Green Treefrogs as Vectors of *Colletotrichum gloeosporioides*

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ABSTRACT

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Green treefrogs (*Hyla cinerea*), commonly observed in southern Arkansas rice fields, were efficient dispersal vectors for *Colletotrichum gloeosporioides* f. sp. *aeschynomene*, a causal agent of anthracnose of northern jointvetch. In four experiments, frogs captured from rice fields infested with diseased northern jointvetch were placed in containers with healthy northern jointvetch plants. An average of 90% of the northern jointvetch plants became infected by the pathogen with up to 10 lesions per plant. Dispersal by frogs was further quantified in greenhouse experiments by monitoring disease development from a point source in simulated rice-weed patches. The frogs moved the pathogen from a source plant to healthy plants, resulting in 95% infection. Densities of treefrogs in 16 surveyed weed patches of eight rice fields varied from 0.3 to three per northern jointvetch plant. Shelter selection experiments showed that in patches consisting of 336 rice tillers and 10 northern jointvetch plants, green treefrogs preferred northern jointvetch to rice at a ratio of 133:1, presumably because northern jointvetch is taller than rice. The fact that green treefrogs prefer northern jointvetch may contribute to the efficacy of the treefrogs as vectors of *C. g. aeschynomene*.

Additional keywords: biocontrol, epidemic

Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. in Penz. f. sp. *aeschynomene* causes anthracnose of northern jointvetch (*Aeschynomene virginica* (L.) B.S.P.), a tall leguminous weed in rice and soybean fields in Mississippi River delta states. Collego, a mycoherbicide, was developed from this fungal pathogen. The ecology of the pathogen has been extensively studied as a model system for *Colletotrichum*. Because species of *Colletotrichum* are important agents in weed biocontrol (6), understanding the dispersal of this fungus is important for the development of mycoherbicides. Information on dispersal mechanisms for this pathogen is limited to rain splash (10). Rain-splash dispersal results in infections on lower parts of the stem (10), which could not explain the upward dispersal of *C. g. aeschynomene* on higher parts of northern jointvetch plants in rice fields.

Insects and nematodes are the most common biological vectors of plant pathogens. Little attention has been paid to the role of amphibians in the spread of fungal pathogens. Green treefrogs (*Hyla cinerea* Schneider) are distributed from Louisiana to southern Illinois (9) and are commonly observed in rice and soybean fields in Mississippi River delta states. Treefrogs have been frequently observed in contact with infected northern jointvetch plants in rice fields. The objective of this study was to determine if green treefrogs can act as dispersal vectors of *C. g. aeschynomene* and to investigate the ecological behavior of this vector with regard to the dispersal of the pathogen.

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MATERIALS AND METHODS

Treefrog transmission tests. Frogs were captured four times during the 1991 rice growing season from 10 differant rice fields infested with diseased northern jointvetch plants in Stuttgart, Arkansas. In the first experiment, 15 frogs were caught from one patch of northern jointvetch and 18 from another. For the second and third experiments, 12 frogs were caught per patch. The fourth sampling was taken during the harvesting season from two rice fields. One of the two sampled fields did not contain northern jointvetch but was adjacent to a field infested with diseased northern jointvetch plants. In the field without northern jointvetch, the green treefrogs were captured from rice plants. The treefrogs from each sampling were returned to the laboratory in Fayetteville, Arkansas, in plastic bags or plastic bottles on the same day. Each patch was considered as a sampling unit, and all treefrogs from one patch were bulked into one sample.

In the laboratory, the treefrogs from each patch were put into glass containers 45 cm in height and 22 cm in diameter or into plastic containers 45 cm in height and 35 cm in diameter for 24 hr. Each container had three or four pots of healthy northern jointvetch plants about 40 cm tall. The number of plants per container differed for different experiments (Table 1). After 24 hr, the frogs were removed and the plants were placed into a dew chamber at 28 C for 24 hr. A control treatment in which plants were not placed in contact with treefrogs also was established for each test. After incubating the inoculated plants in growth chambers at 28 C for 5 days, the number of diseased plants and the number of lesions per plant were determined.

Dispersal in controlled conditions. To determine if frogs vectored inoculum of *C. g. aeschynomene* from plant to plant, simulated rice-weed patches were assembled in a greenhouse. Each patch was sealed with screen in a frame of 122 × 81 × 100 cm, and the base of each frame contained a water pool 76 × 115 cm and 2 cm deep. Twenty-four rice plants at heading stage were grown in each frame. The average number of tillers per rice plant was 14, and there were 336 rice tillers per patch. Ten healthy northern jointvetch plans taller than the rice plants were evenly distributed in each patch. Treatments consisting of 0, 2, or 10 frogs per patch were established, and each treatment had two plots (two replicates). To rid them of spores before being introduced into the patches, the frogs were washed 15 min with tap water, placed in a dew chamber at 28 C for 24 hr, then put in glass containers in contact with healthy northern jointvetch plants. There were no symptoms 6 days after incubation of these plants in a dew chamber

Table 1. Infections of northern jointvetch plants after contact with green treefrogs (*Hyla cinerea*) captured in 1991 from rice fields infested with northern jointvetch infected by *Colletotrichum gloeosporioides* f. sp. *aeschynomene*

Date	No. of fields	No. of patches	Percent infected plants	Lesions per plant
8 August	1	2	100	8.3
29 August	4	6	77	2.7
6 September	3	6	100	5.4
6 October	2	2	68	1.4

Table 2. Dispersal of *Colletotrichum gloeosporioides* f. sp. *aeschynomene* by green treefrogs (*Hyla cinerea*) in simulated rice-weed patches as indicated by number of diseased northern jointvetch plants

No. of frogs per patch	First observation[a]			Second observation[b]		
	No. of plants infected	No. of plants killed	No. of lesions per plant	No. of plants infected	No. of plants killed	No. of lesions per plant
0	0.0 ± 0.0[c]	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
2	2.0 ± 0.8	0.0 ± 0.0	1.2 ± 0.2	6.8 ± 0.5	2.8 ± 0.5	1.7 ± 0.0
10	3.8 ± 2.4	0.5 ± 0.6	2.8 ± 0.2	9.5 ± 0.6	5.0 ± 0.8	4.9 ± 1.8

[a] Eight to 10 days after introduction of source inoculum.
[b] Sixteen to 18 days after introduction of source inoculum.
[c] Mean values and standard error.

at 28 C for 24 hr, indicating that there were no detectable residual spores on these frogs.

After the introduction of frogs, a diseased plant with five to seven lesions was placed in the center of each patch. Temperature in the greenhouse was maintained at 25 C, and free moisture was provided every 2 days by covering the frame with plastic sheets and by using humidifiers and misters. The number of diseased plants, killed plants, and lesions per plant was counted twice for each patch during the test. The treefrogs were fed live crickets (*Acheta domestica* (Linnaeus)) every 2 days. The experiment was repeated twice.

Treefrog observations. The behavior of frogs was observed in both commercial and experimental rice fields infested with diseased northern jointvetch during the rice growing season. Observations focused on shelter selection, movement, and predation of green treefrogs from morning (7:30 a.m.) to evening (8:30 p.m.). The activities of treefrogs in different weather conditions, such as hot, windy, and rainy days, were recorded.

Frog populations. Populations of treefrogs in weed patches of northern jointvetch in rice fields were determined in two surveys during the growing season. Commercial fields with patchily distributed northern jointvetch were selected. Investigations also were conducted in experimental field plots with northern jointvetch at the Rice Research Center at Stuttgart, Arkansas. Each patch was examined by randomly dividing the area into 1 × 2 m quadrats. The number of sampled quadrats per patch depended on the size of the patch. In each quadrat, the number of northern jointvetch plants and the number of frogs observed on these plants were counted. Means of plants per square meter, frogs per plant, and frogs per square meter for each patch were calculated.

Shelter selection. Previous studies (9) showed that *H. cinerea* chose tall plants as shelters. This may be an important behavior for the efficacy of frogs as vectors in this pathosystem because northern jointvetch plants are normally taller than rice by midseason. To confirm this, green treefrog movement and shelter selection were monitored and quantified in simulated weed-rice patches (described above). Each patch contained 10 treefrogs, 10 northern jointvetch plants, and 336 rice tillers. Numbers of frogs sitting on the 336 rice tillers, on the 10 northern jointvetch plants, or on the screen of the frame were counted two to four times a day from 8 a.m. to 8 p.m. A total of 56 observations were recorded. Observations were averaged by time, and the number of treefrogs on rice or on northern jointvetch was plotted against time.

To quantify the shelter preference of green treefrogs, each rice tiller or northern jointvetch plant was assumed to be a potential shelter for a treefrog. If the frogs had no preference, the number on rice would be about 33 times greater than the number on northern jointvetch, based on the number of the two plants. To determine preference, a preference index (I) was calculated as: $I = P_{total} Y_i / P_i$, where Y_i was the number of frogs sitting on i-type plants. P_i was the number of i-shelters (336 rice tillers and 10 northern jointvetch plants), and P_{total} was the sum of rice tillers and northern jointvetch plants in the patch (i.e., 346).

RESULTS

In four separate experiments, northern jointvetch plants were infected by *C. g. aeschynomene* after being in contact with frogs obtained from 16 patches from 10 different fields (Table 1). Percentages of plants infected after being in contact with frogs ranged from 25 to 100, with percentages greater than 90 for most patches. None of the control plants became infected in the four tests. The number of lesions per plant ranged from 1.0 to 10. The number of infections was lower in the second experiment than in the first and third experiments, perhaps because the frogs were caught after a rain lasting from early morning to noon. In the fourth experiment, plants put in contact with frogs sampled from a field without northern jointvetch plants also were infected.

The pathogen was dispersed by treefrogs in the simulated rice-weed patch experiment after introduction of diseased northern jointvetch plants (Table 2). New disease lesions were first observed in the first experiment 6 days after diseased plants were introduced. In the second experiment, infected plants were observed 8 days after the introduction of source plants. Sixteen days after the introduction of source plants, the number of diseased plants averaged 9.5 of 10, with up to 5.2 lesions per plant. The number of northern jointvetch plants killed in the first and second experiments averaged 4.5 and 5.5, respectively, for the 10-frog treatment. There were significant differences in lesions per plant between two-frog and 10-frog treatments. No infection occurred in the treatment without frogs.

Fig. 1. A green treefrog (*Hyla cinerea*) sitting on an anthracnose lesion caused by *Colletotrichum gloeosporioides* f. sp. *aeschynomene* on a stem of northern jointvetch in a rice field.

Green treefrogs were found in rice fields in early May when rice was planted. Close observations were possible because the treefrogs generally sat motionless. In the middle of the growing season, after the rice flowered and northern jointvetch plants were taller than rice, large numbers of treefrogs were observed. Treefrogs often were seen sitting on the upper parts of northern jointvetch above the rice canopy on clear days and on anthracnose lesions on northern jointvetch plants above or inside the rice canopy (Fig. 1), especially later in the growing season when disease incidence was high. In the early morning or on windy days, treefrogs were frequently observed sitting lower on northern jointvetch plants beneath the rice canopy. Active predation was observed in the evening.

Sixteen weed patches from eight different fields were examined in two surveys. Average plant densities among fields varied from 1.3 to 3.0/m^2 (Table 3). The number of frogs per northern jointvetch plant varied from 0.3 to 3.0 in individual patches and averaged 0.32 to 2.0 among fields. The average densities of treefrogs in northern jointvetch patches varied from 0.4 to 3.1/m^2 among the fields (Table 3). As many as five frogs were observed on one plant in a commercial field.

Table 3. Average densities of green treefrogs (*Hyla cinerea*) and northern jointvetch plants in rice fields

Fields[a]	No. of patches	Plants/m^2	Frogs per plant	Frogs/m^2 [b]
Survey 1				
Com 1	3	3.0	0.56	1.7
Expt 1	1	1.8	0.43	0.8
Com 2	3	1.8	2.0	3.1
Expt 2	3	1.8	1.4	2.7
Com 3	2	2.4	0.7	0.6
Survey 2				
Expt 2	2	2.8	0.39	1.1
Com 4	1	1.3	0.32	0.4
Com 5	1	1.45	0.43	0.6

[a] Survey 1 was conducted on 29 August 1991 and survey 2, on 6 September 1991. Com = commercial, expt = experimental.
[b] Frog densities within northern jointvetch patches.

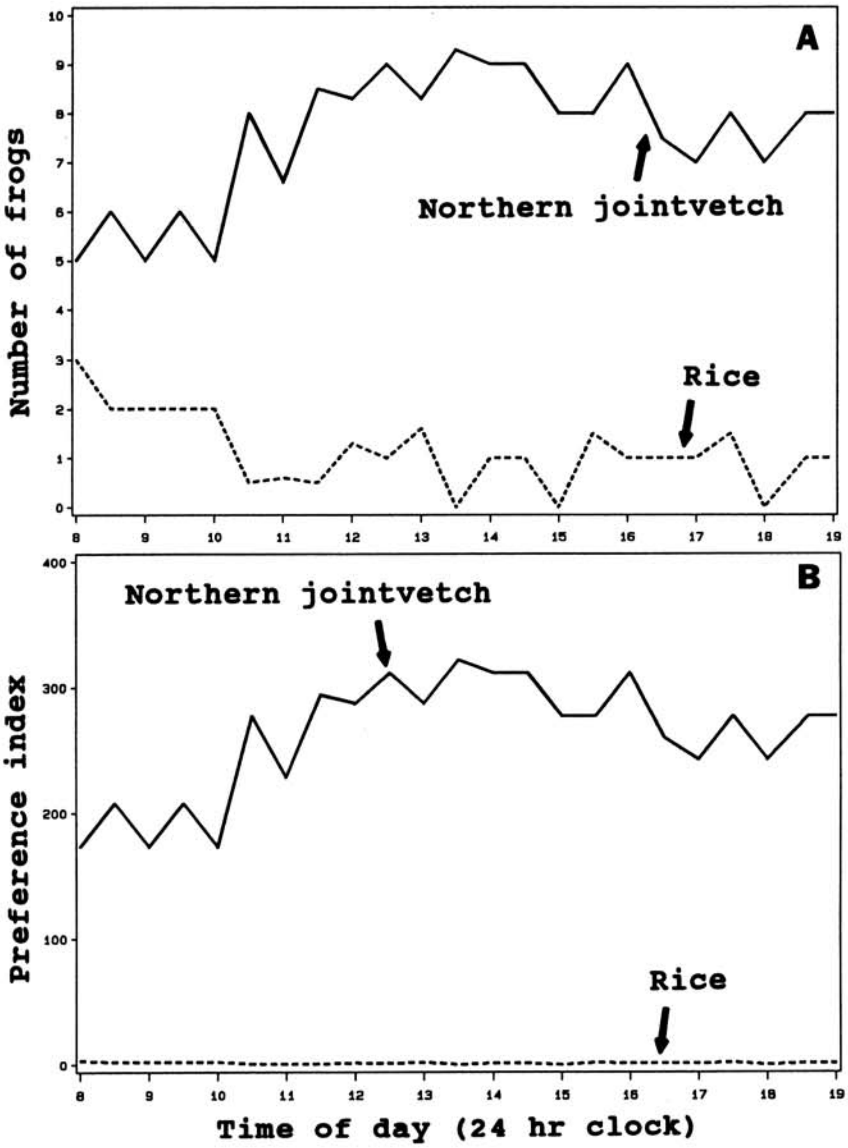

Fig. 2. **(A)** Number of green treefrogs (*Hyla cinerea*) sitting on 336 rice tillers or on 10 northern jointvetch plants during day hours in simulated rice-weed patches and **(B)** relative preference indices for shelter on rice or northern jointvetch.

The behavior of treefrogs in simulated rice-weed patches in a greenhouse was similar to that in field observations. Green treefrogs usually sat motionless on upper parts of northern jointvetch plants with their abdomens firmly in contact with the plant stem. The frogs appeared to prefer northern jointvetch plants as shelters, with more than eight of 10 frogs observed on the northern jointvetch plants (Fig. 2A). Usually, treefrogs remained in the same position for several hours. Most disease lesions were found on the upper portions of the stems. Often one or two frogs were found sitting on the screen in a position above the rice canopies during the day. The preference indices of northern jointvetch to rice were approximately 133:1 (Fig. 2B) instead of 1:33, which would be assumed if treefrogs did not show a shelter preference. The treefrogs were very active during feeding and when they were calling.

DISCUSSION

Our studies show that green treefrogs are efficient dispersal vectors for *C. g. aeschynomene* in rice fields and, on the basis of infection results, that they carry a considerable amount of inoculum. Field observations and infection results indicated that the treefrogs acquired inoculum through physical contact with infested plants. The density of treefrogs in weed patches was high, and the treefrogs preferred northern jointvetch plants as shelters.

Green treefrogs may be efficient vectors in this pathosystem because of their behavior. Ecological studies (1,5,9) as well as our data show that treefrogs use tall plants as shelters. This behavior prevents attacks from snakes and fish (3,9) and provides better vision for predation (2). Northern jointvetch is one of several taller weeds in rice and can dominate an infested rice field. The observed density of treefrogs in patches of northern jointvetch was very high compared with previously reported numbers in different habitats (1). Because green treefrogs selectively take northern jointvetch as shelters, the density of the frogs may be higher in a weed patch than in other parts of a rice field. In a weed patch in a rice field, the chance of moving inoculum from a diseased plant to neighboring plants is very high because selection of northern jointvetch plants for shelter by treefrogs could result in target-specific horizontal movement of inoculum. It has been often observed in rice that many disease lesions on northern jointvetch plants are in positions taller than rice (Yang, *unpublished*). This may be because of frog movement and because the upper parts of plants are more susceptible. In the field, direct contact with lesions may not be a necessary condition for treefrogs to obtain inoculum. *C. g. aeschynomene* produces high numbers of conidia (8), and large areas of the lower stem can become contaminated when rain washes these spores down from upper lesions. As the treefrogs move up and down on the plants, the chance to acquire inoculum increases. Furthermore, the vertical movement of frogs observed in our study as well as by others (1) provides a means of vertical dispersal, with inoculum being moved from lower stems to upper plant parts if the initial infection by seedborne (7) or rainsplash inoculum (10) occurs at the base of a plant.

Any significance of green treefrogs to the spread of this pathogen also may be attributed to the rice ecosystem in Arkansas. A stable population of *H. cinerea* relies on a permanent water body because the frog needs an aquatic environment for reproduction (1,3). The rice ecosystem in part of southern Arkansas consists of permanent irrigation reservoirs surrounded by rice fields, which may provide ideal refuges for stable populations of green treefrogs. When green treefrogs reproduce during May through August (3,9), the irrigated rice fields also provide the frogs with an additional large reproduction area. To quantify the importance of treefrogs to disease dynamics, we need to monitor treefrog populations during growing seasons and determine the percentage of inoculum-carrying treefrogs in the population.

The case of treefrogs as dispersal vectors may be unique because of the unusual conditions of a rice ecosystem, or it may suggest a role of biological vectors in the formation of spatial patterns of plant diseases. Northern jointvetch is patchily distributed in rice. Rain is not likely to move the pathogen from one patch to another because the maximum dispersal distance in rice is 1.5 m in a single rain episode (10). Frogs are known to migrate as far as several hundred meters during reproduction or when food is scarce (1). Such migration may provide a dispersal mechanism from patch to patch.

Although frogs may not be important vectors for most plant diseases, they may be important vectors to be considered in biological risk assessment, a new area in biological science (4). Many species of *Colletotrichum* have been studied as mycoherbicides, and genetic-engineering methods could be used to improve efficacy (6). These organisms eventually will be subjected to field tests. The presence of frogs in test plots may increase the chances of escapes of the organisms being tested. Furthermore, shelter selection by treefrogs can direct the movement of pathogens and may increase the chance of gene exchange among different fungi. For example, mating has been observed between strains of *C. g. aeschynomene* and *C. g. jussiaeae*, two special forms infecting northern jointvetch and winged waterprimrose (*Jussiaea decurrens* (Walt.) DC.), respectively (TeBeest et al, *unpublished*). Both these plants are tall weeds in rice fields and are favorite shelters for treefrogs. The fact that the two weeds are frog shelters may greatly increase the chance of contact and gene exchange between these two pathogens.

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