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Classical biological control of *Aphis gossypii* (Homoptera: Aphididae) with *Neozygites fresenii* (Entomophthorales: Neozygitaceae) in California cotton

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Abstract

In August 1994 and 1995 classical biological control releases were made in cotton in the San Joaquin Valley, California, with an Arkansas strain of the entomopathogenic fungus, *Neozygites fresenii*, a pathogen of the cotton aphid, *Aphis gossypii*. Pre-release samples in both years indicated that *N. fresenii* was not naturally present in *A. gossypii* populations in the San Joaquin Valley. Two release methods were compared: dried *N. fresenii*-infected cotton aphid "cadavers" and chamber inoculation of *A. gossypii*. Both methods were successful in introducing *N. fresenii* to cotton aphids in California; however, higher prevalence of fungal infection resulted with the cadaver treatments. *N. fresenii* persisted and spread in the aphid population until early October 1994 and late September 1995. The highest mean percentage infection in the cadaver treatment in 1994 reached a level (14%) considered imminent for epizootics (12–15%). The use of predator exclusion cages resulted in higher *N. fresenii* prevalences.

Keywords: Cotton; Cotton aphid; *Aphis gossypii*; Biological control; *Neozygites fresenii*; Entomophthorales

1. Introduction

Until the late 1980s the cotton aphid, *Aphis gossypii* Glover (Homoptera: Aphididae), was considered a sporadic pest of cotton grown in the San Joaquin Valley, California (Grafton-Cardwell, 1996). However, since 1991 it has ranked as one of the top three insect pests in California cotton most years, based on number of hectares infested, percentage yield reduction and number of bales lost due to aphid damage (Head, 1993; Williams, 1998). The cotton aphid was ranked as the most damaging pest of the year in 1994, and in 1995 and 1997 it was responsible for the greatest estimated yield losses in the San Joaquin Valley (Godfrey and Rosenheim, 1996; Williams, 1998).

Re-invasion of treated fields by winged aphids and development of insecticide resistance have resulted in low insecticide efficacy, increases in aphid population densities, and increased insecticide use (Grafton-Cardwell, 1996). In field trials with 19 insecticide treatments, Godfrey and Rosenheim (1996) found aphid densities in insecticide-treated plots were up to four times greater 21 days after treatment than in untreated plots, despite up to 98% population reduction in some treatments at 7 days after treatment (DAT). In addition, insecticide applications often destroy natural enemies. Therefore, increased biological control of *A. gossypii* would be desirable in San Joaquin Valley cotton.

Neozygites fresenii (Nowakowski) Batko (Entomophthorales: Neozygitaceae) is an entomopathogenic fungus which occurs naturally in Midsouth and Southeast cotton-growing regions of the US, where it has provided effective natural control of cotton aphid populations since 1989 without detriment to plants, beneficial arthropods or vertebrates. Epizootics caused by *N. fresenii* have been reported in these regions of the United States and in Africa during relatively dry periods (Silvie and Papierok, 1991; Steinkraus et al., 1991; Steinkraus et al., 1995; Weathersbee and Hardee, 1994). Gustafsson

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(1969) reported epizootics of *N. fresenii* in *Aphis fabae* Scopoli (Homoptera: Aphididae) on sugar beets during unusually dry periods in Sweden where the fungus did not seem dependent on amount of precipitation or number of days with precipitation. Based on the literature and our observations, *N. fresenii* does not appear to occur naturally in California cotton aphid populations, though there is some evidence that Steinhaus (1951) found *N. fresenii* in aphids from willow in California. Because *N. fresenii* has the ability to cause epizootics in relatively dry regions it is a good candidate for introduction into the San Joaquin Valley to help control cotton aphids. Even though the natural conditions in the San Joaquin Valley are fairly dry, cotton is grown with irrigation and once the cotton canopy closes over the furrows, levels of humidity within the canopy can be very high. Thus, the irrigated agroecosystem may represent a suitable habitat for a fungal pathogen.

In August 1994 and 1995 field trials were conducted to introduce *N. fresenii* into San Joaquin Valley cotton aphid populations. The objectives of these trials were to develop and compare introduction methods and initiate epizootics.

2. Materials and methods

2.1. Field locations and descriptions

Permission was obtained from USDA-APHIS (permit #942402) and the California Department of Food and Agriculture to allow the introduction of the Arkansas strain of *N. fresenii* in California. The first release of *N. fresenii* was made on 16 August 1994, in a 'Pima' (cultivar S-7) cotton field at the Shafter UC Cotton Research Station in Kern County, California. In 1995, low aphid populations prevented repeating the test at Shafter. Therefore, the second release was made on 17 August 1995 in Madera County near Chowchilla, California, in a 'Maxxa' cultivar commercial organic upland cotton field. Both fields had generalized, moderate to heavy aphid populations (Rosenheim, unpublished data) and were furrow irrigated.

2.2. Release methods

Because *N. fresenii* has not been successfully cultured in vitro, it was necessary to use in vivo produced fungus for introductions. Fungal inoculum (dried, *N. fresenii*-killed aphids, hereafter called "cadavers") was produced by exposing laboratory-reared *A. gossypii* to *N. fresenii* capilliconidia (the infective stage) as described by Steinkraus et al. (1993). The fungus originated from infected cotton aphids collected from epizootics in Arkansas cotton in 1991 and was kept dry and frozen until used.

In 1994 two methods of releasing the fungus (cadaver and chamber) were compared with a control. Each replicate consisted of one aphid-infested cotton plant onto which we released *N. fresenii*. Each treatment was replicated 35 times in a randomized complete block design. In 1995 only the cadaver method was compared with a control; with each treatment replicated 45 times in a randomized complete block design. Controls consisted of untreated aphid-infested cotton plants. Cadavers or fungus-inoculated aphids from the chambers were placed on aphid-infested 8th node (1994) or 7th node (1995) mainstem leaves beneath the plant terminals. Release and control plants were 3-m apart within and between rows.

The cadaver release method consisted of 25 cadavers placed on the underside of an aphid-infested cotton leaf on each replicate plant in the release fields. Immediately prior to release, the leaves were lightly misted with water using a hand plant mister to increase adherence of the cadavers.

The chamber treatment consisted of 25 cadavers placed in 60 by 15 mm plastic Petri dishes and allowed to sporulate for 14 h at 26 °C, 100% relative humidity. Sporulation and formation of capilliconidia in the chambers were confirmed microscopically before use. To inoculate field aphids with the fungus, cotton aphids from naturally infested cotton plants from the Shafter release field were collected and 50 feral *A. gossypii* apterae were transferred from the plants with a fine (00) brush to each inoculation chamber. The chambers were sealed with Parafilm (American Can, Neenah, WI) and held for 2.5 h at 26 °C. During this time the feral aphids contacted infective capilliconidia in the chambers. Then the inoculated aphids were transferred to the undersides of cotton leaves in the release field. The chamber treatment was omitted in 1995 because 1994 data indicated that the cadaver method was as effective and required less labor.

2.3. Predator exclusion cages

Predatory arthropods (coccinellid and chrysopid eggs, larvae, and adults) were located by visual inspection and removed from all treatment and control leaves on all replicates immediately before release of cadavers or chamber-inoculated aphids. In addition, predator exclusion cages made of 1-mm^2 mesh nylon netting were placed over certain replicates to determine whether predators, by feeding on infected aphids and/or reducing the population of healthy aphids, had an adverse effect on the survival and dispersal of the fungus. In 1994 all treatment and control leaves were caged with predator exclusion cages at 0 day after treatment (DAT), then at 8 DAT cages were removed from replicates 26–35. In 1995 treatment leaves in replicates 1–30 were caged at 0 DAT while replicates 31–45 were not caged. Because the

cages might have had a slight effect on the microclimate surrounding the infected aphids, any treatment effect could be due to some combination of excluding predators and enhanced survival of the fungus.

2.4. Sampling and diagnosis for N. fresenii

Neozygites fresenii prevalence in the California *A. gossypii* populations was determined prior to fungus introduction by collecting aphid-infested 7th node mainstem leaves from plants adjacent to release or control plants from replicates 1–15 in 1994 and 1995. These pre-release aphid-infested leaves and all other aphid-infested leaves collected throughout the sampling periods were placed into individual vials containing 70% ethanol. Additional aphid samples were collected 4 and 8 DAT in replicates 1–15 both years to assess the effect of climatic conditions on fungus sporulation and infectivity. We collected one half of each treatment leaf on 4 DAT, leaving the mid-vein intact on the half leaf remaining on the plant. The second half of these leaves was collected 8 DAT. Subsequent aphid-infested leaves in replicates 16–35 (1994) and 16–45 (1995) were collected weekly. The first mainstem leaf below the treatment leaf was collected first. Thereafter, each week a successively lower leaf was collected. If no mainstem leaf was available, a lateral leaf from the same plant height as the missing mainstem leaf was collected instead.

From each leaf sample a randomly chosen subsample of 50 aphids was squash mounted in acid fuchsin-lactophenol stain on microscope slides. Each aphid in a subsample was examined with phase microscopy at 200× for signs of *N. fresenii* infection. Aphids were diagnosed as positive for *N. fresenii* infection if they exhibited any of the following stages of infection: 1 = capilliconidia attached, 2 = protoplasts or hyphal bodies present, 3 = conidial stage (sporulation) or 4 = *N. fresenii*-killed + saprophytic fungi (Steinkraus et al., 1995).

2.5. Fate of released cadavers

To determine how well released cadavers adhered to leaves and sporulated, an additional test was conducted adjacent to and upwind of the test site at 0 DAT in 1995. The purposes of this test were to determine: (1) the percentage of cadavers that adhered to and sporulated on the undersides of release leaves and (2) the percentage of feral aphids infected by this release. The 7th node mainstem leaf on each of 10 cotton plants was hand-cleaned of predators and misted with water using a hand plant mister. Twenty-five cadavers were dispersed near the base of the petiole of each leaf. These leaves were not caged. One DAT, a piece of leaf (4 × 5 cm) containing and surrounding the cadaver release site was collected from 5 of the 10 leaves. The remaining 5 leaves were collected in the same manner 4 DAT. Diagnoses, as previously described, were made of all introduced cadavers and wild aphids in these samples.

2.6. Environmental data and statistical analysis

Environmental data were recorded daily at weather stations at the Shafter UC Research Station (1994) and at Los Banos near Chowchilla (1995) and were used to obtain maximum, minimum, and mean temperatures and relative humidities.

The number of aphids at each stage of infection was tabulated by replicate and treatment in each year. Percentage prevalence levels were determined for each replicate and mean prevalence for each treatment for each sampling date. Percentage prevalence is the percentage of cotton aphids infected with *N. fresenii* in the random subsample of aphids from each sample. The overall effects of the cadaver, chamber, and control treatments in 1994 and the cadaver and control treatment in 1995 were studied by combining the data from caged and uncaged replicates then comparing the means with ANOVA and the Tukey–Kramer HSD test (JMP, 2000). The mean prevalence of caged versus uncaged treatments was compared using ANOVA and the Student's *t* test (JMP, 2000).

3. Results and discussion

The absence of *N. fresenii*-infected aphids in all pre-release samples (16 Aug 1994, $n = 2246$; and 17 Aug 1995, $n = 2249$), early 1994 control samples ($n = 1500$) and all 1995 control samples ($n = 4871$) suggested that *N. fresenii* does not naturally occur in *A. gossypii* in San Joaquin Valley cotton (Table 1). This idea was further supported by an absence of published reports of natural *N. fresenii* infections from *A. gossypii* in California. In addition, no other species of entomopathogenic fungi were observed in all the diagnoses of aphids ($n = 19{,}374$ in 1994; and $n = 9739$ in 1995). Surveys during 1997, 1998, 1999, 2000, and 2001, for fungal pathogens of *A. gossypii* in the San Joaquin Valley (Godfrey et al., 2002) found none infected with *N. fresenii*. These studies suggest that from mid-August to late October entomopathogens do not naturally play a significant role in aphid control in San Joaquin Valley cotton.

3.1. 1994 Release

Though not significantly different, the overall mean percentage *N. fresenii* prevalences in 4 DAT (20 Aug) samples were 13.1 and 8.0% in the cadaver and chamber treatments, respectively (Table 1). The highest prevalence in any one replicate at 4 DAT was 30 and 20% in cadaver and chamber treatments, respectively (Table 2).

Table 1
Effect of cadaver, chamber, or control treatments on percentage of *A. gossypii* infected with *N. fresenii* in San Joaquin Valley, California, Cotton in 1994 and 1995

Date	Mean[a] % (SE) *A. gossypii* infected with *N. fresenii*				
	Cadaver	Chamber	Control	*F*	*P*
1994					
16 Aug[b]	0	0	0	—	—
20 Aug[c]	13.1 (2.7) a	8.0 (1.6) a	0 b	13.2	0.0001
24 Aug[d]	9.7 (2.9) a	7.6 (2.6) a	0 b	5.5	0.008
30 Aug	1.9 (1.1) a	2.4 (1) a	0.2 (0.1) a	1.6	0.203
6 Sep	11.7 (3.2) a	7.6 (3) a	0.5 (0.4) b	5.4	0.008
13 Sep	13.0 (4.8) a	7.9 (2.2) a	1.6 (1.1) b	3.4	0.041
27 Sep	14.1 (3.8) a	6.3 (2.2) a	0.9 (0.5) b	6.7	0.002
3 Oct	2.4 (0.7) a	3.9 (1.1) a	0.4 (0.2) b	5.2	0.008
1995					
17 Aug[b]	0	—	0	—	—
21 Aug[c]	4.1 (1) a	—	0 b	16.9	0.0003
25 Aug[d]	4.0 (1.6) a	—	0 b	6.6	0.016
5 Sep	4.7 (1.4) a	—	0 b	10.2	0.002
11 Sep	5.2 (2.2) a	—	0 b	5.6	0.021
21 Sep	2.5 (2.5) a	—	0 a	1.0	0.321
1 Oct	0	—	0	—	—

[a] Means followed by the same lower case letter within a row are not significantly different, Tukey–Kramer HSD, $\alpha = 0.05$.
[b] Pre-release sampling date.
[c] Four days after treatment (DAT).
[d] Eight DAT.

Table 2
Highest percentage *N. fresenii* infection in any one replicate in each treatment for each sampling date

Date	% Infection[a]		
	Cadaver	Chamber	Control
1994			
16 Aug	0	0	0
20 Aug	30	20	0
24 Aug	44	38	0
30 Aug	22	16	2
6 Sep	22	36	6
13 Sep	84	30	22
27 Sep	50	32	8
3 Oct	8	19	3
1995			
17 Aug	0	—	0
21 Aug	14	—	0
25 Aug	22	—	0
5 Sep	28	—	0
11 Sep	50	—	0
21 Sep	75	—	0
1 Oct	0	—	0

[a] Fifty aphids diagnosed in each replicate.

Overall mean prevalences in 8 DAT (24 Aug) samples were 9.7 and 7.6% in the cadaver and chamber treatments, respectively (Table 1). The highest prevalence in any one replicate at 8 DAT had increased to 44 and 38% in the cadaver and chamber treatments, respectively (Table 2). These results indicate that *N. fresenii* had successfully sporulated from the release cadavers in both treatments and infected feral California *A. gossypii*.

The highest overall mean percentage infection in samples collected after 8 DAT was 14.1% on 27 Sep and 7.9% on 13 Sep in cadaver and chamber treatments, respectively (Table 1). The highest percentage infection in any one replicate after 8 DAT was 84% on 13 Sep and 36% on 6 Sep in cadaver and chamber treatments, respectively (Table 2). The data indicated that the cadaver treatment may be more effective than the chamber treatment as a method of releasing the fungus. Because the cadaver method of release was more effective and much simpler than the chamber method, we did not use the chamber method in 1995.

In 1994 from 30 Aug to 27 Sep, the mean prevalence showed a general increase in all three treatments (cadaver, chamber and control). On 13 and 27 Sep, mean *N. fresenii* prevalences in the cadaver treatment reached the levels of 12–15% considered imminent for epizootics to develop (Hollingsworth et al., 1995). However, prevalences of the fungus did not continue to increase to levels of 30–100% as generally occur in natural epizootics in the Midsouth (Steinkraus et al., 1995).

The first fungal infections in control aphids were detected in 30 Aug samples (Table 1). On this date two control replicates each contained 2% infected aphids (Table 2). By 13 Sep, infection in one control replicate reached 22%. While percentages of *N. fresenii*-infected aphids found in control aphids were not high, with a maximum mean prevalence of 1.6% on 13 Sep, the presence of *N. fresenii* in aphids from controls is important because it indicates that the fungus spread from

Table 3
Total number of aphids in each stage of *N. fresenii* infection for cadaver and chamber treatments for each sampling date

Date	Cadaver				Chamber			
	Capilliconidia only	Protoplasts/ hypal bodies	Conidial stage	*N. fresenii* + saprophytes	Capilliconidia only	Protoplasts/ hypal bodies	Conidial stage	*N. fresenii* + Saprophytes
1994								
16 Aug[a]	0	0	0	0	0	0	0	0
20 Aug	42	18	18	20	18	13	10	16
24 Aug	12	23	23	10	13	17	15	8
30 Aug	14	1	2	2	15	4	2	0
6 Sep	43	13	24	11	25	9	11	5
13 Sep	12	5	29	89	14	14	23	28
27 Sep	5	2	3	131	2	3	7	51
3 Oct	1	3	1	12	2	4	5	24
1995								
17 Aug[a]	0	0	0	0	—	—	—	—
21 Aug	14	2	14	0	–	—	—	—
25 Aug	9	1	20	1	—	—	—	—
5 Sep	19	9	12	19	—	—	—	—
11 Sep	37	2	15	16	—	—	—	—
21 Sep	0	0	0	3	—	—	—	—
1 Oct	0	0	0	0	—	—	—	—

[a] Pre-release samples.

release plants to control plants that were a minimum of 3 m distant.

The life cycle of *N. fresenii* requires 3–4 days from the time a host contacts a capilliconidium until death of the host and sporulation of the fungus (Steinkraus et al., 1991). The data in Table 3 show that sampling dates with high numbers of aphids in early stages of the infection process (capilliconidia only) were followed by sampling dates with high numbers of aphids in the middle (protoplasts/hyphal bodies) and late stages (conidial and *N. fresenii* + saprophytes) of *N. fresenii* infections. These data show that the fungus successfully went through numerous cycles of asexual reproduction in the feral aphid population.

3.2. 1995 Release

The overall mean prevalences in 4 DAT (21 Aug) and 8 DAT (25 Aug) cadaver treatment samples were 4.1 and 4.0%, respectively (Table 1). The highest percentage infection in any one replicate at 4 and 8 DAT were 14 and 22%, respectively (Table 2). The highest overall mean percentage infection in the cadaver treatment after 8 DAT was 5.2% on 11 Sep (Table 1). The highest percentage infection in any one replicate after 8 DAT was 50 and 75% on 11 and 21 Sep, respectively (Table 2).

Infection levels in 1995 were lower than in 1994. The reasons for this are unclear but may have been related to large numbers of coccinellids observed in the organic cotton field in Chowchilla. Predation on aphids could negatively impact survival and spread of *N. fresenii* in the aphid population by removing inoculum and potential hosts.

Our release of *N. fresenii* was not the first attempt at using this fungal pathogen for aphid control. Wilding (1981) made releases of *N. fresenii* into populations of the black bean aphid, *A. fabae* infesting *Vicia* beans, by distributing living infected aphids or fungus-killed aphids. The introduced fungi became established in the aphid populations and after the release in some years significantly reduced their numbers. Wilding (1981) found that the yield of beans was twice as high in fungus-treated plots as in untreated beans, but only half that in plots treated with insecticides.

3.3. 1995 Fate of release cadavers

An overall mean of 73.6% ($n = 125$) cadavers remained attached to undersides of treatment leaves 1 DAT (Table 4). The overall mean percentage sporulation from cadavers recovered 1 DAT was 84.6% ($n = 92$) and no feral aphids were found infected (an expected result because signs of infection are not observable this early). At 4 DAT, an overall mean of 39.4% ($n = 125$) introduced cadavers remained attached to leaves. The mean percentage sporulation of cadavers at 4 DAT was 100% ($n = 48$) and the mean percentage infected wild aphids was 62.4% ($n = 21$). These data show that the cadaver release method effectively places fungal inoculum amid the feral aphid population. Laboratory studies have shown that conidial discharge begins about 1 h after cadavers are removed from frozen storage and placed at room temperature in a humid chamber. Within 5 h most of the primary conidia have been formed, discharged, and formed infective capilliconidia (Steinkraus and Slaymaker, 1994). Therefore, sprinkling cadavers

Table 4
Fate of released cadavers and wild aphids (1995)

Replication	% Cadavers sporulated	% Cadavers recovered	% Wild aphids infected (*n*)
Day 1			
1	69	52	0 (23)
2	60	80	0 (10)
3	100	84	0 (19)
4	100	84	0 (15)
5	94	68	0 (57)
Day 4			
6	100	24	0 (3)
7	100	25	75 (4)
8	100	76	80 (5)
9	100	24	57 (7)
10	100	48	100 (2)

among aphid colonies on misted leaves (the cadaver method) is a simple and effective method for releasing the fungus.

3.4. Effect of predator exclusion cages 1994 and 1995

In all cadaver (1994 and 1995) and chamber (1994) treatments, caged treatments had higher mean percentages of infected aphids than uncaged, though usually the differences were not significant (Table 5). In 1994 the difference between caged and uncaged replicates became significant after 27 Sep. The increased infection in caged treatments most likely was due to reduced coccinellid predation on both healthy and infected aphids. This result suggests that introductions of *N. fresenii* and perhaps other aphid pathogens, could be enhanced by protecting release sites from aphid predators. The interaction of multiple natural enemies on a prey population is complex and may be synergistic, additive, or nonadditive (Rosenheim et al., 1995; Roy et al., 1998). Similane (1996) found that larvae and adults of *Coccinella septempuncta* L. (Coleoptera: Coccinellidae) readily consumed *N. fresenii*-infected *A. gossypii*. Pell

Table 5
Effect of predator exclusion cages on mean percentage *A. gossypii* infected with *N. fresenii* in 1994 and 1995

Date	Mean[a] % infected with *N. fresenii* (SE)					
	Treatment	Cage	No cage	DF	*F*	*P*
1994						
20 Aug	Cadaver	13.1 (2.6)	—	—	—	—
	Chamber	8.0 (1.6)	—	—	—	—
	Control	0	—	—	—	—
24 Aug	Cadaver	0	—	—	—	—
	Chamber	0	—	—	—	—
	Control	0	—	—	—	—
30 Aug	Cadaver	3.2 (1.5) a	0.6 (1.5) a	18	1.46	0.243
	Chamber	2.5 (1.4) a	2.2 (1.4) a	18	0.03	0.873
	Control	0.4 (0.2) a	0.0 (0.2) a	17	2.01	0.174
6 Sep	Cadaver	14.2 (4.2) a	8.5 (4.8) a	16	0.79	0.388
	Chamber	8.7 (4.3) a	6.3 (4.6) a	13	0.15	0.700
	Control	0.7 (0.6) a	0.4 (0.6) a	16	0.08	0.785
13 Sep	Cadaver	19.8 (6.5) a	6.2 (6.5) a	18	2.15	0.160
	Chamber	11.1 (3.0) a	4.8 (3.0) a	18	2.18	0.157
	Control	2.8 (1.6) a	0.4 (1.6) a	18	1.16	0.296
27 Sep	Cadaver	21.8 (4.9) a	6.4 (4.9) b	18	4.94	0.039
	Chamber	10.6 (2.8) a	2.0 (2.8) b	18	4.67	0.044
	Control	0.6 (0.7) a	1.4 (0.7) a	18	0.59	0.449
3 Oct	Cadaver	4.0 (0.9) a	0.8 (0.9) b	18	5.60	0.029
	Chamber	6.7 (1.3) a	1.2 (1.3) b	18	8.94	0.008
	Control	0.5 (0.3) a	0.4 (0.3) a	18	0.02	0.879
1995						
21 Aug	Cadaver	4.1 (0.7)	—	28	—	0.0003
	Control	0	—	28	—	0.0003
25 Aug	Cadaver	4.0 (1.1)	—	28	—	0.016
	Control	0	–	28	—	0.016
5 Sep	Cadaver	7.3 (1.9) a	2.0 (1.9) a	28	3.65	0.066
	Control	0 a	0 a	28	—	—
11 Sep	Cadaver	9.4 (3.0) a	1.1 (3.0) a	28	3.78	0.062
	Control	0	0	28	—	—
21 Sep	Cadaver	5.0 (3.5) a	0 a	28	1.00	0.326
	Control	0	0	28	—	—
10 Oct	Cadaver	0	0	28	—	—
	Control	0	0	28	—	—

[a] Means within a row followed by the same lower case letter are not significantly different, Student's *t* test, (JMP, 2000).

et al. (1997) and Roy et al. (2001) found that *C. septempunctata* fed on aphids infected with *Erynia neoaphidis* Remaudiére and Hennebert (Zygomycetes: Entomophthorales)-infected *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae), reducing the amount of available fungal inoculum, but the coccinellids were also able to vector the fungal pathogen, enhancing its movement within fields.

3.5. Environmental factors

In 1994, the mean maximum and minimum daily temperatures were 33.1 °C (range = 23.9–40.0 °C) and 13.6 °C (range = 8.9–18.9 °C), respectively. The mean maximum and minimum daily relative humidities were 79.9% (range = 60.7–95.6%) and 22.6% (range = 7.0–59.4%), respectively. In 1995, the mean maximum and minimum daily temperatures were 31.3 °C (range = 24.4–36.7 °C) and 12.7 °C (range = 8.3–20.6 °C), respectively. The mean maximum and minimum daily relative humidities were 88.4% (range = 71.8– 97.1%) and 33.5% (range = 20.4–44.6%), respectively.

The low relative humidities found in the San Joaquin Valley release fields could adversely affect the survival and dispersal of *N. fresenii* within a field. Steinkraus et al. (1999) showed that aerially dispersed primary conidia of *N. fresenii* were of importance in the rapid spread of the pathogen within fields and subsequent rapid development of epizootics. It is also known that primary conidia survive poorly at relative humidities below 75% (Steinkraus and Slaymaker, 1994). In the 1994 and 1995 release fields, minimum relative humidities were below 75% each day during the trial. This could reduce survival of aerial primary conidia and thus reduce long-range aerial dispersal of the fungus and may limit the potential of *N. fresenii* in San Joaquin Valley cotton. Survival and short range spread of the fungus in aphid populations on leaves within plants and between plants under the canopy may be less affected by low humidity due to higher humidities present within the boundary layer of leaves (Ferro and Southwick, 1984).

4. Conclusions

Neozygites fresenii from Arkansas cotton aphids was infective to California *A. gossypii* and was successfully introduced and established in cotton aphid populations. Both cadaver and chamber inoculation release methods were successful, but the cadaver release was much less labor intensive and generally resulted in higher infection levels, so it is preferable as a release method. Protecting released fungal inoculum and aphid populations within release sites with cages or other predator exclusion methods may lead to higher infection levels. The pathogen releases in 1994 and 1995 in the San Joaquin Valley did not result in epizootics. This could have been due to an inhibitory effect of relative humidities lower than 75% that occurred at some time each day during the 1994 and 1995 trials, predation by aphid predators, insufficient quantities of release inoculum, lower than optimum aphid numbers for epizootic development, or other unknown factors.

After introduction in 1994 and 1995 *N. fresenii* prevalence increased, new cycles of infection occurred and infection in aphid populations spread within release plants, and at least 3 m from release plants in 1994. Infection levels considered imminent for natural epizootics (ca. 15%) in Midsouth and Southeast cotton-growing regions of the US (Hollingsworth et al., 1995) were attained in mean percentage infection in 1994 and in individual replicates in 1994 and 1995. Our data show that *N. fresenii* persisted in the trial fields until early October in 1994 and late September in 1995, but *N. fresenii* has not been detected subsequently in the San Joaquin Valley (Godfrey et al., 2001). Long-term persistence of the fungus has not been determined. Establishment and persistence of entomophthorales in areas where they do not naturally occur may require multiple releases and several to many years before full establishment occurs (Hajek and Roberts, 1991).

The release methods for *N. fresenii* developed in this study may have application for the recently discovered pest of soybeans, *Aphis glycines* Matsumura (Homoptera: Aphididae), in midwestern soybean fields. Preliminary data from *A. glycines* populations in Michigan have shown that *N. fresenii* was found naturally infecting *A. glycines* and causing epizootics (D. Steinkraus, C. Difonzo, unpublished data). Early season releases of *N. fresenii* into *A. glycines*-infested Midwestern soybean could help control this new pest.

Introduction methods were developed and compared, levels of *N. fresenii* infection that can initiate epizootics were attained and *N. fresenii* persisted until the end of the sampling period. Regular irrigation, early season introduction and low predator pressure may benefit pathogen enhancement and successful establishment of *N. fresenii* in areas where it does not occur naturally. Future progress in in vitro culturing methods and in resting spore biology may greatly enhance introduction methodology. Such advances may lead to the ability to produce amounts of inoculum sufficient for additional, larger-scaled epizootiological studies which could increase the area of establishment of *N. fresenii* and improve aphid control.

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