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Effect of Fire Ant (*Solenopsis invicta*) Venom Alkaloids on the in Vitro Germination and Development of Selected Entomogenous Fungi

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Solenopsis invicta venom alkaloids were evaluated for antimycotic activity against *Beauveria bassiana* isolates, AF-4 and 447, *Metarhizium anisopliae*, and *Paecilomyces fumosoroseus*. Conidial germination of all isolates after 24 hr decreased with an increase in venom alkaloid concentration in both solid and liquid culture. Percentage germination of all isolates except *P. fumosoroseus* increased within 48 hr, indicating that the venom alkaloids act fungistatically on *B. bassiana* and *M. anisopliae*. Both *P. fumosoroseus* and *M. anisopliae* conidia were more sensitive to venom alkaloids than either of the *B. bassiana* isolates. *P. fumosoroseus* conidia were the most sensitive to venom alkaloids, since complete inhibition occurred at all concentrations >0.41 μg of alkaloid/cm^2. *B. bassiana* (AF-4) was more tolerant to alkaloids than the 447 isolate with significantly higher ($P = 0.05$) germination rates at 10, 20, and 30 μg of alkaloid/cm^2. Vegetative growth of the *B. bassiana* isolates was also modified by the alkaloids in liquid culture. The hyphal body phase was induced at concentrations above 5 μg/cm^2 after 48 hr. No hyphal bodies were observed in media containing *M. anisopliae* or *P. fumosoroseus* at any alkaloid concentration.

KEY WORDS: *Solenopsis invicta; Beauveria bassiana; Metarhizium anisopliae; Paecilomyces fumosoroseus;* piperidene alkaloids; venom alkaloids; antifungal.

INTRODUCTION

Since its accidental importation from South America, the fire ant, *Solenopsis invicta*, has attained pest status in the southeastern United States (Lofgren et al., 1975). Numerous strategies for controlling this pest have been pursued using various control methodologies (Lofgren et al., 1975). Presently, the entomogenous fungus *Beauveria bassiana* is being evaluated as a potential microbial control agent of this ant (Alves et al., 1988).

Potential disease-limiting mechanisms are found in the fire ant mound. For example, fire ants, like other social insects, remove debris, including ant cadavers, from their nest within 24 hr (Wilson, 1971). Sporulation of entomopathogenic fungi typically occurs 2 to 3 days after host death. Fungus-infected cadavers are removed before sporulation, thereby limiting dissemination of conidia within the mound. In preliminary assays where laboratory fire ant colonies were inoculated with *B. bassiana*, worker ants removed ant cadavers to one corner of the assay unit prior to fungal sporulation. Furthermore, the cadavers were covered with sand by fire ant workers, which limited conidial dispersion.

In addition to behavioral responses, fire ants produce venom alkaloids that have significant antimicrobial activity in in vitro laboratory assays (Blum et al., 1958; Jouvenaz et al., 1972; Blum, 1988). The venom alkaloids are thought to have evolved in part as disinfectants of the ant cuticle and mound soil (Obin and Vander Meer, 1985; Blum, 1988). Venom can be dispersed into the mound as an aerosol via venom release during the rapid vibration of the sting,

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gaster flagging, or a worker can fling venom droplets at a perceived intruder (Obin and Vander Meer, 1985). The actual amount of venom alkaloids released into the mound environment by this mechanism is unknown. However, it is evident that large amounts of venom alkaloids are available per colony, since each worker's venom sac has ca. 10–15 μg of alkaloids (unpubl. data). The potential presence of high concentrations of antimicrobial alkaloids in mound soil and on the ants makes them likely candidates as inhibitors of *B. bassiana* and, therefore, could have a detrimental impact on the success of the fungus as a biological control agent.

The objectives of this study were, therefore, to quantify the fungistatic effects of *S. invicta* venom alkaloids on entomopathogenic fungi in both liquid and solid culture and to determine the effect of venom alkaloids on the morphological development of *B. bassiana*. The results should have broad applicability to other potential fire ant microbial control agents since the antimicrobial activity of the venom affects several pathogens (Blum, 1988).

MATERIALS AND METHODS

Culturing Fungi and Determining Conidial Viability

A monosporal isolate of *B. bassiana* (strain AF-4), originally isolated from *Artipus floridanus* (Coleoptera: Curculionidae), was obtained from the culture collection of the Citrus Research and Education Center, University of Florida. A monosporal isolate of *B. bassiana* (strain 447) isolated from *S. invicta* was obtained from Dr. Jerry L. Stimac (Entomology and Nematology Department, University of Florida, Gainesville). Monosporal isolates of *Metarhizium anisopliae* from the mole cricket (*Scapteriscus vicinus*) and *Paecilomyces fumosoroseus* from an unidentified host insect were obtained from the fungal culture collection of the Entomology and Nematology Department, University of Florida. Pure cultures of all fungi were grown on Sabouraud's dextrose agar in Petri dishes (15 × 100 mm) at 27°C. After 10 days, conidia were harvested by scraping the plates with a sterile microscope slide. Conidia were then placed in 10 ml of sterile deionized water (SDW), vortexed 1 min, and washed twice in SDW using centrifugation (5000*g*, 5 min) to remove agar residue. Conidial concentration was determined using a Brite Line hemacytometer.

Conidial germination was evaluated at the beginning of each experiment by inoculating 100 μl of Sabouraud's dextrose broth (SDB) with 100 μl of the conidial preparation (5×10^4 conidia/ml) and incubating the broth for 24 hr at 27°C. Conidial germination, defined by the presence of a germ tube, was quantified by counting ca. 100 spores in each of four replicates.

Isolation of Venom Alkaloids from Fire Ants

Venom alkaloids were obtained from laboratory-colony fire ant workers collected and maintained according to the procedures of Banks et al. (1981). Large worker ants were killed in either methanol or hexane and the venom sac was removed by tearing the last two dorsal abdominal sclerites, grasping the sting apparatus with forceps, and pulling the venom pouch free of the abdomen. The venom sac was separated from the sting by dissection and placed in a vial containing either methanol or hexane and the venom was released by crushing the sac with forceps.

Quantification of Venom Alkaloids via Gas Chromatography

Venom alkaloids were quantified using either a Varian 3700 gas chromatograph (Varian Associates, Sunnyvale, California) equipped with a 30-m DB-1 column or a Tracor 540 gas chromatograph (Tracor Instruments Austin, Austin, Texas) equipped with a 15-m DB-5 column. Both GCs were equipped with flame ionization detectors.

Chromatographs were programmed from 150°C to 275°C or 285°C at 5°C/min and data collected using either a Varian Vista 401 data processor or a Perkin-Elmer LCI-100 integrator. Six microliters of a 0.1% hexane solution of tetracosane was added to each alkaloid preparation as an internal standard prior to analysis. Sample quantification was determined by dividing the total alkaloid peak area by the peak area of the internal standard and multiplying by the amount of standard (in μg) added to the sample.

Effect of Venom Alkaloids Applied to Solid and Liquid Substrates on Conidial Germination and Growth

The effect of venom alkaloids on the germination and development of *B. bassiana* conidia on solid substrates was quantified on Milicu (MC) agar (0.36 g of KH_2PO_4, 1.05 g of Na_2HPO_4, 0.6 g of $MgSO_4*7H_2O$, 1 g of KCl, 10 g of glucose, 0.7 g of NH_4NO_3, 5 g of yeast extract, 20 g of agar per liter) prepared with double distilled deionized water. Tenfold serial hexane dilutions of alkaloids were filter sterilized using an HPLC solvent syringe filter (0.45 μm, MSI, Honeoye Falls, New York). A template, constructed by cutting the bottom from a 96-well microtiter plate, was surface sterilized with ethanol and used to hold 10 μl of the venom alkaloid dilutions within a 0.332-cm^2 area. Six concentrations (0, 3.8, 7.6, 15.1, 30.1, and 60.2 μg/cm^2) were evaluated. The hexane was evaporated from the agar surface and the plates were sprayed with an aqueous suspension containing 5×10^6 viable *B. bassiana* conidia per milliliter. Plates were incubated at 27°C. Conidial germination was quantified after 24 and 48 hr by counting ca. 100 spores per replicate for each treatment concentration and identifying the presence or absence of a germ tube using phase-contrast microscopy (400×). Each treatment was replicated four times.

The effect of venom alkaloids on the germination and growth of *B. bassiana* in liquid culture was quantified using the solid media methodology described above with the following exception. Ten-microliter aliquots of the venom alkaloid dilutions were spot plated in individual wells of a 96-well microtiter plate. Seventy-two venom alkaloid concentrations ranging from 0 to 39.6 μg/cm^2 were tested and germination quantified for 105 observations. Concentrations tested and the number of replicates per concentration depended upon the amount of venom alkaloids recovered from the ants and therefore varied from experiment to experiment. Alkaloid concentration per unit area was based on the surface area of the well bottom (0.332 cm^2). *B. bassiana* conidia (5×10^4 conidia/ml) were suspended in SDB and added to the treated wells in 100-μl aliquots. Plates were incubated at 27°C and observed after 24 and 48 hr for conidial germination by inverted phase microscopy (400×).

The effect of different concentrations of venom alkaloids on in vitro conidial germination of AF-4 and 447 *B. bassiana* isolates, *M. anisopliae* and *P. fumosoroseus,* in liquid culture was compared using the previously described protocol. Eight concentrations of venom alkaloids ranging from 0 to 29.7 μg/cm^2 were tested. Each concentration was replicated four times.

Statistical Analyses

Data were analyzed using the linear regression and general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, North Carolina). Mean separation analysis was conducted using Duncan's (1951) multiple range test.

RESULTS

Effect of Venom Alkaloids on Conidial Germination and Growth

Percentage conidial germination of *B. bassiana* (AF-4) on MC agar after 24 hr decreased with an increase in venom alkaloid concentration (Fig. 1a) according to the

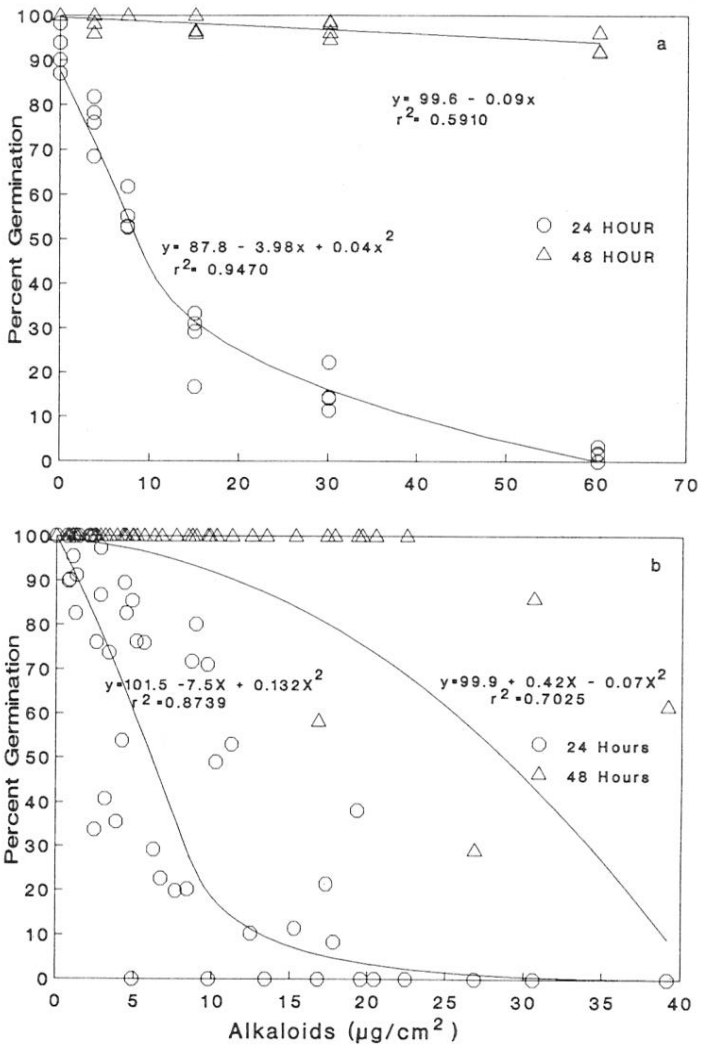

FIG. 1. Percentage conidial germination of *Beauveria bassiana* (AF-4) on (a) MC agar surfaces and (b) in liquid culture in microtiter wells treated with different concentrations of *Solenopsis invicta* venom alkaloids at 24 and 48 hr.

quadratic relationship, $y = 87.76 - 3.98X + 0.04X^2$ ($r^2 = 0.9470$; $P = 0.0001$). At the highest concentration of alkaloids (60 µg/cm^2), less than 5% germination occurred after 24 hr. Concentrations of >10 µg of alkaloids/cm^2 caused over 50% inhibition of conidial germination.

After 48 hr, venom alkaloids had a minimal effect on conidial germination with <10% inhibition observed at any concentration, indicating that the alkaloids have only a temporary fungistatic effect on the germination process. Percentage conidial germination was poorly defined, but strongly associated with alkaloid concentration ($r^2 = 0.5910$; $P = 0.0001$).

Percentage conidial germination in liquid culture, where the microtiter wells were precoated with venom alkaloids, decreased with an increasing alkaloid concentration after 24 hr (Fig. 1b) according to the quadratic relationship, $y = 101.5 - 7.5X + 0.132X^2$ ($r^2 = 0.8739$; $P = 0.0001$). Alkaloid concentrations >20 µg/cm^2 caused complete inhibition of germination at 24 hr. As in the solid media assays, percentage ger-

mination increased within 48 hr, confirming the temporary fungistatic effect of venom alkaloids on germination of AF-4. Percentage conidial germination at 48 hr was highly correlated ($r^2 = 0.7025$) to the concentration of alkaloids according to the quadratic relationship, $y = 99.9 + 0.42X - 0.07X^2$. Concentrations <25 μg of alkaloids/cm^2 did not inhibit conidial germination at 48 hr.

Conidial germination was inhibited by venom alkaloids at 24 hr (Fig. 2a) while conidia in the control produced normal germ tubes (Fig. 2b). At 48 hr, concentrations of venom alkaloids higher than 4 μg/cm^2 induced hyphal body formation in *B. bassiana* in liquid culture. Venom alkaloid-treated conidia developed hyphal bodies and mycelia (Fig. 2c) while conidia in the control produced mainly vegetative mycelia (Fig. 2d). The hyphal body stage of development was not induced on solid agar surfaces treated with any concentration of venom alkaloids.

For *B. bassiana* isolates 447 and AF-4, *M. anisopliae*, and *P. fumosoroseus*, percentage conidial germination decreased with increasing concentrations of venom alkaloids after 24 hr. Concentrations of alkaloids >20 μg/cm^2 gave a total inhibition of conidial germination of all fungal species and isolates (Fig. 3a). Both *P. fumosoroseus* and *M. anisopliae* conidia were more sensitive to venom alkaloids than either of the *B. bassiana* isolates. *P. fumosoroseus* conidia appeared the most sensitive to venom alkaloids with complete inhibition occurring at all concentrations >0.41 μg of alkaloid/cm^2. *M. anisopliae* was more sen-

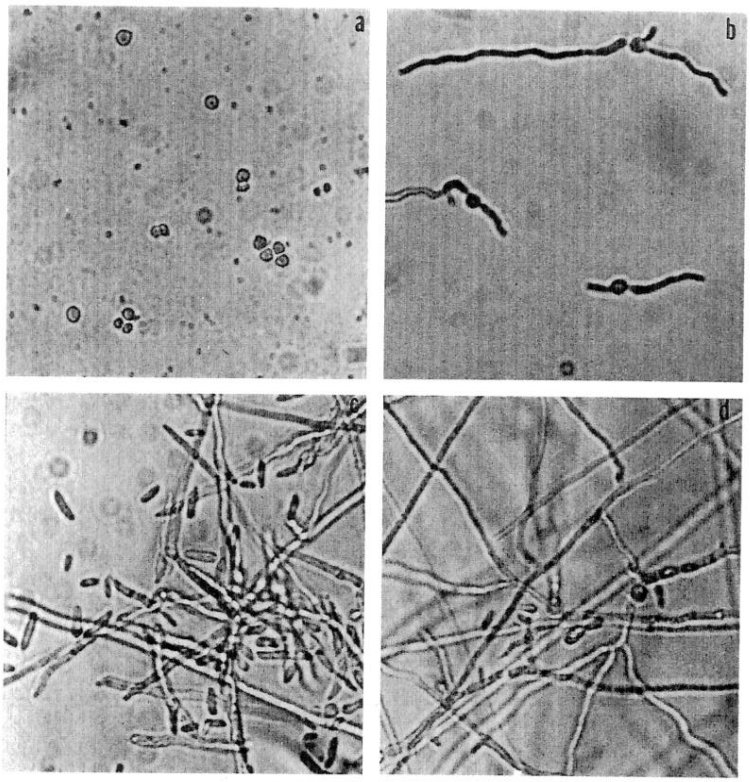

FIG. 2. Germination and development of *Beauveria bassiana* (AF-4) in Sabouraud's dextrose broth culture in microtiter wells with (a) complete inhibition of germination in *Solenopsis invicta* venom alkaloid-coated wells (20 μg/cm^2), (b) normal germ tube development in control wells after 24 hr, (c) induction of hyphal bodies in wells coated with *S. invicta* venom alkaloids (20 μg/cm^2), and (d) normal mycelial growth in control wells after 48 hr incubation at 27°C. (400×.)

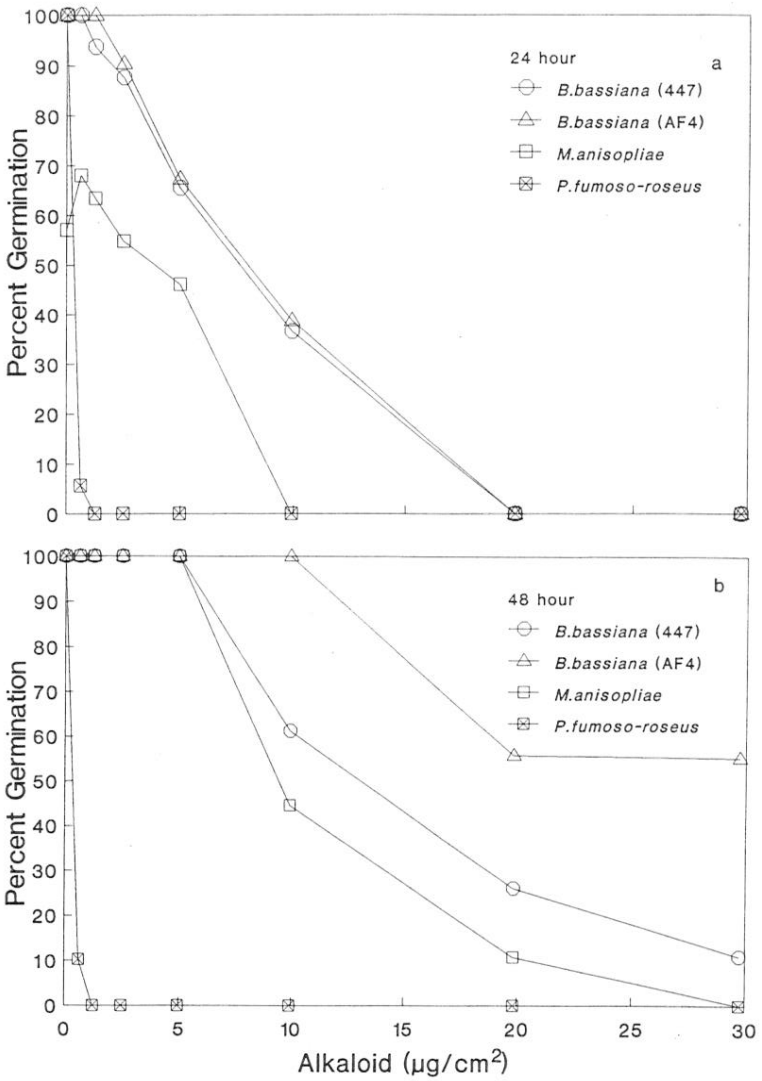

FIG. 3. Comparison of percentage germination of *Paecilomyces fumosoroseus, Metarhizium anisopliae, Beauveria bassiana* strain 447, and *B. bassiana* strain AF4 in liquid culture in microtiter wells coated with different concentrations of *Solenopsis invicta* venom alkaloids after (a) 24 hr and (b) 48 hr incubation at 27°C.

sitive than either of the *B. bassiana* strains, with concentrations >10 µg of alkaloid/cm^2 causing complete inhibition. Percentage conidial germination was similar for both AF-4 and 447 isolates of *B. bassiana* at 24 hr.

After 48 hr, only *P. fumosoroseus* germination rates did not increase compared to 24-hr germination rates. *P. fumosoroseus* conidia were highly sensitive to the venom alkaloids, with no germination at concentrations >0.41 µg of alkaloids/cm^2 (Fig. 3b). Total inhibition of *M. anisopliae* occurred at an alkaloid concentration of 30 µg/cm^2 compared to 90% inhibition of the 447 isolate at the rate. *B. bassiana* (AF-4) was more tolerant to alkaloids than 447 with significantly higher ($P = 0.05$) germination at 10, 20, and 30 µg of alkaloid/cm^2. Recovery of three of the four fungal isolates from the inhibitory effects of venom alkaloids by 48

hr is consistent with the fungistatic mode of activity observed in solid and liquid media assays with *B. bassiana* (Figs. 1a,b).

The hyphal body stage of both AF-4 and 447 was observed at concentrations above 5 $\mu g/cm^2$ after 48 hr. No hyphal bodies were observed in media containing *M. anisopliae* or *P. fumosoroseus* at any alkaloid concentration.

DISCUSSION

The fungicidal activity of venom alkaloids has recently been reported by Blum (1988) where individual or mixed synthetic venom alkaloids incorporated into solid medium (0.8 ng/μl) inhibited >90% of colony growth of eight fungal species, including two species, *Zygorhyncus vuilleminii* and *Mucor* sp., isolated from fire ant larvae. A *Penicillium* sp., also isolated from the larvae, was only slightly inhibited (<30%) by the alkaloid preparations after 53 days. In the present study, ant-derived venom alkaloid concentrations over 80-fold higher (20 $\mu g/cm^2$ = 66.4 ng/μl) than those used by Blum (1988) appear to be only fungistatic to *B. bassiana* and *M. anisopliae,* delaying germination for 24 hr. Only *P. fumosoroseus* remained inhibited by relatively low alkaloid concentrations at 48 hr.

The increased sensitivity of *P. fumosoroseus* to venom alkaloids compared to *B. bassiana* and *M. anisopliae* suggests that differences in venom alkaloid activity observed between this study and that of Blum (1988) are due to differential sensitivity of the fungal isolates to alkaloids. *B. bassiana* appears to be relatively tolerant to other alkaloids as well. Costa and Gaugler (1989) report that the glycoalkaloids, tomatine and solanine, have only moderate effects on the growth of the fungus compared to their effects on other nonentomopathogenic fungi. The delayed conidial germination response observed in *B. bassiana,* where venom alkaloids are applied to media, supports the suggestion of Costa and Gaugler (1989) of an induced detoxification system to deal with alkaloids in this fungus. Further research should be conducted on the mechanisms involved in fungal resistance to the alkaloids.

B. bassiana is a dimorphic fungus and as such has the ability to change growth habits between mycelia and hyphal bodies (yeast-like cells) (in Aoki and Yanase, 1970). Hyphal bodies normally occur as the vegetative stage of the fungus in the hemolymph of infected insects but also occur in artificial culture. Induction of the hyphal body stage of dimorphic fungi is thought to be dependent upon temperature, nutrition, or a combination of temperature and nutrition (reviewed by Garraway and Evans, 1984). Hyphal bodies apparently result from pattern alteration of cell wall synthesis which leads to fungal cell budding instead of germ tube elongation. *B. bassiana*'s dimorphic growth is also affected by nutrition. For example, Aoki and Yanase (1970) altered the number, size, and shape of *B. bassiana* hyphal bodies in liquid culture by the addition of different amino acids. Thomas et al. (1987) report that developmental stages of *B. bassiana* in submerged culture can be altered by manipulating the amounts of phosphate and nitrate in the liquid media. Our study indicates that the hyphal body stage of this fungus can also be induced at 48 hr by the addition of an antagonist, specifically venom alkaloids, to liquid media.

Following germination of conidia in alkaloid-treated media, there was no indication of cellular lysis of either hyphal bodies or mycelia. This suggests that the effect of the venom alkaloids on the fungus is restricted to conidial germination. Venom alkaloids appear to interfere with cell wall synthesis during germination since hyphal bodies are produced instead of elongated germ tubes. However, the exact mechanism involved cannot be deduced from the data collected in this study.

In conclusion, fire ant venom alkaloids act fungistatically on *B. bassiana* AF-4 and 447 isolates, *M. anisopliae,* and *P. fumoso-*

roseus by delaying in vitro germination. The alkaloids also modify the development of the *B. bassiana* isolates resulting in hyphal body formation. These fungal responses were elicited by relatively high alkaloid concentrations. Larval fire ants have only 1 ng of alkaloid present on the cuticle (Obin and Vander Meer, 1985), which is well below the microgram quantities needed in this study to inhibit the fungi in vitro. However, the concentrations of alkaloids present on the adult cuticle and in mound soil may be significantly higher. Studies are being conducted presently to determine the concentrations of venom alkaloids present in mound soil, as well as the amount of alkaloids released by fire ants in response to treatment of mound soil by *B. bassiana*. This information will be useful in determining the actual impact of venom alkaloids on disease development in the mound.

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