Synergism and Antagonism in Fungicide Mixtures Containing Sterol Demethylation Inhibitors

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Fungicides can be combined in mixtures to extend their spectrum of antifungal activity and to counteract resistance development. Another advantage of mixtures may be synergism, by which the efficacy of the individual components is increased and the amount of active ingredients can be reduced (9). Antagonism has the opposite effect. Little has been published on interactions in mixtures of fungicides containing inhibitors of sterol 14α-demethylation (DMIs). At present, these fungicides constitute the largest group of modern products meeting new toxicological and environmental standards (7,29). Avoidance of antagonism and exploitation of synergism in mixtures would provide additional advantages for their successful use. This especially would be the case if synergists also could restore activity of DMIs against DMI-resistant plant pathogens.

MECHANISMS OF SYNERGISM AND ANTAGONISM

The mechanisms involved may be classified as pseudo- or true synergism or antagonism (9). In the case of pseudosynergism, the interaction influences the performance of the parent fungicide by affecting its distribution on the plant surface or uptake into plant tissue. It also may result from the presence of mixed pathogen populations or from populations with both fungicide-sensitive and -resistant subpopulations. In the case of true synergism, the components of a mixture directly react with each other, or the companion compound influences the physiology of the pathogen in such a way that the toxicity of the parent fungicide is changed. This paper only covers mechanisms of true synergism or antagonism.

Reactions between compounds in mixtures containing DMIs have not been described. The only example is imazalil, which can be protonated at low pH, resulting in reduced accumulation of the fungicide in mycelium and decreased antifungal activity (32). Activity of most DMIs depends not on activation (except for triadimefon) or detoxification reactions in plant pathogens (8); most directly inhibit their target enzyme, sterol 14α-demethylase (24, 27,33). This probably explains why interactions based on interference with either one of these processes have not been reported. In fungi, phosphorothiolate fungicides induce mixed function oxidases that also may have affinity to DMIs. Hence, these fungicides may antagonize toxicity of DMIs by interfering with binding to sterol 14α-demethylase (34). Mixtures of stereoisomers of cyproconazole or tebuconazole display synergistic fungicidal activity (19). This is ascribed to preferential binding of the most active isomer in these mixtures to the P450 moiety of sterol 14α-demethylase, whereas the less active isomers may saturate other P450s (33). Most of the literature published on interactions in DMI-containing mixtures describes the enhancing effect of compounds on accumulation of DMIs in fungal mycelium, resulting in synergism.

This mechanism operates under laboratory conditions and can be exploited rationally to develop mixtures that display synergism under field conditions. The biochemical mechanism involved in synergism is described in detail in the major part of this paper. In a number of cases interactions in mixtures containing DMI fungicides are not understood. As an example, the synergism in mixtures of pyrazophos and propiconazole in controlling *Erysiphe graminis* f. sp. *hordei* is mentioned (36).

ACCUMULATION MECHANISM

Accumulation of DMI fungicides in fungal mycelium is the result of two processes: passive influx and active efflux (10,11, 15). Passive influx is caused by diffusion and is probably determined by partitioning of DMIs between the extracellular medium and intracellular compartments of mycelium. Active efflux from mycelium into the surrounding medium is an energy-dependent process. It does not operate in the absence of a carbon source in the medium, at low temperatures, under anaerobic conditions, or in the presence of various metabolic inhibitors. The efflux has an inducible character, because at particular DMI concentrations accumulation appears to be transient in time (12). Equilibrium between influx and efflux at a relatively low level of accumulation is attained after about 1 h of incubation. It is assumed that the initially high levels of accumulation result in complex formation between the P450-dependent sterol 14α-demethylase and DMIs. Concentrations of potent DMIs necessary to inhibit the demethylase in cellfree conditions are extremely low, indicating an extremely high binding activity (24,27,33). Therefore, induced efflux activity probably does not readily affect a fungicide bound to its target site, and demethylase activity remains inhibited under equilibrium conditions. Energy-dependent accumulation has been demonstrated for most DMIs tested (17) and operates in all 10 filamentous fungi investigated (10,15,26,30; M. A. De Waard, *unpublished data*). Hence, energy-dependent efflux of DMIs seems to be a common phenomenon.

Accumulation by DMI-Resistant Mutants

Accumulation by mycelium of DMI-resistant mutants is relatively low and constant in time. As for wild-type isolates, accumulation increases in the presence of various metabolic inhibitors and under other conditions mentioned above. This indicates that accu-

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mulation by resistant isolates also is the result of passive influx and active efflux. However, efflux in resistant isolates seems to be constitutive, thereby preventing DMIs from accumulating to fungitoxic levels at their site of action and inhibiting sterol 14α-demethylase activity. Increased energy-dependent efflux as a mechanism of resistance to DMIs has been described for laboratory-resistant mutants of *Aspergillus nidulans* (10,11), *Penicillium italicum* (15,17), *Candida albicans* (31), *Monilia fructicola* (30), and *Nectria haematococca* var. *cucurbitae* (26).

Inhibitors of Efflux

Chemicals inhibiting efflux of DMIs in DMI-sensitive and -resistant isolates are diverse (11,14–16,23) and comprise antibiotics (cycloheximide), calmodulin inhibitors (calmidazolium, chlorpromazine, and triflupromazine), cationic agents (cetylpyridinium bromide, Cu^{2+}, and dodine), inhibitors of mitochondrial respiration (azide, carbonyl cyanide 3-chlorophenylhydrazone, and cyanide), inhibitors of membrane ATPase (diethylstilbestrol, *N,N'*-dicyclohexylcarbodiimide, and orthovanadate), ionophoric antibiotics (nigericin and valinomycin), and multisite-inhibiting fungicides (phthalimides).

SYNERGISM

Compounds that increase the accumulation of DMIs in fungal mycelium can be regarded as potential synergists (5). Because these compounds have the same effect in mycelium of DMI-resistant isolates, they also may alleviate resistance to DMIs in DMI-resistant isolates. Results of crossed–paper-strip bioassays with DMI-sensitive and -resistant strains of *A. nidulans* indicate this hypothesis is valid for a number of compounds (13–15). Synergism also has been demonstrated in isobolograms for radial growth of *P. italicum*. Attempts to demonstrate synergism in disease control on citrus fruit have failed (6,15). In contrast, synergism in control of *Rhizoctonia solani* on wheat has been described for mixtures of cyproconazole and herbicides. Some of the herbicides used were inhibitors of mitochondrial respiration and, consequently, the synergism may have been caused by inhibition of energy-dependent efflux of cyproconazole (28). Therefore, it is important that the practical significance of synergism in mixtures of DMIs and respiration inhibitors in disease control of plant pathogens be evaluated.

MULTIDRUG RESISTANCE

Multidrug resistance (MDR) is the simultaneous resistance of organisms to multiple chemically unrelated drugs. It can be acquired by stepwise selection with a single product (3,21,22). The drugs have no common chemical feature other than their hydrophobic and amphipathic character. MDR has been described in detail in tumor cells resistant to a wide range of antitumor drugs. In many instances, MDR is due to overproduction of a multidrug transport protein known as P-glycoprotein (P = permeability), which is encoded by specific *MDR* genes (22). This multidrug transporter directly uses the energy of ATP to extrude a large variety of drugs from drug-treated cells. Overproduction of the transporter results in reduction of the concentration of drugs in the cytoplasm and decreased activity. Hence, the biochemical mechanism of resistance is very similar to the one described for fungi resistant to DMIs: energy-dependent efflux.

Standard compounds to test the inhibitory effect on drug efflux from tumor cells are verapamil and quinidine. Both compounds also have a moderate effect on DMI efflux from mycelium (M. A. De Waard, *unpublished data*). Other compounds inhibitory to both drug efflux from tumor cells and DMI efflux from mycelium include calmodulin inhibitors, cycloheximide, ionophoric antibiotics, tetraphenyl phosphonium, and orthovanadate (16,22,23). Compounds that inhibit outward membrane transport of drugs are regarded as potential synergists (reversing agents) for antitumor drugs. The reversing activity can be based on competitive inhibition of substrate (drug) transport by P-glycoprotein. Intensive efforts are being made to develop such synergists for clinical use.

Is DMI Resistance in Fungi a Case of MDR?

Phenotypically, resistance to DMIs can be regarded as MDR because it may be accompanied by resistance or increased sensitivity to unrelated compounds such as acriflavine, cycloheximide, chloramphenicol, and neomycin (35). Resistance to DMIs also develops stepwise (18) and is polygenic (20,26,35). Because of this phenotypic and genetic resemblance, the similarity in the biochemical mechanism of resistance to DMIs and antitumor drugs, and the similarity in the nature of synergists, it is likely that DMI resistance in fungi is, in fact, a case of MDR such as that demonstrated in drug-resistant tumor cells.

Like antitumor drugs, DMIs have a hydrophobic and amphipathic character. In addition, *MDR* genes are widespread in nature and occur in both prokaryotic and eukaryotic organisms. A family of *MDR* genes, described as *PDR* (pleiotropic drug resistance) genes, have been identified in *Saccharomyces cerevisae* (1). Some of these genes have been cloned and fully characterized (2). Current research in our department aims to detect and characterize similar genes in filamentous plant pathogens, such as *Botrytis cinerea*, and to unravel the role of the encoded P-glycoproteins in resistance to DMIs and fungal physiology.

P-GLYCOPROTEINS AS TARGETS OF SYNERGISTS

Because P-glycoproteins probably play a major role in resistance to DMIs, this knowledge may be of use in the development of compounds that synergize the toxicity of DMIs. Two classes of compounds can be distinguished.

The first class includes synergists that inhibit P-glycoprotein activity indirectly by interfering with the supply of ATP, the energy substrate necessary to drive DMI transport. This can be achieved with mitochondrial respiratory inhibitors. Because many multisite-inhibiting fungicides and herbicides act in this way, it is worthwhile to investigate their potency in this respect. The fact that multisite-inhibiting fungicides and herbicides are already registered as pesticides would make their use as synergists relatively simple compared to experimental chemicals that interfere with energy metabolism.

The second class includes synergists that interfere with the activity of P-glycoproteins. Many of these chemicals have been described in the medical literature as reversing agents. They can be divided into (i) noncytotoxic analogues of anthracyclines and vinca alkaloids; (ii) calcium channel blockers (verapamil) and calmodulin inhibitors; and (iii) other compounds that do not clearly belong to the above groups (3,22). Noncytotoxic analogues of anthracyclines do not have cytotoxic effects of their own at the concentrations used but rather appear to enhance cytotoxicity by inhibiting efflux of cytotoxic drugs. The mode of action of these compounds is based on competitive inhibition of drug transport. The reversing activity of calcium channel blockers and calmodulin inhibitors is still incompletely understood, but both classes of chemicals affect intracellular calcium levels, which leads to increased cytoplasmic concentrations of drugs. The third group of chemicals is very diverse and includes chloroquin, cyclosporin, detergents, lipophilic cations, propanolol, quinidine, and synthetic isoprenoids. Many of these compounds have a hydrophobic and amphipathic nature, and some of them are membrane active. Reversal of MDR is correlated with affinity of P-glycoproteins to these compounds. These three groups of reversing agents can be useful as leads in the search for synergists among DMI fungicides. In particular, the search for nontoxic analogues of DMIs as synergists of DMIs may be appropriate because these chemicals already may be available within com-

panies and, therefore, do not require new synthesis programs to test their synergistic activity.

The Physiological Function of P-Glycoproteins

The role of P-glycoproteins in cellular physiology is barely understood (21,22). Only for a very limited number of P-glycoproteins has the physiological substrate been identified (22,25). Tissue-specific expression in mammals suggests that P-glycoproteins involved in MDR may function in secretion of cytotoxic natural products that occur in the diet (22). Therefore, the function of P-glycoproteins has been indicated as a hydrophobic vacuum cleaner. If such a function also would apply to other eukaryotic organisms, the physiological role of P-glycoproteins in plant pathogens may be the secretion of plant defense factors (phytoalexins and phytoncides) that accumulate in fungal cells upon colonization of plant tissue.

These plant defense factors are produced by host plants to kill invading organisms. Successful pathogens obviously are able to cope with these toxins. Various mechanisms may be involved, but a new mechanism, not identified so far, may be effective efflux mediated by P-glycoproteins. This hypothesis would corroborate the observation that *N. haematococca* possesses an inducible and energy-dependent mechanism that excludes pisatin from its site of action (4). Another role of P-glycoproteins in pathogenesis may be the secretion of pathogenicity factors from plant pathogens (toxins, peptides, and proteins). If the hypotheses are true, impaired P-glycoprotein activity can result in enhanced accumulation of plant defense factors in pathogens or reduced secretion of pathogenicity factors. In this way, inhibitors of P-glycoprotein activity can achieve plant disease control by exploiting the natural plant defense response or by annulment of the action of pathogenicity factors. For a long time, the development of nontoxic disease-control agents has been considered, but it could not be realized due to a lack of fundamental knowledge and rational leads. Inhibitors of P-glycoproteins may provide a means to achieve this goal. Such inhibitors can be developed and tested in conjunction with screening for synergism with DMIs.

CONCLUDING REMARKS

Research on the rational development of synergists in mixtures of DMIs offers a challenging way to improve their efficiency and to counteract resistance development. The latter goal depends on the mechanism of resistance operating in plant pathogens resistant to DMIs. At the same time, the mechanism of synergism may provide basic insight in cellular processes, such as expression of genes encoding P-glycoproteins and their function in transport of xenobiotics (DMIs).

Also related to this question is the identity of the physiological substrate. P-glycoproteins of plant pathogens may play a role in secretion of plant defense factors that accumulate in mycelium during pathogenesis or in secretion of pathogenicity factors. Hence, inhibitors of P-glycoprotein activity may not function only as synergists of DMIs but also may be active as nontoxic disease-control agents. Our current research program concentrates on these topics.

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