Bull. Environ. Contam. Toxicol. (1991) 47:97–103

Bulletin of Environmental Contamination and Toxicology

Interactive Effects of the Fungicide Chlorothalonil and the Herbicide Metribuzin towards the Fungal Pathogen *Alternaria solani*

Tammy L. Hill and Glenn W. Stratton

Environmental Microbiology Laboratory, Department of Biology, Nova Scotia Agricultural College, Truro, Nova Scotia, B2N 5E3, Canada

The effects of chemicals on non-target organisms in the environment is becoming an increasingly active area of research, given the increased environmental awareness of society. One area that has become the focus of major public and regulatory concern deals with the environmental impact of pesticides. Presented with the long-term prospect of continued use of pesticides in agriculture and forestry, we must develop a full knowledge of both the adverse and beneficial effects of pesticides and their combinations on the biosphere (Altman and Campbell 1977). Although considerable data have been published on the effects of individual pesticides towards non-target organisms and ecological processes, few data are available on the ecotoxic effects of pesticide combinations (Schuster and Schroder 1990). Most agricultural and forestry operations employ a number of pesticides and, as a result, both target and non-target organisms could be exposed to mixtures of these compounds.

Although the importance of research on interactions between the various components of xenobiotic mixtures is generally recognized (National Research Council 1982), attempts at addressing this problem have been hampered by a lack of adequate methodology (National Academy of Sciences 1981). However, new methods have recently been developed to study the effects of toxicant combinations on microorganisms (Stratton et al. 1982; Stratton 1988). These methods can theoretically be applied to any given test system (Stratton 1988) and have been used extensively to study the toxic interactions between solvents and pesticides towards non-target microorganisms in bioassays (reviewed in Stratton 1989). In addition to ecotoxicity studies, another aim of interaction research with pesticides should be to determine particular combinations of pesticides that will give acceptable pest control at doses lower than those normally used in single applications (Akobundu et al. 1975). This problem also has yet to be adequately addressed. The purpose of the present study was to investigate interactions between the fungicide chlorothalonil and the herbicide metribuzin towards growth of the fungus *Alternaria solani*. These pesticides are commonly employed to control pests in potatoes and *A. solani* is a disease pathogen of that crop. The data obtained from this study addresses both of the above deficiencies in information regarding pesticide combinations.

Send reprint requests to G.W. Stratton at the above address.

MATERIALS AND METHODS

The test pesticides included chlorothalonil (tetrachloroisophthalonitrile; Bravo® W-75; wettable powder; SDS Biotech Corp.; 720 g active ingredient L^{-1}) and metribuzin (4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(*4H*)-one; Lexone® DF; dispersible granules; E.I. Dupont de Nemours Co. Inc.; 75% active ingredient by weight). The pesticide formulations were dissolved in glass distilled water and all pesticide concentrations are given as ppm (mg L^{-1}) of active ingredient.

Alternaria solani, the causative agent of early blight on potatoes, was used as the test organism. Stock cultures were maintained on Potato Dextrose Agar (Difco Laboratories, Detroit, Mn, U.S.A.; pH 5.6±0.5) at a temperature of 25± 1 °C. Preliminary studies were performed using the method of Stratton (1989) to determine the optimum growth medium, pH, and incubation temperature to use in interaction studies. This method identifies test conditions that increase the magnitude of toxicant interactions, thereby making the interactions easier to identify (Stratton 1989). Media tested included Potato Dextrose Agar (PDA), Corn Meal Agar (CMA), Lima Bean Agar (LBA), and Nutrient Agar (NA) (Difco Laboratories). Test pH values ranged from 5.0 to 7.5 in 0.5 unit increments. The pH of sterile molten agar at a temperature of 45°C was adjusted aseptically using filter sterilized 0.1 N NaOH or HCl (Stratton 1989). Test temperatures ranged from 15 to 35±0.5°C in five degree increments. The effects of test parameters on fungal growth were determined in the absence of toxicants, as outlined below.

The fungitoxicity of chlorothalonil, metribuzin, and chlorothalonil-metribuzin mixtures were determined in petri plates using a poisoned agar technique, as outlined in detail elsewhere (Stratton et al. 1982; Stratton 1989). Plates were incubated until control growth reached a diameter of 50-70 mm, at which time all plates were examined and growth recorded as colony diameter. All treatments were replicated six times. Based upon the preliminary experiments noted above, all bioassay systems used PDA, pH 5.5, and an incubation temperature of 30°C. Chlorothalonil and metribuzin were individually tested against *A. solani* at concentrations of 0, 0.1, 0.5, 1.0, 5.0, 10, 50, 100, 300, 500, 600, 700, 800, 900, and 1000 ppm. EC_{50} values were determined as outlined below. Mixtures of chlorothalonil and metribuzin were tested for interaction effects towards *A. solani* using the method outlined in detail by Stratton (1988). The treatment terminology is also defined in that reference. This was accomplished by interacting chlorothalonil concentrations of 0, 500, 600, 700, 800, 900, and 1000 ppm with a metribuzin level of 750 ppm, and by interacting metribuzin concentrations of 0, 500, 600, 700, 800, 900, and 1000 ppm with a chlorothalonil level of 750 ppm (interaction series of treatments). In each interaction experiment the pesticide whose concentration was being varied was also tested individually at the same levels to provide the control data necessary to calculate interaction effects (control series of treatments). The treatment containing 750 ppm of either chlorothalonil or metribuzin only is known as the reference treatment. As well, pesticide-free control systems were included in all experiments. Interaction effects were calculated as outlined below.

For experiments determining the optimal test conditions to use in interaction studies, the data were summarized as net colony diameter (colony diameter in mm minus the size of the inoculum plug) and significant differences were determined using an analysis of variance procedure followed by a Tukey's studentized range test at $P \leq 0.05$ and 95% confidence limits (SAS Statistics Software, SAS Institute

Inc., Cary, N. Carolina, U.S.A.). Data from chlorothalonil and metribuzin dose-response experiments were used to calculate EC_{50} values, which are defined here as the pesticide concentration in ppm required to cause a 50% inhibition of fungal growth, measured as an increase in colony diameter. Dose-response data were plotted and curves fitted to the data using polynomial regression techniques (SAS Institute). Significant differences at P=0.05 were determined using either an analysis of variance procedure followed by a Tukey's range test or a Student's t-test, where applicable. Interaction effects were analyzed as outlined elsewhere (Stratton et al. 1982; Stratton 1988, 1989). The interaction data were presented graphically by plotting the net interaction effect, given as a corrected percent inhibition value, versus concentration of the pesticide whose level was varied in the interaction treatments (see Stratton 1988 for calculation details). The zone of additive interaction was determined by calculating a net reference effect due to the pesticide whose level was held constant in the interaction treatments and indicated on the graph. Significant deviations of the interaction curve above or below this additive zone are indicative of synergistic or antagonistic interactions, respectively (Stratton 1988). Each point on the curve was tested to see if it differed significantly from the reference value used to indicate an additive response using an analysis of variance procedure followed by an LSD test at P=0.05 (SAS Institute).

RESULTS AND DISCUSSION

Chlorothalonil is a broad spectrum agricultural fungicide used to control late blight and early blight in potatoes. Recommended application rates of the formulation used in the present study are approximately 1.1 to 1.7 kg ha^{-1} at regular intervals throughout the growing season (Chemical Pharmaceutical Press 1986). Metribuzin is a herbicide used for both the preemergence and postemergence control of weeds in potatoes. Recommended application rates of the formulation used here are approximately 0.7 to 1.5 kg ha^{-1} for preemergence control of weeds and 0.7 kg ha^{-1} for postemergence control, both applied as a single treatment (Chemical Pharmaceutical Press 1986). *Alternaria solani* is a fungal pathogen of potatoes. Consequently, it can be expected that both of the pesticides tested will be found in the same microenvironment and could have interactive effects towards *A. solani* and other microorganisms found associated with the cultivation of potatoes.

Preliminary experiments were used to determine the bioassay conditions to employ in the interaction studies. Stratton (1989) reported that changes in microbial bioassay parameters, such as pH, temperature, and medium composition, that result in increases in culture growth rate also elicit larger interaction magnitudes. Larger interaction magnitudes make the identification and classification of interaction responses easier, especially when using microbial bioassay procedures designed specifically for toxicant interaction studies (Stratton et al. 1982; Stratton 1988). With *A. solani*, growth was statistically greater when the fungus was grown on PDA, followed by CMA, LBA, and then NA, regardless of the incubation temperature and pH used (data not shown). Since *A. solani* is a pathogen of potatoes, it is logical that this growth medium would supply the organism's nutritional requirements most effectively. Growth was also greatest at a temperature of 30°C (data not shown). This culture's optimum temperature for spore germination and mycelial growth is around 26 to 28°C and the temperature range for growth is from 1 to 45°C (Harrison et al. 1965). With regards to pH, the culture evidenced slowest growth at pH 5.0 and the growth rate increased gradually as the pH was raised from 5.0 to 7.5 (data not shown). However, this increase was not statistically significant at pH values from 5.5 to 7.5. Therefore, a pH of

5.5 was chosen, since this is the pH of PDA without adjustment. An increase in culture growth from pH 4.5 to 7.5 is common in fungi, and is probably due to pH effects on either the biochemistry of cell surfaces (Griffin 1981), the availability of metallic ions (Moore-Landecker 1982), and/or cellular permeability (Moore-Landecker 1982). Based upon these data and the recommendations provided by Stratton (1989), Potato Dextrose Agar, at a pH of 5.5, and an incubation temperature of 30°C were the bioassay conditions used in all subsequent toxicity experiments with *A. solani*.

Both chlorothalonil and metribuzin were tested for toxicity towards growth of *A. solani* in individual bioassays. All concentrations greater than 1.0 to 10 ppm caused a significant inhibition of growth. The EC_{50} values calculated for chlorothalonil and metribuzin were approximately 950±25 ppm and 850±25 ppm, respectively. The correlation coefficients for the polynomial regression curves were >0.990. There are relatively few bioassay data to compare these results with. The effects of herbicides towards soil fungi were reviewed by Anderson (1978). Many soil fungi exhibit a high degree of tolerance to herbicides and, with few exceptions, herbicides applied to growth media at levels approximating the recommended field application rates are not severely inhibitory to fungal isolates (Anderson 1978). The triazine and substituted urea herbicides have been investigated more extensively than any other group. Metribuzin is a triazine herbicide. Triazines have been shown to both inhibit and stimulate fungal growth at concentrations up to 100 to 1000 ppm, with the actual data being dependent upon the herbicide and fungus tested (reviewed in Anderson 1978).

The information available on herbicide effects towards plant pathogenic fungi deal primarily with effects on the incidence of disease and have been reviewed by Altman and Campbell (1977). Richardson (1959) tested several herbicides for their effect on *A. solani*. The number of lesions on treated and untreated tomato plants were compared. 2,4-D caused an increase in the number of disease lesions. The insecticide chlordane did not affect disease development, while the herbicide dalapon and the insecticides endrin and aldrin consistently reduced the number of lesions. Richardson (1970) also tested the effects of atrazine on *A. solani*. All concentrations of atrazine, ranging from 4.4 to 140 ppm, retarded radial growth of *A. solani* when tested using PDA, although the inhibition was pronounced only at atrazine levels greater than 35 ppm. Metribuzin generally increases the severity of plant diseases (Altman and Campbell 1977). The mechanisms involved in a herbicide causing an increase in disease may include direct stimulatory effects on growth and reproduction of the pathogen (Richardson 1970; Ware 1980), effects on the virulence of the pathogen (Ware 1980), or herbicide-induced changes in the physiology of the host plant which increases its susceptibility to disease (Hodges 1981). The results presented here for metribuzin indicate that this herbicide is relatively nontoxic towards *A. solani* when tested *in vitro*.

Chlorothalonil was also relatively nontoxic during laboratory bioassays. Apparently *A. solani* is more susceptible to this fungicide *in vivo,* since chlorothalonil is effectively used as a control agent. Many microorganisms show a higher tolerance for pesticides when grown under optimal conditions in the laboratory. In particular, the bioassay conditions used here were chosen to enhance any interactions that might occur between chlorothalonil and metribuzin, however, conditions which increase the magnitude of toxicant interactions in fungal bioassays also cause a reduction in the toxicity of the compounds tested (Stratton 1989). This has clearly been demonstrated in interaction bioassays employing the fungicide captan and organic solvents tested towards the growth of various fungi (reviewed

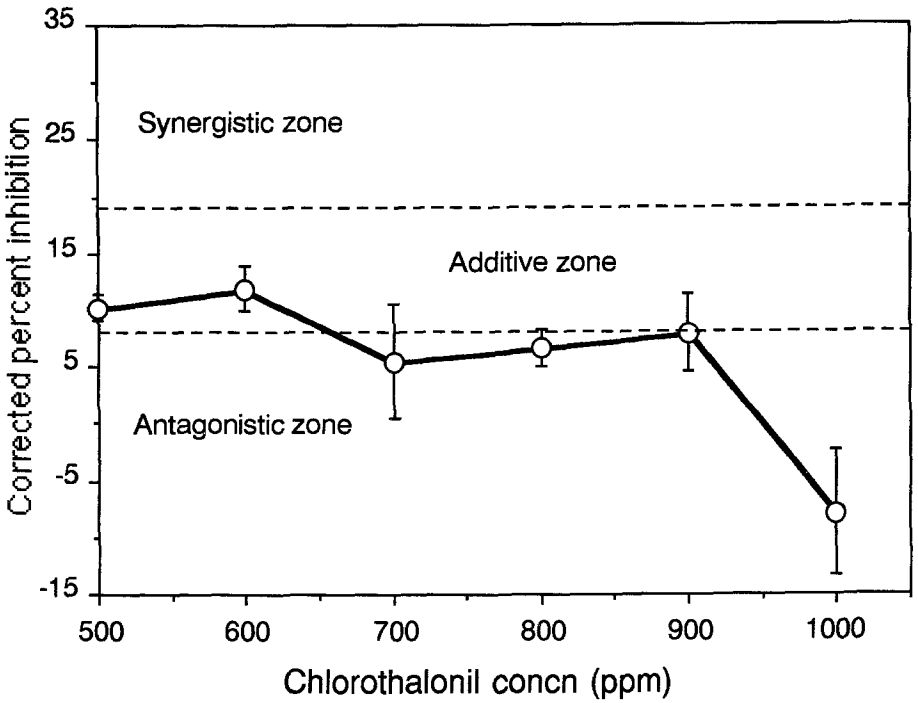

Figure 1. Interaction effects between 750 ppm metribuzin and varying concentrations of chlorothalonil.

in Stratton 1989). For example, with the fungus *Pythium ultimum*, adjusting the incubation temperature and pH from sub-optimum to optimum values induces increases of from 40 to 80% in the magnitude of captan-acetone interactions while inducing a decrease of from 50 to 90% in the toxicity of captan (Stratton 1989).

Interactions between metribuzin and chlorothalonil towards growth of *A. solani* are summarized in Figures 1 and 2. Figure 1 contains the data for interactions where metribuzin was held constant at a concentration of 750 ppm while the level of chlorothalonil was varied from 500 to 1000 ppm. Figure 2 contains the data obtained when the concentration of chlorothalonil was held constant while the level of metribuzin was varied. In both cases similar results were obtained; the fungicide and herbicide interacted in an additive manner at concentrations up to 900 ppm and antagonistically at levels greater than 900 ppm. The additive zone indicated on the graphs was calculated from the data supplied from bioassay systems containing only the pesticide whose level was held constant in that series of experiments. The error bars are standard deviations. The details of the interaction calculations are summarized elsewhere (Stratton et al. 1982; Stratton 1988, 1989). There are no other data on chlorothalonil-metribuzin interactions to compare these data with. As well, there are relatively few data available on pesticide interactions in general (Anderson 1978; Schuster and Schroder 1990). The interaction patterns obtained here, where the type of interaction is dependent upon the concentration of the components of the mixture, are routinely encountered in toxicant interaction experiments employing fungi and other microorganisms (reviewed in Stratton 1989). Reasons for this are unknown and can only be

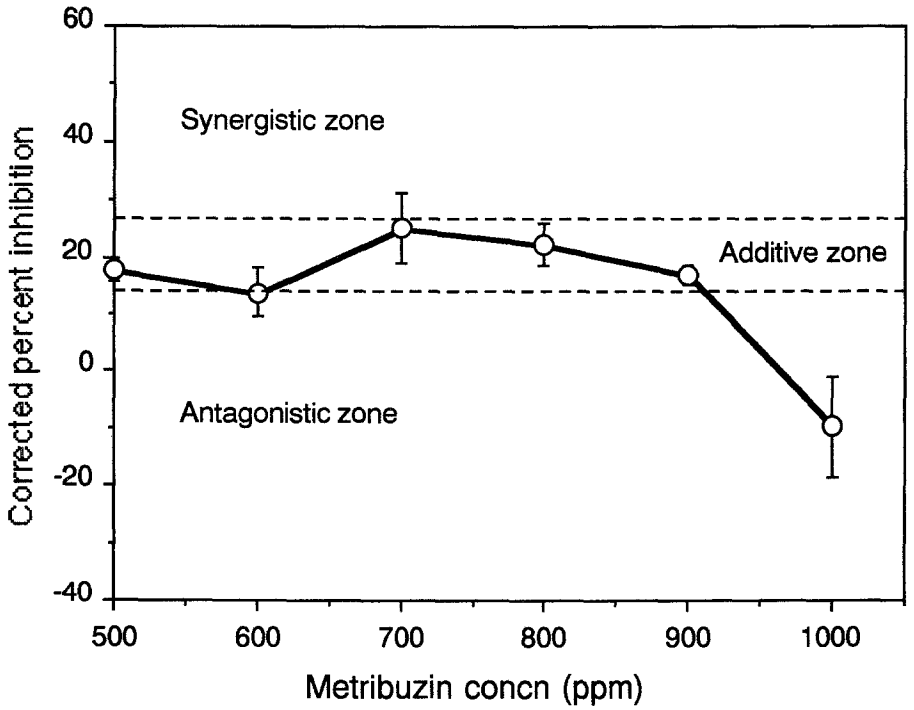

Figure 2. Interaction effects between 750 ppm chlorothalonil and varying concentrations of metribuzin.

addressed with further research (Stratton et al. 1982). With regards to the data presented here, the simultaneous use of chlorothalonil and metribuzin to control disease and weeds in potatoes could lead to antagonistic interactions between the pesticides. This could cause a reduction in the efficacy of chlorothalonil in controlling *A. solani* in potatoes. It would be more desirable to use a herbicide that interacts synergistically with chlorothalonil. This would allow lower concentrations of the fungicide to be used, at least during the early part of the growing season when herbicide residues were still present.

The problem with using laboratory bioassays to predict interactions is in determining what would likely happen in the field. Interactions between metribuzin and chlorothalonil would probably occur only when metribuzin was applied as a postemergence spray, since chlorothalonil is always used as a foliar spray. The recommended application rate for the herbicide under these conditions is 0.7 kg of formulated product ha^{-1} (approximately 525 g of metribuzin) applied in a volume of 95 to 375 L of water (Chemical Pharmaceutical Press 1986). This would yield a concentration of between 1400 and 5500 ppm of metribuzin in the spray. With the fungicide, the recommended application rate is 1.1 to 1.7 kg of product ha^{-1} (approximately 800 to 1200 g of chlorothalonil) applied in a volume of 190 to 1400 L of water (Chemical Pharmaceutical Press 1986). This would yield a concentration of between 570 and 6300 ppm of chlorothalonil in the spray. The amount of pesticide that would come into contact with *A. solani* propagules cannot be predicted, but the spray concentrations fall within the interaction levels discussed above. This indicates that there is a potential for *in situ* interactions between metribuzin and chlorothalonil towards the potato pathogen *A. solani*.

The data presented here emphasize the need for more research on the ecotoxic interactions that occur in mixtures of pesticides. Pesticides can interact synergistically and/or antagonistically towards both non-target microorganisms and target pests. In either case, these interactions can alter the effects of the individual pesticides in the mixtures, thereby causing unpredicted responses from test organisms. In order to accurately predict the environmental impact of pesticides, it is essential that interactions be studied in more detail.

Acknowledgments. This research was supported by the Natural Sciences and Engineering Research Council of Canada.

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Received October 30, 1990; accepted January 11, 1991.