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Concentration of the Systemic Gametocide, TD-1123, in Cotton Nectar, and Honeybee Response[1]

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ABSTRACT

The production of hybrid cotton (*Gossypium hirsutum* L.) seed requires both a system of sterilizing one parent in the crossing block and the effective use of a pollen vector. This paper reports results from studies in which a systemic gametocide was used to produce male-sterile flowers and also reports behavioral responses of honeybees (*Apis mellifera* L.) to the gametocide. Field and greenhouse tests were conducted during 1984 and 1985 to determine the quantity of the foliar-applied systemic gametocide, TD-1123, that was translocated to the floral and bracteal nectar of cotton (*G. hirsutum* L. and *G. barbadense* L.). Field observations and a laboratory bioassay evaluated honeybee response to TD-1123 in nectar or to TD-1123-laced sucrose solutions. The gametocide was found in floral nectar in the field (<1–542 mg L^{-1}) (bracteal nectar not collected) and in both floral and bracteal nectar (11–151 mg L^{-1}) in the greenhouse studies. The highest concentrations of TD-1123 were found in the floral nectar of upland cotton sampled 1 day after spraying (542 mg L^{-1}). Honeybee consumption of 0.84 *M* sucrose solutions that included TD-1123 was not significantly increased at 50 mg L^{-1} but significantly increased at 75 mg L^{-1} of the gametocide. Floral visitation by foraging honeybees as measured in the field was not affected by the gametocide. We concluded that although the gametocide occurs in the nectar of treated cotton plants, it should not pose a threat to adequate pollination activity by the honeybee.

Additional index words: Hybrid cotton, Pollination, Nectar physiology.

CONSIDERABLE research effort has been directed toward the development of breeding stocks and techniques needed to produce hybrid cotton (*Gossypium hirsutum* L. and *G. barbadense* L.) using genetic-cytoplasmic male sterility (cms) (3, 4, 6). Generally, the techniques are successful but significant costs are incurred in developing and maintaining breeding lines. Two, 2-way crosses must be made in sequence; first the cms female parent (A line) is crossed [usually by honeybees, *Apis mellifera* L. (5)] with a pollen fertile maintainer (B line) in order to increase the quantity of A-line seed. This single cross progeny is then crossed, again usually by honeybees, in larger fields with the line incorporating fertility restoration (R line) to produce the quantity of hybrid seed needed for sale to the cotton producer. This procedure follows an intense and costly period of initial development and testing of A- and R-line parents, which has resulted in real concern about the economic feasibility of hybrid cotton using cms systems.

Parent selection and breeding procedures to produce hybrid cotton seed are simplified (genetically) when using a selective gametocide. A broad-spectrum of plant types can be used as parents, thus eliminating much of the time devoted to development of parental lines and increasing the likelihood of obtaining desired characters and hybrid vigor in the progeny (6). The induction of male sterility in cotton by chemical gametocides was first tried in 1956 (1) using 2,3-dichloroisobutanoate (FW-450) as a foliar spray. This was unsuccessful because FW-450 lacked selectivity, causing male and female sterility. More recently, potassium, 3,4-dichloro-5-isothiazolecarboxylate [TD-1123, (7, 9)], another chemical gametocide, has been tested to determine application techniques and rates (6). This chemical is systemic, accumulates in bracts and developing bolls (9), and has male gametocidal properties. It also causes some phytotoxicity. The frequency and rates of application of TD-1123 necessary to maintain male sterility (due to inhibition of pollen initiation) depend on the cultivar and temperature (6). Generally, the plants must be treated every 10 to 12 days. The plants are first sprayed when they are in the initial "pin-head" square stage. These flowers open about 2 weeks later and the pollen-sterilizing effect is active for the 14 to 28 days prior to anthesis. An indicator that treatments have been inadequate is the development of pollen in, and the dehiscence of, anthers located near the base of the style.

Honeybees have been used successfully to accomplish cross-pollination and hybrid cotton seed production in largescale field studies in Arizona and the High Plains of Texas (2, 4, 8, 10, 11, 12). The experimental use of TD-1123 raised questions about what quantities of TD-1123 might accumulate in bracteal and floral nectar and what effect TD-1123 in floral nectar might have on honeybee visitation to cotton flowers. Our objectives were to determine if (i) foliar-applied TD-1123 was translocated to the floral and bracteal nectar and, (ii) if so, how honeybees would be affected. This paper reports the results of field, greenhouse, and laboratory experiments conducted in Arizona in 1984 and 1985.

MATERIALS AND METHODS

Greenhouse Experiment

In a glass greenhouse, previously established plants (one per pot) of Pima (*G. barbadense*) cotton (either 'Pima S-6' or a glandless Pima) were selected for gametocide treatments. Treatments of 0, 1.0, 1.5, and 2.0 mg L^{-1} of TD-1123 were applied in irrigation water every other day to each of five replications (one plant each) selected randomly (completely

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randomized design). Each plant received 460 mL of water per irrigation, which was adequate to prevent wilting for about 2 days. No other water was applied during the test. The greenhouse was kept on a 12-h photoperiod using high intensity Na lamps, and temperatures were controlled at 26 to 28°C at night and 32 to 36°C during the day. Treatments were applied on 20 Nov. 1984 when the plants were in first flower stage. By 17 December (27 days after treatment initiation), minor phytotoxic symptoms (reddening) were noted on the bracteal tips of the 1.5 and 2.0 mg L^{-1} treatments of the Pima plants. Since flowers on treated plants were still producing pollen, and plant mass had increased, treatment concentrations were doubled on 22 December (using the same volume of liquid per plant) and continued until the experiment was terminated on 4 Jan. 1985. By this time, the two highest treatment levels (3 and 4 mg L^{-1}) had sterile flowers and plant height was being stunted.

Nectar was collected by calibrated 20 or 50-μL microcapillary pipets from the greenhouse plants as it became available from flowers and bracts (inner-bracteal and sub-bracteal nectar was combined). All pipets containing samples were plugged with CritoSeal® (Sherwood Medical Industries, Lancer Division, St. Louis, MO)[3] and placed in a freezer until high performance liquid chromatography (HPLC) analysis. The method of analysis was liquid chromatography of the "raw" nectar using a Tracor® 4 Model no. 995 pump with a Model no. 970A variable wavelength detector (Tracor, Austin, TX). The elution solvent (1 *M* HOAC: Methanol; 75/25) was pumped (approximately 9.8 MPa) at 1.425 mL min^{-1} through either a Whatman Partisil PXS 10/25 or an IBM® 5/25 C18 column. Results were quantitated by comparison with values obtained from a standard curve derived from similar concentrations of the authentic chemical obtained as a white powder from the Pennwalt Corp., Tacoma, WA.

Field Experiment

Sixteen rows (188-m long with a row spacing of 1 m) of upland cotton (*G. hirsutum*, 'DP-41') were planted at the University of Arizona Marana Exp. Farm. The soil on this farm is classified as a Pima clay loam, which is a member of the fine-silty, mixed, Thermic family of Typic Torrifluvents. The cotton was treated with TD-1123 approximately every 2 weeks beginning at early flowering (25 June 1985). There was no replication in the experiment. The gametocide was applied by spraying the plants with a solution using a 11.36-L sprayer at 187 to 443 L ha^{-1} water depending on the size of the cotton plants. Actual rates of TD-1123 ranged from 0.08 to 0.11 kg ai ha^{-1} depending on stage of growth. As the plants grew, rates and water volumes were increased to cover the greater leaf area and to wet all the available foliage. Honeybee visitation observations were made twice weekly between 1000 and 1200 h by walking along the rows and counting the number of open flowers and flowers with bees in them; at least 400 flowers were counted for each treatment at each observation period. Two observers walked through the plots alternating between treatments during each observation period; flower and bee count data were averaged and analyzed by ANOVA.

Floral nectar samples were collected (using calibrated microcapillary pipets) on eight dates. Approximately 20 flowers $treatment^{-1}$ were bagged (nylon netting) in the early morning before they opened. These flowers were picked in the early afternoon (1330–1345 h), placed in a plastic bag on ice, and taken to the laboratory. The nectar was removed by capillary pipet between 1430 and 1700 h each sampling day. These pipets were sealed and frozen until chemical analyses were performed. Nectar from only two to four flowers (≃ 20 μL) was sufficient for an individual HPLC analysis. A Kratos model 400 pump and model 773 detector was used with a Brownlee SPERI I-5, RP-18 microbore (0.2 × 22 cm) column. The elution solvent was changed slightly to 1 *M* HOAC/Methanol/Acetonitrile (80:17.5:2.5) pumped at 0.30 mL min^{-1}. Detection was at 280 nm at either 0.01 or 0.02 absorbance units full scale (AUFS) with automatic integration and recording of the detector signal.

Bioassay of Honeybee Response

Two queen-right colonies of bees were moved into an indoor flight room (6.1 × 2.8 × 2.4 m) held at 24 to 28°C. The room was illuminated for 12 h each day with fluorescent lights. Each colony contained four frames of bees (ca. 5000) with queen and brood. Both colonies were fed bee-collected pollen (approximately 70% saguaro) mixed with type 50 sugar (equal parts glucose and fructose, 4.27 *M*), 3:1 wt/wt. Water was available ad libitum throughout the test period. Bees were trained to collect a solution of 0.84 *M* sucrose at sites on a continuously rotating table 3.5 m from the hives.

Treatment solutions of 0, 50, and 75 mg TD-1123 L^{-1} dissolved in 0.84 *M* sucrose solution were prepared using gametocide obtained from the Pennwalt Corp. For the tests, the training sites were removed and replaced with three sites identical in appearance to the training sites. The sites were machined from clear Plexiglas® (4.9-cm diam × 4.8-cm high). The bottom section had six holes drilled to hold six 1-mL glass vials. The top section had six smaller holes allowing only individual bees to access one vial at a time (Fig. 1).

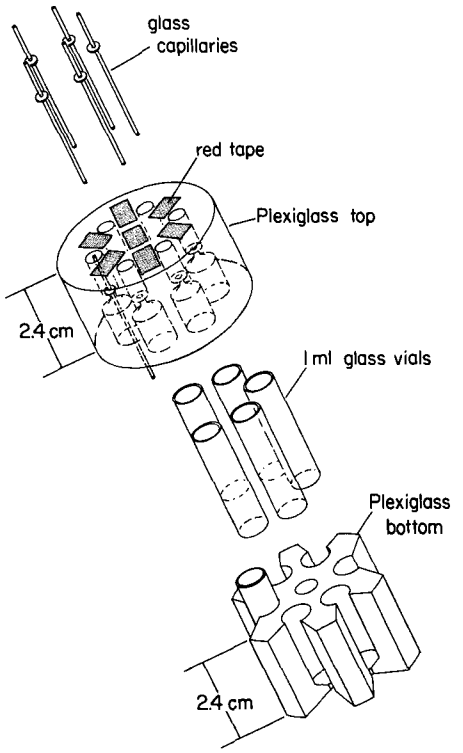

Fig. 1. Diagramatic sketch of Plexiglas® training and test site used in the gametocide bioassay.

[3] Mention of a trademark, proprietary product or vendor does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

The individual vials were centered 13 mm apart in a circle in the holder, and the bees could choose among the vials simply by turning from one to another. The top of the Plexiglas® holder served as the landing platform. To obtain the sucrose solution, the bee had to extend her proboscis into a glass capillary that extended into the vial. This test site design restricted access to the sugar solutions and inhibited the development of a frenzied ("robbing") behavior among the foragers. For testing bee response, three vials in each test site contained only 0.84 *M* sucrose and alternated between the three treatment vials. As bees consumed the solutions, each test site was removed as soon as one vial was nearly empty. Seven tests were conducted over a 4-day period. We used a completely randomized experimental design with three treatments each replicated six times (vials) per test. Elapsed time for tests averaged 26 ± 6 min with 10 to 15 bees per test. Tukey's (Studentized range) test was used to compare treatment means and ANOVA performed to determine significance of results.

RESULTS AND DISCUSSION

Greenhouse Experiment

Under greenhouse conditions, the Pima plants secreted both floral and bracteal nectar, but samples had to be pooled (across dates) to obtain sufficient quantities for analysis by the Tracor HPLC system. The gametocide was secreted into both the floral and bracteal nectar (Table 1). While higher concentrations of TD-1123 were found in the floral nectar, they were not obviously related to treatment level. In contrast, there was a significant ($p < 0.01$) positive relationship between treatment level and TD-1123 concentrations in bracteal nectar ($r^2 = 0.78$).

Field Experiment

Previous analyses of floral nectar samples collected from preliminary (agronomic) tests (1982–1984) established that the TD-1123 gametocide was secreted in floral nectar in amounts generally ranging from 3 to 64 mg L^{-1} (Loper, unpublished data). The 0.08 to 0.11 kg ha^{-1} treatment levels in the 1985 study caused reddening of the leaves, a typical phytotoxic symptom (7), and the gametocide levels in the floral nectar soon after application were high, ranging from 264 to 542 mg L^{-1} (Table 2). The concentration of TD-1123 declined rapidly with time, decreasing to 20% of the initial level after only 6 days. Spraying every 2 weeks was not frequent enough to maintain full male sterility; for example on 26 August, only 88% of the flowers were fully male-sterile. Nectar concentrations of the gametocide 10 to 11 days after spraying were in the range of 20 to 37 mg L^{-1} (Table 2).

Six separate honeybee visitation counts were made over an 11-day period. Bee visitation to untreated cms A-line plots averaged 2.45% (63 bees per 2574 flowers) and treated plots averaged 2.90% (87 bees per 2997 flowers). On five of the six counts, bee visitation was greater on the TD-1123 treated plots. Although these differences are not statistically significant, they do indicate that visitation was good [i.e., exceeding 0.5%, generally considered necessary for good pollen dispersal (12)].

Bioassay Studies

The results of the bioassay (Table 3) indicated that the bees consumed more sucrose solution when the gametocide was present at concentrations similar to those found in floral nectar. Consumption was not significantly higher at 50 mg L^{-1} but was significantly ($p < 0.01$) greater at 75 mg L^{-1} compared to the sucrose control having no TD-1123. No behavioral effects were noticed during either the laboratory bioassay or in the field on 26 August when TD-1123 in floral

Table 1. Concentration of the gametocide TD-1123 in floral and bracteal nectar of Pima Cotton, greenhouse experiment, 1984 to 1985.

Nectary	Treatment†	Days after first application (20 Nov.)	Concentration
	mg L^{-1}		mg L^{-1}‡
Flower	0	45	0
	2	45	129
	3	45	151
	4	34	145
Bract	0	38	0
	1	29	11
	1.5	31	17
	2.0	16	27
	2.0	45	86
	4.0	36	86

† Total quantities of TD-1123 ai added from 20 Nov. 1984 to 4 Jan. 1985 for treatment levels 0, 1, 1.5, 2, 3, and 4 were 0, 12.42, 18.63, 24.84, 37.26, and 49.68 mg $plant^{-1}$, respectively.

‡ Data are results of individual analyses on samples pooled to provide sufficient sample size for HPLC analysis. Rank correlation between treatment and bracteal nectar concentrations were significant; $r^2 = 0.78$, $p < 0.05$, $n = 6$.

Table 2. Concentration of gametocide TD-1123 in floral nectar of upland cotton, field experiment, 1985, Marana, AZ.

Sampling date	No. of sprays	Days since last spray†	TD-1123 concentration	
			Means‡	Range
			mg L^{-1}	
21 August	5	1	426.0	264.6–542.5
26 July	3	4	156.2	100.0–184.9
26 August	5	6	90.0	43.7–172.4
16 August	4	10	24.4	20.1– 29.8
19 July	2	11	33.6	28.8– 37.0
19 August	4	13	17.1	16.0– 17.8
21 August	4	15	16.1	12.1– 22.1
26 August	4	20	1.5	0 – 3.0

† Spray application dates: 25 June, 8 and 22 July, 6 and 20 August (not all rows treated on 20 August).

‡ Average of three samples. Rank correlation between days since last spray and concentration was highly significant; $r^2 = -0.799$, $p < 0.001$, $n = 24$.

Table 3. Amount of 0.84 *M* sucrose solution with three gametocide concentrations consumed by honeybees selecting among sugar solutions with and without added gametocide TD-1123.

Date	Test no.	Replications	Concentration (mg L^{-1})		
			0	50	75
			μL		
11 Jan. 1982	1	6	310	300	400
	2	6	350	350	420
12 Jan. 1982	3	6	300	380	430
	4	6	360	390	410
13 Jan. 1982	5	6	390	430	460
	6	6	380	460	470
15 Jan. 1982	7	6	370	390	420
		Mean†	350a	390ab	430b

† Means within row with letter(s) in common are not significantly different, according to Tukey's (Studentized range); $p < 0.01$, $n = 126$.

nectar averaged 90 mg L^{-1}, and bee visitation was very high (3.7%).

CONCLUSIONS

We have demonstrated that the gametocide TD-1123 is translocated within the cotton plant and secreted in both floral and bracteal nectar. Although the initial application of the gametocide caused some phototoxicity and therefore high concentrations of TD-1123 in the nectar, the rate of degradation or dilution by increase of plant mass over time is relevant. In actual practice, application levels that are gametocidal, but only minimally phytotoxic, would result in TD-1123 levels more in line with those detected at 6 to 10 days after spray levels. Our analytical results suggest that when floral nectar levels of TD-1123 drop below 25 to 35 mg L^{-1}, the plant may begin producing at least partially male-fertile flowers (anthers lower on the style dehisce some pollen while others contain no pollen).

We found that the presence of 75 mg TD-1123 L^{-1} in sucrose solution increased consumption by honeybees. Also, the flowers on treated plants were at least as attractive to foraging honeybees as those on untreated plants. Since the gametocide was secreted in the nectar, we analyzed for TD-1123 in honey from a colony whose workers were fed TD-1123 sugar water. We found TD-1123 and a metabolite in the ripened honey (Loper, unpublished data). However, we would not expect to be able to find it from field colonies where workers could be collecting nectar from a possible +25 000 ha foraging area. In actual practice, the honey from any colonies rented for use as pollinators in gametocidally treated cotton will be destroyed. While this could increase colony rental fees, it would be cheaper than having to obtain an EPA registration for this use of the chemical. We conclude that although the gametocide is secreted into the nectar of treated cotton plants and may be present in the honey, it should pose no threat to adequate pollination activity by the honeybee.

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