THE TOBACCO RINGSPOT VIRUS OF WATERMELON IN OKLAHOMA

By

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INTRODUCTION

The watermelon (*Citrullus vulgaris* Schrad.) is one of Oklahoma's more important horticultural crops. During recent, dry years in this area, fungus diseases of this crop have been relatively unimportant, yet many pathological symptoms have continued to appear. Fruits and foliage have continually shown symptoms characteristic of anthracnose, a disease incited by a fungus (*Colletotrichum lagenarium* (Pass.) E. & H.). Symptoms have been manifest during prolonged periods of drouth, conditions very adverse for the spread of this fungus. Black, necrotic lesions on foliage and small, circular intumescences on watermelon fruits have been frequently observed. Both conditions are diagnostic features of plants invaded by the anthracnose fungus. Lesions on the fruit surface seldom become necrotic and sunken, however, as is typical with the anthracnose disease. Several attempts, all unsuccessful, were made to isolate the fungus from fruit and foliage lesions.

Pound (33) described a virus disease of watermelon in Wisconsin which somewhat resembled anthracnose on the foliage. Rosberg (37) found a similar virus associated with a watermelon fruit disease in Texas. Both viruses, on inoculated foliage, induced lesions which were indistinguishable macroscopically from those of anthracnose.

Both watermelon and muskmelon (*Cucumis melo* L.) were found naturally infected in Wisconsin. Pound (33) described symptoms of the disease in field plantings and on susceptible greenhouse plants. The virus was identified by symptomatology, properties, and cross-protection tests as a yellow strain of the tobacco ringspot virus. Naturally infected watermelon plants were severely stunted. Chlorosis and coarse mottle, accompanied by irregular black lesions identical with those caused by Colletotrichum lagenarium were common symptoms on infected leaves. Vines appeared bunchy and compact as a result of shortened internodes. Fruits were misshapen and distorted with necrotic spots. Infected plants produced no marketable fruits; however, vines tended to recover late in the season.

A similar virus was found to be involved in a watermelon fruit disease in Texas (37). Symptoms were confined primarily to fruits, without obvious foliage symptoms being manifest. Others have reported tobacco ringspot viruses in naturally infected cucurbits. Henderson and Wingard (24) observed 30-40% of the plants in a cantaloupe field in Virginia to be infected with the tobacco ringspot virus. These workers also reported naturally infected squash (Cucurbita maxima Duchesne). Johnson (25) found the virus occurring naturally on cucumber (Cucumis sativus L.) and muskmelon in Kentucky, and Valleau (44) reported it commonly causing a mosaic disease in commercial plantings of cucumber in the same state. Pound (33) was the first to find watermelon naturally infected with the tobacco ringspot virus.

A watermelon fruit disease similar to that described in Texas has been common in Oklahoma for a number of years and has sundry names, the more common being: bumps, measles, pimples, pox, sandbumps, sugarbumps and warts. Such names arise by virtue of the pimple-like nature of the fruit lesions. Usually these show as small, blisterlike warts slightly raised above the normal fruit surface. Fruit lesions resemble initial stages of invasion by the anthracnose fungus; however, the two may be distinguished microscopically as no fungus hyphae are present in the virus incited lesions. Fruits do not rot when held in storage as they frequently do when anthracnose is present.

Investigations began in the summer of 1954 in Oklahoma likewise indicated a virus allied with the pimple disease. Inoculations of primary leaves of cowpea with sap from infected fruits resulted in the production of many reddish-brown lesions (Fig. 6). This suggested the presence of a mechanically transmissible virus. Inoculations from 24 of these fruits from several fields over the state, in every case gave the same characteristic lesions on cowpea. Inoculations using expressed sap from watermelon foliage failed to reveal virus. Havana tobacco plants, similarly inoculated with sap from diseased fruits, produced symptoms characteristic of a ringspot disease. Symptoms were very similar on both cowpea and tobacco with all virus isolates obtained from the several fields.

The disease has been observed in all plantings of the Black Diamond variety visited in Oklahoma. In some fields almost 100 percent infection was found. In the light of these observations it seemed appropriate that the nature of the disease be investigated and its importance evaluated.

The watermelon variety of ranking importance, as judged by acreage, is Black Diamond; therefore, investigation was, in the main, confined to the disease on this variety. Florida Giant, Texas Giant, Cannon Ball and Clara Lee are other varietal names for this melon.

MATERIALS AND METHODS

One of the watermelon virus isolates was selected for use in further studies. This virus was recovered from a naturally infected fruit from a planting near Yale, Oklahoma. Stock cultures of this and all other viruses[2] used were maintained in aphid proof cages on Havana tobacco plants. Greenhouses were dusted frequently for insect control. The warmer greenhouse usually fluctuated from 22°C at night to 31°C during the day; the cooler house correspondingly varied approximately from 16°C to 28°C. Test plants were grown, when possible, at the temperatures favoring more rapid growth.

Cucumber plants, variety Boston Pickling, were used in indexing hosts for presence of the watermelon virus. Cowpeas, variety Black, served as assay plants in tests where estimations of virus activity were needed.

All test plants were grown in a soil-sand mixture in four-inch clay pots. Light applications of a balanced, mineral fertilizer applied occasionally insured vigorous plant growth. Test plants in use were observed daily and their reactions noted.

The rubbing method was used in all test inoculations. Generally, when only a small number of plants was to be inoculated, leaves containing the virus were rolled into a small square of non-absorbent cotton and

[2]The tobacco mosaic virus was from naturally infected tomato plants at Stillwater, Oklahoma. Dr. J. P. Fulton of the University of Arkansas generously supplied his A,C,D,E, and F strains (16) of the cucumber mosaic virus. A green strain of the tobacco ringspot virus and the yellow strain from watermelon were obligingly provided by Dr. Glenn S. Pound of the University of Wisconsin.

mascerated with the finger tips. When the cotton became soaked with sap, it was rubbed over leaves lightly dusted with 600 mesh carborundum. Where larger numbers of test plants were to be inoculated, a mortar and pestle were used to mascerate virus-infected tissue and the juice was taken up in a cotton pad used in inoculation. Small, ground glass spatulas were employed in making inoculations during physical property and inhibition tests. Plants were rinsed with tap water after inoculation to remove excess inoculum.

Techniques applicable only to a particular phase of the investigation are described in the appropriate section.

SYMPTOMATOLOGY AND HOST RANGE OF THE WATERMELON VIRUS IN GREENHOUSE STUDIES

Reactions to the watermelon virus were observed and described in detail on tobacco, watermelon and various other selected species representing several plant families. All species in these studies were grown and inoculated in greenhouses during the winter months. Plants were inoculated in the seedling stage when growing rapidly. In every case at least 9 plants of each species were inoculated with the watermelon virus. Three plants of each species were retained uninoculated to serve as healthy controls. Recovery of the virus was attempted from all inoculated plants regardless of the presence or absence of symptoms. Cucumber plants were used in recovery inoculations to observe if multiplication had occurred in each species. Cucumber was chosen for this purpose as it is apparently less affected by inhibitors of infection from higher plants (18), and it shows distinctive symptoms when systemically infected with the watermelon virus.

Symptoms on tobacco

The reaction of Havana tobacco plants when inoculated with the watermelon virus was noted on several occasions. With favorable temperatures, local symptoms appeared on rapidly growing plants in 72 hours. Initial reaction consisted of small chlorotic or necrotic rings or spots. This was rapidly followed by additional concentric rings which were usually necrotic. Usually, the necrotic rings were discontinuous and only rarely consisted of unbroken rings (Fig. 1,A). Often, only solid necrotic spots developed. Symptoms varied somewhat with the age of plant inoculated and temperature. On younger inoculated plants, as a rule, numerous necrotic flecks appeared upon inoculated sites (Fig. 1,A). Older inoculated plants seemed prone to produce solid necrotic lesions in place of rings (Fig. 1,B). At higher temperatures primary symptoms rarely appeared after inoculation, even though infection had occurred. Primary symptoms never appeared on tobacco plants at temperatures above 32°C.

First signs of systemic development of the virus in tobacco were chlorotic spots of a rather bright yellow color. Subsequently, inter-veinal leaf areas became yellow in varying degrees until the leaf showed a prominent chlorotic mottle (Fig. 1,C). Bright yellow, and often almost white, jagged lines developed in association with the larger veins resulting in the formation of oak-leaf patterns (Fig. 1,D). Oak-leaf patterns frequently became necrotic.

Chlorosis of systemically infected leaves became gradually less evident until, eventually, those present at the time of inoculation appeared almost a normal green. Usually, yellowing was much more persistent on leaf margins and these remained faintly chlorotic for an indefinite time. Recovery was perceptibly more rapid at higher temperatures.

Reactions on tobacco were typical of the tobacco ringspot virus (51). However, the Oklahoma virus, according to symptoms on tobacco at least, appeared to be a yellow strain of the virus and similar in this respect to the virus described by Pound (33). Development of yellow spots and mottle did not occur in tobacco inoculated with a green strain of the tobacco ringspot virus. Instead, chlorotic leaf tissue remained a light green color and recovery was more prompt and complete than with the watermelon virus.

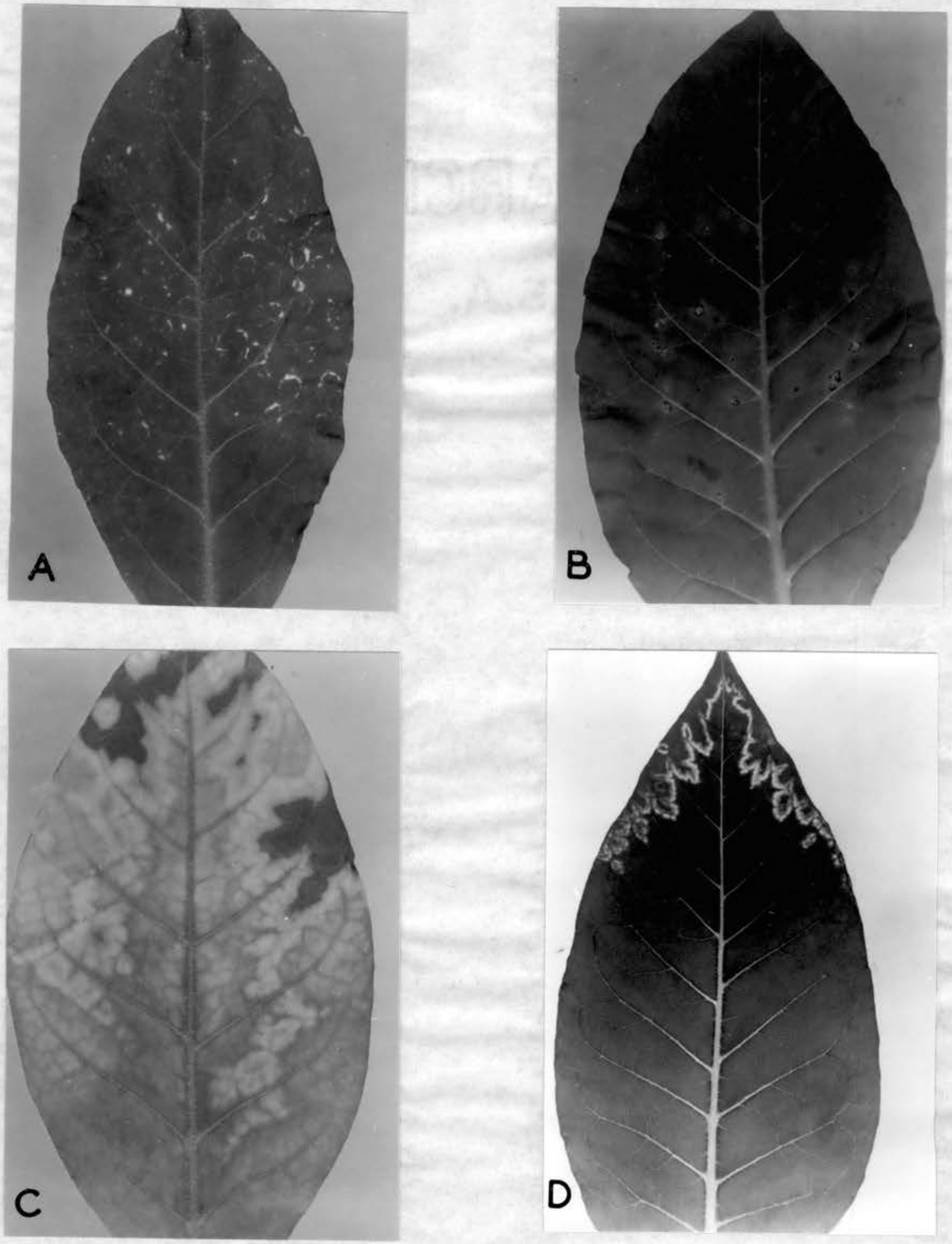

Fig. 1. Local and systemic symptoms on tobacco after greenhouse inoculations with the watermelon virus.
A. Necrotic rings and flecks as primary symptoms.
B. Solid necrotic spots on inoculated leaf. C. Systemic chlorosis and, D. Oak-leaf pattern on systemically infected leaves.

Valleau (44) was the first to note such a yellow strain of the tobacco ringspot virus and found prominent yellowing in tobacco a distinguishing feature of this strain. Price (34) stated that complete masking in older leaves of recovered plants did not occur.

Symptoms on watermelon

Reactions of Black Diamond watermelon plants were studied in the greenhouse. In addition, several other watermelon varieties were inoculated and their reactions noted. Only a few plants of each of these other varieties were used, and a complete description of the disease in these varieties was not attempted. Symptoms on Black Diamond in field plantings are described in a later section.

The initial reaction on inoculated leaves and cotyledons of Black Diamond consisted of black, necrotic lesions appearing at sites of inoculation. These lesions in incipient stages were circular and indistinguishable macroscopically from those of anthracnose. Lesions soon enlarged and became irregular and were very black and necrotic (Fig. 2,B). Local symptoms usually appeared about 72 hours after inoculation.

As primary lesions enlarged the affected portion of the leaf often died if many lesions were present. Inoculated cotyledons invariably died soon after producing local lesions. Systemic infection never resulted from inoculations on cotyledons. Thus, cotyledons appeared to be hypersensitive to the watermelon virus.

Inoculated leaves grown at air temperatures above 32°C seldom developed local symptoms. However, a few chlorotic spots, usually indefinite and scarcely discernible, appeared occasionally on inoculated areas.

Systemic symptoms arose first on immature leaves near the terminal growing point. These symptoms appeared during the initial stages of systemic development of the virus and first showed as small yellow spots (Fig. 2,B). Spots of this sort developed at random over interveinal areas of leaves and frequently were sufficient in number to give the leaf a chlorotic mottled appearance. Chlorotic lesions of this nature often had a tiny, pale center associated with their development.

Irregular chlorotic areas often appeared on very young leaves just beginning to expand in the terminal rosette. Frequently the entire growing point was blighted (Fig. 2,A), and occasionally systemic infection was fatal to plants having initially received a large amount of inoculum. In many cases vines were killed back to the first or second basal node. Axillary buds at those remaining nodes began growth and produced a stunted vine with greatly shortened internodes and undersized foliage.

At higher temperatures plants never died as a result of systemic infection. Instead, plants usually recovered promptly. In general, at very high temperatures infection was totally masked.

Symptoms were also noted on other selected watermelon varieties. These included Klondike, Dixie Queen, Charleston Gray, Congo and Fairfax. All varieties reacted similarly to Black Diamond in the production of both local and systemic symptoms, although Fairfax was never observed to produce the characteristic yellow spots on young leaves. The virus was readily recovered from all varieties to cucumber.

In the main, these observations agree with those of Pound (33), although his descriptions were not detailed enough for an adequate comparison of systemic symptoms. However, local lesions formed after inoculation

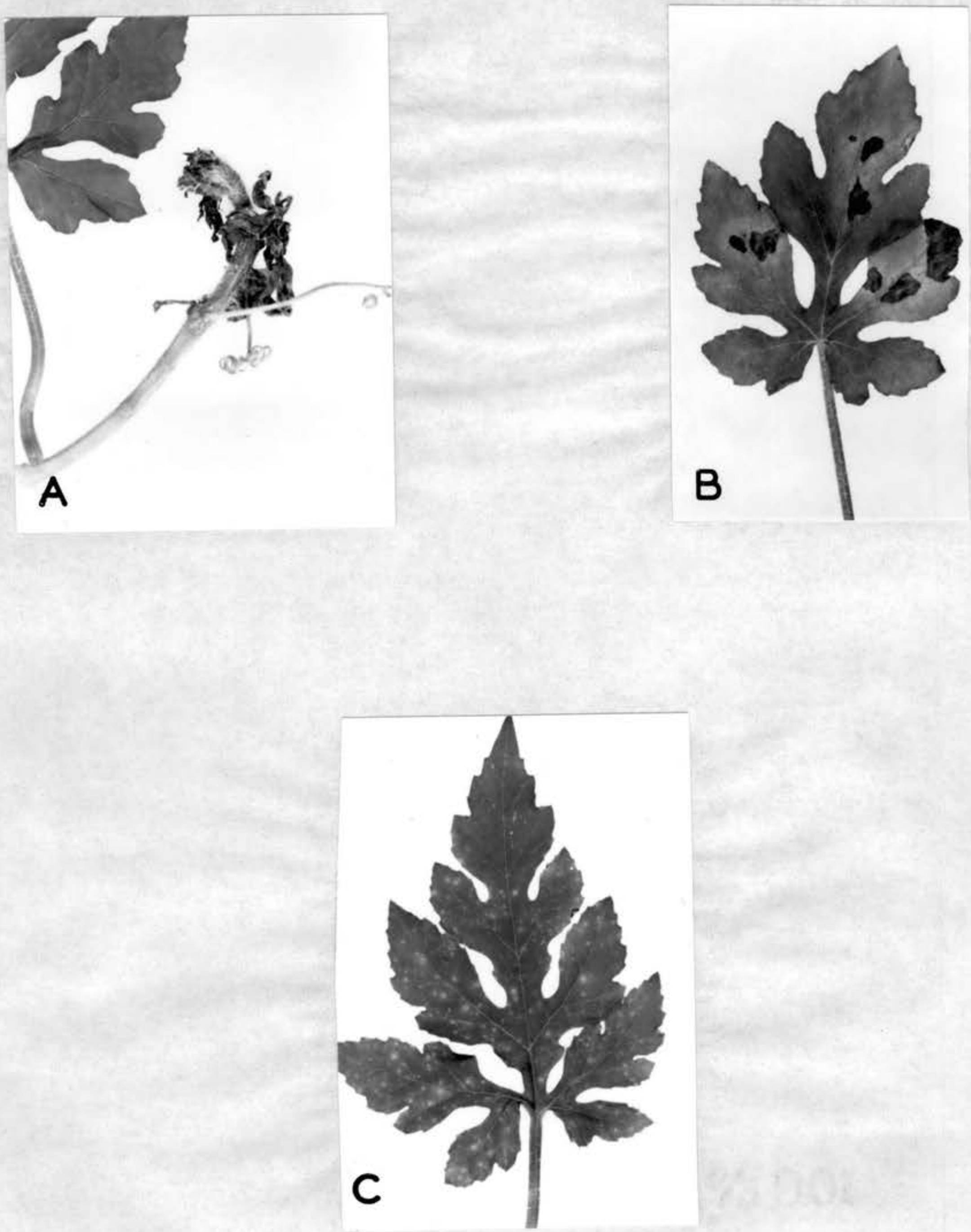

Fig. 2. Symptoms on Black Diamond watermelon after greenhouse inoculation. A. Necrosis of terminal growing point in systemically infected watermelon. B. Necrotic local lesions on inoculated leaf. C. Chlorotic spots in systemically infected leaf.

and stunting in vines systemically infected with the Oklahoma watermelon virus were notably similar to the symptoms described by Pound (33).

Reaction of other hosts to the watermelon virus

Results of these tests are summarized in Table 1. Both local and systemic symptoms are recorded in the table. The virus was recovered from all plants unless noted otherwise.

Host range and symptoms are similar to those reported for the tobacco ringspot virus (51). Some variation in symptoms is understandable on the basis of differences in varieties of host species used and environmental conditions.

Rosberg (37) found petunia not susceptible to the virus he recovered from naturally infected watermelon in Texas and noted this as a difference between the Texas virus and the one with which Pound (33) worked. In inoculations here, however, petunia invariably produced very prominent local and systemic symptoms upon infection with the Oklahoma watermelon virus (Fig. 3,C,D). Even retarded, senescent petunia plants in the greenhouse showed abundant symptoms. Reaction of this species was observed on several occasions. Of the many watermelon virus isolates recovered from naturally infected fruits from widely scattered localities in Oklahoma, all seemed to constitute a rather constant entity and appeared to be very similar or identical to the viruses from watermelon which Pound (33) and Rosberg (37) have described.

As shown in Table 1 all cucurbitaceous plants tested were found susceptible to the virus. Interestingly enough, reactions were somewhat similar on all species and varieties. Local symptoms commonly appeared

Table 1. Host range and symptoms on greenhouse plants after inoculation with the watermelon virus.

HOST SPECIES	PRIMARY SYMPTOMS	SYSTEMIC SYMPTOMS
CHENOPODIACEAE:		
Beta vulgaris L. (garden beet, var. Blood turnip).	Chlorotic lesions followed by etch-like necrotic rings and lines.	General chlorosis, with green color masked by anthrocyanin pigment.
Spinacia oleracea L. (spinach, var. Giant Thick Leaved).	Circular chlorotic lesions, often having necrotic centers.	General chlorosis.
CONVOLVULACEAE:		
Calonyction aculeatum House (moonflower).	. . . None . . .	None; virus not recovered.
Ipomoea purpurea Lam. (morning glory).	Zonate, black necrotic lesions.	Chlorotic mottle followed by irregular black necrosis.
COMPOSITAE:		
Callistephus chinensis Nees. (China aster, var. American Branching).	Zonate, reddish-brown necrotic lesions.	Coarse chlorotic mottle with irregular necrosis.
Helianthus annuus L. (sunflower, var. Double Sun Gold).	Small chlorotic lesions, often becoming necrotic.	General but faint chlorosis.
CRUCIFERAE:		
Brassica oleracea L. (cabbage, var. Stein's Flat Dutch).	. . . None . . .	None; virus not recovered.
B. rapa L. (turnip, var. Purple Top, White Globe, Seven Top).	. . . None . . .	None; virus not recovered.
Raphanus sativus L. (Radish, var. Crimson Giant).	. . . None . . .	None; virus not recovered.

Table 1. (con't.) - Host range and symptoms in greenhouse plants after inoculation with the watermelon virus.

HOST SPECIES	PRIMARY SYMPTOMS	SYSTEMIC SYMPTOMS
CUCURBITACEAE:		
Citrullus vulgaris Schrad. (watermelon, var. Black Diamond, Klondike, Charleston Gray, Congo, Dixie Queen, Fairfax).	Chlorotic spots or circular black necrotic lesions enlarging and becoming irregular; masked at high temperatures.	Small yellow spots appearing first on immature leaves; occasional necrosis of growing tip with subsequent outgrowth of axillary buds; shortened internodes giving compact, bunchy growth habit with stunted and often malformed leaves; symptoms masked at high temperatures.
Cucurbita maxima L. (squash, var. Early Prolific Straight Neck, Giant Crookneck, White Bush Scallop).	Circular chlorotic or necrotic lesions	Yellow spots followed by chlorotic mottle; stunting.
C. pepo L. (pumpkin, var. New England Pie).	. . . ditto . . .	. . . ditto . . .
Cucumis sativus L. (cucumber, var. National Pickling, Boston Pickling, Improved Long Green).	Chlorotic lesions	. . . ditto . . .
C. melo L. (muskmelon, var. Hale's Best, Honey Dew Melon).	. . . ditto . . .	. . . ditto . . .

Table 1. (con't.) - Host range and symptoms in greenhouse plants after inoculation with the watermelon virus.

HOST SPECIES	PRIMARY SYMPTOMS	SYSTEMIC SYMPTOMS
LEGUMINOSAE:		
Phaseolus vulgaris L. (pinto bean)	Necrotic flecks and rings.	Black necrotic cankers on veins, petioles and stems; coarse chlorotic leaf mottle followed by irregular necrosis; necrosis of growing point followed by death of entire plant.
P. coccineus L. (Scarlet Runner Bean).	. . . None . . .	None; virus not recovered.
Pisum sativum L. (pea, var. Alaska, Dwarf Marvel).	Small chlorotic lesions, later becoming necrotic.	Irregular necrosis of leaves and stems; occasional death of entire plant.
Glycine max Merr. (soybean).	Small chlorotic spots.	Coarse chlorotic mottle with some necrosis of stems.
Vigna sinensis Savi. (cowpea, var. Black).	Reddish-brown necrotic lesions.	Chlorotic leaf mottle; dark necrotic cankers on stems, petioles and leaf veins; necrosis of terminal growing point with subsequent death of entire plant.
SOLANACEAE:		
Datura stramonium L. (Jimson weed).	Zonate, chlorotic spots becoming necrotic.	Coarse chlorotic mottle and necrotic spots.
Lycopersicon esculentum Mill. (tomato, var. Rutgers).	. . . None . . .	None; virus not recovered.

Table 1. (con't.) - Host range and symptoms in greenhouse plants after inoculation with the watermelon virus.

HOST SPECIES	PRIMARY SYMPTOMS	SYSTEMIC SYMPTOMS
Nicotiana tabacum L. (tobacco, var. Havana, White Burley).	Necrotic spots and etch-like necrotic and chlorotic rings; masked at high temperatures.	Chlorotic spots and coarse yellow mottle; often prominent chlorotic, oak-leaf patterns frequently becoming necrotic; plants eventually recover; masked at high temperatures.
N. glutinosa L.	Occasional faint chlorotic spots; often masked.	None; virus recovered.
Petunia hybrida Vilm. (petunia).	Zonate, necrotic spots and rings.	Faint chlorotic mottle; necrotic flecking of leaves; necrosis of stems.

as either chlorotic or necrotic lesions. Systemic symptoms usually showed in the form of characteristic yellow spots on leaves (Fig. 3,B). Systemically infected leaves later became slightly chlorotic before plants began a slow recovery. Cucumber usually produced innumerable tiny yellow rings over systemically infected leaves. Most symptoms disappeared after a time although plants at lower temperatures never fully recovered but remained slightly chlorotic and markedly stunted for an indefinite period.

Primary symptoms on bean and pea, as with cowpea, were largely necrotic. Necrotic flecks and occasional rings were produced on inoculated leaves of bean. Systemic infection resulted in a characteristic blighting of the browing point of both bean and cowpea with dark, necrotic cankers appearing on stems, petioles and leaf veins. Infection was usually fatal on both species. On occasion, a coarse, chlorotic mottle developed on trifoliate leaves of bean with systemic development of the virus. At times, an irregular necrosis subsequently appeared on these leaves. This necrosis was particularly evident on veins and veinlets.

The Oklahoma watermelon virus was compared with the Wisconsin watermelon virus in the symptoms each caused on inoculated tobacco, cowpea and cucumber. Reactions on these hosts with each virus were almost identical and indicated that the two viruses were similar or identical.

Wingard (51) has investigated the host range and symptomatology of the tobacco ringspot virus. The host range of the Oklahoma watermelon virus corresponds rather well, although differences are evident. This may be explained to some extent by differences in virus strains. For example, pea (<u>Pisum sativum</u> L.) was apparently not susceptible to Wingard's green strain, whereas, the watermelon virus studied here and the one in Wisconsin (33) were pathogenic on this host.

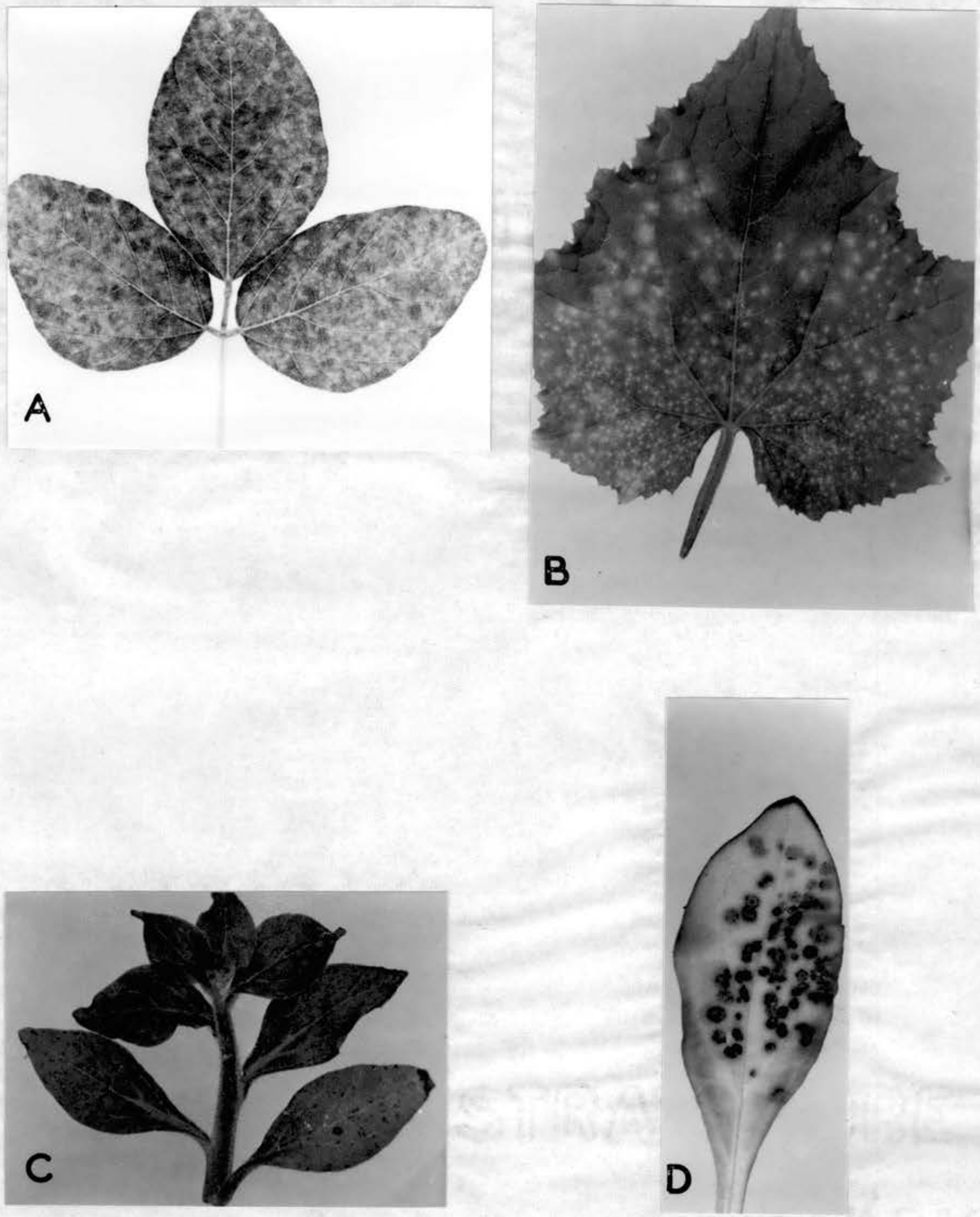

Fig. 3. Reaction of other hosts to the watermelon virus.
A. Systemic mottle on soybean. B. Systemic reaction on cucumber. C and D. Systemic and local necrotic reactions, respectively, on petunia.

In general, other investigators have found few cruciferous plants susceptible to the tobacco ringspot virus. Similarly, the watermelon virus was not found to multiply in any of these plants. On the whole, however, differences, though not great, seemed sufficient to warrant further identification of the watermelon virus by physical properties and more specific means such as cross-immunization and serological tests.

PHYSICAL PROPERTIES OF THE WATERMELON VIRUS

Methods used in determination of physical properties were similar to those described by Walker, LeBeau and Pound (47). Tests on thermal-inactivation-point, tolerance-to-dilution and aging *in vitro*, were repeated three times.

All tests were made using expressed sap from young Havana tobacco plants inoculated 2 weeks previously with the watermelon virus. Stanley (43) and Price (34,35) found the tobacco ringspot virus to reach its highest concentration in tobacco during such an interim after inoculation when plants were showing a maximum amount of necrosis. Since preliminary results had indicated a relationship to the tobacco ringspot virus, a similar period of multiplication was assumed optimum for the watermelon virus. This point of concentration is possibly more important in working with the ringspot viruses as their concentration apparently decreases in tobacco as the plants recover (35,43). However, Fulton (15) has found evidence that this may not always be the case. Leaves of systemically infected tobacco were washed and allowed to air dry before mincing in a meat grinder and extracting the juice. This juice was filtered through several layers of non-absorbent cotton, pipetted into tubes and held in an ice bath until used.

Boston Pickling cucumber seedlings were used as test plants. Cotyledons of these plants were inoculated just previous to expansion of the first true leaves. Small ground glass spatulas were dipped in inoculum and rubbed lightly over carborundum dusted cotyledons. When the inoculum contained active virus, systemic symptoms of small chlorotic spots appeared on the first true leaves about seven days later. Twenty-four cucumber plants were inoculated on both cotyledons with each test preparation. Test plants were observed daily; those showing symptoms were promptly discarded and recorded as positives. Spatulas used in inoculation were cleaned and sterilized before use by washing in soapy water, rinsing in clear water and flaming with alcohol.

In tests determining thermal-inactivation-point, small, thin-walled glass tubes 1.5-2mm in diameter and 10 cm in length were filled with expressed sap leaving approximately 2 cm of each end empty. Both ends were sealed in a small flame. A water bath with a mechanically driven stirring rod and thermostatically controlled to vary not more than $\pm$ 0.1°C was used. Tubes containing the sap were placed in a small wire basket constructed to hold the tubes well separated. The basket with tubes was completely immersed in the water bath at the desired temperature and allowed to remain for 10 minutes. Upon removal, basket and tubes were returned to an ice bath until inoculations were made. For inoculations tube ends were removed and contents blown out onto a small glass surface, then glass spatulas were dipped previous to rubbing over cotyledons of test seedlings.

Dilutions of plant sap with cold distilled water were used in the tolerance-to-dilution tests. Serial logarithmic dilutions of sap, ranging from 1 part sap per 10 parts water to 1 part sap per 1,000,000 parts water, were used as inoculum. Each higher dilution was made by pipetting 1 ml of the previous lower dilution into 9 ml of water. A new dilution was mixed well and allowed to stand for several minutes before removal of an aliquot for the next.

A constant temperature incubator set at 20°C served as storage for plant saps during longevity *in vitro* tests. Undiluted expressed sap was pipetted into tubes stoppered with cotton and placed in the incubator. A single tube was removed daily at about the same hour and used as inoculum.

Results of the physical property tests are presented in Table 2.

Table 2. - Results of physical property tests with the watermelon virus on cucumber.

TREATMENT OF EXPRESSED SAP	RATIO OF PLANTS INFECTED TO THOSE INOCULATED IN TRIAL:			
	1	2	3	TOTAL
Thermal inactivation (10 minutes duration):				
Unheated	24/24	24/24	24/24	72/72
60°C	19/24	20/24	24/24	63/72
65°C	0/24	19/24	3/24	22/72
70°C	0/24	0/24	0/24	0/72
Tolerance to dilution:				
Undiluted	23/24	21/24	24/24	68/72
1-10	23/24	24/24	24/24	71/72
1-100	22/24	20/24	21/24	63/72
1-1000	0/24	8/24	10/24	18/72
1-10,000	0/24	1/24	2/24	3/72
1-100,000	0/24	0/24	0/24	0/72
Aging *in vitro* (hours at 20°C):				
0	24/24	17/24	24/24	65/72
24	24/24	19/24	23/24	66/72
48	24/24	24/24	23/24	71/72
72	23/24	19/24	20/24	62/72
96	21/24	16/24	22/24	59/72
120	24/24	22/24	23/24	69/72
144	22/24	17/24	5/24	44/72
168	7/24	0/24	0/24	7/72
192	——	5/24	0/24	5/48
216	——	0/24	0/24	0/48

These results show properties very similar to the virus with which Pound (33) worked. However, aging _in vitro_ was significantly longer, being 8 days in contrast to the 4 days required for inactivation of the Wisconsin virus. Dilution-end-point of the Oklahoma virus, with a critical point of 1 part expressed sap per 100,000 parts water, was the same as with the Pound virus (33). Similarly, thermal-inactivation-point was approximately the same for the two. These properties are similar to those for the tobacco ringspot virus (8,24,33). Cheo and Zaumeyer (8) have found a virus causing a disease of bean which appears to be a strain of the tobacco ringspot virus. This virus has an aging _in vitro_ time of 15 days at 18°C. Conditions under which tests are made, as well as different strains of the virus, are no doubt partially responsible for differences reported. It is believed by some that processes normally associated with fermentation are operative in inactivating viruses while in degenerating host tissue (30). These same processes are doubtlessly active in expressed saps. Thus, other factors not always considered in aging tests play their part and conceivably may cause wide variation in experimental results.

CROSS-PROTECTION TESTS WITH THE WATERMELON VIRUS

After symptoms and physical properties had indicated a relationship of the Oklahoma watermelon virus to the tobacco ringspot virus, cross-immunization tests were used to confirm this point.

Tobacco plants systemically infected with a typical green strain of the tobacco ringspot virus served nicely for this purpose. Plants were used only after having reached the "acquired tolerance" (AT) stage of infection when no symptoms were present.

Young Havana tobacco plants were inoculated with the green strain of tobacco ringspot virus as obtained from Pound. Both local and systemic symptoms were shown before normal appearing leaves began expansion. Several symptomless leaves were present and all new growth appeared normal after approximately one month. Older basal leaves of each plant, still showing some residual necrosis, were removed leaving only the symptomless younger leaves. Several healthy plants of the same age were treated similarly to serve as controls.

Recovered plants were divided into groups of 10 each. Two groups of 10 recovered plants and 2 healthy control plants each were inoculated with tobacco mosaic and cucumber mosaic viruses. A third group of 20 recovered plants and 4 healthy plants were inoculated with the watermelon virus. Several recovered plants, maintained uninoculated, served as additional controls.

Recovered plants inoculated with either the tobacco mosaic or cucumber mosaic viruses produced symptoms characteristic of the new virus (Fig. 4,C,D). Uninoculated recovered plants remained symptomless.

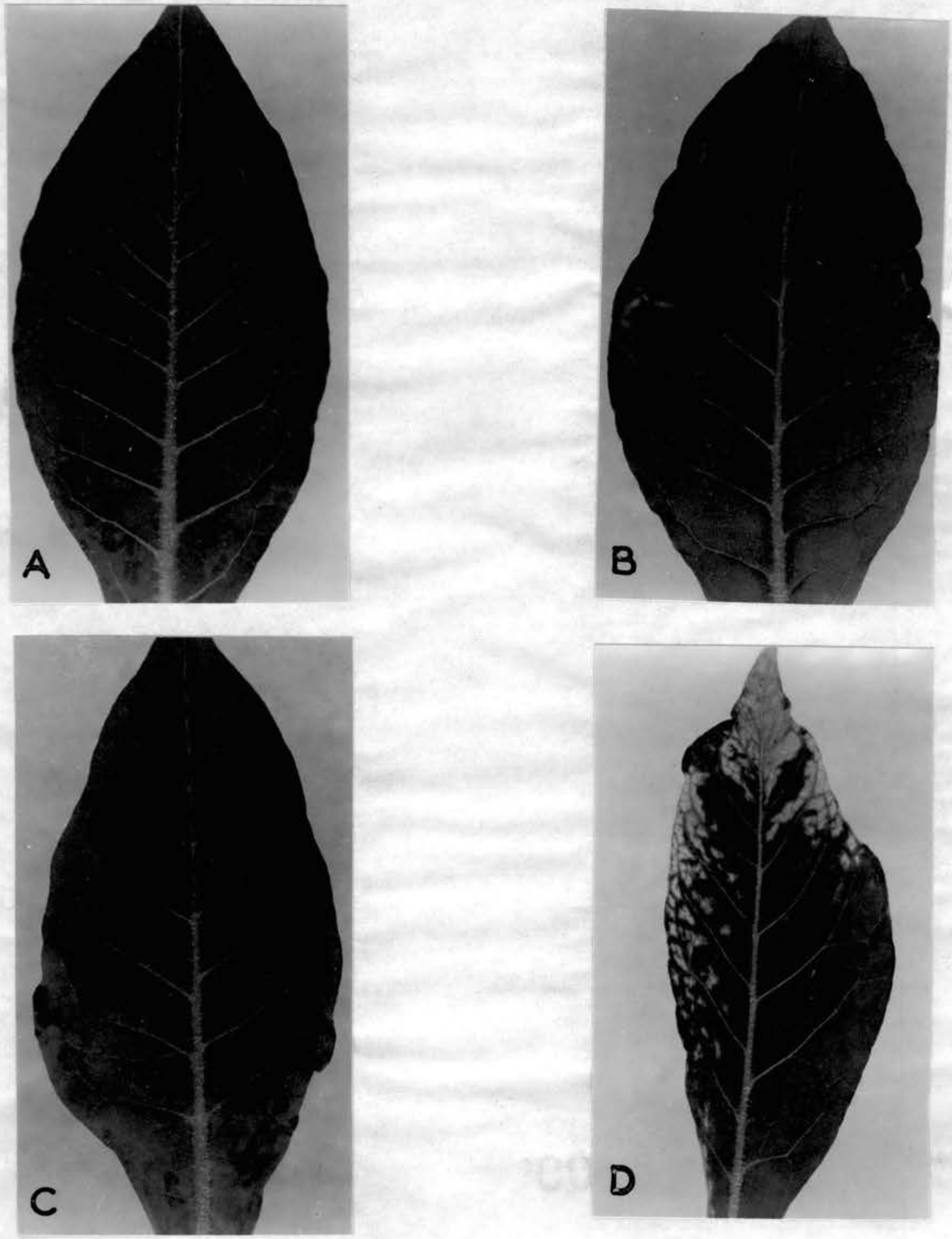

Fig. 4. Results of cross-protection tests on tobacco leaves recovered from tobacco ringspot virus infections. A. Recovered leaf after inoculation with the watermelon virus. B. Healthy leaf of the same age inoculated with the watermelon virus. C and D. Recovered leaves inoculated with tobacco mosaic and cucumber mosaic viruses, respectively.

All inoculated healthy control plants including those inoculated with the watermelon virus produced abundant symptoms (Fig. 4,B). Recovered plants inoculated with the watermelon virus remained symptomless except for a few which produced faint mosaic-mottlelike symptoms on the inoculated leaves (Fig. 4,A). These plants indicated either complete cross-immunization was not occurring, or that these plants were still producing some chronic symptoms (31,45) of the green strain. Price (36) has found that neither the green tobacco ringspot virus of Valleau (44), nor the one of Wingard (51), will protect completely against Valleau's yellow strain of the virus. Similar lack of cross-protection between strains is known in cases of the sugar-beet curly-top viruses (19), and tomato-spotted-wilt viruses (6).

As an additional check on the above noted results, the experiment was repeated using recovered and healthy plants grown from cuttings. This allowed a longer period for the plants to attain the acquired tolerance stage of infection and provided rapidly growing young plants to be inoculated.

Stem cuttings, consisting of one leaf with its axillary bud, were made from both healthy and recovered plants. These were placed in moist sand until roots and a new shoot began growth. Cuttings were transplanted to soil and fertilized. These plants, infected with the green strain, were showing no symptoms and appeared perfectly healthy. Groups of these plants were inoculated as in the original experiment.

In these tests complete cross-immunization was apparent between the watermelon and green tobacco ringspot viruses as recovered plants remained symptomless after inoculation with the watermelon virus. Healthy controls and recovered plants inoculated with either cucumber mosaic or tobacco mosaic viruses, displayed symptoms characteristic of the new virus with which they were inoculated.

Because certain similarities between the watermelon virus and the cucumber mosaic virus had been shown with respect to thermal-inactivation-points and host range, additional cross-protection tests were made with the cucumber mosaic virus using cowpea (16). These plants, variety Black, were inoculated with Fulton's strain F (16) of cucumber mosaic. This strain induces a chlorotic mottle on systemically infected trifoliate leaves of cowpea. Infected plants exhibiting such symptoms after inoculation and several healthy control plants of the same age were inoculated with the watermelon virus on their trifoliate leaves. Several of the same cucumber mosaic plants were retained uninoculated as controls.

Newly inoculated plants soon produced reddish-brown primary lesions characteristic of the watermelon virus. Lesions appeared only on inoculated leaflets. Like symptoms were shown on healthy control plants inoculated with the watermelon virus. No lesions were observed on the uninoculated mosaic plants. Thus, cross-immunization was not shown between the two viruses; hence, the two are not closely related.

SEROLOGICAL TESTS WITH THE WATERMELON VIRUS

Serological tests, because of their specificity, were used to confirm the identity of the virus.

Male rabbits, New Zealand Whites weighing approximately 3.5 lb., were used in preparation of antisera to the watermelon virus. Three of these rabbits were used. Fifteen ml of blood were taken from each by heart puncture previous to injection with antigen. Serum was collected from this blood and frozen for use later as a normal serum control. The heart puncture technique of drawing blood was abandoned after the death of one rabbit. Instead, the method of Markham, Matthews and Smith (41) was used. With this method blood was taken from the marginal vein of the ear after use of a xylene irritant to insure a steady flow. A small incision was made into this vein after irritation, using the corner of a sharp razor blade. Blood was allowed to drip directly into a test tube.

Serum was collected after allowing blood to clot for about 1 hour at room temperature. The clots were then carefully ringed and allowed to set undisturbed for an additional short period. During this second interim the clot collapsed and sank to the bottom of the tube. Supernatant serum was decanted and centrifuged to remove red cells. All serum was preserved frozen until used. Only clear, non-hemolyzed serum was retained.

Clarified sap from Havana tobacco plants, inoculated 2 weeks previously with the watermelon virus, was used as antigen. This juice was assayed on cowpea to insure that a highly active virus preparation was being used for injection. Infected tobacco leaves were collected and rinsed in several changes of distilled water and allowed to air dry. Leaves were then wrapped in aluminum foil and frozen. Immediately before making an injection, leaves were thawed, macerated, and the juice filtered through cotton. This juice was heated for 10 minutes in a waterbath at 55°C for further clarification. Juice was centrifuged to remove coagulum and the supernatant was used for injections.

Both intravenous and intraperitoneal injections of heat clarified sap served to stimulate antibody formation. One rabbit was used in each case. An initial injection of 1 ml followed by 7 injections of 5 ml each of the clarified sap with virus were used intraperitoneally at three-day intervals in one rabbit. The second rabbit received only intravenous injections of the same sap. These were placed in the large, marginal vein parallel to the hind edge of the ear. An initial injection of 0.5 ml was followed 2 days later with an injection of 1 ml of plant sap.

Bleedings were begun on the rabbit injected intraperitoneally 9 days after the last injection. A total of 120 ml of blood was taken from this rabbit during 4 bleedings at one-day intervals. The second rabbit, which received two intravenous injections, was bled on two successive days about 12 days after the last injection. Sixty ml of blood were taken from this rabbit. Serum from either rabbit was found to contain an antibody titer sufficient to give a positive precipitin test. Therefore, as others have found (41), intravenous injections, where possible, provide a more efficient and less laborious means of securing a potent antiserum.

The precipitin test was used to demonstrate virus relationships. As recommended by Chester (9), both normal and immune serum were absorbed with 3 parts healthy plant sap per 1 part serum immediately before use in a precipitin test. Healthy tobacco sap, after clarification, was added to the normal and immune sera in separate tubes and mixed well. These tubes were held at 37°C for two hours, centrifuged to remove precipitates and the supernatant used in the precipitin tests.

Antiserum to the watermelon virus was tried against several known viruses to see if any would give a positive precipitin test, thus, indicating a relationship to the watermelon virus. Other viruses used were those of tobacco mosaic, cucumber mosaic and a green strain of tobacco ringspot. Each virus was inoculated onto young Havana tobacco plants. Two weeks later the leaves were harvested, rinsed in distilled water and frozen. Freezing effected partial clarification of the juices. No heat clarification was employed as this treatment was found partially to decrease activity of the tobacco ringspot virus. Ratios of sap to serum found optimum were the same as Chester's (10) for the tobacco ringspot virus. This ratio, 1 part absorbed serum per 4 parts infected tobacco sap, was found to give a reliable precipitin test with the watermelon virus.

Precipitin tests were repeated 3 times. In each test, tubes were used containing the following:

Tube 1 - One part absorbed normal serum plus 4 parts tobacco sap containing the watermelon virus.

Tube 2 - One part absorbed immune serum plus 4 parts healthy tobacco sap.

Tube 3 - One part absorbed immune serum plus 4 parts tobacco sap containing the watermelon virus.

Tube 4 - One part absorbed immune serum plus 4 parts tobacco sap containing tobacco mosaic virus.

Tube 5 - One part absorbed immune serum plus 4 parts tobacco sap containing cucumber mosaic virus.

Tube 6 - One part absorbed immune serum plus 4 parts tobacco sap containing a green strain of the tobacco ringspot virus.

These tubes were held at 37°C. It was found necessary to observe tubes 2 hours after mixing sap and serum as spontaneous precipitation of plant saps would later obscure positive tests. In all tests positive results were noted in tubes 3 and 6. Precipitates in these tubes were granular, flaky masses which gradually settled upon standing. In general, precipitates did not form in other tubes until some hours later, and when appearing were less granular or aggregated and of a darker color.

In conclusion, it may be stated that the watermelon virus is serologically related to the tobacco ringspot virus as the two form precipitation complexes when present with the same specific antisera. However, as symptoms on tobacco have shown, the watermelon virus is probably a yellow strain, and therefore, very similar or identical to the virus described by Pound (33) from watermelon in Wisconsin.

SYMPTOMS ON WATERMELON IN FIELD PLANTINGS

During summer months the ringspot virus in watermelon appeared primarily as a fruit disease. Foliage and vine symptoms were less evident than on plants grown at cooler temperatures in the greenhouse. However, foliage symptoms were observable on some plants; particularly evident were the irregular, necrotic lesions on foliage. These lesions were very similar to those resulting from greenhouse inoculations (Fig. 2,B) with the ringspot virus.

Death of foliage near centers of hills was also noticed in many plantings. Leaves along basal portions of vines were dead and dried leaving the vine bare and exposed. Foliage slightly farther out on vines tended to become chlorotic and considerable marginal necrosis was evident. This condition was widespread in fields by late July in Oklahoma. Similar symptoms are again associated with anthracnose, a disease seldom encountered when many of these observations were made during the extremely dry weather in the summer of 1954.

A short portion of stunted growth interposed between normal appearing portions on either side was another feature of common occurrence on infected vines. This portion had shorter internodes and severely stunted foliage. This symptom was probably analogous to that in greenhouse plants in which stunting occurred soon after systemic development of infection with plants eventually recovering and resuming their normal growth habit. In general, however, foliage and vine symptoms were sporadic in occurrence in field plantings during warmer weather.

Infection was apparently almost completely masked in most plants in midsummer, except for fruit symptoms which were evident at all times. Rosberg (37) described the disease in Texas as principally one of watermelon fruits. However, with arrival of cooler nights during mid-August and early September in Oklahoma, foliage symptoms became very apparent. Marginal necrosis of leaves was common. A similar increase in severity of fruit symptoms occurred at this time. Accordingly, temperature is likely of prime importance in severity of symptoms associated with the ringspot virus infection. Hence, this disease is likely of greater importance in cooler, northern states as Pound (33) has described a more severe reaction in field plantings than commonly observed in Oklahoma except in late season. During late summer and early fall in Oklahoma field symptoms approximated those described in Wisconsin.

Symptoms rarely appeared on the first small fruits set during early summer. When present, however, these symptoms were often more severe than on mature fruits. Small, discrete lesions, slightly elevated above the normal surface of the fruit, developed more or less evenly distributed over the fruit surface. Many lesions on these small fruits became necrotic (Fig. 5,6). As a rule, however, few fruits showed lesions when young and loss here was not great.

Lesions on mature fruits were usually relatively inconspicuous during hot summer weather. Virus lesions on these fruits appeared as small, pimple-like pustules sparsely scattered over the fruit surface. There seemed to be no tendency for lesions to form on any particular portion of the fruit; however, several lesions often occurred near one another. Lesions on mature fruits were generally rather flat-topped, instead of papillate, and when few in number did not significantly detract from the marketability of the fruit. Hence, during high temperatures of midsummer, fruit lesions due to malformation were of little economic importance.

Fruit lesions resembled early stages of anthracnose infection. Slight elevation of fruit lesions is common with both diseases. Virus lesions on mature fruits seldom became necrotic except in late season; however, a small amount of internal necrosis was evident in all lesions. This will be described in more detail later. Virus lesions rarely led to rotting of the fruit as often occurs when anthracnose is present.

As a rule, anthracnose fruit lesions become necrotic and sunken due to collapse of external rind tissues. This results in formation of characteristic crater-like depressions in the rind. Such a condition was never observed in infections with the virus. Only rarely was necrosis evident on surfaces of mature melons and virus lesions never became crater-like. Virus lesions in some instances in late season became slightly sunken due to retarded growth beneath areas with lesions.

Virus lesions on mature fruits appeared as small translucent areas when cut in cross-section. Usually, a tiny brown, necrotic spot was visible on the external edge of the translucent area.

After arrival of cooler nights in late season both very young and mature melons, if they were still growing, became greatly distorted. Most melons in late season became totally worthless and unmarketable. In the summer of 1954 these more prominent symptoms began to appear during mid-August. Symptoms grew progressively more severe until plants were killed by frost in November. Initially during this period fruit symptoms were similar to those occurring in warmer weather but were more numerous and conspicuous (Fig. 5,A). Symptoms progressively became worse; often the entire surfaces of fruits were pimpled. These lesions were more papillate, and some took the form of elevated rings. Night temperatures during this period were often below 20°C while day temperatures usually reached 30°C.

Fruit lesions developing in late season were invariably much larger and many formed as raised, irregular circles and lines (Fig. 5,C). Considerable surface necrosis in lesions appeared concurrently with cooler nights. Many virus lesions, slightly elevated during their initial stages, became necrotic and slightly sunken. Such necrosis appeared as hard, corky scabs on fruit surfaces (Fig. 5,D). Such scabby areas gradually became depressed as normal growth was apparently inhibited beneath these necrotic areas (Fig. 5,E). As a result of such differential rates of growth in adjoining areas, marked deformation of fruits occurred.

Most fruits at this time were unmarketable; however, the economic value of the crop in late season is slight as high temperatures are a usual prerequisite of good markets. Nevertheless, it becomes apparent that the ringspot virus could potentially cause vastly greater losses during cool seasons. This potentiality seems to be realized in cooler northern states as Pound (33) has described very significant losses in the crop in Wisconsin.

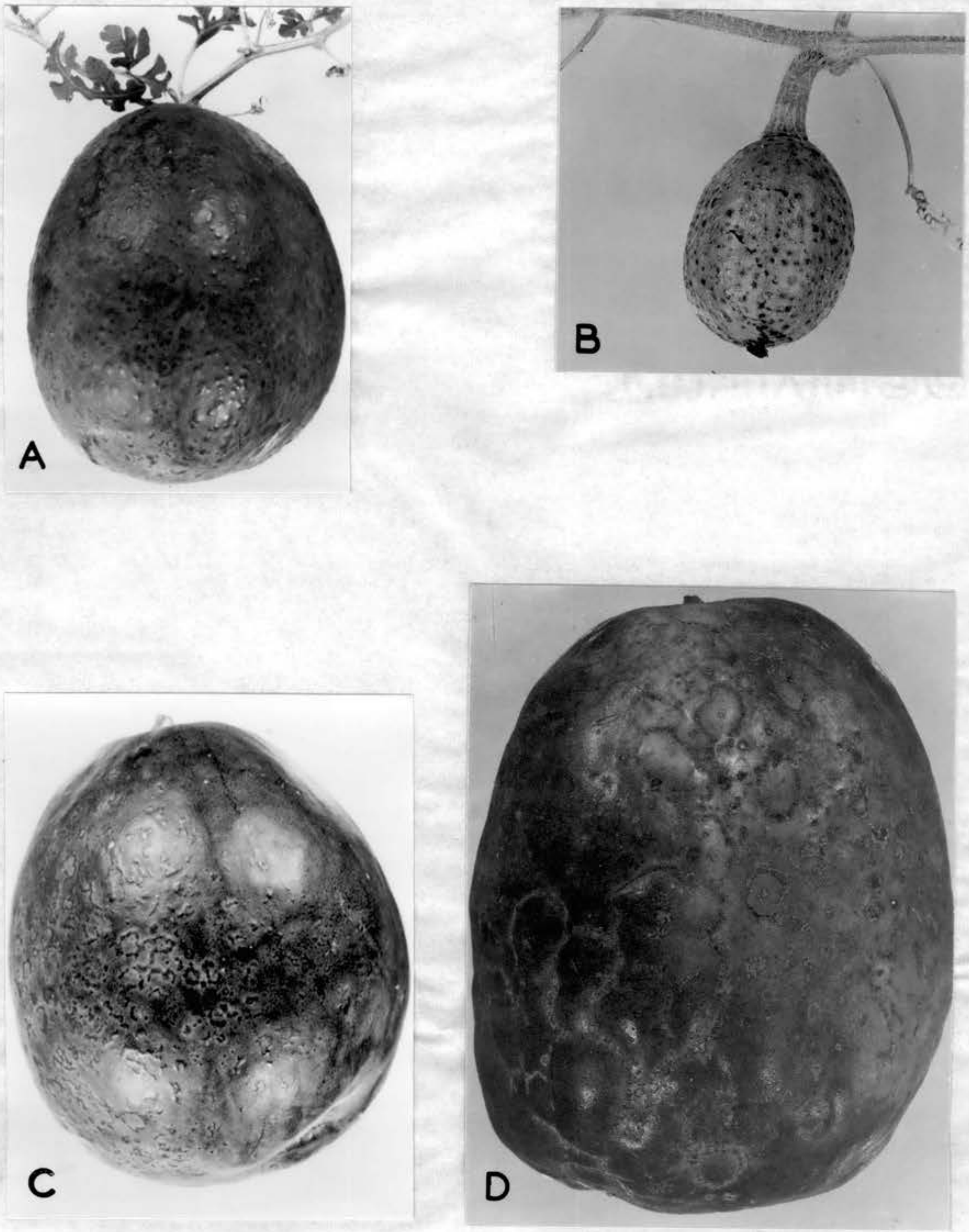

Fig. 5. Symptoms on naturally infected watermelon fruits. A and B. Immature fruits showing raised lesions. C. Fruit showing raised rings and lines previous to becoming necrotic. D. Fruit showing necrotic rings with greatly distorted surface due to differential growth rates of adjoining areas.

HISTOLOGICAL NATURE OF WATERMELON FRUIT LESIONS

Investigation of ringspot virus lesions on watermelon fruits showed these lesions to be small galls in localized areas of the fruit cortex. The histological nature of these galls on Black Diamond fruits was investigated using tissue from naturally infected melons.

Representative lesions were excised from naturally infected fruits throughout the growing season and preserved in formal-acetic-alcohol. Tissues were dehydrated using an ethanol-tertiary butanol series and embedded in paraffin (26). Microtome sections 15 microns in thickness were stained for routine observation with Foster's tannic acid-ferric chloride stain (14). Flemming's triple stain (38) was used in preparing sections for photomicrography.

Barber (3) has described the normal histology of watermelon fruits. Fruits are divided into successive layers (Fig. 6,A). The outermost layer is epicarp and made up of a single transparent sheet of epidermal cells. Hypoderm comprises the next layer which is many cells thick. Hypodermal cells contain numerous chloroplasts and form the green tissue of the rind. These cells are isodiametric except for a few cell layers just beneath the epicarp which appear somewhat flattened tangentially (Fig. 6,A). Numerous small intercellular spaces occur throughout this tissue. The innermost layer is mesocarp, the outer cells of which are sclerenchymatous with numerous pits. These sclerenchymatous cells form an almost continuous sheath around the fruit. This sheath is of varying thickness, but usually consists of 4-5 cell layers. These cells are lignified and contain many simple pits. The sclerenchymatous sheath is broken at intervals by thin walled parenchymatous cells. Due to the varying cell thickness of this layer and its discontinuous occurrence, the inner contour is very irregular (Fig. 6,A). Mesocarp cells below this layer are thin walled and isodiametric. Cells increase in size from the sclerenchymatous sheath inward until those of the watery flesh are of enormous size, some being 1.25 mm in diameter and easily visible to the naked eye. These cells contain no chloroplasts. Large intercellular spaces occur throughout this tissue due to its loosely packed nature (Fig. 6,A).

Typical fruit lesions appeared as small intumescences on the fruit surface. Such lesions on mature fruits were usually hemispherical although many were somewhat flattened on the top. Lesions were found to consist of small galls in the fruit rind. These galls were of a characteristic pathological nature and their formation in the fruit cortex caused the typical outward bulging bumps on infected fruits.

A tiny brown necrotic spot was observable in most lesions when cut into thin sections with a razor blade. This necrotic spot appeared as a tiny corky area in the outer rind, and was often barely discernible to the unaided eye. A small translucent area was usually visible immediately below this necrotic spot. This small, water-soaked area was usually only 1-2 mm in diameter and located just under the necrotic spot. Such areas were observed only under bulging surface lesions and always occurred in the outer region of the melon rind.

Microscopic observation of fruit lesions showed their gall-like nature. The elevated condition of fruit lesions was found attributable to hypertrophy of several mesocarp cells just beneath the sclerenchymatous sheath. A necrotic mass of cell debris was usually visible between these hypertrophied cells and the sclerenchymatous sheath (Fig. 6,A).

Hypertrophied mesocarp cells were apparent in all galls sectioned and observed. These cells elongate in a direction perpendicular to the necrotic spot. During enlargement mesocarp cells became long and asymmetrical and lose their normal isodiametric nature. Necrotic areas were usually evident just beneath the layer of stone cells in the outer mesocarp. This necrotic mass appeared to consist primarily of cell debris from collapsed dead cells which were compressed against the sclerenchymatous sheath. Mesocarp cells below and on both sides of the necrotic mass were elongated directly toward the necrosis. Thus, hypertrophy appeared to be in response to some material emanating from the necrotic area. During elongation mesocarp cells appeared to become tightly compressed as large intercellular spaces, normally present in this tissue, were totally absent (Fig. 6,B). The compact nature of this tissue probably accounted for the translucent appearance of this area when viewed with the naked eye.

Necrosis was always apparent immediately adjacent to the layer of stone cells. Often necrosis extended into the parenchymatous rifts in the sclerenchymatous sheath (Fig. 6,B). The necrotic mass appeared to be highly lignified in most cases. As a general rule, necrosis did not extend across the layer of stone cells and hypodermal cells appeared almost normal (Fig. 6,B).

Elongated mesocarp cells appeared to retain their thin walls and remained non-lignified; however, in a few lesions secondarily pitted walls appeared to develop in a few hypertrophied cells along the periphery of lesions. Hypertrophied mesocarp cells of lesions in later stages of development, had apparently undergone several transverse divisions. Many cross-walls were visible perpendicular to the long axis of hypertrophied cells, and in many cases, long tiers of these cells resulted. Such tiers of mesocarp cells invariably appeared to radiate directly inward toward the necrotic center of the gall (Fig. 6,D).

Hyperplastic development in lesions showing considerable necrosis resulted in the formation of a cambial-like zone of cells surrounding the necrotic center. As a result, necrosis appeared to be occluded or corked out. Such a reaction was always more prominent when considerable necrosis was evident and appeared most often in lesions collected during late season when necrosis often extended through the hypoderm and to the surface of fruits.

Fruit lesions that appeared during periods of warmer weather seldom became necrotic exterior to the sclerenchymatous sheath, although necrosis was always evident in the outer mesocarp of lesions. When surface necrosis appeared on fruits, however, it was evident that hypodermal as well as mesocarpal areas had become necrotic (Fig. 6,D). Lesions developing in late season often appeared slightly sunken due to death and shrinkage of cortical tissues.

Virus lesions on melons apparently develop in a manner similar to the epidermal blisters on tomato fruits infected with a severe type of mosaic (17). Lesions probably begin with the death of a few cells immediately below the sclerenchymatous sheath in the rind of watermelon fruits. Hypertrophied mesocarp cells were always found associated with this necrosis. Hypertrophied cells usually occurred in parallel columns

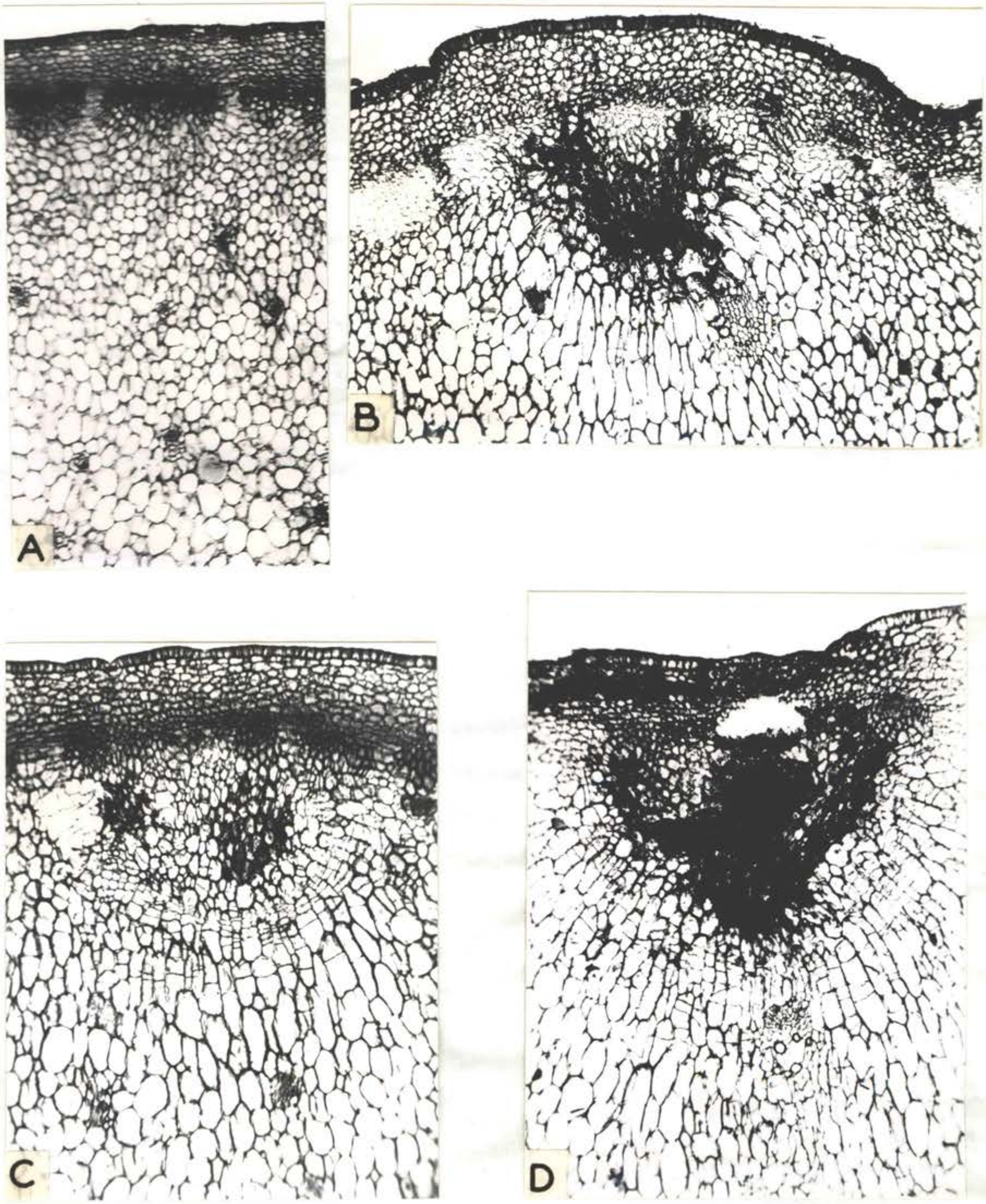

Fig. 6. Histological nature of tobacco ringspot virus lesions on Black Diamond watermelon fruits. A. Section through a healthy fruit. B. A lesion after development in warm weather and showing necrosis and hypertrophied cells in mesocarp. C and D. Lesions after development in late season and showing hyperplasia of cells near the necrotic area. Note extensive necrosis extending through sclerenchymatous sheath and hypoderm in D.

growing in and directly toward the necrotic center. Hence, these cells are apparently produced as a response to the necrosis and as Gardner (17) has stated this suggests that some material diffusing out radially from the necrosis causes the hypertrophy of cells. This condition of necrosis with subsequent hypertrophy, was the final stage of development in most lesions appearing during warm weather. However, lesions in late season appeared to develop one step further. This usually appeared as more extensive necrosis involving the hypodermal tissue. At the same time hypertrophied mesocarp cells adjacent to the necrotic mass became meristomatic and produced a zone of hyperplastic tissue around the necrosis.

Marked malformation occurred on fruits that developed lesions in late season. This appeared to be due to cessation of growth immediately beneath necrotic surface lesions. Such fruit lesions were elevated during early stages of development, but later, appeared to collapse and shrink. Such areas of the fruit seemed to cease growth whereas adjacent areas continued unchecked. This inhibited symmetrical growth of fruits and caused most of them to be warty and misshapen.

Apparently the ringspot virus was the sole incitant of fruit lesions as the two were in constant association and neither bacterial nor fungal organisms were isolated. Also temperature seemed to be of prime importance in symptomatology; a factor well evidenced as being important in many virus infections, particularly with the ringspot viruses. Again, moisture conditions, so very important with most bacterial and fungus diseases, seems to have little effect on incidence of this disease. Symptoms became radically more severe with a simple change in temperature, while moisture conditions remained extremely dry with correspondingly low humidities. Neither were bacteria nor fungi discernible in many lesions sectioned and stained during histological observations. Similarly, fruits never decayed, as commonly results with bacterial and fungal diseases.

In attempts to substantiate evidence that fruit lesions were of virus nature, sterile tissue culture of fruit areas with lesions were grown on White's (50) medium. This medium, plus a yeast extract, gave rather slow growth, but many tissue cultures were still growing after 10 months. A semi-solid medium was found to give better growth.

Areas of rind with lesions were removed aseptically after surface sterilization and placed in sterile culture flasks containing medium. Several dozen of these cultures were made and held at ordinary temperatures. Bacteria or fungi were found only as contaminants in some of the tissue cultures. Thus, the ringspot virus was the sole incitant of fruit lesions. Rosberg (37) was able to induce lesions on watermelon fruits grown in the greenhouse and inoculated with the ringspot virus.

Wingard (51), in early studies with the tobacco ringspot virus, noticed abnormalities on infected fruits of various cucurbitaceous hosts. Fruit symptoms were described as small depressions initially, but gradually became elevated and appeared as pimples on mature fruits. Such symptoms were observed on <u>Cucurbita pepo</u> L. varieties Egg Nest gourd and Golden Summer Crookneck squash. The cucumber mosaic virus is also known to cause intumescences on fruits of cucurbits (12), but as was proven in the cross-immunization and serological tests, the ringspot virus from watermelon is not related to this virus.

Pimpled fruits, of the type described above with the tobacco ringspot virus, apparently were not associated with the "internal-browning"[1] condition described by Gilbert and Artschwager (20); although, the two may occur in the same fruits. However, pimples are often present without internal-browning and vice versa. Internal-browning was very common during the dry summer of 1954 and is probably a physiological disease due to water deficiency in fruits as originally described by Gilbert and Artschwager.

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[1]This condition is also called "cork-rind" and dynamite" by Oklahoma watermelon growers.

ATTEMPTS TO FIND NATURAL MEANS OF TRANSMISSION OF THE RINGSPOT VIRUS

Means were not available for indexing infected hosts during periods of high temperatures when symptoms were masked. Indexing hosts such as cucumber and cowpea gave unreliable results at high temperatures as reactions were sporadic or absent. Thus, insect transmission trials were of necessity confined to greenhouse tests during winter. Insects were not available for these tests unless reared or colonized. For this reason only differential grasshoppers (Melanopus differentialis Thomas) and melon or cotton aphids (Aphis gossypii Glover) were used in transmission trials. Transmissions were attempted only from watermelon to watermelon.

Another means of transmission suggested by Pound (33) as a possibility is watermelon seed. This was tested, although not exhaustively, with seed collected from naturally infected fruits.

Insect transmission trials

Differential grasshoppers were used in these trials because of their availability and as Walters (48) has found are potential vectors of the virus from tobacco to tobacco, although grasshoppers are rather inefficient in this respect. It seemed possible that the insect might be more efficient in transmission to watermelon; however, this insect is not usually a prominent feeder on this crop.

Another insect of greater importance on watermelon is Aphis gossypii. This insect is found in all fields in this area and often causes losses when uncontrolled. These insects were available because of the ease with which they could be colonized on watermelon plants in the laboratory.

When possible, insects were kept caged in the laboratory to prevent accidental infestations in greenhouses. Insects were taken into greenhouses only when used in transmission trials.

Differential grasshoppers were reared in the laboratory using the method of Haydak (21). Females heavy with eggs were collected in field during early fall and caged. Small containers with damp sand were placed in the cages. Females oviposited in the sand and died soon afterward. Egg capsules were collected and placed in damp sand at room temperatures for about two weeks, and then stored in an icebox at a few degrees above freezing for approximately one month. Eggs were then placed in containers at laboratory temperatures beneath a shallow layer of damp sand until the young hoppers emerged about three weeks later.

Young hoppers were collected and placed in a small cage in constant light. A food mixture consisting of one part dried baker's yeast, two parts dried skim milk and two parts dried alfalfa meal was kept in small flat containers in the cages at all times. Water was provided by laying large test tubes stoppered with absorbent cotton on the floor of cages. Hoppers grew rapidly and matured in about thirty days.

Systemically infected watermelon plants served as virus source plants in all transmission trials. Young, healthy watermelon plants in vigorous growing condition were used as test plants.

Grasshoppers were starved approximately 24 hours previous to use in transmission trials. This insured that hoppers would feed readily on test plants and possibly increased the likelihood of transmission.

Hoppers were grasped by their large rear legs and wing-tips with thumb and forefinger during feeding periods on both the source and healthy test plants. In this way hoppers could be controlled and manipulated during feeding. Test plants were not touched with the hands during these manipulations. Insects were allowed to feed 1-2 minutes upon source plants before removal to healthy test plants. Hoppers were moved about over leaves of healthy test plants and allowed to make several small holes in two or three leaves. A single hopper was used on each plant. Feeding holes made in leaves of test plants were approximately 3-5 mm in diameter. Five and usually more of these holes were allowed on one or two leaves of each healthy watermelon seedling.

Melon aphids used in attempted transmissions were colonized on watermelon systemically infected with the ringspot virus. They were then transferred in varying numbers to healthy seedlings. The saliva-moistened tip of a camel's hair brush was used in making transfers. All aphids were cautiously disturbed with the tip of the brush in order that mouth parts were withdrawn from host tissues previous to moving. In this manner, injury to the insects was prevented. Aphids were placed in cages made of 12-inch pot labels (32) on leaves of healthy plants for three days before removal. Test plants were then dusted with parathion to kill the aphids.

Usually, at least fifteen mature hermaphroditic females were placed on each healthy test plant. In some cases mass transfers were made in which several dozen insects were moved from infected to healthy plants. In all trials both apterous and winged individuals were used.

All healthy test plants to which transmission had been attempted were held for about two weeks to allow any infections to become systemic in the plant. After this period, when virus would have been present in sufficient quantity to be transmissible mechanically, all test plants were indexed on cucumber plants. Cucumber proved to be the only reliable host for indexing watermelon plants for presence of the tobacco ringspot virus as cucumber was apparently either non-sensitive to the inhibitor in watermelon foliage (18) or more susceptible to the ringspot virus (31). Due to the occasional masking of infection in watermelon, particularly at warmer temperatures, symptoms alone were not relied upon to indicate presence of virus in the test plants.

Only one plant became infected from forty-eight attempts to transmit the virus with differential grasshoppers. This single transmission was evidence that these insects may occasionally act as vectors but that their efficiency is probably much too low to account for the high percentages of infection in field plantings. Also, the relatively early spread of the disease in the field and the number of hoppers ordinarily found in fields at this time do not seem sufficient to implicate this insect as an important vector. From tobacco to tobacco, Walters (48) reported only six percent transmission of the tobacco ringspot virus with this insect.

Of the 45 attempts to transmit the virus with melon aphids, all were negative. Thus, neither does the melon aphid seem to be a vector of tobacco ringspot virus, at least not under the circumstances existing during these tests.

To date, Walters' (48) data are the only instance of insect transmission reported for the tobacco ringspot virus. The vectors responsible for natural spread of the virus are unknown. These viruses do not appear to be transmitted to any extent by mechanical contact because of their low concentration in host plants (42). In the present studies not a single case of accidental transmission of the ringspot virus was observed in the greenhouse tests.

Several unsuccessful attempts at insect transmission of tobacco ringspot virus have been reported. However, existence of natural vectors is only circumstantial. Pound (33) obtained negative results with *Myzus persicae* Sulzer, yet others have found circumstantial evidence which points to this insect as a possible vector. Smith and Brierley (4) have reported on accidental infection of gladiolus with tobacco ringspot virus as explainable only by the fact that plants had been exposed to *M. persicae*. Valleau (46) believes that *Thrips tabaci* Lindeman may be a potential vector but that it probably transmits the virus in only a small percentage of cases. He has suggested that the manner in which sulfur dusting controls eggplant yellows, tobacco ringspot on eggplant, is probably explainable by the fact that sulfur acts as a repellant to *T. tabaci*, a vector of tobacco ringspot virus.

An attempt was made to hold field collected twelve-spotted cucumber beetles (*Diabrotica undecimpunctata howardi* Barb.) at low temperatures in an icebox for use in transmission trials as the insect is known to overwinter as adults; however, all died before transmission trials could be made.

Seed transmission trials

Tobacco ringspot virus has been shown to be seed transmitted in tobacco (44), petunia (22) and soybean (11). With this fact in mind it seemed possible that watermelon seed might also harbor the virus.

Seed were harvested during the summer from naturally infected melons. These melons were all exhibiting numerous lesions when collected; however, as an additional check a small piece of rind tissue from each was frozen for use later in recovering the virus. This would prove that the melon was infected. This recovery of the virus was made to cucumber during the winter. Only the seed of fruits from which the virus was recovered were used in these tests.

During winter months seed from infected melons was planted in flats in the greenhouse. All resulting seedlings were observed during the first several weeks of growth and all those appearing abnormal or stunted were used to inoculate cucumber. This manner of indexing systemically infected watermelon plants by transmitting the virus to cucumber was found reliable in other tests where symptoms were masked in watermelon. Approximately 900 seedlings were grown from the seed of infected melons and suspected plants were indexed. None of these seedlings proved to be infected.

As the above method seemed somewhat inadequate because of the likelihood of symptoms being entirely masked in seedlings, more seed from infected fruits were planted and all resulting seedlings indexed on cucumber. Approximately 300 seedlings were indexed in this manner with negative results.

Several thousand watermelon seedlings were used in other phases of this investigation. These seedlings were from certified seed from growers in Oklahoma. During harvest of this seed many infected melons are used. Several fields of certified seed growers were visited during harvest and a high incidence of infection was noted on the mature fruits. Hence, many of the certified seed used in this study were undoubtedly from infected melons, yet, in no instance were seedlings found infected during the greenhouse studies unless inoculated.

Watermelon seed apparently do not transmit the virus or at least have not been demonstrated to do so with the experimental procedures used in this investigation.

AN INHIBITOR OF INFECTION IN WATERMELON TISSUES

During early tests, attempts to transmit the virus from watermelon foliage to cowpea were unsuccessful. However, expressed saps from fruits of the same infected plant would cause lesions when inoculated onto cowpea, although only a few lesions usually resulted. These anomalies suggested either that an inhibitor of infection (4) was present, or that the virus was not systemic in foliage and present in only low concentration in fruits as evidenced by the few lesions appearing on cowpea. Later, inoculations to tobacco from melon foliage resulted in transmission in only a few instances. Transmission of the virus from tobacco to cowpea usually resulted in abundant infection. As a consequence of the results just outlined an investigation was initiated to demonstrate whether or not an inhibitor was present in watermelon foliage and fruits.

Initial tests for inhibition were made by comparing the virus activity of infective tobacco saps mixed with distilled water on one hand and with watermelon foliage sap on the other. In one case the tobacco sap was diluted with an equal volume of distilled water to serve as control; sap from the same source was diluted equally with watermelon foliage sap. Thus, each mixture contained the same amount of virus and if no inhibition occurred due to the watermelon foliage juice, each virus preparation should cause about the same number of lesions when inoculated onto cowpea. These two test preparations were compared by the half-leaf method using the primary leaves of cowpea. One half of each leaf was inoculated with the tobacco sap-water control solution and the other half-leaf inoculated with the tobacco sap-foliage sap mixture. Several leaves were inoculated in this manner in each of several tests. In every case many lesions appeared on the control half-leaves, while in no instance did a single lesion result on the half-leaves inoculated with the preparation containing watermelon foliage sap. Thus, it became apparent that melon foliage sap did contain an inhibitor and could completely destroy activity in a very infective preparation of the virus. A similar but less marked inhibitive action was found with sap from watermelon fruits.

Subsequent tests were standardized in order that more reliable estimates of degree of inhibition could be had. Primary leaves of cow-pea, variety Black, were used to assay preparations containing the tobacco ringspot virus or cucumber mosaic virus, and Nicotiana glutinosa L. was used to assay preparations containing tobacco mosaic virus. In all tests equivalent preparations were compared with a standard inoculum consisting of sap from systemically infected Havana tobacco mixed with equal amounts of distilled water. Virus activities of all test preparations were compared with this control by the half-leaf method. Inoculations were made with a glass spatula which was washed and flamed in alcohol before use with each different test inoculum. This spatula was passed twice across each half-leaf previously dusted as evenly as possible with carborundum; hence, each half-leaf received approximately the same amount of inoculum. A small pad of cotton was held under the leaf during rubbing to serve as support and absorb excess inoculum.

The effect of dilution upon watermelon foliage sap was studied in attempts to evaluate more accurately the degree of inhibition. Various dilutions of foliage sap were made with distilled water in the same manner as when studying dilution-end-point of the virus in the physical property tests. An aliquot of each of these dilutions was then mixed with equal amounts of infective tobacco sap and compared with the control by inoculations of half-leaves. Similar tests were made using expressed saps from the rind areas of watermelon fruits. Sample data are given in Table 3 and Fig. 7. Three of these tests were made; all gave similar results.

Dilution-end-point of the inhibitive action of foliage sap was between 10^{-2} and 10^{-3}. Inhibition was, in all cases, still significant at the 10^{-2} dilution. Similar results were had with expressed sap from watermelon fruits; however, dilution-end-point was approximately ten-fold lower. The inhibitor may have been present in lower concentration in the watery fruit. Inhibitive action of fruit sap was never sufficient to neutralize completely the highly active virus preparation from tobacco.

Watermelon foliage saps were found similarly to inhibit infection with tobacco mosaic virus preparations in transmissions to *Nicotiana glutinosa*. Although the effect of dilution was not studied, undiluted foliage sap mixed with an equal volume of virus solution were found to give 33.3 lesions per half-leaf as compared to 244.7 lesions per half-leaf inoculated with a mixture of equal amounts of virus solution and water. This amounted to a reduction of 89.7%. In no instance was the virus activity completely neutralized, however, as it was with ringspot virus. This may have been due to the unusually higher concentration that tobacco mosaic virus reaches in tobacco as compared with the ringspot viruses. Watermelon foliage sap was similarly found to inhibit transmission of cucumber mosaic virus to cowpea. In this case inhibition seemed complete when undiluted foliage sap was mixed with equal amounts of cucumber mosaic in tobacco sap as no lesions were produced on any of the half-leaves inoculated with this mixture. The effect of dilution was not studied in this case.

The inhibitor contained in watermelon foliage was found to be destroyed by heating the sap to 80°C for 10 minutes. The inhibitive action of sap was partially destroyed after heating at 75°C for a similar period; however, lesion counts showed approximately 50% reduction capacity still remained in the foliage juice. Similar effects of heat are known with other inhibitors (29,49) and suggest that these may be of a proteinaceous nature similar to the glycoprotein isolated and described by Kassanis and Kleczkowski (28) from *Phytolacca esculenta*.

The inhibitor from watermelon was found ineffective in preventing transmissions of tobacco ringspot virus to cucumber. Cendron and Kassanis (18) found infection of cucumber less affected than any of several other species tried when investigating the importance of host species to which transmission was attempted in presence of various inhibitors of infection from higher plants. Bawden (4) has stated that an obvious explanation would be that cucumber also contains the inhibitors, as it is commonly known that inhibitors are ineffective in preventing infection on the same species from which the inhibitor came. However, it may be that cucumber is simply more sensitive to infection by tobacco ringspot virus. McKinney and Clayton (31) believe cucumber to be more susceptible than tobacco to the ringspot virus.

Cucumber proved to be the only reliable host for indexing watermelon plants for infection with the ringspot virus in these investigations.

Table 3. Effect of dilution on the inhibitive action of watermelon sap before mixing 1:1 with sap of tobacco infected with the ringspot virus and comparing with a standard inoculum[1] by half-leaf inoculations on cowpea.

Inoculum	Average lesions per half-leaf in 6 replications	Reduction by sap
	No.	Per cent
Watermelon foliage sap:		
Undiluted	0.0	100.0
Standard inoculum	65.0	
1:10 dilution	0.3	99.5
Standard inoculum	62.2	
1:100 dilution	2.3	86.1
Standard inoculum	16.5	
1:1000 dilution	54.8	0.0
Standard inoculum	39.8	
Watermelon fruit sap:		
Undiluted	3.2	95.9
Standard inoculum	52.9	
1:10 dilution	3.2	73.6
Standard inoculum	12.1	
1:100 dilution	170.9	0.0
Standard inoculum	179.1	

[1]Standard inoculum consisted of sap of tobacco infected with the ringspot virus mixed 1:1 with distilled water.

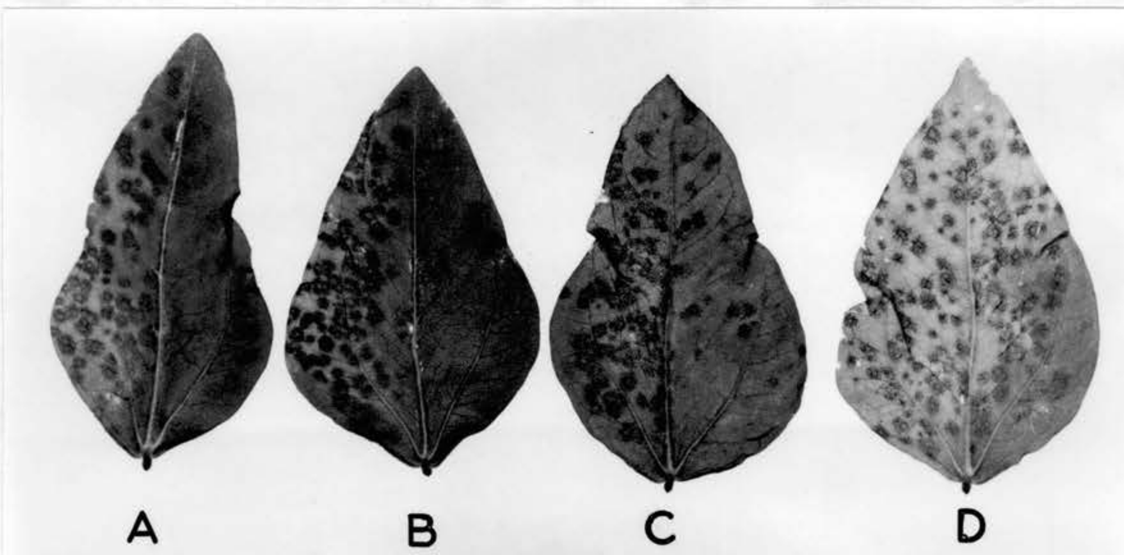

Fig. 7. Effect of dilution on the inhibitor of infection from watermelon foliage in preventing infection with the tobacco ringspot virus on cowpea. All left half-leaves inoculated with standard inoculum consisting of sap of infected tobacco mixed 1:1 with water. All right half-leaves inoculated with sap of infected tobacco mixed 1:1 with watermelon foliage sap after dilution: A. - Undiluted, B. - 1:10, C. - 1:100, D. - 1:1000.

DISCUSSION

The present investigation has demonstrated the common occurrence of the tobacco ringspot virus in association with the pimple disease of watermelon in Oklahoma. The identification of this virus from watermelon substantiates the work of Pound (33) and Rosberg (37) and provides further evidence on the widespread occurrence of the tobacco ringspot virus on watermelon.

Several viruses causing ringspot-like diseases on tobacco are known and are usually distinguishable on the basis of host range and physical property studies (26,42). However, in some cases, more specific methods such as cross-immunization and serology are necessary for distinguishing some of the viruses within the ringspot group (2).

From observations during the course of the present studies it would seem that some host other than watermelon is acting as a reservoir from which the tobacco ringspot virus is being transmitted to watermelon via an insect vector or vectors. Several possible natural hosts occur abundantly in and around Oklahoma watermelon fields. Among these sweet clover (13,23), Jimson weed (24), and horse nettle (25,44) have been found naturally infected with the tobacco ringspot virus. While soybean (1) has been demonstrated as a common host for this virus it seems unlikely that it serves as a virus reservoir in Oklahoma because of the limited acreage of soybeans and the fact that the two crops are usually not grown in the same areas. In the present investigation the ringspot virus has not been demonstrated as occurring in nature in tests of several possible weed hosts. Since these tests were limited and since difficulties were encountered in handling the virus, the present results do not preclude the possibility that the virus does occur commonly in some host other than watermelon in Oklahoma.

Only differential grasshoppers (48) and the green peach aphid (39) have been reported as vectors for the tobacco ringspot virus. In the present study the differential grasshopper was found to transmit this virus from watermelon to watermelon in only one of 48 trials. Few of the insects commonly encountered in Oklahoma watermelon plantings have been tried as vectors. Melon aphids were tried in the present work with negative results. An insect which appears as a likely suspect is the twelve-spotted cucumber beetle (Diabrotica undecimpunctata howardi Barb.) At this time the vector or vectors responsible for introducing the virus to watermelon and responsible for spread from watermelon to watermelon remains unknown.

The evidence from present work is that the virus is not carried in watermelon seed. Since, however, only a few thousand seed were tested this evidence is not conclusive. The problem remains then to explain the widespread, uniform occurrence of this disease in Oklahoma watermelons. The only reasonable explanation seems to be that a rather common host is serving as a virus source and that one or more insects are involved as vectors.

It should be pointed out that the extent and thoroughness of the studies presented have been limited by two important factors. Temperature has been a limiting factor throughout these studies. At 30°C and above it is difficult or impossible to obtain symptom expression with tobacco ringspot virus on the hosts tried. Whether the effect of temperature is on the virus, the host or both has not been determined. The virus has been recovered in a few instances from inoculated hosts showing no symptoms at high temperature. The other principal limiting factor has been the presence in watermelon foliage and fruits of an inhibitor of infection. The demonstration here of this inhibitor constitutes the first known report of watermelon sap having this property.

The tobacco ringspot virus in watermelon plantings in southern areas, as described herein, does not appear to be a disease of great importance. However, losses may well be greater than commonly realized as, to date, no yield data are available with which to evaluate losses. Also worthy of mention are the greater and very obvious losses which occur in late season with cooler temperatures. Similar losses might well occur with cooler seasons in these southern areas as greenhouse tests have shown the virus capable of causing lethal infections on watermelon at cooler temperatures.

Some losses to southern watermelon growers are at times due to confusion of the ringspot disease with anthracnose. Rosberg (37) has mentioned that due to the indistinguishable nature of the fruit lesions with both the ringspot virus and anthracnose diseases, that many virus infected fruits are culled by inspectors at rail shipping points. Anthracnose is known to be associated with a rapid decay of fruits. For this reason, all melons showing the very similar virus lesions are also culled. This may at times represent a substantial loss to growers.

SUMMARY

A mechanically transmitted virus was found constantly associated with a disease of Black Diamond watermelons in Oklahoma. This virus was identified as a yellow strain of the tobacco ringspot virus through a study of symptomatology, host range, physical properties, cross-immunization, and serology.

Host range and symptomatology were studied and described on watermelon, tobacco, cucumber, bean, petunia, and cowpea in addition to several other plants. Physical properties as determined on cucumber were as follows: thermal inactivation, 10 minutes at 70°C; dilution, 1 to 100,000; longevity <u>in vitro</u>, 8 days at 20°C. The watermelon virus effectively protected tobacco plants against subsequent infection with tobacco ringspot virus but not against tobacco mosaic virus or cucumber mosaic virus. Confirmation of the identification of the watermelon virus as tobacco ringspot was obtained through precipitin tests.

The nature and development of the virus induced lesions on Black Diamond watermelon fruits was investigated. Histologically these lesions were found to be gall-like. Lesions were always in the mesocarp region of the fruit and were composed of necrotic tissue with an area of hypertrophied cells immediately beneath. The hypertrophy appeared as a response to necrosis.

Attempts to transmit the tobacco ringspot virus from watermelon to watermelon with differential grasshoppers or melon aphids failed except in one trial in which grasshoppers successfully transmitted the virus. Transmission of tobacco ringspot virus through watermelon seeds was not

demonstrated.

An inhibitor of virus infection was demonstrated in watermelon foliage and fruits. Infection was inhibited by sap from watermelon foliage in transmission of the ringspot and cucumber mosaic viruses to cowpea and with tobacco mosaic virus on *Nicotiana glutinosa*. The inhibitive action of watermelon foliage sap was destroyed by heat, 80°C for 10 minutes, and by dilution, 1 to 1,000.

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