Effect of Alternating Temperature Regimes on Reduction or Elimination of Viruses in Plant Tissues

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Accepted for publication 30 December 1981.

ABSTRACT

Lozoya-Saldana, H., and Dawson, W. O. 1982. Effect of alternating temperature regimes on reduction or elimination of viruses in plant tissues. Phytopathology 72:1059-1064.

The effect of environmental temperature regimes alternating between optimal and restrictive temperatures on reducing or eliminating virus in tobacco mosaic virus (TMV)-infected tobacco or cowpea chlorotic mottle virus (CCMV)-infected cowpea was examined. Infected plants were incubated in temperature regimes that were alternated 4 hr at 40 C and 4 hr at 25 C, 6 hr at 40 C and 2 hr at 25 C, or 4 hr at 45 C and 4 hr at 25 C. The alternating temperatures allowed host growth whereas constant restrictive temperatures did not. These temperature regimes greatly reduced the accumulation of TMV in inoculated and newly developing leaves. However, TMV moved into and multiplied in newly developing plant parts and treatment did not facilitate obtaining plants free of virus by tip culture. The alternating temperature regimes more severely limited CCMV, reducing virus in some leaves below detectable levels. Although CCMV moved into and multiplied in some newly developing tissue, large areas were free of virus as demonstrated by a high percentage of virus-free plants which were obtained from 2- to 5-cm-long shoot segments. Alternating temperature regimes also affected the production of symptoms by both viruses. Leaves that developed in the temperature regimes had either milder or no symptoms and some that normally were symptomless developed unusual symptoms.

One of the more effective measures to control virus diseases of vegetatively propagated plants is the use of virus-free propagation material. Viruses are eliminated from propagation stocks by thermotherapy, propagation of virus-free areas of the plant (ie, shoot tips, green islands, etc.), or by combinations of both procedures (7,10). Thermotherapy usually involves maintaining plants at temperatures near the maximum that they will withstand with the hope that the virus will be inactivated. Although these procedures often are effective, some viruses are difficult to eliminate. Alternative procedures to obtain virus-free propagation materials would be useful.

Effects of elevated temperatures on the replication of two different types of viruses, tobacco mosaic virus (TMV) (1,2) which is a stable rodshaped virus and cowpea chlorotic mottle virus (CCMV) (5) which is an isometric virus that is unstable in vivo, particularly at elevated temperatures (4), were examined previously. Neither virus multiplied in infected hosts maintained constantly at 40 C. When shifted to 40 C from temperatures that allowed the viruses to multiply, synthesis of viral genomic RNAs ceased almost immediately. This was paralleled by a similar

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0031-949X/82/08105906/$03.00/0

cessation of synthesis of several species of host RNA (3). In plants that were shifted back to 25 C after incubation at 40 C, host RNA synthesis resumed immediately. However, there were delays of 4–8 hr for CCMV (5) and 16–20 hr for TMV (2) before viral RNA synthesis resumed. This suggests that host growth could continue while virus multiplication was inhibited by alternating the environmental temperature between optimal and restrictive temperatures if plants were maintained at high temperatures long enough to destroy the capacity for virus replication and at low temperatures for periods that allow resumption of host growth, but are too short to allow recovery of virus replication. This perhaps would allow newly developing plant parts to be free of virus, thus facilitating the production of virus-free propagation materials.

In this paper we examine the effectiveness of different alternating temperature regimes in reducing or eliminating virus from TMV-infected tobacco and CCMV-infected cowpea plants.

MATERIALS AND METHODS

Cultural conditions. The U1 strain of TMV was examined in tobacco (*Nicotiana tabacum* L. 'Xanthi') and CCMV in cowpea (*Vigna unguiculata* (L.) Walp. 'California Blackeye'). Plants were grown in the greenhouse and experiments were conducted in plant growth chambers at designated temperatures (± 1 C) with a 14-hr photoperiod of 15,000 lux. In the alternating temperature regimes, the light period and the high-temperature treatment began at 0600 hours. Each treatment was applied to 25 plants, of which five were utilized for each sampling. Each experiment was repeated four times.

Infectivity assays. Infectivity was assayed on half leaves of *Nicotiana tabacum* L. 'Xanthi-nc' for TMV and soybean (*Glycine max* L. 'Harosoy'; a gift of Don Lindahl, Pioneer Hybrid Seed, St. Joseph, IL 61873) for CCMV in a random block design with six or eight repetitions per sample. Ten 7-mm-diameter disks were removed from leaves of treated plants and stored at −20 C until all samples of each treatment could be assayed together. Frozen leaf disks were homogenized and diluted with 0.05 M glycine-0.03 M potassium phosphate buffer, pH 9.6, containing 1% Celite for TMV and 0.01 M potassium phosphate buffer, pH 7.0, containing 1% Celite for CCMV. Included in each assay were four dilutions of a purified virus standard. The relative infectivity of assays of different treatments were plotted on the same graph by normalizing the numbers of local lesions produced by the standards.

Propagation of explants. Shoot tips (200–700 μm long) from axillary buds of TMV-infected tobacco plants were placed in semisolid medium containing Murashige and Skoog (9) salts supplemented with 0.3 mg indoleacetic acid, 10 mg N^6-isopentenyladenine, and 0.8% agar per liter. Plants developed at 25 C in a 14-hr photoperiod of 1,000 lux and the presence of virus was determined by infectivity assays.

The bases of cowpea shoot tips (2–5 cm long) were placed in distilled water and incubated at 25 C in a 12-hr photoperiod of 1,000 lux. After development of roots, plants were transferred to sterile soil and maintained in a greenhouse. The presence of virus was determined by infectivity assays.

Screening for heat resistant mutants. Infectivity assay hosts were inoculated with TMV or CCMV from heat-treated plants or plants maintained at 25 C and incubated at 25, 32, 35, 40, or 43 C. Soybean plants inoculated with CCMV developed lesions at these temperatures. Xanthi-nc tobacco plants were shifted to 25 C after 48 hr at the higher temperature to allow necrotic lesions to form. The ratio of lesions produced at each temperature by virus from plants in alternating temperature regimes and virus from plants at 25 C was determined.

RESULTS

Effect of alternating temperature regimes on plant growth. Tobacco and cowpea plants 9–12 cm tall were exposed to several temperature regimes in plant growth chambers for 2 wk. Tobacco plants maintained constantly at 25 C grew an average of 14.5 cm in height while cowpea plants grew 15.6 cm. Growth of tobacco plants was reduced 75% when incubated constantly at 40 C and new leaves were distorted (Fig. 1A). Cowpea plants incubated at 40 C usually developed an irreversible wilt that preceded death (Fig. 1B). Plants incubated in temperature regimes alternating between optimal and restrictive temperatures grew much more than plants maintained constantly at 40 C. Plants contained in an alternating temperature regime of 4 hr at 40 C and 4 hr at 25 C (4–40 C:4–25 C) grew almost as much as plants maintained at 25 C (Fig. 1). Plants incubated in temperature regimes of 6 hr at 40 C and 2 hr at 25 C (6–40 C:2–25 C) or 4 hr at 45 C and 4 hr at 25 C (4–45 C:4–25 C) grew less than plants at constant 25 C, but considerably more than plants maintained constantly at 40 C.

Effect of alternating temperature regimes on virus accumulation. To examine the effect of alternating temperatures on virus multiplication, lower leaves of tobacco plants were mechanically inoculated with TMV and incubated 15 days at 25 C or in alternating temperature regimes of 4–40 C:4–25 C, 6–40 C:2–25 C or 4–45 C:4–25 C and increases of virus infectivity were determined. In the more stressful treatments, 6–40 C:2–25 C and 4–45 C:4–25 C, the amount of TMV infectivity in inoculated leaves after 15 days of treatment was 20–25% the level in leaves maintained continuously at 25 C (Fig. 2A). However, virus infectivity increased in leaves in all temperature regimes and the virus was not eliminated from inoculated leaves of any treatments.

The ability of the virus infection to become established in newly developing leaves was determined by monitoring infectivity in these leaves. Only in leaves in the 4–45 C:4–25 C treatment was the level of infectivity greatly reduced in comparison to that in leaves maintained at 25 C (Fig. 2B). None of the newly developing leaves greater than 1 cm in length were free of TMV.

Similar experiments were conducted with CCMV in cowpea. In mechanically inoculated cowpea leaves, infectivity of CCMV in all of the alternating temperature regimes increased only slightly compared to that at constant 25 C (Fig. 2C). After 15 days of 4–40 C:4–25 C or 6–40 C:2–25 C treatments, only 5–8% as much

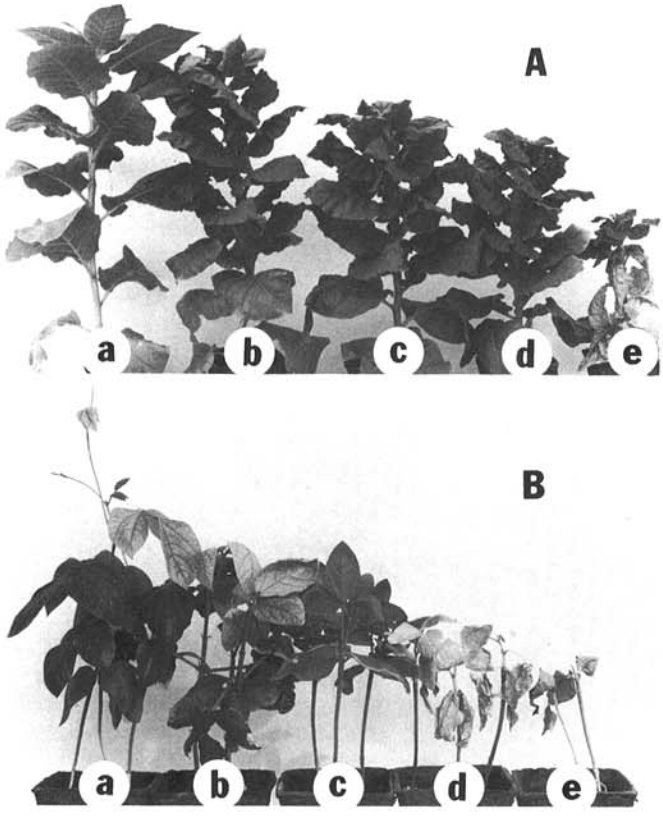

Fig. 1. Effect of alternating temperature regimes on growth of **A,** tobacco and **B,** cowpea plants. Temperature regimes were constant 25 C (a), 4 hr at 4 C and 4 hr at 25 C (b), 6 hr at 40 C and 2 hr at 25 C (c), 4 hr at 45 C and 4 hr at 25 C (d), and constant 40 C (e).

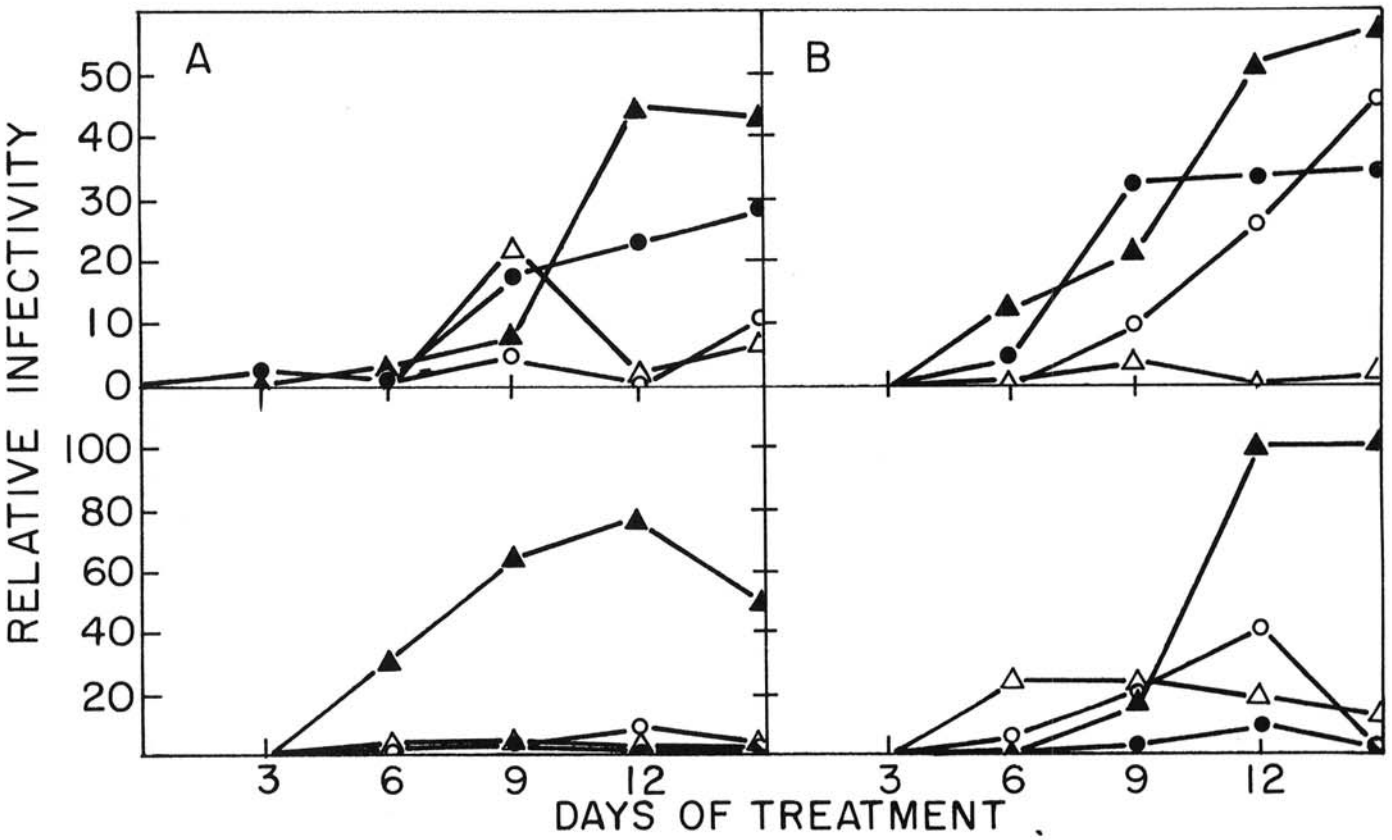

Fig. 2. Effect of alternating temperature regimes on infectivity of TMV in tobacco (**A and B**) and CCMV in cowpea plants (**C and D**) when treatments began immediately after mechanical inoculation of the lower leaves. Infectivity was monitored in inoculated leaves (**A and C**) and the upper, noninoculated leaves (**B and D**). Temperature regimes were 4 hr at 40 C and 4 hr at 25 C (●——●), 6 hr at 40 C and 2 hr at 25 C (o——o), 4 hr at 45 C and 4 hr at 25 C (Δ——Δ), and constant 25 C (▲——▲).

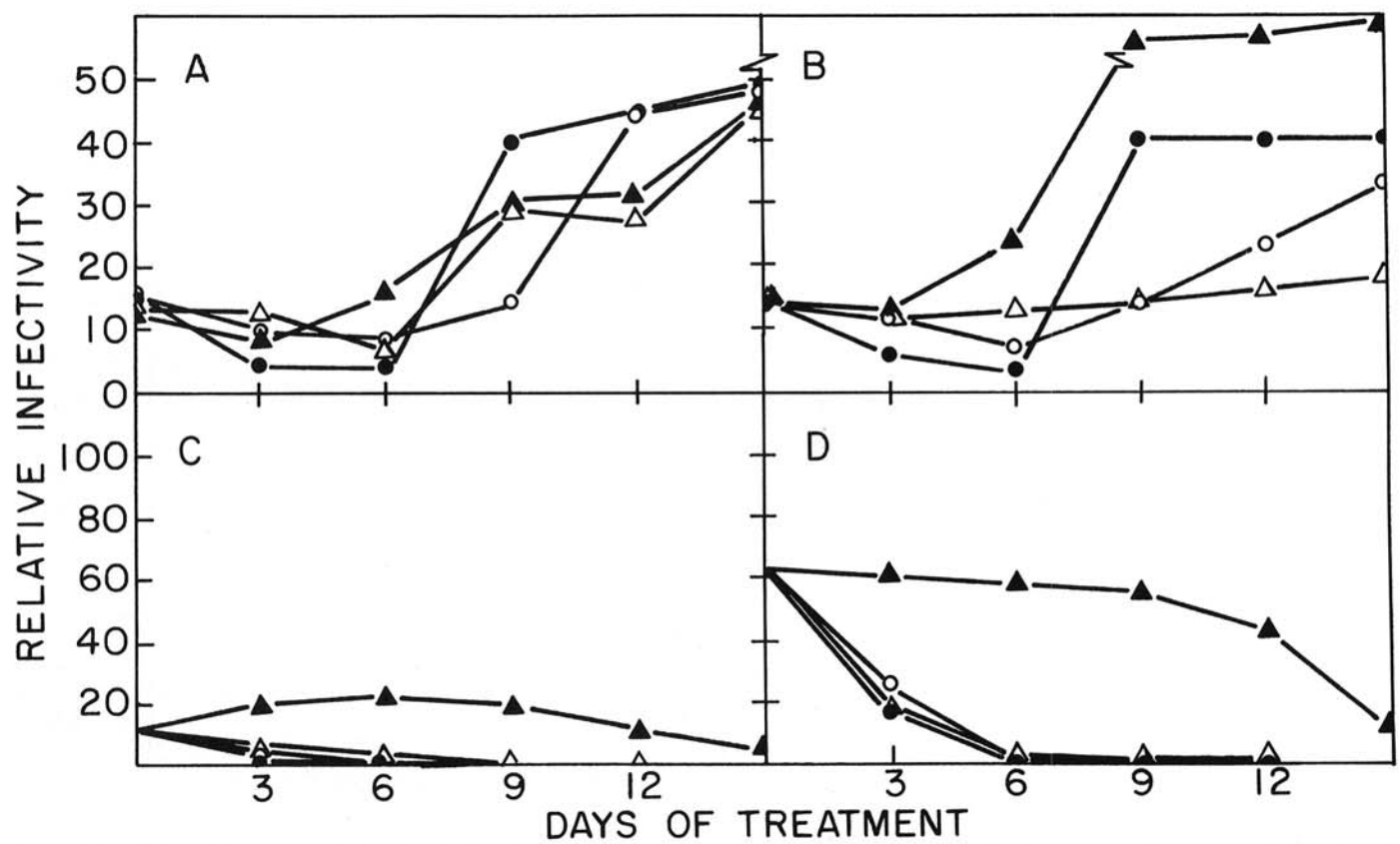

Fig. 3. Effect of alternating temperature regimes on infectivity of TMV in tobacco (**A and B**) and CCMV in cowpea plants (**C and D**) when plants systemically infected for 12–15 days were treated. Infectivity was monitored in inoculated leaves (**A and C**) and the upper, newly developed leaves (**B and D**). Temperature regimes were 4 hr at 40 C and 4 hr at 25 C (●——●), 6 hr at 40 C and 2 hr at 25 C (O——O), 4 hr at 45 C and 4 hr at 25 C (Δ——Δ), and constant 25 C (▲——▲).

infectivity was present compared to that in controls. In primary leaves from the 4–45 C:4–25 C treatment, no virus was detected after 15 days.

Although the alternating temperature regimes greatly reduced the accumulation of CCMV in mechanically inoculated leaves, the virus did move systemically into upper cowpea leaves in 4–40 C:4–25 C and 6–40 C:2–25 C treatments (Fig. 2D). However, after 15 days no virus was detected in the upper leaves of 4–45 C:4–25 C plants treated.

Effect of alternating temperatures on the infectivity of virus formed in systemically infected plants. Plants that were systemically infected containing high levels of virus in both mechanically inoculated and upper leaves at the beginning of treatment were incubated in alternating temperature regimes to determine whether virus infectivity was reduced and whether newly developing leaves became infected and accumulated virus. Tobacco plants mechanically inoculated with TMV and cowpea plants mechanically inoculated with CCMV were maintained at 25 C for 12–15 days, after which they were further incubated at 25 C or in temperature regimes of 4–40 C:4–25 C, 6–40 C:2–25 C, or 4–45 C:4–25 C.

None of the temperature regimes significantly reduced the amount of TMV in mechanically inoculated leaves compared to that in leaves maintained at 25 C (Fig. 3A). Infectivity also was monitored in the upper leaves that developed during the alternating temperature regimes. As the plants grew, the amounts of infectious virus extracted from the upper leaves of plants in all of the alternating temperature regimes were markedly less than that in leaves at 25 C (Fig. 3B). However, TMV was not eliminated from the upper leaves in any of the treatments.

In cowpea plants infected with CCMV, the infectivity in the inoculated primary leaves decreased from the high level at day zero to undetectable levels by day 9 in all of the alternating temperature regimes (Fig. 3C). Although substantial amounts of CCMV were detected in the upper leaves at the beginning of treatments, by 9 days the amount of virus in these leaves declined to barely detectable levels (Fig. 3D). The virus was eliminated from older leaves, but not from the younger leaves.

Presence of virus in shoot tips of heat-treated plants. Shoot tips from axillary buds that developed during the alternating temperature treatments were isolated from TMV-infected tobacco plants and regenerated on agar medium. Infectivity assays demonstrated that all of the resulting plants were infected with TMV.

Cowpea shoot tips were transferred to several media in an attempt to regenerate new plants without success. Alternatively, 2–5 cm shoots were placed in distilled water where roots developed. Shoots from plants grown at 25 C developed roots within 2 wk, while those from the alternating temperature regimes required 3–4 wk to develop roots. Cowpea plants that were incubated at

TABLE 1. Effect of different temperature regimes on production of CCMV-free plants from rooted shoot tips

Temperature regime	Virus-free plants/shoot tips rooted from plants: mechanically inoculated[a]	Virus-free plants/shoot tips rooted from plants: systemically infected[b]
4 hr at 40 C, 4 hr at 25 C	0/12	2/16
6 hr at 40 C, 2 hr at 25 C	0/10	10/12
4 hr at 45 C, 4 hr at 25 C	8/8	9/9
Constant 25 C	0/15	0/20

[a] Cowpea plants were mechanically inoculated immediately before incubation in the alternating temperatures for 14 days.
[b] Cowpea plants were mechanically inoculated and incubated at 25 C for 12–15 days prior to incubation in the temperature regimes for 14 days.

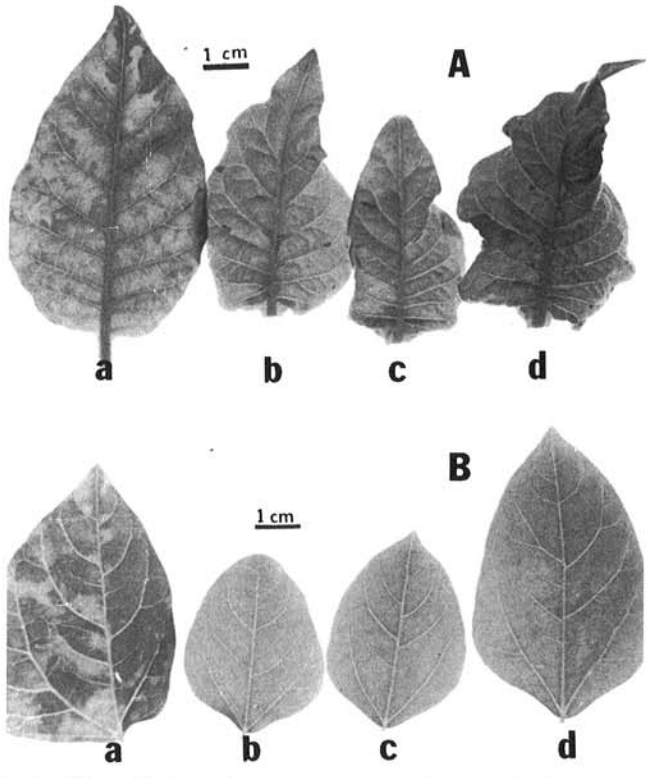

Fig. 4. Effect of alternating temperature regimes on development of systemic symptoms in **A,** TMV-infected tobacco and **B,** CCMV-infected cowpea leaves. Leaves developed at 25 C (a) or in temperature regimes of 4 hr at 40 C and 4 hr at 25 C (b), 6 hr at 40 C and 2 hr at 25 C (c), and 4 hr at 45 C and 4 hr at 25 C (d).

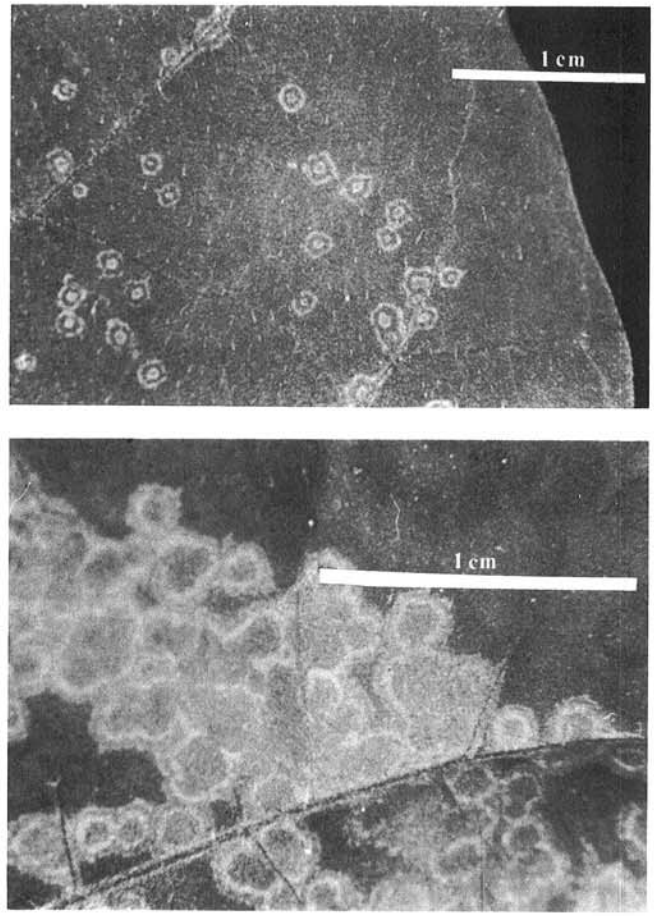

Fig. 5. Two examples of symptoms that developed on tobacco leaves mechanically inoculated with TMV and incubated in a temperature regime of 6 hr at 40 C and 2 hr at 25 C. Photographs were taken 5 days after inoculation.

constant 40 C did not survive 2 wk. A large number of virus-free plants were obtained from shoots that developed in the alternating temperature regimes (Table 1), whereas none of the shoots from plants grown at 25 C were free of CCMV. The 4–45 C:4–25 C treatment was particularly effective; 100% of the plants obtained from shoots were virus-free.

Temperature-resistant virus mutants. In TMV-infected tobacco plants in the alternating temperature regimes, substantial amounts of virus accumulated. One possibility was that this production of virus at high temperatures was due to the development of temperature-resistant mutants. However, TMV and CCMV from heat-treated plants were compared to that from plants maintained only at 25 C for their ability to multiply at high temperatures as described in Materials and Methods. No temperature-resistant mutants of TMV or CCMV were found.

Effect of alternating temperature regimes on symptom expression. Leaves of both healthy and infected tobacco plants that developed in the alternating temperature regimes exhibited different degrees of rugosity (Fig. 1A). However, the development of mosaic symptoms was strongly inhibited in the alternating temperature regimes even though TMV multiplied in these leaves (Fig. 4A). Cowpea leaves infected with CCMV that developed in the alternating temperature regimes exhibited no mottle symptoms (Fig. 4B).

Several atypical types of symptoms were observed on TMV-infected tobacco plants incubated in the alternating temperature regimes. Ringspotlike symptoms developed within about 5 days on mechanically inoculated leaves that normally develop no symptoms (Fig. 5). The type and size of lesions were similar to those resulting from hot water treatment reported by Foster and Ross (6). These ringspots were shown to be proportional to infectivity and the same amount of virus produced more visible ringspots on Xanthi than necrotic lesions on Xanthi-nc plants (Fig. 6). The large number of ringspots produced on half-leaves of Xanthi at low dilutions of inoculum (approximately 600) was much greater than the maximal number of local lesions produced on half-leaves of Xanthi-nc (approximately 150–200) suggesting that a greater number of measurable susceptible sites occur in Xanthi leaves.

Irregular white lines, often in an oak-leaf pattern (Fig. 7), developed on leaves that normally remained symptomless between the inoculated leaves and the young leaves that showed vein-clearing or mosaic when tobacco plants systemically infected with TMV were shifted to the alternating temperature regimes. The white lines delineated the interface between healthy and infected areas of the leaf.

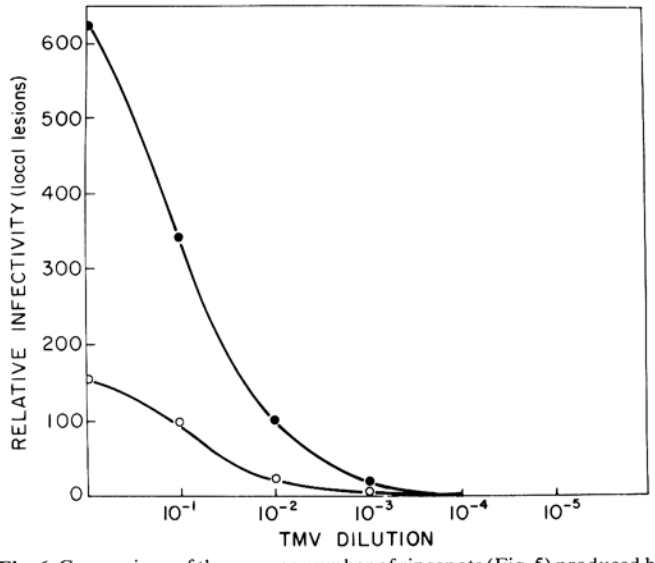

Fig. 6. Comparison of the average number of ringspots (Fig. 5) produced by TMV on half-leaves of tobacco (cultivar Xanthi) incubated in a temperature regime of 6 hr at 40 C and 2 hr at 25 C beginning one day after inoculation (•——•) compared to the average number of necrotic local lesions produced on half-leaves of Xanthi-nc (o——o). Sap from plants infected with TMV was diluted and assayed on half-leaves in a random block design with eight repetitions per sample.

DISCUSSION

Incubation of virus-infected plants in temperature regimes alternating between temperatures restrictive for virus multiplication and host growth and temperatures optimal for both greatly inhibited virus accumulation while allowing somewhat reduced growth of the host. Although the concentration of TMV in tobacco plants incubated in the alternating temperature regimes was greatly reduced, the virus moved into and accumulated in newly developing leaves. The failure to obtain TMV-free plants from buds of heat-treated plants suggests that the alternating temperature regimes did not cause appreciably greater areas near growing tips to be free of TMV. The temperature regimes were much more effective, however, in restricting the replication and movement of CCMV in cowpea. Accumulation of CCMV infectivity was inhibited and preexisting virus in systemically infected plants was greatly reduced and sometimes eliminated from leaves. The high frequency of virus-free plants obtained from newly developing shoots 2–5 cm long demonstrates the effectiveness of the alternating temperature regimes in restricting CCMV in newly developing areas of the plants.

Based on the effect of high temperatures on viral RNA synthesis, TMV multiplication was expected to be controlled by alternating temperature regimes more effectively than that of CCMV. The genomic RNA synthesis of both viruses was totally suppressed at 40

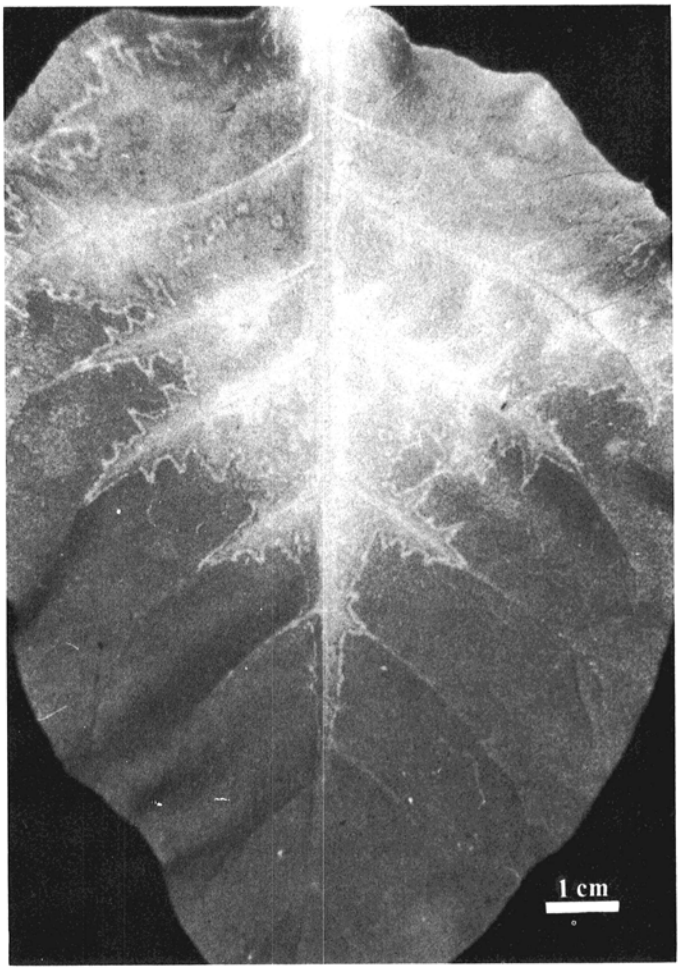

Fig. 7. Symptoms that developed on subsystemic leaves of TMV-infected tobacco plants when systemically infected plants were incubated in a temperature regime of 6 hr at 40 C and 2 hr at 25 C. Photographs were taken 7 days after beginning of treatment.

C as was double-stranded RNA synthesis of TMV, whereas CCMV RF synthesis continued at reduced rates (1,5). Also, the lag period before recovery of virus replication after the shift from 40 C to 25 C was 16–20 hr for TMV compared to 4–8 hr for CCMV (2,5). However, alternating temperature regimes controlled CCMV more effectively than TMV. Despite prolonged exposure to the alternating temperature, considerable amounts of TMV accumulated. One possible explanation may have been the production of temperature-resistant mutants. However, no such mutants were found.

A major component of the effect of the alternating temperature regimes on CCMV infections may be inactivation of virions, since it is known that CCMV is unstable in vivo at 32 C (4). When systemically infected plants with high levels of virus were moved to an alternating temperature regime, the level of CCMV infectivity rapidly decreased whereas that of TMV did not. However, the high temperatures also affected other processes in that the virus was able to move into and multiply in newly developing leaves, but this movement was restricted as demonstrated by the frequency of virus-free plants that developed from 2–5 cm shoot tips.

Although cucumber mosaic and alfalfa mosaic viruses were shown to be inactivated in cell cultures incubated at alternating temperatures of 40 C for 8 hr and at 22 C for 16 hr (11,12), the difference in the effectiveness of the alternating temperature regimes in preventing movement of TMV and CCMV into growing areas of plants suggests that this procedure is effective for only certain viruses. However, the large size (2–5 cm) of the cowpea shoot tips that were free of virus relative to the normal 0.1–0.3 mm shoot tip usually employed (8) should be a major advantage in propagating virus-free plants. Virus-free cowpea plants were obtained from these relatively large shoot tips, whereas, we were unable to induce generation of plants from shoot tips of the size normally used to obtain plants free of virus.

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