Characterization and Field Studies of a Cucumber Mosaic Virus Isolate from Spinach in the Winter Garden Area of Texas

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ABSTRACT

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An isolate of cucumber mosaic virus (CMV) was identified from spinach in the Winter Garden area of Texas. The isolate was very closely related serologically to strain S of CMV and is designated the Texas spinach isolate of CMV-S. The virus infected 39 species of crop plants and wild hosts in 12 of 13 families tested. The green peach aphid efficiently transmitted the virus experimentally. The isolate had a sedimentation coefficient of 91.8 ± 0.1 S as determined by analytical ultracentrifugal analysis. Virions with a mean diameter of 28.9 ± 0.3 nm were found in purified preparations with electron microscropy. A single protein subunit with a mean molecular weight of 25,300 ± 255 daltons was found by sodium dodecyl sulfate polacrylamide gel electrophoresis (SDS-PAGE). Four separate RNA species were resolved by agarose-gel electrophoresis under denaturing conditions. The mean molecular weight distribution of the viral genome was 1.22, 1.09, 0.77, and 0.36 × 10^6 daltons. CARNA 5 was not jointly encapsidated with the viral genome. In the field studies, planting date and plant age at inoculation had no significant effect on crop yield, although CMV infection was highly significant. The isolate significantly affected yields of three spinach cultivars, with reductions ranging from 23.8 to 47.4%.

Additional key words: spinach blight, *Spinacia oleracea*

Spinach L.) was the third most economically important vegetable crop for fresh market in Texas for 198 1. About 22,000 t of fresh-market spinach were produced in Texas in 198 I with a market value exceeding $18 million (11). The two major production areas in the state, the Winter Garden and the Lower Rio Grande Valley, account for about 45% of the total fresh-market spinach acreage harvested in the United States (1).

Five viruses are presently known to contribute consistently to yield losses of spinach each year in Texas. They are beet curly top virus, cucumber mosaic virus (CMV), spinach yellow dwarf virus, tobacco ringspot virus (9), and a newly described isometric virus, as yet unnamed, which has severely affected spinach production in southern Texas (5). In a survey during the 1979–1980 winter growing season, a new virus isolate was obtained. Infected spinach plants not only showed the familiar spinach blight symptoms most commonly associated with CMV but also showed systemic mottling, acute veinal distortion, or severe leaf narrowing, distortion, and leaf curling. The range and variability of symptoms expressed in different spinach cultivars were examined, and the effect of the virus on yields of selected spinach cultivars was determined under field conditions.

In this paper, we characterize the spinach blight virus with host range, vector transmission studies, analytical ultracentrifugal analysis, electron microscopy, serology, and electrophoresis. Furthermore, we demonstrate yield losses of commercial spinach cultivars attributed to the CMV isolate.

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MATERIALS AND METHODS

Virus isolate source. The isolate characterized in this paper was obtained in February 1980 from naturally infected spinach plants. The virus was collected within the Winter Garden area in Zavala County, TX, and maintained on *Nicotiana tabacum* L. 'Samsun NN.' For purification purposes, the virus was propagated in tobacco cultivar Xanthi NN.

Host range and aphid transmission. Test plants were selected from host ranges of spinach viruses and from weed species common around spinach fields. All plants were grown in 25-cm pots in the greenhouse. Mechanical inoculations utilized sap from apical leaves of the maintenance host buffered with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1 % 2-mercaptoethanol and 600-mesh Carborundum. Control plants were maintained and recovery tests were made on *Cucurbita maxima* Duch. 'Zucco' to detect latent infections in hosts that remained symptomless after inoculation.

Green peach aphids (*Myzus persicae* Sulz.) reared on spinach were held for a 1-hr preacquisition starvation period, then transferred to strongly symptomatic and caged spinach plants infected with the spinach isolate. After I-min to 3-hr acquisition feeding periods, the aphids were placed on six healthy caged spinach plants (IO-15 cm high) overnight. The test plants were observed 2 wk for symptom development.

Purification, analytical ultracentrifugation, and electron microscopy. The spinach isolate was purified using a modification of the procedure described by Lot et al (8). Triton X-100 was added to the extraction buffer to 2% at the beginning of the procedure rather than after clarification with chloroform extraction (13). In addition, the virus was finally resuspended in 5 mM sodium borate buffer, pH 9.0, with sodium metaphosphate added to 0.5% to reverse aggregation.

Sedimentation analyses were carried out with purified virus fixed with formaldehyde (4), dialyzed against 5 mM borate resuspension buffer to equilibrium, and analyzed using a Spinco An-D rotor run at 32,000 rpm and at 23.4 C.

Virion morphology was determined with a Hitachi HS-7S electron microscope. Purified virus particles were fixed with formaldehyde, freeze-dried on a Parlodion-coated EM grid, and stained with 1% uranyl acetate in 95% ethanol. Mean virion diameter was determined from measurements of 100 virus particles.

Antiserum production and serology. Antisera against purified and formaldehyde-fixed CMV were obtained by intramuscular and subcutaneous injections of rabbits, 0.5 mg of virus per injection, at weekly intervals. The preparations were emulsified with an equal volume of Freund's complete adjuvant (Difco).

Antiserum prepared against the spinach isolate and CMV-S antiserum (ATCC PVAS 242a) was tested against purified and formaldehyde-treated antigens of the spinach isolate and CMV-Commelina (ATCC PV 30) to compare homology between the antigens.

Analysis of virion protein and nucleic acid. CMV capsid protein molecular weights were determined with sodium dodecyl sulfate-polyacrylamine gel electrophoresis (SDS-PAGE) (6). Molecular weight marker proteins, 14,400–92,500 daltons, were obtained from Bio-Rad.

The viral genomic RNAs were analyzed by electrophoresis in 1.4% agarose gels under denaturing conditions (7). Three markers, including the genomic RNAs of tobacco mosaic virus, brome mosaic virus, and Hind 111 restriction endonuclease DNA fragments of phage λ, were used as molecular weight standards.

RNA was extracted from purified virus preparations following the protocol of McMaster and Carmichael (10). The molecular weights of viral RNAs were determined by plotting the logarithms of the molecular weights of marker RNAs and DNAs against migration distances.

Field studies. Two field studies were conducted to determine the effect of planting date, plant age, and cultivar selection on plant density and final crop yield of CMV-infected spinach plants. Four smooth-leaf spinach cultivars from Del Monte, designated A, B, C, and D, were tested in each study. In study 1, the cultivars were planted three times during the growing season and inoculated at the four-leaf stage. In study 2, the cultivars were planted at the beginning of the growing season and inoculated at different stages of growth. Three field plots 46.9 × 13.4 m were used as replicates for each study. Three double-bedded rows 0.97 m wide were planted for each cultivar per plot, and border rows were planted to encompass each plot. Each row was divided into six 3.66-m sections, each separated by a 3-m section of unplanted vacant row, except for border rows, which were planted solid. Disulfoton (Di-Syston) and diazinon (Diazinon) were applied to the test plots to minimize vector transmission of the virus.

The virus inoculum was prepared by blending CMV-infected tobacco leaf tissue with 0.05 M potassium phosphate buffer, pH 7.0, containing 0.1% thioglycolic acid, at a ratio of 4 ml of buffer per gram fresh weight of tissue. The extract was filtered twice through cheesecloth, and 600-mesh Carborundum was added to 1% (w/v) to enhance inoculation. The inoculum was applied with a 250-ml DeVilbiss paint sprayer and propelled with CO_2 at 45-50 psi of tank pressure. The sprayer was calibrated to a flow rate of 258 ± 28.9 ml/ min. A spray nozzle-to-leaf distance of 5 cm was maintained. For each inoculation, a 3.66-m row section of each cultivar was spray-treated. The cultivars were arranged randomly in a diagonal pattern across each field plot. One bed of each row section was spray-inoculated, and the other bed was sprayed with extraction buffer containing only 1% Carborundum. Double rows of untreated plants on each side of the sprayed rows served as additional controls.

RESULTS

Host range and aphid transmission. The virus was readily sap-transmissible to a wide range of hosts. It infected 39 species of crop plants and wild hosts in I2 of 13 families tested. The susuceptible species included Aizoaceae, *Tetragonia expansa* Thunb.; Amaranthaceae, *Amaranthus* ***caudatus*** L. and ***Gomphrena globosa*** L.; Asclepiadaceae, *Asclepias syriaca* I,.; Chenopodiaceae, ***Beta vulgaris*** L. 'Early Wonder,' ***Chenopodium amaranticolor*** Coste & Reyn., C. ***quinoa*** Willd., and *Spinacia* ***oleracea*** 'Hybrid 62 I'; Compositae, ***Chrysanthemum morifolium*** (Ramat.) Hemsl.; Cruciferae, *Brassica rapa* L. 'Tendergreen'; Cucurbitaceae, *Citrullus lunatus* (Thunb.) Matsum et Nakai 'Congo,' *Cucumis melo* L. 'Hale's Best Jumbo,' C. *sativus* L. 'Chipper,' *Cucurbita* ***maxima 'Zucco,'*** *C. pepo* L. 'Early Sugar,' *Melothria pendula* L., and ***Momordica*** *balsamina* L.; Euphorbiaceae, *Croton* ***capitatus*** Michx.; Leguminosae, *Glycine* ***mux*** (L.) Merr. 'Bragg,' *Vicia* ***faha*** L. 'Long Pod,' and *Vigna unguiculata* (L.) Walp. 'California Blackeye'; Polygonaceae, ***Rumex*** *crispus* L. and ***R.*** *obtusifolius* L.; Solanaceae, ***Capsicurn*** *annuum* L. 'California Wonder,' *C. frutescens* ***I.,*** *Datura metel* L., ***D.*** *stramonium* L., *Lycopersicon esculentum* Mill. 'Petoearly,' ***Nicotiana*** *clevelandii* Gray, N. *glutinosa* L., ***N.*** *rustica* L., N. ***sylvestris*** Speg. et Comes, ***N. tahacum*** L. 'Samsun NN,' N. *tabacum* L. 'Xanthi NN', ***Petunia hyhrida*** Vilm. 'Pink Cascade', *Physalis floridana* Rydb., *Solanum elaeagnifolium* Cav., and S. *melongena* L. 'Japanese Long Purple'; and Umbelliferae, ***Apium*** *graveolens* L. 'Utah 52-70.' No symptoms were observed and recovery tests were negative with *Brassica juncea* (L.) Coss. 'Florida Broadleaf,' ***Avena*** *sativa* L. 'Rodney,' *Zea mays* L. 'Silver Chief,' *Arachis* ***hypogaea*** L. 'Tamnut,' ***Phaseolus*** *lunatus* L. 'Fordhook ***242,' P.*** *vulgaris* L. 'Pinto,' and *Pisum sativum* L. 'Little Marvel.'

The green peach aphid efficiently transmitted the spinach isolate to spinach plants at each acquisition feeding period. In every case, all test plants developed symptoms and eventually blighted within 30 days of inoculation.

Analytical ultracentrifugation and electron microscopy. The sedimentation coefficient curve obtained for the virus isolate using polynomial least-squares regression analysis very closely fit the data ($R^2 = 0.9999$) and is expressed by the function: $S_{20,w} = 91.80 + 0.165C - 0.271C^2$), where C is the virus concentration in milligrams per milliter. Linear regression analysis did not fit the data as closely ($R^2 = 0.9577$.

Small isometric particles were found in purified virus preparations with electron microscropy. The mean diameter of the virions, determined from measurements of 100 particles, was 28.9 ± 0.3 nm.

Antiserum production and serology. Precipitin titers of all antisera prepared against the spinach isolate did not exceed a dilution end point of I /32. Antigen fixed by dialysis with formaldehyde before injection was not significantly more immunogenic than unfixed antigen.

Homology tests between purified antigens of the spinach isolate and known CMV-Commelina at the same concentration were compared using antiserum prepared against the spinach isolate and CMV-S antiserum (PVAS 242a). No spur formation was observed between antigens of the spinach isolate and CMV-Commelina when antiserum against the spinach isolate was used (Fig. IA). However, spur formation did occur between the spinach isolate and known antigens when CMV-S antiserum was used (Fig. 1 B).

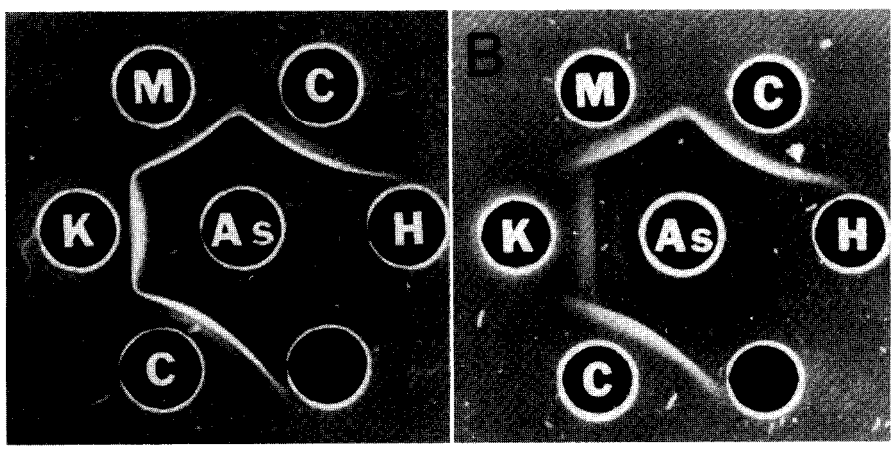

Fig. 1. Serological comparison of the Texas spinach isolate of CMV-S with CMV-Commelina in agarose-gel double-diffusion tests. Well H contained healthy tobacco sap and the unlabeled well was blank. The remaining wells contained I .28 mg/ml of purified virus. Wells C and M represent two separate isolations of the Texas spinach isolate that were found to be identical. Well K contained purified virions of CMV-Commelina (ATCC PV 30). Central wells contain antisera to (A) the Texas spinach isolate and (B) CMV-S (ATCC PVAS 242a).

Analysis of virion protein and nucleic acid. The viral capsid consisted of a single protein subunit. The mean subunit molecular weight estimate of eight determinations was calculated to be about 25,300 ± 255 daltons.

Four separate RNA components (RNAs 1-4) of the viral genome were resolved. The mean molecular weight distribution of the viral genome, estimated from four determinations, was 1.223 ± 0.016, 1.086 ± 0.018, 0.774 ± 0.010, and 0.356 ± 0.008 × 10^6 daltons. The fifth RNA component, CARNA 5, was not detected in the RNA preparations.

Field studies. Symptoms of CMV-infected spinach in the field ranged from blight of seedlings to mottling, stunting, upward leaf cupping, narrowing of leaves, and mild veinal distortion of spinach at intermediate growth stages to severe veinal distortion with no noticeable stunting or chlorosis of mature plants. Most plants surviving inoculation at the four-leaf stage did not show the severe symptoms associated with the seedling and intermediate growth stages, although they did eventually develop the veinal distortion symptom associated with mature plants. Plants usually developed the severe symptoms associated with the intermediate growth stages only when they were inoculated at those stages. Visual detection of diseased plants increased with plant age and approached 100% in fully mature plants. The relatively low detection rate of < 10% for seedlings was due to the rapid decomposition of blighted plants and the low frequency of observations.

Three planting dates were evaluated to determine the effect of planting date, plant age, and cultivar selection on final crop yield after inoculation. In study 1, plants were inoculated at the four-leaf stage after consecutive plantings on 29 September, 27 October, and 30 December 1982, respectively. In study 2, all plants were planted on 29 September and inoculated at different growth stages on the same inoculation dates selected for study 1. Comparisons between yield of inoculated and control plants in plantings 1 and 2 indicated that planting date, plant age, and cultivar selection had no significant effect on final crop yield. However, CMV-infection did significantly affect yield ($P \leq 0.001$). CMV-infected plants had significantly lower yield (1.9 kg) compared with about 2.8 kg/m of row in untreated and Carborundum-treated controls ($P = 0.05$). The third planting was not compared with plantings 1 and 2 because it was harvested before maturity because of incipient bolting tendency.

Plantings 1 and 2 were also analyzed individually to determine the effect of plant age and cultivar selection on final yield. Again, plant age at inoculation had no significant effect on yields of plantings 1 and 2 from comparisons of studies 1 and 2. Similarly, no significant difference was found between yields of the four cultivars tested in both plantings. CMV infections had a highly significant effect on yields of cultivars B, C, and D when cultivars were analyzed individually (Table 1). Inoculated or infected plants of the three cultivars had significantly lower yields than the Carborundum-treated and untreated controls (Table 2).

Planting 3 represented a late winter crop that was harvested before the plants were fully mature to avoid bolting. Significant differences were found between yields of the cultivars at this earlier stage of growth (Table 3). Results of Duncan's multiple range test indicated that cultivar A had the highest yield (0.95 kg), whereas cultivars B and C showed no significant difference in yield (0.71-0.67 kg) and cultivar D had the lowest yield (0.44 kg/ 1.651 m of row) ($P = 0.05$).

The effect of the virus on plant density was evaluated for all three plantings. Planting date, plant age, and CMV infection had significant effects on plant density ($P \leq 0.05$). Infected plants showed significantly higher percent mortality than the healthy controls. However, no significant difference in percent reduction in stand density was observed for the four cultivars. Percent mortality was significantly higher in planting 3 than in planting 2, albeit the mortality rate in planting 1 could not be distinguished from mortality in planting 2 or 3.

DISCUSSION

Homology test results between purified antigens of the spinach isolate and known CMV-Commelina using antisera prepared against the spinach isolate and CMV-S were different. Spur formation did not occur between antigens of the spinach isolate and CMV-Commelina when antiserum against the spinach isolate was used, although spurs did form between these antigens when CMV-S antiserum was used. Thus, the antiserum prepared against the spinach isolate was less specific than the CMV-S antiserum. However, antigen of the spinach isolate

Table 1. Effect of CMV-S infection on yield of each spinach cultivar in plantings 1 and 2 on 29 September and 27 October 1982

Cultivar	df[a]	Mean square[b]	*F* value[c]	GLM[d] (PR > *F*)
A'	2	1,205	1.73	0.1965
B	2	11,148	10.77**	0.0004
C	2	5,669	3.50*	0.0445
D	2	10,123	25.77**	0.0001

[a] Degrees of freedom.
[b] Dependent variable (yield) measured in kilograms.
[c] * = Significant at $P = 0.05$, and ** = significant at $P = 0.01$.
[d] Analysis of variance using general linear models.
'Seed of the cultivars tested provided by the Del Monte Corp. and are not commercially available.

Table 2. Reductions in mean yield of spinach cultivars resulting from CMV-S infections in plantings 1 and 2 on 29 September and 27 October 1982

Planting	Cultivar	Mean yield' Inoculated	Mean yield' Untreated	Difference[b] (%)
1	A'	2.482	2.996	-17.2
	B	1.877	3.239	−42.1*
	C	1.967	2.996	−34.3*
	D	1.392	2.648	−47.4*
2	A	2.368	2.497	-5.2
	B	1.634	2.656	−38.5*
	C	2.126	2.792	−23.8*
	D	1.756	2.421	−27.5*

"Kilograms per 1.651-m row sections sampled.
[b] Percent differences in yield followed with an asterisk are significant at $P = 0.05$.
'Seed of the cultivars tested provided by the Del Monte Corp. and are not commercially available.

Table 3. Effect of cultivar selection on yield of CMV-S-infected spinach in planting 3 on 30 December 1982

Source	df[a]	Mean square[b]	*F* value[c]	ANOVA[d] (PR > *F*)
Model	7	299	7.27	0.000 I
Cultivar	3	1,154	9.33*	0.0002
Treatment	2	504	6.11*	0.0063
Error	28	41	"	"

[a] Degrees of freedom.
[b] Dependent variable (yield) measured in kilograms.
[c] * = Significant at $P = 0.0$ 1.
"Analysis of variance.

reacted very strongly to both antisera. Since the spinach isolate was strongly related serologically to strain S of CMV, we place the isolate in the ToRS serotype group as defined by Devergne and Cardin (2,3). The development of necrotic local lesions on tobacco cultivar Xanthi NN in addition to systemic symptoms provided further confirmation that the isolate belongs to this group. On the basis of serological results, morphology, and physical characteristics, we conclude that the spinach isolate is very closely related to strain S of CMV and we designate it the Texas spinach isolate of CMV-S.

Field evaluations of symptomatology with experimentally inoculated spinach provided evidence that symptom severity was age-dependent. Symptom severity appeared to be inversely proportional to plant age. Since symptoms were more severe when plants were inoculated at earlier stages of growth, disease detection in younger plants was often difficult because the blighted plants decomposed rapidly. Symptoms of mature plants were generally milder with no noticeable stunting or chlorosis, yet the conspicuous veinal distortion symptom was most developed and obvious in mature plants. Planting date and plant age at inoculation had no significant effect on final crop yield. The effects of planting date on yield were compared with mean daily temperature highs during the studies, since temperature is known to be a major factor affecting resistance to CMV (12). Pound and Cheo (12) demonstrated that under experimental conditions, blight resistance of spinach cultivar Virginia Savoy failed at air temperatures of 28 C and higher. For all three plantings,, the mean daily temperature highs during the studies were 28.3 C. The lack of significant yield differences for these planting dates suggests that the critical temperature for breakdown of resistance in the field may be higher than 28 C.

Plant age at inoculation did not significantly affect yield of three cultivars. This is contrary to the observation that symptom severity is age-dependent. The discrepancy may be explained as mortality increases, competition between plants for available resources, including space, decreases. Therefore, high mortality at the seedling stage may be offset by increased growth of surviving plants. This is a major reason why the virus effects on plant density do not necessarily result in reduced yield. Significant differences between yield of the cultivars tested were found only in planting 3 when the crop was harvested before maturity.

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