EFFECTIVE AEROSOL TREATMENT OF MOULD MITES AND ONION THRIPS IN TISSUE CULTURE

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ABSTRACT

Ethyl formate applied as an aerosol killed almost 100% of the mould mites (*Tyrophagus putrescentiae*) present in tissue culture tubs. Mite mortality showed dose responses to the chemical and mortality increased with duration of exposure. Other aerosol formulations, and vacuum and controlled atmosphere treatments were also tested but were not effective in controlling mites. Onion thrips (*Thrips tabaci*) in open and closed tissue culture tubs were killed by ethyl formate, 65% and 85% CO_2 atmospheres, or dichlorvos plus CO_2 propellant. Further work is needed to define effective dose rates and exposure times and to determine the possible phytotoxicity of the aerosols to various plant species.

Keywords: fumigation, *Tyrophagus putrescentiae, Thrips tabaci*, tissue culture.

INTRODUCTION

Contamination of tissue cultures during micropropagation of plants is often the result of fungal infections introduced by mites and insects. Mould mites are more likely to be found inside tissue culture tubs containing agar than in temperature-controlled tissue culture rooms as the relative humidity in these rooms is much lower than the RH of 70% and higher favoured by the mites. Control of mites and thrips in tissue culture laboratories tends to be based on information shared informally between laboratories rather than on published information. Various control measures for mites and thrips are available but many are unsuitable for application to tissue culture material.

The fumigant, phosphine, is used to control mites in stored grain (Anon. 2003). However, it requires specialist application and cannot feasibly be applied in tissue culture laboratories because it is highly toxic (Blake 1988) and application vessels must remain sealed for 7 days. Furthermore, mite eggs may survive fumigation with phosphine.

Aerosol fly sprays containing pyrethrum insecticide have been recommended for use in tissue culture (Kirk 1997). CABI Bioscience (2002) recommends use of 70% ethanol on work surfaces and incubators to avoid mite infestations, and treatment of infested tissue culture tubs using a microwave was found to kill mould mites (van Epenhuijsen & Koolaard 2004).

This paper describes a series of experiments aimed at developing effective controls for mould mite (*Tyrophagus putrescentiae*) and onion thrips (*Thrips tabaci*), which are two of the most common invaders in tissue culture laboratories in New Zealand. Control measures tested included aerosol formulations, elevated CO_2 and vacuum atmospheres. One of the aerosols included ethyl formate, the active ingredient in VAPORMATE™, which occurs naturally in a range of products such as vegetables, stored grains, animal products, and beer and wine (Desmarchelier 1999).

MATERIALS AND METHODS

Rearing mites and thrips

A mould mite culture was started using mites collected from contaminated tubs of tissue-cultured calla plantlets. The mites were reared on a 50:50 mix of yeast and rat food (van Epenhuijsen & Koolaard 2004).

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Onion thrips were collected from onions and reared in 500 ml jars at 70% RH at 27ºC. The lid of each jar contained filter paper for aeration; a piece of paper towel was placed in the bottom of the jars. Twice a week, pieces of leek (*Allium porrum*) leaves were added. Adult thrips for experiments were collected using a device designed to collect small insects (van Epenhuijsen 1995). For the treatments, 15 thrips were placed in 35 ml jars with very fine mesh covering 18 mm vents at both ends of the jars. A small piece of leek was added to each jar. The jars were placed directly into the tissue culture tubs.

All the treatments in each experiment were replicated four times by carrying out runs on four different days. All treatments were applied at 22ºC.

Aerosols, vacuums and controlled atmospheres

A series of experiments examined the effects of aerosol formulations, elevated CO_2 levels and vacuum atmospheres on mite and thrips survival in infested tissue culture tubs sealed with lids. For comparison, a second set of tubs without lids (open tubs) was also used. For each fumigation, four tubs were placed inside the fumigation chamber; two tubs (one open, one closed) contained mites, and two (one open, one closed) contained thrips. Wide-mouthed, plastic tissue culture tubs (290 ml capacity) with snap-on lids, commonly used for tissue culture in New Zealand, were used for the experiments.

Mould-mite infested diet (4 g) was spread over the bottom of open and closed tubs without agar. After treatment, the infested diet was stirred with a needle and poured through a kitchen sieve (approximately 850 µm mesh). The sieved material (a fine dust) was stuck on 50 mm wide black polyethylene self-adhesive tape (Sealed Air Insultape) by laying a piece of tape on the sieved material and rolling metal a piece of pipe over it. All live mites within in two randomly chosen 7 mm diameter circles (77 mm^2) cut out of a thin aluminium sheet overlay were counted on the tape per treatment. Live mites, and dead and live thrips, were counted 2 h after treatment.

Aerosols. The treatments were:

- Aerosol 1: ethyl formate, (16.7% ethyl formate: VAPORMATE™) in a liquid carbon non-flammable formulation, which formed a vapour.
- Aerosol 2: dichlorvos, (50 g/kg: Insectigas) with carbon dioxide as the propellant, which formed a vapour.
- Aerosol 3: permethrin+pyrethrum+piperonyl butoxide with carbon dioxide as a propellant (4.0+1.0+5.0 g/kg: Permigas).
- Aerosol 4: EVI-500, pyrethrins + piperonyl butoxide: with dill added.
- Aerosol 5: tetramethrin + allethrin + d.phenothrin (2.35+0.53+0.53 g/l: Raid Fly & Insect killer) with hydrocarbon as a propellant, was applied as a 3 second spray.
- Control. For each run of the trial, untreated tubs containing mites or thrips were kept at ambient temperature in the laboratory.

Aerosol 1 and 5 treatments were applied to a 92-litre (389 x 608 x 393 mm) stainless steel chamber. The lid on the chamber was placed on high-density sponge rubber (Skellerup EPDM, 25.4 x 12.7 mm) cut into a rectangular shape to seat the lid. The lid was tightly fastened down during fumigation using two elasticised tie-downs. A handgun was used to introduce the gas through an 8 mm diameter port in the side of the chamber. The amount of gas introduced to each chamber was calculated by weight loss in the gas cylinder, which was placed on a scale. Aerosol 5 was applied from canisters (534 ml) purchased from retail stores. Aerosols 2, 3 and 4 were applied to jars placed on the floor of a sealed, lined enclosure (30 m^3).

Vacuum treatments. Vacuum treatments were carried out in a 3.83 m^3 tank under 9.63 kPa and 2% oxygen, with the balance being Aerosol 2, Aerosol 1 or air (control). Assessments of mites and thrips were carried out 1-2 h after treatment.

Controlled atmospheres. In the controlled atmosphere experiment, 70 ml jars containing mites were placed in open or closed plastic tissue culture tubs without agar in plastic sealable containers (240 x 180 mm) containing wetted capillary matting to maintain high humidity. A plastic hose for the supply of CO_2 was inserted in each container through a rubber bung. Two syringe needles acted as vents. Carbon dioxide (100%) was delivered from pressurised cylinders (BOC Gases, Palmerston North) and mixed with

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air supplied via a compressor to give 65% or 85% CO_2 atmospheres. Treatments were applied for 24 h. The gas mix was regulated to supply a constant stream of 300 ml/min to the containers. Regular checks of CO_2 levels were made using a Dansensor O_2/CO_2 gas sensor. The chambers were kept closed for 2 h after the treatment. The untreated control consisted of air supplied to a plastic container. The set of treatments was replicated four times on different days.

Ethyl formate treatments

Rates and exposure trial

A trial was conducted to determine suitable exposure times and rates of ethyl formate to use in future trials.

Jars (70 ml) containing mites were open during fumigation. Each jar contained 10 g mite-infested diet (approximately 8 mm deep) with a 30 x 30 mm piece of black filter paper (Schleicher & Schuell) on the bottom. The jars were placed in 40 litre plastic bags. An appropriate quantity of ethyl formate (Merck-Schuchardt, Hohenbrunn), matching the rates deliverable by Aerosol 1, was poured into a glass Petri dish and also placed in the plastic bag, which was then sealed with tape. Bags were fumigated with 0, 2, 4, 6, 8, 10, 20 or 40 g/m^3 ethyl formate. An air stream was introduced into the bag through a septum over the Petri dish to completely vaporise the ethyl formate and fill the bag. Bags were held at 20ºC for 2, 4 and 6 h. Assessments were carried out after 24 h by counting all live mites in four randomly chosen circles (diameter 7 mm) of the filter paper.

Efficacy of Aerosol 1 in tissue culture tubs

Open tissue culture tubs without agar and closed tubs with agar were fumigated in 200 litre drums at approximately 20ºC. The rates of ethyl formate used were 0, 10, 20 and 40 g/m^3. Mite-infested diet (1.5 g) was spread over the bottom of the open tubs and 3 g of diet was spread over the agar in the closed tubs. In addition, begonia plantlets in tissue culture tubs containing agar were added to the 20 or 40 g/m^3 treatments or left untreated for phytotoxicity assessment.

The diet in the closed tubs could not be assessed because it became wet after absorbing moisture from the agar. All mites on the inner side of the lids on the closed tubs were counted. For the open tubs, live mites in 12 disk areas (each 38 mm^2) of diet were counted. The assessments were carried out 24 h after treatment.

RESULTS

Aerosols, vacuums and controlled atmospheres

All the treatments, both in open and closed tubs, failed to suppress mite numbers, apart from the non-vacuum Aerosol 1 treatment, which had almost no surviving mites in the open tubs. This treatment gave only a moderate reduction of mites in the closed tubs. (Table 1).

Thrips

For the open tubs, Aerosol 1, Aerosol 2 vacuum, Aerosol 1 vacuum and 65% CO_2/ 35% air all gave 100% thrips mortality, while 85% CO_2/15% air gave 98.5% mortality (Table 2). The same five treatments that gave high mortality in the open tubs gave 100% mortality in the closed tubs. Aerosol 2 had some effect on the thrips in the open tubs but it did not penetrate into the closed tubs sufficiently to give any mortality. The other treatments were ineffective against thrips in either open or closed tubs (Table 2).

Ethyl formate treatments

Rates and exposure trial

When ethyl formate was applied to infested jars, numbers of live mites decreased with exposure time, but there was evidence of a greater rate of decrease in mite numbers in response to increasing ethyl formate dose (Fig. 1). At exposure times of 2 and 4 h, 40 g/m^3 ethyl formate was required to reduce mite numbers. A statistically significant ($P<0.05$) reduction in mite numbers was seen at 20 g/m^3 with an exposure time of 6 h.

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TABLE 1: Mean numbers of surviving mites (no. mites/77 mm^2) in open and closed tissue culture tubs after treatment with various aerosols and controlled atmospheres.

Treatment	Open tubs		Closed tubs
Aerosols			
Aerosol 1	1		76
Aerosol 2	224		222
Aerosol 3	191		211
Aerosol 4	211		225
Aerosol 5	297		252
Control (aerosols)		213	
Vacuum			
Aerosol 2 vacuum	235		234
Aerosol 1 vacuum	223		262
Control (vacuum)		255	
Controlled atmosphere			
65% CO_2, 35% air	185		202
85% CO_2, 15% air	145		228
Control (air only)		253	
SEM[1]	35.9		40.1

[1]Standard error of a treatment mean (pooled over all treatments) calculated from four replicate measures for each treatment. Aerosol 1 was excluded from this calculation since its counts were almost all zero.

TABLE 2: Mean proportion (with SE where appropriate) of onion thrips killed in open and closed tissue culture tubs treated with various fumigation, aerosol or controlled atmosphere treatments.

Treatment	Rate (g/m^3)	Open tubs		Closed tubs
Aerosols				
Aerosol 1	20	100		100
Aerosol 2	9	50.0 (6.0)[1]		0
Aerosol 3	8	3.4 (2.4)		3.8 (2.5)
Aerosol 4	9	1.7 (1.7)		3.6 (2.5)
Aerosol 5	27.2	7.8 (3.8)		1.8 (1.7)
Control (aerosols)			1.5 (1.5)	
Vacuum				
Aerosol 2 vacuum		100		100
Aerosol 1 vacuum		100		100
Control (vacuum)			1.6 (1.6)	
Controlled atmosphere				
65% CO_2, 35% air		100		100
85% CO_2, 15% air		98.4 (1.5)		100
Control (air only)			1.8 (1.8)	

[1]Data in parentheses are SEM.

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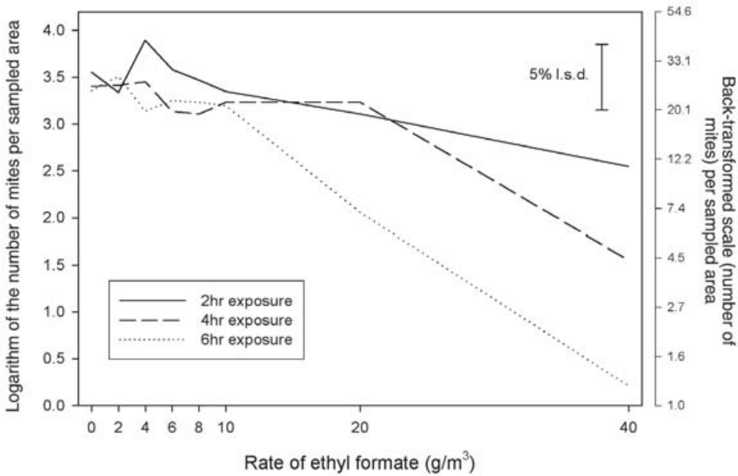

FIGURE 1: Number of mites found in jars after exposure to ethyl formate at various rates and durations.

Efficacy of Aerosol 1 in tissue culture tubs

Aerosol 1 applied to open tubs resulted in fewer mites ($P<0.05$) on average than the nil dose rates for all of the four rates tested (Fig. 2). There was no difference ($P<0.05$) in mite numbers between the four rates of Aerosol 1 used.

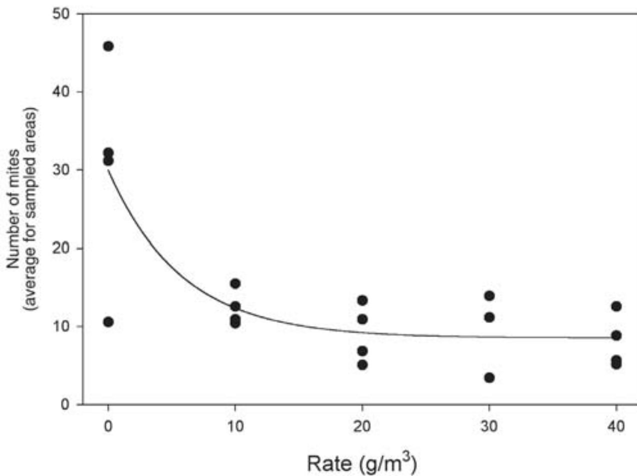

FIGURE 2: Numbers of mites in open tissue culture tubs after treatment with different ratios of ethyl formate. The fitted line is Number of mites = 8.5 + 21.42 (0.84^{Rate}), and the standard error of observations about the fitted line is estimated to be 7.03.

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Diet placed directly on agar in the closed tubs became wet and so live mites could only be counted on the inside of the lids. As with the open tubs, mite numbers in closed tubs decreased with increasing rates of Aerosol 1. Rates of 30 and 40 g/m^3 halved mite numbers compared to the nil rate (Fig. 3).

No phytotoxicity was seen on begonia plants treated with Aerosol 1 at rates of 20 and 40 g/m^3.

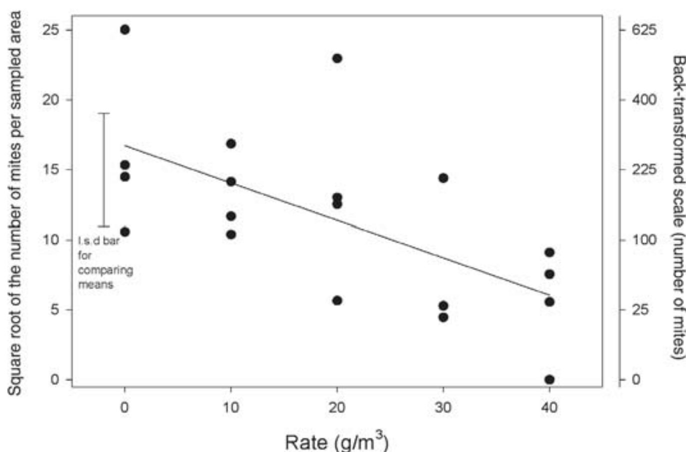

FIGURE 3: Numbers of mites in closed tissue culture tubs treated with different rates of ethyl formate. The LSD (P=0.05) = 8.08 for comparing mean numbers of mites between different rates of Aerosol 1. The fitted regression line shown has the equation: (Mite count)$^{1/2}$ = 16.7-0.27 Rate.

DISCUSSION

The practice of bringing plant material into a laboratory is the main reason for contamination of tissue culture rooms with mites or thrips. Normally, when tissue culture tubs become contaminated, the infested material is thrown out, but often there is only a limited supply of plants and this practice is costly for laboratories. When plant material that may contain mites is received for tissue culture, careful hygiene should be the first step in preventing mite and thrips infestation. Most other measures for preventing mite infestation are difficult to adapt to a tissue culture situation or may be hazardous to the health of those working with tissue culture material.

This work has shown that ethyl formate was effective in killing both mites and thrips in simulated and real tissue culture situations. This treatment would be applied to high value material only. Ethyl formate is a relatively safe aerosol treatment. The Food and Drug Administration of the USA reviewed the use of ethyl formate and concluded that there is no evidence to indicate that it is a hazard to the public when the chemical is used as a flavouring agent (Desmarchelier 1999).

Ethyl formate showed both a dose response and increased mortality over time, indicating that there is potential for further work to define optimum levels of this chemical for control of mites and thrips in tissue culture. Our experiments indicated that ethyl formate is not phytotoxic to begonia plantlets. However, it is phytotoxic to many cut flowers and ornamental species (Weller et al. 1995) so phytotoxicity potential should be further investigated prior to its use with other types of tissue-cultured plants.

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