *Cytochrome P450 8th International Conference*; Lechner, M. C., Ed.; John Libbey Eurotext: Paris 1994; 23-30.

91. Sladek, N. E.; Mannering, G. J. Evidence For A New P-450 Hemoprotein In Hepatic Microsomes From Methylcholanthrene Treated Rats. *Biochem. Biophys. Res. Commun*. **1966**, *24*, 668-674.

92. Reichhart, D.; Salaun, J,-P.; Benveniste, I.; Durst, F. Time Course Induction Of Cytochrome P-450, NADPH-Cytochrome *C* Reductase, And Cinnamic Acid Hydroxylase By Phenobarbital, Ethanol, Herbicides, And Manganese In Higher Plant Microsomes. *Plant Phys*. **1980**, *66*, 600-604.

93. Cross, J. W. Phytoremediation Processes. http://www.mobot.org/jwcross/phytoremediation (accessed 6/12/2003), Missouri Botanical Garden.

94. Keresztesi, B. The Black Locust *Robinia Pseudoacacia*, Silvicultural Aspecs, Utilization. *Unasylva* **1980**, *32*, 22-33.

95. Carpenter, S. B.; Eigel, R. A. Reclaiming Surface Mines With Black Locust Fuel Plantations. *Canadian Reclam. Assoc*. **1979**, *4*, 239-254.

96. Fletcher, J. S.; Nagel, D. Microflora Involved In Phytoremediation Of Polyaromatic Industrial Contaminants-Final Report Executive Summary. <http://ipec.utulsa.edu/3.d/3_final.html (accessed 9/20/2006), University of Oklahoma, 2002.

97. Strand, S. E.; Gordon, M. P. Using Trees To Remediate Groundwater Contaminated With Chlorinated Hydrocarbons. http://emsp.em.doe.gov/emspprojects1996_2003/subsurface/ 548891998.pdf (accessed 9/20/2006), Environmental Management Science Program, 1998.

98. Demnerova, K.; Stiborova, H.; Leigh, M. B.; Pieper, D.; Pazlarova, J.; Brenner, V. Macek, T.; Mackova, M. Bacteria Degrading PCBs And CBs Isolated From Long-Term PCB Contaminated Soil. *Water, Air, & Soil Pollution: Focus* **2003**, *3*, 47-55.

99. Boring, L. R.; Swank, W. T. Symbiotic nitrogen fixation in regenerating black locust (*robinia pseudoacacia* l.) Stands. *Forest Sci*. **1984**, *30*, 528-537.

100. Georgiev, G.; Atanasov, B.; Kalmuckov, K.; Alexandrova, E.; Uzunova, A. Assimilates And Nutrients Partitioning And Efficiency Of Nitrogen Fixation Of Black Locust (*Robinia Pseudoacacia* L.) Grown In The Excess Of Cu In Soil. *Bulg. J. Plant Physiol.* **1996**, *22*, 40-55.

101. Werck-Reichhart, D.; Benveniste, I.; Teutsch, H.; Durst, F.; Gabriac, B. Glycerol Allows Low-Temperature Phase Separation Of Membrane Proteins Solubilized In Triton X-114: Application To The Purification Of Plant Cytochromes P-450 And $B_5$. *Anal. Biochem*. **1991**, *197*, 125-131.

102. Hefner, J.; Croteau, R. Microsome Preparation From Woody Plant Tissues. *Meth. Enzymol.* **1996**, *272*, 243-250.

103. Chen, R.F. Removal Of Fatty Acids From Serum Albumin By Charcoal Treatment. *J. Biol. Chem*. **1967**, *242*, 173-181

104. Mizutani, M.; Ohta, D.; Sato, R. Purification And Characterization Of A Cytochrome P450 (*Trans*-Cinnamic Acid 4-Hydrozylase) From Etiolated Mung Bean Seedlings. *Plant Cell Phys*. **1993**, *34*, 481-488.

105. Yoshida, S. Changes In Microsomal Enzymes And Phospholipid During Dehardening In Stem Bark Of Black Locust. *Plant Phys*. **1976**, *57*, 710-715.

106. O'Keefe, D. H.; Ebel, R. E.; Peterson, J. A. Purification Of Bacterial Cytochrome P-450. *Meth. Enzymol.* **1972**, *52*, 151-157.

107. Omura, T.; Sato, R. The Carbon Monoxide-Binding Pigment Of Liver Microsomes I. Evidence For Its Hemoprotein Nature. *J. Biol. Chem.* **1964**, *239*, 2370-2378.

108. Omura, T.; Sato, R. The Carbon Monoxide-Binding Pigment Of Liver Microsomes II. Solubilization, Purification And Properties. *J. Biol. Chem.* **1964**, *239*, 2379-2385.

109. Omura, T. (2002) Kyushu University (personal communication).

110. Rodgers, M. W.; Zimmerlin, A.; Werck-Reichhart, D.; Bolwell, G. P. Microsomally Associated Heme Proteins From French Bean. Characterization Of The Cytochrome P450 Cinnamate-4-Hydroxylase And Two Peroxidases. *Arch. Biochem. Biophys*. **1993,** *304*, 74-80.

111. Zapata, J. M.; Sabater, B.; Martin, M. Identification of Thyalkaloid Peroxidase of Barley which Metabolizes Hydroquinone. *Phytochemistry* **1998**, *48*, 1119-1123.

112. Adler, P. R.; Arora, R.; El Ghaouth, A.; Glenn, M. D.; Solar, J. M. Bioremediation Of Phenolic Compounds From Water With Plant Root Surface Peroxidases. *J. Environ. Qual*. **1994**, *23*, 1113-1117.

113. Bradford, M. M. A Rapid And Sensitive Method For The Quantification Of Microgram Quantities Of Protein Utilizing The Principle Of Protein-Dye Binding. *Anal. Biochem.* **1976**, *72*, 248-254.

114. Loomis, W. D. Overcoming Problems Of Phenolics And Quinones In The Isolation Of Plant Enzymes And Organelles. *Meth. Enzymol.* **1974**, *54*, 528-544.

115. Shang, T. Q.; Doty, S. L.; Wilson, A. M.; Howald, W. N.; Gordon, M. P. Trichloroethylene Oxidative Metabolism In Plants: The Trichloroethanol Pathway. *Phytochemistry* **2001**, *58*, 1055-1065.

116. Ohta, D. (2003) International Research Laboratories (personal communication).

117. Jefcoate, C. R. Measurement Of Substrate And Inhibitor Binding To Microsomal Cytochrome P-450 By Optical-Difference Spectroscopy. *Meth. Enzymol*. **1978**, *27*, 258-276.

118. Hansikova, H.; Frei, E.; Anzenbacher, P.; Stiborova, M. Isolation Of Plant Cytochrome P-450 And NADPH:Cytochrome P-450 Reductase From Tulip Bulbs (*Tulipa fosteriana* L.) Oxidizing Xenobiotics. *Gen. Physiol. Biophys*. **1994**, *13*, 149-169.

119. Patel, N.; Cardoza, V.; Christensen, E.; Rekapalli, B.; Ayalew, M.; Stewart, Jr, C. N. Differential Gene Expression Of *Chlanydomonas reinhardtii* In Response To 2, 3, 6-Trinitrotoluene (TNT) Using Microarray Analysis. *Plant Sci.* **2004**, *167*, 1109-1122.

120. Ekman, D. R.; Lorenz, W. W.; Przybyla, A. E.; Wolfe, N.L.; Dean, J. F. D. SAGE Analysis Of Transcription Responses In Arabidopsis Roots Exposed To 2,4,6-Trinitrotoluene. *Plant Phys*. **2003**, *133*, 1397-1406.

121. Mezzari, M. P.; Walters, K.; Jelinkova, M.; Shih, M-C.; Just, C. L.; Schnoor, J. L. Gene Expression And Microscopic Analysis Of Arabidopsis Exposed To Chloroacetanilide Herbicides And Explosive Compounds. A Phytoremediation Approach. *Plant Phys*. **2005**, *138*, 858-869.

122. Dharmaraj, S. RT-PCR The Basics. http://www.ambion.com/techlib/basics/rtpcr (accessed 1/22/2003), Ambion.