A Lethal Disease of Tomato Experimentally Induced by RNA-5 Associated with Cucumber Mosaic Virus Isolated from Commelina from El Salvador

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This is the 4th paper in a series on Cucumber mosaic virus-associated RNA-5(CARNA 5).

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ABSTRACT

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The virus which causes a mosaic disease in *Commelina diffusa* in El Salvador was identified as cucumber mosaic virus (CMV) by host range, serology, and electrophoresis of the ribonucleic acid in polyacrylamide gels. Newly isolated virus from commelina (CMV-Com) did not contain detectable amounts of the nongenomic RNA-5 (cucumber mosaic virus associated RNA-5 = CARNA 5). However, CMV-Com was capable of producing substantial amounts of CARNA 5 when CARNA 5 from the CMV-S strain was added to the inoculum; CMV-Com also produced by itself CARNA 5 when passed serially through 'Xanthi' tobacco plants. CMV-Com without detectable CARNA 5 (one transfer in tobacco) incited only a mild systemic mosaic in *Lycopersicon esculentum* 'Rutgers' tomato plants, which developed a fernleaf condition without necrosis or death of the plants. Plants inoculated with CMV-Com that contained CARNA 5 (6 transfers in tobacco) developed necrosis, collapsed, and died. Seedlings inoculated when 8 cm tall (20 days old) were dead after 18 to 20 days; those that were 45 cm tall (60 days old and in flower) died within another 60 days. They did not bear fruit, whereas, noninoculated control plants and plants infected with RNA 1-2-3 did bear fruit. This disease appears to be identical to that which devastated the tomato crop in the French Alsace in 1972. Production of CARNA 5 is supported by French and South African strains of CMV. This is the first report of CARNA 5 in the Western Hemisphere.

Mosaic diseases of *Commelina* spp. have been reported from Hawaii (1) and Florida (12, 16). Cucumber mosaic virus (CMV) was reported to be the cause of a mosaic condition of *C. nudiflora* L. in 1940 (13). In 1975, F. F. Smith observed mosaic symptoms in *C. diffusa* Burm. f. growing alongside irrigation ditches in El Salvador. Cuttings from diseased plants were taken to the quarantine facility at the Plant Introduction Station, Glenn Dale, MD for virus indexing.

The initial purpose of this study was to identify and characterize the causal agent. When it was identified as CMV, it was incorporated into another project for the purpose of testing for the presence of CMV-associated ribonucleic acid-five (CARNA 5). Evidence that CARNA 5 is a small satellite-like RNA, encapsidated within CMV, and dependent for its replication on, but not part of, the viral genome, has been published (2, 7). When present along with RNA 1-2-3 it causes an experimentally induced lethal necrotic disease in tomato plants (8). After determining that CARNA 5 was not present in detectable amounts in CMV in fresh cultures from commelina (CMV-Com), a further objective was to determine whether CMV-Com supports the production of CARNA 5 as the S strain from South Africa does (5, 7). Our final objective was to study the disease caused in tomato plants by the commelina isolate and CARNA 5.

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MATERIALS AND METHODS

Sources of materials, equipment, and methods employed to study host range and serological identity of this virus, along with the RNA extraction procedures employed, have been described (15).

Virus purification and serology.—We purified the Com, S (South African), and D (French) (5) strains of CMV from *Nicotiana tabacum* L. 'Xanthi', inoculated 7 days previously, according to the procedure of Lot et al. (10). To determine host effects on the proportions of viral RNA's 1-2-3-4, CMV-Com also was purified from *Chenopodium quinoa* Willd. 10 to 14 days after inoculation. Electrophoretic migration of CMV-Com was studied in 2.4% polyacrylamide gels as described for other strains of CMV (9). Partially purified CMV-Com was serologically tested with antisera to 28 spherical plant viruses in 0.75% Ionagar gels with wells 5 mm apart. Antisera specific to the S and D strains of CMV were gifts of J. C. Devergne, France. Antiserum to CMV-C was

produced by H. Scott, University of Arkansas.

Ribonucleic acid.—The number and relative amounts of RNA components were determined by polyacrylamide gel electrophoresis as previously described (7, 9). Gels containing electrophoresed RNA were scanned in a Gilford spectrophotometer (9).

Progressively larger proportions of CARNA 5 are produced by some CMV-strains during successive passages in 'Xanthi' tobacco (5). To determine whether CMV-Com would support production of CARNA 5, it also was mechanically transferred to tobacco plants in the 3- to 4-leaf stage.

We separated CARNA 5 of CMV-S from its viral RNA by rate-zonal centrifugation on sucrose gradients as described by Kaper and Tousignant (5). Pathogenicity of CMV-Com without CARNA 5, CMV-Com that contained CARNA 5, and CARNA 5 alone (from the S strain) was determined by inoculating Rutgers tomato seedlings (*Lycopersicon esculentum* Mill.). In most experiments we used CMV-Com at 10 μg/ml and CARNA 5 at 2.5 μg/ml in 0.03 M Na_2HPO_4. Depending on the season, plants were grown either in controlled environmental chambers at 22 C (during the summer) or on open greenhouse benches. Disease development was recorded up to 3 mo after inoculation on surviving plants.

RESULTS

Symptoms and host range.—Most of the leaves of the source commelina plants displayed chlorotic streaks, spots, and small ringspots that were observed within 6 to 10 days on new leaves of the rapidly growing shoots (Fig. 1). Symptoms persisted throughout the year in the greenhouse. Leaves were not visibly deformed nor were plants noticeably stunted. Virus was readily isolated from commelina by triturating leaves in 0.025 M phosphate buffer pH 7.2 and rubbing the juice onto leaves of *C. quinoa*, *N. tabacum*, or *Vigna unguiculata* (L.) Walp. 'Blackeye'.

Fig. 1-2. Leaves of *Commelina diffusa* showing a chlorotic pattern due to infection with cucumber mosaic virus. **1)** From source plant of the virus and **2)** from plant inoculated with purified CMV-Com with CARNA 5 in the inoculum. Presence of CARNA 5 in purified inocula had no observable effect on symptoms.

Healthy seedlings of *C. diffusa* developed chlorotic symptoms about 2 wk after being rubbed with juice from infected commelina plants or with purified virus (Fig. 2), plants rubbed with extracts from healthy commelina seedlings remained symptomless. Purified virus containing relatively large amounts of CARNA 5 (14%) incited symptoms that were indistinguishable from those incited by inoculum without CARNA 5. Amount of CARNA 5 produced in commelina was not determined by purifying the virus from infected plants; however, symptomatic plants which had been inoculated with CMV with and without CARNA 5 did not incite necrosis when back-indexed on tomato seedlings. This suggested that CARNA 5 was not produced in commelina plants. The behavior of CMV-Com was similar to that of common strains of CMV (4) with regard to host range and stability in vitro.

Purification, serology, sedimentation, and electrophoretic mobility.—The commelina isolate sedimented as a single opalescent band in 10 to 40% sucrose density gradients. Yields were 100-400 mg/kg of tobacco tissue and was 400 mg/kg of *C. quinoa* tissue. Purified virus reacted with antisera to the C, D, and S strains of CMV. It did not react with antisera to any of the 27 other viruses. Purified CMV-Com electrophoresed as a single component (Fig. 3) at the same rate as CMV-D and considerably slower than the S strain. In the analytical ultracentrifuge its sedimentation behavior was the same as that described for other CMV strains (9).

Ribonucleic acid.—The proportions of genomic RNA differed according to the host plant in which CMV-Com was propagated. Virus purified from tobacco was always comparatively high in RNA's 1 and 2 and low in RNA 4 (Fig. 4), whereas virus from *C. quinoa* was low in RNA's 1 and 2 and consistently high in RNA 4 (Fig. 5).

Purified CMV-Com from tobacco one passage after its isolation from commelina plants showed no trace of CARNA 5 (Fig. 4). However, like other CMV strains (5), 14% of the total RNA was CARNA 5 when CMV-Com was purified from tobacco inoculated after five successive passages through tobacco plants (Fig. 6). Comparable levels of CARNA 5 were attained after the first passage only when the tobacco plants were inoculated with purified CMV-Com (10 μg/ml to which was added 2.5 μg/ml of CARNA 5 obtained from the S strain (5). These experiments demonstrated that CMV-Com could support the production of CARNA 5 under these two conditions.

Symptoms in tomato plants.—Purified CMV-Com which contained its own CARNA 5 or S strain CARNA 5, shown to be identical by Kaper and Tousignant (6) killed 95% of inoculated Rutgers tomato seedlings in six replicated experiments involving 34 plants over the 4-mo duration of this study (Table 1). None of the control plants rubbed with CARNA 5 alone (from the S strain),

or with RNA 1-2-3-4 alone (e.g., devoid of CARNA 5) developed necrosis or died.

Plants inoculated with CARNA 5 alone did not become infected or show any disorder (Fig. 7). Plants inoculated with purified CMV-Com containing only RNA 1-2-3-4 developed a mild mosaic (Fig. 8, Table 1). These plants later developed a fern-leaf condition like that reported for certain other strains of CMV (4). Although slightly stunted after 50 days, none of these plants displayed any necrosis.

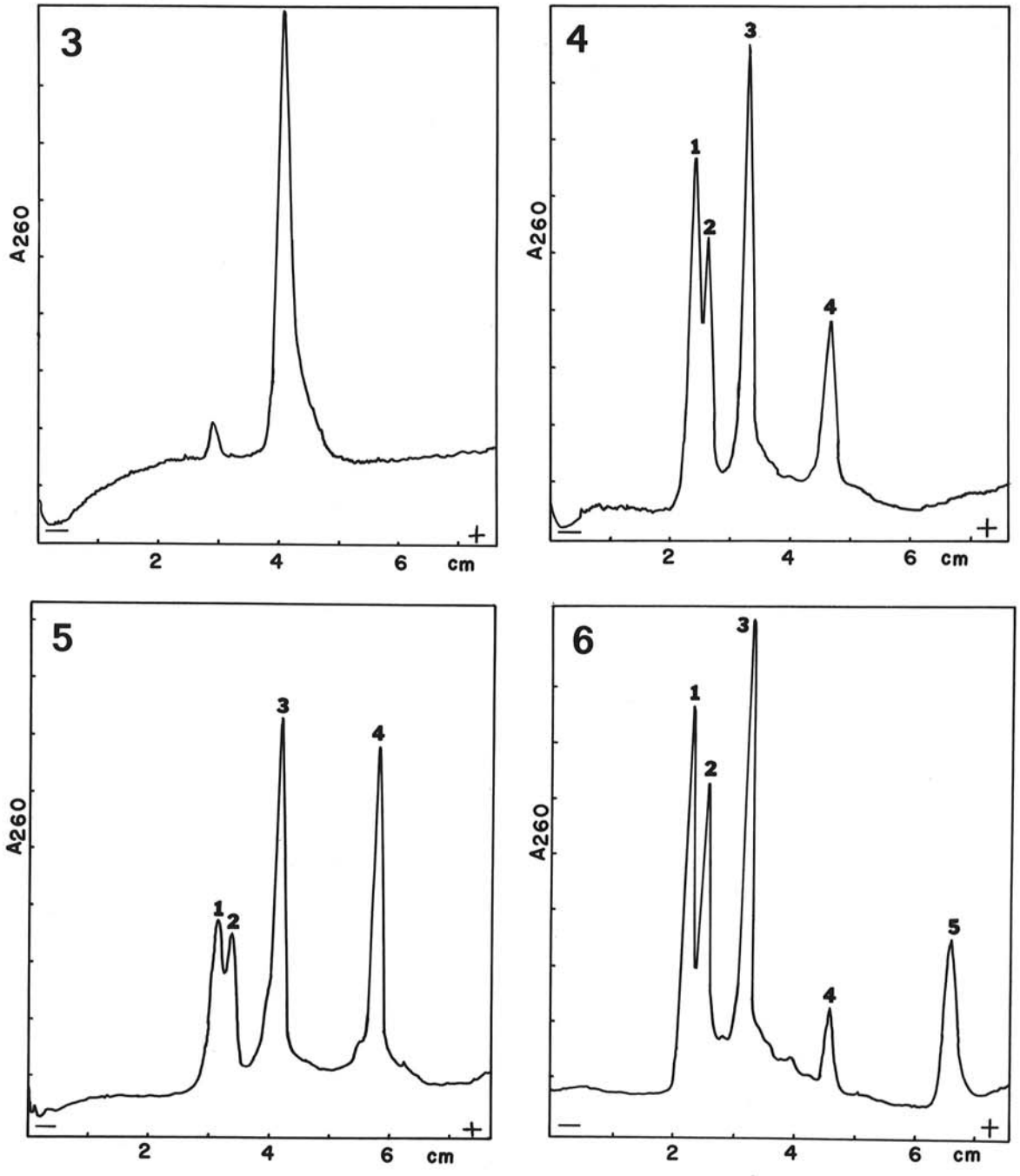

Fig. 3-6. Polyacrylamide gel electrophoresis patterns of 3) CMV-Commelina strain; the smaller peak at 2.9 cm is aggregated virus particles; and (4-6) the RNA from CMV-Commelina. **4)** A typical profile of RNA from CMV propagated in first passage tobacco plants; 5) represents the RNA profile of CMV propagated in *Chenopodium quinoa*. **6)** The RNA profile of virus from tobacco after the sixth passage. Preparations of this RNA composition are lethal to tomato plants at all sizes tested, up to 45 cm.

Plants inoculated with both RNA 1-2-3-4 and CARNA 5 became necrotic and died.

The development of the lethal disease in plants inoculated with CMV-Com which contained CARNA 5 varied even among a "homogeneous" population of seedling plants of a given experiment. The one symptom that 95% of these plants had in common was premature death. Death occurred in spite of variations in temperature, the size and age of plants when inoculated, the concentration of inoculum, and duration of the disease. Most plants inoculated at the first-true-leaf stage were dead after 18 days, whereas flowering 60-day-old plants which were 45 cm tall when inoculated were dead within another 60 days. None of the plants infected with CMV containing CARNA 5 fruited but those infected with only CMV-Com RNA 1-2-3-4 did.

First symptoms of infection with CMV-Com that contained CARNA 5 were necrotic blotches (Fig. 9-a) on one or more inoculated leaves, or a necrotic midrib in one or more systemically infected leaflets. The necrosis appeared in many leaves in another 5-10 days. Usually plants showed leaf epinasty (Fig. 9-b and 10-a, b) and general chlorosis. In small plants (under 10 cm) advanced systemic chlorosis, necrosis, and leaf epinasty (Fig. 11-a) lead to the death of most plants in less than 2 wk. Larger plants, on the other hand, did not die as rapidly. When most of the veins and midribs became sufficiently necrotic, the upper 50% to 90% of the plant died, after a day or two with wilted appearance, leaving only a green stem and one or more of the lowest leaves. Since axillary buds did not develop, the remaining portion of the plant died during the following 2 to 4 wk (Fig. 11-b).

Occasionally the necrosis appeared only in a single inoculated leaf. The necrosis spread down the petiole to the stem, causing a necrotic streak on one side of the stem. Sometimes the necrosis girdled the stem and caused otherwise healthy-appearing plants to break over (Fig. 12).

TABLE 1. Response of Rutgers tomato seedlings to mechanical inoculation with two strains of cucumber mosaic virus purified from 'Xanthi' tobacco plants

Inoculum CMV strain and RNA composition	Response of plants to inoculation
Commelina strain, intact virus purified from first transfer [RNA-5 (= CARNA 5) undetectable]	mosaic and fern- leaf (Fig. 8)
from sixth serial transfer (14% of RNA was CARNA 5) (Fig. 6)	death (Fig. 11)
from first transfer plus CARNA 5 added from S strain (Ref. 5)	death (Fig. 11)
S strain (from sixth serial transfer; RNA extracted and separated into two parts[a]) Part 1 RNA 1-2-3	mild mosaic
Part 2 CARNA 5	no infection
Parts 1 + 2 (RNA 1-2-3 + 5)	death

[a]See reference 8 (J. M. KAPER and H. E. WATERWORTH, 1977. Virology 74:209-222) for details.

In previous work (8) we confirmed the association of CARNA 5-containing CMV with necrosis in tomato plants by purifying the virus from infected tomatoes and electrophoresing its RNA. In necrotic plants CARNA 5 was abundant but it was undetected in plants without necrosis. This association was further confirmed by indexing CMV-Com-infected tomato plants by mechanical inoculation to young tomato and *C. quinoa* seedlings. Virus from tomato plants without necrosis incited distinct chlorotic lesions in *C. quinoa* after 6 days, proving that the tomato plants contained virus, and only a mild mosaic in tomato plants – usually after 2 to 3 wk. Virus from necrotic tomato plants also incited chlorotic lesions in *C. quinoa*, but also incited necrosis in tomato plants within 2 to 3 wk.

DISCUSSION

So far as we can determine, the experimental necrotic disease described is the same as the naturally occurring disease which devastated field tomato crops in the French Alsace region in 1972 (11, 14). Whether this disease has ever occurred in El Salvador or the USA is not known. Surveys are being made in order to answer this question.

To the casual observer, the CMV-induced necrotic disease could be confused with certain bacterial- and fungus-incited diseases, especially early blight, Fusarium or Verticillium wilts, southern bacterial wilt, and even late infections by *Pythium*. However, distinct differences in symptoms and disease development exist.

Although CMV-Com supported production of CARNA 5 in tobacco and presumably in tomato plants, our data suggested that there was very little CARNA 5 in the source commelina plants from El Salvador and that it was not produced when plants were inoculated with CMV-Com + CARNA 5. Consequently the primary problem in El Salvador is that this perennial weed, which grows in major agricultural regions, serves as a continuous reservoir of aphid-transmissible virus which could serve as a helper for the production of CARNA 5.

In surveying for virus disease problems, F. F. Smith noted that some of the common crops near the commelina-infested irrigation ditches are melons, cucumbers, beans, and cowpeas. Unless these crops or other plant species nearby would themselves actively support the multiplication of CARNA 5 in the presence of CMV, the danger of sudden outbreaks of CARNA 5 disease(s) would be small. Research is being conducted on these aspects of the CARNA 5 problem.

The symptoms we observed in commelina leaves that had been innoculated with purified CMV-Com looked more like the symptoms Morales et al. (12) attributed to infection by a potyvirus than the symptoms he attributed to his isolate of CMV from commelina. However, our isolate and Morales' isolate were alike in that each incited a kind of chlorotic ringspot in commelina although the types of ringspot produced were quite different.

Doolittle and Webb (3) reported that one (American Type Culture Collection Virus #30) of six strains of CMV from commelina was lethal to spinach cultivars that were "highly resistant" to their five other strains of CMV. It is not likely that the CARNA 5 was the cause for its lethality in spinach since tomato plants were not killed by their isolate.

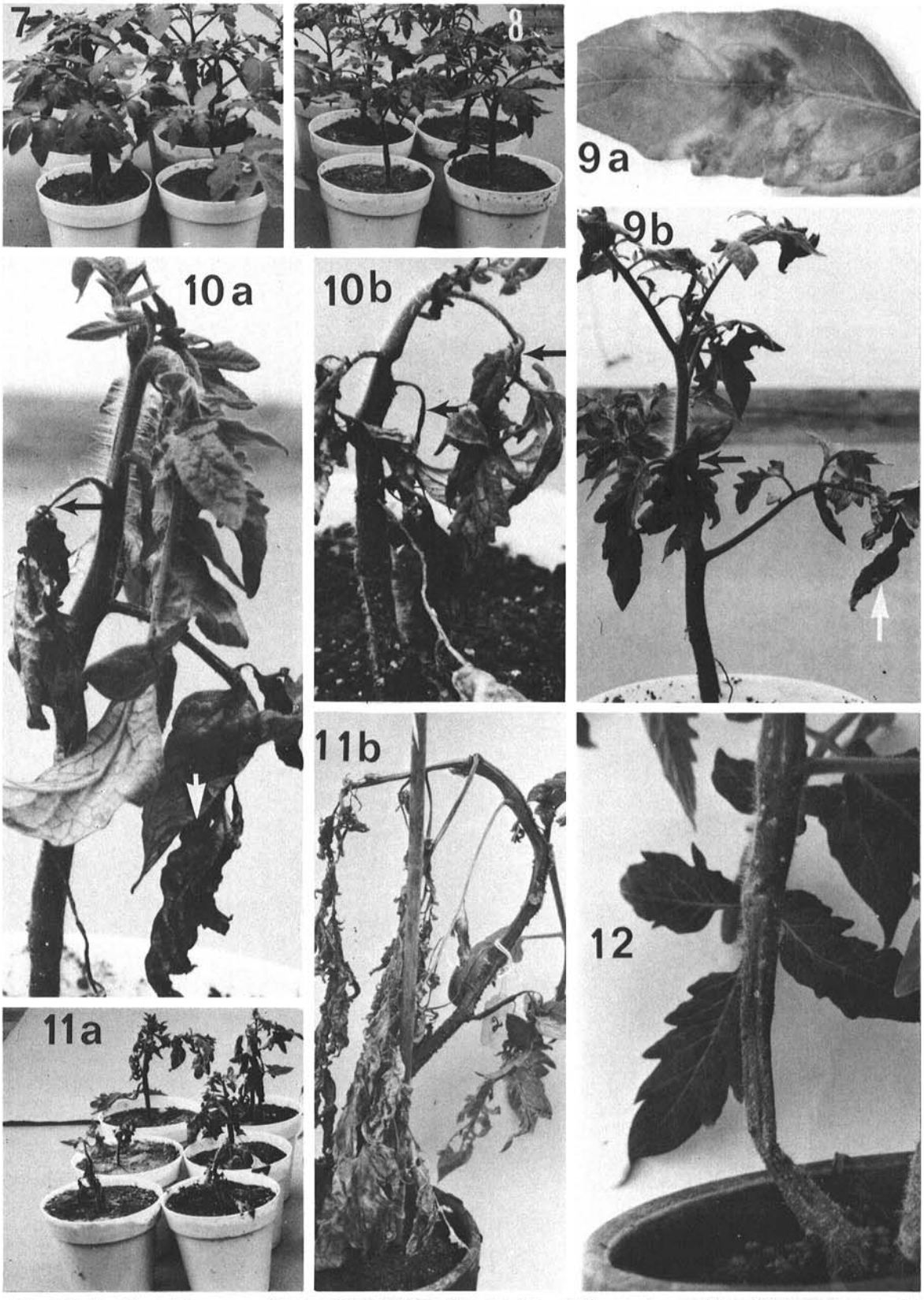

Fig. 7-12. Seedlings of tomato cv. 'Rutgers' that had been inoculated by rubbing the leaves with purified preparations of: **7)** CARNA 5 only (from the S strain), **8)** CMV-Commelina strain in which no CARNA 5 was detected. Plants in Fig. 7 show no symptoms and those in Fig. 8 show early stages of typical CMV-induced narrowing of leaflets. Fig. (9-12) plants, (inoculated with CMV-Com that included CARNA 5) showing progressive stages of the disease from **9)** early symptoms through **10)** intermediate to **11)** advanced disease. Symptoms begin with 9a) local necrotic areas and 9b) entire necrotic leaflets (arrows), leaf epinasty, and later include 10-a, b) necrotic petioles (arrows), wilting, and general chlorosis, and finally 11) death of the infected plants. Those in 11a) were 10 cm tall and those in 11-b) were 45 cm tall when inoculated. Figure **12** shows a less common localized reaction in the stem due to infection with CMV and CARNA 5. These plants break over and die. Plants in Fig. 7, 8, and 11-a were inoculated at the same time.

Yarwood described a reversible host adaptation phenomenon in serially transferred CMV cultures (17). Several of the host reactions that he described among his experiments were attributed to selection of mutants. Yet a buildup of CARNA 5 during his serial transfers in cucumber, for example, could easily explain his "reversible" host reaction when the virus was returned to cowpea where CARNA 5 may not have been produced as abundantly or not at all.

Serial transfers of CMV-Com in tobacco plants results in the presence of CARNA 5 even though none was detected in the initial inoculum. Attempts to explain this have been given (5) and studies are in progress to determine if CARNA 5 originates in specific host plants.

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