SEED AND POLLEN TRANSMISSION OF CHERRY RING SPOT VIRUS IN BUTTERCUP SQUASH

by

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INTRODUCTION

The nature of spread of viruses from an infected plant to a healthy plant and their method of survival from one season to the next has become an important phase of Plant Pathology. Infected seeds and even infected pollen have been found to be involved in this problem.

The spread of virus in tree fruits is especially important, as the removal of infected plants and the replanting of healthy plants as commonly employed with other crops is not feasible. In many instances a fruit tree will not produce an economical crop until it is 5 to 10 years old. Therefore growers are reluctant to remove an infected tree as long as it continues to produce some marketable crop.

Cherry ring spot virus is very common in stone fruit trees. Considerable evidence has been presented to indicate the value of planting virus free trees and keeping them virus free after they are planted in the orchard. In order to provide the orchardists with virus free trees, both the rootstock and the variety used on these roots must be free from virus. After the trees are planted in orchards, methods of preventing these trees from becoming infected must also be evolved.

A review of the literature indicates that cherry ring spot virus is transmitted through the seed. Transmission of the virus

from an infected tree to a healthy tree has also been observed. The lack of a known insect vector for this virus suggested that pollen from infected flowers might be important not only in seed transmission of the virus but also may be a method by which the virus spreads from tree to tree.

The ring spot virus can be transmitted mechanically to squash. The strains from sour cherry trees cause a bright golden mottle that is very easily recognized and which becomes uniformly systemic. Squash was selected for these studies because of the ease of growing larger numbers of plants under more controlled conditions than would be possible if cherry trees were used.

In the present investigations, attempts have been made to determine (A) whether cherry ring spot virus is transmitted through the seeds of infected squash plants, (B) the effect of diseased or healthy ovaries and pollen combinations on subsequent seed transmission, and (C) whether pollen from diseased plants can produce infection in healthy plants and thereby serve as one of the means of spread in the field.

REVIEW OF LITERATURE

Several viruses have been found to be seed transmitted but, in general, a protective mechanism has evolved in most plants that apparently provides immunity to embryo infection by the great majority of the plant viruses. Often, the percentage of seeds through which a virus is transmitted is very low.

There are only a few reports in the literature on the seed and pollen transmission of stone fruit virus but these all are concerned with the virus complex used in this study. Both seed and pollen transmission has been reported for other viruses and they are reviewed under their respective crop headings.

The review of literature has been broadly classified into the two headings of seed transmission and pollen transmission.

I. SEED TRANSMISSION

Stone fruit ring spot virus

Cochran (5, p. 269-270) first reported that the ring spot virus can invade and be carried in Mazzard cherry seeds. In one commercial lot of Mazzard cherry seed he reported 25 of the 469 seedlings were infected with virus because they showed ring patterns in the leaves. Cation (7, p. 40) observed that 10 percent of Mahaleb seed transmitted ring spot virus and 8.7 percent transmitted cherry yellows complex. Sour cherry yellows virus complex was not transmitted through the seeds of Montmorency but at

least 30 percent of the seed carried the ring spot virus when the seeds were collected from trees known to be infected with sour cherry yellows. In a later study on the transmission of ring spot and sour cherry yellows through seeds, Cation (8, p. 4) found 41 and 24 percent transmission respectively from two different Mahaleb trees infected with sour cherry yellows. When a commercial source of Mahaleb seedlings was indexed it showed 11 percent virus infection, with a sour cherry yellows - ring spot ratio of 1 to 2. Montmorency seedlings produced from a sour cherry yellows tree showed 46 percent infection, with a sour cherry yellows - ring spot ratio of 1 to 4. Nyland (38, p. 517-518) observed that seeds from five different sources of Stockton Morello known to be carrying the sour cherry yellows and ring spot complex of viruses produced many seedlings that showed symptoms resembling sour cherry yellows in the first growing season. In the following year, additional seedlings developed sour cherry yellows symptoms. However, when 35 seedlings which showed yellows-like symptoms were indexed on Shiro-fugen, only 11 were positive for ring spot.

Evidence indicating the passage of the ring spot virus through seeds of Mazzard and Mahaleb cherries suggested a trial to determine whether there was similar passage through peach seeds. Cochran (6, p. 964) collected a few seeds from an experimentally infected Lovell and four naturally infected Rio Oso Gem peach trees. Three of the seedlings from the Rio Oso Gem and one of the seedlings from

the Lovell were infected with ring spot virus. The lots were too small to indicate the percentage of transmission. Millikan (35, p. 84) reported that random indexing of unbudded Lovell peach seedling stock to Montmorency and Shiro-fugen indicators revealed about 16 percent infection by peach ring spot virus. The observations of Wagon, et al. (51, p. 117) suggested seed transmission of both peach ring spot and peach necrotic leaf spot viruses. Seed transmission of peach ring spot virus occurred in from 1.1 to 11.7 percent and of peach necrotic leaf spot virus in from 3.0 to 9.0 percent of the seed tested.

Tobacco ring spot virus

Henderson (25, p. 227) reported the transmission of tobacco ring spot virus through the seeds of Petunia violacea Lindl. When 810 seeds from Petunia plants inoculated with tobacco ring spot virus were planted, 160 or 19.8 percent of the seedlings developed the ring spot.

Valleau (46, p. 28) reported that the two ring spots of tobacco, designated as green and yellow, are both transmitted through tobacco seeds in percentage up to 15 percent. In his studies (47, p. 78) on seed transmission and sterility of these two strains of tobacco ring spot he noted that seed infection with yellow ring spot ranged from about 1 to 17 percent. He later reported (48, p. 550-551) that the yellow ring spot virus of tobacco was active in seeds that had been stored for 5½ years.

Desjardins, et al. (19, p. 86) have demonstrated that tobacco ring spot virus is also transmitted through 54 to 78 percent of the seed of Lincoln variety of Soybean.

Athow and Bancroft (1, p. 698-699) reported that when seeds from Harosoy Soybean plants infected with tobacco ring spot virus were germinated in the greenhouse, 10 percent of the seedlings were infected. They also showed that the virus remains active in seeds for at least 9 months under ordinary storage condition.

Tobacco mosaic virus

Doolittle and Beecher (18, p. 993-994) reported that the greenhouse tomatoes at Beltsville, Maryland, have occasionally suffered from a strain of tobacco mosaic which caused foliar shrivelling and necrosis. Seed transmission occurred in 5 out of 342 tomato seedlings when grown from freshly extracted seeds of diseased plants. No seed transmission occurred in those seeds subjected to more than 10 days drying. They also reported (17, p. 800-801) that freshly extracted tomato seeds from ordinary mosaic infected plants showed cotyledonary deformity which developed into typical mosaic symptoms. When stored for 3 to 12 months before planting, none of the seedlings showed any evidence of mosaic during the seedling stage, but some developed mosaic symptoms after they had developed 4 to 6 leaves. The infection in the plants grown from stored seeds

could not be definitely attributed to seed transmission of the virus.

Tomato ring spot virus

Kahn (29, p. 295) reported seed transmission of the tomato ring spot virus to 76 percent of the seedlings of Lincoln variety of Soybean.

Cucumber mosaic virus

McClintock (31, p. 786) reported that both field observations and experimental tests indicated that cucumber mosaic virus was transmitted through seeds. Doolittle and Gilbert (15, p. 77) reported that one plant out of 5500 seedlings obtained from seeds from diseased plants showed cucumber mosaic in a field experiment. Later they reported (16, p. 327) that a certain percentage of seed from mosaic plants of the wild cucumber, Micrampelis lobata, Torr. and Gray. produced diseased plants the following season. Kendrick (28, p. 823) reported that cucurbit mosaic virus was transmitted through the seeds of muskmelon. Middleton (33, p. 409-410) reported the seed transmission of cucumber mosaic virus through squash. Poor quality seeds carry 0.96 percent of infection whereas good quality seeds had only 0.14 percent. He also reported that the virus remained viable in seeds that had been stored for three years. There were no apparent difference of transmission between

seed samples sown shortly after harvest or about three years later.

Legume virus

McClintock (32, p. 61) recorded the seed transmission of a mosaic virus in Lima bean, which was the first report of seed transmission in Leguminosae.

Stewart and Reddick (41, p. 61) reported the occurrence of a mosaic disease of Pea beans (Phaseolus vulgaris L.) in the field. Bean seeds from mosaic diseased plants developed diseased seedlings. Pierce and Hungerford (39, p. 2) observed that bean plants which have become infected with mosaic during the growing season - secondary infection - produced seeds which averaged 33.7 percent mosaic when indexed in the greenhouse, whereas plants which originated from mosaic infected seeds - primary infection - produced seeds which averaged 48.6 percent mosaic.

Snyder (44, p. 523) found that mosaic of asparagus bean (Vigna sesquipedalis Wight.) was transmitted through 37.0 percent of the seed.

Zaumeyer and Harter (52, p. 326) reported that two new closely related viruses of beans - bean mosaic virus 4 (southern bean mosaic virus 1) and bean mosaic virus 4A (southernbeans mosaic virus 2) were both isolated from seeds in the milk and dough stages and from freshly ripened seeds, but bean mosaic virus 4A

only was isolated from seeds stored in the laboratory for 7 months. About 5 percent of such seeds produced diseased plants.

Cheo (9, p. 18-19), during a study on the effect of seed maturity on the inhibition of southern bean mosaic virus in beans, found that if fresh immature seeds from systematically infected bean plants were germinated immediately after harvest the virus was transmitted to the seedlings but completely ripened or dried seeds did not transmit the virus.

Gardner (21, p. 417) reported that 21 out of 34 seedlings grown from seeds collected from a mosaic infected plant of the Progressive white variety of Cowpea were infected.

Lettuce mosaic virus

Newhall (37, p. 105-106) first reported that mosaic of lettuce was frequently transmitted through the seed. Mosaic symptoms were distinctly apparent on 27 out of 563 plants. Also, Grogan, et al. (22, p. 939) reported that 1 to 3 percent of all commercial seed lots tested transmitted the lettuce mosaic virus. Couch (10, p. 63) found that the rate of seed transmission of lettuce mosaic virus of variety Bibb was distributed among the various floral heads in a randomized manner irrespective of topographical relationship or time or order of floral initiation. He also noted that lettuce plants inoculated just before flowering produced fewer virus infected seeds than those inoculated soon

after planting. Plants that became infected after flowering had started did not transmit the virus through the seeds.

Barley false stripe virus

McKinney (34, p. 48) noted that seeds from Chevron variety barley plants infected with false stripe virus produced 132 virus infected plants out of 226 seedlings.

Elm mosaic virus

Bretz (4, p. 3), observed that the seedlings from approximately one percent of the seeds which were separated from fruit coverings and from $3\frac{1}{2}$ percent of those seeds whose fruit coverings were left intact prior to planting, developed characteristic mosaic symptoms. There was no such correlation of seed transmission between malformed and normal fruits and the percentage of seedlings showing mosaic symptoms was approximately the same in each case.

Miscellaneous viruses

Jones (26, p. 948-949) from Washington state, reported that greenhouse Cineraria (Senecio cruentus, D.C.) suffers from two virus diseases, streak and mosaic. Both are transmitted through the seeds and all commercial lots of seeds tested carried the virus. The streak virus remained active in four year old seeds but mosaic was inactivated by one year of storage of the seeds in one sample,

whereas, in another case mosaic was not inactivated in one year old seeds.

Bennett (3, p. 87) discovered a new virus in dodder, Cuscuta californica, Choisy, in the vicinity of Riverside, California. The virus was transmitted through 4.8 percent of the seed from infected plants of Cuscuta campestris, Yuncker.

Keur (27, p. 20) mentioned that the virus which produces variegation in Abutilon thompsonii, Veitch and Abutilon mulleri is in a limited way transmissible to some of the seedlings obtained by crossing these two clones. Of 536 seedlings obtained from this cross, 287 were green and 249 showed transmissible variegation.

Blakeslee (2, p. 31) reported that the graft transmitted disease he described as Quercina of Jimson weed (Datura stramonium, Linn.) was transmitted to 79 percent of the seeds when infected plants were pollinated with pollen from normal plants.

Wallace and Drake (49, p. 20), in their investigations of the seed transmission of the Avocado sun-blotch, reported that the virus can be transmitted readily through seeds. Very probably this source of infection has been responsible for the high percentage of diseased trees in certain nursery lots where scion budwood originated from sources which on past performance was believed to be healthy.

Salmon and Ware (42, p. 729-730) observed that of 228 seedlings raised from hop plants affected with a chlorotic disease,

development of the disease occurred in 28 plants in the first year. Of the remaining 200 plants, 33 plants showed the disease in the second year. Thus, of the 228 seedlings raised, 61 developed chlorotic symptoms. They concluded that transmission of the disease was through the seed.

Reddick (40, p. 124) reported a low percentage of seed transmission of the potato acropetal necrosis virus.

Traversi (45, p. 349) mentioned that the varieties of sunflower that are planted in Argentina are highly susceptible to an unidentified virus. The percentage of seed transmission in different varieties varied from 17.0 to 43.33 percent.

II. POLLEN TRANSMISSION

Stone fruit ring spot virus

Way and Gilmer (50, p. 1222-1223) demonstrated pollen transmission of necrotic ring spot virus in cherry. Flowers of virus free English Morello cherry trees were emasculated at the balloon stage of development to prevent self- or cross-pollination. Pollen from infected Montmorency cherry trees was applied to the stigmas of the emasculated flowers 2 to 5 days after emasculation. Seedlings were indexed for virus on National pickling cucumber seedlings and 5 out of 18 seedlings were infected. Later Gilmer and Way (24, p. 624-625) demonstrated by mechanical inoculation on cucumber and squash (var. Butternut) that pollen from Montmorency sour cherry

trees was infected with necrotic ring spot virus and prune dwarf virus. They also demonstrated the transmission of both necrotic ring spot virus and prune dwarf virus by pollen to seeds produced on healthy English Morello trees. About 25 percent of the seeds resulting from fertilization of healthy ovules with pollen from diseased trees were infected with necrotic ring spot virus, prune dwarf virus, or both viruses. Ehlers and Moore (20, p. 519-520) collected pollen from Shiroplum, Early Richmond, Montmorency, Dyehouse sour cherries known to be infected with various stone fruit virus diseases. Inoculum was prepared by grinding the pollen in a small quantity of 0.01M sodium phosphate buffer at pH. 8. The virus was found to be mechanically transmissible from pollen.

Elm mosaic virus

Callahan (12, p. 5) made four types of pollinations between healthy and mosaic infected parents of elm. Branches of each parent were forced to flower in cans of water in the greenhouse. Pollinations were made at post-anthesis with a pollen gun. The percentage of infected seedlings from each type of pollination is given below.

(1) Healthy female x Healthy male - 0 percent.

(2) Infected female x Infected male - 48 percent.

(3) Healthy female x Infected male - 30.5 percent.

(4) Infected female x Healthy male - 75 percent.

From this he concluded that the elm mosaic virus was pollen transmitted to seeds.

Barley false stripe virus

Gold, et al. (23, p. 115-117) demonstrated that seed and pollen transmission of barley false stripe is associated with the presence of distinctly rod-shaped virus particles. These particles are found in leaves, embryos, endosperm, pollen, unfertilized pistils and anthers from diseased barley plants. Pollen transmission of the disease was suggested by the presence of the rods in seeds produced from healthy pistils pollinated by pollen from diseased plants. Seeds from diseased pollen and healthy pistils produced a small percentage of diseased seedlings.

Potato acropetal necrosis virus

Reddick (40, p. 122) observed that progeny of some potato crosses were infected with acropetal necrosis virus. In 4 of 7 cases involved, the pollen parents were definitely known to be infected with acropetal necrosis virus. He believed that this demonstrated pollen transmission of the virus.

MATERIALS AND METHODS

Plant materials

Buttercup squash plants (Curcurbita maxima Duchesne var. Buttercup) were used in all the experiments conducted during this investigation.

Squash plants were selected because of the ease of growing them in large numbers. Buttercup squash variety was chosen in this study as it produces very prominent and easily recognizable symptoms with the RS 31 strain of cherry ring spot virus.

Seedlings used either for maintenance of culture or as test plants were grown in the greenhouse in number 10 cans filled with soil. After planting, the seeds were covered with a thin layer of sand to give uniform moisture and germination.

Source of virus

The RS 31 strain of cherry ring spot virus which caused a bright golden mottle on the leaves of Buttercup squash, was obtained from Dr. J. A. Milbrath and used throughout these studies. Fresh inoculum was obtained by inoculating the cotyledonary leaves of this variety of squash and by maintaining the virus in young actively growing plants.

Preparation of inoculum and methods of inoculation

Inoculum was prepared by grinding young diseased leaves, fruit parts or pollen with a few drops of 0.5 percent potassium phosphate buffer solution in a mortar with a pestle. Carborundum powder was dusted on the cotyledons before any inoculation and they were washed with water a few minutes after the inoculation.

All inoculations except those otherwise mentioned were made by rubbing the cotyledons with the finger after dipping it into the inoculum.

Method of pollination

All pollinations were done by hand in the field experiments of 1959 whereas a pollen gun was also employed in some experiments in 1960. To control pollination, the tips of the female flowers which were expected to open the following day were tied by means of a string (Figure 1). Before pollination the flower was opened and after the pollination the string was again tied round the petals to prevent further pollination from other sources.

Hand pollination was done by collecting the male flowers from diseased or healthy plants, the petals were removed and anthers with the pollen were rubbed on the stigmas. The male flowers used for pollination were also tied in the same way as the female flowers to prevent insect damage. Fresh pollen was used in all cases and

all pollinations were made between 8 and 11 o'clock in the morning. A number of tied female flowers were left at random without pollination as checks in controlled pollination plots. The object of having check flowers was to determine whether there was any possibility of these flowers being pollinated and thus developing into normal fruits. All the flowers pollinated were numbered and the dates of pollination recorded.

A pollen gun was devised to collect pollen as well as to pollinate the flowers without any possible contact of the parent tissue with the stigma other than pollen (Figure 2). The pollen was collected in the glass vial by creating a vacuum by sucking through the long arm of the gun and holding the short arm in close proximity of the pollen grains. The pollen thus collected in the vial was blown back through the short arm onto the stigma.

Seed selection and planting

Fruits were harvested after maturation and all seeds were taken from the fruits after two weeks storage. Many infertile and light seeds were floated off during washing. The remaining seeds were then dried and counted for each fruit. They were kept in separate small paper bags and stored at 35°F. until they were planted.

The seeds were planted either in number 10 cans and placed on greenhouse benches or in ground beds in the greenhouse. In all

cases the seeds were treated with Spergon before planting. The seedlings were kept under observation for about six weeks after which the number of diseased seedlings and number of healthy seedlings were recorded.

EXPERIMENTAL RESULTS

I. SEED TRANSMISSION

A preliminary test on the seed transmission of cherry ring spot virus in Buttercup squash was made with the seeds collected from 22 fruits supplied by Dr. J. A. Milbrath. These fruits were harvested from diseased plants which were naturally pollinated in the year 1958. The seeds were planted in soil beds in the greenhouse during December 1958 to June 1959. Thirty-four of the 1316 seedlings developed the characteristic symptoms of the virus which indicated that a total of 2.58 percent of the seed transmitted the cherry ring spot virus.

Another lot of seeds of the year 1958 was planted on June 28, 1960 in a field plot. Of the 3675 seeds planted, 2216 germinated and developed plants. Eleven of the 2216 plants, or 0.49 percent developed typical symptoms of cherry ring spot virus. These seeds had been stored for 18 months at 35°F. The percentage of seed transmission of the virus from the seeds of fruits from the same plot which had been planted the preceeding year was 2.58 percent. This suggests that the activity of the virus declines during storage.

A. Seed transmission studies of cherry ring spot virus in 1959

A field experiment was designed in 1959 to compare the effects of diseased or healthy ovaries and pollen on the subsequent seed transmission of cherry ring spot virus in Buttercup squash. In plot I, the plants were to be diseased and the flowers pollinated with healthy pollen. In plot II the plants were to be healthy and the flowers were to be pollinated with pollen from diseased plants. In Plot III all plants were to be infected with cherry ring spot virus and natural pollination allowed to occur from these infected plants.

The seeds in all plots were planted on May 21, 1959. In plots I and II the seeds were planted in 12 hills in each plot with the hills 10 feet apart in each direction. In plot III seeds were planted in a single row of 15 hills. This plot was isolated from other squash plots to avoid cross pollination with normal plants. The plants in plots I and III were inoculated with the RS 31 strain of cherry ring spot virus in the cotyledon stage to insure uniform virus infection of all plants (Figure 3).

All male flowers in plots I and II were removed until after the pollinations were completed to prevent natural pollination. As an added precaution, all female flowers used in these plots were tied with a string just previous to their natural opening.

Controlled pollination in plots I and II was started July 23, 1959 and continued until September 2, 1959. All the flowers

pollinated were numbered and the dates of pollination were recorded. Since all of the plants were diseased in plot III and natural pollination was allowed, the fruits formed on these plants were not numbered.

In plot I, 192 flowers were pollinated from which 38 fruits developed to maturity. In plot II, 248 flowers were pollinated and 72 developed into mature fruits. Fifty-seven fruits developed in the naturally pollinated plot III. Of the check flowers which were left with petals tied and not pollinated artificially, none developed into normal fruits. This suggests the technique employed to control pollination in this investigation was dependable. All the fruits from all plots were harvested on November 5, 1959.

The seeds from each fruit were removed, cleaned, dried and stored in separate packages. The number of seeds and the percentage of seed transmission could thus be determined for each fruit. There was not sufficient greenhouse space to plant all of the seeds at any one time. Therefore the period of planting extended over a 5 months period, from December 25, 1959 to June 10, 1960. Five different planting dates were necessary before all the seeds had been tested.

The data as presented in Table I suggest that ovule infection prior to fertilization was essential for seed transmission of the virus. None of the 5974 ovules which were not infected at the time of fertilization produced infected seeds when pollinated with infected pollen.

Seed transmission did occur from plants in plots I and III where the ovules were infected at the time of fertilization. This suggests that infected ovules are responsible for seed transmission rather than infected pollen.

Some of the fruits which developed on an infected plant did not produce infected seeds. Thirteen of 38 fruits which developed on 32 diseased plants pollinated with healthy pollen carried some infected seeds, while 25 fruits failed to produce any infected plants. Fifty-seven fruits developed on 65 diseased plants pollinated with diseased pollen and only 5 of these fruits carried infected seeds (Table II). The percentage of seed which carried the virus in any individual fruit was quite variable whether the fruits developed from diseased ovaries and healthy pollen or diseased ovaries and diseased pollen (Table II).

The seeds that were collected from fruits developed as a result of fertilization between diseased or healthy ovaries and pollen showed varying degrees of seed development. Some were well developed while others consisted only of the seed coat with little or no development of embryo. The last type was classified as flat seed. The fruits which developed from healthy ovaries and diseased pollen produced 4.58 percent flat seed, while the fruits developed from diseased ovaries and healthy pollen produced 5.87 percent flat seed.

Table I. Number of infected seedlings derived from seeds of fruits resulting from different combinations between diseased or healthy ovaries and pollen.

*Lot No.	No. seedlings	No. diseased seedlings	% seed transmission
Plot I. Infected flowers pollinated with normal pollen			
I	258	1	0.38
II	410	4	0.97
III	833	11	1.32
IV	701	1	0.14
V	1184	3	0.25
Total	3386	20	0.59
Plot II. Normal flowers pollinated with infected pollen			
I	818	0	0
II	985	0	0
III	354	0	0
IV	1106	0	0
V	2711	0	0
Total	5974	0	0
Plot III. Infected flowers pollinated with infected pollen			
I	625	1	0.15
II	750	4	0.53
III	183	2	1.09
IV	-	-	-
V	-	-	-
Total	1558	7	0.44

*Lot number

I. Planted on December 25, 1959 and grown in cans.
II. Planted on February 27, 1960 and grown in cans.
III. Planted on March 21, 1960 in soil beds.
IV. Planted on April 14, 1960 and grown in cans.
V. Planted on June 10, 1960 at Plant Pathology Farm.

Table II. Variation in the percentage of infected seeds in individual fruits when infected flowers were pollinated with healthy or infected pollen.

Fruit No.	No.of seeds	No. germinated	No. infected	* % infected
Fruits resulting from normal pollen				
1	115	80	4	5.0
2	96	46	2	4.34
3	107	72	1	1.38
4	54	17	2	11.76
5	133	60	1	1.66
6	59	15	1	6.66
7	167	110	3	2.72
8	122	44	1	2.27
9	86	43	1	2.32
10	163	102	1	0.98
11	151	141	1	0.70
12	212	186	1	0.53
13	113	93	1	1.07
14 to 38	3283	2377	0	0.00
Fruits resulting from infected pollen				
1	79	40	2	5.0
2	215	67	2	2.98
3	104	27	1	3.70
4	82	25	1	4.0
5	101	47	1	2.12
6 to 57	3632	1352	0	0.00

* Percent of infected seed was calculated on the basis of seed germination.

The fruits developed from diseased ovaries and diseased pollen produced 19.76 percent flat seed. This suggests the possible sterility of pollen or abortion of ovules due to virus infection.

B. Further studies on seed transmission of cherry ring spot virus in 1960.

In order to obtain more informations as to the nature of the seed transmission of cherry ring spot virus in Buttercup squash, a field experiment similar to that of 1959 was again planned for 1960. For this purpose two widely separated plots were selected.

In plot I, only healthy plants were grown and none of the plants were emasculated. All flowers to be pollinated were prevented from opening by tying each flower with a string a day before the flower was expected to open. The plants for this experiment were established by planting the seeds on June 8, 1960 in two rows 30 feet apart. When the plants began to grow they were spaced to give 66 plants in each row. The plants in this plot were used as part of the pollen transmission studies described in a later section.

In plot IA, 145 flowers were pollinated with a pollen gun using pollen from diseased plants, and in plot IB, 130 flowers were pollinated by rubbing the stigmas with anthers bearing pollen from diseased plants. Of the 145 flowers pollinated in plot IA, 82 normal fruits developed and the 130 flowers pollinated in plot IB developed 59 normal fruits.

In plot II there were 40 plants in 17 hills planted in a single row. The seeds were also planted on June 8, 1960 and all plants were inoculated at the 2 to 3 leaf stage with cherry ring spot virus strain RS 31 by the air brush technique as reported by Lindner and Kirkpatrick (30, p. 507-509). All plants used in this plot showed the typical symptoms of the cherry ring spot virus.

Controlled pollination by rubbing the stigmas with anthers bearing healthy pollen from healthy plants was started August 2, 1960 and completed by September 2, 1960. The female flowers were tied previous to opening to prevent natural pollination as in the previous experiment.

Of the 111 flowers pollinated in plot II by the above method, 29 developed fruits. Thirty seven fruits which had resulted from natural pollination were also developed in this plot. Since there were no diseased Buttercup squash plants near this plot these fruits probably developed from flowers pollinated with pollen from infected plants.

The fruits from these plots were harvested October 25, 1960 and stored for further studies on seed transmission. Unfortunately time allowed for completion of this thesis terminated before the seeds could be planted and the percentage of seed transmission determined. However, these data will be collected and used in future studies and included in a technical paper.

II. POLLEN TRANSMISSION

Das, Milbrath and Swenson (14) reported that transmission of cherry ring spot virus occurred when Buttercup squash plants were pollinated by rubbing the stigmas of healthy plants with pollen bearing anthers from diseased plants. In a field experiment 8 out of 49 plants thus pollinated became infected with the virus. In another controlled greenhouse experiment, 3 of 81 plants pollinated with pollen from diseased plants became infected with cherry ring spot virus, while 81 non-pollinated plants in the same greenhouse remained healthy throughout the experiment. The possibility of mechanical transfer of the virus by rubbing the stigma with infected host tissue had not been eliminated in either case.

Two experiments were conducted in 1960 in order to investigate the possibility of pollen transmission of cherry ring spot virus in Buttercup squash when only pollen was applied on the stigmas. The field experiment was combined with an experiment for further investigations of seed transmission and the other experiment was conducted in a greenhouse where only pollen transmission was studied.

A. Pollen transmission studies in the field

In 1960 a field experiment was designed to study the possibility of pollen transmission of cherry ring spot virus in Buttercup squash.

Controlled pollination was started on August 2, 1960 and completed on September 2, 1960. In one of the rows the female flowers were pollinated with diseased pollen by means of a pollen gun, whereas in the other row the flowers of alternate plants were pollinated by rubbing the stigma with an anther bearing infected pollen. The other plants were left as checks to observe whether these plants became infected from natural agencies such as insect transfer of diseased pollen or virus from other plots where infected plants were grown. The object of having two types of pollination was to determine whether the pollen transfer of the cherry ring spot virus as previously noted was due to pollen or accidental mechanical transmission by rubbing the infected host tissue on the stigmas.

That the pollinations were successful was evidenced by fruit setting on 82 of the 145 flowers pollinated with the pollen gun and 59 of the 130 flowers which were pollinated by rubbing the stigmas with the pollen bearing anthers. None of the tied flowers left for checks developed into normal fruits, again verifying that no natural pollination had occurred.

No visible virus transmission was apparent in any plants in this plot 46 days after the last pollinations were made. Time did not permit the testing of these plants on young indicator plants for virus infection.

B. Pollen transmission experiment in the greenhouse

This experiment was designed exclusively to determine whether pollen transmission of cherry ring spot virus in Buttercup squash occurs when only pollen from infected flowers is applied to the stigmas of healthy flowers. The plants were grown in ground beds in the greenhouse. Seeds were planted on June 18, 1960. Five hills were planted 2 feet apart in each row and the rows were also 2 feet apart (Figure 4). There were 27 rows with 135 plants, of which 21 rows with 105 plants were pollinated with the pollen gun and 6 rows of 30 plants were left as checks. There were no infected plants in this greenhouse or near the outside of the greenhouse. The only source of infection was the diseased pollen from the plants used to pollinate these healthy plants.

When the plants were in the 8 to 10 leaf stage they were pruned to force branching. Each plant was limited to two branches which were trained to grow on separate strings (Figure 5). This was done in order to keep the plant to a manageable size, also to get several flowers in a small space.

The controlled pollination was done from August 18 to September 16, 1960. The plants were kept under observation until October 8, 1960 when they had to be removed as the space was needed for other work.

Two hundred four flowers were pollinated on 97 plants. Eight plants could not be pollinated because they had not developed

normally or flowers were not formed in time to be pollinated. Of the 97 pollinated plants 9 plants became infected with typical virus symptoms at the growing tips (Figure 6). The symptoms in these plants usually became visible two to three weeks after the last pollination. This showed that 9.27 percent of the healthy plants became infected when pollinated with diseased pollen. None of the 30 check plants in this experiment showed any virus symptoms during this period. Table III summarizes the data of this experiment.

Table III. Number of flowers pollinated on each plant with infected pollen and the number of plants which became infected as a result of these pollinations.

No.flowers pollinated each plant	No.plants pollinated	No.plants infected	% infection
1	25	1	4.0
2	47	5	10.63
3	17	2	11.76
4	7	1	14.28
6	1	0	0.0
Total	97	9	9.27

During the pollination the number of female flowers pollinated varied on different plants. In general there was an increase in percentage of infection when more flowers on the plants were pollinated. One plant became infected when a single flower was pollinated, while another plant did not become infected when six flowers were pollinated.

(i) Plant growth and symptom expression

Nine plants with new growth after pollination developed positive virus symptoms while 78 plants having similar growth did not. In 10 plants there was little or no growth after pollination and neither was there any visible virus symptom. The tips of these 10 plants were cut and tested individually for the possible presence of virus by inoculating on young Buttercup squash plants, but the presence of virus was not detected in any case. Most of these plants had been cut back during the experiment, in order to keep these plants from becoming too large. Vigorous growth had not developed on these 10 plants which might have influenced the development of the virus.

In summary, definite infection was noted on 9 of the 97 plants pollinated with pollen from plants infected with cherry ring spot virus.

(ii) The development of fruits resulting from the pollinations made in this experiment

Of the 204 flowers pollinated, 92 developed into normal fruits, 56 developed partially and 54 showed little or no development (Figure 7). In check plants a similar ovary development was also observed.

All of the 9 plants which became infected from the pollination showed some fruit development. These data are presented in Table IV.

Table IV. Fruit development on the plants which became infected

Infected plant	Number of pollination	Normal fruit	Partially developed	Little or no development
1	2	0	1	1
2	1	1	0	0
3	2	1	1	0
4	2	1	1	0
5	2	1	1	0
6	3	2	1	0
7	4	2	1	1
8	3	1	1	1
9	2	1	1	0
Total 9	21	10	8	3

At least one ovary developed into a normal fruit on each of 8 infected plants but in one case no normal fruit developed although 2 flowers were pollinated. Normal fruit development of the pollinated ovary may not be necessary to cause infection in these plants, but further observations are needed to have conclusive evidence in this respect.

(iii) Detection of virus in fruits developed on artificially pollinated plants

Thirty-four fruits, including normal and partially developed ones, were collected at random from the controlled pollination plot. The presence of virus in flesh and immature seeds from individual fruits was tested by mechanical inoculation on squash plants. Of these 34 fruits, 13 were from infected plants and 21 fruits were from pollinated plants without virus symptoms. Ten normal appearing fruits from infected plants showed the presence of virus in flesh

and seeds. Four such fruits showed the presence of virus in flesh only, 1 fruit in seed only, and in 5 fruits the virus was detected in both flesh and immature seeds. No virus was detected in 3 partially developed fruits from infected plants and no virus was recovered from fruits from pollinated plants without any virus symptoms.

The presence of virus in the flesh and immature seeds from the fruits developed on infected plants showed that the virus was transmitted to the developing ovaries during fertilization by infected pollen. Apparently the virus moves out of the ovary to the vegetative parts of the plant and causes infection. The failure to recover virus from fruits developed on plants without symptoms was possibly due to failure of the pollen to transmit virus to the ovaries. Apparently no successful pollination occurred with infected pollen grains on these fruits.

(iv) Mechanical transmission of virus from infected pollen

Mechanical transmission of virus from pollen of infected flowers was also tested on an individual flower basis. For this purpose pollen was collected either by tapping the pollen into the mortar or by a pollen gun from freshly opened male flowers on diseased plants maintained in the greenhouse.

Inoculum was prepared by grinding the pollen so collected in a mortar with a pestle with one or two drops of 0.5 percent potassium phosphate buffer solution and carborundum powder. Inoculation was

done on the cotyledons of young squash plants.

Of the 54 flowers tested during July to September, 1959 and again during June to September, 1960, only pollen from 2 flowers demonstrated the presence of infected pollen grains as expressed by symptoms on the test plants. Apparently the concentration was too low to infect squash under these conditions or the pollen in some infected flowers may not have been carrying the virus.

DISCUSSION

Seed transmission of the ring spot virus in stone fruit has been well established by Cochran (5, p. 269-270) and others. Similar seed transmission in Buttercup squash plants provided a much more desirable host for the studies reported in these investigations. The monoecious habit of producing male and female flowers and the development of many ovules in a single fruit makes squash an ideal host for these studies.

The problem of infected seed and transmission of the virus in pollen becomes an important phase of a nursery program to produce virus free trees. If virus free trees in a cherry seed orchard can become infected from diseased pollen, or if virus infected pollen can cause infection through the seed, the problem of isolation of these seed orchards becomes of major importance. By using squash plants the desired information was obtained in a much shorter time than could have been done if mature cherry trees were used for these studies.

Way and Gilmer (50, p. 1222-1223) obtained 5 infected seedlings out of 18 from seeds of English Morello cherry. These seeds were developed when pollen from infected Montmorency cherry trees were applied to the stigmas of virus free English Morello cherry trees. Gilmer and Way (24, p. 624-625) also recorded about 25 percent of seed resulting from fertilization of healthy ovules of

English Morello trees with pollen from diseased Montmorency sour cherry were infected with necrotic ring spot virus, prune dwarf virus or both viruses. Their observations are in contrast with the observations made in the present investigations with Buttercup squash plants. Pollen from squash plants infected with cherry ring spot virus when used to pollinate flowers on healthy squash plants did not produce any infected seed as evidenced by 5974 healthy plants grown from such seeds. However, 8 of these 49 squash plants became infected with cherry ring spot virus during this experiment apparently from the infected pollen.

The present investigation showed that when healthy Buttercup squash plants were pollinated with infected pollen some infection in the plants could occur. Nine out of 97 plants thus pollinated became infected with typical cherry ring spot virus under the greenhouse conditions, whereas no visible virus transmission was apparent in plants which were also pollinated by the same method in the field. Callahan (11, p. 40) working with elm mosaic virus, did not find infection in plants when pollinated with diseased pollen, although he (12, p. 5) obtained seed transmission of elm mosaic virus by infected pollen.

The presence of virus has been detected in immature seeds from fruits developed on infected plants as a result of fertilization between healthy ovaries and infected pollen. This indicates possibly the presence of virus in seed coat rather than in embryo

because none of 5974 seeds resulted from fertilization of healthy ovaries by infected pollen transmitted by the virus. This may possibly be explained by inability of virus from infected pollen to pass beyond the integuments of the ovules. So the question of embryo infection in Buttercup squash lies in the fact that ovules should be infected in its early stage of development which is only possible when it developes in an infected plant. Apparently the embryo cannot be infected in its later stage of development by way of infected pollen. Sheffield (43, p. 507-508) suggested that if the virus were present in the "embryo" prior to fertilization, it would be expected to multiply in the meristematic tissue of the developing embryo. If it were not present in the ovule prior to fertilization it would be unable to reach the embryo later unless it was brought by the pollen tube. Crowley (13, p. 122) reported that some seed transmitted virus can infect the embryo either by way of pollen or to some extent by infecting the young embryo in the early stages of its development.

All seeds produced in any individual fruit developed on a diseased plant did not carry the virus. Percentage of infected seed was quite variable in such fruit. A similar phenomenon has also been observed by Nelson (36, p. 23) with the mosaic disease of bean (Phaseolus vulgaris L.) which he believed to be due to localization of the virus in certain tissue.

The seeds produced in these experimental fruits showed varying degrees of development; some were normal seeds and others

were flat with little or no development. A greater percentage of flat seed was observed in the fruits developed from diseased ovaries and diseased pollen. This may be best explained on the basis of certain pollen sterility or abortion of ovules due to virus infection. Valleau (47, p. 78) found that the seed production in tobacco affected with tobacco ring spot virus was abnormally low which he thought was due to partial sterility of the pollen due to microspore infection with virus. He reported that seed production was increased markedly through the use of pollen from healthy plants or plants affected with virus other than ring spot. He (48, p. 550) also noted that both yellow and ordinary green strains of ring spot virus of tobacco have a marked effect on pollen following so-called recovery. There were varying degrees of abortion as indicated by reduced set of seeds. Some strains caused complete pollen sterility and affected plants fail to set any seed.

SUMMARY

These studies have demonstrated that cherry ring spot virus can be transmitted in seeds from infected Buttercup squash plants.

The seed transmission that occurred under different experimental conditions of these studies ranged from 0.44 to 2.58 percent.

When seeds from infected fruits were stored for 18 months at 35°F. there was a decline in the percentage of seed transmission.

The seeds produced in fruits that resulted from different crosses between diseased or healthy ovaries and pollen showed a mixture of well developed and poorly developed flat seeds. The greater number of flat seeds developed in fruits that resulted from the fertilization of diseased ovaries by diseased pollen.

In a greenhouse experiment when healthy Buttercup squash plants were pollinated with cherry ring spot virus infected pollen, 9.27 percent of the plants became infected with typical virus symptoms. No apparent virus transmission was observed in plants in a similar experiment in the field.

When a single flower on each plant was pollinated, some plants became infected. As more flowers on a plant were pollinated a higher percentage of infection did occur.

Fruits developed on infected plants due to pollination with infected pollen carried the virus in fleshy portions of the fruit. Seeds which were immature at the time of testing also were carrying the virus which may have been only on the seed coat.

The presence of virus within the pollen from infected flowers was demonstrated when inoculum was prepared of the pollen grains by grinding them and inoculating on young squash plants.

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APPENDIX

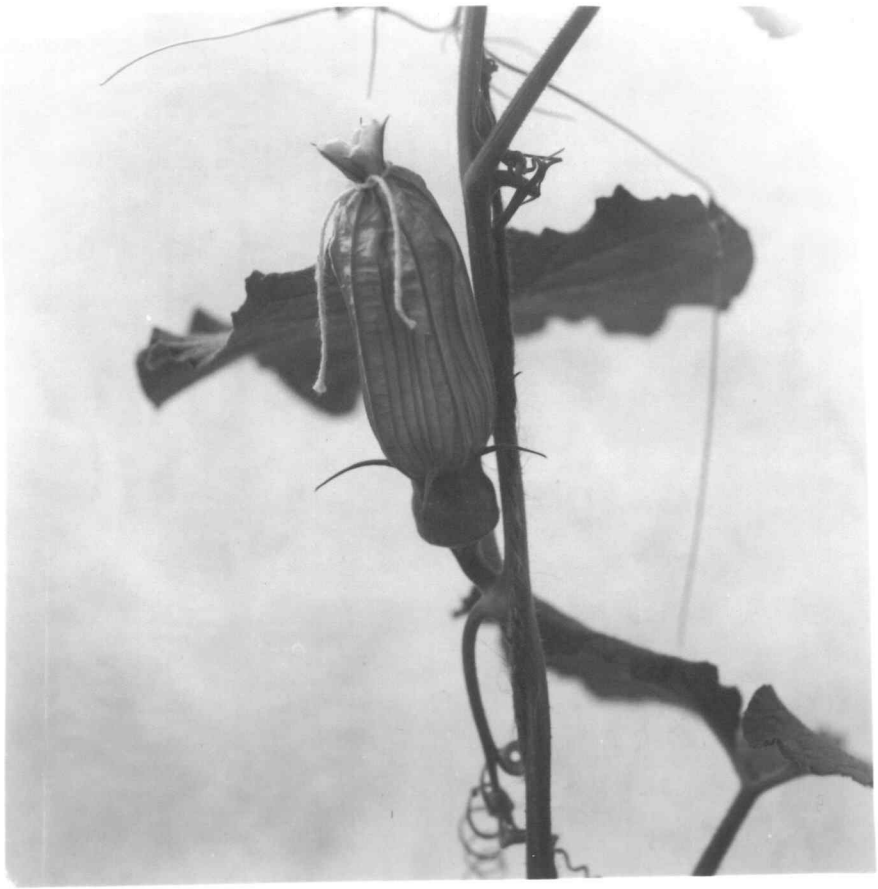

Figure 1. A female squash flower ready for pollination. The petals were tied with a string the previous day to prevent natural pollination.

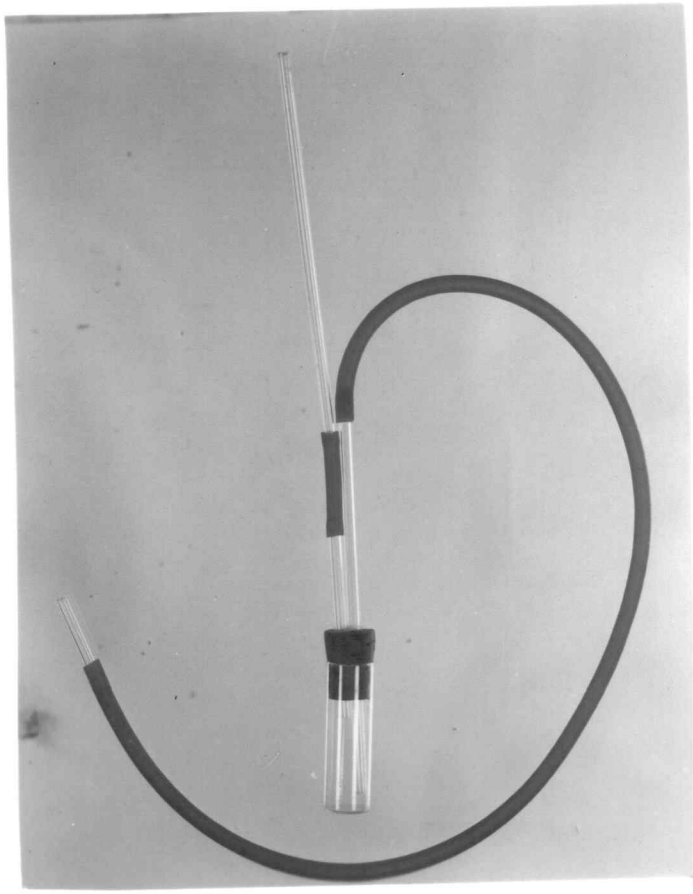

Figure 2. The pollen gun designed to collect pollen and also used for pollinating the flowers in the pollen transmission studies.

Figure 3. Buttercup squash plants showing typical cherry ring spot virus symptoms at well advanced stage of infection.

Figure 4. General view of the greenhouse plot used for pollen transmission studies.

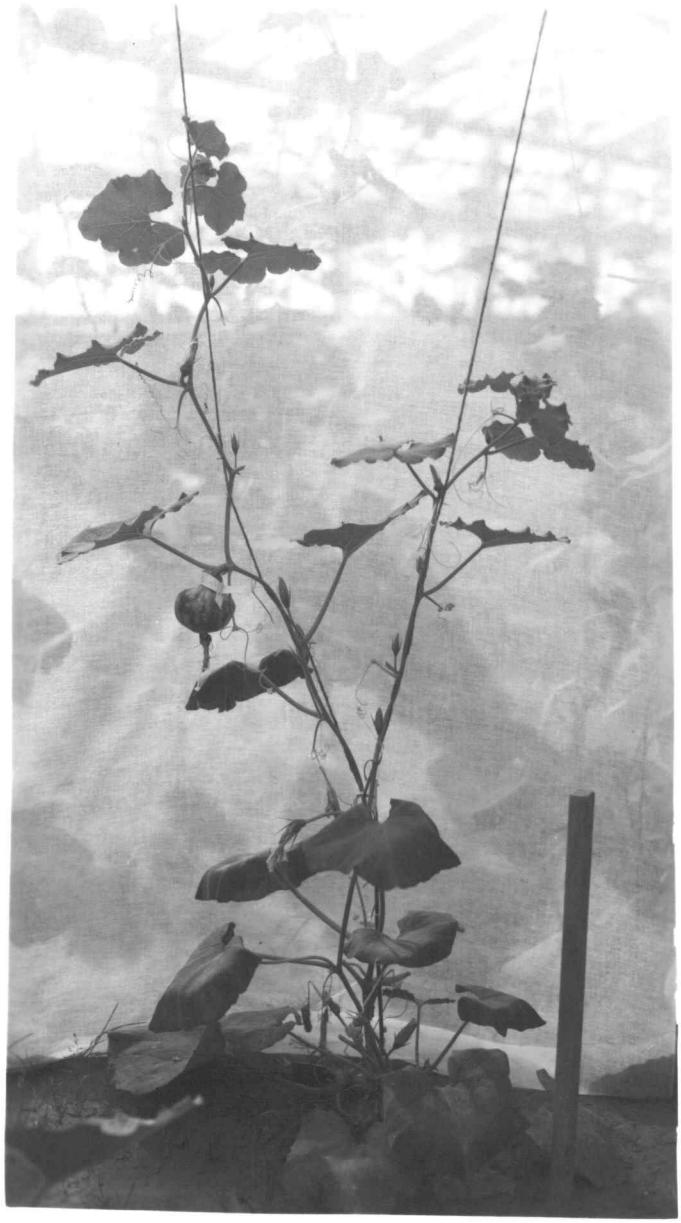

Figure 5. Squash plants grown in the greenhouse and pruned at the 8 to 10 leaf stage to give two branches which were trained on strings. Such plants developed numerous flowers which were used for pollen transmission studies.

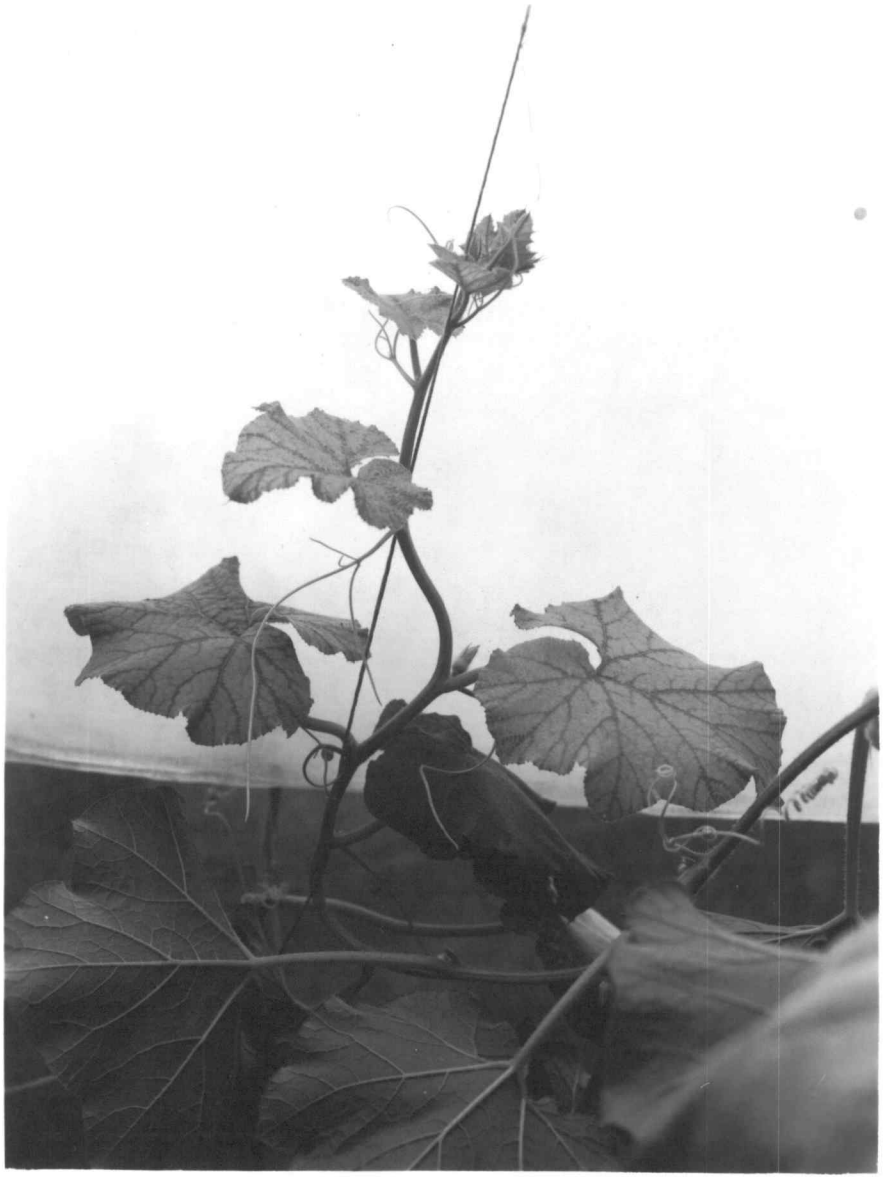

Figure 6. Terminal growth of a Buttercup squash plant showing typical cherry ring spot virus symptoms. This plant became infected when pollinated with pollen from a diseased plant.

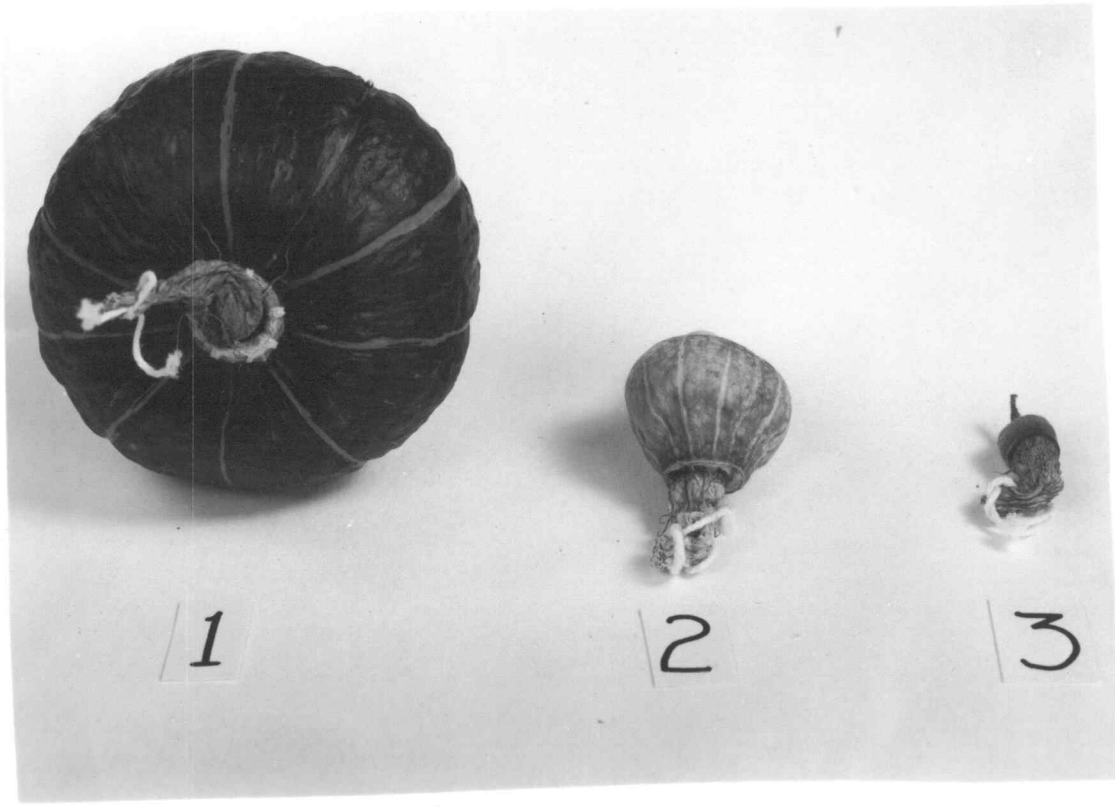

Figure 7. Three squash fruits which illustrate the ovary development on the plants during the pollen transmission studies. The fruit marked 1. shows normal ovary development, 2. partially developed ovary, and 3. shows little or no development of the ovaries following pollination.