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Xerocomus chrysenteron's Potential as a Bioremediation Agent of DDT.

ABSTRACT:

As interest in the state of our environment increases, so goes the study of bioremediation, the use of biological organisms to clean up pollution. While at Peking University in China, we studied the potential for an ectomycorrhizal fungus, *Xerocomus chrysenteron*, to degrade the environmental toxin DDT. We used a series of assays to determine the presence and activity of two fungal enzymes, laccase and lignin peroxidase, produced by the organism, and then used gas chromatography-mass spectrometry (GC-MS) to identify and quantify the metabolites formed in *X. chrysenteron* exposed to DDT. While a qualitative assay using fungi plated on agar indicated *X. chrysenteron* produces lignin peroxidase, a quantitative assay was not able to measure enzymatic activity in liquid medium. The GC-MS tests, however, identified a lower concentration of DDT as well as increased concentrations of its degradation products, DDE and DDD. The relationship between DDT and fungal enzymes, specifically laccase, needs to be examined more thoroughly to determine if DDT induces enzyme activity and if the fungal enzymes can effectively degrade DDT.

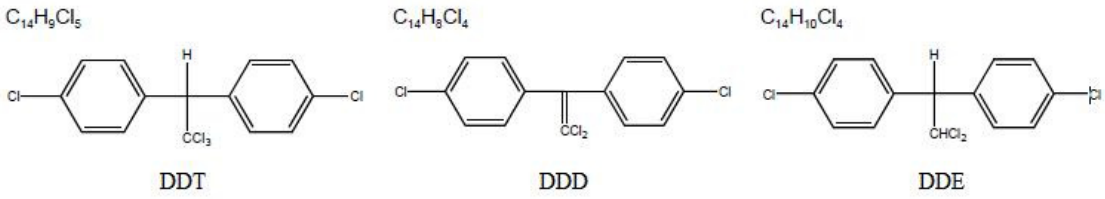
INTRODUCTION:

Our abundant use of chemicals has had an effect on our world. As our knowledge of cause and effect expands and global consequences manifest themselves, it is only a matter of time before we must take full responsibility for our actions. To improve our living environment, we must clean it.

Even mankind's ingenuity pales beside nature's accomplishments. Thus, the concept of bioremediation is a promising one. Bioremediation, as defined by the American Academy of Microbiology is "the use of living organisms to reduce or eliminate environmental hazards resulting from accumulations of toxic chemicals and

other hazardous wastes" (Gibson et al, 1992). Essentially, it is a process that utilizes natural solutions for environmental problems. Often times, bioremediation is more effective, less costly and less dangerous than other potential solutions.

To observe the process of bioremediation, it is best to examine a case-study: one contaminant and one solution. One of the most notorious environmental chemical contaminants is Dichloro-Diphenyl-Trichloroethane (DDT). DDT is not a naturally occurring chemical compound. It was first synthesized in 1874 by using a sulfuric acid catalyst to combine chloral and chlorobenzene (WHO, 2005). The two phenyl rings lend the compound an unusually high stability and resistance to degradation due to the delocalized electrons that form electron clouds both above and below the ring structure while the chlorine atoms cause it to resist oxidative breakdown. This results in DDT's lengthy half-life of 2-15 years (EPA, 2008). DDT's metabolites, Dichloro-Diphenyl-Chloroethylene (DDE), and Dichloro-Diphenyl-Chloroethane (DDD), are unfortunately almost just as stable as the original molecule.



(ATSDR, 2007)

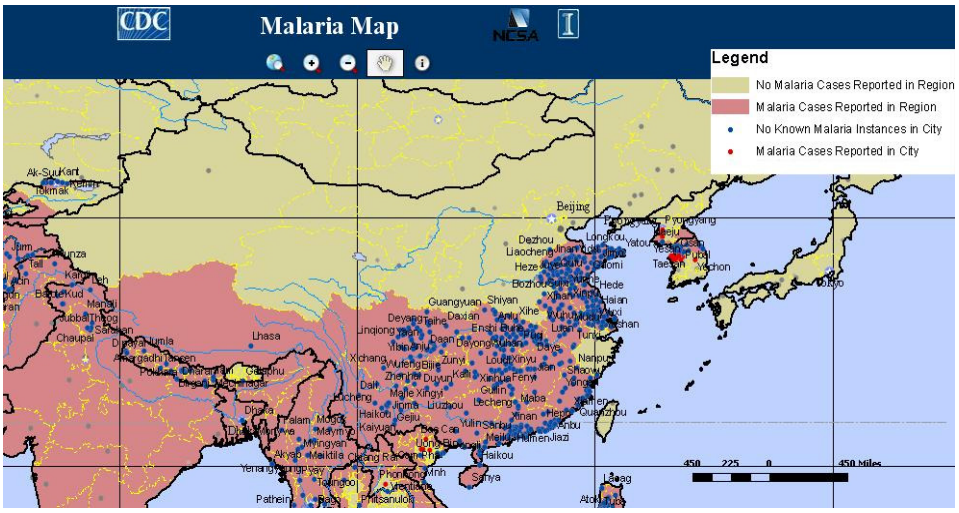
Since DDT and its metabolites linger in the environment so long after initial application, they have collectively been classified as Persistent Bioaccumulative Toxins (PBTs) by the US Environmental Protection Agency (EPA) and as Persistent Organic Pollutants (POPs) by the World Health Organization (WHO).

DDT's prevalent use was due to its effectiveness as an insecticide. However, when DDT was first synthesized in 1874, its insecticidal properties were unknown. Paul Hermann Muller was the first to discover DDT's ability as a very effective insecticide in 1935 and was awarded the 1948 Nobel Prize (WHO, 2005).

As an insecticide, DDT made its début during World War II (WHO, 2005). Armies have always been plagued by diseases born of reduced sanitation, increased

contact between individuals, and unclean drinking water. Dire physical conditions are also weaken immune systems, allowing for the easy transmission of pathogens. WWII coupled modernized warfare with modern advancements in defense against disease. Mosquitoes and lice were two disease vectors that were particularly worrisome during WWI and were heavily targeted during WWII. Mosquitoes carried malaria while lice easily passed typhus.

Malaria is actually caused by a mosquito-borne parasite that infects the human host. Symptoms of the disease include fever, chills and other flu-like symptoms. The Center for Disease Control estimates that there are 350-500 million cases of malaria worldwide every year (CDC, 2008). As many as one million of those cases are fatal each year (CDC, 2008*).* Malaria is transmitted in 41% of the world's environments and, in 2002, was marked the 4th leading cause of children's deaths in developing countries (CDC, 2008). The WHO's Global Malaria Programme was created to manage a global response to Malaria and promote preventive measures, including residential spraying and mosquito nets. Below is the CDC's malaria map, showing the amount of impacted land in China. Note that the majority of China's population is concentrated in the red malaria zones.



(CDC, 2008)

By WWII, Malaria was almost entirely eliminated in Europe so DDT was used to primarily to eliminate lice. The South Pacific, however, still struggled with a rampant

population of Malaria-carrying mosquitoes so DDT was used there to control the disease vector. DDT proved to be an exceedingly effective insecticide and, after the war, was used extensively throughout 1945-1975 in agricultural practices.

The advent of aerial spraying increased the application of DDT insecticides far above that of any other. Easy and effective application over large tracts of land, combined with DDT's deadly effect on arthropods and supposed benignity to humans, resulted in huge usage. However, in 1962, environmental concerns were prompted by Rachel Carson's *Silent Spring* and the American public took an active role in crying for a ban on the use of DDT. *Silent Spring* exposed many of DDT's effects on the environment—specially its detrimental effects on birds—and alluded to potential effects on humans in the form of cancer. Recognizing the legitimate concern, the EPA delisted DDT in 1972, just after Hungary, Norway and Sweden. Currently DDT is still banned and the EPA lists the compound as a Class B2 probable carcinogen and a Level 1 PBT chemical (EPA, 2008).

Most developed countries have discontinued use of DDT in agricultural practices but some developing countries that still suffer from malaria do use DDT as a preventive measure because it is one of the cheapest, most effective methods of disease vector control if the local mosquito populations are not resistant.

In 2001, the Stockholm Convention was officially ratified as an international regulation for the usage of DDT and other similar POPs. Under the Convention, DDT used in agricultural practices is prohibited but DDT may be used in efforts to control disease vector insect populations. China is one of the few countries that is allowed to manufacture DDT. It is estimated that "the two DDT manufacturers in China... [produce] a total annual output of about 4,000 tons" (Chuan, 2004). As shown on the CDC's Malaria map, malaria is still problematic in areas of Southern China and DDT is used to control the local mosquito population.

The desire for an international regulation on the usage of DDT and other POPs was two-fold. Developed countries, able to afford others methods of protecting crops, expressed concern for the lingering effects of DDT in the environment—from both ecological and anthropomorphic stances. Developing countries as a whole, however, tend

to be less concerned with the effect of human actions on the environment in favor of progress and many might have continued to use DDT.

The worldwide use of DDT, however, was having a negative effect on the DDT's insecticidal properties. This was a consequence that concerned developing countries. The world wide usage was destroying large amounts of arthropods—but some were surviving. Those survivors were breeding and the following generations were found to have evolved a resistance to DDT. When such a strong selection pressure (insecticide) is exerted, beneficial adaptations (resistance to insecticide) are amplified in subsequent generations because only the survivors live to reproduce and pass their genes along. It is an unnatural form of natural selection due to anthropomorphic influence. In essence, it fast-forwards evolution.

The WHO Global Malaria Programme cites insecticide resistance as a major threat to the effort of controlling the spread of malaria. In addition to documenting the local mosquito populations susceptibility to DDT, the WHO declares that the "possibility of insecticide resistance calls for careful monitoring of the susceptibility of malaria vectors to the selected insecticide" (WHO, 2006). There is a desire to avoid potential development of resistance so that developing countries will not have to resort to more costly chemicals. The documented cases of resistance in Africa have proven to be a sufficient warning to other countries (WHO, 2006).

Not willing to jeopardize the effectiveness of DDT by continuing to use it on a large scale, the Convention agreed that DDT use would be limited to disease vector control, a direct benefit to human lives in areas afflicted by malaria (WHO, 2008). This reduction in DDT use has been shown to also reduce the rate of resistance emergence among the arthropod species. Thus, developing countries still have access to a cheap and readily available method of controlling malaria that is still highly effective.

Even though DDT's use has been strictly regulated, the history of DDT's application and its penchant for lingering in the environment and even accumulating in the food chains warrants a bioremediation program. China is a particularly important area for bioremediation opportunities. With a history of environmental abuse and a tendency to sacrifice everything for the sake of development, China really needs a cost-efficient

and simple solution to its environmental pollutions. A member of the Office for the Implementation of the POP Convention observed that “the monitoring and evaluation system of POPs is not perfect” (Chuan, 2004). Furthermore, the mysterious disappearances of government files pertaining to DDT usage suggests extensive and even possible continued agricultural use of the insecticide (Chuan, 2004).

While DDT's ecological consequences have been well documented because of its detrimental effects on many wildlife species, particularly apex predators, DDT's direct health concerns towards humans are still in dispute. Currently there is no consensus among the scientific community. Restrictions on DDT's use in most developed countries have largely decreased the amount of research being conducted on this particular POP. However, some researchers maintain that the optimal time to begin conducting research on the effects of DDT is now.

Barbara Cohn has been involved in researching the correlations between POPs, among them DDT, and breast cancers. DDT is of interest because of its lipid-solubility. It has been hypothesized that women exposed to DDT just prior to puberty are at a greater risk for developing breast cancer as they age (Cohn, 2007).

During puberty, the average woman gains fat as the body matures. Thus any DDT that would be sequestered during the formation of these fat reservoirs would accumulate, often in the breasts. After giving birth to a child, milk production relies heavily on the fat stores of the breasts, breaking down the tissue and releasing some sequestered DDT. A recent study in China by Shu et al demonstrated traceable levels of DDT in the women of two different regions, Beijing and Shenyang. The levels were 125 ng/g of lipid and 132 ng/g of lipid, respectively (Shu et al, 2008). Of these samples, it was noted that there was a higher ratio of DDD and DDE as compared to DDT, indicating that the DDT exposure was historical and that the populations at large were probably at a similar risk and would continue to be so for years to come (Shu, 2008).

Barbara Cohn is more interested in how exposure to DDT may affect the risks of developing breast cancer as a woman ages. She is currently running a study in California to track women who grew up in California in the 1940s and would have had exposure to DDT either before, during or after puberty. Cohn suggests that these women would be at

a greater risk of developing breast cancer because of the DDT toxins sequestered in their fatty breast tissue as their breasts developed (Cohn, 2008). As the body ages, it becomes less able to cull off mutated cells and potential cancerous cells. As with many kinds of cancers, negative effects on health are not immediately noticed upon exposure but, rather, require 10-30 years to develop (Cohn, 2007).

Preliminary results of Cohn's study have shown a statistically significant five-fold increased risk of developing breast cancer in women born after 1931 (Cohn, 2007). In agreement with her hypothesis, these women were mostly under 14 when DDT began to be utilized agriculturally and under 20 when DDT application was at its peak (Cohn, 2007). Similarly, individuals who were not exposed before the age of 14 had no showed no increased probability in developing breast cancer (Cohn, 2007). Knowing that DDT was banned in 1972 in the US, many of those women who were at that vulnerable stage of puberty during the period of greatest DDT application should begin to show an increased incidence of breast cancer.

While some researchers focus on the question of *if* DDT causes breast cancer, other researchers focus on *how* DDT could cause breast cancer. The DDT used as an insecticide actually contained a few variations of DDT. One particular isomer of DDT has been proven to be an estrogen-mimicker. As a hormone, estrogen serves to stimulate the growth of breast cells when regulated correctly. The addition of an environmental estrogen compound to a female's system would, for all intensive purposes, cause the same effect as true estrogen—growth of breast cells, potentially cancerous breast cells, in addition to other disruptions of the endocrine system. This hold significant implications for women who develop estrogen-sensitive breast cancer because the additional "estrogen" can exacerbate the cancer.

However DDT does not just affect females. Due to its estrogen-like characteristics, it is also hypothesized to affect males as well. McGlynn hypothesized that, due to endocrine-disrupting properties, some POPs may increase risks for testicular germ cell tumors (TGCTs) (McGlynn, 2008). The results of the study, conducted on 754 subjects, suggested that "increased exposure to p ,p '-DDE may be associated with the risk of both seminomatous and nonseminomatous TGCTs" and also that, since "TGCT is

initiated in very early life, it is possible that exposure to these persistent organic pesticides during fetal life or via breast feeding may increase the risk of TGCT in young men" (McGlynn, 2008).

Further research to definitively determine DDT's carcinogenic properties is important because if the results suggest exposure to DDT does increase the incidence of cancer, individuals can be targeted for pre-screening with the goal of early detection of any cancerous cells. Additionally, it may increase the support for bioremediation projects.

DDT however, is obviously not the easiest compound to break down. Few organisms are capable of degrading such a resistant compound but fungi look promising. A diverse section of the evolutionary tree, fungi specialize in degradation—it is merely a matter of finding the right enzymes, and thus the right fungus, to utilize.

As arbuscular fungi have been extensively studied, we sought to investigate the potential of ectomycorrhizal fungi as bioremediation agents. We specifically focused on *Xerocomus chrysenteron* (XC) and its pathways that involved the laccase and lignin peroxidase enzymes. Unlike arbuscular mycorrhizal fungi, where the hyphae enter the plant cell wall, ectomycorrhizal fungi form a protective sheath around a plant's root without penetration. Both types of fungi live in close association with plants in a symbiotic relationship. XC's potential to be utilized in bioremediation projects was first suggested when XC showed “good tolerant ability to DDT stress” by demonstrating normal growth mode under various concentrations of DDT (Chao, 2007).

White rot fungi have shown great potential in the bioremediation field because of their unique ability to degrade stable compounds like lignin, which is responsible for the structural strength of wood. These fungi can excrete peroxidase enzymes (specifically lignin peroxidase) that can oxidize the lignin compound completely to CO_2 (Aust et al, 1993). Then the fungus can access cellulose, and use it as a carbon source. In a similar manner, the white rot fungus may also be able to oxidize environmental contaminants (Aust et al, 1993).

XC, however, has been hypothesized to oxidize environmental contaminants, like DDT, through a different pathway (Chao et al, 2007). Laccase, an enzyme associated with the degradation of lignin, is an oxidase enzyme that can oxidize and open phenyl

rings. We decided to research the relationship between XC and DDT. In particular, we were interested in: whether XC could degrade DDT; what mechanism it used to degrade DDT if it did; how the presence or absence of DDT affected the activity of that mechanism and the overall fungus. This research was conducted with the goal of providing China with a naturally occurring bioremediation agent to utilize against DDT contamination.

METHODS:

CULTURE MAINTENANCE:

Culture Materials:

0.1 g/L Yeast extract powder

2.0 g/L Glucose

1 L distilled H_2O

1.8% of total, agar powder

The majority of laboratory time was spent making new batches of culture for the fungal experiments. After mixing all of the dry ingredients and the distilled water, we added 0.1 M HCl dropwise until the pH was 5.5, the pH level of optimal fungal growth. Depending on the experiment's requirements, we either plated the fungus or cultured it in a suspension of liquid culture, without agar. All fungus samples had a10 day incubation at 25°C prior to experimentation.

ENZYME ASSAYS:

Over the course of the project, several different types of enzyme assays were performed to assess the activity of different enzymes, such as laccase and lignin peroxidase.

QUALITATIVE AZURE B CULTURE:

Purpose: To provide a rough, qualitative estimation of lignin peroxidase production by the fungus. During the fungus' metabolism of nutrients in the wild,

theoretically it would use lignin peroxidase. If lignin peroxidase is produced by the fungus grown in the laboratory, it will oxidize Azure B, resulting in a fading of the blue color directly surrounding the sample. This provided a general baseline of lignin peroxidase activity in the absence of DDT.

Materials and Procedure: Following the procedure for *Culture Maintenance,* we conducted the Azure B Assay by adding 0.1 g/L of Azure B to the culture. We plated samples and then incubated them at 25°C for 10 days. At the end of 10 days, the color change surrounding the sample was qualitatively assessed.

QUALITATIVE COLOR ASSAY:

Purpose: To observe lignin peroxidase activity by color change and prioritize fungal cultures based on the qualitative observations of color change.

Materials:

2.2 mL culture filtrate

0.1 mL of 1 mM Methylene Blue

0.3 mL of 0.5 mM sodium tartarate buffer (pH=4.0).

0.1 mL of 4.5 mM hydrogen peroxide

Procedure:

Samples of filtrate, experimental and control, were prepared as stated above and blanks—use of water instead of hydrogen peroxide—were allowed to stand for 10 minutes before we compared color changes of the solutions to the blanks. Samples that gave significant color change received priority testing.

AZURE B ASSAY:

Purpose: Assess the enzymatic activity of lignin peroxidase by quantitatively measuring the color change of the sample; a decrease in absorbance would signify laccase-catalyzed break down of the Azure B compound in an oxidation-reduction reaction.

Materials:

1 mL of 125 mM sodium tartarate buffer (pH=3.0)

500 µL of 0.160 mM Azure B

500 µL of culture filtrate

500 µL of 2 mM hydrogen peroxide (test) or water (blank)

Procedure: We used a spectrometer set at 651 nm to compare the samples of culture filtrate and hydrogen peroxide against a blank of filtrate and water. Any change in absorbance would be due to the activity of the enzyme. We took readings once a minute for 3-6 minutes, beginning at the addition of the hydrogen peroxide. This was performed on the three specimens as prioritized by the *Qualitative Color Assay*.

METHYLENE BLUE ASSAY:

Purpose: To assess the enzymatic activity of laccase by quantitatively measuring the color change of the sample; a decrease in absorbance would signify lignin enzymes oxidizing Methylene Blue in the presence of hydrogen peroxide.

Materials:

0.1 mL of 1.2 mM of Methylene Blue

0.6 mL of 0.5 mM sodium tartarate buffer (pH = 4.0)

2.2 mL of culture filtrate

0.1 mL of 2.7 mM hydrogen peroxide (test) or water (blank)

Procedure: We used a spectrometer set at 664 nm to compare the samples of culture filtrate and hydrogen peroxide against a blank of filtrate and water. Any change in absorbance would be due to the activity of the enzyme. We took readings once a minute for 3-6 minutes, beginning at the addition of the hydrogen peroxide. This was performed on the three specimens as prioritized by the *Qualitative Color Assay*.

LACCASE ASSAY:

Purpose: To determine the activity of the laccase enzyme in the presence

of DDT, its metabolites, DDD, and a similar chemical, Dicofol (D_f), via absorbance

Materials and Procedure: We used filtrate samples of XC exposed to DDT, DDD, or Dicofol for seven days, in addition to a control, to observe the activity of the laccase enzyme. The solutions analyzed were prepared as follows, where ABTS is a substrate mediator believed to aid in the oxidation process (Amitai, 1998). The pH of the solution was 5. Data was collected every two minutes for five minutes for each sample, at a wavelength of 420 nm.

Blank	Experimental
2.7 mL Acetate Buffer; unknown concentration	2.6 mL Acetate Buffer; unknown concentration concentration
0.3 mL ABTS; unknown concentration	0.3 mL ABTS; unknown concentration
	0.1 mL Filtrate

ASSAYS WITH DDT:

A series of assays were performed using fungus samples in liquid culture that either were exposed to DDT (experimental) or not (control) for a given amount of time. Using a similar procedure to the one above (See *Culture Maintenance*), fungal samples were cultured. Each day, we took four submerged fungus samples from incubation. We added 10 ppm DDT to two experimental samples and left the remaining two as controls. This was repeated for each of the 10 days the fungus was incubating. To perform the assays, we used vacuum filtration to filter the fungus from each flask, collecting the filtrate while the dried mycelia were discarded with the filter paper.

ANALYSIS OF DDT METABOLITES AND CONCENTRATIONS

Purpose: To determine if DDT and/or its metabolites were present and if so, in what concentrations in relation to length of exposure.

Materials and Procedure: We extracted 2 mL samples of fungus from three

individual suspensions that had been incubating for 20 days. Each suspension had an initial concentration of 10 ppm DDT. We poured these samples into a titration buret along with 5 mL of hexane, shaking vigorously. The DDT dissolved readily in the hexane and the hexane and water separated based on density and immiscibility. The heavier water was drained out and the hexane with DDT was placed in a beaker and the procedure was repeated 2 more times, resulting in a final volume of approximately 15 ml of hexane with DDT for each sample.

We evaporated off the hexane, leaving only the DDT. We added approximately 1mL of hexane to the beaker and poured the solution into a small 2 mL brown vial; this was followed by a second rinse with hexane. Then we added 0.02 mL of PCNB, Pentachloronitrobenzene, an internal standard, resulting in a concentration of 1 ppm PCNB.

A Gas-Chromatograph Mass-Spectrometer was employed to separate, identify and quantify the compounds (DDT, DDE, and DDD). "Hexane extracts were analyzed using an Agilent 6890N GC equipped with a capillary column (30m*0.25mm ID, HP-DB5 column) and a 59731 mass spectrometer detector. The column oven was programmed from an initial temperature of 50 C to 200 C at a rate of 15C/min, after holding for 1 min, and then ramped at a rate of 10C/min to 280 C with a final hold time of 5 min. The detector and injector were maintained at 280 and 250 C, respectively." (Yang, 2009)

RESULTS:

Figure 1: XC's ability to Oxidize Azure B, indicating Lignin Peroxidase Activity.

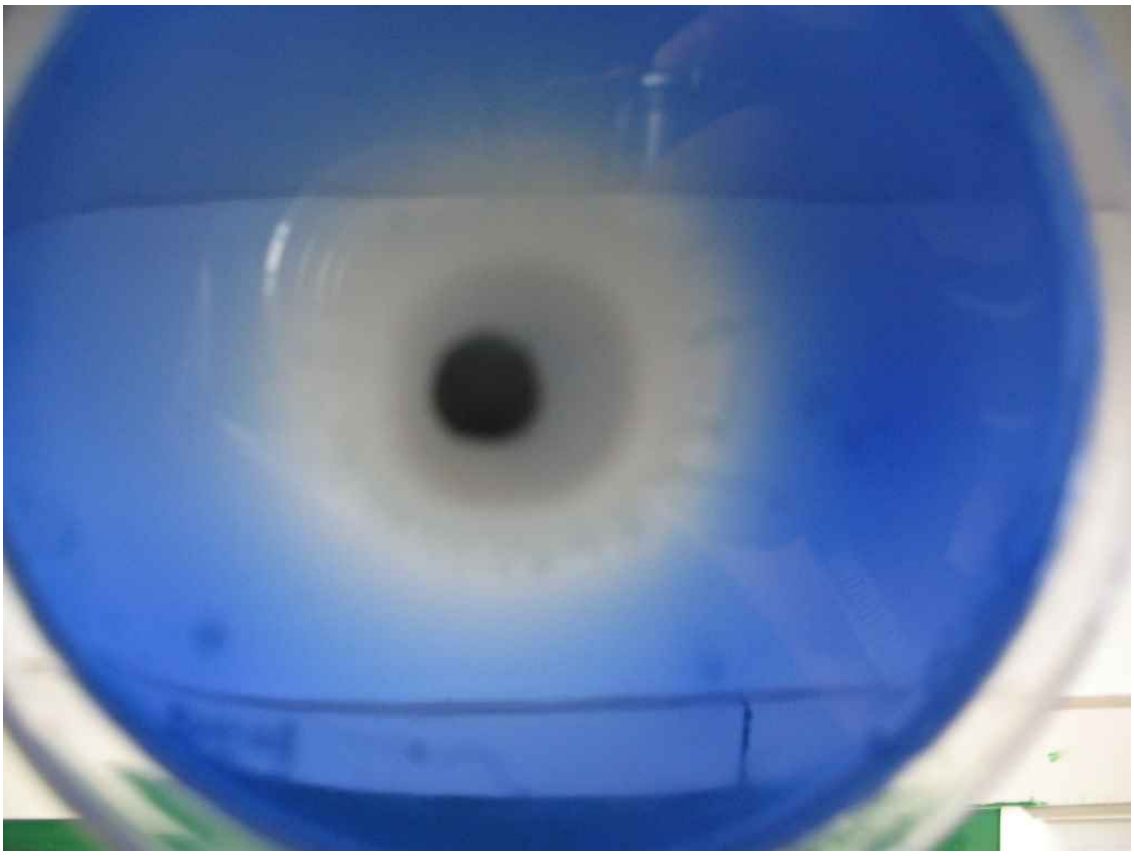


Figure 1 shows a plate of agar and Azure B exposed to a sample of XC. The white ring around the sample of fungus signifies the fungus' oxidation of Azure B, by lignin peroxidase. This distinct pattern of oxidation led to the following series of experiments which attempted to analyze enzyme activity.

Table 1: Lignin Peroxidase Induction by DDT in Fungi Grown in Liquid Culture, as Observed through Azure B Assay

Samples Date	Trial # and Time (min)	Absorbance @ 651 nm
9.19.07	Trial 1: Sample Zero (0:00)	-0.001
	Trial 2 (1:00)	-0.006
	Trial 3 (2:00)	-0.007
	Trial 4 (3:00)	-0.008
9.18.07	Trial 5: Sample Zero (0:00)	-0.005

	Trial 6 (1:00)	-0.005
	Trial 7 (2:00)	-0.005
	Trial 8 (3:00)	0.006
9.24.07 DDT	Trial 9: Sample Zero (0:00)	0.006
	Trial 10 (1:00)	0.005
	Trials 11-16 (2:00-7:00)	0.004
	Trials 17-20 (8:00-11:00)	0.005

Table 1 shows the absorbance at 651 nm of cultured filtrate samples grown over a course of a week, tested in order of significance as judged from the qualitative assessment. The first trial of each sample was used as a zero point and the absorbance from that zero point was recorded. The first two samples (dated 9.19.07; 9.18.07) were not exposed to the DDT but the third sample (9.24.07) was exposed to DDT. None of the samples saw a significant change in absorbance that would indicate that lignin peroxidase was being produced in the culture medium.

Table 2: Lignin Peroxidase Induction by DDT as Observed through Methylene Blue Assay

Samples Date	Trial # and Time (min)	Absorbance @ 664 nm
9.18.07 DDT	Trial 48: Sample Zero (0:00)	0.050
	Trial 49 (1:00)	0.047
	Trial 50 (2:00)	0.047
	Trial 51 (3:00)	0.047
	Trial 52 (4:00)	N/A

9.22.07 DDT	Trial 53: Sample Zero (0:00)	0.201
	Trial 54 (1:00)	0.199
	Trial 55 (2:00)	0.202
		Absorbance @ 611 nm
	Trial 56: Sample Zero (0:00)	0.182
	Trial 57 (1:00)	0.184
	Trial 58 (2:00)	0.186
	Trial 59 (3:00)	0.185
	Trial 60 (4:00)	N/A
9.24.07 DDT	Trial 61: Sample Zero (0:00)	-0.125
	Trial 62 (1:00)	-0.126

Table 2 shows the absorbance at 664 nm and then the absorbance at 611 nm of cultured filtrate samples grown over a course of a week, tested in order of significance as judged by the result of the qualitative assessment. The first trial of each sample was used as a zero point and any change in the absorbance from that zero point was recorded. The first wavelength corresponded to Methylene Blue and the second wavelength to a degradation product, Azure C. None of the samples demonstrated any significant change in absorbance that would indicate lignin peroxidase production or activity.

Table 3: Lignin Peroxidase Induction by DDT as Observed through Methylene Blue Assay and Conducted with New H_2O_2 Solution

Sample Date	Trial # and Time (min)	Absorbance @ 611 nm

9.21.07 DDT	Trial 64: Sample Zero (0:00)	0.165
	Trial 65 (1:00)	0.167
	Trial 66 (2:00)	0.169
	Trial 67 (3:00)	N/A
9.23.07	Trial 68: Sample Zero (0:00)	-0.087
	Trial 69 (1:00)	-0.092
	Trial 70 (2:00)	N/A
9.18.07	Trial 71: Sample Zero (0:00)	0.035
	Trial 72 (1:00)	0.033
	Trial 73 (2:00)	0.029

Table 3 shows the absorbance at 611 nm of cultured filtrate samples grown over a course of a week, tested in no particular sample order. The first trial of each sample was used as a zero point and any change in the absorbance from that zero point was recorded. This data was collected in conjunction with Table 2, however a new H_2O_2 solution was prepared and used. Trials 67 and 70, with an absorbance of N/A, were not actually conducted. None of the samples demonstrated any significant change in absorbance that would indicate the presence and activity of lignin peroxidase. The difference in initial absorbance readings may have been due to the particular composition of the actual filtrate.

Table 4: Laccase Induction in XC by DDT, Dicofol, and DDD.

Test Filtrate	Trial # and Time (min)	Absorbance at 420 nm
XC	Trial 1: Sample Zero (0:00)	-0.004

	Trial 2 (1:00)	-0.004
DDT	Trial 1: Sample Zero (0:00)	N/A
Dicofol 1	Trial 1: Sample Zero (0:00)	0.008
	Trial 2 (1:00)	0.021
	Trial 3 (3:00)	0.038
Dicofol 2	Trial 1: Sample Zero (0:00)	0.008
	Trial 2 (1:00)	0.021
	Trial 3 (2:00)	0.038
DDD	Trial 1: Sample Zero (0:00)	-0.007

Table 4 shows the absorbance at 420 nm of cultured filtrate samples grown over the course of a week. The first trial of each sample was used as a zero point and the absorbance from that zero point was recorded. We were unable to take a reading for the DDT sample. The initial test with Dicofol (Dicofol 1) showed an increase in absorbance, which corresponds to an increase in laccase activity. A second test (Dicofol 2) from the same culture produced similar results. This indicates that Dicofol may play a role in inducing laccase activity.

DISCUSSION:

Environmental systems are intricate and complex. The entire interaction between the fungus, the plants, the toxins and the enzymes can be best understood by gaining an insight into each component of the relationship. To best aid bioremediation efforts in this case, emphasis should first be placed on understanding the relation between the fungal enzymes and the environmental toxins, like DDT. This relationship must be examined from both ends: how does the enzyme affect the concentration of DDT and how does the presence of DDT affect the level of the enzyme produced by the organism?

The presence and activity of the laccase or lignin peroxidase enzyme was first

assessed qualitatively by using a colored dye, Methylene Blue or Azure B. The laccase enzyme and lignin peroxidase oxidized the Methylene Blue and Azure B, respectively. After qualitative analysis, the enzymes' activity in liquid culture was assessed quantitatively by using a spectrometer. We anticipated that the quantitative results would reflect the qualitative.

The targets of this particular study were the extracellular fungal peroxidases and laccase enzymes produced by XC. These enzymes are renowned for their degrading abilities although it is believed that they proceed through very different pathways. Peroxidases, like lignin peroxidase (LiP) and manganese peroxidase (MnP), are heme proteins that are composed of an iron complex of polyphrin (Ikehata et al, 2004). They are oxidoreductases, serving to catalyze oxidation and reduction reactions. During this reaction, the primary food source is oxidized and a peroxide, like hydrogen peroxide, is reduced. In white rot fungus, which can produce both LiP and MnP, there is a clear preference to produce MnP when there is an increased concentration of Mn available (Ikehata et al, 2004).

Laccase, however, is believed to be a p-diphenol di-oxygen oxidoreductase. Instead of iron, laccase anchors multiple copper atoms at the active site (Ikehata et al, 2004). Laccase catalyzes oxidation-reduction reactions, but may also use molecular oxygen as the oxidant to oxidize phenols, benzene rings and aromatic compounds (Ikehata et al, 2004)).

All of these enzymes are ligninolytic because they catalyze the breakdown of the lignin in wood fibers. Laccase specifically has a strong oxidative ability as observed in its ability to break degrade lignin compounds (Ikehata et al, 2004). However laccase's oxidative ability hinges on other variables. When low molecular weight lignin compounds were treated with laccase, polymerization resulted. When high molecular weight lignin compounds were treated with laccase, especially in the presence of glucose oxidase, depolymerization resulted. These conflicting processes compound the research being conducted on laccase's effects. Additionally, in laboratory settings laccase substrates like ABTS (2-2-azinobis-(3-ethylbenzothioaline-6-sulfonic acid ammonium salt) are added and act as a mediator, to see maximum depolymerization of lignin

(Ikehata et al, 2004). It has been suggested that addition of these mediating substrates may increase the oxidation of xenobiotics (Ikehata et al, 2004).

Unfortunately, the assays performed did not yield conclusive data. The Azure B Culture suggested there was some oxidation occurring (Figure 1). The white ring surrounding the fungus sample denotes where the dye has been degraded (Figure 1). These results suggested that XC could perform oxidative processes, potentially by using the lignin peroxidase enzyme. The Qualitative Color Assay, however, showed no distinct color change. This conflicted with the earlier Azure B Culture. Since both tests were qualitative, we pursued quantitative enzyme assays. Both the Azure B Assay and the Methylene Blue Assays proved to be inconclusive because no change in absorbance was seen beyond experimental error (Tables 1, 2, 3).

The Azure B Assay to test the activity of lignin peroxidase was conducted on three samples. Plates were named for the date they were created and whether or not the sample was exposed to DDT. When the filtrate was exposed to H_2O_2 and ABTS, it was expected that the absorbance would decrease over time. All three samples were zeroed and there was no significant change from that initial zeroing point (Table 1). The readings of the spectrometer varied between -0.008 and 0.006 however the last digit of the spectrometer is not significant (Table 1). For all intensive purposes, the absorbance remained at zero for the duration of the tests on all three samples.

The first Methylene Blue Assay was conducted on three samples, all of which were exposed to DDT (Table 2). A total of 14 readings were taken over three samples. The first sample, 9.18.07 DDT, had a range of absorbance readings from 0.050 to 0.047 (Table 2). The first reading was 0.050 but the remaining three readings were all 0.047, with the difference being within experimental error (Table 2).

The second sample, 9.22.07 DDT, was tested a total of seven times. Initially the sample was read at a wavelength of 651 nm and the readings ranged from 0.201 to 0.202 (Table 2). These data indicate no change in absorbance. We then changed the wavelength to 611 nm, a reading that corresponds with Azure C. Both Methylene Blue and Azure B can potentially degrade to Azure C. The absorbance of these readings actually increased over time, from 0.182 to 0.186 but this increase was not large enough to be outside

experimental error (Table 2). The last sample, 9.24.07 DDT, only had two readings, both of which were negative, indicating no laccase presence (Table 2).

The second Methylene Blue Assay was actually an extension of the first, but with a fresh solution of H_2O_2. Three samples were tested, one of which was exposed to DDT. The 9.21.07 DDT sample showed an absorbance increase from 0.165 to 0.169, but this increase was not deemed significant (Table 3). The sample 9.23.07 showed only a negative absorbance and was determined to lack the lignin peroxidase enzyme. The final sample, 9.18.07, like the first sample, also showed some decrease in absorbance from 0.035 to 0.029 but, again, this was not a significant decrease (Table 3).

Acknowledging that our research thus far was inconclusive, we performed another set of assays in a new, but related, direction. Since DDT itself did not seem to be activating laccase, we examined one of DDT's degradation products, DDD, and a chemical similar to DDT, Dicofol. Laccase's response to these chemicals would suggest that laccase plays a role in a secondary step of DDT degradation. In fact, laccase activity increased in response to Dicofol, suggesting that it is Dicofol that plays a role in inducing laccase (Table 4). This consistent increase in laccase activity was immediately replicated with a second sample (Table 4). However, this was the only assay conducted with a focus on the relationship between laccase and Dicofol so additional research is necessary.

However, there was evidence of DDT's degradation. We analyzed the concentration of DDT from the filtered, dried and ground up mycelium of the fungus using GC-MS. Although no data from the experiment was recorded (it was done solely as a demonstration), the laboratory had shown in the past that DDT, DDD and DDE were all identified (Chao et al, 2007). As DDD and DDE are common degradation products of DDT and the concentration of DDT had decreased from the initial 10 ppm, it suggests that DDT was possibly being degraded by the fungus.

Before crediting the fungus, additional experiments would need to be conducted to make sure that the DDT is actually being degraded. It is possible that the fungus is sequestering the toxin or even that the walls of the container are retaining some of the DDT residue. The DDT metabolites could be due to an impure initial solution of DDT. The actual amounts of DDT in all possible locations (solution, fungus, container walls)

would have to be compared to the initial starting dose of DDT and the difference would have to be substantial to acknowledge a successful degradation project. One possible method of determining this would be to analyze initial and final masses.

Adjustment of research direction also needs to be considered. Our premise needs to be strongly reconsidered. It should be definitively proven that XC does produce either lignin peroxidase or laccase enzymes. The amount of inconclusive tests suggests that perhaps XC may not produce enough of those enzymes of interest, despite being an ectomycorrhizal fungus. Huang et al have previously done spot tests for oxidative enzymes and different ectomycorrhizal fungi proved to produce different amounts of oxidative enzymes (Huang et al, 2007). A similar test should be conducted on XC to determine which enzymes—and at what levels—are expressed.

The difficulty of learning how DDT and the fungal enzymes interact was compounded by the rate of bacterial infection in the fungus. As the laboratory was a shared resource for several different research projects, the fungus often suffered from exposure to the bacteria-based experiments. Even when plated in a sterile environment, the fungus demonstrated a high risk of infection. Sometimes several different infections would plague one sample. Infections were noted by white or yellowed colored colony growths on the agar plates. As the bacteria growth rates were significantly faster than the fungal growth rates, the competition between bacteria and fungus often favored the bacteria. For efficiency's sake, we need to devise a manner to better protect the samples' integrity, possibly through the application of antibiotics.

With or without the involvement of XC, research should continue to determine the relationship between DDT and laccase enzymes. Does the presence of DDT induce the laccase enzyme? There has already been some preliminary research on this issue, but nothing yet has been accepted for publication (Chao et al, 2007). The primary opposition for this correlation is the length of the time delay between the addition of DDT and the increase in laccase production; 3 months is a long time to draw such a direct correlation as cause and effect. There would be great benefit in closely analyzing the process by which the fungus absorbs or neutralized the toxins, specifically where in the mechanism the enzymes of interest are utilized.

In addition to determining if DDT induces laccase enzyme production or activation, we would like to continue researching how the enzyme affects DDT concentration. This would involve purifying the enzyme and allowing it to interact *in vitro*, directly with DDT.

The relationship between DDT and the fungal enzymes may be bidirectional. Our preliminary research indicates that DDT might induce laccase and laccase might be able to degrade DDT. It is possible that the presence of DDT may promote activity of the laccase enzyme and allow it to degrade more DDT. If we are able to elicit and fully understand the interaction between the environmental contaminant and the bioremediation agent, we should be able to increase its effect and, hopefully, make it an environmentally safe and economically-smart method of DDT bioremediation for China.

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