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Interactions of the Solvent Acetone with the Fungicides Benomyl and Captan in Fungal Assays

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The poisoned-agar technique for assessing toxicity involves the addition of volumes of stock solutions of a compound to specific volumes of an agar medium. The differences in radial growth, generated from inoculated mycelial discs on control and experimental plates, are used to assess the toxicity of the chemical. Water-insoluble compounds are usually added to the medium in organic solvents, such as acetone or ethanol. The solvents used, and the volumes added, are sometimes defined (MANTEN et al. 1950; EDGINGTON et al. 1971), or such information may not be specified (ALI et al. 1979; BUCHENAUER and ERWIN 1976).

In spite of the frequent use of solvents in bioassays, little attention has been given to their possible interactions with the candidate pesticide and the organism being tested. MANTEN et al. (1950) reported that 2% acetone in a medium did not affect the growth of the fungi used in their studies. However, evidence has been presented to show that acetone markedly affects both the replication of vaccinia and rabbit-pox viruses (GHENDON and SAMOILOVA 1968; CHERNOS et al. 1972) and the ultrastructure of the alga Chlorella (PARASHER et al. 1978). DALELA et al. (1979) observed that solvents influenced the degree of inhibition of ATPase activity induced by insecticides in tissues from a fresh-water teleost. They concluded that it was obligatory when report-ing experimental data to clearly define details of the solvents used.

In this paper, the effects of acetone on the growth of fungi are reported. A theoretical discussion of possible interactions between a solvent system, a toxic chemical, and a test organism, which result in additive, antagonistic and synergistic responses, is presented. Experimental data to illustrate the interactions of acetone and two fungicides, benomyl and captan, on the growth of four fungi are detailed.

MATERIALS AND METHODS

The phytopathogenic fungi, Sclerotinia homeocarpa, Fusarium oxysporum f. lycopersici, Polyporus hirsutus and Pestalotia sp. were used in this study. The effects of acetone on growth were determined by adding the solvent to 100 mL of melted Difco potato-dextrose agar medium to give final levels of 0 (control), 0.1, 0.2, 0.3, 0.4, 0.6, 0.8, 1.0, 1.5 and 2% (v/v). Bottles of medium were shaken for 2 minutes on a rotary shaker and 10 mL volumes of control and solvent-treated medium were dispensed into petri dishes.

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The plates of medium (replicates of five) were inoculated with mycelial discs (8 mm diameter) taken from the outer edge of 6-day fungal colonies. The cultures were incubated at 30°C and colony diameters were measured when control colony diameters exceeded 40 mm. Percent inhibitions were calculated and data were plotted on logarithmic-linear graph paper as log concentration of acetone versus percent inhibition. The concentration of acetone producing 50% inhibition of radial growth (EC_{50}) was determined from each plot.

Interactions between acetone and the fungicide were determined by holding the amounts of captan or benomyl constant while varying the acetone concentration over the range of 0.1 to 2.0%. Benomyl (methyl 1-(butyl carbamoyl) benzimidazol-2-ylcarbamate) was used at a concentration of 0.1 ppm with S. homeocarpa and Pestalotia sp., and 1.0 ppm with P. hirsutus and F. oxysporum. Captan (N-(trichloromethyl thio)-cyclohex-4-ene-1,2-dicarboximide) was used at a level of 10 ppm with all cultures. Inoculated plates, containing the above range of solvent concentrations only, were used as the solvent controls. The percent inhibitions due to the solvent (solvent effects) and the solvent-fungicide combinations (solvent-fungicide effects) were calculated with respect to growth on control plates (no solvent and no fungicide). The percent inhibition due to solvent-fungicide combinations was also calculated with respect to growth on solvent control plates. The resulting data were designated as net fungicide effect. The data on percent inhibition were plotted on logarithmic-linear graph paper. A Student's t test ($P = 0.02$) was used to conclude if the responses elicited by acetone concentrations greater than 0.1% differed significantly from inhibition at 0.1% acetone.

The toxicity of benomyl and captan over the ranges of 0.01-5 ppm, and 1-30 ppm, respectively was determined at acetone concentrations of 0.1% and 1.0%. Percent inhibition was calculated from the appropriate solvent controls, and data were plotted on logarithmic-linear graph paper from which EC_{50} values were determined.

RESULTS AND DISCUSSION

Theory of interactions

The Gowing equation (GOWING, 1960) has been used to obtain the expected additive response when two herbicides are interacted on a test plant system. In this paper, the equation is used to examine data obtained by interacting a solvent and a fungicide on a test fungus as follows:

$$E = A + \frac{(100-A)}{(100)} \times B$$

Where: E is the expected additive effect
A is the inhibition due to the fungicide alone, and
B is the effect due solely to the solvent.

An additive response occurs when the inhibition due to the solvent-fungicide combination is the same as the expected additive effect calculated by the equation. An antagonistic interaction is when the solvent-fungicide combination results in an inhibition significantly less than the expected response. A synergistic response is one which is significantly greater than that which is expected.

The theoretical graphs of interactions between a solvent and a fungicide are presented in Figures 1, 2 and 3. When the response is additive (Fig. 1), the net fungicide effect is the same as the expected additive response. In this type of interaction, the true inhibitory effect of the fungicide can be calculated with respect to growth on solvent control plates. However, when the interaction of the solvent and fungicide is one of synergism, the net fungicide effect will deviate significantly from the expected additive response and this is shown as an increase in the percent inhibition due to one concentration of the fungicide (Fig. 2). An antagonistic response is denoted by a significant decrease in the percent inhibition calculated for the net fungicide effect as the solvent concentration increases (Fig. 3).

Effects of acetone on fungal growth

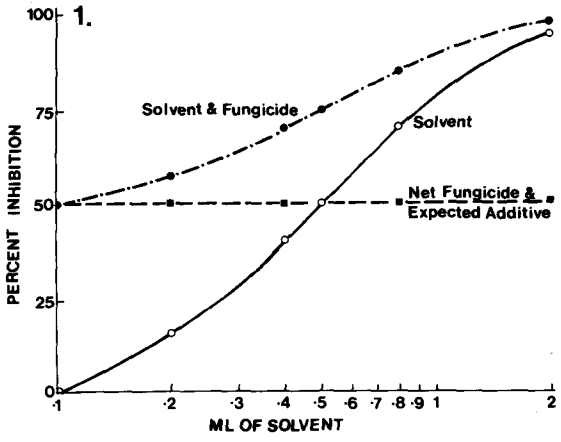
The effects of acetone on the growth of the test fungi are summarized in Table 1 and Figures 4, 5 and 6 (see curves labelled Solvent). The least sensitive fungus was P. hirsutus (Basidiomycotina). The radial growth of this culture was inhibited 30% at the maximum acetone concentration used (2% v/v) and an EC_{50} could not be calculated. Pestalotia sp. and F. oxysporum (Deuteromycotina) and S. homeocarpa (Ascomycotina) responded in a similar fashion to acetone (compare solvent curves in Fig. 5 and 6 and the EC_{50} values in Table 1). A rapid change in sensitivity to acetone began at approximately 0.7% acetone (v/v) with these three cultures. This change in sensitivity was denoted by a point of inflection on the percent inhibition vs mL of solvent plot (Fig. 5 and 6--solvent curve). This change in sensitivity may be indicative of a second mode of action associated with the higher acetone concentrations.

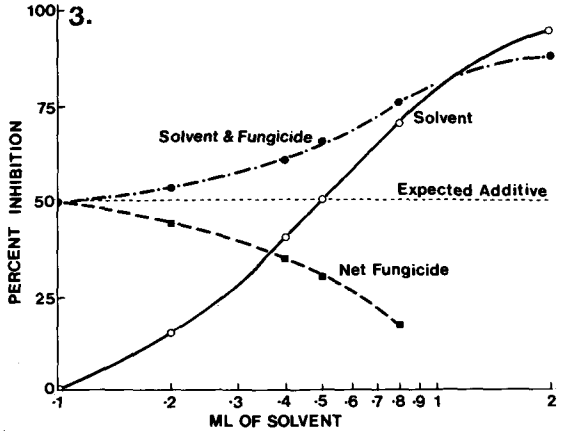
It was evident that acetone was a moderately fungitoxic compound, but the specific mode of action was not elucidated. Toxicity may involve the lipid/sterol fraction of the cell membrane. This hypothesis was based upon the work of LAMPEN (1966) which showed that the antibiotic nystatin, which binds to the sterol portion of a fungal cell membrane, was released upon treatment of the cells with acetone.

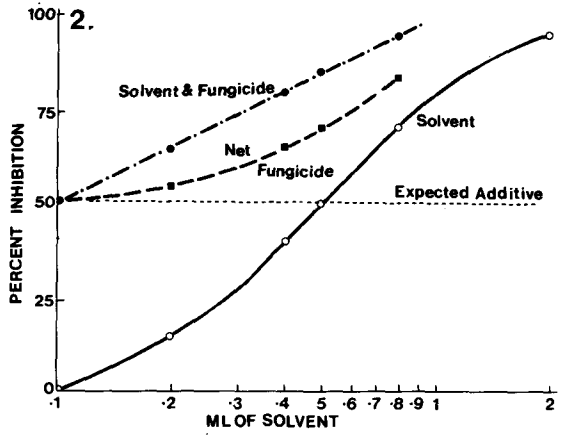
TABLE 1
Influence of acetone on fungal growth

Fungus	% inhibition of growth due to acetone[1]				EC_{50} of acetone
	0.1	0.5	1.0	2.0	
Polyporus hirsutus	10	10	17	30	>2.0
Pestalotia sp.	4	7	36	97	1.25
Sclerotinia homeocarpa	11	36	58	99	0.88
Fusarium oxysporum	4	5	25	58	1.8

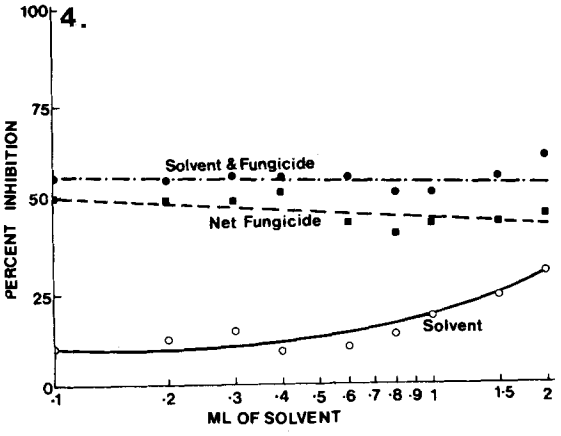
[1]Concentrations of acetone as mL/100 mL of medium (% acetone) Percent inhibition was calculated from the diameter of growth on acetone-treated medium with respect to growth on control medium (no solvent). All treatments were done in replicates of five.

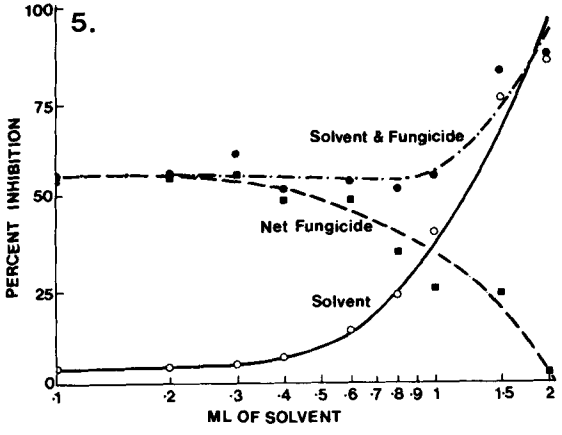


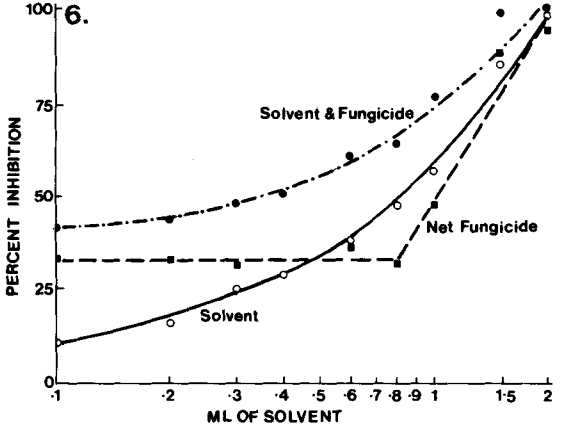




Figures 1, 2 and 3. The theoretical responses which are obtained when a fungicide and solvent are used in combination.
1--Additive
2--Synergistic
3--Antagonistic
Solvent as mL/100 mL of medium (% solvent)







Figures 4, 5 and 6. The effect of acetone (Solvent); acetone and 10 ppm captan, (Solvent and Fungicide); acetone and captan corrected for the solvent effect (Net Fungicide)
4--Polyporus hirsutus
5--Pestalotia sp.
6--Sclerotinia homeocarpa
Acetone solvent as mL/100 mL of medium (% acetone)

Effects of solvent-fungicide combinations on fungal growth

The interactions of captan and benomyl with acetone resulted in inhibition patterns that approximated the theoretical patterns previously discussed (Fig. 1, 2 and 3).

P. hirsutus grown on potato-dextrose agar treated with captan (10 ppm) and acetone (0.1%-2.0% v/v) produced responses (Fig. 4) which fit the criterion for an additive interaction (Fig. 1). There was no significant difference (P = 0.02) in the percent inhibition due to captan plus 0.1% or 2.0% acetone (Table 2). The EC_{50} values for captan obtained with 0.1% and 1.0% acetone were 10.6 ± 0.8 and 13.0 ± 1.9 ppm which were not significantly different at P = 0.02 (Table 3). Similarly, benomyl (0.1 ppm) and varying levels of acetone interacted additively with P. hirsutus (Table 2). The calculated EC_{50} values of 3.0 ± 0.14 and 2.9 ± 0.2 ppm with 0.1% and 1.0% acetone, respectively, verified this conclusion (Table 3).

TABLE 2

The solvent-fungicide interactions of acetone-benomyl and acetone-captan mixtures.

Fungus	Fungicide (ppm)	% inhibition of growth due to acetone[1] 0.1	2.0	Response
P. hirsutus	Captan (10 ppm)	49	45	Additive
	Benomyl (0.1 ppm)	6	10	Additive
Pestalotia sp.	Captan (10 ppm)	54	3	Antagonistic
	Benomyl (0.1 ppm)	61	95	Synergistic
S. homeocarpa	Captan (10 ppm)	32	97	Synergistic
	Benomyl (0.1 ppm)	24	82[2]	Synergistic
F. oxysporum	Captan (10 ppm)	33	53	Antagonistic
	Benomyl (1.0 ppm)	54	78	Synergistic

[1]Concentration of acetone as mL/100 mL of medium (% acetone)

[2]This percent inhibition was determined with 1.5% acetone

An antagonistic interaction was observed with Pestalotia sp. captan and acetone (Fig. 5). There was a significant reduction (P = 0.02) in inhibition (3%) at 2% acetone when compared to the inhibition (54%) at 0.1% acetone (Table 2). The antagonistic interaction noted was further substantiated for captan and acetone by the calculated EC_{50}s at 0.1% acetone (7.6 ± 0.4 ppm) and 1.0% acetone (11.7 ± 1.8 ppm). In contrast, benomyl and acetone interacted synergistically yielding EC_{50} values of 0.09 ± 0.003 and 0.065 ± 0.004 ppm calculated for 0.1% and 1.0% acetone, respectively (Table 2 and 3).

Captan and acetone interacted synergistically with S. homeocarpa (Fig. 6), as did benomyl and acetone (Table 2). These conclusions were supported by the EC_{50} values for captan and benomyl calculated at 0.1% acetone (17.8 ± 1.0 and 0.22 ± 0.02 ppm) and 1.0% acetone (3.4 ± 0.4 and 0.11 ± 0.001 ppm) respectively (Table 3). Fusarium oxysporum produced responses indicative of synergism for both captan and benomyl (Table 2) and this was verified by the calcula-

tion of EC_{50} values for 0.1% and 1.0% acetone (Table 3).

TABLE 3
Toxicity of benomyl and captan with 0.1% and 1.0% acetone to four test fungi

Fungus tested	Fungicide	EC_{50} (ppm) at acetone concentrations of 0.1%	1%	Response
P. hirsutus	Captan	10.6±0.8	13.0±1.9	Additive
	Benomyl	3.0±0.14	2.9±0.2[1]	Additive
Pestalotia sp.	Captan	7.6±0.4	11.7±1.8[1]	Antagonistic
	Benomyl	0.09±0.003	0.065±0.004[1]	Synergistic
S. homeocarpa	Captan	17.8±1	3.4±0.4[1]	Synergistic
	Benomyl	0.22±0.02	0.11±0.01[1]	Synergistic
F. oxysporum	Captan	>30	20.5±0.7[1]	Synergistic
	Benomyl	1.05±0.02	0.87±0.03[1]	Synergistic

[1] EC_{50} values are significantly different between 0.1 and 1% acetone ($P \leq 0.02$)

These studies show that additive, antagonistic and synergistic interactions can occur when an organic solvent is used in a fungicide bioassay. Moreover these interactions are not only limited to fungicides, since STRATTON et al. (1980) have shown that the insecticide permethrin, acetone and blue-green algae (Anabaena spp.) also can interact to yield these same complex responses. Additive responses are of little concern in a bioassay as corrections can be made using solvent controls to obtain an accurate measure of a compound's toxicity. Synergistic and antagonistic responses are more difficult to interpret. The determination of correction factors for each fungus, fungicide, solvent and solvent concentration would be necessary to obtain accurate results. However, the calculation of correction factors can be avoided if only those concentrations of solvents which interact additively are chosen for use in the bioassay. For example, from these studies the selection of a concentration of 0.1% acetone (v/v) for use in a bioassay would be justified, since it interacted additively with both fungicides and all test fungi.

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