EFFICACY OF ENCAPSULATED LAGENIDIUM GIGANTEUM
(OOMYCETES: LAGENIDIALES) AGAINST CULEX QUINQUEFASCIATUS AND Aedes aegypti LARVAE IN ARTIFICIAL CONTAINERS

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ABSTRACT. Presporangial mycelia of Lagenidium giganteum cultured on sunflower seed extract were encapsulated in calcium alginate and added once (July 18) to outdoor (Raleigh, NC) caged tires, wood and concrete containers populated with first instars of Culex quinquefasciatus or Aedes aegypti. First instars were added twice weekly (for 10 wk) to simulate natural oviposition. The fungus persisted for 10 wk and recycled in the mosquito larvae of both species. The overall reductions of Cx. quinquefasciatus and Ae. aegypti immatures were higher in tires (55 and 45%, respectively) and wood (67 and 38%) than in concrete containers (17 and 14%). There were low correlations of the numbers of mosquito immatures with measurements of water quality (chemical oxygen demand, ammonia nitrogen and conductivity) in the containers.

INTRODUCTION

The use of biological control, either alone or integrated with other mosquito control strategies, to suppress mosquito populations that develop in artificial and/or natural containers would be advantageous. A potentially useful biological control agent is the fungus Lagenidium giganteum Couch (Jaronski and Axtell 1983, 1984; Lacey and Undeen 1986; Guzman and Axtell 1987a, 1987b; Kerwin and Washino 1988). Alginate capsules of presporangial mycelia of the fungus are effective and convenient to store, handle and apply to mosquito breeding habitats (Axtell and Guzman 1987, Patel et al. 1990). These capsules controlled Culex quinquefasciatus Say in outdoor plastic wading pools (Axtell and Guzman 1987). The fungus, however, has not been evaluated against mosquitoes in other artificial containers over a long period. In addition, no information has been published on the effects of water quality and temperature in artificial containers on the ability of encapsulated L. giganteum to infect mosquito larvae. Therefore, we evaluated encapsulated L. giganteum outdoors from July to September 1989 against Cx. quinquefasciatus and Aedes aegypti (Linn.) in tires, wood and concrete containers.

MATERIALS AND METHODS

Fungal culture: The California isolate (obtained in 1987 from J. L. Kerwin, University of California, Davis, CA) of L. giganteum was maintained in our laboratory on extracts of sunflower seeds (SFE) as previously described (Jaronski and Axtell 1984, Guzman and Axtell 1986). Prior to encapsulation, the fungal isolate was passed 22 times through Cx. quinquefasciatus larvae (2- to 3-day-old).

Alginate capsules, containing the asexual (presporangia) stage of L. giganteum, were prepared following the procedure of Axtell and Guzman (1987) using 1.5% KELGIN HV* (Kelco, Division, Merck & Co., San Diego, CA) sodium alginate. This procedure results in capsules with a 3-4 mm diam and containing 1,500-2,000 sporangia. The encapsulated fungus typically produces zoospores for 7-14 days after immersion in water (Patel et al. 1990). Newly prepared capsules were dried overnight between paper towels at room temperature (ca. 27°C), placed in closed plastic containers and stored for 4 days at 15°C prior to their introduction into the artificial containers.

Test containers: Three types of artificial containers (tires, wood and concrete) were used outdoors during July to September 1989, in the vicinity of Raleigh, NC. The automobile tires (sizes P165/80R13 and P185/80R13) were obtained from a used-tire shop in Raleigh. Each tire was cut in half (along the diameter), and each piece was covered with a black plastic sheet. Wood boxes (inside dimensions: 19.5 x 18.5 x 15 cm height) were made from kiln-dried red oak (2 cm thick) and put together with screws and marine glue. The wood containers were a modification of the fabricated tree holes described by Lewis and Tucker (1978), but without covers. The round concrete containers (diam 18 cm, height 12 cm, thickness 2 cm) were made from Sakrete® mortar mix cement (6 kg/liter water). Like the wood containers they had open tops, but each container was placed in a plastic bucket. One half of each container type were placed outdoors in a wooded, shaded area for either a short (1-2 months) or long (4-9 months) time for aging and weathering prior to the experiments. Containers with the short or long duration outdoors were subsequently referred to as "new" or "old" containers, respectively. At the end of the weathering period, the containers
were rinsed with rain water that was previously collected and stored in plastic drums.

At the start of the experiment, 3 liters of rain water were added to each half-tire and 1.5 liters to each concrete or wood container. The water level in each container was maintained throughout the experiment by adding rain water as needed. Three new and 3 old containers of each type were placed in each of 4 screened cages (1.8 x 1.8 x 1.8 m) outdoors. The cages were used to prevent oviposition in the test containers by wild mosquitoes. Two cages were used for monitoring the populations of Cx. quinquefasciatus immatures (larvae and pupae). Containers in one cage were treated with a single addition of encapsulated L. giganteum (20 capsules/container on July 18) and those in the second cage were untreated controls. The other 2 cages were similarly used for monitoring Ae. aegypti immature populations with treated containers in one cage and untreated containers in the other cage.

About 75 first instars (1- to 2-day-old) of Cx. quinquefasciatus or Ae. aegypti were initially introduced into each container on July 17, and twice weekly (Monday and Thursday) thereafter until September 27. About 2 ml of a liver slurry (35 mg liver powder/ml water) were added into each container twice weekly for the first 4 wk and as needed thereafter to support larval survival. The larvae of Cx. quinquefasciatus and Ae. aegypti used in this study were obtained from laboratory colonies established in March 1985 and June 1988, respectively, from larvae collected in the vicinity of Raleigh, NC.

Counting methods: The numbers of live larvae and pupae in each container were determined by absolute counts at weekly intervals (11 dates, July 18-September 27, 1989). The water in each container was poured through a fine mesh screen positioned on top of a plastic bowl. The recovered larvae were counted while the sieve was submerged in clear water and classified as small (first and second instars) or large (third and fourth instars). After counting, the immatures and the water in the bowl were returned to the original container.

The presence of L. giganteum in the containers was determined by: 1) collecting and examining dead resident larvae, and 2) testing for zoospore activity in water samples. When available, up to 4 dead resident larvae were recovered from each container and examined microscopically for fungal infection. Zoospore activity was monitored weekly by placing 20 larvae (1- to 2-day-old) of Cx. quinquefasciatus into a water sample (50 ml) 3-4 h after it was taken from each container. The samples were held for 6 days (ca. 27°C) to determine the number of larvae infected by L. giganteum due to zoospores present in the water samples. Dead larvae were removed daily from these samples to prevent zoospore production and fungal recycling from the cadavers.

Water quality was monitored by obtaining a water sample (100 ml) from a container at weekly intervals and measuring chemical oxygen demand (COD) and ammonia nitrogen (NH₃-N) (Hach Company 1985). High levels of COD and NH₃-N have been shown to reduce infection of mosquito larvae by L. giganteum (Jaronski and Axtell 1982). Conductivity and pH of the water were recorded at the site using a portable meter (Model 33, Yellow Springs Instrument Co., OH). Water temperatures were recorded hourly in 4 containers of each type using a 21X Micrologger (Campbell Scientific, Inc., UT) with wire probes placed ca. 4 cm below the water surface.

To further verify the suitability of the container water for fungal survival and zoosporogenesis, 10 alginate capsules of L. giganteum were immersed in 25 ml of water from each of the samples taken weekly for water quality analysis and 20 larvae (1- to 2-day-old) of Cx. quinquefasciatus were added. The capsules and the dead larvae were observed microscopically for vesicle formation and infection, respectively.

Statistics: Split plot analysis of variance with container age as a main plot factor and counting interval as the subplot factor was used to test the significance of differences in numbers of mosquito immatures in new (1- to 2-month weathering) versus old (4- to 9-month weathering) containers. Similar analyses with species as replicate, container age and container type as main plot factors, and counting interval as the subplot factor were used to test the significance of differences in the reduction of the total number of mosquito immatures among the three types of containers. Regression analyses were applied to the data from both new and old containers treated with encapsulated L. giganteum to evaluate relationships between the total number of immatures of Cx. quinquefasciatus or Ae. aegypti and COD, NH₃-N and conductivity. The fitted models included effects for replicates, container type, water quality parameters and water quality by container type interactions. Nonsignificant ($P \geq 0.10$) effects were eliminated and reduced models were run to get prediction equations using PROC GLM (SAS Institute 1985). The Statistical Analysis System (SAS Institute 1985) was used for all statistical analyses.

RESULTS

For each container type, those treated one time with the encapsulated fungus had a significantly ($P \leq 0.05$) lower mean number of live
immatures (larvae and pupae) of a species than did untreated containers of the same type except for Ae. aegypti immatures in concrete containers (Table 1). Overall, there were significantly fewer mean numbers of live immatures of Cx. quinquefasciatus than of Ae. aegypti in each type of treated container. In the treated containers there was no significant difference in the mean number of immatures in old versus new containers except for Cx. quinquefasciatus in tires and Ae. aegypti in wood. The mean number of immatures of Cx. quinquefasciatus differed significantly among the types of containers treated with fungus; the number was highest in concrete, intermediate in tires and lowest in wood. The mean number of Ae. aegypti immatures was not significantly different among the types of treated containers.

The overall percentage reduction in the numbers of immatures of Cx. quinquefasciatus in untreated containers compared to fungus-treated containers of the same type was not significantly (P ≤ 0.05) different for tires (55%) and wood (67%) and was lower for concrete (17%) containers in the 10 wk following the addition of capsules. Small (first and second instar) larvae, large (third and fourth instar) larvae and pupae were reduced by 59, 52 and 47%, respectively, in treated tires; 59, 73 and 74% in treated wood containers. In concrete containers, no reduction of the number of large larvae and pupae was observed, but there was a 28% reduction of small larvae. Similarly, the overall reduction of the total number of Ae. aegypti immatures (larvae and pupae) averaged 45, 38 and 14% in treated tires, wood and concrete containers, respectively. Except for large larvae in concrete containers, small and large larvae and pupae of Ae. aegypti were reduced in the treated containers of all three types.

The weekly mean numbers of immatures in fungal-treatments for both species in tires, wood and concrete containers are shown in Fig. 1 (A–F). For both species, the numbers of immatures fluctuated similarly in the treated and untreated tires and wood containers throughout the study period. There were significantly fewer Cx. quinquefasciatus immatures in the treated tires than in the untreated tires in all 10 wk. Aedes aegypti immatures were also lower in tires in all 10 wk although not significantly so during the last 2 wk. Culex quinquefasciatus immatures were also significantly lower in the fungus-treated wood containers except in the last week. In wood, Ae. aegypti immatures were significantly fewer in number in the treated containers compared with the untreated containers in the first 5 wk. The poorest control of larvae with the one time fungus treatment was in the concrete containers. Numbers of Cx. quinquefasciatus were significantly reduced in the treated concrete containers only during weeks 1 and 10. Numbers of Ae. aegypti immatures were also significantly lower in treated concrete containers only in these 2 weeks and in week 7.

The presence of L. giganteum in the treated tires, wood or concrete containers was routinely confirmed by microscopic examination of samples of resident dead larvae, when available. In tires, 128 of 138 dead resident larvae examined were infected with L. giganteum; 103 of 112 dead larvae in wood containers, and 46 of 59 dead larvae in concrete containers.

The data on water quality (COD, NH_3-N, conductivity and temperature) for the tires, wood and concrete containers are presented in

<table>
<thead>
<tr>
<th>Container</th>
<th>Mosquito stage</th>
<th>Untreated</th>
<th>Treated</th>
<th>Percent reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tire</td>
<td>Small larvae</td>
<td>53.5</td>
<td>22.0</td>
<td>58.9</td>
</tr>
<tr>
<td></td>
<td>Large larvae</td>
<td>48.9</td>
<td>23.5</td>
<td>51.9</td>
</tr>
<tr>
<td></td>
<td>Pupae</td>
<td>10.9</td>
<td>5.8</td>
<td>46.8</td>
</tr>
<tr>
<td></td>
<td>Total immatures</td>
<td>113.4</td>
<td>51.1</td>
<td>54.9</td>
</tr>
<tr>
<td>Wood</td>
<td>Small larvae</td>
<td>48.4</td>
<td>20.0</td>
<td>58.7</td>
</tr>
<tr>
<td></td>
<td>Large larvae</td>
<td>50.4</td>
<td>13.7</td>
<td>72.8</td>
</tr>
<tr>
<td></td>
<td>Pupae</td>
<td>9.3</td>
<td>2.4</td>
<td>74.2</td>
</tr>
<tr>
<td></td>
<td>Total immatures</td>
<td>108.0</td>
<td>36.0</td>
<td>66.7</td>
</tr>
<tr>
<td>Concrete</td>
<td>Small larvae</td>
<td>51.5</td>
<td>37.2</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>Large larvae</td>
<td>22.2</td>
<td>22.5</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Pupae</td>
<td>6.0</td>
<td>7.8</td>
<td>-30.0</td>
</tr>
<tr>
<td></td>
<td>Total immatures</td>
<td>81.2</td>
<td>67.4</td>
<td>17.0</td>
</tr>
</tbody>
</table>

Table 1. Mean number of immatures per container per week and overall percent reduction of Culex quinquefasciatus and Aedes aegypti in tires and wood and concrete containers during the 10-wk period following treatment with encapsulated Lagendidium giganteum on July 18, 1989, and weekly additions of first instar larvae.
Table 2. The pH values had a narrow range, 6.9-7.3, in all containers for both species throughout the study period and, therefore, the data are not presented. Water temperature ranges were 17.0-32.6, 16.4-31.6 and 16.6-33.0°C in tires, wood and concrete containers, respectively, in the study period, except in the last week with cooler temperatures. There were only minor differences in the hourly temperatures among the 3 types of containers at any time during the 10 wk of the study. The temperatures in all containers, except during the last week, should have been appropriate for fungal growth and zoosporogenesis (Jaronski and Axtell 1983, Guzman and Axtell 1987b).

Of the several regression analyses tested, including univariate, multivariate and multivariate with higher order interactions, no single model showed a strong correlation between any of the water quality parameters measured and the number of immatures in the fungus-treated containers. All models tested had low $r^2$ values (generally less than 0.5). Relationships at these low $r^2$ values, if significant, were considered weak. There was no consistent pattern in relationships. For example, in a multiple linear regression model to relate total immatures and all 3 water quality parameters, the numbers of Ae. aegypti immatures in all fungus-treated containers were significantly and positively correlated with COD ($P \leq 0.05$, $r^2 = 0.13$), but the numbers of Cx. quinquefasciatus were negatively correlated with COD ($P \leq 0.05$, $r^2 = 0.09$).

Throughout the study period, quality of water from tires and wood containers, and to a lesser extent from concrete containers, was apparently appropriate for vesicle formation since alginate capsules of L. giganteum that were added to samples of water collected from the containers were observed microscopically to produce vesicles. Vesicles were produced from the capsules in 97 of 112 samples in tires, 87 of 112 samples in wood containers and 74 of 111 samples in concrete containers. Furthermore, fungal infec-
tion was observed in Cx. quinquefasciatus larvae added to the water samples along with alginate capsules of L. giganteum which produced vesicles. Zoospore activity, as indicated by mean percent infection of Cx. quinquefasciatus larvae assayed in water samples removed weekly from different containers, was relatively low (tires, 18%; wood, 11%; concrete, 5%), but confirmed fungal activity throughout the study period in tires and wood containers and to a lesser extent in the concrete containers.

Following termination of the experiment in September due to cold weather, the containers were left outdoors in the cages over the winter. During the following early summer (July 20 to July 4, 1990), first instars of Cx. quinquefasciatus were added to each container at 2-5 day intervals. Larvae were removed 2-5 days after adding to the containers, and the dead larvae were examined for infection. Four infected larvae were recovered on two different dates from one concrete container but none from any other container. Thus, there was evidence, although only slight, that the fungus overwintered, probably in the oospore stage.

**DISCUSSION**

The tires and wood and concrete containers exhibited different water quality characteristics that apparently affected L. giganteum activity and thus the density of immature mosquito populations in those containers. In this study, water quality, particularly in tires and wood containers, was suitable for the establishment of L. giganteum in the immature populations of both mosquito species. The overall reduction in immature populations of both species was higher in wood containers and tires than in concrete containers. Although COD levels greater than 250 mg/liter occurred, particularly in wood containers, L. giganteum zoosporogenesis persisted. In previous studies (Jaronski and Axtell 1982), the infectivity of L. giganteum (NC isolate) against Cx. quinquefasciatus was lost in water with the COD = 119-186 mg/liter. In another study (Guzman and Axtell 1987b), the fungus (CA isolate) caused 80-92% larval infection in the same mosquito species in water with the COD = 97-214 mg/liter. The inoculum used in our experiment was obtained after 22 serial passages of the fungus (CA isolate) through Cx. quinquefasciatus larvae, which may have increased its vigor and tolerance of higher COD levels. Lord and Roberts (1986) reported that 15 serial passages through mosquito larvae restored the vigor of L. giganteum (CA isolate) in terms of zoosporogenesis, oosporogenesis and infectivity.

In concrete containers, the slight decline in the number of immatures of *Ae. aegypti* could be attributed to factors other than those measured by us. Kramer (1990) showed a strong negative correlation between water hardness (as measured by mg/ml CaCO₃) and mortality of mosquito larvae due to L. giganteum. Lord and Roberts (1985a, 1985b) observed that various species of bacteria and other microorganisms affected zoosporogenesis and zoospore survival. Jaronski and Axtell (1982) showed that TKN and P affected infection of mosquito larvae by the fungus. Guzman and Axtell (1987a) reported adverse effects of turbidity on zoosporogenesis. Other factors could have contributed to the fluctuations in the numbers of immatures.

The encapsulated fungus was effective against Cx. quinquefasciatus and Ae. aegypti in tires and wood containers, and to a lesser extent in concrete containers. The fungus was able to recycle for 10 wk in the artificial containers and perhaps would recycle longer, but the experiment was terminated due to cool weather. Although our study demonstrated the ability of the encapsulated fungus to reduce the immature populations of Cx. quinquefasciatus and Ae. aegypti in wood containers, similar trials should be done in actual tree holes and tires containing organic debris. Tree holes are complex mosquito breeding habitats, with leaf litter content and water quality parameters varying enormously (Carpenter

Table 2. Mean (±SE) water quality parameters (COD, NH₃-N, conductivity and temperature for 10 wk in tires and wood and concrete containers with *Aedes aegypti* or *Culex quinquefasciatus* following introduction of encapsulated *Lagenidium giganteum* on July 18, 1989, and weekly additions of first instars.

<table>
<thead>
<tr>
<th>Species</th>
<th>Container</th>
<th>COD  mg/liter</th>
<th>NH₃-N mg/liter</th>
<th>Conductivity umhos/cm</th>
<th>Temperature °C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ae. aegypti</em></td>
<td>Tires</td>
<td>67.7 ± 10.5</td>
<td>1.7 ± 0.2</td>
<td>52.6 ± 2.8</td>
<td>29.1 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>Wood</td>
<td>145.2 ± 39.6</td>
<td>1.1 ± 0.2</td>
<td>29.2 ± 1.5</td>
<td>29.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Concrete</td>
<td>105.8 ± 17.7</td>
<td>0.4 ± 0.0</td>
<td>196.1 ± 8.3</td>
<td>30.8 ± 0.5</td>
</tr>
<tr>
<td><em>Cx. quinquefasciatus</em></td>
<td>Tires</td>
<td>47.6 ± 9.3</td>
<td>1.5 ± 0.2</td>
<td>50.6 ± 2.9</td>
<td>30.2 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>Wood</td>
<td>144.3 ± 36.4</td>
<td>1.0 ± 0.2</td>
<td>30.1 ± 1.2</td>
<td>25.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Concrete</td>
<td>88.8 ± 15.2</td>
<td>0.5 ± 0.1</td>
<td>220.9 ± 11.6</td>
<td>30.3 ± 0.5</td>
</tr>
</tbody>
</table>

Note: Tires and wood and concrete containers with *Aedes aegypti* or *Culex quinquefasciatus* following introduction of encapsulated *Lagenidium giganteum* on July 18, 1989, and weekly additions of first instars.

In concrete containers, similar trials should be done in actual tree holes and tires containing organic debris. Tree holes are complex mosquito breeding habitats, with leaf litter content and water quality parameters varying enormously (Carpenter
(1982), which could affect fungal activity. In concrete containers, encapsulated L. giganteum may not be efficacious as evidenced by the low reductions in immature mosquito populations in treated containers in this study. However, this should be tested further in large concrete water storage containers.

Encapsulated L. giganteum can be conveniently applied to natural and artificial containers. Ideally, the application should be done when the mosquito larvae are in early instars and the water quality is suitable. Changes in water quality, host density and age-structure influence the reduction of mosquito larval population over a period of time (Guzman and Axtell 1987a). Although all species of mosquitoes can be infected by L. giganteum, differences in species susceptibilities may cause variations in persistence and cycling of the fungus. There should be further evaluations of encapsulated fungus against other species of mosquitoes in tires, wood and concrete containers as well as in other types of natural and artificial containers.

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