Reduction of the Severity of Verticillium Wilt of Cotton By The Growth Retardant, Tributyl [(5-chloro-2-thienyl)methyl] phosphonium Chloride

D. C. Erwin, S. D. Tsai, and R. A. Khan

Department of Plant Pathology, University of California, Riverside, 92502.

Funds for this research were provided by the United States Department of Agriculture made available through Cotton Incorporated, Raleigh, N.C. We wish to acknowledge with thanks the assistance of H. B. Cooper and associates, Agricultural Research Service, U.S. Department of Agriculture, in the field research.

Accepted for publication 22 July 1975.

ABSTRACT

The growth retardant, tributyl[(5-chloro-2-thienyl)-methyl]phosphonium chloride (TTMP), reduced the growth of cotton by about one third when used in soil in pots (50 mg/2 kg). Foliar application at 40-80 μg/ml had little effect on growth of plants in the greenhouse or in the field. Wilt symptoms were delayed when TTMP was applied to growing plants by soil drench in the greenhouse and subsequently inoculated with *Verticillium albo-atrum*. There were fewer propagules of *V. albo-atrum* in petioles of treated plants than in petioles of control plants. Foliar application in the field with TTMP (40 and 80 μg/ml) in June also consistently reduced the number of *V. albo-atrum* propagules in petiole tissue when assayed as late as September. In addition, the number of bolls per meter of row and the yield of cotton seed and lint were significantly increased.

Phytopathology 66:106-110

Buchenauer and Erwin (5) reported that *N,N*-dimethylpiperidinium iodide (DPII) and *N,N*-dimethylpyrrolidinium iodide (DPYI) acted as growth retardants on cotton. The properties of growth retardants similar to these compounds were also recently reported by Zeeh et al. (19). Buchenauer and Erwin (6) also reported that DPYI and DPII were not fungitoxic to *Verticillium albo-atrum* in vitro, but when used as a soil drench on potted cotton plants, suppressed the severity of symptoms expression of Verticillium wilt, and also reduced the numbers of propagules of *V. albo-atrum* in artificially inoculated plants. Hemigossypol, which is considered to be a phytoalexin in cotton (2, 3, 17, 18), was detected to a larger extent in ethanolic extracts from inoculated plants treated with DPYI and DPII than from nontreated plants (6). These results strongly suggested that these compounds induced or stimulated production of hemigossypol.

We report here that the nonfungitoxic growth retardant, tributyl[(5-chloro-2-thienyl)methyl]phosphonium chloride (TTMP), reduced the severity of Verticillium wilt and concurrently the number of *V. albo-atrum* propagules in naturally infected and in artificially inoculated cotton plants, and that TTMP increased the yield of seed and lint. A part of this study was reported as an abstract (10).

MATERIALS AND METHODS.—Cotton (*Gossypium hirsutum* L. 'SJ-1') plants were grown in the greenhouse at 21 C night and about 28 C day temperatures in sandy loam soil in 15 × 15 cm pots. Each week plants were fertilized with a nutrient solution which consisted of (ml/liter) the following molar solutions: $Ca(NO_3)_2 \cdot 4H_2O$, 30.0; KH_2PO_4, 6.0; $NaH_2PO_4 \cdot H_2O$, 1.0; and $MgSO_4 \cdot 7H_2O$, 2.0. Minor elements were added to provide (μg/ml) B, 0.5; Mn, 0.5; Zn, 0.05; Cu, 0.02; Mo, 0.01; and Fe, 1.0. Reference to use of M $NaHPO_4 \cdot H_2O$ at 10.0 ml/liter in a previous paper (9) was an error. Each plant was inoculated by stem puncture with about 10-20 spores of *V. albo-atrum* Reinke & Berth. (defoliating microslerotial isolate V-3H) produced on a shaker in the Malca synthetic medium (12). Concentration was determined with a hemocytometer. In some experiments the number of spores was verified by streaking aliquots (0.01 ml) on sodium polypectate agar (SPA) (11).

The growth regulant TTMP (CHE 8728, obtained from the Chemagro Division of Baychem Corporation), was water soluble and 90% active. Dosages expressed are of the active ingredient. In the greenhouse TTMP was applied as a drench to soil. In vitro fungitoxicity was determined by measuring radial growth of *V. albo-atrum* at 25 C on potato dextrose agar amended with varying concentrations of TTMP.

TTMP was applied in the field (Wilcox farm, Tulare County, California) as a foliar spray with a pressurized (1 kg/cm^2) hand sprayer (AgriTechnical Associates, Riverside, California) equipped with a Spraying Systems® T-jet 8001 fan nozzle until foliage was thoroughly wetted on 19 June (one time) and on both 19 June and 27 June (two times). On 19 June plants were in the initial square (flower bud) formation stage. The field was heavily infested with *V. albo-atrum*.

The method for determination of the population of *V. albo-atrum* propagules in petiole tissue was a modification of that of Saalting (13) and Buchenauer and Erwin (4, 6). Petioles collected from the uppermost fully expanded leaves were surface decontaminated in 0.5% sodium hypochlorite for 5 minutes and washed in sterile water. The end of each petiole was removed to ensure that xylem tissue in which the sodium hypochlorite might have killed *V. albo-atrum* was not assayed. The petiole tissue was blotted on paper towels, weighed (2.0 g green weight), and blended in 200 ml sterile distilled water in a Sorvall Omnimixer® at full speed (three 20-second periods). Aliquots (1 ml of 1:10 dilution) were spread on the surface of each of at least five petri plates of SPA (11), and incubated at 21-24 C for 8-12 days. *V. albo-atrum* colonies producing typical microsclerotia were counted by use of a dissecting microscope. When stem tissue was

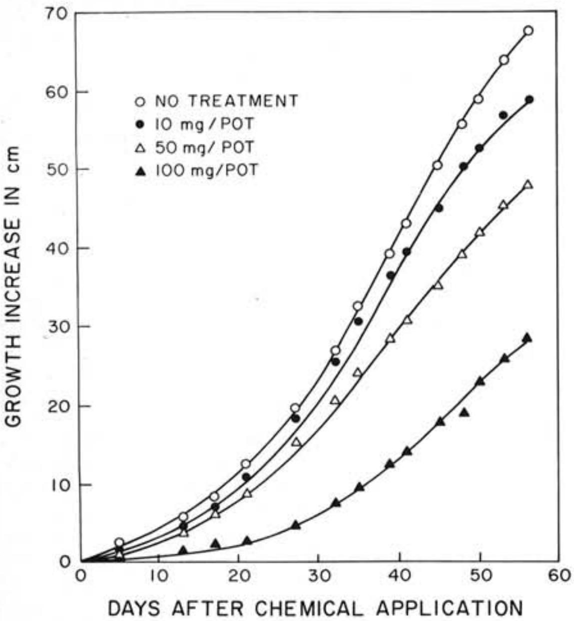

Fig. 1. Effect of tributyl[(5-chloro-2-thienyl)methyl]phosphonium chloride (TTMP) at different concentrations on growth of cotton plants (var. SJ-1). Chemical was applied as a soil drench to 4-week-old plants in 15 × 15 cm diameter pots of sandy loam soil. Each value is the average of four replicates.

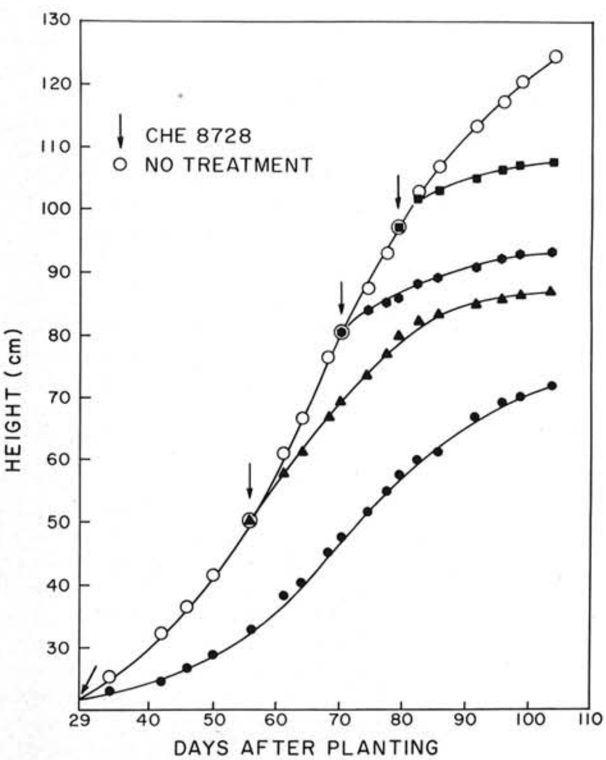

Fig. 2. Effect of applying tributyl[(5-chloro-2-thienyl)methyl]phosphonium chloride (CHE 8728) (50 mg) by soil drench to cotton plants growing in 15 × 15 cm diameter pots (2 kg soil) on growth. Time of treatment is indicated by an arrow. Each value is the average of four replicates.

assayed, it was treated similarly.

RESULTS.—*Fungitoxicity of TTMP*.—When TTMP was added to potato dextrose agar at concentrations of less than 500 μg/ml, there was no reduction in growth of *V. albo-atrum*. At 1,000 μg/ml there was about 50% reduction in diameter of the colonies. It did not appear that TTMP was fungitoxic at the levels used in plants.

Effect of TTMP on growth of cotton.—To determine the dosage effect of TTMP on cotton, water suspensions were drenched on each pot of soil (10, 50, and 100 mg/2 kg of soil) containing one plant 4 weeks of age. Increasing the dosage of TTMP to 100 μg increasingly retarded growth (Fig. 1). At 100 mg/pot TTMP was toxic and caused distortion of the leaves, but at 10 and 50 mg/pot growth was only moderately retarded. Since the average number of internodes on treated plants were similar to that on control plants, growth reduction was due to shortening of the internodes. Leaves on treated plants were a darker green color than on control plants.

In another experiment, plants in the greenhouse were treated by soil drench with TTMP (50 mg/2 kg soil) at 29, 56, 68, and 79 days of age. Growth was retarded in proportion to concentration and was detected within 5 days after each treatment (Fig. 2).

Effect of TTMP on the incidence and severity of Verticillium wilt.—In initial experiments, in which stems were inoculated with the conventional level of inoculum (10^6 spores/ml) which were suitable for assaying the degree of control by systemic fungicides (9), TTMP had little or no effect on the onset of disease. With the rationale that high inoculum concentration might overcome the more subtle physiological effects of the nonfungitoxic growth regulator TTMP, studies were made on the effect of inoculum concentration on disease severity (to be reported elsewhere). These results indicated that stem puncture inoculation of a plant with about 10 spores in a drop was sufficient to induce mild but reproducible symptoms. All subsequent inoculations were made with this level of inoculum.

Plants were treated with TTMP at different times from 29 to 78 days after planting (Fig. 2) and inoculated. When samples of petiole tissue were assayed 24 days later, the numbers of *V. albo-atrum* propagules in treated plants were much fewer than in nontreated plants up to 78 days after treatment (Table 1).

In another experiment, plants were treated with TTMP by soil drench (50 mg/pot) 87 days prior to inoculation by stem puncture. The onset of disease as measured by the foliar symptom index was delayed, and the severity, as measured by the vascular discoloration index, was reduced. Propagule counts from stem tissue were also slightly but significantly reduced by TTMP only at 50 mg/pot (Table 2).

In another experiment TTMP applied at 50 mg/pot delayed the onset of Verticillium wilt and reduced the number of propagules in petiole tissue.

Effect of TTMP on Verticillium wilt in the field.—TTMP as a foliar spray (40 μg/ml) in June decreased the severity index of Verticillium wilt only slightly from 0.45 for the nontreated control to 0.25 based on a visual rating of foliar symptoms (0 = no disease; 5 = severe chlorosis and defoliation) on 1 August. The numbers of *V. albo-atrum* propagules were consistently lower in the treated than in non-treated plots

TABLE 1. Suppression of propagules of *Verticillium albo-atrum* in cotton plants growing in soil treated with TTMP[x] (50 mg/2 kg soil) at different stages of growth

Age of plant when treated (days)	Length of treatment (days) prior to time of inoculation	Inoculation[y]	Propagules/g of petiole tissue[z]
	No treatment	+	131,242 a
29	80	+	7,456 b
56	53	+	14,516 b
68	41	+	16,561 b
78	31	+	37,930 b
	No treatment	0	0 b

[x]TTMP is tributyl[(5-chloro-2-thienyl)methyl]phosphonium chloride.

[y]Plants were inoculated by stem puncture with 10 spores/plant 24 days previous to analysis for propagules.

[z]Petioles from near the tops of plants were blended, diluted, and plated on sodium polypectate agar 24 days after inoculation. Figures with a common letter are not different ($P = 0.05$) (LSD = 49,048).

TABLE 2. Effect of TTMP[v] applied 87 days prior to stem puncture inoculation of cotton on the severity of Verticillium wilt

		Foliar symptom index[x]			Vascular discoloration index[y]	
TTMP[w] (mg/pot)	Inoculation	12 days	17 days	20 days	34 days	Propagules/g stem tissue[z]
0	+	6.7	28.3	42.1	70.9	38,618 a
10	+	0	7.2	26.0	38.1	24,333 ab
50	+	0	0	13.5	35.7	14,382 bc
100	+	0	0	17.3	36.1	25,194 ab
0	0	0	0	0	0	0 c

[v]TTMP is tributyl[(5-chloro-2-thienyl)methyl]phosphonium chloride.

[w]Applied by drenching soil (2 kg) 87 days before inoculation of plants by stem puncture (approximately 10 spores per plant).

[x]Foliar symptom index = $\frac{\text{Sum of leaf symptom values (0-4)}}{\text{number of leaves examined} \times 4} \times 100$, based on a symptom-rating scale of: 0 = normal; 1 = epinasty; 2 = slight chlorosis; 3 = severe chlorosis; and 4 = defoliation.

[y]Vascular discoloration index = $\frac{\text{Sum of vascular discoloration rating (0-3)}}{\text{number of nodes on each plant} \times 3} \times 100$, based on a vascular discoloration rating scale of: 0 = no discoloration; 1 = 30% discoloration; 2 = 30-60% discoloration; and 3 = 60-100% discoloration of a cross section of the xylem tisues.

[z]At end of the experiment (34 days after inoculation) stems were blended, diluted, and plated on sodium polypectate agar. Figures with a common letter are not different ($P = 0.05$) (LSD = 19,608).

when assayed on 7, 19 August, 7 and 22 September (Table 3). The number of bolls per meter of row and yield of seed and lint was increased 28% by TTMP applied one time (1×) and 30% by TTMP applied two times (Table 3).

In another experiment in the same field TTMP was applied two times at 40 μg/ml (39.6 g/ha) and two times at 80 μg/ml (79.2 g/ha). The reduction of propagules of *V. albo-atrum* as measured by the petiole dilution assay was similar to that reported in Table 3. Yield was increased 21% over the control and there was no significant difference due to dosage.

After the cotton was harvested in November 1973 and the number of unopened green bolls which were not harvestable were counted, there were 11 per plot in the nontreated control, but in the TTMP treated plots there were only three to eight per plot which indicated that TTMP either increased the rate of maturity or induced an earlier set of bolls.

DISCUSSION.—The suppression of Verticillium wilt by TTMP appears to be of enough significance to warrant further research on the effects of growth regulators on plant disease. The possibility that a growth regulator such as TTMP might increase resistance of the cotton plant to the vascular wilt disease caused by *V. albo-atrum* spp. is of potential significance.

In 1974, in a field experiment similar to the one reported here, foliar treatment with TTMP (40 g/ha) caused a decrease in the number of propagules of *V. albo-atrum* (5 September) from 20,000 to 900 per gram of petiole tissue (significant, $P = 0.05$). Yield of seed and lint was increased 20% (significant, $P = 0.05$). In addition, in the same experiment, chlormequat (chlorocholine chloride) at 10 and 25 g/ha applied by foliage spray increased yield of seed and lint 6 and 12% respectively (significant, $P = 0.05$) and decreased the *V. albo-atrum* propagule count to 900-2,000/g petiole tissue (differences from control significant, $P = 0.05$) (8).

The evidence obtained by the petiole dilution assay on reduction of the population of *V. albo-atrum* propagules in treated plants suggests that physiological resistance of the plant might be increased by foliar treatment with TTMP in the field and by soil application in greenhouse tests. There is not as yet any information as to what the

TABLE 3. Effect of TTMP[x] applied to foliage at the initial square formation stage on cotton in one application 19 June (1×), or in two applications 19 June and 27 June, 1973 (2×), on yield of seed cotton

	Bolls/meter	*Verticillium albo-atrum* (propagules/g petiole tissue)[y]				Yield of seed and lint	
Treatment	Aug 5	Aug 7	Aug 19	Sept 7	Sept 20	(kg/plot)[z]	(% of control)
TTMP 40 μg/ml 2×	48	0	401	400	501	2.8 a	130
TTMP 40 μg/ml 1×	50		21,654	250	3,910	2.7 a	128
No treatment	40	1,733	51,528	7,840	49,123	2.1 b	100

[x]TTMP is tributyl[(5-chloro-2-thienyl)methyl]phosphonium chloride.

[y]Ten randomly selected petioles (5 cm in length) from each plot were combined, weighed, surface-decontaminated with sodium hypochlorite (0.52%) for 5 minutes, washed, and from this sample 2 g were blended in an Omnimixer, in 200 ml of water, diluted 1:10, and plated on sodium polypectate agar. Since assays were not made from each replication, propagules per gram of tissue on the different dates of assay were analyzed. The TTMP treatments were significantly different ($P = 0.05$) than no treatment.

[z]Two rows 4 meters (13 feet) in length, spaced 96 cm (38 inches) apart, four replicates in a randomized block design. Duncan's multiple range test used for the statistical analysis; values followed by the same letter do not differ significantly, $P = 0.05$.

physiological nature of this role might be. However, there are some reasonable hypotheses.

There is increasing reason to believe that some form of biochemical resistance in cotton might be inducible. Bell was the first to suggest that phenolic materials related to gossypol were involved in resistance to Verticillium wilt of cotton (2, 3). Later Zaki et al. (18) characterized and identified hemigossypol in tolerant inoculated cultivars of *G. hirsutum*. In a later paper Zaki et al. (17) reported that the antifungal hemigossypol was associated with the tolerance of SJ-1 cotton to a defoliating isolate of *V. albo-atrum* following inoculation with the mild nondefoliating isolate SS-4. This indicated that cross protection induced by SS-4 to the defoliating strains as reported by Schnathorst and Mathre (14) could be due to production of phytoalexins. Cross protection against a severe isolate by previous inoculation with a mild isolate also was demonstrated in a field experiment reported by Barrow (1); however, in practice there seems little chance that this method could be made practical.

If phytoalexins could be induced in cotton by use of growth regulators, this method might be of practical significance. In recent work from our laboratory the compounds *N,N*-dimethylpyrrolidinium iodide and *N,N*-dimethylpiperidinium iodide delayed the onset of disease and reduced the population of *V. albo-atrum* in inoculated treated cotton plants (6). Both compounds or their metabolic by-products induced in inoculated treated plants the production of a compound with a comparable R_f value on thin layer plates to hemigossypol. These compounds also reduced the population of *V. albo-atrum* propagules in the field (D. C. Erwin and S. D. Tsai, *unpublished*).

The reduction of numbers of propagules of *V. albo-atrum* in inoculated plants that were previously treated with TTMP (which is not fungitoxic in vitro) suggests that antifungal compounds produced in the plant might be involved.

In addition to the antifungal response induced in plants TTMP has marked physiological effects. Stutte and Rudolph reported that TTMP applied to foliage at bloom time stimulated an increase in yield of soybeans (15, 16). Increased soybean yields were correlated with an increase in number of pods per plant. In our work, TTMP also increased the numbers of bolls set and the yield of cotton in the field. Stutte and Rudolph (15, 16) reported that in TTMP-treated plants there was a reduction in the photosynthesis-respiration equilibrium point, which indicated that the treated soybean plant retained more fixed carbon. An increase in stomatal resistance, which indicated partial closure of stomates, was also associated with an increase in yield of treated soybean plants. Cothren and Stutte (7) noted in soybean that TTMP and tetrahydrofurfuryl isothiocyanate caused a relative increase in the percentage of sucrose in relation to fructose and glucose in leaf tissue. They postulated that yield increases might be related to the increase in sucrose which is the principle sugar involved in the leaf-to-sink (seed) system in plants.

LITERATURE CITED

1. BARROW, J. R. 1969. Cross protection against field infestation of Verticillium wilt. Pages 32-33 *in* Proc. Beltwide Cotton Prod. Res. Conf., 29th Cotton Dis. Council, 7-8 January. New Orleans. 134 p.
2. BELL, A. A. 1967. Formation of gossypol in infected or chemically irritated tissues of Gossypium species. Phytopathology 57:759-764.
3. BELL, A. A. 1969. Phytoalexin production and Verticillium wilt resistance in cotton. Phytopathology 59:1119-1127.
4. BUCHENAUER, H., and D. C. ERWIN. 1972. Control of Verticillium wilt of cotton by spraying with acidic solutions of benomyl, methyl 2-benzimidazole carbamate, and thiabendazole. Phytopathol. Z. 75:124-139.
5. BUCHENAUER, H., and D. C. ERWIN. 1973. N,N-dimethylpyrrolidinium iodide and N,N-dimethylpiperidinium iodide as new plant growth retardants. Z. Pflkrankr. Pflanzenpathol. Pflanzenschutz 80:567-575.
6. BUCHENAUER, H., and D. C. ERWIN. 1973. Reduction of the severity of Verticillium wilt of cotton by the growth retardants N,N-dimethylpyrrolidinium iodide and N,N-dimethylpiperidinium iodide associated with induction of antifungal compounds. Z. Pflanzenkr. Pflanzenpathol. Pflanzenschutz 80:576-586.
7. COTHREN, J. T., and C. A. STUTTE. 1973. Influence of water stress and growth regulator applications on sugar composition of soybean leaves. Phyton Rev. Int. Bot.

Exp. (Argentina) 31:137-143.
8. ERWIN, D. C. 1976. Growth retardants: evidence for their effect on suppression of Verticillium wilt of cotton. *In* N. McFarlane, ed. Evaluation of biological activity. Academic Press, London. (In press).
9. ERWIN, D. C., J. J. SIMS, and J. PARTRIDGE. 1968. Evidence for the systemic, fungitoxic activity of 2-(4′-thiazolyl)benzimidazole in the control of Verticillium wilt of cotton. Phytopathology 58:860-865.
10. ERWIN, D. C., S. D. TSAI, and R. A. KHAN. 1974. Reduction of the severity of Verticillium wilt of cotton by the growth retardant tributyl [(5-chloro-2-thienyl)methyl]phosphonium chloride (CHE 8728) Proc. Am. Phytopathol. Soc. 1:56 (Abstr.).
11. HUISMAN, O. C., and L. J. ASHWORTH, JR. 1974. Verticillium albo-atrum: quantitative isolation of microsclerotia from field soils. Phytopathology 64:1159-1163.
12. MALCA, I., D. C. ERWIN, W. MOJE, and B. JONES. 1966. Effect of pH and carbon and nitrogen sources on the growth of Verticillium albo-atrum. Phytopathology 56:401-406.
13. SAALTING, G. F. 1963. De ontwickeling van Fusarium oxysporum in resistence en vatbare rassen van Lupinus luteus. Tidschr. Plantenziekt. 69:3-75.
14. SCHNATHORST, W. C., and D. E. MATHRE. 1966. Cross-protection in cotton with strains of Verticillium albo-atrum. Phytopathology 56:1204-1209.
15. STUTTE, C. A., and R. D. RUDOLPH. 1971. Growth regulators increase soybean yields. Ark. Farm Res. 20:16.
16. STUTTE, C. A., and R. D. RUDOLPH. 1973. Use of yield-enhancing chemicals on soybeans. Ark. Farm Res. 22:3.
17. ZAKI, A. I., N. T. KEEN, and D. C. ERWIN. 1972. Implication of vergosin and hemigossypol in the resistance of cotton to Verticillium albo-atrum. Phytopathology 62:1402-1406.
18. ZAKI, A. I., N. T. KEEN, J. J. SIMS, and D. C. ERWIN. 1972. Vergosin and hemigossypol, antifungal compounds produced in cotton plants inoculated with Verticillium albo-atrum. Phytopathology 62:1398-1401.
19. ZEEH, B., K. /H. KÖNIG, and J. JUNG. 1974. Development of new plant growth regulators with biological activity related to CCC. Kemia-Kemi 1:621-623.