Efficacy of Antifungal Agents to Control *Aspergillus niger* Contamination in an Artificial Diet for *Lygus hesperus* Knight (Heteroptera: Miridae)[1]

Janet Alverson

Biological Control and Mass Rearing Research Unit, USDA, Agricultural Research Service, Mississippi State, MS 39762 USA

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Abstract The ability of four antifungal agents to suppress growth of *Aspergillus niger* (USDA, ARS Robert T. Gast Rearing Laboratory, Mississippi State, MS isolate) in an artificial diet for *Lygus hesperus* Knight (Heteroptera: Miridae) was tested by inoculating a standard number of conidial spores into artificial diet with different antifungal agents added and determining the amount of ensuing microbial growth. The effects on biological fitness of *L. hesperus* also were measured. Measured characteristics of biological fitness included total number of surviving adults, mean biomass (dry weight) accumulated per cage over the total treatment period, egg production, time to adult emergence, and time to the beginning of egg laying. Benzoic acid, high concentrations of formalin, and a high concentration of sorbic acid suppressed the growth of *A. niger*. Propionic acid and low concentrations of sorbic acid and formalin were less effective at suppressing *A. niger* growth. Biological fitness was negatively affected in insects reared on diets containing high levels of *A. niger* growth and in diets with high concentrations of formalin. This study demonstrates how the antifungals that are added to the artificial diet of *L. hesperus* to control contamination by *A. niger* must be chosen carefully with consideration not only of their effectiveness at controlling contamination, but also to the tolerance level of the insect to the chemical compound.

Key Words Plant bugs, *Aspergillus niger,* artificial diet, antifungal, mass rearing

Western tarnished plant bugs, *Lygus hesperus* Knight (Heteroptera: Miridae), are major pests in many cropping systems in North America. They are reared at the USDA, ARS Robert T. Gast Rearing Laboratory to provide insects for entomologists to conduct research on biological control, physiology and behavior of these insects. The artificial diet used to rear *L. hesperus* is susceptible to contamination by microorganisms, especially the fungus, *Aspergillus niger* (USDA, ARS Robert T. Gast Rearing Laboratory, Mississippi State, MS isolate). Therefore, sterile techniques must be strictly adhered to in diet preparation and handling. It is common for antimicrobial agents to be added to artificial diets to aid in the prevention of microbial contamination. The diet (referred to as NI diet) presently used to rear *L. hesperus* (Cohen 2000) contains formalin and propionic acid for mold control. Many antimicrobial agents, however, have been shown to have detrimental effects on the insects being reared

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Offprint requests may be made electronically (janeta@vetmed.wsu.edu). Current address: USDA, ARS, ADRU, P.O. Box 646330, Pullman, WA 99164-6630.

(Ouye 1962, Kishaba et al. 1968, Bass and Barnes 1969, Toba et al. 1969, Singh and House 1970a, b, Alverson and Cohen 2002). The detrimental effects of microorganisms on insect health also have been well documented (Beard and Walton 1969, Howell 1971, Singh and Bucher 1971, Toscano and Reeves 1973, Thompson et al. 1977, McLaughlin and Sikorowski 1978, Thompson and Sikorowski 1978, 1982, MacGown and Sikorowski 1980, Wiygul and Sikorowski 1981, 1986, 1991, Sikorowski et al. 1992). Therefore, care must be taken in the selection of an antimicrobial dietary additive to ensure that it is not only effective against microbial dietary contaminants, but also nontoxic to the insect being reared.

Previous work in this laboratory determined the effects of five antifungal agents on biological fitness of *L. hesperus* (Alverson and Cohen 2002). Biological fitness characteristics measured included total number of surviving adults, mean biomass (dry weight) accumulated per cage over the total treatment period, egg production, time to adult emergence, and time to the beginning of egg laying. Methyl paraben was the most toxic to *L. hesperus* of the antimicrobials tested, so much so that it was dismissed as a possible dietary additive for antimicrobial control for this insect and was not tested in the present experiment. Formalin also was found to be somewhat toxic to the insects, especially in terms of the number of eggs produced per female. Benzoic acid, propionic acid, and sorbic acid showed very low levels of toxicity, even at high concentrations. Thus, selection of one antifungal agent from among these for use in rearing *L. hesperus* will depend on their relative effectiveness against the microbe(s) to be targeted, their relative costs, and the convenience of their use.

The effects of *A. niger* on biological fitness of *L. hesperus* also were tested (Alverson 2002). Insects reared on artificial diet inoculated with *A. niger* without antifungals showed a high mortality of nymphs, a decrease in mean biomass, delayed development time, and a decrease in egg production.

The purpose of the present study was to determine the efficacy of these antifungal agents in controlling *A. niger* contamination in an artificial diet for *L. hesperus.* The effects of the treatment diets on the development, survival, and fecundity of *L. hesperus* as ascertained by the measurements of biological fitness listed above also were determined. It was hypothesized that the insects reared on diets with the most efficacious antifungal additives, and thus the least amount of *A. niger* contamination, would exhibit the fewest negative effects on biological fitness. Because formalin was found to be somewhat toxic when used in artificial diets for *L. hesperus,* it was anticipated that formalin would be somewhat toxic to *L. hesperus* in this study as well.

Materials and Methods

Insects. *Lygus hesperus* used in these studies were derived from a colony from Biotactics, Inc. (Riverside, CA), reared on the "C diet" at the Gast Rearing Laboratory for 1.5 yrs (Cohen, unpubl. data), and then on the new NI diet (Cohen 2000) for 29 generations (as of January 2001) previous to this experiment. The NI diet, used in these experiments, was a combination of whole eggs and egg yolks, soy flour, wheat germ, lima bean meal, yeast, and vitamins (Cohen 2000). *Lygus hesperus* adults were placed in the Mississippi State University Entomology Museum to serve as voucher specimens for this study.

Experimental protocol. Cages used for all of the insects' life stages were Rubbermaid® 1.7-L Servin'Saver™ (Wooster, OH) rectangular plastic storage boxes. Cages were topped with organdy cloth (0.4-mm mesh) held tight by the box's snap-on

top that had a 15.7 × 26.5 cm opening cut into it. The organdy cloth was changed to a 1.0-mm mesh fiberglass screen when the nymphs were large enough (approximately third instar) to be contained by the larger mesh. Growth chamber (Powers Scientific model DS33SD, Pipersville, PA) conditions were a light-dark cycle of 16:8 h, temperature of 27°C (±1.0°C), and relative humidity of 60% (±2%). Cages were placed on wire racks allowing air circulation and light to reach each cage. The testing colony was treated according to the recommendations of Debolt and Patana (1985) and Cohen (2000). Original gel oviposition packets used to begin the test were placed intact, or cut to achieve the correct egg count, into cages. To reduce cannibalism, egg packets were placed inside cages with shredded paper (0.6 × 28.4 cm) rather than loosely-wadded paper towels. Feeding packets and gel packets for oviposition were made from 20.8 × 10 cm strips of Parafilm® (Pechiney Plastic Packaging, Menasha, WI) folded and sealed with a heat sealer (Deni Freshlock Turbo II, Keystone Manufacturing, Co., Buffalo, NY) along two sides to create a 10.4 × 10 cm packet with an open top for filling. The first feeding packets provided to newly-eclosed nymphs were stretched by hand to facilitate feeding, and one stretched packet was placed inside the cage as well as on top of the organdy. Feeding packets on the tops of the cages were replaced every 48 h and stretched until the nymphs were second instars (approximately post oviposition day 9). The feeding packets inside the cages and the original egg packets were removed between days 10 and 12, and the organdy was replaced by larger mesh screening, as nymphal size allowed. Once adults emerged, a 2% Gelcarin® (FMC-Food Ingredients Division, Rockland, ME) gel packet was placed on top of the cage for oviposition and changed daily. Previous work (Cohen 2000) indicated that a higher percentage gel than formerly used (Debolt and Patana 1985) increased egg hatch.

Antifungals and diet. The diets consisted of NI ingredients prepared as previously described (Cohen 2000) except the antimicrobial agents formalin and propionic acid were omitted. All diet preparations were conducted in a laminar flow hood with the exception of formalin which was handled within a chemical fume hood. The warm diet (approximately 45°C) was poured into a sterile glass beaker, and the antifungal agents were added as follows: no antifungal (control); benzoic acid (4000, 3000, and 2000 ppm); formalin (37% formaldehyde at 1500, 1000, and 500 ppm); propionic acid (1600, 1200, and 800 ppm); and sorbic acid (1600, 1200, and 800 ppm). The concentrations chosen for testing were based on previous use of these antifungals against *A. niger* (Funke 1983). All chemicals were purchased from Sigma (St. Louis, MO), except the formalin which was purchased from Fisher Scientific (Pittsburgh, PA). Sterile deionized water was the solvent for the benzoic acid, propionic acid, and sorbic acid. The pH of the test diets was 5.5 ± 0.2. Approximately 20 ml of diet was dispensed into each diet packet, and the tops were heat sealed.

The *A. niger* was isolated at the Gast Rearing Laboratory and, therefore, represents a microorganism that is a known contaminant of artificial diet at this laboratory. *Aspergillus niger* conidia were harvested after 5 to 8 days of growth at 27°C on Sabouraud dextrose agar (SDA) (Difco, BD Diagnostics Systems, Sparks, MD). They were harvested by lightly scraping the surface of the mold with a sterile cotton swab dampened in sterile water. The black conidia were rinsed off the swab into a sterile tube containing 2 ml of sterile water. The diet packets for the fungal challenge were inoculated with a working solution of *A. niger* (2000 spores in 0.3 ml) via syringe just prior to heat sealing the tops. This resulted in a final dilution of 100 spores per ml of

diet. This inoculum dose was chosen because it is within the range likely to occur when insect diets are contaminated by chance (Singh and Bucher 1971).

Diet packets on top of the cages were changed every 48 h and mold growth was visually scored as follows: heavy growth (+++) such as found in the control diet with no antifungals added; moderate growth (++) somewhat suppressed as compared to the control; light growth (+) suppressed compared to the control; and no growth (0). *Aspergillus niger* growth was confirmed by plating the diet on SDA and incubating the plates at 27°C.

Experimental design. A randomized complete block design with 13 treatments was used to separate differences in effectiveness in reducing *A. niger* growth. Four antifungal agents were tested at three concentrations each, plus a control with no antifungal chemicals added. All diets were inoculated with a standard inoculum of *A. niger* as described above. Three replications were performed. Blocks consisted of replication in time.

To begin all tests, rearing units consisting of individual cages, described above, were set up and inoculated with a Parafilm® gel oviposition packet containing 200 eggs. All test eggs for each replication were oviposited within a 2 h period. The following parameters were measured for each treatment group: (1) total number of surviving adults; (2) the insect biomass (dry weight) accumulated per cage over the total treatment period, including adults, deceased nymphs, and exuviae; (3) time to adult emergence; (4) time to the beginning of egg laying; and (5) number of eggs produced by each cage of adults per day for 5 d. The experiment was terminated 26 d after hatch of the test insects (Debolt and Patana 1985). Surviving adults were collected and killed by freezing at –20°C for 4 to 6 h. Dead nymphs and exuviae also were collected and frozen. Dry weights were obtained after drying both collections at 70°C for 48 h. The collections were combined for each cage to determine a total biomass. Hatch rates from the original egg packs used to begin the experiment and from the F1 egg packs were calculated based on counts of microscopically observed opened opercula and empty eggs. Data were analyzed using analysis of variance (ANOVA), and treatment means were compared by Fisher's protected least significant difference (LSD) (SAS Institute 2000).

Results and Discussion

Benzoic acid (2000, 3000, and 4000 ppm), higher concentrations of formalin (1000 and 1500 ppm), and a high concentration of sorbic acid (1600 ppm) suppressed growth of *A. niger* (Table 1). Propionic acid (800, 1200, and 1600 ppm), a low concentration of formalin (500 ppm), and lower concentrations of sorbic acid (800 and 1200 ppm) were less effective at suppressing *A. niger* growth.

Biological fitness of *L. hesperus* was negatively affected when reared on several of the treatment diets (Table 2). Survival to adults was lowest among the insects reared on the control diet with no antifungal agent and on the diets containing formalin or propionic acid. Of the diets tested, the control diet and the diets containing the two highest concentrations of formalin, the two lowest concentrations of propionic acid, and the lowest concentration of sorbic acid yielded the least total insect biomass. The lowest egg production was observed for the insects reared on the control diet and the diet containing the lowest concentration of propionic acid. Insects reared on diets containing the two higher concentrations of formalin and the medium concentration of propionic acid also had low egg production, although not as low as the insects reared

Table 1. Effect of antifungals on *A. niger* growth in NI artificial diet used for laboratory-rearing of *L. hesperus*

Antifungal	Concentration	*A. niger* growth*
Control	–	+++
Benzoic Acid	2000 ppm	0
	3000 ppm	0
	4000 ppm	0
Formalin	500 ppm	+
	1000 ppm	0
	1500 ppm	0
Propionic acid	800 ppm	++
	1200 ppm	++
	1600 ppm	+
Sorbic acid	800 ppm	++
	1200 ppm	+
	1600 ppm	0

* Visual rating of fungal growth: 0 no growth, +++ heavy growth (control), ++ moderate growth, somewhat suppressed compared to control, + light growth, clearly suppressed compared to control.

on the control diet. The longest development times (as measured by the number of days until adult emergence and start of oviposition) were observed among the insects reared on the control diet and the diet containing the highest concentration of formalin. The insects reared on diets containing benzoic acid (all concentrations tested) and the two higher concentrations of sorbic acid showed the least detrimental effects on biological fitness.

The insects that suffered the most detrimental effects on biological fitness were those that were reared on diets that supported heavy or moderate levels of *A. niger* growth and diets with formalin added, thus supporting the hypothesis stated earlier that insects reared on diets with the most efficacious antifungal additives would have the fewest negative effects on biological fitness. Previous studies on *L. hesperus* reared on artificial diets containing antifungal agents showed methyl paraben and formalin to be toxic and the lipophilic acids (benzoic, sorbic, and propionic acids) to be relatively nontoxic to the insects (Alverson and Cohen 2002). *Aspergillus niger* contamination in the artificial diet used to rear *L. hesperus* also has been shown to be detrimental to the biological fitness of the insect (Alverson 2002).

An ideal antimicrobial dietary additive suppresses microbes without harming the insect. In this study it was shown that complete suppression of *A. niger* is best for the biological fitness of the insects. Benzoic acid was shown to be the most efficacious of the antifungals tested against *A. niger* contamination. It also was low in toxicity to the insect. It also has been approved for use in human food (Jay 2000) in contrast to formalin, a known carcinogen (Sun 1981). This is of concern for the safety of insectary

Table 2. Effect of antifungal agents in NI artificial diet and *A. niger* on biological fitness of *L. hesperus

Antifungal	Concentration	Total number of surviving adults	Total biomass (dry weight in mg)	Eggs per female per day	Days to adult emergence	Days to oviposition
Control	–	106.7 ± 3.2 e	546.5 ± 11.9 e	9.1 ± 0.2 g	17.7 ± 0.6 d	22.3 ± 0.3 d
Benzoic acid	2000 ppm	144.7 ± 2.7 ab	753.4 ± 58.9 ab	29.3 ± 0.5 ab	14.3 ± 0.3 a	18.0 ± 0 a
	3000 ppm	151.7 ± 2.2 a	786.8 ± 7.9 a	28.6 ± 0.7 abc	14.7 ± 0.3 ab	18.3 ± 0.3 a
	4000 ppm	150.0 ± 4.0 a	768.7 ± 23.2 ab	27.7 ± 4.6 abcd	15.3 ± 0.3 abc	19.0 ± 0.6 abc
Formalin	500 ppm	125.7 ± 2.2 bcde	669.6 ± 15.8 bcd	28.1 ± 1.2 abc	15.3 ± 0.3 abc	19.0 ± 0.6 abc
	1000 ppm	123.7 ± 1.1 bcde	637.2 ± 9.1 cde	20.1 ± 1.1 def	15.7 ± 0.3 bc	20.0 ± 0 c
	1500 ppm	114.3 ± 8.4 cde	644.8 ± 45.0 cde	20.9 ± 2.5 cde	16.0 ± 0 c	21.7 ± 0.3 d
Propionic acid	800 ppm	111.0 ± 8.5 de	577.3 ± 30.0 de	12.7 ± 2.1 fg	15.3 ± 0.3 abc	19.7 ± 0.3 bc
	1200 ppm	119.0 ± 9.1 cde	609.7 ± 10.8 de	17.1 ± 3.6 ef	15.3 ± 0.3 abc	19.0 ± 0 abc
	1600 ppm	127.7 ± 4.1 bcde	718.2 ± 23.9 abc	23.2 ± 1 abcde	15.3 ± 0.3 abc	19.0 ± 0 abc
Sorbic acid	800 ppm	132.0 ± 7.5 abcd	637.4 ± 26.8 cde	21.6 ± 1.5 bcde	15.3 ± 0.3 abc	18.7 ± 0.3 ab
	1200 ppm	134.0 ± 7.4 abc	737.1 ± 8.0 abc	26.1 ± 0.6 abcd	15.3 ± 0.3 abc	18.7 ± 0.3 ab
	1600 ppm	145.7 ± 3.4 ab	737.8 ± 5.2 abc	30.0 ± 0.9 a	15.3 ± 0.3 abc	18.7 ± 0.3 ab

* Means ± SE within the same column followed by the same letter were not significantly different (LSD test, $P < 0.01$, SAS Institute, Inc., software version 8.01, 2000).

workers preparing and handling the diet. If convenience of use and cost are comparable to the formalin and propionic acid presently used in the NI diet, benzoic acid would appear to be a better choice as an antifungal diet additive for *L. hesperus.* Before a modification in the established NI diet can be made, however, the new diet using benzoic acid should be tested for at least three, and preferably five, generations to assure the continued production of insects with measurements of biological fitness comparable or better than those of insects reared on the NI diet.

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