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Review Article

Fungicide: Modes of Action and Possible Impact on Nontarget Microorganisms

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Fungicides have been used widely in order to control fungal diseases and increase crop production. However, the effects of fungicides on microorganisms other than fungi remain unclear. The modes of action of fungicides were never well classified and presented, making difficult to estimate their possible nontarget effects. In this paper, the action modes and effects of fungicides targeting cell membrane components, protein synthesis, signal transduction, respiration, cell mitosis, and nucleic acid synthesis were classified, and their effects on nontarget microorganisms were reviewed. Modes of action and potential non-target effects on soil microorganisms should be considered in the selection of fungicide in order to protect the biological functions of soil and optimize the benefits derived from fungicide use in agricultural systems.

1. Introduction

Soil is arguably the most important resource for food production. It is a very complex system whose functions not only depend on its physical properties, but also on its biological components. In particular, soil microorganisms are essential players in the cycling of several elements essential to life, including C, N, and P [1].

Understanding the effect of fungicides on the beneficial activities of microorganisms is important to assess the hazards associated with fungicide used in agriculture. Crop productivity and economic returns will be maximized with the use of products controlling well fungal pathogens, but preserving beneficial organisms. Different organisms may possess identical or similar mechanisms and constituents, and fungicides targeting nonspecific binding sites can directly affect nontarget organisms. For example, the toxicity of carboxylic acid fungicides is derived from the ability of these chemicals to bind on DNA topoisomerase II, as common enzyme that unwind, and wind, DNA to allow protein synthesis and DNA replication. This enzyme is found in fungi but also in prokaryotic cells [2]. Some glucopyranosyl antibiotic fungicides are toxic to bacteria, in which they may inhibit the synthesis of amino acids [3]. These fungicides are also toxic to certain nonfungal higher eukaryotic organisms [4].

Indirect nontarget effects are also possible. Microorganisms are either functionally or nutritionally connected with each others, and changes in a component of a microbial community may influence the structure of the whole community. This is particularly true for plant-associated microorganisms, which influence on and are influenced by the plant metabolic status [5–7].

In order to establish a proper regulation for the use of the many fungicidal substances promoted by industry in sustainable agriculture, fungicide action modes and possible side effects on nonfungal microorganisms must urgently be clarified. Fungicide action modes have never been well classified, and the side effects of these important chemicals are not fully understood. Therefore, fungicide use may have negative impacts that are difficult to predict [8]. In this paper, current knowledge on the action modes of fungicides impacting membranes, nucleic acids and protein synthesis, signal transduction, respiration, mitosis and cell division, and Multisite activity, as well as on their side effects on nontarget organisms will be summarized and organized. The framework emerging from this analysis sheds a much needed light on the possible side effects of the numerous fungicidal

products in use and facilitates the assessment of the risks associated with their use. The information summarized here will support the development of efficient agroecosystems where the contribution of naturally occurring bioresources is preserved.

2. Modes of Action and Side Effects of Fungicide Groups

2.1. Effects on the Synthesis of Lipids, Sterol, and Other Membrane Components. The cell membrane is a selectively-permeable wall that separates the cell content from the outside environment. Membranes perform many biological functions in all living cells. They preclude the passage of large molecules, provide the shape of the cell, maintain cell water potentials, and are involved in signal transduction [9]. Negative impacts of fungicide on the membrane of microorganisms were found to alter the structure and function of soil microbial communities.

The structure of lipids, the basic components of cell membranes, was modified by fungicides of the Aromatic Hydrocarbons (AH) group, impacting the functionality of microbial membrane systems. For instance, Dicloran (2,6-dichloro-4-nitroaniline)—an AH fungicide registered in North America, Europe, and South Africa since 1975 for the control of *Basidiomycetes, Deuteromycetes,* and *Rhizopus* species [10]—is phototoxic. The cell membranes of treated fungi become sensitive to solar radiation, which then destroys the structure of linoleic acid, a common membrane lipid. Another active AH fungicide ingredient, etridiazole (5-ethoxy-3(trichloromethyl)-1,2,4-thiadiazole), causes the hydrolysis of cell membrane phospholipids into free fatty acids and lysophosphatides [11], leading to the lysis of membranes, in fungi. Previous research proved that these fungicides have side effects on other soil microorganisms. Dicloran can cause mutation in *Salmonella typhimurium* by disturbing hydrophobic interactions within the membrane [12]. Etridiazole also reduced the nitrification rate of ammonium-oxidizing bacteria in soil [13], with possible effect on this component of the soil microbial community and ramifications on its structure and function.

Sterols are another important component of cell membrane in fungi. Demethylation-inhibiting (DMI) fungicides inhibit sterol biosynthesis in fungal cells. Triadimefon ((*RS*)-1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)butan-2-one) demethylated at C-14, introduced a double bond at C-22, and reduced a double bond at C-24 in the carbon skeleton of sterols in a fungal membrane, causing disfunction and cell lysis [14]. Although bacteria do not have sterols, sterol-targeting fungicides have indirect side effects on these microorganisms. Research found that triadimefon had long-term inhibiting effects on soil bacterial community [7]. Triticonazole ((*RS*)-(*E*)-5-(4-chlorobenzylidene)-2,2-dimethyl-1-(1*H*-1,2,4-triazol-1-ylmethyl)cyclopentanol), a triazoles fungicide, can stimulate bacteria proliferation in soil [15], while two other sterol-targeting fungicides, fenpropimorph (*cis*-4-[(*RS*)-3-(4-*tert*-butylphenyl)-2-methylpropyl]-2,6-dimethylmorpholine) and propiconazole ((2*RS*,4*RS*;2*RS*,4*SR*)-1-[2-(2,4-dichlorophenyl)-4-propyl-1,3-dioxolan-2-ylmethyl]-1*H*-1,2,4-triazole), inhibited overall bacterial activity [16]. Such differential effect may be explained by changes in competition among different soil microorganisms. Recent research of dimethomorph ((*EZ*)-4-[3-(4-chlorophenyl)-3-(3,4-dimethoxyphenyl)acryloyl]morpholine) revealed that this fungicide can influence the activity of bacteria involved in N cycling, with impact on nitrification and ammonification [17], through its different impact on different bacterial ecotypes and changes in bacterial community structure.

Some fungicides target fungal intracellular membrane systems and their biological functions. A widely used fungicidal compound, acriflavine (3,6-diamino-10-methylacridin-10-ium chloride), increases mitochondrial permeability and releases cytochrome c in fungal cells, repressing plasma membrane receptor activation, disordering proton stream and collapsing the electrochemical proton gradient across mitochondrial membranes [18]. As a consequence, ATP synthesis is decreased leading to cell death. It was also shown that acriflavine could thickened both the peripheral and cross cell wall of the gram-negative bacteria *Staphylococcus aureus* [18], suggesting the possibility of nontarget effects of acriflavine on bacterial growth (Table 1).

2.2. Effects on Amino Acids and Protein Synthesis. Proteins are the most important building blocks in living organisms. They have various important biological functions such as making up the cytoskeleton, delivering signals among cells, and catalyzing biochemical reactions [36]. Proteins are made of amino acids. Several fungicides interfere with the biosynthesis of amino acids and proteins, affecting the biological functions of impacted organisms.

Streptomycin (5-(2,4-diguanidino-3,5,6-trihydroxy-cyclohexoxy)-4-[4,5-dihydroxy-6-(hydroxymethyl)-3-methylamino-tetrahydropyran-2-yl]oxy-3-hydroxy-2-methyl-tetrahydrofuran-3-carbaldehyde), an antibiotic produced by *Streptomyces griseus* that has long been used as a fungicide [37], also has bactericidal activity. Streptomycin interferes with amino acid synthesis. In *Escherichia coli*, application of streptomycin caused misincorporation of an isoleucine molecule in the phenylalanine polypeptide chain associated with 70S ribosomes [38]. Another research with a *thermus thermophilus* mutant strain suggested that misreading of the genes coding for amino acid synthesis explains the negative effect of streptomycin on bacteria [3]. Furthermore, Perez et al. [4] found that streptomycin could also be a nonselective excitatory amino acid (EAA) receptor antagonist. This antibiotic selectively blocked amino acid receptors in the anterior vestibular nerve fibers of *Ambystoma tigrinum*, a salamander, suggesting that it could also be toxic to eukaryotes, in addition to fungi and bacteria.

Oxytetracycline ((2Z,4S,4aR,5S,5aR,6S,12aS)-2-[amino(hydroxy)methylidene]-4-(dimethylamino)-5,6,10,11,12a-pentahydroxy-6-methyl-4,4a,5,5a-tetrahydrotetracene-1,3,12-trione) is widely used in agriculture because of its broad-spectrum antibiotic activity. It is also registered as fungicide in New Zealand and VietNam, according to the information

Table 1: Action mode and possible nontarget effects of fungicides.

Action mode		Fungicide chemical group	Common name	Nontarget effects
Lipid, sterol, and other membrane components	Lipid	Aromatic hydrocarbons	Dicloran	Mutagen to *Salmonella typhimurium* [12]
			Etridiazole	Retards nitrification by affecting ammonium oxidizers [13]
	Sterol	Triazoles	Triadimefon	Long-term inhibiting effects on soil bacterial community [7]
			Triticonazole	Increases total number of bacteria in soil [15]
		Cinnamic acid amide	Dimethomorph	Impacts nitrifying and ammonifying bacterial activities in sandy soils [17]
		Triazole	Hexaconazole	Impacts bacterial activities related to N cycling [19]
		Morpholine	Fenpropimorph	Inhibit general bacterial activity in wetland [16]
		Triazole	Propiconazole	
	Tebuconazole			May retard plant-growth-promoting effects of *Azospirillum brasilense* on its hostplant [20]
	Intracellular membrane components	Hydrochloride	Acriflavine	Thickens peripheral and cross cell wall of *Staphylococcus aureus* [18]
Amino acid and protein synthesis		Glucopyranosyl antibiotic	Streptomycin	Inhibits amino acid synthesis in bacteria [3] and is neurotoxic to amphibian [4]
		Tetracycline antibiotic	Oxytetracycline	Also used as bactericide [21]
Signal transduction		Phenylpyrroles	Fludioxonil	Toxic to algae [22] and potential risk to prokaryotes [23]
		Dicarboximides	Iprodione	Affects signal transduction in bacteria [24]
			Vinclozolin	Inhibits total bacterial growth [25]
Respiration	NADH oxido-reductase (Complex I) inhibitors	Pyrimidinamines	Diflumetorim	Unknown
	Succinate-dehydrogenase (Complex II) inhibitors	Pyridine carboxamides	Boscalid	May affect growth of prokaryotes [26]
		Benzamides	Flutolanil	
		Gxathiin carboxamides	Carboxin	Inhibits denitrifying bacterial activity in wetland sediment [16]
	Oxidative phosphorylation uncouplers	2,6-dinitroanilines	Fluazinam	Have a potential risk to environmental microorganisms [27]
		Dinitrophenyl crotonate	Dinocap	Inhibits ammonifying bacterial activity and stimulate general bacterial respiration in soil [28]
Mitosis and cell division	Inhibitor of spindle microtubules assembly	Methyl benzimidazole carbamate	Benomyl	May affect nitrifying bacteria [29] and arbuscular mycorrhizal fungi [30]
			Carbendazim	Reduces the diversity of soil bacteria [31]
		Phenylurea	Pencycuron	May affect metabolically activated soil bacteria in short term [32]

Table 1: Continued.

Action mode		Fungicide chemical group	Common name	Nontarget effects
Nucleic acids synthesis	RNA polymerase I inhibitors	Acylalanines	Metalaxyl	Affects activities of ammonifying and nitrifying bacteria in soil [33]
		Oxazolidinones	Oxadixyl	Unknown
	Adenosin-deaminase inhibitors	Hydroxypyrimidines	Ethirimol	Unknown
Multisite activity		Phthalonitrile	Chlorothalonil	Impacts bacterial activities related to N cycling [29]
		Dithiocarbamate	Mancozeb	Impacts bacterial activities related to nitrogen cycling [17] and carbon cycling [28] in soils
		Phthalimide	Captan	Inhibits denitrifying bacterial activity [16]
		Dithiocarbamate	Thiram	
		Anthraquinone	Dithianon	Reduces bacterial diversity in soil [34]
		Copper	Copper sulfate	Reduces the number of bacteria and streptomycetes in sandy soil [35]

provided by Pesticide Action Network of North America (http://www.pesticideinfo.org/Detail_ChemReg.jsp?Rec_Id=PC38140). Previous research reported inhibitory effects of oxytetracycline on protein synthesis in bacteria through interference with the ternary amino-acyl-tRNA complex binding to the acceptor site of ribosomes [39], leading to retarded bacterial growth, disordered microbial community structure, and limited microbial ectoenzyme activity in the soil system [21, 40]. Therefore, caution must be taken with the application of oxytetracycline to control fungal diseases, as it is antibiotic and impacts bacteria.

2.3. Effects on Signal Transduction. The fungicide affecting microbial membranes or proteins, as we discussed above, may affect signal transduction, which takes place at the level of membranes and involves the function of certain proteins.

Phenylpyrrole fungicidal ingredient fludioxonil (4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1*H*-pyrrole-3-carbonitrile), is a nonsymtemic fungicide, also known to interfere with the signal transduction pathways of target fungi [41]. The work of Rosslenbroich and Stuebler [42] revealed inhibition of spore germination, germ tube elongation, and mycelium growth in *Botrytis cinerea*, by fludioxonil-related interference in the osmoregulatory signal transmission pathway of this fungus. This finding was supported by Ochiai et al. [43] who found that fludioxonil can disturb the CANIKI/COSI signal transduction pathway, leading to the dysfunction of glycerol synthesis and inhibition of hyphae formation in *Candida albicans*. Recently, Hagiwara et al. [44] reported the inhibiting effect of fludioxonil on a large number of genes involved in a two-component signal transduction system, in filamentous fungi. Impact on this system suggests that fludioxonil may have a nontarget effect on bacteria, as this dualistic signal transduction mechanisms is also reported in prokaryotes [45].

Effects on signal transduction are also found in dicarboximide fungicides. Iprodione (3-(3,5-dichlorophenyl)-*N*-isopropyl-2,4-dioxoimidazolidine-1-carboxamide), a contact dicarboximide fungicide widely used in a variety of crops, inhibits glycerol synthesis and hyphal development by cutting off signal transduction [43], as does fludioxonil. Iprodione can modify the structure of the soil bacterial community, as reported in a recent research [24]. Interference with signal transduction by dicarboximide fungicide vinclozolin ((*RS*)-3-(3,5-dichlorophenyl)-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione) caused low growth rate, abnormality, and changes in the productions of hexoses and chitin in treated *B. cinerea* [46]. Vinclozolin also had inhibiting effects on soil bacterial growth and nitrogen metabolism, in soil systems [25]. The metabolite of this fungicidal compound, 3,5-dichloroaniline, is also toxic and persistent [23], further suggesting possible impacts of the fungicide vinclozolin on nontarget soil organisms.

2.4. Effects on Respiration. Several fungicides with different modes of action were reported to inhibit microbial respiration. Some are NADH oxidoreductase (Complex I) inhibitors, others are succinate-dehydrogenase (Complex II) inhibitors, cytochrome bc1 (Complex III) inhibitors, and oxidative phosphorylation uncouplers.

Only few fungicides were reported so far to inhibit respiration by affecting Complex I system in fungal mitochondria. Diflumetorim ((*RS*)-5-chloro-*N*-{1-[4-(difluoromethoxy)phenyl]propyl}-6-methylpyrimidin-4-ylamine), first registered in Japan in 1997 to control powdery mildew

and rust in ornamental plants [47], inhibits NADH oxidoreductase activity leading to fungal death [48]. Very limited research has investigated the mode of action mode of Complex I inhibitors, which remains poorly understood.

Three widely used Complex II inhibitors, boscalid (2-chloro-*N*-(4′-chlorobiphenyl-2-yl) nicotinamide), carboxin (5,6-dihydro-2-methyl-1,4-oxathiine-3-carboxanilide), and flutolanil(*α*,*α*,*α*-trifluoro-3′-isopropoxy-*o*-toluanilide), cause dysfunction of succinate dehydrogenase (SDH) in the tricarboxylic cycle and mitochondrial electron transport chain, inhibiting the activity of Complex II and respiration in fungal cells [49–52]. Significant yield increases were reported with the use of these fungicides, indicating their effectiveness in the control of fungal diseases [53, 54]. Since Complex II is a common enzyme complex system existing in many eukaryotic and prokaryotic organisms [26], nontarget effects of Complex II inhibitors on soil bacteria were repeatedly reported [55, 56], suggesting that cautions must be used with these chemicals.

Whereas some fungicides affect fungal respiration at the level of the enzyme complex system, other fungicides may impact respiration through other targets. Fluazinam (3-chloro-*N*-(3-chloro-5-trifluoromethyl-2-pyridyl)-*α*,*α*,*α*-trifluoro-2,6-dinitro-*p*-toluidine) triggers very unusual uncoupling activity in target cells. The metabolic state of their mitochondria was found to be inhibited after exposure to fluazinam, which may be caused by the conjugation of the chemical with glutathione, in mitochondria [57]. Consequently, ATP production is inhibited and downstream cellular metabolisms is interrupted. In fact, the uncoupling activity of eight fluazinam derivatives was recognized [58], which suggests that fluazinam has complicated ramifications on fungal metabolic pathways and may be toxic in the environment [27]. Another fungicide dinocap (*RS*)-2,6-dinitro-4-octylphenyl crotonates and (*RS*)-2,4-dinitro-6-octylphenyl) showed similar action mode to fluazinam, which inhibited ammonifying bacterial activity [28], suggesting side effects of this fungicide group on bacteria growth.

2.5. Effects on Mitosis and Cell Division. The methyl benzimidazole carbamate (MBC) fungicides are known to impact mitosis and cell division in target fungi [59, 60]. Previous research revealed the inhibitory effects of these fungicides on the polymerization of tubulin into microtubules. These MBC fungicides bind on *β*-tubulin in microtubules inhibiting their proliferation and suppressing their dynamic instability [61–63]. Microtubules are the cytoskeletal polymers in eukaryotic cells and, thus, play a vital role in many cellular functions. The application of MBC fungicides suppresses the assembly of spindle microtubules, disturbs the chromosomal alignment at the metaphase plate and microtubule-kinetochore interacions causing chromatid loss, chromosome loss or nondisjunction in target cells [64], which may also yield side effects on other microorganisms as described below.

Benomyl (methyl 1-(butylcarbamoyl) benzimidazol-2-ylcarbamate) and carbendazim (methyl benzimidazol-2-ylcarbamate), two very popular MBC fungicides widely used in crop production, inhibit mitosis in fungi. They can also influence the beneficial arbuscular mycorrhiza fungi (AMF) [30] and mammalian cells [65, 66]. Although no evidence of a direct effects of MBC fungicides on soil bacteria was reported yet, some research has associated these fungicides to the inhibition of nitrification in soil, a microbially mediated process [29]. The effect of MBC fungicides on bacteria and other soil organisms remains to be clarified.

2.6. Effects on Nucleic Acids Synthesis. Phenylamides (PA) fungicides affect nucleic acids synthesis by inhibiting the activity of the RNA polymerase I system. For example, metalaxyl (methyl *N*-(methoxyacetyl)-*N*-(2,6-xylyl)-DL-alaninate), a widely used PA fungicide, inhibits uridine incorporation into the RNA chain [67]. It interferes with nucleic acid synthesis through inhibition of RNA polymerase I activity thus blocking rRNA synthesis at the level of uridine transcription [68]. PA fungicide applications can increase the prevalence of fungicide resistance in pathogen population and yield more fungicide-resistant isolates, as shown by a recent study using AFLP (amplified fragment length polymorphism) and SSR (simple sequence repeats) markers [69]. Fungicides in the PA group must be used with caution, as the side effect of this fungicide on N cycling associated bacteria was reported [33].

Hydroxypyrimidines fungicides were also reported for their inhibiting effects on adenosine-deaminase. As an example, ethirimol (5-butyl-2-ethylamino-6-methylpyrimidin-4-ol) was reported for its effects on several metabolites such as inosine and adenine nucleotides in barley powdery mildew (*Erysiphe graminis* f.sp. *hordei*.) [70]. Ethirimol caused overexpression of adenine phosphoribosyltransferase, which may further break down the balance of the nucleotide pool. Besides, ADAase, which catalyzes the hydrolytic deamination of adenosine, was inhibited by ethirimol. Consequently, production of inosine was ceased, and synthesis of nucleic acid was impaired. The gene responsible for resistance to ethirimol, *ethIS*, was reported later in *Erysiphe graminis* f.sp. *hordei* [71]; therefore, caution must be taken with the application of hydroxypyrimidines fungicide as fungicide resistance in target populations could be developed by repeatedly fungicide application.

2.7. Fungicides with Multisite Activity. Multisite activity fungicides are widely used in agronomic activities due to the broad spectrum of disease control activity, but may have side effects on other microorganisms due to their multiple biochemical sites impacts. Chlorothalonil (tetrachloroisophthalonitrile), a widely used phthalonitrile fungicide, can block the transformation of alternative special structure of glutathione and reduce enzymes activities which used special conformation of glutathione as their reaction centers. Previous research found that chlorothalonil can influence bacterial growth in soil, which may have ecological consequences on N cycling [29]. Mancozeb (manganese ethylenebis(dithiocarbamate) (polymeric) complex with zinc salt), another Multisite activity fungicide impacting metabolism in target cells, can also affect bacteria involved in both C and N cycling in soil [17, 28]. Other Multisite activity fungicides

such as captan (*N*-cyclohex(trichloromethylthio)-4-ene-1,2-dicarboximide) and thiram (bis(dimethylthiocarbamoyl) disulfide) inhibited the growth of denitrifying bacteria [16], perhaps due to their nonspecific effects on biochemical compounds which contain thiol in target cells. Besides, copper-based Multisite activity fungicide, such as copper sulfate (copper(II) tetraoxosulfate), inhibited bacteria and streptomycetes growth in soil [35] and may have nontarget effects on other soil microorganisms.

3. Conclusion

Fungicidal compounds may have side effects and impact nontarget soil microorganism. The effects of fungicides on soil microorganisms can be important, as the feedback of the soil microbial community can affect crops growth and production in cropping systems. The relationships existing between fungicides, the soil microorganisms, and other environmental factors are complex and difficult to predict. On the other hand, the multiplicity of fungicides' modes of action increases the difficulty of evaluating the risks associated with fungicide use. Since it is desirable to optimize the benefit of natural soil biological functions to crop production, understanding fungicides mode of action and impact on metabolism could help us using fungicide more wisely in agriculture.

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