Mortality of the Cycad Aulacaspis Scale (Hemiptera: Diaspididae) by the Entomopathogenic Fungus *Isaria fumosorosea* Wize under Laboratory Conditions[1]

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Abstract The entomopathogenic fungus *Isaria fumosorosea* Wize (PFR97® strain, Certis USA, Columbia, MD) was tested as a mortality agent of the cycad aulacaspis scale, *Aulacaspis yasumatsui* Takagi, at 2 temperatures in the laboratory. First instars of *A. yasumatsui* were treated with 1 of 4 concentrations of *I. fumosorosea* (5.4×10^7, 9.9×10^6, 6.4×10^5, or 1.8×10^5 blastospores/ml of water) or a water only control. Following treatment, insects were held at either 20 or 30°C. The highest concentration treatment resulted in the highest mean infection rate at 8 d postapplication (73 ± 4.2% at 30°C; 84 ± 3.7% at 20°C). However, there was no interaction between blastospore concentration and temperature for infection rate. The lowest mortality rate was obtained with the highest concentration of blastospores under 20°C (13 ± 3%). The LC_{50} at 20° and 30°C were 6.1×10^6 and 5.3×10^6 blastospores/ml, respectively. The LT_{50} was lower at 30°C than at 20°C for the 3 highest concentrations. The radial growth of the fungus on potato dextrose agar 20 d after inoculation was 37% greater at 30°C than its radial growth at 20°C. These results indicate that *I. fumosorosea* may be a new biological control weapon for suppressing infestations of cycads by *A. yasumatsui*. This is the first report of *I. fumosorosea* being evaluated to infect an armored scale insect.

Key Words invasive pest, armored scale, infection rate, molting rate, biological control, entomopathogenic fungus

The cycad aulacaspis scale, *Aulacaspis yasumatsui* Takagi (Hemiptera: Diaspididae), is a pest of cycads in many countries (Howard et al. 1999, Weissling et al. 1999, Hodgson and Martin 2001, Moore et al. 2005, Germain and Hodges 2007, Segarra-Carmona and Pérez-Padilla 2007). It was accidentally introduced into Florida (USA) from Southeast Asia and quickly spread throughout the urban landscape where it infests cycads in the genera *Cycas* (Cycadaceae), *Dioon, Encephalalartos, Microcycas* (Zamiaceae), and *Stangeria* (Stangeriaceae), feeding on the aerial parts and roots of host plants (Howard et al. 1999). Infestations usually appear first on the base of the rachis, then spread on the leaf until the whole plant is infested, weakens, and dies. When the plant is completely infested with the scale insect, the undersides of leaves are covered with a white waxy layer of scales of different instars, which are difficult to clean up even when the insects are dead (European and Mediterranean Plant Protection Organization 2008).

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The first instar is the primary dispersal stage of the cycad aulacaspis scale and moves to noninfested plants by wind or animal contact (Watson 2005). The optimal temperature for development of nymphs is 30°C; whereas, their development is severely limited at <20°C (Cave et al. 2009b). The fecundity of the cycad aulacaspis scale is not quantified yet, but it is observed to be a very prolific insect and this may be one of the reasons why its natural enemies have not provided effective control on cycads in Florida.

The cycad aulacaspis scale has had a considerable impact on the ornamental plant industry in the countries it has invaded (Watson 2005). It is an unusually difficult pest to control because it forms dense populations and moves quickly to new areas via the plant trade (Howard and Haynes 2006). The most effective method of control has been the application of fish and agricultural oils on the foliage as soon as the first instars (crawlers) begin to appear; these oils do not permit the crawlers to establish on the plant and start feeding. Continuous applications of oil for several weeks may also kill some adult females (Weissling et al. 1999). Horticultural oil must be applied at 2 or 3 wk intervals to be effective, but frequent oil treatments can result in phytotoxicity and an unsightly build-up of oil and dead scales (Hodges et al. 2003). Dinotefuran is a systemic pesticide that is water soluble and can be either drenched or applied twice a year to foliage to give excellent control (Caldwell 2005, Webb 2009). Both oil and dinotefuran are considerably detrimental to natural enemies (Smith and Cave 2006a). Two natural enemies introduced into Florida to control the scale are the parasitoid *Coccobius fulvus* (Compere and Annecke), brought from Thailand in 1998 (Howard et al. 1999), and the predatory beetle *Cybocephalus nipponicus* Endrödy-Younga, introduced in 1998 but already present in the state (Smith and Cave 2006b). In addition, 16 species of predatory lady beetles (Coccinellidae) have been found on scale-infested plants in south Florida, but none have been found to suppress the populations of the cycad aulacaspis scale (Cave 2006).

No entomopathogenic fungi have been observed growing on cycad aulacaspis scale in Florida. *Isaria cinnamomea* (Petch) (= *Paecilomyces cinnamomeus*) was observed and isolated from a scale insect in Thailand (Hywel-Jones 1993, Isaka et al. 2007). The use of a commercially-available entomopathogenic fungus may be an alternative for the management of the cycad aulacaspis scale. *Isaria fumosorosea* (= *Paecilomyces fumosoroseus*) (Hypocreales: Cordycipitaceae) was demonstrated to infect first instars of *Bemisia argentifolii* (Bellows and Perrin) (Lacey et al. 1999). The fungus grows optimally between 20 and 25°C in Europe and between 25 and 28°C in the southern USA and western Asia; in monsoon climates (India), it tolerates temperatures between 32 and 35°C (Vidal et al. 1997). The objective of our study was to evaluate the potential of *I. fumosorosea* as a biological control agent of first instars of *A. yasumatsui* as noted by Cave et al. (2009a). A laboratory bioassay was performed to assess the effectiveness of 4 blastospore concentrations of a commercial strain of *I. fumosorosea* for causing mortality of first instars at 2 constant temperatures of 20 and 30°C.

Materials and Methods

A colony of the cycad aulacaspis scale was maintained at the Indian River Research and Education Center in Fort Pierce, FL, USA. The colony was maintained by placing uninfested cycads (*Cycas revoluta* Thunberg) next to scale-infested cycads to allow first instars to move to new plants. The colony was maintained in an environmental growth chamber at 28°C, 70 - 80% RH, and 12:12 L:D photoperiod.

A blastospore suspension of *I. fumosorosea* (PFR97® 20% WDG; Certis USA, Columbia, MD, USA) was prepared by mixing 1 g of PFR97 with 100 ml of sterile distilled water in a beaker, vortexed, and allowed to stand for 20 min until the excess of inert product precipitated. The liquid part (supernatant) of the suspension, which contained the blastospores, was decanted and used in the experiments. Four different concentrations prepared by serial dilutions of PFR97 in water were used in this study: (1) 5.4×10^7; (2) 9.9×10^6; (3) 6.4×10^5; and (4) 1.8×10^5 blastospores/ml. The initial solution was serially diluted by mixing 5 ml of suspension with 45 ml of sterile distilled water to reach the other 3 concentrations. The number of blastospores per ml in each suspension was confirmed by using a Neubauer hemocytometer. Each 50-ml suspension was poured into separate Nalgene® (Fisher Scientific, Suwanee, GA) spray bottles (170 ml capacity) for application on infested plants.

A factorial design experiment consisted of 5 treatments (4 concentrations of PFR97 and water only control) applied at 2 temperatures (20, 30°C). Fifty leaflets infested with the cycad aulacaspis scale were detached from different cycad plants, and 10 settled first instars were left on each leaflet after carefully removing the remaining scale stages from the leaflet. To standardize the pressure inside the spray bottles, each bottle was pumped the same number of times (30) for each fungal concentration and the control. Ten leaflets per blastospore concentration were sprayed in a laboratory fume hood by making 2 pulls on the spray bottle trigger. Ten leaflets were used as controls and were sprayed with sterile distilled water only.

Treated leaflets (5 leaflets per treatment per temperature) were placed individually inside Petri dishes (10 cm diam) containing moistened filter paper and sealed with Parafilm. Petri dishes were maintained in environmental growth chambers at 20 or 30°C, 70 - 80% RH, and 16:8 L:D photoperiod. Preliminary trials indicated that cycad leaflets placed inside Petri dishes as mentioned above would remain green for 2 wks.

All leaflets were inspected at 3, 5, 6, and 8 d following treatment application. Each leaflet was examined under a dissecting microscope, and the number of cycad aulacaspis scales alive and the number apparently dead (as evidenced by purplish discoloration and/or presence of hyphae after 3 days) were recorded. In addition, the percentage of nymphs molting from first to second instar was recorded for each leaflet. The experiment was performed twice.

The radial growth of PFR97 on potato dextrose agar (PDA) was measured under the 2 experimental temperatures to determine under high moisture conditions (~100% RH) and a constant photoperiod the effect temperature has on the growth of *I. fumosorosea*. Twenty Petri dishes with PDA were inoculated with a 0.4-ml droplet of spore suspension with a concentration of 5.4×10^7 blastospores/ml. Ten Petri dishes were sealed with Parafilm and stored at 20°C and the other 10 at 30°C. The fungal radial growth rings (length and width) on the PDA were measured 4, 7, 10, 13, 15, 18, and 20 d after inoculation. This experiment was performed twice.

Mortality and molting rates 8 d after treatment application were analyzed with an analysis of variance general linear model, and means were separated by the Student-Newman-Keuls method using SAS software (SAS Institute Inc. 2004) ($\alpha = 0.05$). Data were transformed from percentages to a logarithmic scale to reduce variation; non-transformed data are reported. Probit software (Finney 1971) was used to determine LC_{50} and LT_{50}. The radial growth of PFR97 on PDA was analyzed by linear regression using SAS software. All data from the 2 repetitions of each experiment were analyzed together because there were no significant differences between the 2 replicates of the 2 experiments ($P > 0.05$).

Results and Discussion

Mortality rates by PFR97 for first-instar *A. yasumatsui* increased over time in all treatments except the control (Fig. 1), probably because of a progressive germination of the blastospores and the production of new conidia by the fungus. Mortality rates at all sampling points were highest with the concentration of 5.4×10^7 blastospores/ml. At both temperatures, mortality rate with the highest concentration 8 d after treatment application was significantly higher at 20°C ($F = 36.79$; $df = 4$; $P < 0.001$) and 30°C ($F = 37.95$; $df = 4$; $P < 0.001$) than the mortality rate with 9.9×10^6 blastospores/ml, which was significantly higher than the mortality rates with the next lowest concentration

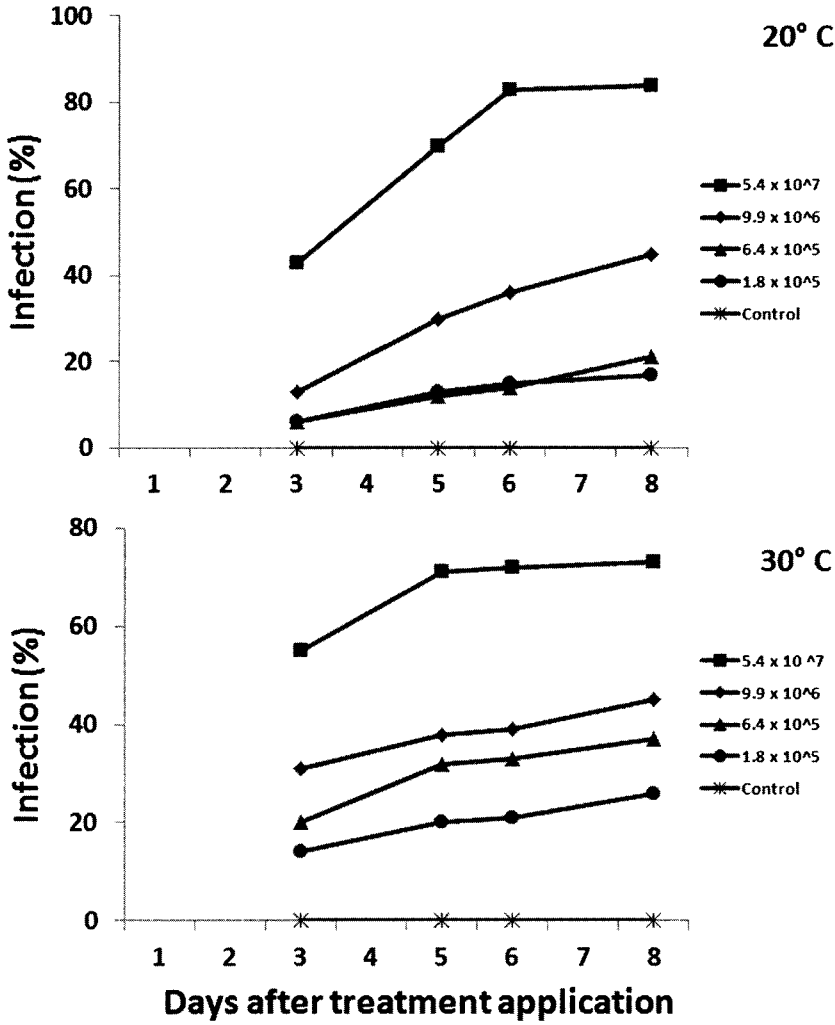

Fig. 1. Infection rates of first-instar *Aulacaspis yasumatsui* by *Isaria fumosorosea* at 2 temperatures following application of 4 blastospore concentrations.

Table 1. Median lethal times (LT_{50}) (days) of *Isaria fumosorosea* (PFR97) infecting first-instar *Aulacaspis yasumatsui* at 2 temperatures and 4 blastospore concentrations.

Temp	Concentration*	LT_{50}	95% fiducial limits	Probit equation**	*P*
20°C	5.4×10^7	3.3	2.7 - 3.8	$M = -1.6 + 3.00\log_{10}T$	<0.0001
	9.9×10^6	8.7	7.4 - 12.3	$M = -2.2 + 2.34\log_{10}T$	<0.0001
	6.4×10^5	23.8	13.3 - 296.3	$M = -2.4 + 1.74\log_{10}T$	0.0020
	1.8×10^5	35.8	15.4 - 40292	$M = -2.2 + 1.39\log_{10}T$	0.0131
30°C	5.4×10^7	2.1	0.3 - 3.2	$M = -0.4 + 1.21\log_{10}T$	0.0038
	9.9×10^6	11.8	$7.4 - 1.3 \times 10_{12}$	$M = -0.9 + 0.84\log_{10}T$	0.0431
	6.4×10^5	14.2	9.0 - 189.0	$M = -1.4 + 1.19\log_{10}T$	0.0067
	1.8×10^5	35.9	$14.0 - 2.1 \times 10_{12}$	$M = -1.6 + 1.00\log_{10}T$	0.0348

* Blastospores/ml

** Mortality rate (M) as a function of time (T)

at 20°C, but not at 30°C. Mortality rates with the lowest concentration were not significantly different from those of the next lowest concentration. No mortality was detected in the control treatment.

The LC_{50} values were not significantly different between the 2 experimental temperatures. The LC_{50} of PFR97 at 20°C was 6.1×10^6 blastospores/ml (95% fiducial limits = 4.0×10^6 - 9.7×10^6). The probit equation at this temperature was $Y = -1.3 + 0.75\log_{10}B$ ($P < 0.0001$), where Y is the estimated mortality rate and B is concentration of blastospores/ml. The LC_{50} at 30°C was 5.3×10^6 blastospores/ml (95% fiducial limits = 2.8×10^6 - 11.8×10^6). The probit equation at this temperature was $Y = -0.8 + 0.44\log_{10}B$ ($P < 0.0001$).

The LT_{50} at 20°C varied from 3.3 d at the highest concentration to 35.8 d at the lowest concentration (Table 1). At this temperature, the LT_{50} at the highest concentration was significantly higher than the LT_{50} at the next highest concentration, which was significantly higher than the LT_{50} of the 2 lowest concentrations. The LT_{50} at 30°C varied from 2.1 d at the highest concentration to 35.9 d at the lowest concentration (Table 1). At this temperature, the LT_{50} at the highest concentration was significantly higher than the LT_{50} at the next highest concentration, which was not significantly higher than the LT_{50} of the 2 lowest concentrations. The LT_{50} values for the highest concentration were similar between the 2 experimental temperatures.

The percentage of first instars molting to second instar decreased with increasing blastospore concentration (Table 2). However, there was an interaction between

Table 2. Percentage molting of *Aulacaspis yasumatsui* from first to second instar after exposure to 4 blastospore concentrations of *Isaria fumosorosea* and incubated at 2 temperatures 8 d after treatment application.

Temperature	Blastospores/ml	% molting*
30°C		
	5.35×10^7	27 ± 4.2 a
	9.90×10^6	55 ± 5.0 b
	6.38×10^5	58 ± 4.7 b
	1.70×10^5	59 ± 7.7 b
	Control	93 ± 2.6 c
20°C		
	5.35×10^7	13 ± 3
	9.90×10^6	27 ± 7.75
	6.38×10^5	42 ± 7.9
	1.70×10^5	46 ± 10.5
	Control	48 ± 9.6

* Means with the same letters are not statistically different ($P > 0.05$, Student-Newman-Keuls). Analysis of variance detected differences among treatments at 20°C, but Student-Newman-Keuls test was unable to separate the means.

blastospore concentration and temperature. The lowest percentage of molting was obtained with the highest concentration at 20°C, but it was not significantly ($F = 4.28$; $df = 1$; $P = 0.0532$) different from the percentage of molting with the same concentration at 30°C. This may be due to the combination of greater mortality at the highest blastospore concentration (this study) and the suboptimal lower temperature for scale development (Cave et al. 2009b).

The radial growth of *I. fumosorosea* on PDA at 15 d at 20 (18.8 mm ± 0.2) and 30°C (23.1 ± 0.7) were significantly different ($F = 30.99$; $df = 1$; $P < 0.0001$) (Fig. 2). The following linear models show the relationship of radial growth with time after inoculation.

Linear model for 20°C $Y = 0.68089 + 1.14243X$ $R^2 = 0.95$

Linear model for 30°C $Y = 1.31583 + 1.45097X$ $R^2 = 0.875$

where:

Y = radius (mm) of fungus

X = days after inoculation

This in vitro technique theoretically indicates which temperature would allow for the fastest rate of germination and subsequent growth either on the leaf surface or on the insect cuticle. Results from this study indicate that the saprophytic phase of *I. fumosorosea* that grew fastest at the higher temperature in vitro also had the fastest rate of infection and death of the scale insects. Positive correlation between the optimum temperature for fungal growth in vitro and infection rate in vivo has been reported by other researchers (Maniania and Fargues 1992, Yeo et al. 2003, Taylor and Khan 2010). Our results confirmed this hypothesis under constant laboratory conditions; however, this needs to be tested under field conditions which can vary considerably.

This is the first report of *I. fumosorosea* infecting an armored scale insect. Results presented here are the first evidence of *I. fumosorosea* as a potential biological control agent for suppressing populations of the cycad aulacaspis scale. The scale matures quickly at 30°C, but *I. fumosorosea* provided a high mortality rate at this temperature,

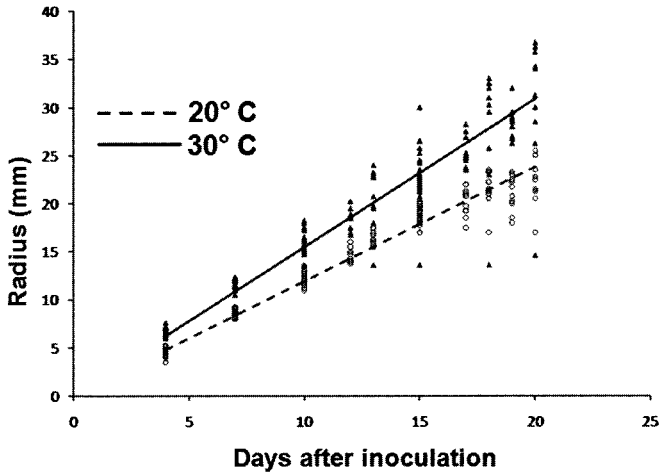

Fig. 2. Radial growth of *Isaria fumosorosea* on PDA at 2 temperatures (open circles = 20°C; closed triangles = 30°C).

which indicates that *I. fumosorosea* may be used under the conditions by which the scale optimally develops. If the temperature is near 20°C, the cycad aulacaspis scale has problems molting (Cave et al. 2009b), to which an application of fungus would result in fewer first instars molting to second instar. However, the pathogen's efficacy is lower at 20°C compared with 30°C.

Because *I. fumosorosea* is a highly infective fungus, it may have negative effects on nontarget organisms. This suggests that the interaction between the fungus and other natural enemies of the cycad aulacaspis scale warrants further study. The interaction between predators or parasitoids and *I. fumosorosea* has been shown to be benign, and the fungus is compatible with beneficial organisms (Sterk et al. 1995a, b, Avery et al. 2008). If there is no antagonism between the fungus and predators and parasitoids of the cycad aulacaspis scale, the fungus may be applied to the initial stages of an infestation so as to render the pest populations more manageable for predators and parasitoids. The fungus should be applied against first-instar cycad aulacaspis scales because the waxy cover that the scale develops when it molts to second instar is likely a formidable barrier to penetration by germ tubes.

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