Inhibition of Tobacco Mosaic Virus by 8-azaguanine and 2-thiouracil in Diploid and Tetraploid Physalis floridana

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ABSTRACT

A tetraploid line of *Physalis floridana* was obtained by treating diploid *P. floridana* seed with colchicine. The 4N and 2N plants were used to test tobacco mosaic virus (TMV) multiplication and infectivity and the effectiveness of 8-azaguanine (8-AG) and 2-thiouracil (2-TU) as TMV inhibitors.

Under greenhouse, laboratory, and growth chamber conditions, the 4N:2N TMV titer ratios were 2.05, 3.12, and 1.34, respectively. There was no difference in the specific infectivity of TMV from the 4N and 2N sources.

Treatment with 8-AG and 2-TU inhibited TMV multiplication and reduced the specific infectivity of the recovered TMV in both 4N and 2N *P. floridana*. The inhibition of TMV multiplication by 8-AG ranged from 48 to 96% in 2N plants, and from 30 to 95% in 4N plants, depending on the method of application and concn of 8-AG.

At 26 and 30 C, a single application of 8-AG stimulated TMV infectivity. At lower temp or with repeated applications, 8-AG reduced TMV infectivity. Under conditions providing a continuous supply of 8-AG to the infected leaves, specific infectivity was reduced 43.3% in 2N plants, and 48.6% in 4N plants. Phytopathology 60:1255-1258.

Nucleic acid base analogues are possible chemotherapeutants of plant virus diseases. Of those studied, 8-azaguanine (8-AG) and 2-thiouracil (2-TU) have been the most effective in reducing virus multiplication and/or infectivity (1, 2, 3, 5, 7, 8, 11, 13).

The mechanism(s) of base analogue action is largely unknown. The analogues are often incorporated into the virus RNA (4, 12, 13, 17), and this has been implicated as a cause of reduced virus infectivity. Using a *Physalis floridana* Rydb.-tobacco mosaic virus (TMV) system, Kirkpatrick & Lindner (*unpublished data*) found that the effects of one application of 8-AG carried over with diminishing effect through three seed-propagated generations, implying some degree of gene-8-AG interaction.

In the current investigation, the effect of varying the amount of host genetic material on the effectiveness of 8-AG and 2-TU as inhibitors of TMV was investigated. Diploid (2N) and tetraploid (4N) *P. floridana* were used as test hosts.

MATERIALS AND METHODS.—*Development of tetraploid lines of Physalis floridana.*—Germinating seeds of diploid *P. floridana* were treated for 24 hr with a 0.2% solution of colchicine. Plants showing abnormalities were selected and increased by vegetative propagation. Several different lines of *P. floridana* were developed.

The acetocarmine root tip smear method (16) was used to make chromosome counts. The root tips were pretreated 1 hr with 3% methanol to facilitate spreading of the chromosomes.

Inoculation of plants with TMV.—Plants were trimmed to three leaves prior to inoculation. Each leaf was inoculated with a suspension of 10 μg/ml TMV and 0.1% 600-mesh Carborundum, using the airbrush method (9).

Purification of TMV.—Frozen infected leaves were homogenized at room temp in 0.01 M phosphate-cysteine HCl buffer, pH 7.0. The homogenate was heated at 60 C for 10 min and centrifuged at 10,000 *g* for 10 min, and the supernatant was subjected to three cycles of differential low-speed (7,000 *g*) and high-speed (81,000 *g*) centrifugation. The final pellet was resuspended in distilled water. Virus preparations with an OD 260/280 value of 1.22 were considered sufficiently pure.

Specific infectivity of TMV.—Young cucumber cotelydons were inoculated with 40 mμg/ml TMV by the airbrush method. After 7 days, the cotyledons were harvested, cleared in 70% ethanol at 80 C, and stained in a lactic acid-iodine solution (10). The starch-rich local lesions produced by virus infection were then counted.

Application of 8-azaguanine and 2-thiouracil.—Different concn of 8-AG in 0.05 M sodium carbonate were applied to the leaves by one of the following methods: 1) Brushing or spraying the leaves: the 8-AG was contained in 0.1% Aerosol O.T. solution. Applications were made 1 day before TMV inoculation and at various periods after inoculation unless otherwise indicated. The leaves were harvested and frozen 14 days after inoculation. 2) Floating leaf method: immediately after inoculation with TMV, the leaves were detached and placed on filter paper supports in petri dishes containing the desired concn of 8-AG. The leaves were then incubated under artificial light for 6 days; 2-thiouracil was applied to infected leaves by the floating leaf method at a single concn of 30 ppm. Virus produced in the presence of 8-AG or 2-TU is designated as virus from treated plants (Vt) and that without treatment as control virus (Vc).

RESULTS.—*Development of tetraploid lines of Phy-*

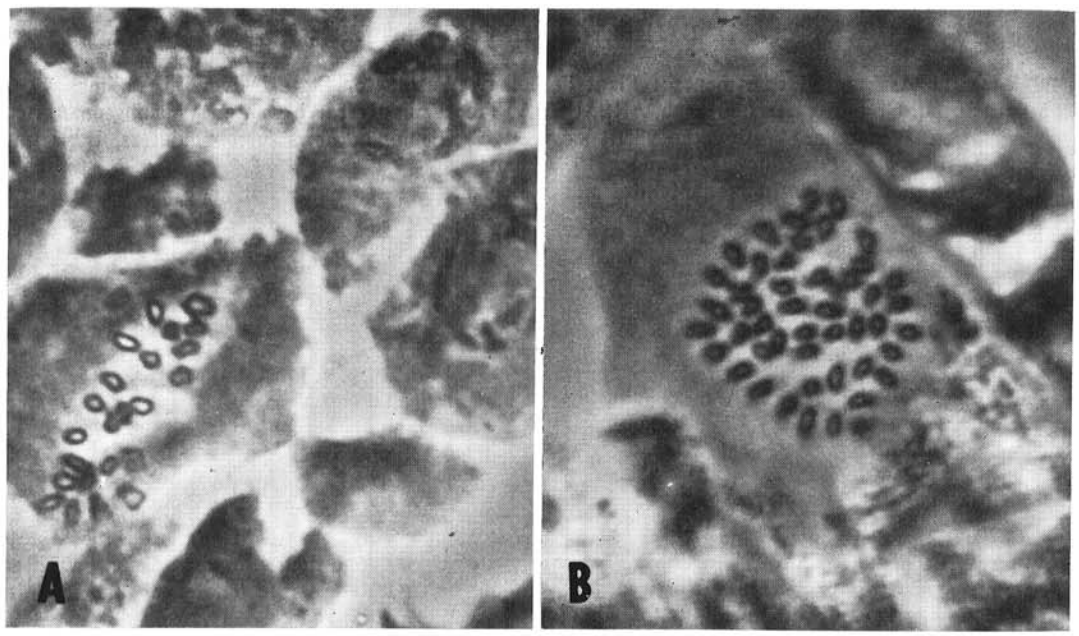

Fig. 1. The chromosomes of a cell of diploid (24 chromosomes) *Physalis floridana* plant (A) and of a tetraploid (48 chromosomes) plant (B) (×900).

salis floridana.—Several lines of plants showing variations in growth habits and appearance were established. All plants grew to maturity and flowered, but only a few were fertile. Seeds from the modified plants were larger and germinated more rapidly than seeds from normal 2N plants.

Chromosome counts of the various lines ranged from the normal 2N = 24 to approximately 96 chromosomes/cell. Those lines which produced viable seed had counts of about 48 chromosomes (Fig. 1-B); seed from one of the modified lines showing 48 chromosomes was used to establish a stable tetraploid line of *P. floridana*. Subsequent generations maintained the 48 chromosome number.

TMV synthesis and infectivity in 2N and 4N P. floridana.—Prior to determining the inhibitory effects of 8-AG and 2-TU, the amounts of recovered virus were compared in 2N and 4N *P. floridana*. Tests for TMV increase were conducted under greenhouse conditions in a growth chamber (16:8-hr light:dark cycle). The mean temp for all growth conditions was 22 C.

Under greenhouse and laboratory conditions, 4N plants generally yielded TMV titer twice that from 2N plants, while in the growth chamber the 4N plants did not show such a consistent increase in virus yield (Table 1). In the growth chamber the TMV titer from 4N plants was greater than that from 2N plants after 8 days, but after 14 days the titer from both sources were nearly equal. The mean 4N:2N titer values were 1.34 under growth chamber conditions and 4.15 under laboratory conditions. The over-all mean 4N:2N titers were 2.05 (greenhouse), 3.12 (laboratory), and 1.34 (growth chamber).

There was no significant difference in the specific infectivity of TMV from the 4N and 2N sources. The mean 4N:2N source specific infectivity ratio of TMV was 1.04.

Treatment with 2-TU.—Treatment with 30 μg/ml 2-TU inhibited TMV synthesis and specific infectivity of recovered TMV in 2N and 4N *P. floridana* leaves; replication of TMV was inhibited 65% in 2N leaves and 59% in 4N leaves. The purified virus from the

TABLE 1. Tobacco mosaic virus (TMV) titers and infectivity in diploid (2N) and tetraploid (4N) *Physalis floridana*

Culture[a]	Days after infection	mg TMV/g tissue[b]			Specific infectivity[c]		
		2N	4N	4N:2N	2N	4N	4N:2N
Greenhouse	8	10.3	20.6	2.0			
	14	11.9	24.9	2.1			
Growth chamber	8	17.0	30.0	1.8	18.3	15.0	0.8
	14	30.3	28.4	0.9	15.3	20.7	1.4
Laboratory[d]	6	2.1	4.4	2.1	69.0	48.3	0.7

[a] Mean temp 22 C.
[b] Virus titers determined spectrophotometrically.
[c] Specific infectivity at 40 mμg/ml.
[d] Detached leaves.

above test was diluted to 40 mμg/ml and inoculated into cucumber cotyledons to determine specific infectivity. Treatment with 2-TU reduced the specific infectivity of TMV from 2N plants 60%, and from 4N plants 57%.

Treatment with 8-AG.—Newly inoculated leaves were detached and floated on solutions containing 25, 50, and 100 μg/ml of 8-AG. In a second test, 100 and 200 μg/ml of 8-AG were brushed onto the leaves 18 hr after inoculation.

Both methods of 8-AG application at all concn resulted in inhibition of virus multiplication (Table 2). The relative inhibition in 2N and 4N detached leaves was similar at all concn of 8-AG. Some 8-AG toxicity to the leaves was evident at 50 ppm, and at 100 ppm the leaves were extensively damaged. With the brush method of 8-AG application, TMV multiplication in 2N plants was inhibited 48.0% at 100 ppm and 55.6% at 200 ppm. In 4N plants, the inhibition at 100 and 200 ppm was 30.0% and 68.3%, respectively. (The 2N plants appeared to be more sensitive to lower concn of 8-AG than were 4N plants, but the max inhibition in 4N plants was greater than in 2N plants.) The floating leaf method was the most efficient means of treating with 8-AG, as the extent of inhibition by 8-AG did not increase at levels above 50 μg/ml.

Treatment with 8-AG inhibited TMV replication, but the effect on TMV infectivity was not clear. Treatment with 8-AG has been found to depress virus infectivity (7, 8, 13, 14), but Matthews (12) pointed out possible difficulties in measurement, and data in the present investigation suggests further irregularities. The effect of 8-AG on specific infectivity of TMV was inhibition in some cases and stimulation in others. In two of three tests with 8-AG applied by the brush method 24 hr after inoculation, the specific infectivity of virus from treated 2N and 4N plants was greater by 38% or more than in nontreated plants, while in the third test the specific infectivity of the virus from treated 2N and 4N plants was reduced 52% and 25%, respectively.

When the infectivities following 8-AG treatment increased, the plants were grown in the greenhouse during the summer months when the temp were high (mean daily temp, 30 C). Therefore, the relation of temp to the effectiveness of 8-AG treatment was investigated.

P. floridana plants were inoculated with 10 μg/ml TMV and grown at 17 C or 26 C in constant-temp chambers under artificial light (16:8-hr light:dark cycle). Fifteen hr after inoculation, 500 ppm of 8-AG were applied by the brush method to each inoculated leaf. Leaf samples were harvested after 5, 11, and 20 days of growth.

Treatment with 8-AG inhibited TMV synthesis in 2N and 4N plants at 17 C and 26 C (Table 3). At 17 C, in both 2N and 4N plants the inhibition was greater in the earlier stages of growth, and became less at later stages. There was a similar trend at 26 C, but the results were more variable. The degree of inhibition was greater at 17 C than at 26 C, and at 17 C the inhibition was somewhat greater in 2N plants than in 4N plants, especially in later stages of growth. At 26 C, the results were too variable to warrant comparison of inhibition in 2N and 4N plants.

The specific infectivity of TMV recovered from *P. floridana* was greater when the plants were grown at 26 C than at 17 C, regardless of time of incubation. In comparison of the 26 C and 17 C ratios in 2N plants, there was a 39% increase in the infectivity of Vc and 92% increase of Vt. In 4N plants the infectivity increase was less, with values of 26% and 19%. The inhibition of infectivity of Vt in 2N plants was 32% at 17 C, but only 6% at 26 C, and in 4N plants the inhibition at 17 C and 26 C was 15% and 20%. The effects of 8-AG treatment appear to be modified at 26 C. The effect on TMV replication was less at 26 C than at 17 C in both 2N and 4N plants, and there was modification of the effect on specific infectivity at 26 C in 2N plants, but none in 4N plants.

The modification of the effect of 8-AG treatment at 26 C may be due to breakdown of 8-AG into less inhibitory substances (17). The infectivity of TMV was tested in experiments where the application method provided a continuous supply of 8-AG to the infected leaves. In one trial, detached infected leaves were floated on 25 μg/ml 8-AG, and in a second test 500 μg/ml of 8-AG were applied every 2nd day to

TABLE 2. The effect of method of application and concentration of 8-azaguanine on tobacco mosaic virus (TMV) synthesis in 2N and 4N *Physalis floridana*

		Detached floating leaf method		Brush method	
Virus source	μg/ml 8-AG	mg TMV/g fresh tissue	%[a] Inhibition	mg TMV/g fresh tissue	%[a] Inhibition
2N	0	1.06		0.83	
	25	0.26	75.5		
	50	0.04	95.9		
	100	0.07	93.1	0.43	48.0
	200			0.37	55.6
4N	0	4.40		1.00	
	25	0.84	80.8		
	50	0.25	94.4		
	100	0.35	92.1	0.70	30.0
	200			0.32	68.3

[a] mg TMV of control less those of treatment/control × 100 = per cent inhibition.

TABLE 3. The effect of 8-azaguanine treatment of tobacco mosaic virus (TMV) synthesis in 2N and 4N *Physalis floridana* at 17 C and 26 C; 5, 11, and 20 days after infection

Days after infection	Virus source	μg/ml 8-AG	mg TMV/g fresh tissue		% inhibition[a]	
			17 C	26 C	17 C	26 C
5	2N	0	0.13	0.44		
		500	0.05	0.37	61.5	15.9
11	2N	0	0.54	1.20		
		500	0.37	1.10	32.1	8.3
20	2N	0	0.88	1.45		
		500	0.66	1.28	25.0	11.7
5	4N	0	0.17	0.74		
		500	0.07	0.56	58.8	24.3
11	4N	0	0.62	1.80		
		500	0.30	1.05	51.6	47.2
20	4N	0	1.10	1.62		
		500	0.62	1.59	43.6	1.8

[a] mg TMV of control less those at 500 μg/ml 8-AG/control × 100 = per cent inhibition.

intact leaves by brushing. With each method, there was a comparable reduction of specific infectivity. The inhibition in 2N plants was 43.3%; in 4N plants, 48.6%. The 2N and 4N plants did not differ significantly in their response to 8-AG treatment.

DISCUSSION.—Tetraploid *P. floridana* more efficiently supported TMV multiplication than did comparable diploid plants, but this differential efficiency is affected by the environment. The effects of increased amounts of genetic material on TMV infectivity are not clear. There were wide differences in the 4N:2N infectivity ratios from otherwise similar materials. The mean 4N:2N ratio was 1.04, indicating no difference in specific infectivity of TMV from the 2N and 4N sources.

Both 8-AG and 2-TU were effective inhibitors of TMV synthesis and infectivity. The host chromosome composition generally did not influence the effectiveness of base analogue treatment. When the compounds were applied continuously to infected plants, both TMV titer and infectivity inhibition occurred to a similar extent in 2N and 4N plants. But a single application of 8-AG at ambient greenhouse temp during the summer months enhanced rather than inhibited TMV infectivity. In plants, 8-AG is deaminated to 8-azaxanthine (17), and higher temp may accelerate this process, thus modifying the effects of the chemical. This effect may also be related to the finding of Sander (15) that low concn of 8-AG stimulated TMV synthesis in tobacco leaves.

The temp experiments did not clarify the effects of 8-AG on infectivity. Virus recovered from plants grown at 26 C was more infective than that from plants grown at 17 C, regardless of time of infection.

Kassanis & McCarthy (6) found that the proportion of defective particles of *Dolichos* enation mosaic virus (serotype of TMV) was greater in French bean plants kept at 20 C than at 32 C. The rate of multiplication and the infectivity/unit weight were also greater at the higher temp. It may be that more defective TMV particles are produced at 17 C than at 26 C, or that the higher temp selects a more infective strain of TMV.

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