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Research Papers

Efficacy of mefenoxam is affected by a lag period between application and inactivation of *Pythium* species

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Summary. A study was undertaken to investigate whether inactivation of *Pythium* by mefenoxam takes place as soon as it comes in contact with the fungicide. Laboratory experiments were conducted which involved three sensitive isolates of *P. aphanidermatum* (EC_{50}<1 μg mL^{-1}), three sensitive isolates of *P. spinosum* (EC_{50}<1 μg mL^{-1}) and two resistant isolates of *P. aphanidermatum* (EC_{50}>100 μg mL^{-1}). In liquid cultures, inactivation of the sensitive *Pythium* isolates by mefenoxam took place between 12 hr and >96hr at concentrations from 1 to 100 μg mL^{-1}. The time required for inactivation was negatively correlated with the concentration of mefenoxam (P<0.05). Colonization of cucumber seeds placed at different distances from the sensitive *Pythium* inoculum in soil amended with 1 and 5 μg mL^{-1} mefenoxam occurred in the first 12 hr. However, colonization was reduced after 24 hr and was completely inhibited after 96 hr. Efficiency of colonization in the sensitive *Pythium* populations was found to have a negative correlation with the concentration of mefenoxam used. Growth and colonization by the resistant *Pythium* isolates were not found to be affected with mefenoxam. This study appears to be the first report of presence of a lag period between application of mefenoxam and time taken to inactivate *Pythium*.

Key words: cucumber, metalaxyl-M.

Introduction

Pythium species cause several diseases on cereals, grasses, vegetable crops, ornamental plants, fruit trees and forest trees. The type, symptoms and severity of these diseases depend on the pathogen species, their aggressiveness, the host plants, plant parts infected as well as the prevailing environmental conditions. Most *Pythium* species infect juvenile tissues of seedlings and plants, but can also attack feeder roots or root tips of older plants, stems and foliage of some grasses and fruits, resulting in damping-off, wilt, stunting, decline and fruit rot diseases (Hendrix and Campbell, 1973; Aegerter *et al*., 2000; Al-Sa'di *et al*., 2007; Al-Sadi *et al*., 2011a). Losses can vary from reductions in vigor and yield of infected plants, as exemplified by *P. pyrilobum* infecting rice in Australia (Cother and Gilbert, 1993), to completely eradicating whole fields, as with *P. deliense* infecting watermelon and muskmelon in Oman (Al-Rawahi *et al*., 1998; Al-Sadi *et al*., 2011a). Severity of this disease was reported to increase under stress induced by salinity on cucumbers (Al-Sadi *et al*., 2010a) and the disease is affected by inoculum which is introduced into greenhouses via unknown sources (Al-Sa'di *et al*., 2008d; Al-Sadi *et al*., 2011b)

Several strategies have been developed and implemented to manage *Pythium* induced diseases. These include cultural practices (e.g. solarization and soil replacement) (Christensen and Thinggaard, 1999) and the use of biocontrol agents and defense inducers (Deadman *et al*., 2006; Al-Hinai *et al*., 2010; Al-Sadi *et al*., 2010b). However, chemical control, dominated by the use of metalaxyl (mefenoxam) fungicides, continues to be the most preferable and

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efficacious way in different places (Stephens, 1985; Brantner and Windels, 1998; Al-Sa'di *et al*., 2008b). Metalaxyl is a commonly used fungicide for the control of *Pythium* induced diseases in many greenhouses in Oman (Al-Toobi *et al*., 2005). Most of the metalaxyl fungicides (racemic mixture of R- and S-enantiomers) depend nowadays on the active ingredient mefenoxam (Metalaxyl-M), which is the active R-enantiomer.

Extensive use of mefenoxam in Oman and elsewhere has resulted in problems with the use of this fungicide. Development of resistance to metalaxyl and mefenoxam was reported in several oomycete pathogens, including *Pythium* species (Cook and Zhang, 1985; Lou *et al*., 2001; Moorman and Kim, 2004). In addition, metalaxyl and mefenoxam can be rapidly biodegraded in soils following repeated use (Droby and Coffey, 1991; Al-Sa'di *et al*., 2008b).

Although recent studies in Oman indicated that no resistance is present against metalaxyl or mefenoxam among *Pythium* species (Al-Sa'di *et al*., 2008a, c), metalaxyl was reported to suffer from rapid biodegradation in greenhouse soils (Al-Sa'di *et al*., 2008b). However, field observations showed delayed suppression of *Pythium*-damping-off of cucumber following application of mefenoxam. This issue has not been previously addressed, so the main objective of the present study was to investigate the time required for inactivation of *Pythium* species following application of mefenoxam. Specific objectives were to: (1) determine the time required for inactivation of sensitive and resistant *Pythium* propagules following application of mefenoxam and (2) study the effect of mefenoxam concentrations and time on the ability of *Pythium* to colonize cucumber seeds. Findings from this study should provide valuable information to assist development of effective management strategies for root diseases caused by oomycetes.

Materials and methods

Inactivation of *Pythium* by mefenoxam

A laboratory experiment was conducted to determine the time required for mefenoxam to inactivate *Pythium* growth *in vitro*. Eight isolates of *Pythium* were used in this study. Five isolates were *P. aphanidermatum* and three were *P. spinosum*. Two of the *P. aphanidermatum* isolates have resistance to mefenoxam (EC_{50}>100 μg mL^{-1}) and were provided by Gary Moorman, University of Pennsylvania, USA. The other isolates were from Oman from cucumber seedlings showing damping-off symptoms and they are sensitive to mefenoxam (EC_{50}<1 μg mL^{-1}). Identity of the isolates, sources and levels of sensitivities to fungicides were determined previously (Al-Sa'di *et al*., 2007, 2008a, c) (Table 1). All isolates were maintained on 1% cornmeal agar (CMA) slants at room temperature (18–20˚C) for further use.

In this experiment, the time required for inactivation of *Pythium* growth by mefenoxam was investigated following a modified method of Kim *et al.* (2004) using the eight isolates of *Pythium*. Plugs (4 mm diam.) of 3-d-old cultures on potato dextrose agar (PDA) of each of the isolates were placed in 50

Table 1. Isolates of *Pythium aphanidermatum* and *P. spinosum* used in the inactivation and colonization experiments.

Isolate	Species	Origin	State/District	Host	Collected/ provided by
P002	*P. aphanidermatum*	Oman	Barka	Cucumber	A. Al-Sadi
P011	*P. aphanidermatum*	Oman	Musanaa	Cucumber	A. Al-Sadi
P014	*P. aphanidermatum*	Oman	Barka	Cucumber	A. Al-Sadi
P004	*P. spinosum*	Oman	Barka	Cucumber	A. Al-Sadi
P006	*P. spinosum*	Oman	Seeb	Cucumber	A. Al-Sadi
P009	*P. spinosum*	Oman	Barka	Cucumber	A. Al-Sadi
P124	*P. aphanidermatum*	USA	Pennsylvania	Chrysanthemum	G. Moorman
P125	*P. aphanidermatum*	USA	Pennsylvania	Poinsettia	G. Moorman

mL centrifuge tubes filled with 20 mL of sterile distilled water amended with mefenoxam at concentrations of 0, 1, 5, 10 or 100 μg mL^{-1}. Cultures were incubated at 27°C on a rotary shaker at 110 rpm for the following time periods: 1, 6, 12, 24, 48, 72 and 96 h. Plugs were then washed in sterile distilled water and placed on 2.5% PDA in Petri plates for 24 h. Four replicates were used for each incubation time × mefenoxam concentration combination. The tubes and the plates were incubated at room temperature (25ºC). No mycelium growth at a given time and concentration was used to indicate that the isolate was inactivated (Kim *et al*., 2004).

Influence of mefenoxam on colonization of cucumber seeds by *Pythium*

This experiment aimed to assess the influence of different mefenoxam concentrations on efficiency of colonization of cucumber seeds by the eight *Pythium* isolates, following a modified method of Otten *et al.* (2004). Sand (40 g) which had been previously autoclaved for 1 h was placed in Petri plates (90 mm diam.) and saturated with water and amended with 0, 1, 5 or 10 μg g^{-1} a.i. mefenoxam. The sand surface was leveled and a 4 mm diam. mycelial plug of *Pythium* was placed at one end of each Petri plate. Seven autoclaved cucumber seeds were placed at each of the following distances from the *Pythium* plug: 2.5, 5, 10, 20, 30, 40 and 50 mm. The colonized cucumber seeds were assessed after 12, 24, 48 and 96 h. The removed seeds were replaced with new sterilized seeds in the same place from which they were removed. The removed seeds were each washed in 25 mL sterile distilled water and placed on PDA for 24 h. Seeds from which mycelium growth was detected were considered colonized. At least four plates were used for each time × concentration × isolate combination and the plates were incubated at room temperature. Each experiment was repeated at least twice.

Results

Inactivation of *Pythium* by mefenoxam

Assessment of the time required for inactivation of growth of *Pythium* isolates differing in sensitivity to mefenoxam revealed that *P. aphanidermatum* isolates resistant to mefenoxam (EC_{50}>100 μg mL^{-1}) were not inactivated after incubation in 100 μg mL^{-1} for 4 d. However, isolates sensitive to mefenoxam were inactivated in less than 12 h after incubation at 100 μg mL^{-1}a.i. mefenoxam. At concentrations of 1 to 10 μg mL^{-1}, *P. aphanidermatum* and *P. spinosum* isolates took between 12 and more than 96 h until they became inactive (Table 2). The time required for inactivation was negatively correlated with the concentration of mefenoxam (P<0.05).

Table 2. Time required for inactivation of *Pythium* at different concentrations of mefenoxam.

Isolate[a]	EC_{50} (µg mL^{-1})	Time (h)				
		0 µg mL^{-1}	1 µg mL^{-1}	5 µg mL^{-1}	10 µg mL^{-1}	100 µg mL^{-1}
P002	0.36	–	72	48	24	1
P011	0.29	–	72	48	48	1
P014	0.35	–	–	48	48	1
P004	0.24	–	48	48	12	1
P006	0.24	–	48	24	24	1
P009	0.21	–	48	24	12	1
P124	>100	–	–	–	–	–
P125	>100	–	–	–	–	–

[a] Isolates P002, P011, P014, P124 and P125 are *P. aphanidermatum*, while P004, P006 and P009 are *P. spinosum*.
–, Isolate was not inactivated.

Influence of mefenoxam on colonization of cucumber seeds by *Pythium*

Application of mefenoxam to sand reduced the rate of colonization of cucumber seeds with time and distance in mefenoxam sensitive but not resistant isolates (Figure 1). All *Pythium* isolates grew on the mefenoxam-free sand and colonized cucumber seeds at a rate of approximately 15–20 mm per day. The rate of growth of all *Pythium* isolates was not affected by time in the mefenoxam-free sand.

The rate of growth of the resistant *Pythium* isolates (P124 and P125) in mefenoxam-free sand as well as in sand amended with 1, 5 and 10 μg mL^{-1} mefenoxam was found to be 15–20 mm. No significant differences were found in growth between the two isolates at the different mefenoxam concentrations or for the same isolate on the different mefenoxam concentrations (P>0.05) (Figure 1).

Colonization of cucumber seeds placed 10 mm away from the sensitive *Pythium* inoculum in sand amended with 1, 5 and 10 μg mL^{-1}a.i. mefenoxam occurred in the first 12 h, but was reduced after 24 h and completely inhibited after 96 h. After 12 h, *Pythium* isolates P002 and P011 colonized seeds placed

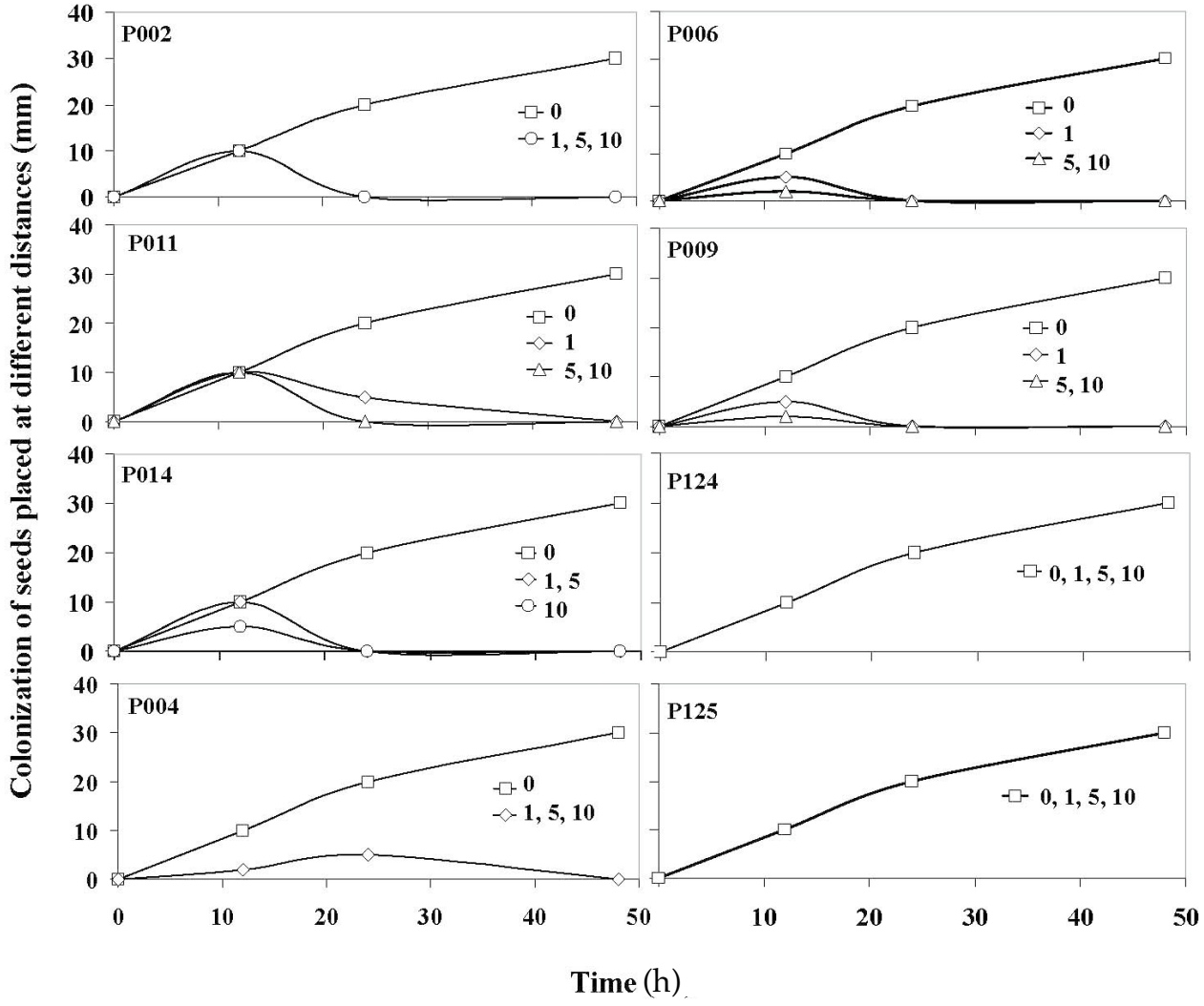

Figure 1. Influence of different concentrations of mefenoxam on the time required to colonize cucumber seeds by *Pythium* isolates differencing in sensitivity to mefenoxam (see Table 1 for characteristics of isolates).

at 2.5, 5 and 10 mm distances from the source of inoculum in plates with no mefenoxam as well as in plates with 1, 5 and 10 μg mL^{-1} mefenoxam. After 24 h, *Pythium* isolate P004 colonized cucumber seeds placed at 2.5 and 5 mm from the inoculum source in sand with mefenoxam concentrations of 1, 5 and 10 μg mL^{-1}. Although efficiency of colonization in the sensitive *Pythium* isolates was not affected by mefenoxam in the first 12 h, there was a negative correlation with the concentration of mefenoxam used after 24 h of incubation in sand amended with mefenoxam (P<0.05).

Discussion

Metalaxyl (mefenoxam) acts on Peronosporales by inhibiting ribosomal RNA synthesis (Davidse, 1988), thus suppressing mycelial growth and sporulation (Hamm *et al*., 1984; Cohen, 1986). Following application, metalaxyl suppresses diseases caused by Oomycete pathogens by inactivation rather than by eradication of the pathogen (Hamm *et al*., 1984; Benson, 1987). Since its introduction in the late 1970s, the fungicide has significantly contributed to the control of species of Peronosporales by offering low effective rates, post-infection curative control, high activity under high infection pressure and systemic control of diseases caused by these pathogens, for which there had been often limited effective chemical control options (Morton and Urech, 1988).

The present laboratory investigations provide evidence that there is a delay in the inactivation of *Pythium* following application of mefenoxam. *Pythium* species required at least 12 to 94 h to become inactive under concentrations of 1 to 10 μg mL^{-1} mefenoxam. These concentrations were reported to be suppressive to *Pythium* growth in previous studies (Al-Sa'di *et al*., 2008a, b, c). Also, *Pythium aphanidermatum* and *P. spinosum* isolates which are sensitive to mefenoxam were found to colonize cucumber seeds placed close to the inoculum sources within 12–24 h following application of 1 to 10 μg mL^{-1} mefenoxam, but colonization was greatly reduced after 24 h. Thus, laboratory data provide evidence that inactivation of *Pythium* by mefenoxam is not achieved as soon as mefenoxam is applied, but usually occurs within the first 24 h after application. This may therefore allow some infection to occur, or for infected cucumber seedlings to develop symptoms as has been shown from laboratory and field experiments (Al Sadi, 2007).

The time required by mefenoxam to suppress *Pythium* infection can be affected by soil characteristics, amount of irrigation water or concentration of mefenoxam in the rhizosphere. Hickey and Coffey (1980) related the delayed complete suppression of *Peronospora pisi* sporulation on *Pisum sativum* to 6 d after application of metalaxyl as a soil drench compared to within 24 h when metalaxyl was supplied via plant root systems to the possible adsorption of metalaxyl to soil particles. Similarly, Stein and Kirk (2003) related differences between laboratory and field studies in the efficacy of dimethomorph for control of *Phytophthora infestans* to the possible incomplete coverage and/or dilution or metabolism of dimethomorph *in planta*.

The present study reports for the first time a lag period between application of mefenoxam and complete inactivation of *Pythium* isolates. To overcome the problem resulting from delayed inactivation of *Pythium* inoculum by mefenoxam, earlier applications of mefenoxam (2 d prior to transplanting) are advised (Al-Sa'di, 2007). However, whether other fungicides used for control of other diseases caused by *Pythium* and other oomycete and fungal pathogens are affected by this problem is an issue which deserves investigation in future studies.

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