Effect of Plant Hormones on Virus-Replicating Capacity of Cotton Infected with Tobacco Mosaic Virus

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ABSTRACT

Cotton (*Gossypium hirsutum*) is highly resistant to tobacco mosaic virus infection. The low virus-replicating capacity in cotton can be increased by dark treatment, and by application of kinetin, indole-3-acetic acid, 2,4-dichlorophenoxyacetic acid, Ethrel (2-chloroethylphosphonic acid), and 4-dichlorophenyl-1-1-dimethylurea. The virus increase due to dark treatment is counteracted by a supplementing of sucrose in the excised leaf culture. Ethrel releases ethylene; and its application, similar to the dark treatment, is to accelerate tissue senescence. Ethrel treatment favors virus increase in cotton and cucumber, but not in *Physalis floridana*. 4-Dichlorophenyl-1-1-dimethylurea produces virus increase in cotton and cucumber but not in *P. floridana*. Phytopathology 61:869-872.

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Cotton plants (*Gossypium hirsutum* 'Acala 4-42') were reported to be subliminally infected when artificially inoculated with tobacco mosaic virus (TMV) (3). Subliminal infection is considered to be a highly resistant reaction to TMV infection, and the virus-replicating capacity (VRC) in cotton is low. However, a hundredfold increase in VRC in cotton can be obtained by culturing the excised infected seedling under continuous dark. It has been postulated (3) that rapid senescence of cotton tissue in darkness provides a net gain in TMV biosynthesis, due to a simultaneous breakdown of some induced metabolic resistance. Plant hormones regulate metabolic activities of plant growth and senescence. This paper reports the results of an investigation of the effects of some plant hormones used in the growing media for excised cotton seedlings on the development of TMV.

MATERIALS AND METHODS.—Cotton (*Gossypium hirsutum* L. 'Acala 4-42') seedlings were grown in 3-inch clay pots in an air-cooled greenhouse fitted with carbon-filters for smog control with diurnal temperature range from 21 to 29 C. Seedlings were excised just above the soil level at the cotyledon stage, before expansion of terminal true leaves. The cotyledons were then air-brush inoculated (7) with 0.5 μg/ml of purified TMV in distilled water. After inoculation they were rinsed with tap water, and the stems of one or two seedlings were inserted into glass vials (4-dr screw cap, 20 × 70 mm) containing 0.5% purified agar in distilled water. The materials tested were kinetin (K), indole-3-acetic acid (IAA), 2,4-dichloro-phenoxyacetic acid (2,4-D), gibberellic acid (GA), 2-chloroethylphosphonic acid (Ethrel), 4-dichlorophenyl-1-1-dimethylurea (DCMU), and sucrose. Plant hormones to be studied were incorporated into 0.5% water agar at indicated concentrations. The inoculated seedlings were divided into two groups, each group containing hormone treatments and a control. One group was cultured under continuous light at room temperature in a Lucite box (103 cm × 39.5 cm × 25.5 cm) illuminated with four rows of cool-white fluorescent lights (40 w) at an elevation of 76 cm. The second group of inoculated seedlings was placed in plywood boxes coated with epoxy resin for continuous dark treatment. Water mist spray was introduced, and the incubation chambers were aerated by fans twice a day.

Infected cotyledon samples were collected for virus determination at 7 days and at 10 to 14 days after virus inoculation, depending on the condition of the culture in the dark. For each collection interval, 40 cotyledons were harvested, weighed, and frozen. The frozen samples were homogenized (Virtis "45" homogenizer) with 3 volumes of 0.01 M neutral phosphate buffer containing 0.01 M cysteine-HCl/fresh wt of tissue. The homogenate was filtered through one layer of cheesecloth, clarified at 60 C for 10-20 min, and given one cycle of low- (3,200 *g*), high- (54,000 *g*), and low-speed centrifugation (Beckman Model L ultracentrifuge). The final 8-ml preparations were used to inoculate cucumber seedlings (*Cucumis sativus* L. 'Chicago Pickling') for quantitative determination of virus activity. The average number of starch lesions per cotyledon (from 14 to 20 cotyledons/sample) can be counted 7 days after inoculation. The starch lesions were developed in cotyledons stored 24 hr in the dark, cleared in 70% alcohol, and immersed in an IKI-lactic acid mixture.

RESULTS.—Sucrose (1%), kinetin (5 ppm), IAA (10 ppm), 2,4-D (10^{-3} M), GA (12.5 ppm), Ethrel (500 ppm), and DCMU (28.5 ppm) were supplied separately to excised TMV-inoculated cotton seedlings grown either under continuous light or under continuous dark after inoculation. Results of their relative virus content are listed in Table 1.

Among the control plants for all treatments, 89-100% of the increase in VRC could be attributed to the continuous dark treatment after inoculation. Among the hormone- or sucrose-treated plants, the increase in VRC was similarly due to continuous dark treatment except for the early harvest sample from plants treated with sucrose and the sample from plants treated with Ethrel (Table 1). There was a slight increase in VRC

TABLE 1. Effect on tobacco mosaic virus replication in excised cotton plants grown in media containing six plant hormones and sucrose under continuous light or dark

Treatment	Days after inoculation	Continuous light: Avg. no. of lesions		Continuous dark: Avg. no. of lesions		% Effect attributed to continuous dark	
		Check	Treated	Check	Treated	Check	Treated
Sucrose, 1%	7	0.4	1.5	4.2	1.6	90.5	6.3
	12	0.4	0.5	158.7	16.5	99.8	97.0
Kinetin, 5 ppm	8	2.0	3.0	119.0	391.0	98.3	99.2
	11	5.0	6.0	282.0	616.0	98.2	99.0
Indole-3-acetic acid, 10 ppm	7	6.0	20.0	64.0	212.0	90.6	90.6
	12	3.0	12.0	131.0	459.0	97.7	97.4
	14	0	9.0	104.0	649.0	100.0	98.6
2,4-D 10^{-3} M	7	0	13.0	103.0	84.0	100.0	84.5
Gibberellic acid, 12.5 ppm	7	3.0	1.0	43.0	80.0	93.0	98.8
	10	2.0	3.0	249.0	166.0	99.2	98.2
Ethrel, 500 ppm	7		396	266.0	1,100.0		64.0
4-dichloro-phenyl-1-1-dimethylurea, 28.5 ppm	7	4.0	3.0	179.0	132.0	97.8	97.7
	11	1.0	7.0	463.0	552.0	99.8	98.7

in plants treated with sucrose and grown under light, but only in 7-day samples. The VRC of sucrose-treated plants grown in the dark was inhibited to one-third of the control at 7-day sample and one-tenth of the control at the 12-day sample. Kinetin stimulated VRC of plants grown in the light to a slight extent, and to a proportional extent for kinetin-treated plants grown in the dark. Indole-3-acetic acid effectively stimulated VRC in treated plants grown in the light (59-100%) as well as plants grown in the dark (25-83%). 4-Dichloro-phenyl-1-1-dimethylurea and 2,4-D were effective stimulants to VRC in treated plants grown in the light, but not so to those plants grown in the dark. The effect of GA on VRC of cotton was not clear.

Ethrel effect on VRC in cotton was pronounced. It stimulated VRC in both light and dark cultures (Table 1). The effect of Ethrel was additive to the effect of the dark. Aqueous Ethrel is stable at pH 3.5 or below, but releases ethylene gas when pH rises above 3.5. Ethylene is generally considered to contribute to plant tissue senescence. Virus-replicating capacity of TMV-infected cotton seedlings put in a light box with wounded apples was increased sevenfold over that of infected cotton seedlings in continuous light without a source of ethylene.

Ethrel at 250 ppm was sprayed on individually potted cotton seedlings 3 hr and 20 hr after TMV inoculation. The seedlings were maintained under greenhouse-growing conditions with a control series of inoculated water-sprayed cotton seedlings. After 7 days, 40 cotton cotyledons from each of the treated and control plants were collected and assayed for virus content. An average of 505 starch lesions/cotyledon was obtained from Ethrel-treated tissue, and an average of 15 starch lesions/cotyledon was obtained from water-treated tissue on cucumber test plants. Ethrel, therefore, promotes VRC in cotton under greenhouse-growing conditions. Ethrel promotes VRC in cotton more effectively than other plant hormones tried. To see whether or not this effect is only limited to cotton, because of its subliminal infection, other host plants with different susceptibility to TMV replication were also tested.

Cucumber cotyledons, a starch lesion host of TMV, inoculated with 1 μg/ml TMV, were sprayed 4 hr and 20 hr after inoculation with 500 ppm Ethrel. After 9 days, cotyledons were collected from Ethrel-treated and water-treated cucumber plants, and the virus was purified from these tissues. A virus content of 12.5 μg/cotyledon or 24 μg/g fresh wt cucumber tissue was obtained from water-treated control plants. The Ethrel-treated tissue yielded 32.6 μg/cotyledon or 54 μg/g fresh wt cucumber tissue, more than a twofold increase in virus. Biological assay of these preparations indicated the infectivity of virus from Ethrel-treated cucumber tissue to be 100 to 150% more active than that from the control tissue.

Physalis floridana Rydb. is a highly susceptible systemic host to TMV. Seedlings of *P. floridana,* pruned to 4-6 middle leaves with the terminal growth removed, were sprayed 4 hr and 20 hr after TMV inoculation (0.15 μg/ml TMV) with Ethrel at concentrations of 100 ppm, 200 ppm, 400 ppm, and 800 ppm. Leaves were collected 4, 8, 12, and 15 days after inoculation, and an area 4.0 × 5.0 cm from each leaf was used to determine TMV concentration per unit leaf area. A total of 6 to 8 leaves was collected for each sample. No significant stimulation of VRC occurred in *P. floridana*; rather, an inhibition is evident (Table 2).

4-Dichloro-phenyl-1-1-dimethylurea is reported to inhibit photosynthetic CO_2 fixation; or physiologically, it counteracts the effect of light (12). While this effect was not particularly noted with the excised cultured cotton seedlings (Table 1), further studies with DCMU were made with greenhouse-grown seedlings of cotton, cucumber, and *P. floridana.* A solution of DCMU was sprayed on TMV-inoculated plants of these species 4 hr and 20 hr after inoculation at the following dilutions: 10^{-6} M; 10^{-5} M; 2.5×10^{-5} M; 5×10^{-5} M, and 10^{-4} M. The TMV content was analyzed as previously indicated. 4-Dichloro-phenyl-1-1-dimethylurea at about

TABLE 2. Effect of 2-chloroethylphosphonic acid (Ethrel) on virus-replicating capacity of *Physalis floridana*, a susceptible systemic host plant of tobacco mosaic virus (TMV)

Days after inoculation	TMV content—μg/leaf area at various concentrations of Ethrel				
	Control	100 ppm	200 ppm	400 ppm	800 ppm
4	49.1	23.0	29.0	28.3	24.3
8	1,692.7	1,137.7	1,961.9	1,140.5	1,689.9
12	5,228.0	4,915.0	5,261.4	5,028.3	5,581.0
15	6,820.0	5,383.6	5,561.3	4,917.7	6,349.2

10^{-5} promoted VRC in cotton and cucumber, and there was an inhibition of VRC in *P. floridana* (Table 3).

DISCUSSION.—Cotton seedlings support only subliminal infection by TMV. This low yield of virus replication in cotton can be interpreted, in general, as cellular resistance. Two types of cellular resistance may occur. One type may be passive and nonspecific. The cellular environment may be unfavorable for TMV biosynthesis, because of the presence of certain inhibitors or the lack of raw materials essential for TMV biosynthesis. The other type may be a specific antiviral response incited by the process of virus infection. The defense mechanism of the cell works against the intrusion of the virus. This latter activity is then considered as metabolic resistance. The ability of susceptible hosts to produce large amounts of viral progeny as compared with a subliminal host, which produces very little, is indeed intriguing. Factors that are favorable to virus biosynthesis and factors that are favorable to host resistance could be separately responsible for the eventual VRC of a plant.

Plant diseases, caused by fungi, bacteria, or viruses, are stochastic causes of plant senescence (13). The general yellowing of diseased plants typifies the progress of the senescent process. Since infection accelerates senescence (4), there is a possibility that senescence might favor infection to a certain extent. Senescence in excised leaves accelerated rapidly in darkness (11). Ethylene, produced by Ethrel, a regulator for plant metabolism, is known as an endogenously produced substance which promotes fruit ripening and senescence of plant tissue (1). The present results indicate that darkness and ethylene separately, or in conjunction, promote VRC in cotton. Therefore, it can be assumed that senescence might favor virus infection in cotton. Udvardy et al. (12) indicated in a study on *Avena* leaves that sucrose supplied in darkness had an effect similar to illumination. The addition of sucrose, therefore, retards senescence and increases metabolic resistance to TMV infection. Khudairi (6) reported chlorophyll degradation by light in leaf discs in the presence of sugar. Such degradation process could be responsible for slightly reducing resistance in cotton when sucrose was added under the light culture (Table 1).

The fact that Ethrel application does not favor VRC in *P. floridana* indicates that Ethrel has no direct stimulating effect on the process of virus biosynthesis. Nakagaki et al. (10) also reported that exogenously introduced ethylene has no effect on TMV synthesis in *Nicotiana tabacum* plants, another susceptible systemic host of TMV. Therefore, the effect of net gain in VRC by Ethrel treatment in cotton and cucumber can only be explained by a resulting breakdown of metabolic resistance. In *P. floridana* there is none or little active metabolic resistance during virus biosynthesis; therefore, the effect of induced senescence to virus production is not evident. Nakagaki et al. (10) reported the ethylene production by detached leaves infected with TMV. It is possible to have different rates of ethylene production caused by TMV infection among different plant species. This, in turn, may influence the eventual resistance to TMV infection.

Metabolic resistance could be directly related to photosynthetic activities. Light provides resistance to TMV infection in cotton, as do sucrose supplements in dark (Table 1). The application of DCMU, a known photosynthesis inhibitor, also inhibits virus resistance in cotton and cucumber. The application of DCMU on *P. floridana*, however, did not increase susceptibility, but on the contrary, had some inhibitory effect on TMV biosynthesis. Therefore, neither Ethrel nor DCMU stimulate virus biosynthesis in *P. floridana*. The increase in VRC in cotton and cucumber by Ethrel and DCMU treatments can be explained as a repression of metabolic resistance against virus infection.

Morgan & Hall (8, 9) reported evidence of the

TABLE 3. Effect of 4-dichloro-phenyl-1-1-dimethylurea (DCMU) when applied after tobacco mosaic virus inoculation on virus-replicating capacity of different plants

Plants[b]	Days after inoculation	Concentration of DCMU				
		10^{-6} M	10^{-5} M	2.5×10^{-5} M	5×10^{-5} M	10^{-4} M
Cotton	7		8.0[a]	8.0	3.0	1.0
	14		2.5	2.5	3.0	1.5
Cucumber	10	2	145			
Physalis floridana	4		0.43			0.43
	6		0.66			
	8		0.34			1.3

[a] Virus index = $\frac{\text{virus content of treated sample}}{\text{virus content of control sample}}$.

[b] Virus content in cotton and cucumber was expressed by the average number of starch lesions per cotyledon, and the virus content in *P. floridana* was expressed by spectrophotometric readings at 260 nm of purified preparation/leaf area.

stimulation of ethylene synthesis by the auxins, 2,4-D and IAA, in cotton. Even though the auxins cause an increased synthesis of cellular RNA and protein (5), the stimulation by 2,4-D and IAA of VRC in cotton was probably due to ethylene production. Cheo (2) reports an enhancement of TMV biosynthesis in both cucumber and *P. floridana* by application of 2,4-D. Therefore, auxins could act in both directions by stimulating TMV biosynthesis through a higher rate of RNA and protein turnover, and by repressing metabolic resistance through ethylene production in resistant hosts.

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