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Selection of Virulent and Persistent *Metarhizium anisopliae* Isolates to Control Black Vine Weevil (*Otiorhynchus sulcatus*) Larvae on Glasshouse *Begonia*

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The virulence of 10 *Metarhizium anisopliae* isolates against black vine weevil, *Otiorhynchus sulcatus*, larvae was examined in the glasshouse using potted *Begonia*. The most virulent isolate, 275-86, reduced larval numbers by 86% compared to the control population (pots treated with 0.05% Triton X-100 only) and two other *M. anisopliae* isolates reduced the larval population by over 80%. Only one isolate, 313-87, failed significantly to reduce the larval population. There were no significant differences between the six most virulent strains, however, four of these were significantly more virulent than 313-87. There was no significant correlation between larval number and plant biomass. Three *M. anisopliae* isolates were further examined for persistence in peat compost. All three strains significantly reduced larval numbers on *Begonia* compared with the control population. The highest levels of control were recorded with strain 100-82 (67–80%) which was significantly more virulent than strain 35-79. The fungal drenches were most effective when they were applied 8 weeks before the application of *O. sulcatus* eggs, however, significant levels of control were also recorded with spores which were applied 16 weeks before the eggs. Control with conidia applied 4 weeks after the eggs was significantly lower than that with the application 8 weeks before the eggs, suggesting that there might be differences in the levels of control achieved using curative and prophylactic spore applications. The results of these experiments demonstrate that *M. anisopliae* has considerable potential as a prophylactic microbial control agent for *O. sulcatus* in the glasshouse.

KEY WORDS: *Metarhizium anisopliae; Otiorhynchus sulcatus;* biological control; strain virulence; glasshouse studies; persistence.

INTRODUCTION

The black vine weevil, *Otiorhynchus sulcatus*, is a serious pest of glasshouse ornamentals and significant damage has been reported on species, such as *Cyclamen*, *Primula*, *Begonia*, and *Impatiens*. Larval populations have also been recorded on *Coleus*, *Gazinia*, *Kalanchoe*, *Pelargonium*, and *Sinningia* (Moorhouse, 1990). Populations of *O. sulcatus* larvae were traditionally controlled by preventative application of aldrin, however, this chemical has now been replaced by more environmentally acceptable products, such as insect parasitic nematodes. These new products have limited persistence and consequently can only be used effectively as curative treatments. Many growers would prefer to use a prophylactic control agent for black vine weevil larvae because of the difficulties in predicting the size and significance of the soil-dwelling larval populations. There is therefore a need to evaluate the potential of alternative control agents for *O. sulcatus* larvae with specific reference to efficacy and persistence.

Entomogenous fungi, including *Metarhizium anisopliae*, have shown considerable potential as microbial control agents for *O. sulcatus* larvae. Moorhouse *et al.* (1993) demonstrated that *M. anisopliae* could successfully infect *O. sulcatus* in the laboratory and Prado (1979) and Zimmermann (1981) obtained good control in glasshouse pot plant experiments. Most of the published work on *O. sulcatus* control on pot plants using fungi has involved the comparison of one or two isolates from a number of entomogenous species. The only exception to this was the work of Gillespie (1989) who examined 21 isolates from three fungal genera in small-scale pot experiments. Five of the 11 *M. anisopliae* strains examined by Gillespie (1989) were also included in the present experiment because the results justified a more detailed evaluation. The remaining strains were selected because they had shown potential in bioassay experiments (Moorhouse *et al.*, 1993).

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These initial studies warrant further investigation of the potential of different *M. anisopliae* strains under glasshouse conditions. The earlier bioassays demonstrated that there were significant differences in the virulence of the *M. anisopliae* isolates examined. In order to interpret fully the results from the bioassay assessments, it is necessary to demonstrate consistency between the bioassay and glasshouse assessments. It will then be possible to base a larger strain selection program on simple laboratory procedures in the knowledge that the results will be applicable to a commercial glasshouse situation.

Prolonged persistence of *M. anisopliae* in the soil has been reported by Milner and Lutton (1976). These results, as with many other reports on spore persistence, were based on *in vitro* assessments and there is a need to relate persistence to infectivity. This was done by Fargues and Robert (1985) using scarabeid larvae and they found that there were significant differences between the persistence and infectivity of different *M. anisopliae* isolates. Similar experiments need to be conducted with *O. sulcatus* larvae to demonstrate that *M. anisopliae* might be sufficiently effective and persistent to be a biological replacement for aldrin.

MATERIALS AND METHODS

Strain Selection Experiment

Begonia miniplugs (cv. Happy Choice) were potted into peat compost in 0.5 l square plastic pots. After 19 days, 144 plants were moved into a shaded glasshouse and arranged on capillary matting in a 12 × 12 block. The minimum glasshouse temperature was set at 15°C and cooling by automatic ventilators helped to keep summer temperatures below 25°C.

O. sulcatus adults were collected from an outdoor caged population and transferred to plastic plant propagators. The adults were maintained on strawberry leaves at 20–22°C under a 16:8-hr photoperiod (Moorhouse *et al.*, 1992b). The eggs for the initial experiment were collected over 1 week and placed on moist filter paper in a 9-cm petri dish and maintained at 20°C for 72 hr. Ten "tanned" eggs were placed on the compost surface (around the base of the plant stem) of each pot using a camel hair brush. White and partially melanized eggs were not used in the experiment because earlier work had demonstrated that these eggs were nonviable (Moorhouse, 1990). Five batches of 100 eggs were also placed on moist filter paper in petri dishes and maintained under glasshouse conditions so that egg hatch could be determined. Ninety-three percent of the eggs hatched within 4 weeks and the remaining eggs were considered nonviable.

Ten *M. anisopliae* multispored wild-type isolates from a range of insect species and geographical locations (Table 1) were removed from liquid nitrogen and plated onto Sabouraud's dextrose agar (SDA; Oxoid Ltd., Basingstoke, UK). These plates were incubated at 23°C for 10 days and then conidia were subcultured onto fresh SDA plates and incubated for a further 10 days at the same temperature. The original SDA plates were placed in plastic bags and maintained at 5°C and these were used as stock cultures for the persistence experiment (below). Conidia were harvested by flooding the plates with sterile 0.05% Triton X-100 (BDH Chemicals Ltd., Poole, UK) and agitating with a metal rod. The conidial suspensions were filtered through four layers of sterile, coarse-mesh cheesecloth to remove the hyphal debris. The suspensions were then centrifuged (10 min, 3000 rpm) and resuspended in fresh 0.05% Triton X-100. Spore concentrations were determined using an improved Neubauer hemocytometer and adjusted to 10^7 conidia ml^{-1} by dilution with 0.05% Triton X-100.

TABLE 1
Origins of *M. anisopliae* var. *anisopliae* Isolates

Isolate number[a]	Original host	Location
35-79	*Otiorhynchus sulcatus*	England
37-80	*Otiorhynchus sulcatus*	England
100-82	*Melolontha melolontha*	France
101-82	*Melolontha melolontha*	France
108-82	*Wisena* sp.	New Zealand
159-83	A scarabeid pasture pest	New Zealand
189-83	*Otiorhynchus sulcatus*	England
275-86	*Cydia pomonella*	Germany
276-86	*Otiorhynchus sulcatus*	Germany
313-87	Unknown	Tasmania

[a] This refers to the isolate number in the Horticulture Research International culture collection.

The weevil populations on the experimental plants were allowed to develop for 15 days before the treatments were applied. Twenty-four *Begonia* plants were then removed from the glasshouse and treated with a drench of sterile 0.05% Triton X-100 (25 ml/pot). A group of 12 plants was then treated with a similar drench containing 10^7 conidia ml^{-1} of strain 35-79. This process was repeated using drenches of the remaining 9 *M. anisopliae* strains on further batches of 12 plants (taking care to avoid cross contamination). The plants were then returned to the glasshouse and arranged in a 12 × 12 randomized complete block design (one pot of each fungal treatment and two replicate Triton X-100 pots per block). Germination of 10^6 conidia ml^{-1} suspensions of each strain was assessed on SDA at 25°C using the technique developed by Hall (1977). Conidial viability was determined after 24 hr by observing three groups of 100 conidia using phase contrast microscopy. The germination after 24 hr ranged from 89% for strain 108-82 to 98% for strains 101-82, 189-83, and 275-86.

The experimental pots were maintained in the glasshouse for a further 9 weeks and the plants were then

severed at compost level and the fresh weight of each plant (except those in block I) was recorded and the results were assessed by analysis of variance. The larval populations were then determined by destructive assessment.

Strain Persistence Experiment

Three *M. anisopliae* strains, 35-79, 100-82, and 275-86, were selected for further evaluation of the interaction between virulence and persistence. Two hundred forty *Begonia* seedlings (cv. Happy Choice Mix) were planted in peat compost in 0.5 l square plastic pots and placed in the glasshouse in 12 blocks of 20 plants. A treatment plan was then drawn up by randomly allocating one pot in each block to each treatment. SDA plates were inoculated with conidia from the stock culture plates (prepared above) and incubated at 23°C for 10 days. Spore suspensions containing 10^7 conidia ml^{-1} of each strain were prepared as above and drenched onto 12 replicate pots (1 pot per block) at a rate of 25 ml per pot. This process was repeated 4 weeks later using fresh spore suspensions and a second group of 3 × 12 plants. The application was repeated three more times at 8, 12, and 16 weeks after initial spore application using further groups of 3 × 12 untreated pots. Two additional groups of 12 replicate pots were treated after 16 weeks with a 25 ml per pot drench of either 0.05% Triton X-100 or deionized water. Twenty "tanned" weevil eggs (viability 95.2%) were then applied to all the treated and untreated pots immediately after application of the 16-week treatments. The remaining 36 untreated plants were drenched with fresh spore suspensions after 20 weeks. Germination of all the spore batches was determined and the viabilities of strains 35-79, 100-82, and 275-86 ranged from 93–98, 96–99, and 95–98%, respectively. All the plants were maintained for a further 8 weeks after the final spore application and they were then destructively assessed and larval populations determined.

RESULTS

Strain Selection

The variance-to-mean ratio of the different treatments was close to 1.0, demonstrating a good Poisson fit. The results were therefore analyzed by analysis of variance on the square root-transformed data and the transformed means were compared using *t* tests with an assumed significance threshold of $P = 0.05$.

All the *M. anisopliae* isolates examined with the exception of strain 313-87 significantly reduced weevil numbers ($P < 0.001$) on *Begonia* compared to the Triton X-100 population (Table 2). The reduction in larval numbers ranged from 86 to 20% for strains 275-86 and 313-87, respectively. The other eight strains reduced larval numbers by over 50%. There were no significant differences between the reductions in larval numbers by the leading group of six strains. However, four of these, 37-80, 101-82, 189-83, and 275-86, were significantly more virulent than 313-87 ($P < 0.001$) and 108-82 ($P < 0.05$). Strain 275-86 was also more virulent than both 276-86 and 35-79.

The mean weight of the plants treated with Triton X-100 was significantly lower than those treated with strains 35-89, 100-82, 276-82, and 313-87 ($P < 0.05$) and 101-82 ($P < 0.01$), however there were no significant differences between strains (Table 2). Further analysis using larval number as a covariate demonstrated that there was no significant interaction between plant weight and larval number. The mean weight of the plants from the pots treated with Triton X-100, with a larval population of 4.88 larvae/pot, was 159.1 g, whereas the mean weight of the plants from the pots treated with strain 275-86 was 173.4 g/pot

TABLE 2

Control of *O. sulcatus* Larvae on *Begonia* and Mean Plant Weight 9 Weeks after Treatment with Different *M. anisopliae* Strains

Treatment	Live larvae recovered/pot	Mean square root transformation[a]	Percentage control[b]	Mean plant weight (g)[c]
Triton X-100	4.88	2.174	0	159.3
35-79	1.67	1.081	66	215.5
37-80	0.92	0.819	81	178.7
100-82	1.33	0.976	73	210.7
101-82	1.08	0.774	78	226.1
108-82	2.00	1.294	59	175.3
159-83	1.17	0.854	76	195.0
189-83	0.83	0.638	83	205.7
275-86	0.67	0.569	86	173.4
276-86	1.75	1.207	64	213.7
313-87	3.92	1.935	20	219.6

[a] The SEDs of the transformed means (with 122 *df*) were Triton X-100 vs fungal strain = 0.1999; between fungal strains = 0.2308.
[b] Percentage control is based on the reduction in the treated population compared to the mean of the two Triton X-100 populations.
[c] The SEDs of the mean plant weight (11 replicates, 110 *df*) were Triton X-100 vs fungal strain = 24.48; between fungal strains = 28.26.

with a population of only 0.67 larvae/pot. The individual plant weights were highly variable, possibly as a result of the mixed cultivar, and this may have masked any link between larval population and plant growth.

Strain Persistence

The variance-to-mean ratio of the persistence data showed a degree of overdispersion (McCullagh and Nelder, 1989), but it was generally distributed. Analysis of variance was used to analyze the count data (subjected to a square root transformation). There was no significant difference ($P > 0.05$) between the larval populations in the pots treated with either 0.05% Triton X-100 (7.08 larvae/pot) or deionized water (7.67 larvae/pot) and therefore the results from the two sets of pots were combined to give an overall control mean of 7.38 larvae/pot. The comparison of the water and Triton X-100 treatments showed that the reductions in larval populations on plants treated with conidia in this experiment (and the strain selection experiment above) was not the result of the wetting agent.

All three strains significantly reduced larval numbers compared to the combined control population (Table 3). The highest reductions were achieved with strain 100-82 which reduced the larval population by 67–80%. This isolate was significantly better ($P < 0.001$) than strain 35-79, but it was not significantly different from strain 275-86 (Table 3). A significant block effect ($P < 0.05$) was observed in this trial and this may have been due to the irregular surface of the glasshouse benches causing uneven water distribution.

The time factor was divided into orthogonal polynomials (to the second order), although it might be claimed that application of eggs prior to spore application reflects a discontinuity in the time factor. Analysis showed a strong quadratic time trend with all the strains being most effective when they were applied 8 weeks before the eggs (Table 3). There were no significant differences between the larval populations on the pots treated with conidia 12, 8, 4, or 0 weeks before egg application. However, the larval population on the pots treated with conidia 8 weeks before egg infestation was significantly lower ($P < 0.05$) than that on the pots treated with conidia 16 weeks before or 4 weeks after egg infestation (Table 3). There were no significant differences between the different aged spores of the same strain, with the exception of the treatments of 35-79 which were applied 8 weeks before or 4 weeks after the eggs ($P < 0.05$). These results demonstrate that *M. anisopliae* conidia can persist in compost and remain active against weevil larvae for at least 16 weeks.

TABLE 3

Larval Populations (and Mean Square Root Transformations) on *Begonia* Plants Which had been Treated with *M. anisopliae* Conidia 16, 12, 8, 4, or 0 Weeks before or 4 Weeks after Egg Infestation

Application time (before or −after eggs)	Mean larval number (and transformed value) *M. anisopliae* strain 35-79	100-82	275-86	Mean
16 weeks	2.92 (1.513)	2.75 (1.377)	2.75 (1.419)	2.81 (1.436)
12 weeks	3.08 (1.543)	1.33 (0.963)	2.50 (1.454)	2.30 (1.320)
8 weeks	2.00 (1.257)	1.25 (0.816)	1.42 (0.858)	1.56 (0.977)
4 weeks	2.58 (1.305)	1.83 (1.066)	2.25 (1.263)	2.22 (1.211)
0 weeks	2.92 (1.624)	1.58 (0.905)	1.83 (1.127)	2.11 (1.219)
−4 weeks	3.83 (1.881)	1.58 (1.003)	1.75 (1.363)	2.39 (1.416)
Treatment means	2.89 (1.521)	1.72 (1.022)	2.08 (1.247)	
Control mean[a] = 7.38 (2.610)				

Note. The SEDs of the transformed means (with 210 *df*) were between control and treatment means = 0.1810; between treatment means = 0.1280; between control and application time means = 0.2024; between application time means = 0.1810; between control and fungal treatments = 0.2715; between fungal treatments = 0.3135.

[a] This is the mean larval population on the pots treated with 0.05% Triton X-100 and water.

DISCUSSION

There have been very few reports of strain selection experiments under commercial conditions; most of the published reports (for example, Feng and Johnson, 1990) involve assessment under bioassay conditions. The data presented in this paper can be directly compared to published bioassay results (Moorhouse *et al.*, 1993) and the usefulness of bioassay strain selection for *O. sulcatus* larvae can be determined. The LT_{50} values of *M. anisopliae* strains calculated in laboratory bioassays was broadly correlated with their performance in the glasshouse strain assessment. The only anomalous results were obtained with strains 159-83 and 313-87. Strain 159-83 was ranked tenth in the bioassay assessment and fifth in the glasshouse experiment. This strain is known to have a low temperature optimum for growth (Moorhouse, 1990) and it is possible that the glasshouse temperatures during the experiment may have enhanced its efficacy. Bioassay data with strain 313-87 demonstrated contact activity, whereas larval control was poor in peat compost. This unexpected result could have been caused by a number of factors such as strong conidial adsorbance on the peat particles and reduced persistence. Similar anomalies have been observed with chemical insecticides. Harris and Mazurek (1964) found that aldrin was four times more toxic to *Gryllus pennsylvanicus* when applied to soil compared with direct contact application, whereas mevinphos was 320 times less toxic under similar conditions.

Bioassay and pot data are not strictly comparable because of the different conidial application techniques. Immersion in a conidial suspension represents a temporary exposure to a very high inoculum concentration. Prolonged exposure of insects to conidia in treated potting media may provide a lethal dose from a sublethal bioassay concentration through the accumulation of spores with time (Ferron, 1985). The mechanisms of contact between conidia and the larval cuticle in soil are poorly understood and further research in this area is required.

The poor correlation between larval population and plant weight is surprising because damage to the root system would normally be expected to reduce growth. Penman and Scott (1976) reported a positive correlation between larval numbers and reductions in leaf number, size, and berry number on strawberry plants. It is possible that *Begonia* plants are able to tolerate and compensate for a certain amount of root damage because they have extensive fibrous root systems. Neiswander (1953) concluded that established plants with large root systems could withstand some feeding without any adverse effects on growth. Species, such as *Cyclamen*, which have a more limited root system are less able to cope with damage by *O. sulcatus* larvae and plants are frequently killed by very low larval populations (Moorhouse, 1990). A number of the larvae in the Triton X-100 pots were beginning to feed around the stem base at the time of assessment and it is likely that severe damage may have occurred if the assessment had been delayed. Moorhouse *et al.* (1992a) reported that the position of larvae within the root system was critical, with one larva causing more damage at the stem base than several around the periphery of the root system.

Persistence is essential for prophylactic control and is also a desirable characteristic for curative products. All three *M. anisopliae* strains were still effective after 16 weeks and this contrasts with the significantly reduced virulence in one strain of *M. anisopliae* which was observed by Fargues and Robert (1985) after 4 months in soil. Another isolate examined by these authors was still highly active after 21 months and it is possible that any differences between the strains used in the present work would have become evident over a longer time period.

Spore application and distribution within a pot is critical to larval control. Zimmermann (1981) and Tillemans and Coremans-Pelseneer (1987) found that prophylactic treatment was more effective than curative application. The latter workers found that larval control was reduced from 84 to 72% when the application of *Beauveria brongniartii* conidia was delayed by 14 days after infestation with *O. sulcatus* larvae. Similar results were obtained in the present study with strain 35-79, but the difference between curative and prophylactic conidial application was less obvious with the other two strains. Moorhouse *et al.* (1992c) observed that spore concentration in peat compost was negatively correlated with depth and it was suggested that larvae at the base of the pots survived because they were not exposed to lethal spore doses. The reduction in control was quantified by Moorhouse (1990) who reported a 10% decrease in control when conidia were applied 4 weeks after the eggs compared with prophylactic application. It is possible that migration of the young *O. sulcatus* larvae during the 4 weeks following egg application in the current work was limited and the majority were in the surface layers and were therefore exposed to lethal spore concentrations when the curative drenches were applied. It is also possible that compost and plant factors, such as moisture status and root density, may have permitted greater spore penetration (Storey and Gardner, 1987).

The results of the initial strain assessment conclusively demonstrate the potential of *M. anisopliae* and the persistence results provide an indication that some *M. anisopliae* isolates can control *O. sulcatus* over a prolonged period.

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