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Interactions of Fungicides and Pesticides with Specific Enzymes

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1. Introduction

This chapter concerns influences of fungicides and pesticides on specific enzymes of various living systems. There is a growing interest on enzyme systems and environmental factors affecting them in the field of biochemistry and molecular biology. As known, fungicides, pesticides and other chemicals can enter rain water, food, irrigation water or rivers in many cases, and may be hazardous for living systems. Many chemical substances including fungicides, pesticides, drugs and metal ions influence metabolism at very low concentrations by altering enzyme activities and disrupting physiological balances. Many biocides are known to interfere with a number of processes as they have neurotoxic, hematotoxic, genotoxic, hepatic and renal effects on vertebrates (Hayes, 1990; Pretty & Hine, 2005; Ecobichon, 1996; WHO, 1967; Eisler, 1996). Although there are numerous examples of applications of widely used pesticides and fungicides, little is known about their effects on specific enzymes in organisms and there is a serious lack of data and information on exposures, effects and biological evaluation that connect them. Whereas the effects of several factors on enzyme levels and activity is reasonably well appreciated, the effects of xenobiotic exposure on specific enzyme systems have not received substantial review yet. Relevant xenobiotics are derived from pharmaceutical, nutraceutical and environmental exposure, and many of the mechanisms involved are highly complex in nature, not easily predictable from existing *in vitro* tests and do not always predict well from *in vivo* animal models. After a detailed review of enzymes, fungicides and pesticides, a framework for considering the different levels of direct and indirect modulation by xenobiotics is developed herein, and areas that still require further investigation are highlighted. It is anticipated that this chapter may help explain some of the variation in levels of specific enzymes, guide the direction of long-term drug/nutraceutical safety trials, and stimulate ideas for future research.

2. Enzymes

2.1 Description

Enzymes are proteins which catalyze biochemical reactions in high yields. The molecules at the beginning of the enzymatic process are called substrates, and the enzyme converts them

into different molecules, called the products. Enzymes are required in almost all processes in a biological cell at significant rates. Enzymes are very selective for their substrates and speed up only a few reactions from among many possibilities. Thus, the set of enzymes made in a cell determines which metabolic pathways occur in the cell. Enzymes lower the activation energy of a reaction like all catalysts, thus they dramatically increase the reaction rates. Most enzyme reaction rates are much faster (millions of times) than those of un-catalyzed reactions. Since enzymes are catalysts, they are not consumed by the reactions they catalyze, nor do they alter the equilibrium of these reactions. However, enzymes do differ from most other catalysts by being much more specific. Enzymes are known to catalyze about 4,000 biochemical reactions (Bairoch, 2000). The macromolecular components of almost all enzymes are composed of proteins, except for a class of RNA modifying catalysts known as ribozymes. Many enzymes consist of a protein and a non-protein (called the cofactor). The proteins in enzymes are usually globular. The intra- and intermolecular bonds that hold proteins in their secondary and tertiary structures are disrupted by changes in temperature and pH. This affects shapes and so the catalytic activity of an enzyme is pH and temperature sensitive. Enzymes are found in all tissues and fluids of the body. Intracellular enzymes catalyze the reactions of metabolic pathways. Plasma membrane enzymes regulate catalysis within cells in response to extracellular signals, and enzymes of the circulatory system are responsible for regulating the clotting of blood. Almost every significant life process is dependent on enzyme activity. Enzymes are classified on the basis of their composition. Those composed wholly of proteins are known as simple enzymes in contrast to complex enzymes, which are composed of protein plus a relatively small organic molecule. Complex enzymes are also known as holoenzymes. In this terminology the protein component is known as the apoenzyme, while the non-protein component is known as the coenzyme or prosthetic group where prosthetic group describes a complex in which the small organic molecule is bound to the apoenzyme by covalent bonds; when the binding between the apoenzyme and non-protein components is non-covalent, the small organic molecule is called a coenzyme. Many prosthetic groups and coenzymes are water-soluble derivatives of vitamins. Although enzymes are highly specific for the kind of reaction they catalyze, the same is not always true of substrates they attack. Generally, enzymes having broad substrate specificity are most active against one particular substrate.

2.2 Enzymatic catalysis

Catalysis of biochemical reactions in the cell is vital because reaction rates of the uncatalysed reactions are much lower. The mechanism of enzymatic catalysis and other types of chemical catalysis are in principle similar. The enzyme reduces the energy required to reach the highest energy transition state of the reaction by providing an alternative reaction route and by stabilizing intermediates. The reduction of activation energy (Ea) increases the number of reactant molecules with enough energy to reach the activation energy and form the product. In order for a reaction to occur, reactant molecules must contain sufficient energy to cross a potential energy barrier, the activation energy. All molecules possess varying amounts of energy depending, for example, on their recent collision history but, generally, only a few have sufficient energy for reaction. The lower the potential energy barrier to reaction, the more reactants have sufficient energy and, hence, the faster the reaction will occur (Bender, 1964). All catalysts, including enzymes, function by forming a transition state, with the reactants, of lower free energy than would be found in the uncatalysed reaction. Even quite modest reductions in this potential energy barrier may

produce large increases in the rate of reaction. There are a lot of mechanisms by which activation energy decrease may be achieved. The most important of these involves the enzyme initially binding the substrate(s), in the correct orientation to react, close to the catalytic groups on the active enzyme complex and any other substrates (Chaplin, 1986). In this way the binding energy is used partially in order to reduce the contribution of the considerable activation entropy, due to the loss of the reactants' (and catalytic groups') translational and rotational entropy, towards the total activation energy. Other contributing factors are the introduction of strain into the reactants, provision of an alternative reactive pathway and the desolvation of reacting and catalysing ionic groups. The energies available to enzymes for binding their substrates are determined primarily by the complementarity of structures. The specificity depends upon minimal steric repulsion, the absence of unsolvated or unpaired charges, and the presence of sufficient hydrogen bonds. These binding energies are capable of being quite large. However, enzymes do not use this potential binding energy simply in order to bind the substrate(s) and form stable long-lasting complexes. If this were to be the case, the formation of the transition state between enzyme-substrate and enzyme-product would involve an extremely large free energy change due to the breaking of these strong binding forces, and the rate of formation of products would be very slow. They must use this binding energy for reducing the free energy of the transition state. This is generally achieved by increasing the binding to the transition state rather than the reactants and, in the process, introducing an energetic strain into the system and allowing more favourable interactions between the enzyme's catalytic groups and the reactants. A description of several ways enzyme action may be affected as follows.

Salt concentration: If the salt concentration is close to zero, the charged amino acid side chains of the enzyme molecules will attract each other. The enzyme will denature and form an inactive precipitate. If, on the other hand, the salt concentration is very high, normal interaction of charged groups will be blocked, new interactions will occur, and again the enzyme will precipitate. An intermediate salt concentration such as that of human blood (0.9%) or cytoplasm is the optimum for many enzymes.

pH: pH is a logarithmic scale that measures the acidity or H^+ concentration in a solution. The scale runs from 0 to 14 with 0 being highest in acidity and 14 lowest. When the pH is in the range of 0-7, a solution is said to be acidic; if the pH is around 7, the solution is neutral; and if the pH is in the range of 7-14, the solution is basic. Amino acid side chains contain groups such as -COOR and $-NH_2$ that readily gain or lose H^+ ions. As the pH is lowered an enzyme will tend to gain H^+ ions, and eventually enough side chains will be affected so that the enzyme's shape is disrupted. Likewise, as the pH is raised, the enzyme will lose H^+ ions and eventually lose its active shape. Many enzymes perform optimumly in the neutral pH range and are denatured at either an extremely high or low pH. Some enzymes, such as pepsin, which acts in the human stomach where the pH is very low, have a low pH optimum.

Temperature: Generally, chemical reactions speed up as the temperature is raised. As the temperature increases, more of the reacting molecules have enough kinetic energy to undergo the reaction. Since enzymes are catalysts for chemical reactions, enzyme reactions also tend to go faster with increasing temperature. However, if the temperature of an enzyme-catalyzed reaction is raised still further, a temperature optimum is reached; above this value the kinetic energy of the enzyme and water molecules is so great that the conformation of the enzyme molecules is disrupted. The positive effect of speeding up the reaction is now more than offset by the negative effect of changing the conformation of more

and more enzyme molecules. Many proteins are denatured by temperatures around 40-50 ^{0}C, but some are still active at 70-80 ^{0}C, and a few even withstand boiling.

Modulators: Many molecules other than the substrate may interact with an enzyme. If such a molecule increases the rate of the reaction it is an activator, and if it decreases the reaction rate it is an inhibitor. These molecules can regulate how fast the enzyme acts. Any substance that tends to unfold the enzyme, such as an organic solvent or detergent, will act as an inhibitor. Some inhibitors act by reducing the -S-S- bridges that stabilize the enzyme's structure. Many inhibitors act by reacting with side chains in or near the active site to change its shape or block it. Many well-known poisons such as potassium cyanide and curare are enzyme inhibitors that interfere with the active site of critical enzymes.

2.3 Enzyme inhibition

A number of substances may cause a reduction in the rate of an enzyme catalysed reaction. Some of these are non-specific protein denaturants. Others, which generally act in a fairly specific manner, are known as inhibitors. Loss of activity may be either reversible, where activity may be restored by the removal of the inhibitor, or irreversible, where the loss of activity is time dependent and cannot be recovered during the timescale of interest. Many drugs and poisons are enzyme inhibitors. If the inhibited enzyme is totally inactive, irreversible inhibition behaves as a time-dependent loss of enzyme concentration, in other cases, involving incomplete inactivation, there may be time-dependent changes in both Km and Vmax. More important for most enzyme-catalysed processes is the effect of reversible inhibitors. These are generally discussed in terms of a simple extension to the Michaelis-Menten reaction scheme (Michaelis & Menten, 1913).

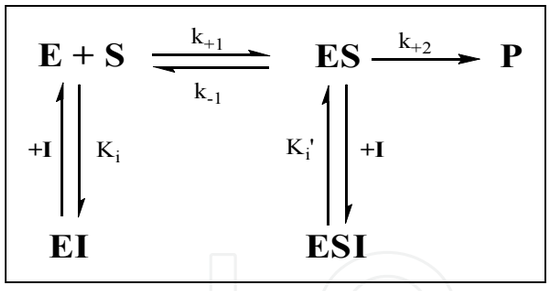


Fig. 1. Reaction scheme E: enzyme, S: substrate, I: inhibitor, P: product

I represents the reversible inhibitor and the inhibitory (dissociation) constants K_i and K_i' are given by

$$K_i = \frac{[E][I]}{[EI]} \qquad K_i' = \frac{[ES][I]}{[ESI]}$$

For the present purposes, it is assumed that neither EI nor ESI may react to form product. Equilibrium between EI and ESI is allowed, but makes no net contribution to the rate equation as it must be equivalent to the equilibrium established through:

$$EI + S \rightleftharpoons E + S + I \rightleftharpoons ES + I \rightleftharpoons ESI$$

In competitive inhibition, K_i' is much greater than the total inhibitor concentration and the ESI complex is not formed. This occurs when both the substrate and inhibitor compete for binding to the active site of the enzyme. The inhibition is most noticeable at low substrate concentrations but can be overcome at sufficiently high substrate concentrations as the Vmax remains unaffected. Normally the competitive inhibitor bears some structural similarity to the substrate, and often is a reaction product (product inhibition), which may cause a substantial loss of productivity when high degrees of conversion are required. A similar effect is observed with competing substrates, quite a common state of affairs in industrial conversions, and especially relevant to macromolecular hydrolyses where a number of different substrates may coexist, all with different kinetic parameters. The reaction involving two co-substrates may be modelled by the scheme (Cornish-Bowden, 1974).

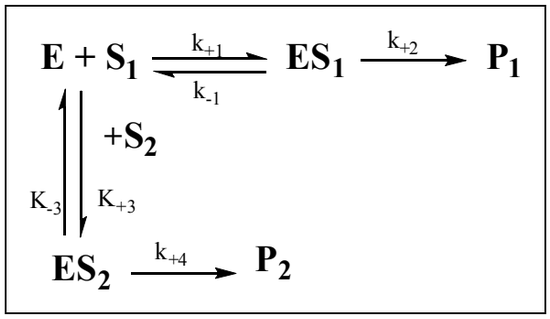


Fig. 2. Reaction scheme for two substrates

Both substrates compete for the same catalytic site and, therefore, their binding is mutually exclusive and they behave as competitive inhibitors of each others reactions. In, uncompetitive inhibition K_i is much greater than the total inhibitor concentration and the EI complex is not formed. This occurs when the inhibitor binds to a site which only becomes available after the substrate (S_1) has bound to the active site of the enzyme (Cornish-Bowden, 1976). This inhibition is most commonly encountered in multi-substrate reactions where the inhibitor is competitive with respect to one substrate (e.g. S_2) but uncompetitive with respect to another (Cornish-Bowden et al., 1978) (e.g. S_1), where the reaction scheme may be represented by:

$$E + S_1 \rightleftharpoons ES_1 + S_2 \rightleftharpoons ES_1S_2 \longrightarrow \text{product}$$

$$E + S_1 \rightleftharpoons ES_1 + I \rightleftharpoons ES_1I \not\longrightarrow$$

The inhibition is most noticeable at high substrate concentrations (i.e. S_1 in the scheme above) and cannot be overcome as both the V_{max} and K_m are equally reduced (Cornish-Bowden & Endrenyi, 1986). A special case of uncompetitive inhibition is substrate inhibition which occurs at high substrate concentrations in about 20% of all known enzymes (e.g. invertase is inhibited by sucrose). It is primarily caused by more than one substrate molecule binding to an active site meant for just one, often by different parts of the substrate molecules binding to different subsites within the substrate binding site (Crompton & Waley, 1986; Fersht, 1985). If the resultant complex is inactive this type of inhibition causes a reduction in the rate of reaction, at high substrate concentrations. It may be modelled by the following scheme:

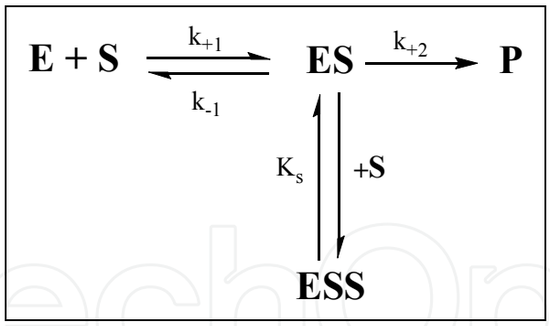


Fig. 3. Reaction scheme for high substrate concentrations

In noncompetitive inhibition, both the EI and ESI complexes are formed equally well (i.e. K_i equals K_i'). This occurs when the inhibitor binds at a site away from the substrate binding site, causing a reduction in the catalytic rate. It is quite rarely found as a special case of mixed inhibition (Henley & Sadana, 1985; Hill et al., 1977; Koshland, 1962). The fractional inhibition is identical at all substrate concentrations and cannot be overcome by increasing substrate concentration due to the reduction in Vmax.

3. Fungicides

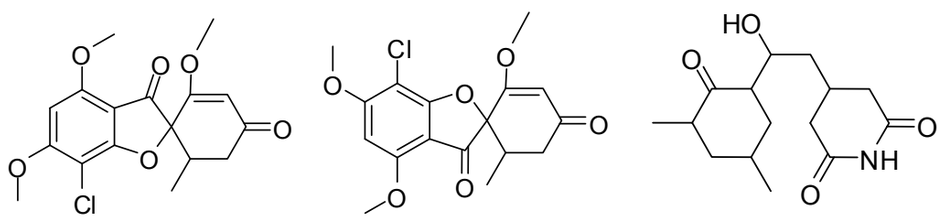
3.1 Description

Fungicides are chemical compounds or biological organisms used to kill or inhibit fungal spores or fungi which can cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. They are used both in agriculture and to fight fungal infections in animals. Fungicides can either be contact, translaminar or systemic. Contact fungicides are not taken up into the plant tissue, only protect the plant where the spray is deposited; translaminar fungicides redistribute the fungicide from the upper, sprayed leaf surface to the lower, unsprayed surface; systemic fungicides are taken up and redistributed through the xylem vessels to the upper parts of the plant. New leaf growth is protected for a short period. Most fungicides that can be bought retail are sold in a liquid form. The most common active ingredient is sulfur, present at 0.08% in weaker concentrates, and as high as 0.5% for more potent fungicides. Fungicides in powdered form are usually around 90% sulfur and are very toxic. Other active ingredients in fungicides include neem oil, rosemary oil, jojoba oil, and the bacterium Bacillus subtilis. Fungicide residues have been found on food for human consumption, mostly from post-harvest treatments (Brooks & Roberts, 1999). Some fungicides are dangerous to human health.

3.2 Structure activity relationship

The advent of organic fungicides was an important milestone in the quest for antifungal compounds capable of exerting a selective effect without damaging the host plant. Selectivity may depend upon differences in biochemistry, cytology, or on differential accumulation. The criticality of the latter process is obvious from the narrow division between fungitoxicity and phytotoxicity in many classes of organic compounds. Protectant fungicides often act through selective accumulation within the pathogen, and many are potentially toxic to both fungal and plant cells, but the latter are protected by the cuticle which acts as a barrier to the passage of foreign chemicals. However, damage can still arise by exceeding a recommended dosage rate or through abnormal climatic conditions

favouring increased absorption. The organic chemist designing candidate fungicides, is also able to achieve a selective margin between fungitoxicity and phytotoxicity by structural modifications affecting partition through barriers such as cuticular and cytoplasmic membranes, by altering molecular size and shape or the lipophilic / hydrophilic balance or hydrogen-bonding capacity. The multiplicity of structural and conformational arrangements of groups of key atoms which are possible in organic compounds has led to important advances in antifungal specificity. This is nowhere more true than with systemic fungicides where it has been variously attributed to structural features of the entire molecule, or to some biologically active centre interacting with specific receptors. Differential fungitoxicity between stereoisomers is known. In griseofulvin, one of the first compounds shown to have systemic antifungal activity (Crowdy et al., 1955), the stereochemistry is critical . Of four isomers resulting from asymmetric centres at carbon atoms 2 and 6', only griseofulvin is active, and the racemic form has only half of the activity of (+)-griseofulvin. The diastereoisomer known as epi(+)-griseofulvin (II) and all transformation products therefrom are inactive.

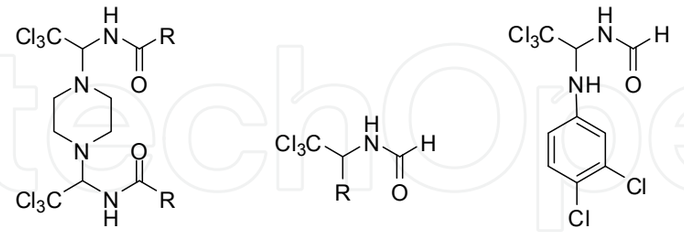


I II III

Fig. 4. Structures of the stereoisomers

Cycloheximide (III) has four asymmetric centres (Fig. 4) (at C-2, C-4, C-6 and C-2'), the compound produced by Streptomyces griseus being 2,6-trans. Probably the simplest example of selective fungitoxicity is that of secbutylamine which has a narrow antifungal spectrum involving a few Penicillium spp. (Eckert et al., 1975), The (-) isomer is considerably more active than the (+) isomer, a fact which cannot be related to selective accumulation by sensitive fungi, and it seems that this selectivity is due to factors closely associated with the mitochondria or pyruvic dehydrogenase which is the site of action. Reasons for the contrasting selectivity of structural isomers are not always obvious. In addition to differing partition parameters, stereochemical or hydrogen-bonding factors are almost certainly involved; additionally special features of the molecule involving particular reactions such as hydrolysis and oxidation are often of considerable importance. Hydrogen bonding is certainly involved in the striking differences that are shown by substituted 2,4- and 2,6-dinitrophenols. Almost without exception the former tend to have high intra- and inter-molecular hydrogen-bonding strengths leading to greater cuticular and epidermal penetration and greater consequent risk of phytotoxicity, whilst the 2,6-isomers are exclusively retained by cuticular waxes. The influence of chemical structure manipulation on biological activity has a fascination for the synthetic organic chemists. Interest in substituted formamides was stimulated after the systemic fungicidal activity of triforine (IV, R=H) was discovered (Ost et al., 1969). Carter et al. (1972) showed that the piperazine moiety was not essential for systemic activity and found that the compound (V, R=methoxy) controlled E. graminis when applied to roots although it was poorly fungistatic or

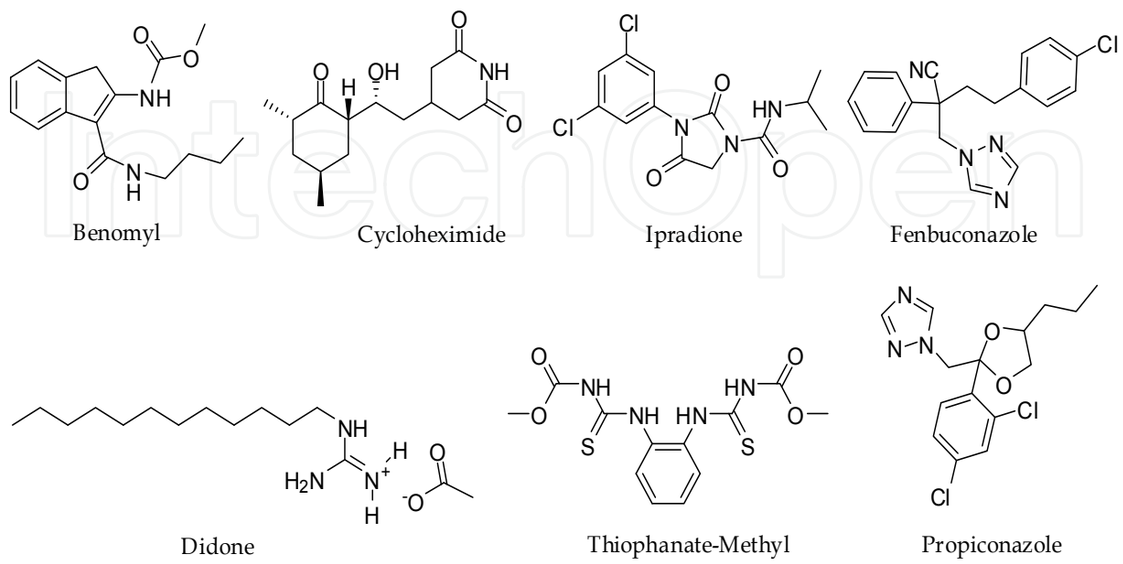
protectant. They subsequently tested about a hundred related compounds and found that the alkoxy and alkylamino analogues (VI, R=alkoxy or alkylamino) were more active systemically than the corresponding alkylthio compounds, and that activity appeared to be greater with the C_3 and C_4 members than with the methyl and ethyl analogues (Fig. 5).



IV V VI

Fig. 5. Structures of the analogues

The work of Brown and Woodcock (1975) revealed further structural specificities. Thus the basic structure (XVI, R=H) was inactive in both leaf-spray and root-drench tests against E. graminis on barley (EDs0 >400 x I0 "s M) but the phytotoxicity at the leaf tips of root-drenched plants suggested that translocation was not prevented. The tribromomethyl analogue exhibited some activity in the root-drench test which suggested that the trihalomethyl group contributed to fungitoxicity by way of lipophilic or inductive-electronic effects rather than through specific receptors. In general their results indicated little correlation between activity in leaf-spray and root-drench tests, thus emphasising that these modes of application had different structural requirements. One structural feature common to the triforine and chloraniformethan molecules that seems significant is the imino group. That the imino hydrogen atom does not appear to be critical , however, seems likely from the comparisons, although steric and conformational factors could account for inactivity . The structures of some widely used fungicides are provided below (Fig. 6, Fig.7).



Benomyl Cycloheximide Ipradione Fenbuconazole

Didone Thiophanate-Methyl Propiconazole

Fig. 6. Structures of some widely used fungicides

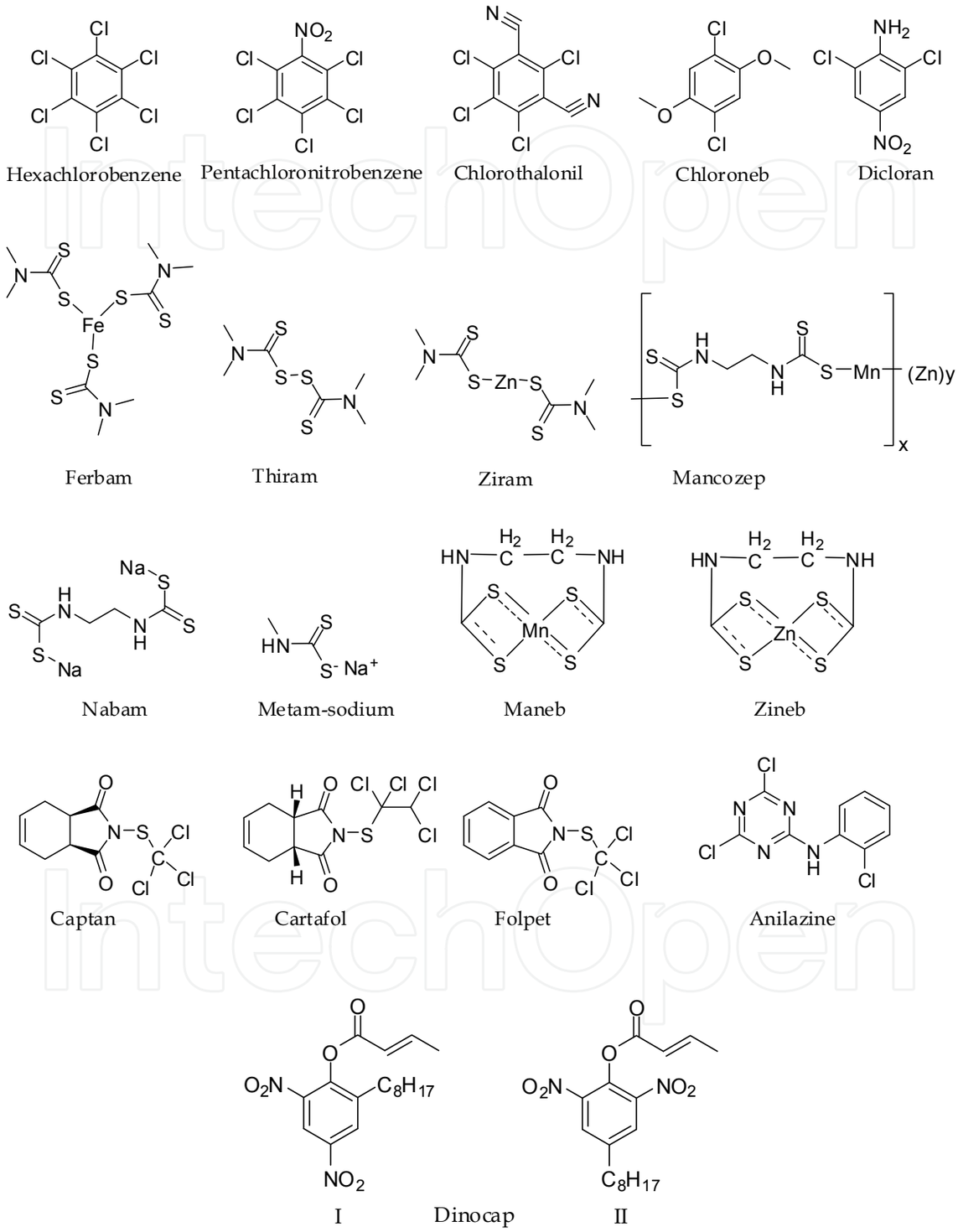


Fig. 7. Structures of some widely used fungicides

4. Pesticides

4.1 Description

Pesticides are substances or mixtures of substances used for preventing, destroying, repelling or mitigating any pest. A pesticide may be a chemical substance, biological agent (such as a virus or bacterium), antimicrobial, disinfectant or device used against any pest. Pests include insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes, and microbes that destroy property, spread disease or are a vector for disease or cause a nuisance.

4.2 Structure activity relationship

Pesticides can be grouped according to chemical structure. Pesticides with similar structures have similar characteristics and usually have a similar mode of action. Most pesticide active ingredients are either inorganic or organic pesticides. From a scientific view, inorganic pesticides do not contain carbon and are usually derived from mineral ores extracted from the earth. Examples of inorganic pesticides include copper sulphate, ferrous sulphate, copper and sulphur. Organic pesticides contain carbon in their chemical structure. Most organic compounds are created from various compounds, but a few are extracted from plant material and are called 'botanicals'. Examples of organic pesticides include: captan, pyrethrin, and glyphosate. Organic pesticides with similar structures are grouped into families of chemicals. Prominent insecticide families incorporates organochlorines, organophosphates, and carbamates. Organochlorine hydrocarbons (e.g. DDT) could be separated into dichlorodiphenylethanes, cyclodiene compounds, and other related compounds. They operate by disrupting the sodium/potassium balance of the nerve fiber, forcing the nerve to transmit continuously. Their toxicities vary greatly, but they have been phased out because of their persistence and potential to bioaccumulate (Kamrin, 1997) Organophosphate and carbamates largely replaced organochlorines. Both function through inhibiting acetylcholinesterase enzyme, allowing acetylcholine to transfer nerve impulses indefinitely and causing a variety of symptoms such as weakness or paralysis. Organophosphates are quite toxic to vertebrates, and have in some cases been replaced by less toxic carbamates (Kamrin, 1997). Thiocarbamate and dithiocarbamates are subclasses of carbamates. Prominent families of herbicides include phenoxy and benzoic acid herbicides (e.g. 2,4-D), triazines (e.g. atrazine), ureas (e.g. diuron), and Chloroacetanilides (e.g. alachlor). Phenoxy compounds tend to selectively kill broadleaved weeds rather than grasses. The phenoxy and benzoic acid herbicides function similar to plant growth hormones, and grow cells without normal cell division, crushing the plants nutrient transport system Triazines interfere with photsynthesis (Kamrin, 1997). The structures of some widely used pesticides are provided below (Fig. 8).

5. Interactions of fungicides and pesticides with enzymes

5.1 Modulation of the enzyme activity

Many chemicals affect the activity of specifif enzymes both *in vitro* and *in vivo* (Coban et al., 2008). For instance, medical drugs (Alici et al., 2008; Ekinci et al., 2007a), metal ions (Ekinci et al., 2007b; Tekman et al., 2008), pesticides and fungicides (Senturk et al., 2009; Ceyhun et al., 2010a) generally inhibit the enzymes at very low concentrations (Ekinci & Beydemir, 2010a). These inhibitions could be very dangerous in some cases (Gulcin et al., 2008; Ekinci

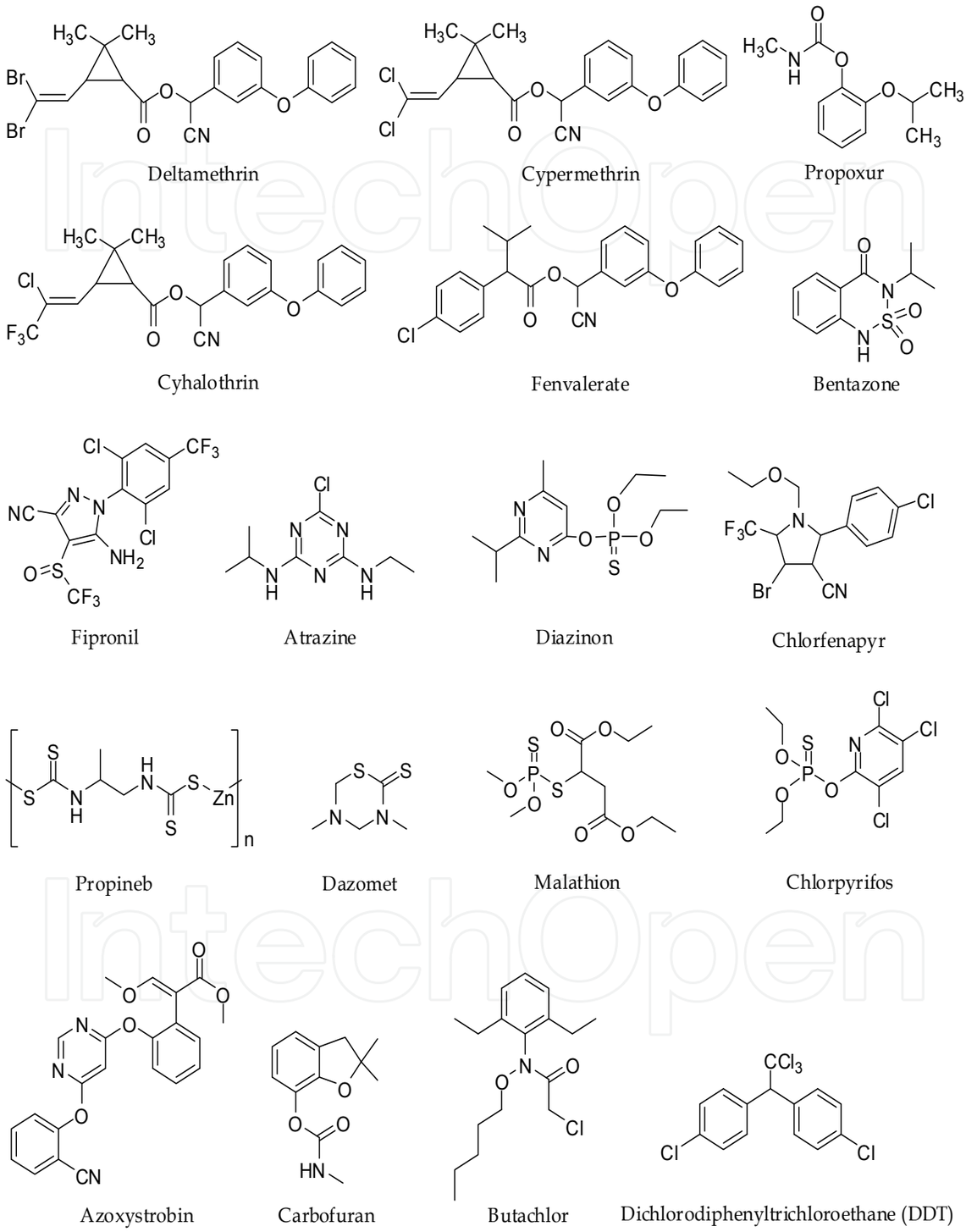


Fig. 8. Structures of some widely used pesticides

& Beydemir 2009a,b) whereas some enzyme inhibitors could be used for the treatment of several diseases (Bayram et al., 2008; Senturk et al., 2009a,b; Coban et al., 2009; Ekinci et al., 2010; Alp et al., 2010). Although investigation of the alterations in the activity of several enzymes have gained considerable attention over the past years (Coban et al., 2007; Ciftci et al., 2008), there is still a deep need of understanding the influences of pesticides and fungicides on specific enzyme systems. We have recently investigated the inhibitory effects of mancozeb, cypermethrin, deltamethrin and dinocap on the pH regulatory enzyme carbonic anhydrase (CA) from rainbow trout (Ekinci & Beydemir, 2010b).The physiological function of the CA isozymes is to facilitate the interconversion of CO_2 and HCO_3^-; therefore, they play key roles in diverse processes, such as physiological pH control and gas balance, calcification, and photosynthesis. In addition, CA plays an important role in ion transport and pH regulation in eye, kidney, central nervous system (CNS) and inner ear. Our findings indicated that pesticides and fungicides dose-dependently decreased *in vitro* carbonic anhydrase activity at micromolar concentrations and that deltamethrin, dinocap, mancozeb and cypermethrin are potent inhibitors for fish carbonic anhydrase enzymes, and might cause undesirable effects with uncontrolled usage by disrupting acid-base regulation as well as salt transport in freshwater or seawater adapted fish. Our results showed that deltamethrin interestingly has a much lower IC_{50} value than cypermethrin, which has a Cl atom instead of a Br. Carbonic anhydrase has Zn^{+2} ion in its active site and it is assumed that electronegative atoms in the inhibitors coordinate to the zinc site at low concentrations. Thus, we concluded that carbonic anyhdrase is very susceptible to alterations in electronegativity of interacting groups. Because deltamethrin was the most powerful inhibitor in *in vitro* experiments, we used it for *in vivo* tests in different doses (0.25 μg/L, 1 μg/L and 2.5 μg/L) and the activities were measured at different time intervals (6^{th},12^{th},24^{th} and 48^{th} hours) for the CA enzymes in rainbow trout tissues (muscle, liver, kidney). For each tissue, inhibition values were calculated and compared with each other. Consequently, deltamethrin inhibited the CA enzymes of rainbow trout tissues with the rank order of muscle > kidney > liver. The pesticides and fungicides were determined to inhibit the CA enzymes of rainbow trout tissues at very low concentrations. The lowest inhibition effect was observed on liver carbonic anhydrase enzyme and we therefore proposed that the inhibitory impact of deltamethrin might be reduced by detoxification enzymes in the liver because detoxification occurs mainly in liver for all living systems (Ekinci & Beydemir, 2010b).

In another study, we aimed to determine the alterations in enzymatic activity of fish antioxidant metabolism in response to deltamethrin administration (Ceyhun et al., 2010b). To this end, three different deltamethrin concentrations (0.25, 1.0, 2.5 μg/L) were administrated to rainbow trout (*Oncorhynchus mykiss*) for different time intervals (6, 12, 24, 48 and 72 h) in order to observe the influences of the pesticide on the activities of glutathione reductase, glucose-6-phosphate dehydrogenase, 6-ghosphogluconate dehydrogenase. Glucose-6-phosphate dehydrogenase (G6PD) is a cytosolic enzyme in the pentose phosphate pathway, a metabolic pathway that supplies reducing energy to cells (such as erythrocytes) by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH). The NADPH in turn maintains the level of glutathione in these cells that helps protect the red blood cells against oxidative damage. (Corpas et al., 1998). Glucose-6-phosphate dehydrogenase deficiency is an X-linked recessive hereditary disease. Individuals with the disease may exhibit nonimmune hemolytic anemia in response to a number of causes, most commonly infection or exposure to certain medications or

chemicals. G6PD deficiency is the most common human enzyme defect (Frank, 2005). 6-Phosphogluconate dehydrogenase (6PGD) is the third enzyme of the pentose phosphate metabolic pathway, catalyzing the conversion of 6-PGA (6-phosphogluconate) to D-riboluse-5-phosphate in the presence of $NADP^+$. The reaction, catalyzed by 6PGD, yields NADPH, which protects the cell against oxidant agents by producing reduced glutathione (GSH) (Bianchi et al., 2001; Lehninger et al., 2000). Glutathione reductase (GR; NADPH: oxidized glutathione oxidoreductase, EC 1.6.4.2), a flavoprotein, is an important enzyme which catalizes convertion of oxidized glutathione into reduced glutathione. The enzyme uses NADPH as electron donor for the reduction of GSSG. GR enables several vital functions of the cell such as the detoxification of free radicals and reactive oxygen species as well as protein and DNA biosynthesis by maintaining a high ratio of GSH/GSSG (Schirmer et al., 1989; Rendón et al., 2004). We observed that the activities of the enzymes decreased with increasing deltamethrin concentrations and exposure time. The pesticide had greater inhibitory effect on gill enzymes than on muscle, liver and kidney enzymes.

Many environmental pollutants including fungicides and pesticides are capable of inducing oxidative stress in aquatic animals. Oxidative stress occurs as a result of the effect of xenobiotics causing disturbances in antioxidant enzyme systems and, as a result, the oxidative stress resulting from the production of reactive oxygen species (ROS) has gained considerable interest in the field of ecotoxicology (Kappus, 1987; Lemaire et al., 1996). The induction of antioxidant expression by the fungicides and pesticides reflects the activation of defense mechanisms in organisms to counteract ROS toxicity. Antioxidant enzymes, such as GR, G6PD and 6PGD, have major direct or indirect effects on antioxidant systems and they are useful biomarkers because they are involved in regenerating reduced glutathione (GSH) from glutathione disulfide (GSSG). Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are indirect antioxidant enzymes in the pentose phosphate pathway and responsible for NADPH production. Because fish tend to adapt to oxidative conditions when exposed to pesticides, fungicides or other pollutants, relatively high levels of GR, G6PD and 6PGD enzymes are expressed in the muscle, liver, kidney, and gills of fish (Stephensen et al., 2000). Nevertheless, because of complex interactions and interrelationships among individual components, the physiological role of these enzymes in the cells is poorly understood. On the other hand, inhibited activity of enzymes caused by exposure to fungicide or pesticide may be due to several reasons; first is production of O_2^- (Bagnasco et al., 2000), second is direct action of fungicides and pesticides on the synthesis of the enzyme (Bainy et al., 1993; Oruç & Uner, 2000), and finally through direct inhibition of enzyme activity both *in vivo* and *in vitro*.

Teisseire and Vernet (2001) showed that the specific activities of enzymes of the Halliwell–Asada pathway, namely ascorbate peroxidase and glutathione reductase, increased after 24 h of exposure to folpet, reaching 155 and 273% of the control level at 96 h, respectively. A fast induction of glutathione S-transferase activity was observed after 6 h of folpet exposure. They were unable to discern whether glutathione S-transferase was involved in folpet metabolism or in peroxide scavenging. The fungicide was also found to stimulate activities of two H_2O_2-scavenging enzymes, catalase and pyrogallol peroxidase. They found the stimulation of catalase was rapid (as early as 12 h after exposure) and strong, since the activity was 252% of the control after 48 h of exposure. According to their data, induction of pyrogallol peroxidase was less important; although it reached 66% at 96 h. The fungicide did not affect guaiacol peroxidase activity. As suggested by the simultaneous and significant

induction (55 to 173%) of antioxidative enzyme defenses of L. minor, generation of reactive oxygen species by the fungicide and involvement of oxidative stress was proposed as a possible mechanism in the phytotoxicity of folpet (Teisseire & Vernet, 2001).

Wu and Tiedemann (2002) reported that two modern fungicides, a strobilurin, azoxystrobin (AZO), and a triazole, epoxiconazole (EPO), as foliar spray on spring barley (Hordeum vulgare L. cv. Scarlett) 3 days prior to fumigation with injurious doses of ozone (150–250 ppb; 5 days; 7 h/day) induced a 50–60% protection against ozone injury on leaves. Fungicide treatments of barley plants at growth stage (GS) 32 significantly increased the total leaf soluble protein content. Additionally, activities of antioxidant enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) were increased by both fungicides at maximal rates of 16, 75, 51 and 144%, respectively. Guiacol-peroxidase (POX) activity was elevated by 50–110% only in AZO treated plants, while this effect was lacking after treatments with EPO. This coincided with elevated levels of hydrogen peroxide (H_2O_2) only in EPO and not in AZO treated plants. The enhancement of the plant antioxidant system by the two fungicides significantly reduced the level of superoxide (O_2^-) in leaves. Fumigation of barley plants for 4 days with non-injurious ozone doses (120–150 ppb, 7 h/day) markedly and immediately stimulated O_2^- accumulation in leaves, while H_2O_2 was increased only after the third day of fumigation. Therefore, O_2^- itself, or as precursor of more toxic oxyradicals, appears to be more indicative for ozone-induced leaf damage than H_2O_2. Ozone also induced significant increases in the activity of antioxidant enzymes (SOD, POX and CAT) after 2 days of fumigation in fungicide untreated plants, while after 4 days of fumigation these enzymes declined to a level lower than in unfumigated plants, due to oxidative degradation of leaf proteins. This was the first report demonstrating marked enhancement of plant antioxidant enzymes and enhanced scavenging of potentially harmful O_2^- by fungicides as a mechanism of protecting plants against noxious oxidative stress from the environment (Wu & Tiedemann, 2002).

Kara and Çelik (1997) investigated the effects of benlate, penncozep, bayleton, cupravit and dithane on human serum enzymes, myocardial creatine kinase (CK-MB), amylase, creatine kinase (CK), aspartate amino transferase (AST), serum glutamyl pruvic transferase (SGPT), alkaline phosphatase (ALK-P), δ-glutamyl transferase (GGT-P) and lactate dehydrogenase (LDH), *in vitro*. They reported that bayleton inhibited only SGPT and it was ineffective on the other seven enzymes. Benlate, penncozep, cupravit and dithane inhibited some enzymes, but activated the others. Benlate was the strongest inhibitor for CK-MB, cupravit for amylase, dithane for ALK-P, penncozep for CK, AST, SGPT and GGT-P. No inhibition was occurred in LDH. Of the fungicides they tested, the most effective one was penncozep whereas the least effective was Bayleton. The most inhibition was shown in SGPT and CK. They considered cupravit as an activator rather than inhibitor (Kara & Çelik, 1997).

5.2 Impact on gene expression

The levels and localization of expression of specific genes is very important for metabolism (Cankaya et al., 2007). In addition to altering enzyme activity, pesticides and fungicides have also strong impacts on expression of several proteins. We demonstrated that deltamethrin causes a significant elevation in the mRNA levels of stress related protein Hsp 70 (heat shock protein 70) in rainbow trout muscles (Ceyhun et al., 2010b). These stress proteins comprise a set of abundant and inducible proteins involved in the protection and

repair of the cell against stress and harmful conditions (Sanders, 1993), therefore, they are very useful biomarkers that have been used to monitor the impact of environmental factors on various animal species, including fish (Lewis et al., 1999). Elevated levels of various heat shock proteins have been measured in tissues of fish exposed to environmental contaminants, such as heavy metals (Williams et al., 1996; Duffy et al., 1999), industrial effluents (Janz et al., 1997; Vijayan et al., 1998), and polycyclic aromatic hydrocarbons (Vijayan et al., 1997; Vijayan et al., 1998). Thus, we evidenced the stress causing effect of deltamethrin.

We have very recently reported the acute and long term influences of deltamethrin on the expression of insulin-like growth factor-I (IGF-I), insulin-like growth factor-II (IGF-II) and growth hormone (GH-I) in rainbow trout muscles (Aksakal et al., 2010). We treated rainbow trouts with different concentrations of deltamethrin (0.25 µg/L, 1 µg/L, 2.5 µg/L) and observed the alterations in mRNA expression levels of IGF-I, IGF-II and GH-I at different time intervals (at 6th, 12th, 24th, 48th, 72th hours and 30th day). The mRNA levels significantly decreased with increasing deltamethrin concentrations thus we demonstrated that deltamethrin has a powerfull impact on the expression of IGF-I, IGF-II and GH-I in rainbow trout which might cause undesirable outcomes not only in growth, but also in development and reproduction. There are similar studies in the literature. Nieves-Puigdoller et al. 2007 reported that exposure to hexazinone (HEX) and atrazine (ATZ), highly mobile and widely used herbicides along rivers in the United States, reduced feeding after 10 days of exposure and had an impaired growth rate in Atlantic salmon. They stated that HEX and ATZ at 10µg l^{-1} exposure had no effect on plasma levels of cortisol, growth hormone (GH), insulin growth factor I (IGF-I). Eder et al. (2008) demonstrated that treatment of juvenile Chinook salmon with chlorpyrifos (CP) and esfenvalerate (EV) led to significantly decreased IGF-I transcription in spleen on days 20 and 60, whereas a short-term increase was seen after CP exposure (day 4). The impact of commonly used pesticides, endosulfan and deltamethrin, on the molecular stress level in black tiger shrimp Penaeus monodon, was investigated using classical oxidative stress biomarkers, protein carbonylation profiles, and levels of heat shock proteins. Results showed that 4 days exposure to 0.1 µg L−1 deltamethrin significantly ($p < 0.05$) increased lipid peroxidation (LPO) level in gills (Dorts et al., 2009).

We also examined whether metallothionein-A (MT-A), metallothionein-B (MT-B) and cytochrome P450 1A (CYP 1A) expressions are induced in response to pesticide administration. For this purpose, we produced muscle metallothionein-A, metallothionein-B and cytochrome P450 1A cDNAs and used quantitative RT-PCR to assay mRNAs in rainbow trout exposed to acute and long-term deltamethrin administration. We observed that deltamethrin exposure significantly ($p<0.05$) increased the expression levels of Cyp1A, MT-A and MT-B in time and dose dependent manner. Polycyclic and halogenated aromatic hydrocarbons (PAHs and HAHs) can enhance the generation of reactive oxygen species (ROS) by inducing cytochrome P450 1A (CYP 1A) *in vivo* and *in vitro*. Metallothionein has been recognised as a useful biomarker for quantifying exposure to heavy metal pollution. Each molecule readily chelates up to seven metal ions through the formation of thiolate bonds with the cysteine residues. It is induced by heavy metals (Cherian & Nordberg, 1983) and its role in the sequestration and detoxification of heavy metals is widely accepted (Vallee, 1979). Metallothionein has been assayed in a range of animals, including many

aquatic organisms, in various tissues. Recently, increased interest has been directed towards the mRNA levels in addition to the levels of the protein itself (Hayes et al., 2004). Although MTs have been widely utilized to identify specific responses to heavy metal pollution, there is now a body of evidence demonstrating that in vertebrates (mammals and fish) MT synthesis is stimulated by different endogenous and exogenous agents (Kägi & Schäffer, 1988), e.g. glucocorticoid hormones, various kinds of stres (cold, heat, extreme exercise), cytokines and in particular compounds leading to production of reactive oxygen species (ROS) (Dalton et al., 1994). Therefore, in mammals and fish, not only inorganic pollutants such as heavy metals but also organic contaminants may activate MT neosynthesis (Wormser & Calp, 1988; Sato et al., 1989; Baumann et al., 1991; Pedrajas et al., 1995). Our study supported the theory that deltamethrin causes a great amount of oxidative stress such that induction of the CYP 1A increases generation of reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical, and hydrogen peroxide, and MT synthesis is stimulated by different endogenous and exogenous agents in particular compounds leading to production of reactive oxygen species (ROS). Our study also contributed to determination of pesticide pollution impact in the freshwater environment and identification of novel inducers of such genes in addition to well known agents (Unpublished data).

6. Conclusion

It is unfeasible to forbid the use of fungicides and pesticides against harmful fungi and pests because of product loss today. Fungicides kill or inhibit fungi or fungal spores which can cause serious damage in agriculture, resulting in critical losses of yield, quality and profit. In addition to agriculture, fungicides are also used to fight fungal infections in animals. Similarly, pesticides are used against any pest including insects, plant pathogens, weeds, molluscs, birds, mammals, fish, nematodes (roundworms), and microbes that destroy property, spread disease or are a vector for disease or cause a nuisance. Although there are benefits to the use of fungicides and pesticides, there are also drawbacks, such as potential toxicity to humans and other animals. It is clear from above discussion that some fungicides and pesticides have useful effects on specific organisms, whereas others have significantly hazardous influences. Therefore, the impacts of fungicides and pesticides must be well defined in order to use the best agents in terms of greater effectiveness and less side effects. It is critically important to explore further interactions of biocides in order to detect compounds with different mechanism of action profiles as compared to dangerous ones, and to find novel applications for the usage of these widespread fungicides and pesticides. On the other hand, due to the complexity and immensity of world-wide pollution, there is a compelling need to develop rapid and sensitive screening methods for monitoring the effects and presence of fungicides and pesticides.

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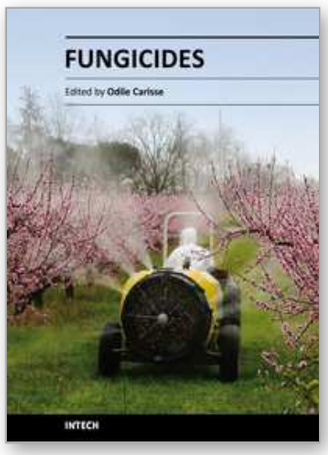
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Fungicides

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Plant and plant products are affected by a large number of plant pathogens among which fungal pathogens. These diseases play a major role in the current deficit of food supply worldwide. Various control strategies were developed to reduce the negative effects of diseases on food, fiber, and forest crops products. For the past fifty years fungicides have played a major role in the increased productivity of several crops in most parts of the world. Although fungicide treatments are a key component of disease management, the emergence of resistance, their introduction into the environment and their toxic effect on human, animal, non-target microorganisms and beneficial organisms has become an important factor in limiting the durability of fungicide effectiveness and usefulness. This book contains 25 chapters on various aspects of fungicide science from efficacy to resistance, toxicology and development of new fungicides that provides a comprehensive and authoritative account for the role of fungicides in modern agriculture.

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