# PATHOGENICITY OF WATER AND OIL BASED SUSPENSIONS OF *METARHIZIUM ANISOPLIAE* (METSCHNIKOFF) SOROKIN AND *BEAUVERIA BASSIANA* (BALSAMO) VUILLEMIN TO CITRUS MEALYBUG, *PLANOCOCCUS CITRI* (RISSO) (HEMIPTERA: PSEUDOCOCCIDAE)

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## Summary

Laboratory bioassays compared the pathogenicity of six isolates of *Metarhizium anisopliae* and *Beauveria bassiana* against second instar citrus mealybugs, *Planococcus citri* under conditions of $26 \pm 1^0$ C, and 85 ± 1% RH in 24 hour darkness. All isolates exhibited pathogenicity. *M. anisopliae* isolate FI-1248 was the most virulent isolate in both water and oil suspensions with $LC_{50}$ values of $6.4 \times 10^5$ conidia/mL and $3.4 \times 10^4$ conidia/mL respectively. *M. anisopliae* isolate FI-0985 was found to be the least virulent.

**Keywords:** citrus mealybug, microbial control, *Metarhizium*, *Beauveria*, horticultural mineral oil

## INTRODUCTION

Citrus mealybug, *Planococcus citri* (Risso) (Hemiptera: Pseudococcidae) is a cosmopolitan pest with a very wide host range, including citrus, vines, ornamentals and glasshouse crops (Al Ali 1959; Cox and Ben-Dov 1986; Smith *et al.* 1997; Bedford *et al*. 1998; Waterhouse 1998). Most crop losses occur through premature blossom and fruit drop, or increased incidence of navel end rot and sooty mould to fruit, leaves and twigs (Smith *et al.* 1997; Bedford *et al.* 1998; Waterhouse 1998). Chemical sprays may be difficult due to the hydrophobic covering on both individuals and colonies of the mealybug (Moore 1988). Current management methods, which emphasise the use of predators and parasites such as coccinellid beetles, lacewings and wasps, have met with some success. However, these natural enemies are susceptible to the insecticides applied to control mealybugs and other pests (Bedford *et al.* 1998). The absence of natural enemies results in resurgence of *P. citri* populations, with few control options available until natural enemies can be re-established. An 'environmentally soft' pesticide to control citrus mealybug that is non-toxic to its natural enemies would, therefore, have widespread appeal for its management on citrus, vines and glasshouse crops.

Microbial pesticides are ideal for use in Integrated Pest Management programs because of their host specificity and environmental safety (Starnes *et al.* 1983). Fungi are the only pathogens reported infecting mealybugs under natural conditions with species of *Cadosporium*, *Neozygites*, *Metarhizium* and *Aspergillus* being recorded from the field (Murray 1978; Samways and Grech 1983; Moore 1988; Martinez and Bravo 1989). However, Le Ru *et al.* (1985) suggests that the record of *Neozygites fresenii* (Nowakowski) infecting *P. citri* is doubtful as *N. fresenii* is probably aphid specific. Perhaps the fungus infecting mealybugs is a new species of *Neozygites*. The fungi recorded from mealybugs in the field are all rare and none appears to be promising for development as a mycoinsecticide. Since products based on the fungi *M. anisopliae* and *B. bassiana* have been developed as mycoinsecticides in the USA and Australia, isolates of these fungi originating from unrelated insect hosts were selected for screening against *P. citri*.

Recent evidence suggests that entomopathogenic fungi are more efficacious under low humidity conditions when formulated in oil suspensions (Bateman *et al.* 1993) and can kill more rapidly (Milner *et al.* 1997). Water-miscible oils are ubiquitous pest management tools used in the citrus industry against mites, scales and leaf miner (Davidson *et al*. 1991; Smith *et al*. 1997). They may also benefit biopesticides by improving the sticking and spread of inoculum, protecting against desiccation and ultra-violet light, thus leading to increased field persistence (Moore and Prior 1993). Therefore, combining fungal pathogens for the selective management of citrus mealybug with an oil used to control other citrus pests is likely to produce multiple benefits. As the first stage of developing a biopesticide for citrus mealybugs, we report on the pathogenicity of six fungal isolates in aqueous and oil-based sprays.

## MATERIALS AND METHODS

Same age, second-instar nymphs of *P. citri* were used for bioassays. These were obtained by collecting mealybug crawlers from a culture maintained on butternut pumpkins. Newly infested pumpkins were incubated for 10–11 days in individual cardboard cages that were covered with fine netting to prevent mealybug escape or reinfestation.

The six fungal isolates were selected from the CSIRO Insect Pathogen Culture Collection and were cultured in 90 mm Petri dishes on Veen's Medium (Veen and Ferron 1966) at 25 ± $1^0$ C. The conidia were harvested dry using a sterile wire loop and aseptic techniques 17-20 days after plate inoculation

Stock aqueous suspensions were prepared by suspending fresh conidia in an aqueous solution of 0.5 mg/L Tween 80® (Sigma Pty Ltd). Stock oil suspensions were prepared by suspending fresh conidia in an emulsion of 1.0 mg/L horticultural mineral oil, D-C-Tron Plus®, (using 0.5 mg/L Tween 80 as a diluent). The concentration of each stock conidial suspension was determined using a haemocytometer and an Olympus® compound microscope at 400x magnification. Serial dilutions of 1 x $10^5$ conidia/mL to 1 x $10^9$ conidia/mL were made from the stock suspensions using 0.5 mg/L Tween 80 solution for aqueous suspensions and 1.0 mg/L D-C-Tron Plus® for oil suspensions. Both an untreated control and control treated with 0.5 mg/L Tween 80 only, were used. Prior to their use in bioassays, the percentage of viable spores in each conidial suspension was determined using the method described by Walstead *et al.* (1970). Germination was defined as the stage when the germ tube length equalled half the spore length (Drummond *et al.* 1991). The conidial suspensions used ranged from 87 to 95% germination.

Each replicate comprised a pest-free, unsprayed leaf from Californian peppercorn, *Schinus molle* (L.). All leaves were similar size and each leaf was trimmed to 17-18 terminal leaflets. Leaves were sterilised by immersing them for 5 s in a 10.0 mg/L sodium hypochlorite solution and rinsing twice in distilled water. They were then dried between layers of paper towel. Petioles were trimmed and immediately inserted through a small hole in the lid of a 300 mL plastic food storage container into vials of distilled water. A piece of Blue-tac® (Bostik Australia Pty Ltd) was placed at the junction of the petiole and container lid to ensure that the leaves remained vertical and to prevent escape of mealybugs. Fifteen mealybugs were introduced onto each leaf using a fine camel-hair brush (size 00) and allowed to settle for 1 h before treating.

An artists' airbrush (Paasche Airbrush Company, Harwood Heights, Illinois, USA), powered by compressed air at 110 kPa and fitted with a broad band nozzle, was used to spray conidial suspensions for each treatment. One mL aliquots of spore suspension were sprayed evenly onto each leaf. The distance from the airbrush to the leaf surface was approximately 20 cm. All treatments were stored in darkness at 26 ± $1^0$ C and 85 ± 1% RH in total darkness, for the duration of the experiment.

Mealybugs were observed daily from days five to ten after treatment for mortality, which was assessed as lack of movement when gently prodded with a camel-hair brush. Dead mealybugs were removed from the leaf during data recording, placed on moist filter paper contained within a sealed Petri dish and incubated at >98% RH and 25 ± $1^0$ C. They were examined later for signs of fungal sporulation. Conidia from the fungal growth on one mealybug per replicate, was isolated onto Veen's medium and cultured as previously described to confirm its identity. Only the numbers of dead mealybugs subsequently showing signs of sporulation after incubation were used in the statistical analysis. The final (day 10) accumulative mortality data were analysed using the Probit procedure of SPSS® for Windows Version 9 (SPSS 1999). The test for statistical significance between appropriate LC values was failure of their 95% confidence limits to overlap.

## RESULTS

All fungal isolates were found to be pathogenic to second instar *P. citri* in both aqueous and oil suspensions, although there was considerable variation in their virulence. Oil emulsion suspensions of conidia were generally found to be more effective at killing second instar *P. citri* than were equivalent aqueous suspensions. However, based on the overlap of 95% confidence limits, these differences were not significant.

*M. anisopliae* isolate FI-1248 was the most virulent against second instar *P. citri* in both aqueous and oil suspensions, with $LC_{50}$ values of 6.4 x $10^5$ and 3.4 x $10^4$ conidia/mL respectively (Table 1). *M. anisopliae* isolate FI-0985 was the least virulent, with $LC_{50}$ values of 1.4 x $10^7$ and 7.4 x $10^6$ conidia/mL in water and oil respectively. Based on individual comparisons of $LC_{50}$s and the overlap of the 95% confidence limits, *M. anisopliae* isolate FI-1248

**Table 1. LC50 and 95% confidence limits of aqueous and oil suspensions of *M. anisopliae* and *B. bassiana* isolates, against *P. citri.***

| Isolate | Fungal species | Original host | Water | | Oil | |
|---|---|---|---|---|---|---|
| | | | Slope (SE) | LC50 (95%CL) | Slope (SE) | LC50 (95%CL) |
| FI-1248 | ***M. anisopliae*** | *Mastotermes darwinienis* | 0.56 (0.09) | $6.4 \times 10^5$ ($2.0 \times 10^5 – 1.5 \times 10^6$) | 0.42 (0.09) | $3.4 \times 10^4$ - |
| FI-1218 | ***M. anisopliae*** | Isolated from BioBlast[R] product | 0.30 (0.07) | $7.8 \times 10^5$ ($5.9 \times 10^4 – 3.3 \times 10^6$) | 0.34 (0.07) | $7.6 \times 10^4$ - |
| FI-1312 | ***B. bassiana*** | *Chalcodermus sp.* | 0.19 (0.07) | $1.3 \times 10^6$ ($7.0 \times 10^3 – 1.1 \times 10^7$) | 0.73 (0.12) | $1.8 \times 10^5$ ($1.1 \times 10^2 – 1.1 \times 10^6$) |
| FI-0023 | ***M. anisopliae*** | *Aeneolamia albofasciata* | 0.18 (0.06) | $4.9 \times 10^6$ ($2.2 \times 10^5 – 8.1 \times 10^7$) | 0.32 (0.07) | $1.2 \times 10^5$ ($3.7 \times 10^3 – 6.3 \times 10^5$) |
| FI-1186 | ***M. anisopliae*** | *Antitrogus parvulus* | 0.30 (0.07) | $1.3 \times 10^7$ ($2.7 \times 10^6 – 6.4 \times 10^7$) | 0.25 (0.07) | $7.9 \times 10^4$ ($2.9 \times 10^2 – 6.6 \times 10^5$) |
| FI-0985 | ***M. anisopliae*** | *Austracris guttulosa* | 0.16 (0.06) | $1.4 \times 10^7$ ($4.6 \times 10^5 – 7.7 \times 10^8$) | 0.37 (0.07) | $7.4 \times 10^6$ ($2.2 \times 10^6 – 2.3 \times 10^7$) |

(aqueous suspension) was significantly more virulent against second instar *P. citri* than *M. anisopliae* isolates FI-1186 and FI-0985 (water suspensions). *M. anisopliae* isolate FI-0023 (oil suspension) was significantly more pathogenic than the oil suspension of *M. anisopliae* isolate FI-0985. No confidence limits were obtained for oil suspensions of *M. anisopliae* isolates FI-1248 and FI-1218 due to heterogenity, therefore, virulence would be considered equal when comparing all six isolates.

## DISCUSSION

These results support the hypothesis that strains of *M. anisopliae* and *B. bassiana* isolated from unrelated insect species produce mortality in *P. citri* nymphs when applied at concentrations ranging from $1 \times 10^5$ to $1 \times 10^9$ conidia/mL. Other authors investigating efficacy of fungal entomopathogens to mealybugs in the laboratory have reported mortality occurring at similar spore concentrations when using fungal pathogens isolated from field-collected mealybug cadavers (Samways and Grech 1983; Martinez and Bravo 1989; Drummond *et al.* 1991).

The most virulent isolate in our investigations, *M. anisopliae* FI-1248, was at least as efficacious against second instar *P. citri* as other isolates of *M. anisopliae* and *B. bassiana* to homopterans reported in other studies (Feng and Johnson 1990; Feng *et al.* 1990; Puterka *et al.* 1994). However, these authors found a wide range of virulence between isolates tested. In our investigations, a wide range of virulence was not recorded, with all six isolates tested having $LC_{50}$ values between $3.4 \times 10^4$ and $1.4 \times 10^7$ conidia/mL. This may be due to differences in insect species susceptibility. *P. citri* produces numerous waxy, dermal filaments which may act as traps for the fungal conidia. If so, this could increase the number of conidia contacting the insects' cuticle. The lipophilic nature of these dermal filaments may also aid adhesion of fungal conidia to the insect's cuticle, as the conidial cell walls of *Metarhizium* and *Beauveria* isolates are also lipophilic. A greater number of conidia contacting and adhering to the insect's cuticle, is likely to increase the chance of infection and subsequent death.

In our investigations, the infectivity of conidia suspended in an oil emulsion was not found to be significantly different to that of conidia suspended in an aqueous solution. Oil and oil-emulsion formulations of fungal conidia are known to significantly increase virulence and enable infection at low field humidities (Prior *et al.* 1988; Bateman *et al.* 1993; Smith 1994; Milner *et al.* 1997; Smith 1997; Milner and Staples 1998).

Our findings can be compared with those of Smith (1994; 1997) who reported that the efficacy of *Paecilomyces fumosoroseus* (Wise) Brown and Smith, against *Bemisia tabaci* (Gennadius) was greater in oil emulsion formulations than in aqueous suspensions. However, Smith (1994) used emulsions of plant-derived pest oil whereas we used a mineral-derived oil in our study. Barson *et al.* (1994) reported that the fungal conidia of *M. anisopliae* and *B. bassiana* suspended in mineral based oils were not as effective in killing house flies as conidia suspended in plant derived oils, although these were in pure oils and not emulsified aqueous suspensions.

Heterogeneity in the data for oil emulsion suspensions of three treatments, namely FI-1248, FI-1218 and FI-1312, may have been a result of several factors. The lipophilic nature of the cell walls makes the conidia naturally gravitate towards the oil particles in the suspension, quickly forming a viscous top layer of conidia and oil. The cryptic habit of *P. citri* may have also added to the heterogeneity of the data. The mealybugs tended to cluster together on the midribs or in leaf axils when transferred, and as a result, a proportion of these may have escaped initial contact with the fungal inoculum. Their sedentary nature, is also likely to minimise secondary contact from treated leaf surfaces.

*P. citri* is likely to be a good candidate for control by fungal pathogens. Ferron (1977) reported that fungal infections could be initiated even at low atmospheric relative humidity because insect cuticles provide a microclimate of high relative humidity. The numerous, waxy, dermal filaments produced by citrus mealybug should provide the high relative humidity microclimate suitable for germination of conidia of entomogenous fungi. The cryptic habit of *P. citri* (Smith *et al.* 1997; Bedford *et al.* 1998) and plant host range provide additional opportunities for its successful management with fungal pathogens. The relative humidity in such microhabitats is likely to be higher than that in the surrounding atmosphere, favouring both the initiation of fungal infections and the sporulation of cadavers. In citrus orchards, high humidity may also occur under the crop canopy, especially at night, and after irrigation or rainfall. This phenomenon is even more frequent in northern Australia. Glasshouse environments are also especially suited to pest control by fungal entomopathogens, due to their elevated atmospheric relative humidity and warm temperatures. The optimal temperature for growth and development of *B. bassiana* and *M. anisopliae*, (25–30$^0$C), generally coincides with optimal conditions for *P. citri* infestations, thereby increasing the chance of epizootics and residual pathogen activity, as the fungi can multiply and persist within their host (Ferron 1977). As *P. citri* is a sap sucking insect there is likely to be sufficient time for fungal spores to germinate, infect and kill mealybugs before they cause significant plant damage.

Further work is required to determine the future role of FI-1248 as a control strategy for *P. citri*. In particular, trials to assess its field efficacy and non-target effects are required together with an assessment of the feasibility and economics of its mass production.

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