MANAGEMENT OF FUNGICIDE TOLERANCE AND SPECIFIC TOLERANCE OF *SCLEROTIUM CEPIVORUM* TO DICARBOXIMIDE FUNGICIDES

by

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Management of fungicide tolerance and specific tolerance of Sclerotium cepivorum to dicarboximide fungicides

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ABSTRACT

Fungicide-tolerance is of increasing concern in agriculture. The problem was recognized following the introduction of benomyl, and threatens the long-term usefulness of many fungicides released since.

Fungicide tolerance is a stable, heritable characteristic of a cell that allows growth and reproduction at fungicide concentrations that inhibit other cells of that species. Cross-tolerance is stable, heritable tolerance to two or more fungicides induced by one of them. Fungicides with selective toxicity or mode of action appear more susceptible to tolerance than those with multiple sites of action. A common characteristic of most recent fungicides is selective toxicity or mode of action.

Fungicide-tolerant plant-pathogenic fungi may be less fit, comparable to, or more fit than intolerant strains. Pathogenic potential of tolerant strains in the presence of a fungicide can be enhanced due to suppression of antagonists. Continued use of a fungicide after tolerance has developed can lead to selection of fitter tolerant strains.

Tolerance management strategies are largely untested in the field. Proposed approaches include development of use patterns that minimize the likelihood of tolerance, and alternating use of or mixing two or more fungicides having different modes of action. Models of the effects of use patterns and alternating *vs* mixed fungicide approaches have been reported.

An obvious need exists to use and improve tolerance detection techniques in the field, and for research on the mode of action of current and experimental fungicides.

Sclerotium cepivorum, a soil-associated fungus causing onion white rot, was tested for tolerance to dicarboximide fungicides *in vitro*. Five isolates of *S. cepivorum* tolerant to one dicarboximide showed cross-tolerance to the dicarboximides iprodione, myclozolin and vinclozolin, and to dichloran and PCNB, but not to benomyl, captan or thiram. The dicarboximides, dichloran and PCNB share a common structural subunit. EC90 values for most tolerant isolates were >1000 times those of the parent isolates. Dicarboximide tolerance left benomyl sensitivity unchanged. Parent isolates varied widely in sensitivity to PCNB and thiram. Frequency of occurrence of dicarboximide tolerance was too low and variable to obtain a reliable estimate. All tolerant isolates infected onions under greenhouse conditions.

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Introduction

Tolerance of plant pathogens to synthetic fungicides is becoming a significant problem. As recently as 1967, fungicide tolerance was not considered to be important (20). However, with the introduction and widespread use of benomyl and the rapid development of resistance to it, the problem has become the subject of a great deal of study.

As research into fungicide tolerance has evolved more or less independently of similar work in entomology and medicine, there is some confusion over terminology. Most authors make no distinction between the terms tolerance and resistance (5,8,9,18,20,41). I will use 'tolerance' in this paper and define it as a stable, heritable characteristic of a cell that allows growth and reproduction at fungicide concentrations that inhibit other cells of that species. Cross tolerance is defined as stable, heritable tolerance to two or more fungicides that has been induced by one of them (7,9,18).

Metal-based fungicides have been widely used for many years, yet reports of tolerance to them are extremely rare (18,41). More modern synthetic compounds such as captafol,

thiram and captan[1] have also produced few problems with tolerance (9,41). Hints of problems to come arose in the early 1960's when tolerance to fungicides such as PCNB and dichloran began to appear, but such tolerance was uncommon in field situations (20,41).

While the introduction of systemic materials such as oxycarboxin and ethirimol in the mid-1960's (50) produced some tolerance problems (9,41), it was the introduction and widespread use of the benzimidazole fungicides in the early 1970's that suddenly thrust chemical control of plant pathogens into the dilemma that had faced entomology and medicine for years: how to make sensible economic use of very effective materials that can quickly become virtually useless due to tolerance? This paper will attempt to address this issue in general and examine some aspects of it relating to control of Sclerotium cepivorum by dicarboximide fungicides.

Mechanisms of Tolerance

It is generally agreed that the difference between fungicides that produce tolerance quickly and those that do not, probably lies in the mode of action. Materials that affect only a single site in the fungal cell may be circumvented by a single genetic change in some cases, whereas tolerance to those with

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[1]Chemical, common and trade names of all fungicides in this paper are given in Appendix I.

multiple sites of action would more likely require multiple genetic changes in the same cell (5,6,9,18,19,25). While this is a useful generalization, the frequency of occurrence of tolerance will also be governed by the mechanism of tolerance and the likelihood of that mechanism arising in the cell. Obviously, if the genetic changes required to circumvent a single-site lesion are complex, development of tolerance will be retarded. Conversely, if a multi-site fungicide can be degraded or excluded by a single genetic change, development of tolerance will be rapid. However, it is extremely unlikely one would know *a priori* which tolerance mechanism will arise or the number and type of genetic changes necessary to implement that mechanism. Therefore the pest manager must of necessity use the mode of action as a crude approximation of the likelihood of the development of tolerance, realizing the limitations of this technique in estimating the likelihood of genetic change.

The following list of known tolerance mechanisms is meant only as an outline. Readers seeking further detail are directed to the descriptions found in references 5,7,9,17,and 18 on which this list is based.

1. Reduced permeability or exclusion - cell membranes or transport systems do not allow passage of the fungicide.
2. Detoxification - the compound is either chemically altered to a less toxic form or is bound to physiologically non-essential components and effectively removed from the metabolic pool.

3. Decreased activation - some fungicides must be converted from a relatively non-toxic form to a more physiologically active form. An organism deficient in the required enzymes will be tolerant.
4. Site alteration - the binding site is altered to decrease its affinity for the toxicant.
5. Compensation - the target molecule is produced in sufficient quantity to "swamp" the fungicide concentration.
6. Circumvention - an alternate biochemical pathway bypassing the inhibited step is used, although this may involve reduced efficiency or biochemical versatility.
7. Reduced requirement for inhibited product.

Note that mechanisms 1,2 and 3 can occur with single-site or multiple-site mode of action fungicides while mechanisms 4,5,6 and 7 are only likely with single-site fungicides.

Emergence of Tolerance in the Field

Genetic Status

Tolerance to a fungicide can pre-exist in an exposed population or arise by mutation. There is evidence, for example, of pre-existing tolerance to benomyl in natural populations (6,9). According to some indications, benomyl may actually increase the frequency of recombination events, thereby

accelerating genetic change (18). The amount of inherent genetic variability of a fungus will affect how rapidly selection can occur, heterocaryosis being one example of such a source of variability (7,40).

Fitness

Before a tolerant strain of a pathogen presents a disease control problem it must survive and compete. There are many examples of fungi that easily develop tolerance *in vitro*, yet have shown little or no tolerance in the field, presumably because the tolerant strains are less fit for survival in the field environment than are the wild-type strains(5,6,7,9,18).

Besides pathogenicity, other factors governing fitness[2] in the absence of a fungicide include :

- the ability to produce resting structures and to survive unfavourable environmental conditions,
- the length of the reproductive cycle and synchrony of inoculum production with the occurrence of susceptible host tissue,
- the amount of inoculum produced,
- saprophytic ability, and

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[2]Fitness is used in this context to mean the overall pathogenic potential of fungicide tolerant and intolerant strains in the absence of the fungicide.

- genetic stability (6,9,18).

It is possible to produce a strain that is quite fit in the presence of a fungicide, but is unable to survive in its absence due to poor competitive ability (41). However, low initial fitness can be improved by continued use of the same fungicide, as selection will then operate on only the tolerant survivors, allowing the fittest of them to survive and reproduce (7). This will of course bear upon fungicide use strategies.

Dispersal

The speed with which a fit, tolerant strain becomes a problem will also depend on the number, mobility and longevity of its dispersal propagules, its host range and distribution, and its competitive ability compared to local or immigrant susceptible strains in the absence of the fungicide. The geographical use pattern of the fungicide will bear upon the emergence of tolerance as well. For example, if all orchards within a valley are sprayed with the same fungicide for control of a mobile, aerial pathogen, tolerance will likely become a problem faster than if different orchards were to use different fungicides (6,9,18).

Selection Pressure

Intensity of selection pressure will influence the emergence of tolerance in the field. In the case of selection of fungicide tolerant strains of plant pathogens, the fungicide acts as a sieve, concentrating any tolerant genotypes. In general, the more intense the pressure applied to a fungus, the more rapid the change will be, provided there are sufficient survivors to maintain a viable population (19). The type of chemical and its persistence in the environment will also affect selection pressure. A persistent chemical poses a greater selection pressure as it makes dormancy a much less effective escape strategy (7,19). For non-obligate parasites, application of a fungicide to the entire potential range of the organism will result in higher selection pressure than application to only the 'at-risk' crop plant, as there will not be any part of the pathogen population left unexposed to provide a source of susceptible competitors to the tolerant strains (18). A reservoir of sensitive strains may be impractical, however, in the case of potentially explosive diseases such as late blight (*Phytopthora infestans*) (51).

Inhibition of antagonists in the environment by the fungicide will affect selection pressure as well, allowing tolerant strains of low initial fitness to survive long enough for the fitter individuals to be selected (7,18).

Implications of Tolerance

There are several concerns related to the development of tolerance in plant pathogens.

First, a disease problem may become worse in the presence of a fungicide when tolerance to that fungicide exists, due to suppression of antagonists (7,18).

Second, most fungicide-tolerant pathogen strains are less pathogenic than their susceptible counterparts (11,27,34,44,48), but in at least one case a tolerant strain of a pathogen was reported to be more pathogenic (6).

Third, many growers are reluctant to abandon the use of a particular fungicide when they have observed good control in the past, especially if suppliers continue to recommend it. Thus selection pressure can continue after the emergence of tolerance, increasing the chances of a permanent population shift to fit, tolerant strains (41). This situation is apparently occurring in Europe with the use of dicarboximide fungicides on *Botrytis cinerea* (51).

Finally, if tolerance makes the useful life of a chemical short or curtails its use, manufacturers will be less willing to invest in new materials that may encounter similar problems (9,41). With the seemingly inevitable loss of some currently registered fungicides to changing health and safety regulations, it would seem prudent to use present and future materials in such a manner that the development of tolerance can be postponed

or prevented entirely.

Strategies for Managing Tolerance

As tolerance to fungicides is now a recognized problem, some authors have set out ideas for managing tolerance.

New Materials

While development of new fungicides is expensive and time consuming, several interesting ideas have been proposed.

1. Use of adjuvants to overcome fungicide tolerance (17).
2. Find inhibitors of alternate pathways that bypass fungicide-inhibited metabolic steps (17).
3. If the binding site has been altered, try to alter the chemical to fit (17). Computer graphics are now being employed to this end (35).
4. Investigate materials that bolster the host plants' resistance to the pathogen (7).

Use Pattern Strategies

In selecting a management strategy, one must first assess the likelihood of tolerance emerging in the field. Many factors are involved. Delp (9), for instance, reports a model wherein development of tolerance to benomyl is dependent on:

- frequency of tolerant propagules
- proportion of tolerant propagules surviving the spray program
- year-to-year survival of tolerant propagules
- number of disease cycles per season
- area treated and extent of coverage
- survival of tolerant populations from year to year.

While it is pointless to reiterate the mathematical details of this model, it provides a useful list of factors to be considered when planning a control program.

In addition, one must consider the development of tolerance to a chemical in other organisms, the frequency of tolerance in laboratory and greenhouse assays and the mode of action of the chemical (19).

For new chemicals, tolerance testing should be done either before introduction or before widespread use, to establish use patterns that would avoid the problems experienced with benomyl and its now severely curtailed usefulness (6,7,18,19).

If a choice of fungicides exists, a non-persistent multi-site mode of action material will be much less likely to induce tolerance (7,9,18,19,30).

In general, use patterns should avoid favouring competitive, tolerant strains at the expense of sensitive strains. To this end, a fungicide should be present for the minimal time and over the minimal space required (7,18,19), and wide-spread, long-term exposure to a single fungicide should be avoided (9,30). Exposure in space can be restricted by the use

of seed or furrow treatment, for example. Limiting use in time is exemplified by the use of ethirimol on powdery mildew of barley in England. Mildew control is more important for the spring crop than the winter crop, so discontinuing use of the fungicide on the winter crop is recommended. As ethirimol tolerance in the pathogen is unstable, the tolerant proportion of the population decreases over the winter, allowing maximal control of mildew from the ethirimol treatment in the following spring's crop (7,19).

Dekker (5) advocates using the highest possible dose of fungicide to kill all but the virtually immune strains, but to be wary of continued selection in favour of these strains if this strategy is continued. Ogawa et al. (41) disagree and advocate minimal fungicide use and alternating chemicals in the treatment schedule.

The use of alternating schedules or mixtures of fungicides with different modes of action is often advocated as a means of limiting exposure to one material alone for long periods of time (9,25,30,41). In the southeastern United States, Cercospora arachidola, a peanut leaf spot pathogen, had become highly tolerant of benomyl after 3 - 4 seasons of use. In areas where a rust fungicide also active against Cercospora was used in a mixture with benomyl, benomyl tolerance has not become a problem after 9 years of use (9). Gilpatrick (22) reports a similar experience from New York state where dodine used alone for control of apple scab (Venturia inequalis) resulted in the

development of tolerance within 9 years, whereas in areas where benomyl, carbamates and captan were applied for control of other diseases that are not controlled by dodine, dodine tolerance has not appeared after more than 20 years of use.

Tolerance of Botrytis to benomyl is a widespread problem (10,23,33) but in Australia, mixtures of captan and benomyl are used to control Colletotrichum acutatum (strawberry black spot) and Botrytis respectively. After 8 years of use, benomyl tolerance is not a problem (9).

Littrell (30) found strains of Fusicladium effusum (pecan scab) tolerant to benomyl in commercial pecan groves where benomyl had been used exclusively for 3 years. In groves where triphenyltin hydroxide had been used alternately with benomyl, no tolerant strains could be found.

Once tolerance is established, mixtures have been of little use in delaying further build-up of tolerant strains (24,31,40).

Several theoretical models of tolerance development under mixed or alternating fungicide regimes have also been proposed (9,26,49) but too little rigourous field testing of the alternating/mixture theories has been published to allow confident generalization. However, growth-room studies with P. infestans on potatoes convinced Ciba-Geigy that metalaxyl was sufficiently treatened by tolerance and that a metalaxyl-mancozeb mixture was sufficiently effective at delaying the development of tolerance, that only the metalaxyl-mancozeb mixture is now marketed for potatoes (51).

A related idea is presented by Wolfe (54), who advocates use of mixtures of cultivars with differing resistances, each component being treated with a different fungicidal seed treatment, or left untreated. He argues that the resulting heterogeneous environment will delay the breakdown of resistance in the plant and the development of tolerance in the pathogen.

The model presented by Delp (9) predicts that the use of another fungicide in a mixture with benomyl will significantly delay the onset of tolerance. The model is quite general and not applicable to a specific situation, but represents one of the early attempts to quantify the dynamics of tolerant populations of pathogens. A more elaborate model by Kable and Jeffery (26), dealing with mixtures of systemic and protectant fungicides, predicts that when the initial tolerant proportion of the population is low, many sprays will be needed to reach the 1% level. From there only a few sprays are needed until tolerance dominates. This could pose significant problems for a monitoring program. The model further predicts that if spray coverage is complete, there is no advantage to using mixtures; an alternating schedule should be used. As coverage becomes less complete, mixtures gain an advantage.

Skylakakis (49) developed a model that allows for differential growth and epidemiological properties of the sensitive and tolerant proportions of the pathogen population, and for differing efficacies of the fungicides. Skylakakis' model largely confirms the predictions of the Kable and Jeffery

model, but does predict an advantage for mixtures when coverage is complete, in contrast to Kable and Jeffery's model. It also predicts that as the efficacy of the second material in the mixture or alternating schedule increases, the delay in emergence of tolerance will increase.

A model recently published by Levy et al. (29) allows more flexibility than the previous models and includes such factors as fungicide weathering (i.e. loss of fungicide from the system), additive effects between fungicides, variations in coverage, competition between tolerant and susceptible subpopulations, initial frequency of the tolerant genotype and apparent infection rate. This model predicts that a mixture will be superior to alternations when there is no additive action of the fungicides and when fungicide weathering is rapid. delay in the onset of tolerance is greatest at high reproductive rates, probably due to greater competition from the sensitive genotype. This model agrees with Kable and Jeffery's (26) prediction that when coverage is incomplete, mixtures are superior in delaying the onset of tolerance.

Levy et al. (29) also predict that when weathering is slow, alternate applications will be superior to mixtures late in the season. Additive mixtures are predicted to provide superior control but will lead to the buildup of tolerance more rapidly than a systemic alone.

Clearly, field experiments are needed to confirm these predictions before widespread recommendations can be made on the

use of mixtures or alternating schedules. In the future, if mathematical models can be verified with field data, they may provide a useful tool for experimentation with different combinations of tolerance avoidance strategies. Currently, models present one of only a few attempts to quantitatively and precisely examine the relationships between the many and various factors contributing to the development of fungicide tolerance. As such, they are a valuable exercise, whether or not they are subsequently proven to be correct. Coupling laboratory studies, such as McPhee and Nestmann's continuous culture experiments (37), to models to test predictions may be a valuable first step.

Instructions for use on fungicide labels should, where possible, contain strategies for avoiding tolerance as part of the use pattern (41). This view is endorsed by both the American Phytopathological Society (55) and the U.S. Environmental Protection Agency (2).

Monitoring

Many authors advocate monitoring for the development of tolerance in a pathogen population and switching to another material with a different mode of action when this occurs (5,8,9,18,19,30,41).

All new chemicals should be screened *in vitro* for the ability to induce tolerance in pathogen populations. While such tests may lack 'realism' (51), they have the advantage of being

reproducible and can test large numbers of propagules in a relatively short time (5,6,7,9,18,19). Some progress is being made toward more realistic in-vitro tests (eg. 37) but further work in necessary. Laboratory studies are also necessary to establish sensitivity levels of previously unexposed populations of pathogens for later use in monitoring programs (30).

Greenhouse studies are seen as a compromise between field studies and laboratory studies. Including host plants increases the realism of the pathogen/host/chemical interactions but other factors that may play a role are absent (7,30).

Ultimately management must include field monitoring, as the predictive value of laboratory and greenhouse assays is never known until it is tested in the field. Ideally one should study fields with different treatment histories to determine the actual time required for the emergence of tolerance in this context. Besides baseline dose responses for tolerant and sensitive strains, tests of virulence and pathogenicity are necessary (30). Two factors should be considered in establishing threshold levels for tolerant strains in the field:

1. What proportion of the pathogen population must be fungicide-tolerant to trigger a disease outbreak in the presence of a fungicide (30)?
2. Will the population revert to sensitivity when the chemical is withdrawn? (5,6,18,19,30).

Field monitoring has been used successfully to avert control failures due to tolerance in Cercospora leafspot on

peanut to benomyl (30), Botrytis cinerea on tomato to benomyl (16), Venturia inaequalis on apples to dodine and Penicillium spp. on citrus to various chemicals (41).

Where possible, monitoring should be integrated with other tolerance avoidance strategies as it is not always a sufficient strategy alone and may be too costly or slow (8,51).

Summary

In designing a fungicide-based control program, there are three basic areas to be considered in assessing the need for a tolerance avoidance strategy.

1. The Fungicide

- What is the mode of action? Fungicides whose mode of action can be circumvented by a single genetic change are more at risk to development of tolerance than are those that require many genetic changes. This may roughly correlate to single-site/multi-site modes of action.

- Has this fungicide produced tolerance problems in other pathogens or in this pathogen on other crops or in other locations?

- Has this fungicide been used on this field before? On other fields in this area?

- Are other fungicides with different modes of action available as alternatives? Are mixtures chemically feasible or would they be unstable? Are they registered for this use?

- How persistent is the fungicide and how susceptible will it be to typical local weathering conditions?

- What affect will the fungicide have on other pathogens on this crop. Will it control more than one pathogen or is it likely to cause problems with control of other pathogens or antagonists?

2. The Pathogen

- Has this pathogen developed tolerance easily to other fungicides? Is there a risk of cross-tolerance in existing tolerant strains?

- How difficult would it be to monitor for tolerance? Has a tolerance threshold been established anywhere?

- Has this pathogen ever been screened to determine frequency of occurrence of isolates tolerant to this fungicide? Are tolerant isolates of this pathogen pathogenic and competitive? Do tolerant populations tend to revert to sensitivity if the fungicide is withdrawn?

- How great is the inoculum potential and dispersal ability? If tolerance appears, how quickly is it likely to become a problem?

- Does the pathogen have any local alternate hosts to provide a reservoir of susceptible competitor strains?

3. The Crop

- How valuable is the crop? How much can be spent on control and tolerance avoidance procedures? What are the risks if control fails?

- Has a damage threshold been established?

- How difficult is it to apply the fungicide? Are many applications possible or is treatment restricted to a one time seed or furrow application? How complete can coverage be?

Much more research is needed before most of the above considerations can be adequately addressed. No one individual, organization or institution can deal with all these considerations simultaneously. Responsibility for them can be broken down into four overlapping groups: governments, industry, independent researchers, and users.

1. Governments

Governments, both federal and provincial, have the power to greatly help or severely hinder progress toward real-world application of tolerance avoidance measures. Of greatest importance are the fungicide registration regulations. If the

use of mixtures, particularly pre-pack mixtures, or alternating schedules is to become common, a sufficiently wide range of fungicides must be available for use on a particular crop. Currently, especially in minor crops, the limited range of fungicides legally available limits choices for mixture components. The registration procedure for particular fungicide/crop combinations should be improved to facilitate mixture choices.

In Canada, provincial governments have great influence over fungicide use patterns through published production guides. If valid strategies exist and have been tested under local conditions, production guides should reflect and promote this approach. A few efforts in this direction are evident (38) but the relative paucity of such recommendations reflects the lack of concrete knowlege and registered alternatives. In California, fungicide use schedules for tree-fruits have been constructed around tolerance-avoidance, but the long-term effect is as yet unknown. California also requires that benomyl be used only in combination with a protectant fungicide in tree-fruit orchards (40).

Care should be taken that the entire fungicide schedule be considered and that control measures for one pathogen do not interfere with tolerance avoidance strategies for another.

Governments can also make contributions in the area of research. Vast gaps exist in general knowlege of tolerance avoidance strategies and specific knowlege of the behaviour of

individual pathogens, crops and fungicides in the tolerance equation. Through government agencies, incentives to companies and research grants, Canadian governments have influence over the majority of pest management related research done in this country. Ensuring that information on the avoidance of tolerance is available for a variety of crops and pathogens will immensely aid plant disease management in Canada.

2. Industry

Promoting tolerance avoidance strategies is a clear case of enlightened self-interest for the fungicide industry. If a fungicide's efficacy is lost to tolerance, there will be no profit from its manufacture, distribution or sale. With the massive investment needed to bring a new fungicide from the laboratory to the marketplace, any decline in use could represent a substantial loss. Besides the development of new materials with the use of increasingly sophisticated new techniques (35), fungicide manufacturers should be investing in research to determine the likelihood of development of tolerance to their fungicides, strategies for avoiding tolerance and development of data to support registration of the materials needed for those strategies. To do this, given the present state of the art, they would also have to fund research into basic techniques to accomplish these goals. Presently, at least some companies are pressing for the adoption of pre-packaged

mixtures, as this neatly circumvents the problem of enforcement of tank-mix or alternating use schemes (51). Some work is also being done on the efficacy of mixtures (31,51). Ensuring that fungicide labels contain adequate information for tolerance avoidance and that agricultural agencies and extension personnel are aware of them is also essential. These are steps that can be taken for both new and existing fungicides. Co-operation between manufacturers of different fungicides that havepreviously shown cross-tolerance is also necessary if growers are being urged to switch materials when tolerance appears (51).

3. Research

This section is directed primarily at 'independent' researchers, those not employed directly by government or industry. There is a need for more information on the question of mixtures vs alternating fungicides, and other factors affecting development, stability and fitness of tolerant strains before generalizations can be made on field strategies. These problems will require input from the disciplines of genetics, population dynamics, physiology and biochemistry, ecology, plant pathology and other fields. There are many unanswered questions here; if interest in them continues to grow, the problem of fungicide tolerance will likely diminish.

For the researcher in pest management, fungicide tolerance presents specific problems. Field experiments are required to

set tolerance thresholds, to develop monitoring techniques, and to compare various use pattern strategies. A French computer program developed for a growers group assists in decision making in the application of herbicides and fungicides on wheat (28). It would be useful to attempt to integrate fungicide tolerance risk factors as parameters of this program. Defining the parameters is the greatest present difficulty.

4. Fungicide Users

Included in this group are growers, field pest managers and extension personnel. While their options are often limited by lack of information and legal restrictions, certain choices certainly remain. Simply being aware of the potential for fungicide tolerance is imperative. Fungicide users can seek out experience in other areas, other crops and other pathogens with a particular fungicide to get a rough idea of what to expect. Where a choice exists, the use of multi-site fungicides, mixtures or alternating schedules containing multi-site materials would appear beneficial in cases where a desired fungicide has been shown vulnerable to tolerant strains of the target pathogen. Treatment of the minimal area required and for the minimum time possible is a recommended procedure for any pesticide, and will reduce the selection pressure for fungicide tolerance. Similarly, the use of resistant cultivars and cultivation techniques that minimize host plant susceptibility

to disease is always a recommended approach. Where tolerance is a threat, perhaps the most important direct action available to a user is vigilance and/or monitoring for tolerance, even if only by damage assessment. Users should quickly discontinue the use of a fungicide when indications of tolerance appear because of the risk of further selection for increased fitness in the tolerant population.

Ideally, most of these options would be tested by local agriculture extension or research personnel.

Fungicide tolerance in the field appears to be an increasingly serious problem. Research stimulated by this problem will undoubtably alter the future approaches to minimizing fungicide vulnerability.

B. PART II: SPECIFIC TOLERANCE OF *SCLEROTIUM CEPIVORUM* TO DICARBOXIMIDE FUNGICIDES

Introduction

Control of white rot of *Allium* spp. caused by *Sclerotium cepivorum* Berk. using the dicarboximide fungicides iprodione and vinclozolin has been reported from various parts of the world (14,15,21,39,53). While these fungicides show real promise for control of this disease, they have also shown a propensity for selecting tolerant strains of other pathogenic fungi (Table 1). The purpose of this study was to assess the potential for development of tolerance to dicarboximide fungicides in *S. cepivorum* and to evaluate the *in-vitro* sensitivity of different isolates of *S. cepivorum* to dicarboximide and other fungicides.

Table 1: Organisms reported tolerant to dicarboximide and other fungicides

Organism	Iprodione	Vinclozolin	Dichloran	PCNB	References
Alternaria alternata	X		X		36
Botrytis cinerea	X	X	X	X	10,23,27, 33,34,42
Botrytis squamosa	X				44
Botrytis tulipae	X	X			3
Fusarium nivale	X	X			4
Monilinia fructicola	X	X	X		46,47,52
Penicillium expansum	X	X			48
Sclerotinia homeocarpa	X				11
Sclerotium cepivorum			X		32
Trichoderma harzianum	X	X	X		1

Materials and Methods

All concentrations of fungicides in this report refer to active ingredient only. All fungicides were added as aqueous suspensions to cooled, autoclaved Difco potato dextrose agar (PDA). Fourteen isolates of *S. cepivorum* were tested for the occurrence of tolerance to dicarboximide fungicides by sprinkling 100 - 500 sclerotia of each source (S) isolate onto PDA containing 100 ug/ml of either iprodione (Rovral 50W, May & Baker Canada Inc.) or vinclozolin (Ronilan 50W, BASF Canada Inc.) using two plates per isolate. Mycelium from germinating sclerotia was transferred to unamended PDA. Mycelial plugs (5 mm

diameter) from the subcultures were placed on PDA amended with 100 ug/ml of the same chemical from which the subculture was originally obtained. Those showing radial growth exceeding 15 mm total colony diameter after 4 days incubation at 22 - 24 C were transferred onto unamended PDA. These subcultures were then subjected to at least 10 transfers, each of at least 10 days duration, on unamended PDA. They were then transferred onto PDA containing 100 ug/ml iprodione or vinclozolin, as appropriate, to test for stability of tolerance. Those showing radial growth exceeding 15 mm in 4 days at 22 - 24 C were designated as tolerant (T) isolates.

All S and T isolates were tested to determine their levels of sensitivity to iprodione, vinclozolin, myclozolin (BCI-100F 50W, BASF Canada Inc.), dichloran (Botran 75W, Tuco Products Inc.), PCNB (Terraclor 75W, Olin Mathieson Chemical), benomyl (Benlate 50W, Plant Products Inc.), captan (Orthocide 50W, Ortho Chemicals), and thiram (Arasan 75W, Dupont), each at 100, 10, 1, 0.1, and 0.01 ug/ml in PDA (Table 2). Any isolates that grew at 100 ug/ml of any active ingredient were also tested at 1000 ug/ml of that chemical. Each combination of isolate, chemical and concentration was replicated five times. Radial growth was recorded after 4 days of incubation at 22 - 24 C. The four parent S isolates (those that yielded T isolates) were tested on vinclozolin-amended PDA at 0.02, 0.04, 0.06, 0.08, 0.10, 0.30, 0.50, 0.70, 0.90 and 1.10 ug/ml with 5 mm diameter plugs for 4 days at 22 - 24 C when colony diameter was measured. They were

also tested on iprodione-amended PDA at 0.06, 0.08, 0.10, 0.30, 0.50, and 0.70 ug/ml as above. Again, each combination of isolate, chemical and concentration was replicated five times.

Appropriate data transformation was determined to be base 10 log(colony diameter) using the P7D program of BMDP (12) and transformed data were subjected to linear regression by the P6D program of BMDP (12). Significance and r^2 values were derived by the method given by Zar (56). EC90 values (concentration which inhibits growth by 90%) were obtained from the regression equations. Where growth occurred at 1000 ug/ml, the EC90 value was recorded as >1000 ug/ml.

The frequency of occurrence of tolerance in the four parent S isolates was estimated in populations of sclerotia produced on PDA and collected after the medium had completely dehydrated. Sclerotia were scattered on PDA plates amended with 100 ug/ml of vinclozolin or iprodione, counted, incubated for 14 days at 22 - 24 C, and the resulting colonies counted. Identity of the colonies was confirmed by plating onto unamended PDA. Viability of the sclerotia was estimated by plating sclerotia onto unamended PDA and counting germination over 14 days. Each viability test was replicated at least 19 times for each isolate.

Pathogenicity bioassays were carried out in the greenhouse using onion sets cv. White Ebenezer in 10-cm plastic pots with Abbotsford sandy soil. Ten plants (2 per pot) were used for each of the four parent isolates and the five T isolates. Two holes

were made in the soil and 100 - 300 sclerotia axenically grown in 20:1 sand-cornmeal medium were added. The bulbs were inserted in the holes on top of the sclerotia and covered with soil. Ten bulbs were planted without sclerotia as a control.

Plants were harvested as they showed top symptoms and all remaining plants were harvested 150 days after planting.

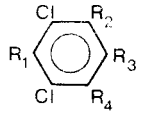
Results

Five isolates able to grow on PDA amended with either 100 ug/ml vinclozolin (J191V, S187bV, S187bVa, S201bV) or iprodione (VDMI) were recovered from four of the 14 source (S) isolates tested. These five isolates all grew to more than 15 mm diameter in 4 days on 100 ug/ml vinclozolin or iprodione, as appropriate, when retested after passage through unamended PDA. After 10 or more subsequent transfers on unamended PDA, all five isolates grew well on vinclozolin or iprodione amended PDA and were designated as tolerant (T) isolates. None of the parent S isolates grew on these media.

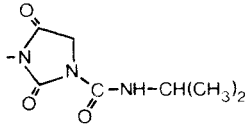
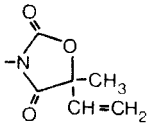
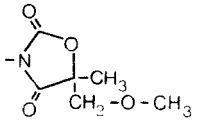
Tolerance to iprodione was observed for the T isolates recovered from vinclozolin-amended PDA and *vice versa*. At this point, the study was enlarged to include myclozolin, dichloran, PCNB, benomyl, thiram and captan (Table 2).

Table 2. Structural relationships among the eight fungicides tested for toxicity to Sclerotium cepivorum

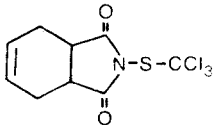
Fungicides containing the structural subunit



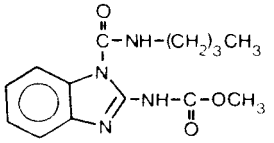
N-substituted 3,5-dichlorophenyl

Fungicide	R_1	$R_2 = R_4$	R_3
PCNB	-Cl	-Cl	
Dichloran	$-NH_2$	-H	
Iprodione	-H	-H	
Vinclozolin	-H	-H	
Myclozolin	-H	-H	

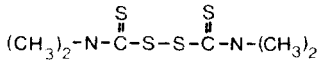
Structurally unrelated fungicides



Captan



Benomyl



Thiram

One of the four T isolates obtained originally from vinclozolin-amended PDA (J191V) and the single isolate obtained from iprodione-amended PDA (VDMI) showed strong cross-tolerance to all five fungicides having in common the N-substituted dichlorophenyl structural subunit (Table 3). Mycelial growth of these two isolates occurred on media amended with 1000 ug/ml of vinclozolin, iprodione, myclozolin, dichloran, or PCNB. The remaining three isolates originally recovered from vinclozolin-amended PDA (S187bV, S187bVa, S201bV) also grew on PDA containing 1000 ug/ml of dichloran or PCNB. They were less tolerant of iprodione and myclozolin, but were nevertheless more tolerant of these fungicides than were their respective parental S isolates (Table 3). None of the S isolates grew at concentrations of dichloran greater than 0.1 ug/ml and no parent S isolate grew at concentrations of PCNB greater than 100 ug/ml while all five T isolates grew at 1000 ug/ml.

The tolerances of the five T isolates for benomyl were completely unaltered from those of the parental S isolates (Table 3). Tolerances of four of the five T isolates for thiram were increased 10-fold over the tolerances of their respective S isolate parents, that for the remaining T isolate (S187bV) decreased 10-fold (Table 3). Tolerances of T and parental S isolates for captan remained the same for three of the five T isolates, and were increased 10-fold over that of the parental isolate for the remaining two T isolates (S187bV, S187bVa).

Table 3: Highest concentrations (ug/ml) of various fungicides in PDA allowing growth of various isolates of *Sclerotium cepivorum*.

Isolate	Source	Vinclozolin	Iprodione	Myclozolin	Dichloran	PCNB	Benomyl	Thiram	Captan
				'S' Isolates					
J191	Hull, U.K.	0.1	0.1	0.1	0.1	0.01	1	10	1000
NZ32	Auckland, New Zealand	1.0	1.0	0.1	0.1	0.01	1	100	1000
BBY	Burnaby, B.C.	0.1	0.1	0.1	0.1	0.01	1	1000	1000
ELS	Fairview, Alta.	0.1	0.1	0.1	0.1	0.01	1	1000	1000
GP	Grand Forks, B.C.	1.0	1.0	0.1	0.1	0.01	1	1000	1000
NZ37	Auckland, New Zealand	0.1	0.1	0.1	0.1	1	1	10	1000
S187a	Australia	0.1	0.1	0.1	0.1	100	1	10	1000
S197a	Australia	0.1	0.1	0.1	0.1	100	1	10	1000
S197b	Australia	0.1	0.1	0.1	0.1	100	1	10	1000
S201a	Australia	0.1	0.1	0.1	0.1	100	1	10	1000
J192	Hull, U.K.	0.1	0.1	0.1	0.1	100	1	100	1000
VDM	Wageningen, Holland	0.1	0.1	0.1	0.1	100	0.1	100	1000
S187b	Australia	1.0	0.1	0.1	0.1	100	1	100	100
S201b	Australia	0.1	0.1	0.1	0.1	100	1	100	1000
				'T' Isolates					
S201bV	S201b	1000	1	10	1000	1000	1	1000	1000
S187bVa[1]	S187b	100	10	10	1000	1000	1	1000	1000
S187bV	S187b	1000	1000	10	1000	1000	1	10	1000
J191V	J191	1000	1000	1000	1000	1000	1	100	1000
VDMI	VDM	1000	1000	1000	1000	1000	0.1	1000	1000

[1] reverted to sensitivity comparable to S187b after 25 transfers through unamended PDA.

The 14 S isolates were highly sensitive to vinclozolin, iprodione, myclozolin, dichloran and benomyl, and highly tolerant to captan. Marked variations (10,000-fold differences) in the sensitivity of S isolates to PCNB were observed, and substantial variation in sensitivity to thiram (100-fold differences) was also noted (Table 3).

With one exception, EC90 values for vinclozolin and iprodione against the four stable T isolates of S. cepivorum were >1000 ug/ml (Table 4).

Table 4: Comparative[1] EC90 values for vinclozolin and iprodione against radial growth of tolerant (T) and parental (S) isolates of Sclerotium cepivorum on fungicide-amended potato dextrose agar.

Isolate	Status	EC90 (ug/ml)	Isolate	Status	EC90 (ug/ml)
		VINCLOZOLIN			
S187b	S	0.39	S187bV	T	>1000
S201b	S	0.38	S201bV	T	>1000
J191	S	0.38	J191V	T	>1000
VDM	S	0.43	VDMI	T	>1000
		IPRODIONE			
S187b	S	0.52	S187bV	T	>1000
S201b	S	0.35	S201bV	T	≤10[2]
J191	S	0.44	J191V	T	>1000
VDM	S	0.40	VDMI	T	>1000

[1] $r^2 > 0.933$ for all regressions, $P \leq 0.025$
[2] Precise value not determined

A determination of a precise EC90 value for iprodione against T isolate S201bV was inadvertantly omitted. Data in Table 3 show that S201bV was substantially more sensitive to iprodione than were the other T isolates, and that the EC90 value for iprodione against this isolate was between 1 and 10 ug/ml. The EC90 values for vinclozolin and iprodione against the four parental S isolates were remarkably similar and ranged from 0.35 to 0.52 ug/ml active ingredient in PDA.

Tolerance to the dicarboximide fungicides in S. cepivorum does not seem to be completely stable as isolate S187bVa reverted to sensitivity comparable to that of its parental S isolate S187b after 25 transfers on unamended PDA.

In spite of screening almost 85,000 sclerotia, the numbers of tolerant sclerotia detected were extremely low and variable. This precluded the calculation of a precise and reliable estimate of the frequency of tolerance. Despite this, the four parent S isolates showed similar ranking of frequencies on either iprodione or vinclozolin amended PDA (Table 5). Isolates VDM, S187b, S201b and J191 yielded 1.90×10^{-1} %, 4.8×10^{-2} %, 2.7×10^{-2} %, and 1.8×10^{-2} % tolerant sclerotia, respectively, averaged over both fungicides.

Table 5: Frequency of occurrence of tolerant sclerotia of *Sclerotium cepivorum*

	VDM	S187b	S201b	J191
VINCLOZOLIN				
No. tested	9828	9040	14497	15059
No. germinated	21	6	4	4
Mean frequency	0.214%	0.066%	0.028%	0.027%
IPRODIONE				
No. tested	4877	7466	11643	12084
No. germinated	7	2	3	1
Mean frequency	0.143%	0.027%	0.026%	0.008%
UNTREATED				
Mean germination	88.1%	70.7%	89.0%	96.1%

All T and S isolates tested produced infections in onion sets in pots. None of the plants without added sclerotia became infected (Table 6).

Table 6: Proportion of onion plants (cv. White Ebenezer) infected with *S. cepivorum*

S Isolates	Prop. Infected	T Isolates	Prop. Infected
VDM	0.7	VDMI	0.8
J191	0.8	J191V	0.6
S187b	0.2	S187bV	0.3
		S187bVa	0.3
S201b	0.8	S201bV	0.5
Check	0.0		

Discussion

Clearly *S. cepivorum* has substantial biological potential to develop tolerance to the dicarboximide fungicides. These data show that even at or above the limits of solubility for vinclozolin or iprodione (50) significant growth of the T isolates occurs, even though this represents concentrations >1000-fold higher than the EC90 values of the parent isolates for these chemicals. The phenomenon of cross-tolerance among the dicarboximides and dichloran and PCNB fungicides noted for other organisms (19,27,36) clearly occurs in *S. cepivorum*.

Although the mode of action of the dicarboximides is still unclear (13,43,45) the widespread occurrence of cross-tolerance within this group (4,27,36,46,48,52) and between the dicarboximides and the other fungicides containing the N-substituted 3,5-dichlorophenyl structural subunit (19,27,36) would suggest that tolerance to these chemicals may have its basis in this structural feature they share (Table 2). This cross-tolerance is of particular interest in the control of onion white rot as tolerance to dichloran has been reported for this pathogen (32). Note that although dichloran would properly be called an N-substituted 2,6-dichlorophenyl compound due to the nomenclatural primacy of the amine group at position R1, its structure fits the same basic pattern as the rest of the N-substituted 3,5-dichlorophenyl compounds shown in Table 2. In the interest of simplicity I have grouped it as an N-substituted

3,5-dichlorophenyl compound for this discussion, realizing that this nomenclature is not strictly correct.

Benomyl would appear to be a good candidate for use in a mixed or alternating chemical approach for control of onion white rot as it was the only fungicide tested to which sensitivity remained unchanged between the T and S isolates, and all isolates were sensitive to low concentrations (Table 2). This lack of correlation in tolerance between benomyl and the dicarboximides and N-substituted 3,5-dichlorophenyl-containing fungicides has been noted in other organisms (13,52), although multiple resistance is possible (52).

While the infectivity assay (Table 6) is unrealistic and should not be taken as representative of the results that would occur in the field, it does show that at least under some conditions the tolerant isolates are pathogenic and should not be dismissed lightly.

Appendix I

List of chemicals mentioned.

Common name	Trade name	Chemical name
benomyl	Benlate	methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate
captafol	Difolatan	(N-(1,1,2,2,tetrachloroethylthio)-3a,4,7,7a-tetrahydrophthalimide
captan	Orthocide	N-trichloromethylmercapto-4-cyclohexene-1,2-dicarboximide
dichloran	Botran	2,6-dichloro-4-nitroaniline
dodine	Cyprex	n-dodecylguanidine acetate
ethirimol	Milstem	5-n-butyl-2-ethylamino-4-hydroxy-6-methylpyrimidine
fentin hydroxide	Du-Ter	triphenyltin hydroxide
iprodione	Rovral	3-(3,5-dichlorophenyl)-N-(1-methylethyl)-2,4-dioxo-1-imidazolinecarboxamide
mancozeb	Dithane M-45	polymer of manganous ethylene-bis-dithiocarbamate with zinc
metalaxyl	Ridomil	methyl N-(2-methoxyacetyl)-N-(2,6-xylyl)-DL-alaninate
myclozolin	BCI-100F	3-(3,5-dichlorophenyl)-5-methyl-5-methyoxymethyleneoxazolidine-2,4-dione
oxycarboxin	Plantvax	5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxanilide
quintozene (PCNB)	Terraclor	pentachloronitrobenzene
thiram	Arasan	tetramethylthiuram disulphide
vinclozolin	Ronilan	3-(3,5-dichlorophenyl)-5-methyl-5-vinyloxazolidine-2,4-dione

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