Suggested short title :

# THE MECHANISM OF SEED TRANSMISSION OF TRSV IN SOYBEAN

by

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# THE MECHANISM OF SEED TRANSMISSION OF TOBACCO RINGSPOT VIRUS IN SOYBEAN

by

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A thesis submitted to the Faculty of Graduate Studies and Research of McGill University in partial fulfillment of the requirements for the degree of Master of Science.

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July 1973

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## ABSTRACT

Virus-like particles of tobacco ringspot virus (TRSV) were detected by electron microscopy in thin sections of the intine of the pollen wall, the wall and cytoplasm of the generative cell, the integuments, nucellus, embryo sac wall and in the megagametophytic cells of soybean, Glycine max cv. Harosoy. Anthers of TRSV-infected soybean produced less pollen than anthers from virus-free plants; the pollen from infected plants was low in germination capacity and its germ tubes elongated more slowly than germ tubes of pollen from virus-free plants. Evidence from cross-pollination experiments suggests that infection of megagametophytes is the principle factor contributing to seed transmission of TRSV in soybean. Analytical density gradient centrifugation of extracts from cotyledons of infected seeds indicated that the ratio of bottom component (B) to middle component (M) of TRSV remained essentially unchanged (B/M = 10) during seedling development whereas little or no middle component was detected in extracts of primary and trifoliate leaves from such seedlings.

Master of Science Degree,
Department of Plant Pathology.

TOBACCO RINGSPOT VIRUS : MOYEN DE TRANSMISSION PAR GRAINSES DANS LE SOJA

par ANN FOOK YANG

## RÉSUMÉ

Des virions du tobacco ringspot virus (TRSV) ont été révélés par observations effectuées au microscope électronique sur des coupes ultrafines de l'intine du paroi pollinique, du paroi et du cytoplasme de la cellule générative, des intéguments, de la nucelle, du paroi du sac embryonnaire et des cellules mégagamétophytiques du soja, Glycine Max cv. Harosoy. Les anthères des sojas infectés par le TRSV produisent moins de pollen que celles des plantes saines; peu du pollen des plantes infectées réussit à germer et les tubes polliniques s'allongent plus lentement que ceux du pollen des plantes saines, Les résultats d'expériences de pollinisation croisée suggerènt que la transmission par graines du TRSV dans le soja est associée principalement à l'infection des mégagamétophytes. L'étude au moyen de centrifugation analytique en gradient de densité d'extraits obtenus des cotylédons de graines infectées indique que, pendant le développement du plant, le rapport du composant inférieur ou "bottom" (B) au composant intermédiaire ou "middle" (M) reste essentiellement le même (B/M = 10) , tandis que dans les extraits des feuilles primaires ou trifoliées de tels plants, il se trouve peu de composant intermédiaire, ou bien aucun.

Maîtrise ès Sciences,
Département de Pathologie Végétale.

## ACKNOWLEDGEMENTS

The author expresses his sincere thanks to Dr. R.I. Hamilton for suggesting the problem and for his advice and encouragement during the course of the study.

Thanks are due to Dr. M. Weintraub, Director, who kindly offered the use of research facilities in the Agriculture Canada Research Station, Vancouver, British Columbia, Canada ; Misses E. Lo and B. Schroeder for their assistance in electron microscopy ; Dr. H.C. Lim and Mr. H. Severson for photographic assistance ; Mr. K.Y. Chong for harvesting soybean seeds ; Mrs. Carol Dodds for translating the abstract into French and to Mrs. Y.S. Choo for typing this thesis.

The author gratefully acknowledges the financial support of the Quebec Agricultural Research Council (Research Grant McA 66-274) which made this work possible.

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# 1. INTRODUCTION

The first instance of seed transmission of a plant virus was reported by Reddick and Stewart (1918), who showed that bean common mosaic virus (BCMV) was seed borne in bean (Phaseolus vulgaris) and that the rate of seed transmission was as high as 50 percent. Since then many plant viruses have been found to be seed transmitted, and several periodic reviews have been written on this method of virus transmission (Fulton, 1964; Baker and Smith, 1966; Bennett, 1969; and Shepherd, 1972). Bennett (1969) lists 132 host-virus combinations involved in seed transmission. In most cases, however, relatively little is known about the virus-tissue relationship in the seeds of infected plants and thus little is known of the mechanism whereby viruses are transmitted through seeds.

The objective of this thesis was to determine the mechanism of seed transmission of tobacco ringspot virus (TRSV), the virus which causes bud blight, a serious disease of soybean.

# 2. LITERATURE REVIEW

## 2.1. SEED TRANSMISSION OF PLANT VIRUSES

Virus-infected seeds can provide for the survival of viruses between generations of their host plants. The infectivity of viruses can be preserved for several months to a number of years in infected seeds. This property ensures the establishment of the diseases and their further spread in the new generation of host plants.

Certain viruses depend more on seed transmission than other methods of transmission for the perpetuation of diseases that they cause. The relative importance of seed transmission depends on the host range of the virus, the presence of an efficient vector and on other factors. Doolittle and Gilbert (1919) pointed out that cucumber mosaic virus in seeds of wild cucumber (Echinocistis lobata) could be the source of inoculum for the commercial cucumber. Seed-borne tobacco mosaic virus (TMV) has been considered to be important in initiating infection and further spread in tomato (Taylor et al., 1961). Barley stripe mosaic virus (BSMV) has caused significant loss in wheat and barley and Slykhuis (1967) stated that the disease in both crops was perpetuated by the seed-borne causal virus.

Nematode-transmitted viruses are often seed-transmitted (Lister and Murant, 1967; Murant and Lister, 1967). The dependency of these viruses upon seed transmission varies from one virus to the other. Lister and Murant (1967) found that the nematode Longidorus elengatus could acquire raspberry ringspot virus and tomato black ring virus from

weed seedlings emerging from infected seeds. The nematode could retain both viruses for about nine weeks, if the soil was left fallow; but it could regain the viruses after this time by feeding on infected seedlings from infected seeds which were allowed to germinate. Therefore, the consistent retention of the viruses by these nematodes depends upon the continuous supply of infected weeds arising from infected seeds. On the other hand, arabis mosaic virus and grapevine fanleaf virus could be retained in the nematode Xiphinema spp. as long as eight months, and the nematodes were often found to be associated with perennial plants. Thus the role of seed transmission of the latter viruses is less significant than that of the two former viruses.

Seedlings which emerge from infected seeds are already infected. These plants serve as inoculum sources and the yield loss can be high if the disease is spread to neighbouring plants by vectors. The instance of economic loss in lettuce caused by lettuce mosaic virus has been well documented (Grogan et al., 1952; Zink et al., 1956). Although seed-transmitted in low percentage, lettuce mosaic virus can be transmitted to a large number of healthy plants in a short period of time by aphids. Seed-borne virus has been considered to be the most important primary source of inoculum.

Seed transmission facilitates the even distribution of the virus in the crop. Since the infected seeds are randomly distributed, numerous centers of primary inoculum are available in the field to provide further spread by vectors. In contrast, viruses that are introduced from external sources usually occur in patches, at the edges of the field (Shepherd, 1972; Hill et al., 1973).

All methods of virus dispersal other than by infected seed or infected plant parts (root stocks) are effective over relatively short distances. Although the circulative type of vector can spread viruses to localities several miles from the source of inoculum, seed transmission is responsible for most long distance spread of the seed-borne viruses. Seed-borne bean mosaic carried in commercial seed lots has been implicated in the wide spread of the disease (Farjardo, 1930). Shepherd and Holdeman (1965) attributed the world-wide spread of sugar cane mosaic virus in corn and Johnson grass to the movement of infected seeds. Since seeds are among the items in international trade, certain diseases may be transported to localities where they do not occur. Wind, birds and other animals may contribute to the dispersion of seeds and the viruses in them.

### 2.1.1. FREQUENCY OF SEED TRANSMISSION

The type or variety of host plant can affect the rate of seed transmission of a virus. Thus, Eslick and Afanasiev (1955) found 63.7 percent seed transmission of BSMV in Compana barley, but only 4.4 percent in the variety Titan, when both were inoculated at the boot stage. Lister and Murant (1967) showed that seed transmission of the nematode-transmitted polyhedral viruses, which have a very wide host range, is very much influenced by the host plant. For example, seed transmission of arabis mosaic virus was 80 percent in Chenopodium album, 21-100 percent in Polygonum persiacria but only 1.8 percent in Lycopersicum esculentum. Kennedy and Cooper (1967) found 20.6 percent seed transmission of soybean mosaic in the soybean variety Harosoy but no seed transmission in the variety Merit.

Different strains of the same virus may not be seed-transmitted in the same host variety. Thus, Grogan and Schnathorst (1955) reported 3 percent seed transmission of TRSV, strain 98, in Paris Island Cos lettuce, but 'calico' virus, another strain of TRSV, was not seed transmitted in this variety. McKinney and Greeley (1965) reported a wide range of seed transmission rates for different strains of BSMV in barley, wheat and oats. Some strains of BSMV were not seed-transmitted while others were transmitted efficiently. Bennett (1969) suggested that host-influenced seed transmission could be an important factor in plant breeding programs designed to reduce or eliminate seed transmission of viruses.

The amount of seed transmission is largely dependent upon the stage of plant development at the time of infection. A high percentage of seed transmission occurs when the plant is infected well before flowering. The frequency of seed transmission of TRSV in soybean can be as high as 100 percent if plants are infected for a long period of time before flowering (Athow and Bancroft, 1959), but it is much lower if plants are infected just prior to or soon after flowering (Crowley, 1959; Owusu et.al, 1968). Similarly, the seed transmission of BCMV in bean (Schippers, 1963) and of lettuce mosaic virus in lettuce (Couch, 1955) is reduced by inoculating the plants at an older stage of growth.

According to Eslick and Afanasiev (1955), BSMV can be seed transmitted at a low level when the plants are inoculated long after flowering. Crowley (1959) obtained similar results but he pointed out that pre-flowering infection was required for high level of seed transmission of BSMV in barley and that the low level of seed transmission

detected in the seeds from plants inoculated at the post-flowering stage could result from late tillering. This view has been supported by Timian (1967) who concluded that seed transmission of BSMV occurred only if plants were inoculated before anthesis.

The temperature at which infected plants are grown can affect the frequency of seed transmission. Crowley (1957) reported that seed transmission of BCMV did not occur in seeds from plants grown between 62-65°F, whereas plants grown at 68°F produced 16-25 percent of infected seeds. Medina and Grogan (1961) could not confirm this observation from their experiments. They suggested that the difference was probably due to a difference in susceptibility of host varieties. According to Singh et al. (1960), among the four varieties of BSMV-infected barley plants grown at 16°C , one of the varieties produced 3 percent infected seeds, while the other three varieties produced healthy seeds ; plants grown at 20°C produced 9 to 27 percent in the same varieties.

2.1.2 DISTRIBUTION OF VIRUS IN SEEDS

Transmission of a seed-borne virus to the seedlings can be achieved via two routes, 1) by virus which is present on the seed surface or in the seed coat, and 2) by virus that infects the embryo. The evidence for the presence of viruses in the seeds has been obtained mainly from infectivity tests of the seed parts, and also by electron microscopy and serological tests.

Chamberlain and Fry (1950) reported that TMV was present on the surface of tomato seed, because seed transmission was prevented by acid or fermentation treatments. Crowley (1958) found that the virus was present in the seed coat in addition to infesting the seed surface. Taylor et al. (1961) repeated the experiments and concluded that TMV present in the endosperm apparently resisted inactivation by treatments which inactivated viruses in or on the seed coat.

Southern bean mosaic virus (SBMV) has been reported to be seed transmitted in bean in about 5 percent of the seeds harvested from infected plants (Zaumeyer and Harter, 1943). Gay (1973) reported that the frequency of seed transmission could be as high as 26 percent in cowpea infected with a cowpea strain of the virus. Cheo (1955) and Crowley (1959) reported that SBMV infectivity was present in the young embryo, but was subsequently inactivated at maturation, although it could be detected in homogenates of the seed coat. However, McDonald and Hamilton (1972) could not detect infectious SBMV in extracts of immature or mature embryos which had been decontaminated by detergent solution or tap water, while extracts from unwashed immature embryos were highly infecticus. Moreover, no infectivity could be associated with unwashed mature embryos, suggesting that the infectivity associated with the embryo was due to virus derived from the seed coat. They further showed that the infectivity of SBMV occurring in the seed coats declined markedly during maturation.

TRSV was found in the embryos but not in the seed coats of infected soybean seeds (Athow and Bancroft, 1959). Schippers (1963) reported that BCMV was inactivated in the pods and seed coats during

maturation and drying but that the virus in the embryos remained infectious even during storage and germination. A similar situation has been reported for pea seed-borne mosaic virus in pea seeds (Stevenson and Hagedorn, 1973).

The presence of BSMV in barley seed has been demonstrated by serological techniques (Scott, 1961; Hamilton, 1965; Carroll, 1969, 1972; Shivanathan, 1970), by infectivity tests (Inouye, 1962, 1966), and by electron microscopy (Gold et al., 1954; Inouye, 1966; Carroll, 1969). Gold et al. (1954) and Inouye (1966) observed BSMV particles in extracts of infected embryos and endosperm. Carroll (1969) further demonstrated the presence of BSMV in thin sections of infected embryo.

### 2.1.3. LONGEVITY OF INFECTIVITY IN SEEDS

Viral infectivity can persist in infected seeds for varying periods of time depending on the virus-host combination (Bennett, 1969). Generally, seed transmission was highest with freshly harvested seed but it declined with storage. Middleton and Bohn (1953) reported that seed transmission of muskmelon mosaic virus dropped from 95 to 5 percent in three years, but it could still be recovered after five years of storage. Valleau (1932) found decreased seed transmission of TRSV in tobacco after storing for 5.5 years.

Several viruses have been reported to maintain a constant level of seed transmission during storage. The seed transmission rate of BCMV, for example, remained unchanged for three years (Nelson, 1932). Scott (1961) reported a similar result for BSMV in seed stored for 6.25 years.

Viral infectivity in seeds can be affected by storage temperature. Laviolette and Athow (1971) reported that the seed transmission level of TRSV in soybean remained unchanged for five years if it was stored at 2°C, but that the level began to drop after storage at room temperature for two years.

## 2.2. SEED FORMATION

A perfect flower is composed of a calyx, consisting of a whorl of sepals, a whorl of petals, a circularly arranged set of stamens and a central pistil. Each stamen consists of a filament and a terminal anther. A pistil consists of a terminal stigma, a middle style and an expanded basal ovary which contains one or more ovules. Each ovule consists of one or two integuments and an internal megasporangium (nucellus). A micropyle leads through the integuments to the megasporangium (Wilson et al., 1971).

One of the nucellar cells develops into a megaspore mother cell which undergoes meiosis to form a tetrad. In most cases, three of the four haploid cells degenerate. In the remaining one, which enlarges to form the functional megaspore, the nucleus undergoes three divisions. Four of the eight nuclei occupy one end of the spore cell and the remainder are at the other end. Three of each group of four nuclei become partitioned off as cells, leaving two polar nuclei at the centre. Thus a seven-celled megagametophyte is formed. One of the three gametophytic cells at the micropylar end becomes an egg and the other two are synergid cells; the three cells on the other end are antipodal cells and the remaining one is the central cell.

In each anther, there are numerous microspore mother cells. These cells are connected to tapetal cells by plasmodesmata which disappear during the onset of meiosis. However, the interconnections among the microspore mother cells are improved by forming cytoplasmic channels. At the same time, deposition of callose within the cell wall of each spore mother cell begins. Each microspore mother cell is isolated when the cytoplasmic connections are plugged by callose at the conclusion of prophase (Heslop-Harrison, 1966; Echlin, 1968).

After meiosis is completed, each microspore mother cell produces a tetrad of microspores. Each microspore is similarly sheathed in the callose. Each of them begins to deposit its own primary exine, with a pattern of species-specific surface sculptures. As development proceeds, the callose sheath becomes thinner and finally disappears, thus liberating the pollen grain in the thecal cavity. The primary exine is then impregnated with sporopollenin, which is secreted from the tapetal cells, to protect the pollen grain. A layer of secondary exine is formed beneath the primary exine. These two layers are separate at first, but fuse together later. The cellulosic intine is formed inside the secondary exine to complete wall formation in the pollen grain. At the same time, the nucleus undergoes a simple mitotic division to form the vegetative and the generative nucleus. A cell wall is formed to enclose the generative nucleus. A pollen grain at this stage is considered to be mature (Echlin and Godwin, 1968, 1969). The generative nucleus may undergo a mitotic division to form two sperm nuclei before germination, depending upon the plant species (Maheshwari, 1950).

Most angiosperms require cross-pollination for proper seed set to occur, although some species are regularly self-pollinated (eg. bean). Numerous pollen grains may land on the stigma of a pistil. The stigma is sticky and traps pollen grains, which eventually germinate. Each pollen grain usually produces one germ tube. The germ tube aided by extracellular enzymes which digest tissues, migrates down the style, into the micropyle and it eventually enters the megagametophyte. If the generative cell has not already divided prior to germination, it divides in the germ tube to form two sperm nuclei, each of which is surrounded by a cytoplasmic sheath. Both of them are discharged into one of the synergid cells ; one migrates into the egg and the other into the central cell. The egg nucleus fuses with the incoming sperm nucleus to form the diploid zygote, while the other sperm nucleus fuses with the two polar nuclei in the central cell to form the triploid primary endosperm. The latter cell develops into endosperm to provide nutrients for the growth of the egg cell which becomes an embryo. The antipodal cells and synergid cells degenerate soon after completion of fertilization.

The endosperm may be consumed by the embryo in plants such as the legumes; but it may become the prominent part of the seeds in some plant species, such as the cereals. The endosperm is utilized during germination. The nucellus is generally reabsorbed during the development of the embryo. However, the nucellus, such as that of beet seeds, may persist until being consumed during germination. A mature seed is covered by the seed coat which develops from the integuments of the ovule. The

seed coat can be thin, thick, tough, stony or fibrous depending upon plant species (Wilson et al., 1971). The vascular system of the plant extends through the funiculus to the seed coat but it does not penetrate into the embryo (Maheshwari, 1950).

## 2.3. MECHANISM OF SEED TRANSMISSION

A seed can be formed only if the fusion of both gametes has occurred in the ovule. Theoretically, an infected seed can arise from one of the following : 1) fertilization of a healthy megagametophyte by infected pollen; 2) fertilization of an infected megagametophyte by healthy pollen; 3) direct invasion of the developing embryo by virus originating in the mother plant; and 4) the invasion of the seed coat by virus from the mother plant.

### 2.3.1. TRANSMISSION THROUGH MICRO- AND MEGA-GAMETOPHYTES

There are several viruses that are known to be transmitted by pollen. Reddick and Stewart (1918) suggested that pollen could transmit BCMV to the ovules of healthy flowers. Nelson and Down (1933) found that pollen and ovules were equally effective in transmitting the virus by cross-pollination, but that self-pollination of infected flowers produced a higher incidence of infected seeds than did crossing between healthy and infected parents. However, Medina and Grogan (1961) and Schippers (1963) reported a higher rate of seed transmission if one of the parents was healthy than when both were infected. Medina and Grogan (1961) also reported that the rate of seed transmission through either parental source varied with the variety of bean.

Various rates of transmission through pollen were reported in BSMV-infected barley. Gold et al. (1954) reported 10 percent pollen transmission of BSMV, but Inouye (1962) reported up to 35 percent, depending upon variety and other factors.

Raspberry ringspot virus, arabis mosaic virus and tomato black ring virus were reported to be transmitted by pollen (Lister and Murant, 1967). Other viruses reported to be pollen-transmitted were elm mosaic (Callahan, 1957), lettuce mosaic virus (Ryder, 1964), prune dwarf virus (Gilmer and Way, 1960), cherry necrotic ringspot virus (Way and Gilmer, 1958) and pea-seed-borne mosaic virus (Stevenson and Hagedorn, 1973).

In many cases, the viability of pollen grains from infected plants is inferior to that from healthy plants. Blakeslee (1921) reported that Datura quercina virus caused almost complete pollen sterility in Datura stramonium plants, but seed transmission was obtained by pollinating infected plants with healthy pollen. This implies that seed transmission occurs through the infected megagametophyte. Valleau (1932, 1939, 1941) reported semi-sterility of tobacco pollen grains infected with TRSV. A strain of TRSV caused complete sterility of pollen so that no seed set occurred unless the infected tobacco plants were crossed with healthy pollen. Semi-sterility of pollen caused by virus infection was also reported in lettuce infected with lettuce mosaic (Ryder, 1964) and in BSMV-infected barley (Yamamoto, 1951, Inouye, 1962). Pollen of low viability has been reported in plants infected with BCMV (Medina and Grogan, 1961) and with some of the nematode-transmitted polyhedral viruses (Lister and Murant, 1967).

Pollen grains infected by a virus do not necessarily transmit the disease. Bennett and Costa (1961) could detect sowbane mosaic virus in pollen of Atriplex coulteri by infectivity assay, but found no seed transmission. However, it was found to be seed-transmitted in other species of Atriplex. Cadman (1965) found apple chlorotic leafspot virus in pollen of both Chenopodium quinoa and C. amaranticolor, but there was no evidence of seed transmission in either species.

In addition to pollen transfer of virus to the ovules of healthy flowers, infected pollen has been implicated in the transmission of virus to healthy plants. Gilmer and Way (1960) reported the spread of prune dwarf virus from tree to tree through pollen. Similarly, cherry necrotic ringspot virus (Das and Milbrath, 1961; George and Davidson, 1963), and sour cherry yellows virus (Gilmer and Way, 1963; George and Davidson, 1963) could be spread by pollen. Cameron et al. (1973) concluded that prunus necrotic ringspot virus-infected pollen was responsible for the spread of the disease in prune and sour cherry orchard, because trees deflowered for seven years did not become infected. The spread of apple chlorotic leafspot virus and black raspberry latent virus in raspberry were also furnished by the infected pollen (Cadman, 1965; Converse and Lister, 1969).

Virus infection of the megagametophyte has been indirectly proven by crossing infected plants with pollen from healthy plants. The evidence for this kind of transmission has been demonstrated in BSMV in barley (Inouye, 1962, 1966), BCMV in bean (Nelson and Down, 1933; Medina and Grogan, 1961; Schippers, 1963), lettuce mosaic virus in lettuce (Ryder, 1964), pea seed-borne mosaic virus (Stevenson and Hagedorn, 1973), elm mosaic in

elm (Callahan, 1957), Lychnis ringspot virus in Lychnis divaricata and Silene noctoflora (Bennett, 1959), raspberry ringspot in strawberry and raspberry (Lister and Murant, 1967) and tomato black ring in raspberry (Lister and Murant, 1967).

In most cases,more seed transmission occurs through infection of the megagametophyte than of the microgametophyte. Ryder (1964), for example, reported 5 percent seed transmission through infection of the megagametophyte but infection of the microgametophyte caused only 0.48 percent seed infection. Stevenson and Hagedorn (1973) observed similar results in pea infected with pea seed-borne mosaic virus. Bennett (1969) suggested that the infection of the ovule or of the embryo sac by one means or another was essential for seed transmission. Although it might not be true in certain cases, such infection apparently greatly increased seed transmission.

The evidence for virus particles present in the gametophytes in situ is lacking in most cases. Although Gold et al. (1954) detected the tubular particles of BSMV in a homogenate of pollen from BSMV-infected barley., the presence of this virus in the pollen grain was not reported until later when it was found to occur as scattered particles and crystalline aggregates in the pollen cytoplasm (Gardner, 1967). Carroll (1972) failed to locate the virus in unfertilized ovules, in spite of circumstantial evidence for its presence.

Schipper (1963) reported that BCMV,another tubular virus , was present in dip-preparations of most ovules which had been collected from plants prior to flowering. He indicated, however, that the virus could not have entered all of the eggs because the percentage of seed transmission was low.

There is no evidence in the literature for the detection of any icosahedral plant virus by electron microscopy of thin sections of microgametophytes, megagametophytes or embryos.

## 2.3.2. DIRECT INVASION OF YOUNG DEVELOPING EMBRYOS

The body of evidence so far has implicated embryo transmission as the main route of seed transmission. The embryo is most likely infected at the time of fertilization by virtue of the infected gametes which participate in the fertilization process. However, there are reports which suggest that embryos can be invaded by viruses as a consequence of inoculating healthy plants during and after the flowering period.

Eslick and Afanasiev (1955) and Crowley (1959) found that BSMV could be seed transmitted in barley seeds from plants inoculated after flowering. However, Hagborg (1954) and Inouye (1962) could not confirm this report from their experiments in wheat and barley respectively. Timian (1967) repeated the experiments in barley and found no evidence of transmission by seeds from the main tillers of plants inoculated seven days prior to anthesis and thereafter, but he found BSMV-infected seeds from late tillers. The result perhaps explains the controversy among the previous reports.

Crowley (1959) reported that SBMV and TRSV were able to invade developing embryos of bean and soybean respectively. McDonald and Hamilton (1972) concluded that the infectivity of SBMV detected in the bean embryo was a result of contamination with the virus from the seed

coat and they suggested the virus could not have entered the embryo. Gay (1973), ignoring the work of McDonald and Hamilton (1972), reported that SBMV in cowpea could be recovered from all parts of the seed, even when the inoculation was performed when the seeds were approaching maturity. This report, therefore, should not be considered as evidence for the direct invasion of developing embryos by SBMV because no decontaminating measures were undertaken.

Nematode-transmitted polyhedral viruses are reported to be seed transmitted (Lister and Murant, 1967). Although several viruses can be transmitted in more than 50 percent of the seeds produced, only the nematode-transmitted polyhedral viruses in certain hosts have attained 100 percent seed transmission. Seed transmission of other vector-transmitted viruses is not as effective. Lister and Murant (1967) suggested that the mechanism of seed transmission of nematode-transmitted viruses could be associated with the ability of the viruses to invade meristem tissue. These viruses characteristically induce acute symptoms follow by chronic symptoms and it has been suggested that this pattern of symptoms is due to the invasion of the meristems by these viruses (Valleau, 1941). TRSV, a member of this group of viruses, has been found in the meristematic cells of bean (Crowley et al., 1969; Atchison and Francki, 1972), and tobacco (Roberts et. al., 1970) by electron microscopy.

Whether there is a correlation relationship between seed transmission and the ability of the virus to invade meristematic tissue remains to be investigated. Potato virus X, for example, is not known to be transmitted in potato seeds, but it has been found in the meristem tips of potato shoots (Appiano and Pennazia, 1972).

2.3.3. TRANSMISSION BY VIRUS PRESENT IN AND ON SEED COATS

Although TMV can be found on the seed surface, in the seed coat and in the endosperm of tomato seed, the main source of inoculum for seedling infection is the virus carried on the seed surface (Taylor et al., 1961). Seedlings grown from infected seeds can be contaminated with TMV, which is common on the root, but the virus occurs on the cotyledons only if the seed coats are elevated during germination. Infection occurs during transplanting, when wounds are created. There is no evidence of seed transmission caused by the virus present in the endosperm or in the seed coat of tomato seed (Taylor et al., 1961).

As mentioned earlier, seed transmission of SBMV occurs in bean in spite of the fact that it does not enter the embryo. McDonald and Hamilton (1972) suggested that seedlings become infected during germination in the manner suggested for seed transmission of TMV in tomato.

Infection of the seed coat does not necessarily result in seed transmission. According to Ford (1966), pea streak virus was associated with the seed coats of immature seeds, but it was not seed transmitted. Similarly, cowpea chlorotic mottle virus was found in the immature seed coats of cowpea, but the virus was inactivated during maturation (Gay, 1969).

2.3.4. APPARENT FAILURE OF SEED TRANSMISSION

The fact that viruses may occur in high concentration in leaves and yet fail to be transmitted in the seed of infected plants has been noted many times. Several theories have been suggested to explain this observation.

For convenience, they are grouped as follows (Matthews, 1970) :

a) Failure to infect the embryo

Caldwell (1934) suggested that the rapid growth rate of the embryo and endosperm could result in the breakdown of plasmodesmata between embryonic tissue and nucellar cells. This view has been supported by electron microscopic evidence in several reports (Nieuwdorp, 1963; Schulz and Jensen, 1968; Carroll, 1972). Bennett (1940) suggested that the lack of vascular connections between the embryo and the mother plant explained the lack of infection of embryos by those viruses which were restricted to vascular tissue. Nevertheless, it could not explain the lack of embryo infection by viruses that invade parenchymatous tissue.

Tomato produces only a few seeds if it is infected with tomato aspermy virus. Caldwell (1952) observed that meiosis of both microspore and megaspore mother cells was prevented by the virus, and suggested that the healthy seeds which were produced arose from spore mother cells that had escaped infection. He stated later (Caldwell, 1962) that meiotic irregularities caused by virus infection might be quite widespread, based on the fact that infected plants generally produce fewer seeds.

Medina and Grogan (1961) reported that a seed-transmitted bean mosaic, BV-1, could be detected in ovules and pollen of infected plants but that BV-2, a non-seed-transmitted strain, could not be found in these tissues. The latter virus was detected in pistils and petals but not in anthers. The non-seed-transmitted strain of BSMV was likewise not found in the ovules and pollen of infected barley (Carroll, 1972). These results

seem to support the theory proposed by Bennett (1940) that some viruses can not invade and survive in haploid tissue.

b) Embryo inactivation

The evidence that viruses are inactivated in the developing embryos is lacking, since the best evidence, that of inactivation of SBMV in the embryo during maturation (Cheo, 1955), has been disproved by McDonald and Hamilton (1972).

Duggar (1930) suggested that inhibitory substances in seeds could be responsible for preventing seed transmission of TMV, based on the inhibitory effect of seed extracts. Kausche (1940) reported that TMV may be present in unripe seeds, but that it was inactivated by a substance evolved during ripening and germination of the seeds. Crowley (1957) found no evidence for TMV being inactivated by developing embryos, using a variety of techniques.

Inouye (1962) reported that the level of seed transmission of BSMV was determined by the amount of infection during the early stage of growth, and that there was no evidence of virus inactivation in the later stages of seed development, storage and germination. However, he subsequently reported (Inouye, 1966) that some infected seeds could develop into healthy plants suggesting that inactivation of the virus occurred in the germinating seeds. Since the observation was based on electron microscopy, it is questionable whether all the observed virus particles were infectious.

Medina and Grogan (1961) demonstrated that pollen from infected susceptible plants could transmit BCMV to the seeds of other susceptible

plants, but seed transmission did not occur in resistant plants when they were crossed with such pollen. Presumably, the virus had entered the embryo sacs but it was inactivated later. However, this account can not be taken as evidence for the inactivation of the virus in the embryo, because the resistant plants would not support virus replication.

Caldwell (1962) suggested that lack of high energy phosphate in embryos could account for the lack of seed transmission. No evidence has been presented to support this view.

## 2.4. THE SOYBEAN

The soybean (Glycine max L. Merr) belongs to the family Leguminoseae, subfamily Papilionlideae. It was originally cultivated in China and later introduced to Korea, Japan and United States.

Soybean flowers are 6-7 mm long, consisting of a tubular calyx terminating in five lobes, (Guard, 1931). The corolla consists of five unequal petals, the posterior petal, two wings and two keel petals . There are 10 stamens, but nine of these are elevated by expansion of their common base during flower development, thus separating a posterior stamen. There are usually 5-16 flowers on each axillary raceme (Guard, 1931).

Soybeans are short day plants (Johnson and Bernard 1963). The critical length of the dark period varies from one variety to the other (Borthwick and Parker, 1939). Usually more flowers are produced than can be supported to seed maturation. Johnson and Bernard (1963) reported that

soybeans could shed as high as 75 percent of its flowers. The factors that caused flower and pod shedding were environmental conditions rather than lack of viable pollen or the failure of fertilization (Kato and Sakaguchi, 1954; Van Schaik and Probst, 1958). However, unfertilized basal ovules were occasionally observed, probably due to their relatively long distance from the stigma and the consequent difficulty in being fertilized (Kato and Sakaguchi, 1954).

Soybeans are strictly self-pollinated plants. Outcrossing in nature is usually less than 1 percent, because anthesis occurs before the petals have completely opened. Kato et al. (1954) reported that fertilization occurred on the day of flowering. Johnson and Bernard (1963) described the procedures for artificial crosses. The best result was obtained the day before flowering. Although stigmas remain receptive for two days after flowering, fertilization takes place within ten hours after pollination and therefore pollen from other flowers would not be able to fertilize the eggs (Johnson and Bernard, 1963). An interesting exception to this is the report that as high as 40 percent outcrossing occurs in TRSV-infected plants by pollen from healthy neighbours (Brim et al., 1964). Outcrossing was determined by the colour of the pubescence or of the cotyledon as genetic markers. Neglegible outcrossing could be detected on healthy plants because TRSV reduced pollen viability (Brim et al., 1964; Athow, personal communication).

Guard (1931) reported that two or three ovules were normally produced in each ovary. Kato et al., (1954) observed four in some ovaries, and late flowers generally produced fewer ovules probably due to insufficient

water and improper nutrition. After fertilization, the zygote divides rapidly for about 16 days to form the proembryo. It grows and forms the cotyledon, plumule, epicotyl and hypocotyl. The endosperm and nucellus, which provide nutrients during this development, are obliterated in the final stages of embryo development. The inner integument is crushed and reabsorbed when the outer integument differentiates into a seed coat (Kamata, 1952).

## 2.5. TOBACCO RINGSPOT VIRUS

TRSV was first described by Fromme et al. (1927). Wingard and Fromme (1928) reported the virus has a wide host range. It was not purified until 1956, when Steere published the procedures for its purification. Later, Corbett and Robert (1962) and Stace-Smith et al. (1965) published their methods for TRSV purification.

The virus particles are spherical in shape, 29 nm diameter, and they consist of 40 percent RNA (Stace-Smith et al., 1965). Chambers et al. (1965) reported that TRSV consisted of 42 morphological units based on the electron mocroscopy of negativily stained preparations. Mayo et al. (1971) reported that TRSV and several other nematode-transmitted viruses shared the common property of possessing 60 protein subunits based on the estimated total molecular weight of coat protein and that of individual protein subunits from polyacrylamide gel electrophoresis. Therefore the number of morphological subunits should be 12.

There are three classes of sedimenting particle, top, middle and bottom with sedimentation coefficients of 51S, 91S, and 126S, respectively

(Stace-Smith, 1970). Particles in top component are empty protein shells; particles of middle component contain one short RNA strand ($1.2 \times 10^6$ daltons) per particle; and each particle of bottom component has two short or one long RNA ($2.2 \times 10^6$ daltons) (Diener and Schneider, 1966). The three TRSV particles are identical morphologically and serologically, differing only in density. The short RNA is not infectious but it enhances the infectivity of the long RNA (Harrison et al., 1972). The rate of replication of the RNA strands appears different. Schneider and Diener (1968) found that the ratio of short to long RNA strands increased with age of infected leaves.

A satellite virus (S-TRSV), coated with TRSV protein, was first reported by Schneider (1969). Schneider (1971) and Schneider et al. (1972) reported that S-TRSV consisted of a population of nucleoproteins with varying numbers of single strands of S-TRSV RNA. Each particle in the S-TRSV population consists of protein encapsulating 12-25 strands of S-TRSV RNA. A regular increase in particle density corresponding to an increase in the number of RNA strands in each particle was observed (Schneider et al., 1972).

The main diseases cause by TRSV are ringspots in tobacco, cucumber, hydrangea, Easter lily, blue berry, and bud blight of soybean. The most destructive disease is soybean bud blight (Stace-Smith, 1970).

Soybean bud blight was first reported in Indiana in 1941 (Samson, 1942). Melhus (1942) found it in Iowa, and Johnson (1943) observed it in Ohio. Allington (1946) first identified the causal agent as TRSV, and described some of the symptoms. Additional symptoms were described by Hildebrand and Koch (1947) and Kahn and Latterel (1955).

Essentially, "bud blight" describes the disease. Many of the flower buds or leaf buds are killed. Young leaves have a bronzed appearance, and they are somewhat darker and small when mature. Sometimes vein clearing, necrotic stippling, and rolling can be noted. Terminal buds become curled, necrotic and brittle. Infected plants are stunted and there is proliferation of the trifoliate leaves. Discoloration of the pith of the stems and branches can be seen in the vicinity of the nodes. Swollen nodes and proliferation of flower buds occur occasionally. However, most of the flower buds are killed in the early stages of their development. Young pods may wither and fall off. Most pods which remain on the plants are single-seeded, whereas the majority of pods on healthy plants are multiple-seeded. Yield losses are not only due to reduced seed and pod numbers, but infected plants produced poor quality seeds and they usually mature late, persisting in the field until killed by frost. Yield loss can be as high as 100 percent (Johnson et al., 1954). According to Athow and Laviolette (1961), combine harvesting is impossible if 60 percent of the plants are infected, because they are still green at the time healthy plants have matured. They also reported that 30 percent infection did not significantly reduce yields since the healthy plants can compensate for the reduced seed production of infected plants because of a competitive advantage. Soybeans infected with TRSV usually produce a large number of seeds infected with purple stain disease caused by Cercospora kikuchii (Crane and Crittenden, 1962; Crittenden et al., 1966) suggesting a predisposition of TRSV-infected plants to other diseases.

TRSV can be seed-transmitted in petunia (Henderson, 1931), lettuce (Grogan and Schnathorst, 1955), Taraxicum officinale (Tuite, 1960), tobacco (Valleau, 1932) and soybean (Desjardins et al., 1954; Kahn, 1956; Athow and Bancroft, 1959). The highest percentage of seed transmission occurs in soybean. Desjardins et al. (1954) reported 54-78 percent seed transmission in the variety Lincoln, and Kahn (1956) reported 82 percent; Athow and Bancroft (1959) reported as high as 100 percent seed transmission in the variety Harosoy. The rate of seed transmission in soybean is not related to the position of the pods on the stems or the position of the seeds in the pods (Athow and Laviolette, 1962), but it is closely related to the earliness of infection (Crowley, 1959; Athow and Bancroft, 1959; Owusu et al., 1968; Schmitthenner and Gordon, 1970).

Several strains of TRSV from different plant species have been reported (Stace-Smith, 1970). Walters (1962) recognised several strains isolated from soybean based on the symptoms produced in a host range assay.

While TRSV can be seed-transmitted, it can also be transmitted by a range of vectors which causes further spread of the disease, especially from weed hosts to plants in the vicinity (Tuite, 1960; Crittenden et al., 1966). Crittenden et al. (1966) and Hill et al. (1973) observed different rates of TRSV infection in soybean plots, depending on the distance from infected weeds. The plots that were closest to the weeds suffered badly from the disease. This indicates the efficiency of aerial vectors. The known aerial vectors of TRSV are grasshoppers (Walters, 1952; Dunleavy, 1957), nymphs but not adults of thrips (Messieha, 1969), mites of the genus Tetranychus (Thomas, 1969), tobacco flea beetle (Schuster, 1963), and the

aphids Myzus persicae and Aphis gossypii (Rani et al., 1969). Hendrix (1961) also indicated that TRSV could be transmitted by nematodes. Fulton (1962) confirmed this and identified the nematode as Xiphinema americanum. Bergeson et al. (1964) reported that TRSV was transmitted readily from soybean or cucumber to cucumber, but rarely from cucumber to soybean. Movement of virus in soybean was rapid from the leaves to the roots but very slow from the roots to the leaves. Viruliferous nematodes that were stored at 10°C for 49 weeks were still able to transmit TRSV, suggesting that such nematodes could overwinter between crops. X. americanum can transmit more than one strain of TRSV at the same time. Thus Sauer (1966) found three strains of the virus in the same nematode and suggested that there was no evidence for cross protection in the nematode. Rush (1970) found that the nematode could transmit four strains of TRSV except the Eucharis mottle strain from Peru and suggested that there was some specific relationship between the virus and its vector.

The virus was found in the embryos but not in the seed coats of mature seeds by infectivity tests (Athow and Bancroft, 1959). The mechanism of seed transmission has not been investigated. The infectivity of TRSV in the seeds did not change for two years if seeds were stored at room temperature. It dropped slowly from then on and then dropped sharply after 3.5 years. However the virus did not lose any infectivity for five years if infected seeds were stored at 2°C (Laviolette and Athow, 1971).

# 3. STUDIES ON THE CENTRIFUGAL COMPONENTS OF TOBACCO RINGSPOT VIRUS IN SOYBEAN SEEDS

## 3.1. INTRODUCTION

The replication of multiple component plant viruses appears to involve discrete replicative RNA species (Van Griensven and Van Kammen 1969) and this would suggest that the proportions of the various components could change during the course of an infection. Hull (1972) reported that the ratio of heavy to light components of broad bean mottle virus, and the ratio of RNA species 1 and 4 to RNA species 2 extracted from the virus increased with age of infection in broad bean. Schneider and Diener (1966) reported that the ratio of middle component plus bottom component of tobacco ringspot virus in bean was higher in samples collected two and one half to three days than in those collected six to seven days after inoculation. Matthews (1958) reported a constant ratio of the protein top component to the nucleoprotein components of turnip yellow mosaic virus in infected chinese cabbage. However, Francki and Matthews (1962) subsequently reported that the ratio of the two components did change with the age of the infection. The ratio of the protein top component to the nucleoprotein components increased rapidly for two days following their first detection, but it decreased slowly thereafter. The empty protein shell was not detected until two to three days after detection of viral nucleoprotein. Paul (1963) reported a constant ratio of the two components of broad bean mosaic virus with the age of infected leaves. Bancroft (1962) found a stable ratio of approximately 1:7:7 for the top, middle and bottom

components, respectively, of bean pod mottle virus in bean.

There are apparently no reports on component ratios of seed-transmitted multiple component viruses in extracts from seeds of their host plants. The experiments in this section were designed to determine the ratio of bottom component to middle component of TRSV by analytical density gradient centrifugation of extracts from infected seedlings and seeds at several stages of germination.

## 3.2. MATERIALS AND METHODS

Both healthy and TRSV-infected soybean seeds were obtained from Mr. F.A. Laviolette, Department of Plant Pathology, Purdue University, Lafayette, Indiana.

The presence of seed-borne TRSV was confirmed by infectivity assay of extracts from a seedling showing symptoms indicative of TRSV. Soybean leaves showing virus symptoms were ground with an equal volume of 0.02 M sodium phosphate buffer pH 7.2 (referred to as buffer henceforth) and the homogenate was inoculated to a range of host plants including Solanum melongena, Petunia hybrida, Citrullus vulgaris var. Charleston Gray, Antirrhinum manus, Phaseolus limensis and Chenopodium amaranticolor. The symptoms produced by these plants were typical of those described for TRSV by McLean (1962). The pathogen was further confirmed as TRSV by a positive reaction between the crude sap and TRSV-antiserum in an agar diffusion test. The TRSV-antiserum was provided by Dr. R. Stace-Smith, Agriculture Canada Research Station, Vancouver, B.C. Canada.

The percentage of soybean seeds infected with TRSV was determined by infectivity assay of the extracts of embryos and seed coats of 100 seeds. The seeds were soaked in water overnight in order to loosen the seed coat so that it could be removed. Extracts of the embryo and seed coat from each seed were inoculated to primary leaves of cowpea (Vigna unguiculata (L) Walp. cv. Early Ramshorn). The cowpea plants were scored for the presence of TRSV-induced local lesions 3 days later. The level of embryo infection was 75 percent, but no infectivity was detected in the seed coats. Therefore, seed coats from mature seeds were not sampled in the experiments to be described in this section.

### 3.2.1. PREPARATION OF PURIFIED TRSV STOCK.

Purified stock preparations were made from bean (Phaseolus vulgaris, cv. Kentucky Wonder Wax), inoculated at the two leaf stage, 10-14 days previously with inoculum from infected soybean. The purification schedule, essentially that of Steere (1955), is as follows :

1. Add equal volume of buffer to each unit weight of leaf tissue.
2. Homogenize in Waring Blendor.
3. Squeeze homogenate through Miracloth ; discard pulp.
4. Add equal volume of a 1:1 mixture of chloroform and butanol ; stir with a magnetic stirrer for 30 mins.
5. Centrifuge at 10,000 g in a SS-34 rotor in the Sorvall RC-2-B centrifuge for 20 mins.

6. Collect aqueous phase and incubate at room temperature overnight in order to complete the denaturation of host proteins.

7. Centrifuge at 10,000 g ; collect supernatant fluid.

8. Centrifuge supernatant fluid at 103,000 g in the No.65 rotor in the Beckman Model L ultracentrifuge for 2 hrs.

9. Resuspend virus with suitable amount of buffer.

10. Repeat steps 7-9 until virus pellets are clear and resuspend the virus in a small volume of buffer.

### 3.2.2. ESTIMATION OF VIRUS CONCENTRATION IN STOCK PREPARATION

The concentration of the virus in the stock preparation was determined spectrophotometrically. Dilutions of the stock were made with buffer and the absorbance was recorded in the range of 220-300 nm in a Unicam SP-800 Spectrophotometer (quartz cuvette, 1 cm light path). The concentration was determined by using the following formula :

$$\text{Conc. (mg/ml)} = \frac{\text{(absorbance at 260 nm)(dilution)}}{\text{Extinction coefficient}}$$

The extinction coefficient ( $E^{0.1\%}_{260nm} = 10$ ) is the absorbance of TRSV at a concentration of 0.1% using a wavelength of 260 nm and a light path of 1 cm (Stace-Smith, 1970) ; it is a standard reference value for TRSV.

### 3.2.3. PREPARATION OF EXTRACTS OF SOYBEAN SEEDLINGS INOCULATED WITH TRSV

Healthy seedlings were grown in peat pots (2 sq. in) which were kept in plastic flats in a growth chamber, maintained at 20°C and 75% relative humidity. The photoperiod was 12 hours with a light intensity of 1800 f.c. at the soil level.

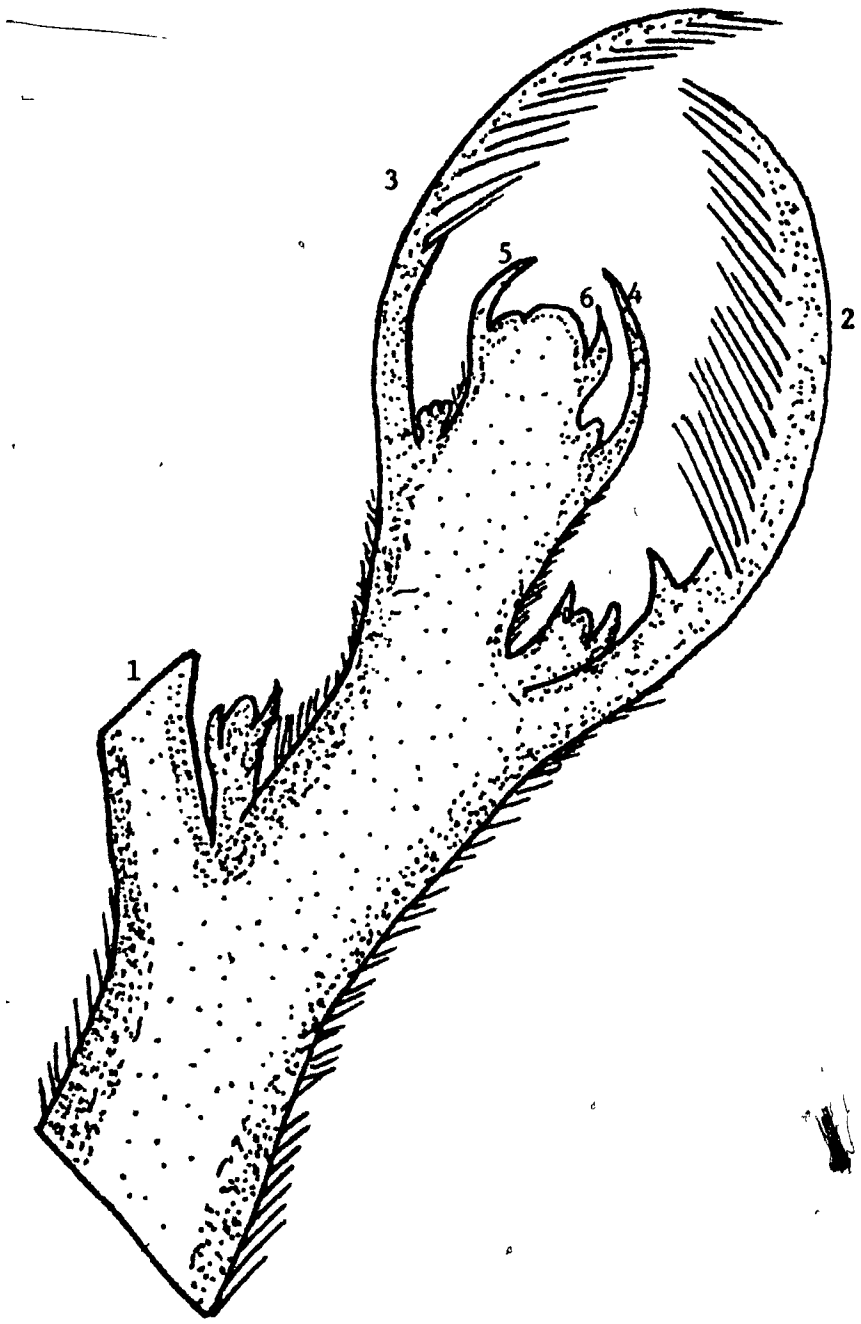
Primary leaves were inoculated five days after seedling emergence with a mixture of purified virus suspension (0.01 mg/ml) and 500 mesh carborundum. The inoculum was rubbed gently on the primary leaves with Q-tips. A sketch of the apical shoot of seedlings at that stage is shown in Fig. 1.

Inoculated seedlings were sampled four days after inoculation and then at three day intervals until 19 days post-inoculation.

Ten seedlings were harvested at a time and divided into groups consisting of cotyledons, simple leaves and compound leaves (first trifoliates). They were separately processed by the following procedure :-

1. Add 1 ml of buffer to each gram of fresh tissue ; triturate with a mortar and pestle.
2. Transfer homogenate to a tube ; mix with an equal volume of 1:1 mixture of chloroform and butanol and emulsify the mixture with a Vortex mixer.
3. Centrifuge at 10,000 g for 20 mins.
4. Tansfer supernatant fluid to a clean tube and incubate at room temperature overnight.

Fig. 1. Sketch of an apical shoot at the time when healthy seedlings were inoculated with TRSV. The numbers represent the sequence of trifoliate leaf buds and primordia.


3
5
6
4
2
1

5. Centrifuge at 10,000 g; discard pellet.

6. Mix supernatant fluid with polyethylene glycol (M.W. 6000) and NaCl to final concentrations of 8% and 0.3 M respectively (Hebert, 1963).

7. Centrifuge at 10,000 g; discard supernatant fluid.

8. Resuspend the pelleted virus in a suitable volume of buffer, 0.15 ml and 0.05 ml for each gram of leaf tissue and cotyledon respectively.

9. Clarify suspension by low speed centrifugation; retain supernatant fluid for analytical sucrose gradient centrifugation.

### 3.2.4. PREPARATION OF EXTRACTS FROM MATURE SEEDS

TRSV-infected seeds were soaked overnight and processed in groups, each consisting of three seeds, by triturating them in 10 ml of buffer and proceeding as described in section 3.2.3. The resuspending buffer was 0.15 ml for each gram of soaked seeds.

### 3.2.5. PREPARATION OF EXTRACTS FROM COTYLEDONS OF GERMINATING SEEDS

TRSV-infected seeds were sown in peat pots in a growth chamber under the same conditions as described in section 3.2.3. Seeds were unearthed 1, 3, and 5 days after sowing. Cotyledons from three seeds were washed with tap water and treated exactly as described for mature seeds in section 3.2.4. Emergence of seedlings occurred 6-7 days after sowing.

### 3.2.6. PREPARATION OF EXTRACTS OF SEEDLINGS FROM INFECTED SEEDS

Seedlings were selected for analysis after a preliminary infectivity assay had established which seedlings were infected. For the assay, one cotyledon from each emerging seedling was homogenized with a small volume of buffer and the homogenate was rubbed onto primary leaves of Early Ramshorn cowpea. Assay plants were incubated in a greenhouse and scored for TRSV lesions 3 days later. Those seedlings that yielded infectious extracts were noted and used for analysis. Ten seedlings were harvested 3 days after emergence at four day intervals until 19 days post-emergence. Preliminary experiments indicated that the concentration of TRSV in leaves was too low to be detected by analytical density gradient centrifugation of extracts from ten plants. The number of plants was increased to 30, but because of limitations in the space available for growing the plants, analysis of extracts was done at 14 and 24 days post-emergence.

Harvested seedlings were processed by the procedure as described in section 3.2.3. The resuspending buffer was 0.05 ml per gram of tissue, including leaves and cotyledons.

### 3.2.7. ANALYTICAL SUCROSE GRADIENT CENTRIFUGATION

Samples from each of the above extracts were subjected to analytical density gradient centrifugation in order to determine the relative amounts of the sedimenting components of TRSV. Sucrose gradient columns were made by sequentially layering 3 mls of 40, 30, 20 and 10% (W/V) sucrose solutions in 3-3/4" × 9/10" cellulose nitrate tubes. The solvent for the

sucrose was the same as the buffer solution used for virus extraction. The columns were incubated at $4^{o}C$ overnight to allow formation of a linear 10-40% sucrose concentration gradient (Brakke, 1960). The sample to be analyzed (0.2 ml) was layered on a sucrose gradient column and centrifuged in the SW 40 rotor for 2 hours at 40,000 rpm in the Beckman L2 65B ultracentrifuge. After centrifugation, the columns were fractionated with a ISCO Model D Density Gradient Fractionator. This apparatus consisted of a syringe-pump which pumped 50% sucrose into the bottom of each gradient column. The contents of each tube were forced through a flow cell and past a UV light source coupled to a UV absorbance monitor. As the contents flowed past the UV light source, the absorbance was automatically recorded on an external strip chart recorder. The absorbance monitor was operated at its most sensitive range (full scale was equivalent to an optical density of 0.25 at 254 nm). Absorbance profiles on the external chart recorder were traced onto graph paper (20 divisions per inch). Areas of the peaks corresponding to UV absorbing fractions of the columns were calculated by counting the squares. Ratios of the sedimenting components of TRSV were determined from the sums of the squares

## 3.3. RESULTS

Preliminary experiments indicated that duplicate samples analysed by sucrose gradient centrifugation gave essentially identical absorbance profiles. The results reported here are based on a single centrifuged column per sample.

Table 1. Ratio of bottom component (B) to middle component (M) of TRSV in extracts of cotyledons[a/] from non-germinated and germinated seeds and from emerged seedlings

| Sample | Relative amount | | B/M Ratio | Average ratio |
|---|---|---|---|---|
| | B | M | | |
| Non germinated seeds | 182[b/] | 21 | 8.6 | |
| | 183 | 15 | 12.2 | |
| | 120 | 10 | 12.0 | 10.1 |
| | 47 | 4 | 11.7 | |
| | 178 | 30 | 5.9 | |
| Germinated seeds 1[c/] | 142 | 8 | 5.1 | |
| | 278 | 49 | 5.6 | 6.8 |
| | 182 | 21 | 8.6 | |
| | 93 | 12 | 7.7 | |
| 3 | 69 | 6 | 11.5 | |
| | 260 | 23 | 11.3 | 10.4 |
| | 131 | 16 | 8.2 | |
| | 211 | 20 | 10.5 | |
| 5 | 81 | 10 | 8.1 | |
| | 178 | 30 | 5.9 | 8.1 |
| | 193 | 36 | 5.3 | |
| | 99 | 7 | 13.2 | |
| Emerged seedlings 3[d/] | 76 | 5 | 15.2 | |
| | 28 | 6 | 4.6 | 9.9 |
| 7 | 177 | 8 | 19.6 | |
| | 106 | 52 | 2.0 | 10.8 |
| 11 | 22 | 5 | 4.4 | |
| | 31 | 2 | 15.5 | 10.0 |
| 15 | 31 | 4 | 7.7 | |
| | 114 | 8 | 14.2 | 11.0 |
| 19 | 140 | 32 | 4.3 | |
| | 110 | 10 | 11.0 | 7.8 |

a/ Cotyledons from three seeds or from ten plants were analyzed in each experiment reported.

b/ The numbers represent the square units on the graph paper.

c/ The number of days after sowing.

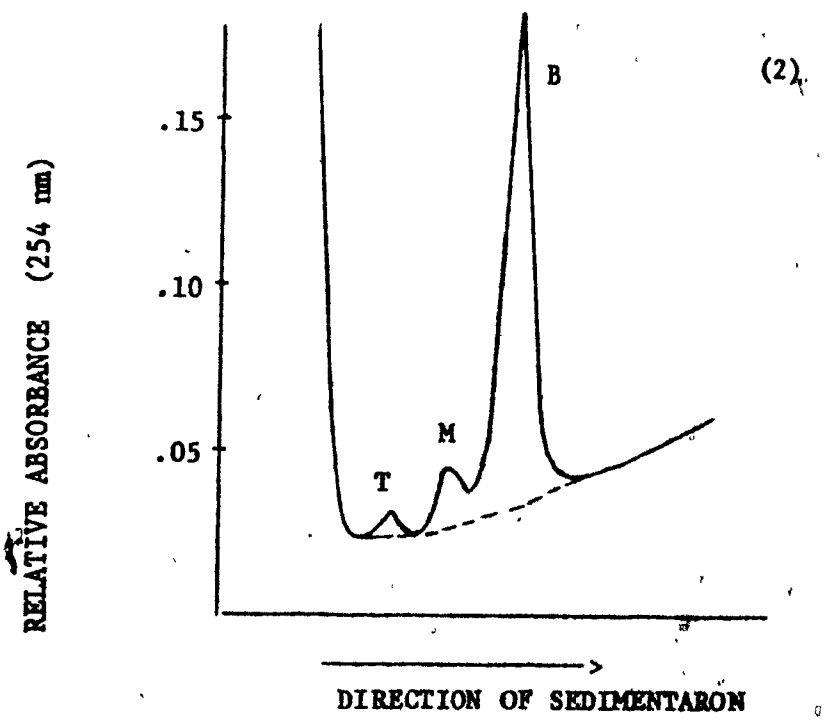
d/ The number of days after emergence of seedlings; the day of emergence was taken as day 6 after sowing.

### 3.3.1. ANALYSIS OF EXTRACTS FROM TRSV-INFECTED SEEDS AND FROM THEIR SEEDLINGS

The characteristic UV-absorbing profile of TRSV showing top, middle, and bottom components was obtained in extracts of cotyledons of TRSV-infected seeds (Fig. 2). A host component, occasionally detected in extracts from healthy plants and which sedimented at about the same rate as TRSV top component, made it impossible to accurately estimate the amount of top component in extracts from virus-infected plants. Consequently, only the results based on the estimates of the relative amounts of bottom and middle components of TRSV are reported here.

The ratios of bottom component to middle component (B/M ratios) in extracts from cotyledons of ungerminated infected seeds, germinated infected seeds and their seedlings are summarized in Table 1. Although individual results varied considerably, an average ratio of about 10 was obtained at various sampling periods. Extracts of cotyledons from samples of 30 seedlings taken 14 and 24 days post-emergence (Table 2) produced a similar B/M ratio. Thus the overall B/M ratios in extracts of cotyledons

Fig. 2. UV absorbance profile of extracts of infected seeds in a centrifuged sucrose gradient, showing characteristic profile of TRSV. T, M, and B represent top, middle, and bottom components respectively. Gradient column was centrifuged at 40,000 rpm for 2 hours.


(2)
B
.15
.10
.05
M
T
RELATIVE ABSORBANCE (254 nm)
DIRECTION OF SEDIMENTARON

indicate no evidence of change in the period of time investigated.

The B/M ratio in systemically infected leaf tissue of seedlings from infected seeds could be calculated in only one of four primary leaf samples and none of the four samples of the first trifoliate leaves because the middle component was detected in only one sample (Table 2). It appeared that a large amount of bottom component must be present before the middle component could be detected.

Table 2. Ratio of bottom component (B) to middle component (M) of TRSV in extracts of cotyledons and leaves of soybean seedlings from TRSV-infected seeds [a/].

| Expt. No. | Days post-emergence | Relative amount of components | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Cotyledon | | | Primary leaves | | | First trifoliates | | |
| | | B | M | B/M | B | M | B/M | B | M | B/M |
| 1 | 14 | 72 | 12 | 6.0 | 17 | 0 | ∞ | 28 | 0 | ∞ |
| | 24 | 70 | 0 | ∞ | 119 | 14 | 8.5 | 24 | 0 | ∞ |
| 2 | 14 | 160 | 21 | 7.6 | 63 | 0 | ∞ | 78 | 0 | ∞ |
| | 24 | 74 | 9 | 8.2 | 46 | 0 | ∞ | 26 | 0 | ∞ |

a/ Thirty seedlings were used for each analysis.

### 3.3.2. ANALYSIS OF EXTRACTS FROM INOCULATED SEEDLINGS

A phenomenon commonly observed in inoculated seedlings was that of severe shock symptoms in the second trifoliate leaves manifested by chlorotic and necrotic margins, malformation, and the blighting of one or

more leaflets (Fig. 3). In severe cases, the leaf bud was totally blighted. This symptom was visible six to seven days after inoculation. Accompanying these symptoms were the proliferation of axillary buds at the cotyledons, and the stunting of the inoculated seedlings (Fig. 3). The trifoliate leaves usually were small with mosaic symptoms, but occasionally marginal necrosis developed.

Table 3. Ratio of bottom component (B) to middle component (M) in extracts from TRSV-inoculated soybean seedlings [a/].

| Expt. No. | Days after inoculation | Relative amounts of components | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Cotyledons | | | Primary leaves | | | First trifoliates | | |
| | | B | M | B/M | B | M | B/M | B | M | B/M |
| 1 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 7 | 0 | 0 | 0 | 110 | 9 | 12.2 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 152 | 10 | 15.2 | 282 | 0 | ∞ |
| | 13 | 0 | 0 | 0 | 268 | 17 | 15.7 | 86 | 1 | 86.0 |
| | 16 | 17 | 0 | ∞ | 279 | 12 | 23.2 | NA[b/] | NA | - |
| | 19 | 0 | 0 | 0 | 380 | 14 | 27.1 | 149 | 3 | 49.0 |
| 2 | 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | 7 | 0 | 0 | 0 | 131 | 26 | 5.0 | 0 | 0 | 0 |
| | 10 | 0 | 0 | 0 | 94 | 10 | 9.4 | 220 | 11 | 20.0 |
| | 13 | 7 | 1 | 7.0 | 160 | 7 | 22.8 | 56 | 1 | 56.0 |
| | 16 | 102 | 7 | 14.5 | 215 | 8 | 26.8 | 196 | 4 | 49.0 |
| | 19 | 44 | 3 | 14.7 | 140 | 3 | 49.5 | 98 | 1 | 98.0 |

a/ Ten seedlings were used for each analysis.

b/ NA indicates results not available because of broken centrifuge tube.

Fig. 3. An inoculated seedling (left) showing blighting symptoms in the second trifoliate leaf bud and proliferated axillary buds at the cotyledons. The infected seedling is stunting as compared with the healthy seedling (right) of the same age.

(3)

In extracts from inoculated primary leaves, the B/M ratio increased with age of infection. A similar trend occurred in the extracts from the first trifoliate leaves. The ratios were higher in the trifoliate leaves than in the inoculated primary leaves (Table 3). It is interesting to note that the concentration of TRSV and the B/M ratios in extracts from inoculated leaves were higher than those of the comparable leaves of seedlings produced by seeds (Tables 2 and 3). Tobacco ringspot virus was not detected in extracts of cotyledons by sucrose density gradient centrifugation until 13-16 days after inoculation. It could be detected in the trifoliate leaves within a shorter period of time (10 days).

## 3.4. DISCUSSION

The experiments reported in this section were designed to determine if the B/M ratio of TRSV varied with the type of infection in soybean (infection of seedlings from infected seed or infection of seedlings by direct inoculation) and the stage of the infection. The sedimentation profile obtained with most of the extracts was typical of that previously reported for this virus (Stace-Smith et al., 1965; Schneider and Diener, 1966). The typical sedimentation pattern was obtained with samples from the cotyledons of ungerminated infected seeds and from cotyledons of germinating seeds and young seedlings (Tables 1 and 2) as well as from primary leaves directly inoculated with the virus (Table 3).

The B/M ratio varied considerably in different cotyledon samples of ungerminated seeds, germinating seeds and young seedlings from infected

seeds, but the average value of two to four experiments was relatively stable up to 19 days postemergence (Table 1). Leaf tissue systemically infected from seed-borne virus (Table 2) produced little middle component, since it could not be detected by sucrose density gradient centrifugation, even when the sample size was increased from 10 to 30 seedlings per sample. In such cases, it appeared that a threshold amount of bottom component had to be present before middle component could be detected.

The B/M ratio in extracts of leaves from inoculated seedlings was higher than that in cotyledons of ungerminated and germinating infected seeds, but it was lower than that in leaves of seedlings systemically infected from infected seeds. The data show that the B/M ratios in inoculated seedlings increased with age of infection indicating a slower rate of accumulation of middle component than of bottom component. The B/M ratio was approaching infinity as infected plants approached the chronic symptom stage. Seedlings grown from infected seeds are in a permanent chronic stage of infection, thus the B/M ratio was infinite. The results are in agreement with the report of decreasing middle to middle plus bottom components of TRSV in bean (Schneider and Diener, 1966). Schneider and Diener (1968) reported that the decreasing ratio of middle to middle plus bottom components was closely related to the reduction in specific infectivity of TRSV with age of infection. The reduction in specific infectivity was thought to be due to an increased amount of short RNA species and to degradation of RNAs. The increased amount of short RNA species increased the probablity of two short RNA strands being encapsulated

in the same particle, thus, effectively increasing the amount of bottom component.

The third trifoliate leaves of inoculated seedlings apparently "recovered" from severe symptoms which were apparent in the second trifoliate leaves. This may be related to the low specific infectivity of the virus in the chronic phase. The "recovery" of plants infected with broad bean mottle (Kadoma and Bancroft, 1964) and bean pod mottle (Gillaspie and Bancroft, 1965) has been associated with the reduction of specific infectivity of these viruses. The fact that cowpea chlorotic mottle virus-infected plants do not recover from virus symptoms in spite of the reduction of specific infectivity of the virus (Kuhn, 1965) indicates that other reasons could be involved. The observation that tobacco, but not bean, (Price, 1932 ; Schneider and Diener, 1968) "recovers" from TRSV infection suggests that it is a result of a genetic interaction between host and virus genomes.

The apical dominancy in association with auxin production has been well documented (Wilson et al., 1971). The proliferation of axillary buds in inoculated seedlings was likely due to the disturbance of auxin production by the apex when the leaf bud was blighted.

# 4. THE MECHANISM OF SEED TRANSMISSION OF TOBACCO RINGSPOT VIRUS IN SOYBEAN

## 4.1. INTRODUCTION.

The frequency of seed transmission of TRSV in soybean depends on the stage of development of the host plant at the time of infection (Crowley, 1959 ; Athow and Bancroft, 1959 ; Owusu et al., 1968) and also on the variety (Athow and Bancroft, 1959 ; Kahn, 1956). Crowley (1959) demonstrated that a small amount of seed transmission occurred in plants inoculated after flowering, presumably by direct invasion of the developing embryos by the virus. Generally, a high frequency of seed transmission occurs when plants are infected prior to flowering. Athow and Bancroft (1959) reported that 100 percent of the seed produced on the variety Harosoy produced infected seedlings.

The distribution of the virus in mature soybean seeds was determined by assaying seed parts for infectivity. Infectious virus was recovered from the embryos of mature seeds but not from the testae of the same seeds (Athow and Bancroft, 1959; Owusu et al., 1968). The demonstration that TRSV can be found in the embryos but not the seed coats of mature seeds suggest that the megagametophytes or the microgametophytes are involved in the transmission of this virus through soybean seeds.

This section of the thesis reports on experiments which describe the mechanism of seed transimission of TRSV. Two main experiments were done : 1) cross-pollination between healthy and virus-infected soybean to determine the relative amounts of pollen or ovule transmission; and

2) electron microscopy of pollen and ovules in various stages of development to determine the location of the virus in these tissues.

## 4.2. MATERIALS AND METHODS

### 4.2.1. PLANTS

Healthy soybean plants were grown in 7 in. pots, in the greenhouse. The potting medium consisted of 3 parts pasteurized soil, 1 part sand and 1 part peat moss together with a suitable quantity of "Osmocote", a granulated fertilizer coated with plastic to facilitate slow release of nutrients in the soil. The ambient temperature in the greenhouse varied between 22° and 30°C. Supplementary illumination was provided by fluorescent lamps for a total photoperiod of 12 hours.

One half of the seedlings was inoculated at the two leaf stage with 0.01 mg/ml purified TRSV mixed with 500 mesh carborundum, using Q-tips as the inoculating device. The other half of the seedlings was not inoculated and was kept on a separate bench. Staking of the plants in order to facilitate access to the flowers was necessary. All precautionary steps were taken to prevent infection of the healthy plants, which were indexed frequently by inoculating expressed sap of leaf tissue onto cowpea, a hypersensitive host. Contaminated plants were removed immediately.

### 4.2.2. CROSS-POLLINATION

Flower buds were ready for crossing one day before flowering (Johnson and Bernard, 1963). At this stage the tips of the petals had just

pushed through the calyx lobes and dehiscence of the anthers had not yet occurred. The node bearing a flower bud was affixed to the stage of a binocular microscope with cellophane tape and all operations pertaining to cross-pollination were observed with a microscope.

Emasculation was performed with two pairs of fine tip forceps. A pair of them was used to hold the base of the calyx, while the lobes of the calyx and the corolla were removed with another pair of forceps. The calyx lobes were removed by pulling downwards and the corolla was grasped with the forceps at a right angle to the axis and pulled off gently. The anthers could be removed together with the corolla in one motion. However, a few anthers were occasionally left behind and these were removed carefully without damaging the ovary.

Flower buds that were to be pollen donors were collected and the calyxes and corollas were removed before those flowers which were to be pollen receptors were emasculated. The water loss resulting from emasculation of the anthers caused them to dehisce. One of these anthers was transfered onto each stigma of a newly emasculated flower bud.

Crosses were made between healthy pollen and TRSV-infected pistils ($H\hat{\sigma} \times T\overset{Q}{+}$); and vice versa ($H\overset{Q}{+} \times T\hat{\sigma}$). Self-pollinations were also allowed in both infected ($T\overset{Q}{+} \times T\hat{\sigma}$) and healthy ($H\overset{Q}{+} \times H\hat{\sigma}$) plants. The crosses were performed on two groups of plants : 1) plants within the first 15 days of the flowering period; and 2) plants that had flowered for 20 days. The flowering period of soybean lasts for about 35 days.

Each crossed flower bud was labelled. Generally, those pistils

that failed to set seeds abscised within one week after crossing. Those that remained green for two weeks were regarded as successfully crossed. New flowers that developed later on the same racemes were removed to avoid confusion.

To determine the extent of seed transmission in cross-pollination experiments, the $F_1$ progeny from each group of crosses was assayed for infectious virus on cowpea. Inocula were prepared by grinding soaked seeds or cotyledons from seedlings with a small volume of buffer.

### 4.2.3. PRODUCTION AND VIABILITY OF POLLEN

The number of pollen grains and their viability in both healthy and infected plants was investigated. Anthers from flower buds were harvested one day before flowering, and triturated with a glass rod in a test tube containing 0.1 ml of water. Anther walls were destroyed easily, but a large number of pollen grains remained intact. The number of pollen grains in each flower was estimated with a hemacytometer. This is a specially made glass slide with a H-shaped trough between two parallel ridges. Two counting areas were available ; each had nine 1 $mm^2$ rulings at 0.1 mm below the level of the ridges. When covered with a cover glass at the level of the ridges, the number of pollen grains in 1 $mm^2$ would represent the number in 0.1 $mm^3$ of the pollen suspension. The number of pollen grains in each flower (0.1 ml) was obtained by multiplying the average number of pollen grains in one unit area by a factor of 1,000. The number of pollen grains in each flower was estimated from the average of five readings ; estimates were made from 25 flowers from healthy plants

and from 25 flowers from TRSV-infected plants.

For pollen viability tests, pollen grains from 10 infected and from 10 healthy plants were pooled separately and germinated overnight at 25°C in 30% sucrose containing 120 ppm boric acid. The percentage of germination was estimated by microscopically observing 10 single drop samples ; pollen grains that had produced germ tubes, irrespective of the germ tube length, were considered to have germinated.

The length of each germ tube was estimated with an occular micrometer which had been calibrated against a stage micrometer. One division on the occular micrometer was equal to 0.526µ, using a 10X objective. Therefore, the length of a germ tube was equal to 0.526µ (X), where X is the number of divisions measured with the occular micrometer.

### 4.2.4. NUMBER OF OVULES AND THEIR DISTANCES FROM THE STIGMA

The ovaries of healthy and infected flowers were dissected with a scalpel under a dissecting microscope, one day before flowering. The number of ovules per ovary was recorded and the distance between the micropylar end of each ovule and the stigma was measured with a ruler.

### 4.2.5. INFECTIVITY ASSAY OF THE FLOWER BUDS AND YOUNG SEEDS

The anthers, ovules and ovary wall of each flower bud were separately surface-disinfested by immersing them in 10% $Na_3PO_4$ for one minute and washing them several times with distilled water. The tissues were ground separately between 2 sterile glass slides with a drop of buffer

and a small quantity of carborundum. Inoculation was done by rubbing the slides gently on the primary leaves of cowpea.

Young seeds were divided into embryos and seed coats and separately disinfested as described above before triturating in a small volume of buffer with a mortar and pestle. The crude sap was inoculated onto primary leaves of cowpea.

4.2.6. ELECTRON MICROSCOPY

Young leaves were cut into thin strips. Ovaries were transversely cut into segments, each containg an ovule, and anthers were sliced open. All tissues were cut in 5% buffered glutaraldehyde solution to facilitate penetration of the ficatives. All ovaries and anthers were sampled either 1 day or 3-4 days before flowering. Some anthers with mature pollen were sliced open and incubated at 25°C in 30% sucrose solution containing 120 ppm boric acid to allow the pollen to germinate. The anthers were then rinsed in buffer prior to fixation. All tissues were fixed and embedded according to the following schedule : -

1. Fix tissues in 5% glutaraldehyde in 0.1M phosphate buffer pH 7.2 for 60 mins.
2. Wash in 0.1M phosphate buffer twice, 15 mins each time.
3. Postfix in 1% osmium tetroxide in Palade's buffer (Palade, 1952) for 90 mins.
4. Dehydrate in graded alcohol series,

   50% ----- 15 mins.

70% ----- 15 mins.

95% ----- 15 mins., twice.

100% ----- 15 mins., twice.

5. Transfer to 1:1 propylene oxide/ 100% alcohol, and then to pure propylene oxide, 30 mins. each.

6. Infiltrate in 1:1 propylene oxide/ Epon overnight.

7. Embed in pure Epon and cure 24-36 hrs. in 60°C oven.

The Epon-embedded materials were sectioned with a diamond knife on a Reichert Om U2 ultramicrotome. Thin sections were picked up with a clean slot grid and placed on a 100 or 200 mesh grid coated with a carbon-supported collodion film. The sections were stained with uranyl acetate and lead citrate (Reynold, 1963 ; Hayat, 1970) and examined with a Philips EM 200 electron microscope operated at 60 KV. All electron micrographs were recorded on 35mm film.

Some tissues were processed with the protein stain method described by Hills and Plaskitt (1968). Essentially, plant tissues were fixed in glutaraldehyde and dehydrated in graded acetone, each solution being saturated with uranyl acetate. Thin sections were stained briefly in lead citrate before being examined with the electron microscope. This method is useful for differentiating small icosahedral virus particles from ribosomes.

## 4.3. RESULTS

### 4.3.1. CROSS-POLLINATION EXPERIMENTS

The results of cross-pollination in terms of seed set and number of mature pods harvested are presented in tables 4 and 5 respectively. The $H\hat{O} \times T\overset{Q}{+}$ cross set 10 times as many pods as the reciprocal cross in the two experiments that were done. The difference in percentage of pod set in the two experiments was probably due to the fact that the first experiment was performed on late flowers, which could have suffered from malnutrition, thus affecting the vigor of pollen grains and the rate of pod set (Kato et al., 1954).

Table 4. Number of pods set in cross-pollination experiments between healthy and TRSV-infected soybean.

| Expt. No. | Type of cross-pollination | | | | | |
|---|---|---|---|---|---|---|
| | $H\hat{O} \times T\overset{Q}{+}$ | | | $H\overset{Q}{+} \times T\hat{O}$ | | |
| | Number of pod set | Number of crosses | % | Number of pod set | Number of crosses | % |
| 1 | 18 | 103 | 17.4 | 2 | 120 | 1.7 |
| 2 | 84 | 205 | 40.9 | 7 | 173 | 4.0 |

The majority (78.4%) of the pods that had set from the $H\hat{O} \times T\overset{Q}{+}$ cross were retained on the infected plants until the pods matured, but only 11% of the pods from the $H\overset{Q}{+} \times T\hat{O}$ cross were harvested from healthy plants. Crossing healthy flowers with TRSV-infected pollen was difficult. Athow (personal communication) had tried similar experiments without success.

Table 5. Number of mature pods obtained from crosses between healthy and TRSV-infected soybean.

| Cross | Number of pod set | Mature pods | |
|---|---|---|---|
| | | Number | % |
| $H\delta^{\uparrow} \times T\varphi^{0}$ | 102 | 80 | 78.4 |
| $H\varphi^{0} \times T\delta^{\uparrow}$ | 9 | 1 | 11.0 |

4.3.2. POLLEN PRODUCTION AND VIABILITY

A significant reduction in pollen production was observed in flowers of infected plants. Partial to complete failure of pollen production was not uncommon. Many anthers were necrotic and puffy looking. Dehiscence of these abnormal anthers usually failed to occur. In healthy flowers, the stigma was found to be encircled by anthers, thus allowing pollen grains immediate access to it when anthesis occurred. Conversely, many of the infected flowers had shorter filaments, which rendered pollen deposition on the stigma virtually impossible.

The number of pollen grains produced in each infected flower ranged from 0 to 2,000 compared with 4,000 to 5,000 per flower in most of the healthy ones (Fig. 4). The germination rates were 47 percent and 77 percent for infected and healthy pollen respectively.

Pollen grains from infected plants produced shorter germ tubes (252 μ mean length) than those from healthy plants (425 μ mean length). The distribution of the lengths of germ tubes is summarised in Fig. 5.

Fig. 4. Frequency distribution of the number of pollen grains per flower estimated from 25 samples each from TRSV-infected (a), and healthy soybean (b).

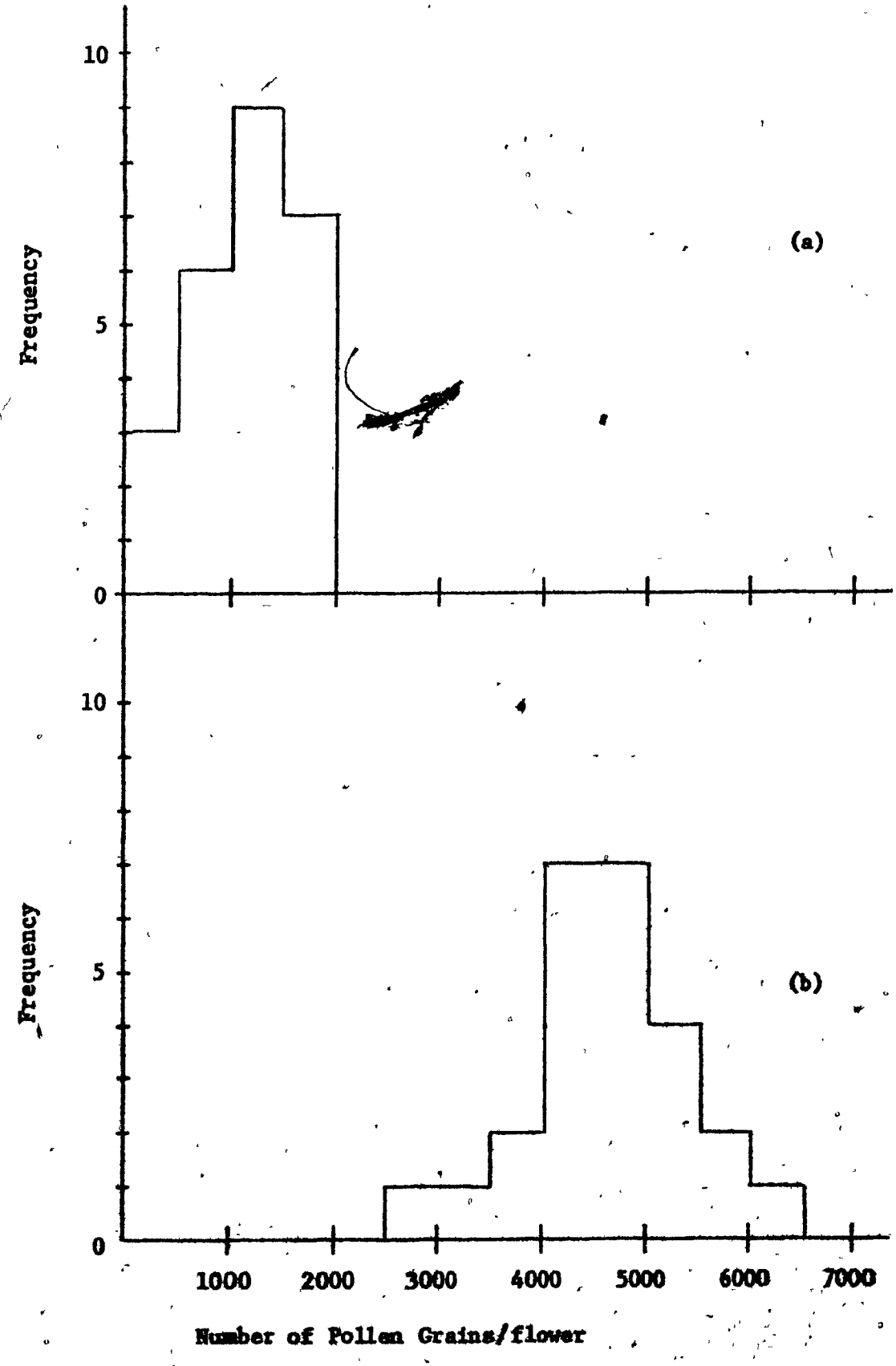
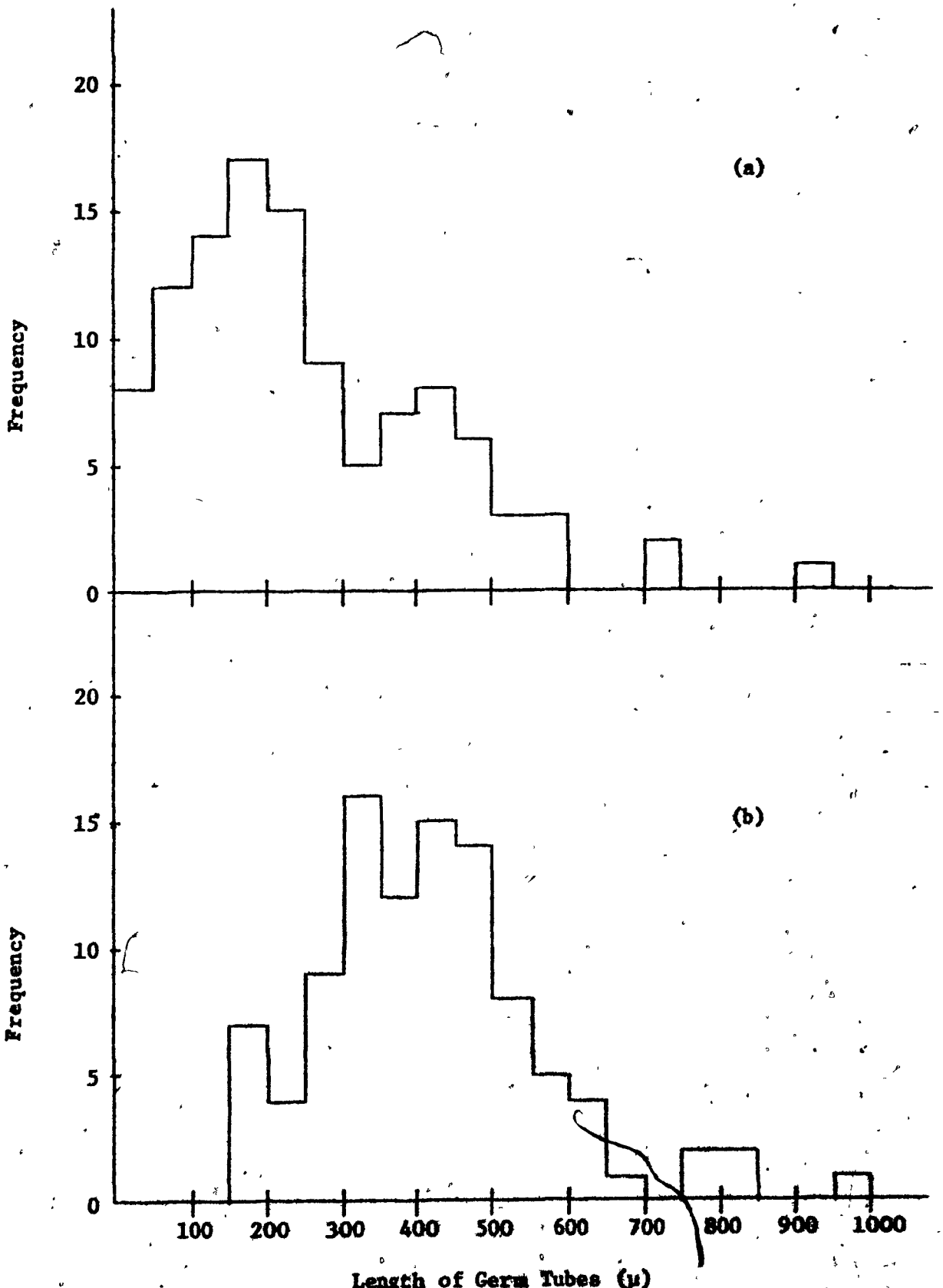

10
5
0
Frequency
(a)
10
5
0
Frequency
(b)
1000
2000
3000
4000
5000
6000
7000
Number of Pollen Grains/flower

Fig. 5. Frequency distribution of the lengths of germ tubes of 100 pollen grains from TRSV-infected (a), and healthy soybean (b); the mean values are 252μ and 425μ , respectively.


(a)
(b)
Frequency
Frequency
0
5
10
15
20
100
200
300
400
500
600
700
800
900
1000
Length of Germ Tubes (μ)

Few pollen grains from infected plants were able to produce long germ tubes. Micrographs of germinated pollen from infected and healthy plants are shown in Figs. 6 and 7 respectively.

### 4.3.3. NUMBER OF OVULES AND THEIR DISTANCES FROM THE STIGMA

TRSV infection did not affect ovule production. The number of ovules in each ovary was commonly 2, occasionally 3. The total numbers of ovules counted in 85 samples were 294 and 299 for healthy and infected overies respectively.

Table 6. Distance (mm) between the stigma and the terminal (T), middle (M) and basal (B) ovules in ovaries of healthy and TRSV-infected soybean.

| Expt. | Ovules from healthy soybean | | | Ovules from infected soybean | | |
|---|---|---|---|---|---|---|
| | T | M | B | T | M | B |
| 1 | 1.5 | 1.8 | 2.2 | 1.8 | 2.0 | - |
| 2 | 1.8 | 2.0 | 2.3 | 1.5 | 1.8 | 2.3 |
| 3 | 1.8 | 2.2 | - | 1.8 | 2.2 | - |
| 4 | 2.0 | 2.3 | - | 1.8 | 2.2 | - |
| 5 | 2.0 | 2.3 | - | 1.5 | 1.9 | - |
| 6 | 1.5 | 1.8 | - | 1.8 | 2.1 | 2.5 |
| 7 | 2.0 | 2.2 | - | 1.8 | 2.2 | - |
| 8 | 1.8 | 2.0 | - | 1.5 | 2.0 | 2.4 |

The distance between a terminal ovule and the stigma was at least 1.5 mm, whereas a basal ovule could be as far as 2.5 mm away from the stigma (Table 6). A pollen grain must, therefore, produce a germ tube 1500μ long in order to fertilize the nearest egg. The distances were apparently not affected by TRSV infection.

### 4.3.4. SEED YIELD AND INFECTIVITY ASSAY

A majority of the pods produced by infected plants was single seeded with the seed developing from the terminal ovules. Although a similar phenomenon was observed in some healthy pods, the majority of them produced two or three seeds.

Table 7. Number of seeds per pod produced by healthy and TRSV-infected soybean and by reciprocal crosses between healthy and TRSV-infected soybean.

| Cross | No. of Pods containing | | | Percentage of Pods containing | |
|---|---|---|---|---|---|
| | 1 seed | 2 seeds | 3 seeds | 1 seed | 2 or 3 seeds |
| H♀ × T♂ | 0 | 0 | 1 | 0 | 100 |
| H♂ × H♀ | 109 | 308 | 40 | 33.9 | 76.1 |
| H♂ × T♀ | 30 | 43 | 7 | 37.5 | 62.5 |
| T♂ × T♀ | 224 | 127 | 18 | 62.7 | 37.3 |

It was interesting to note that, like self-pollinated pods from healthy plants ($H\mathring{O} \times H\mathring{\varphi}$), pods from the $H\mathring{O} \times T\mathring{\varphi}$ cross produced more multiple-seeded pods than single-seeded pods (Table 7). Obviously, poor pod production and seed yield in infected plants were due to poor pollen. Only a single pod was obtained from the $H\mathring{\varphi} \times T\mathring{O}$ cross.

Infectivity assays of seeds produced in these experiments (Table 8) showed that the $H\mathring{O} \times T\mathring{\varphi}$ cross and the naturally pollinated TRSV-infected soybean control ($T\mathring{\varphi} \times T\mathring{O}$) produced 94 and 98 percent TRSV-infected seed, respectively; none of the healthy plants ($H\mathring{\varphi} \times H\mathring{O}$) or the $H\mathring{\varphi} \times T\mathring{O}$ cross produced infected seed.

Table 8. Infectivity of extracts from seeds produced on healthy and TRSV-infected soybean and from reciprocal crosses between healthy and TRSV-infected plants.

| Source plants | Source of inoculum | | | |
|---|---|---|---|---|
| | Seeds | % infected | Cotyledons | % infected |
| $T\mathring{\varphi} \times H\mathring{O}$ | 19/20 [a/] | 95 | 28/30 | 93 |
| $H\mathring{\varphi} \times T\mathring{O}$ | 0/3 [b/] | 0 | - | - |
| $T\mathring{\varphi} \times T\mathring{O}$ | 20/20 | 100 | 29/30 | 97 |
| $H\mathring{\varphi} \times H\mathring{O}$ | 0/20 | 0 | 0/30 | 0 |

a/ Numerator is the number of infectious extracts; denominator is the number of extracts inoculated.

b/ There was only one pod containing 3 seeds matured from the $H\mathring{\varphi} \times T\mathring{O}$ cross.

### 4.3.5. INFECTIVITY ASSAY OF FLOWER BUDS AND YOUNG SEEDS

The results of the infectivity assays were scored on the basis of presence or absence of TRSV lesions on cowpea; no quantitation of lesions was made. TRSV infectivity could be detected in extracts from infected ovary walls, ovules and anthers. Since pollen grains were difficult to homogenize by simple grinding, the infectivity detected in extracts of anthers was likely derived from anther walls. The infectivity detected in extracts of ovules could not be traced to the original tissues, such as the integuments, the nucellus or the embryo sac. Virus infectivity was detected in both embryos and seed coats of young seeds from infected plants. Healthy counterparts were negative in all tests.

### 4.3.6. ELECTRON MICROSCOPY

TRSV virions are morphologically similar to ribosomes and therefore it is difficult to identify individual virions in thin sections (Crowley et al., 1969). The identification of the virus was based on secondary characteristics, such as the presence of virons in tubules and aggregates or crystals of virus-like particles which were not found in healthy tissues.

#### 4.3.6.1. LEAF TISSUE

There were several noticeable differences between infected and healthy leaf tissues, although the fine structure of the organelles in infected leaf cells appeared normal. Rows of virus-like particles in narrow tubules were found extending from one cell to the other through

plasmodesmata (Fig. 8). The diameter of these virus-like particles was about 26 nm, whereas the inner dimension of the tubules measured about 30 nm.

Virus-like particles in crystals bounded with a membrane were found in the sieve tubes of infected leaf tissue. Groups of these particles were also found in the vacuoles of phloem cells. Cell walls often protruded into sieve tubes (Fig. 9). No similar particles could be found in healthy leaf tissues (Fig. 10).

4.3.6.2. OVULES

Each ovule consists of two integuments, a nucellus and a megagametophyte (Fig. 11). A longitudinal section of the megagametophyte usually shows few of the expected seven cells. The synergid cells are located at the micropylar end, with a prominent filiform appartus. The micropylar end of the egg cell is occupied by a vacuole and the nucleus is located at the chalazal end (Figs. 11 and 22). Both the synergid cells and the egg cell contain a small quantity of starch granules (Fig. 27). The central cell contains a large number of starch granules and two fused polar nuclei which can be seen if the sectioning plane is correct. When fertilization takes place this cell becomes triploid and forms the endosperm which provides nutrients for the growth of the embryo. The antipodal cells are located at the chalazal end of the embryo sac. All of the cells are located inside a prominent embryo sac wall. There are plasmodesmata between the cells but none are found in the embryo sac wall itself. The nucellar cells adjacent to the megagametophyte are degenerating. A cutinized, electron-dense layer marks the boundary between the nucellus

and the integuments.

In infected ovules, virus-like particules in crystals were found in the sieve tubes of the integument (Fig. 12), and in the vacuoles of some nucellar cells (Fig. 14). Occasionally, several groups of virus-like particles were found in the vacuoles of some nucellar cells (Fig. 13). Files of virus-like particles in tubules were widely distributed in the nucellus. These tubules were either embedded in the cell walls (Figs. 12, 16, 17), or were traversing them through plasmodesmata (Figs 18 and 19).

The protrusion of cell walls associated with TRSV infection has been reported recently in phloem tissue of TRSV-infected soybean (Halk and McGuire, 1973). This structural change was observed in leaf tissue (Fig. 9) and it was very common in the nucellar tissue (Figs. 15, 16, 17, and 19). The cell wall protrusions were always associated with tubules containing virus-like particles.

In healthy ovules, the organelles of the degenerating nucellar cells around the megagametophyte eventually disappeared and only the plasma membrane and vesicles could be found. Numerous virus-like particles in groups were found among the vesicles in the degenerating nucellar cells of TRSV-infected soybean (Figs. 20 and 21).

In the megagametophytes, virus-like particles in groups or crystals were frequently found in the embryo sac walls (Figs. 22, 23, 24, and 26), and in the walls of megagametophytic cells (Figs. 25, 27, and 28). Some of them were membrane-bounded. Those without a membrane were perhaps due to artifacts. Virus-like crystals embedded in the walls were not found

in the sporophytic tissue of mother plants nor have they been reported in the literature. Virus-like particles in tubules were often found embedded in the walls ; occasionally such particles were located in plasmodesmata (Fig. 29) between the megagametophytic cells.

Virus-like particles in crystalline array were found on one occasion in the vacuole of an antipodal cell (Figs. 30 and 31). Such crystal formation was not observed in other megagametophytic cells in the limited observation reported here, probably because such crystal formation was rare.

4.3.6.3. MICROGAMETOPHYTES

The exine of a mature pollen grain is electron dense, whereas the intine is electron transparent. The cytoplasm of the vegetative cell within a pollen grain contains a large number of organelles and food reserves, such as grey bodies and starch granules but it is devoid of vacuoles (Figs. 32 and 34). The vegetative nucleus is spherical and centrally located. The generative cell is suspended in the cytoplasm of the vegetative cell. Each generative cell has a thin electron-transparent wall, a prominent nucleus but it is devoid of most organelles (Figs 32 and 38). The walls of the generative cell in immature pollen is incomplete; only patches of electron-transparent material can be observed (Fig. 39). A detailed study of pollen formation of Helleborus foetidus (Echlin, 1972) showed that a pair of tightly appressed unit membranes separate the newly formed generative and vegetative nuclei. As development proceeded, the middle space was gradually filled by electron-transparent material. A germinated pollen grain contains a large number of vesicles which developed

from active Golgi bodies. As germination proceeds, all cytoplasmic contents including the generative cell are moved into the germ tube (Larson, 1965).

Virus crystals were consistently found in the intine of pollen grains from infected plants (Figs. 34, 35, and 36). Similar results were obtained by protein staining procedures (Fig. 40). These crystals may be located in cytoplasmic protrusions in the intine (Fig. 37). They may be found in the cell wall and cytoplasm of generative cells (Figs. 38 and 39). Such crystals were not found in the cytoplasm of vegetative cells nor in the vegetative and generative nuclei. On one occasion, virus crystals were found in a germ tube (Fig. 41). It was located close to a nucleus within a thick electron-transparent band. The nucleus was believed to be a generative nucleus based on observations of nuclei in infected pollen grains, and the thick band was probably a tranformed generative cell wall before the onset of nuclear divison in the generative cell. However, positive identification could not be made.

A careful search of thin sections of 116 healthy pollen grains for virus-like particles was unsuccessful. The inner surface of the intine of healthy pollen grains was smooth and it contained small pockets free of particles (Figs. 32 and 33). Conversely, 36 out of 40 (90%) infected pollen grains observed contained virus crystals in their intines.

There were no tubular inclusions found in any infected pollen grains or in their germ tubes. Virus crystals were not located in the cytoplasm of vegetative cells probably because of their scarcity or because virus particles were scattered loosely.

Abbreviations used in the figures :

AC = Antipodal cell

CC = Central cell

CH = Chloroplast

EC = Egg cell

EX = Exine

EW = Embryo sac wall

F = Phytoferritin

FA = Filiform apparatus

G = Golgi bodies

GB = Grey body

GC = Generative cell

IG = Integument

IN = Intine

M = Mitochondrion

N = Nucleus

P = Plasmodesma

PC = Phloem cell

PM = Plasma membrane

PW = Protruded cell wall

ER = Endoplasmic reticulum

S = Starch granule

ST = Sieve tube

SC = Synergid cell

T = Tonoplast

TW = Germ tube wall

V = Virus-like particle

VA = Vacuole

VC = Vegetative cell

VE = Vesicle

VN = Vegetative nucleus

W = Cell wall

Note : All bars are 0.5μ unless otherwise marked.

Figs. 6 and 7.

Germinated pollen grains. The pollen grains were germinated overnight in 30% sucrose containing 120 ppm boric acid overnight in room temperature. The quality of the infected pollen grains (Fig. 6) is poor comparing to the healthy ones (Fig. 7)

X 200

Fig. 8. A thin section of mesophyll cells of a TRSV-infected soybean leaf showing a file of virus-like particles in a tubule extending from one cell to the other through a plasmodesma.

X 48,000

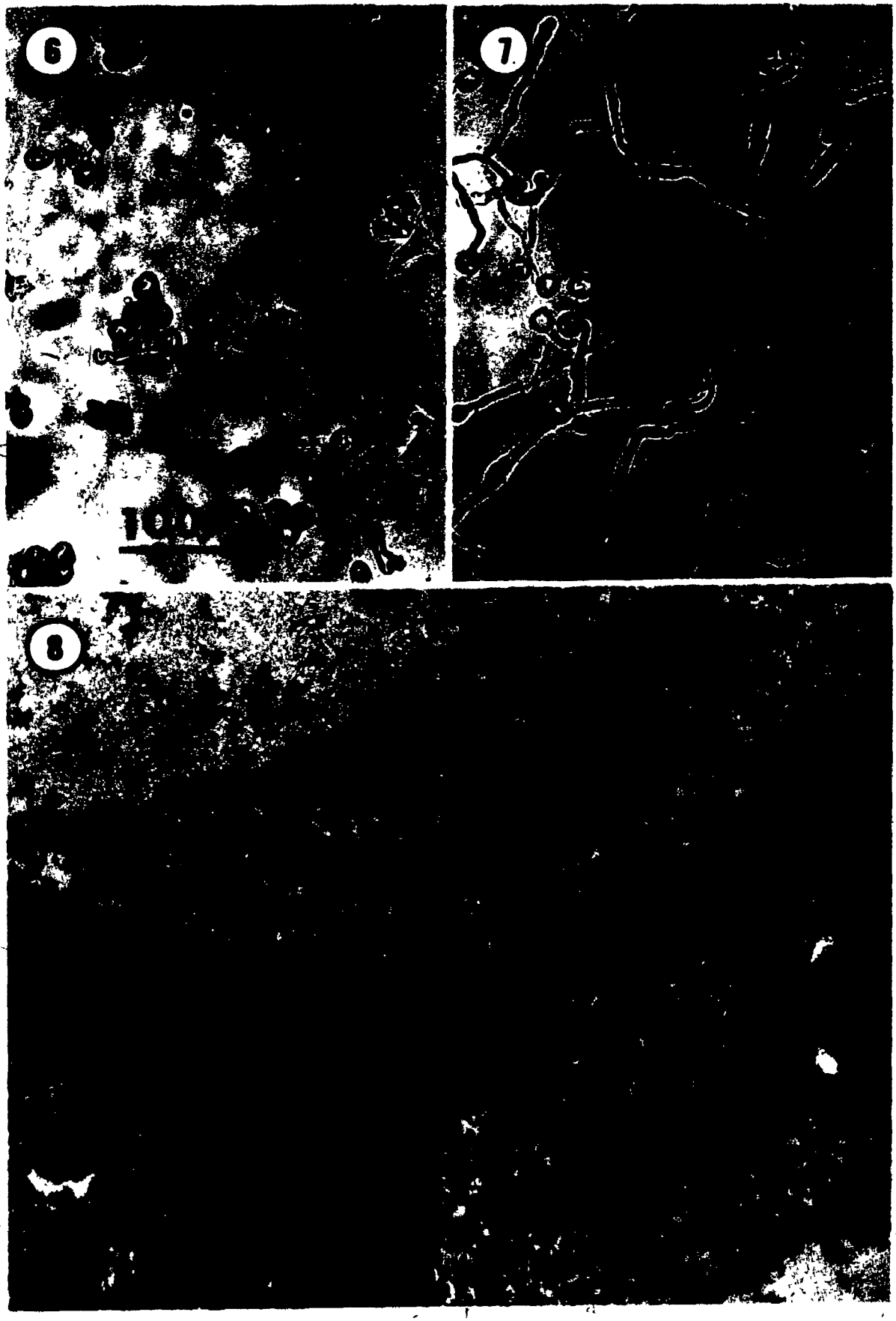

6
100 nm
7
8

Fig. 9. A vascular bundle of a TRSV-infected soybean leaf. Virus-like particles in crystals are located in two sieve tubes. Groups of such particles are present in the vacuoles and several particles are found in the plasmodesmata of phloem cells. A cell wall protrusion can be seen in the lower sieve tube.

X 18,500


ST

Fig. 10. A vascular bundle of a healthy soybean leaf showing the organelles. No virus-like particles are visible.

X 18,500

Fig. 11. A megagametophyte in an ovule of TRSV-infected soybean showing a synergid cell with a prominent filiform apparatus, the polarized egg cell, and the central cell with two fused nuclei and prominent starch granules. The nucellar cells adjacent to the megagametophyte are degenerating (arrow). The nucellus and integuments are separated by a electron-dense cutinized layer.

X 2,800

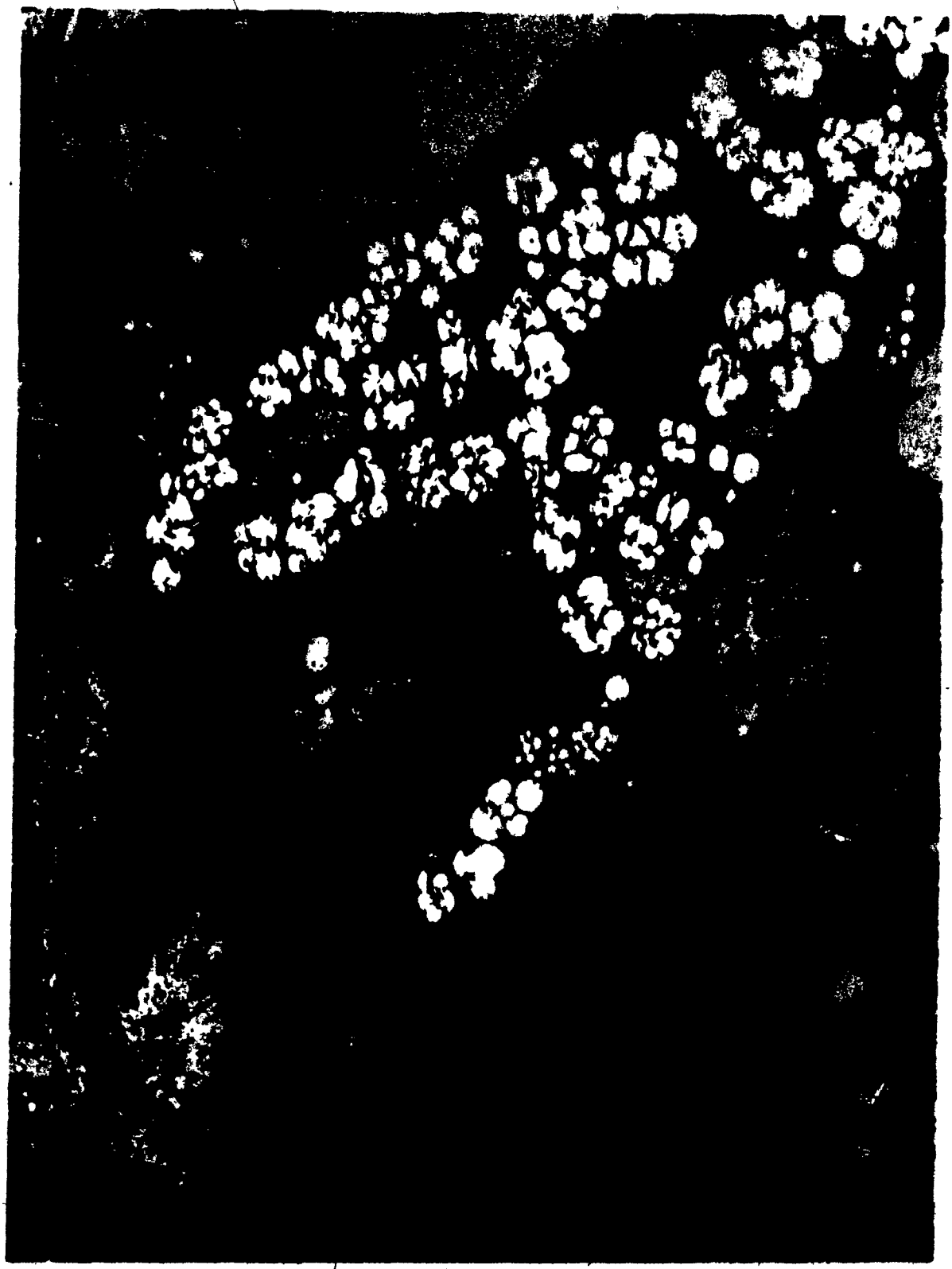


Fig. 12. Integument of an ovule of TRSV-infected soybean. A virus-like crystal is seen in the lumen of a sieve tube in the integument. There are two fragments of a tubule (based on observations of serial sections) containing virus-like particles embedded in the protruded wall.

X 48,200


ST

Figs. 13-15. Nucellus in an ovule of TRSV-infected soybean.

Fig. 13. Clusters of virus-like particles are found in the vacuole of a nucellar cell.

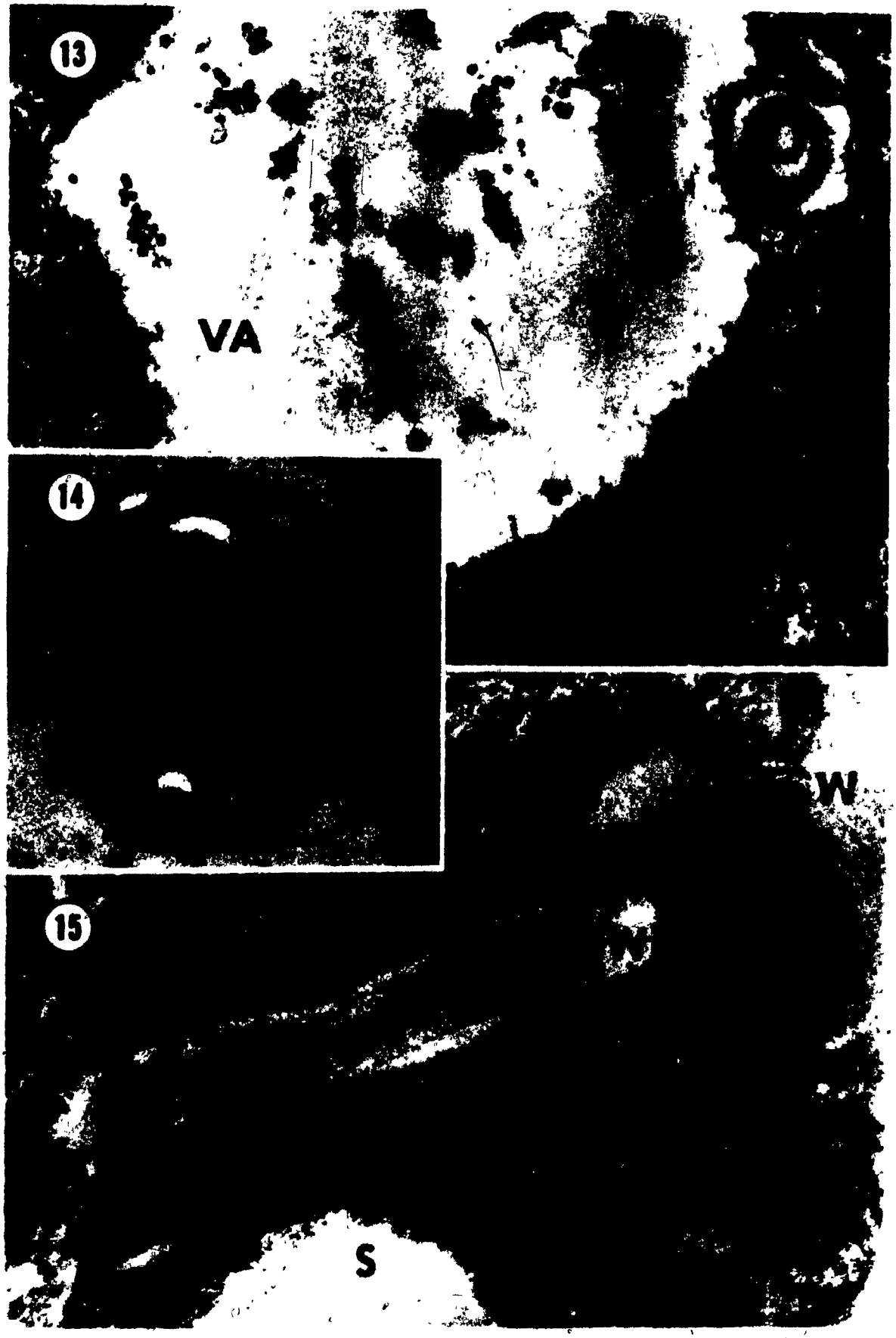
X 63,800

Fig. 14. A virus-like crystal encircled with several layers of membrane is located in a vacuole of a nucellar cell.

X 72,900

Fig. 15. A section of a protruded cell wall with a tubule containing virus-like particles is located in the cytoplasm of a nucellar cell.

X 109,500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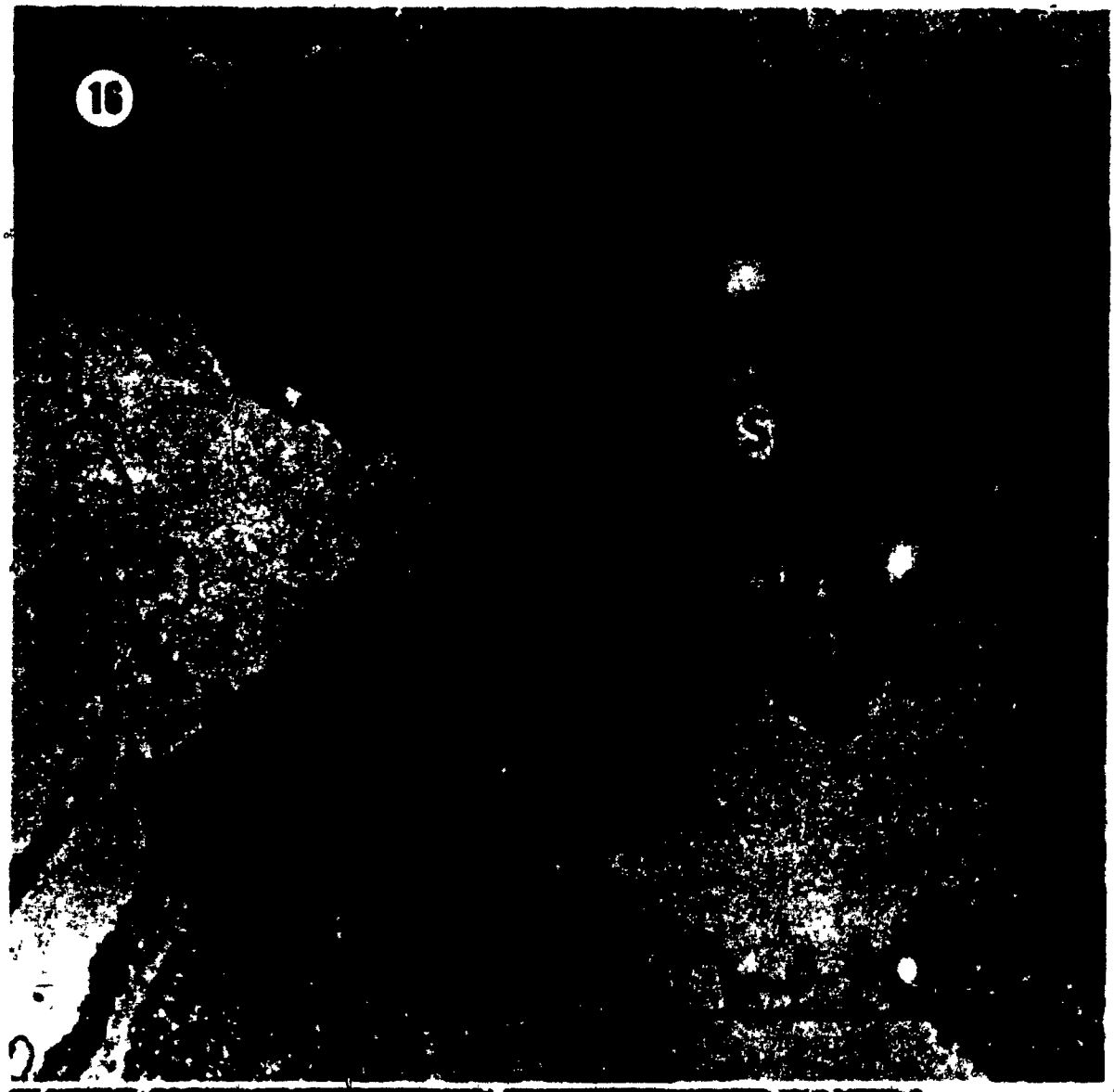
Figs. 16-17. Nucellus in an ovule of TRSV-infected soybean.

Fig. 16. Three tubules containing virus-like particles are embedded in the cell wall protrusions.

X 11,000

Fig. 17. Two tubules, presumably sections of the same one, are embedded in the cell wall protrusion.

X 41,900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Figs. 18-19. Nucellus in an ovule of TRSV-infected soybean.

Fig. 18. A tubule containing virus-like particles is located in the plasmodesma (area "a"), both of which are associated with the cell wall protrusion. The dark line is the boundary between the nucellus and the integument.

X 11,900

Fig. 19. Higher magnification of area "a" of Fig. 18 showing a tubule containing virus-like particles associated with the plasmodesma and the protruded cell wall.

X 117,500

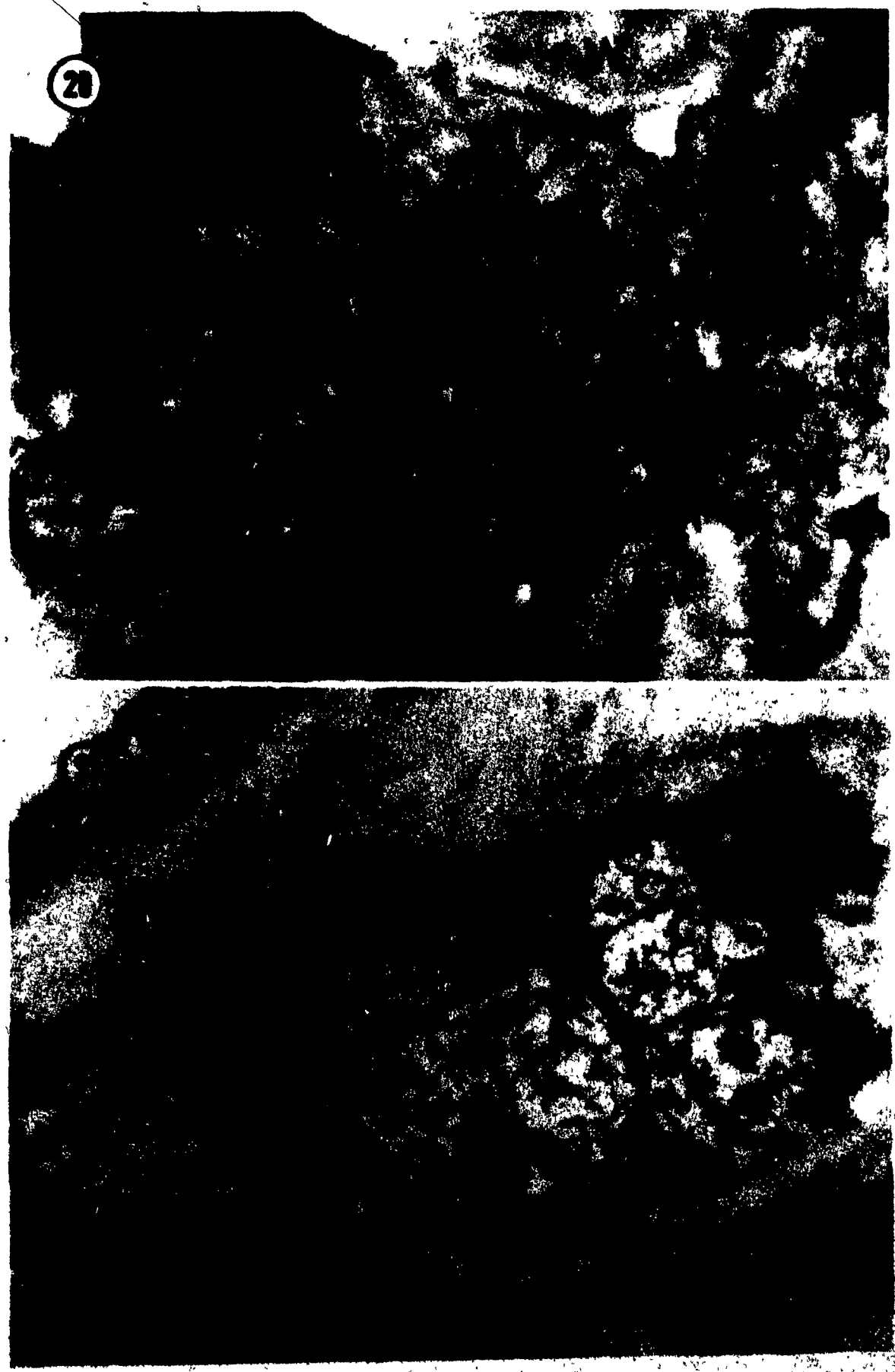
Figs. 20-21. Nucellus in an ovule from TRSV-infected soybean.

Fig. 20. A degenerating nucellar cell containing virus-like particles adjacent to the megagametophyte.

X 43,500

Fig. 21. A group of virus-like particles is located in a degenerating nucellar cell adjacent to the megagametophyte.

X 51,300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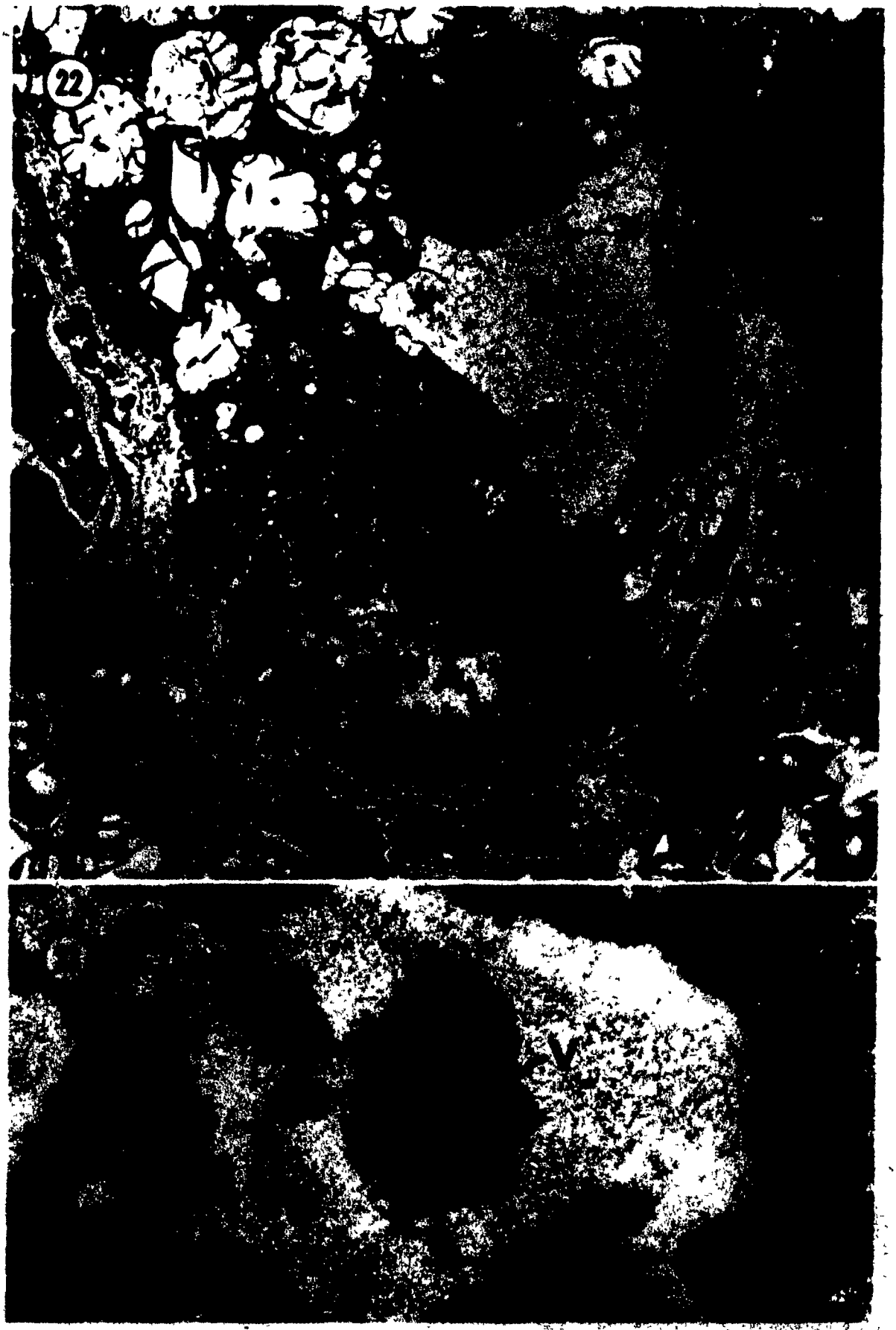
Figs. 22-23. A megagametophyte of TRSV-infected soybean.

Fig. 22. A virus-like crystal is located in the embryo sac wall (area "a").

X 3,500

Fig. 23. Higher magnification of area "a" of Fig. 23, showing the embedded virus-like crystal.

X 118,500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Figs. 24-26. A megagametophyte in an ovule of TRSV-infected soybean.

Fig. 24. Virus-like crystal is located in the synergid cell wall (area "a") and a few groups of virus-like particles are located in the embryo sac wall (area "b").

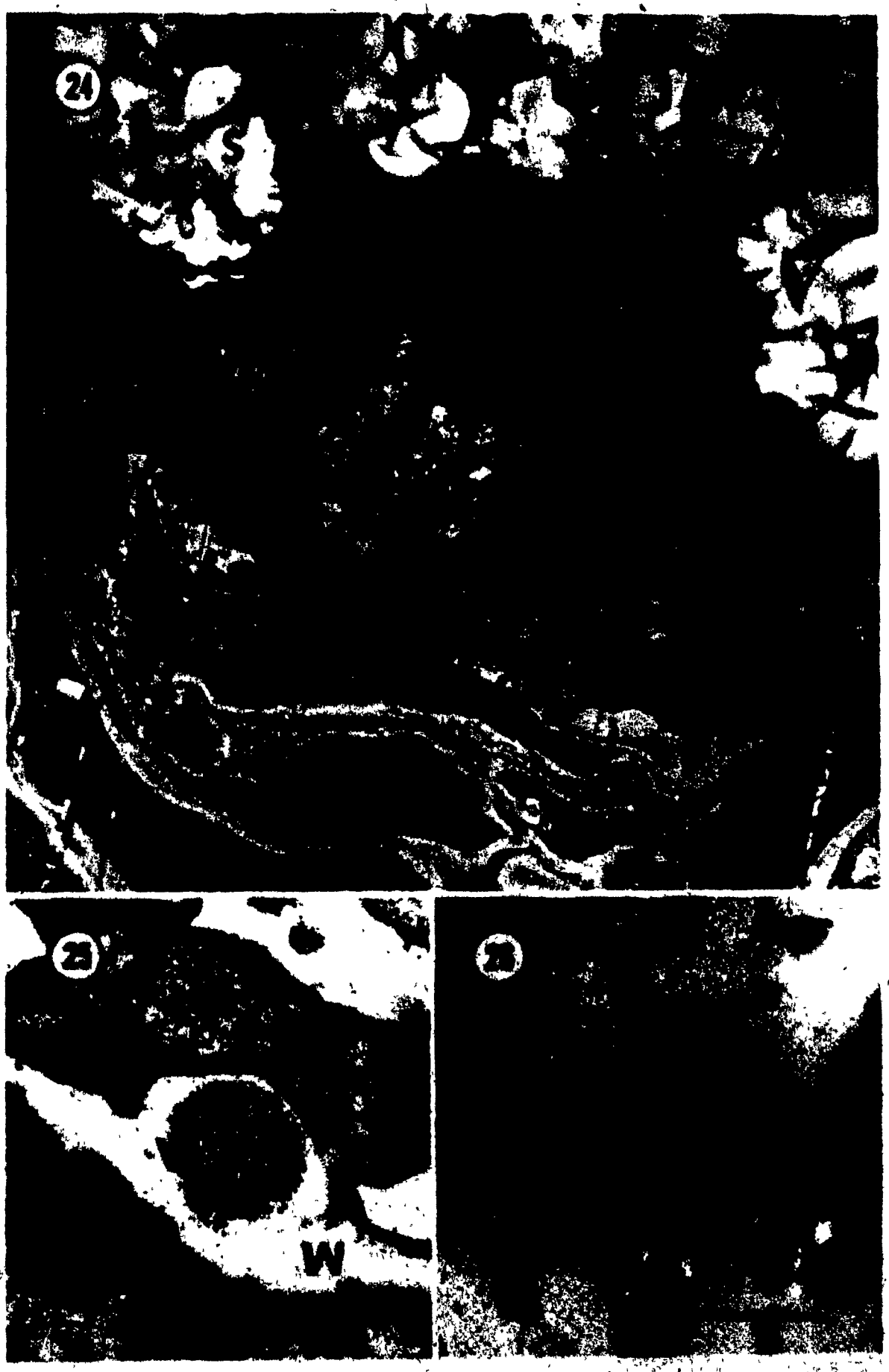
X 7,500

Fig. 25. Higher magnification of area "a" of Fig. 24. There is no evidence of a membrane present.

X 62,000

Fig. 26. Higher magnification of area "b" of Fig. 24. Groups of virus-like particles in the amorphous matrix are trapped in the embryo sac wall.

X 66,000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Figs. 27-29. A megagametophyte in an ovule of TRSV-infected soybean.

Fig. 27. Virus-like particles are located in the wall between the egg and synergid cells (areas "a" and "b"). The nuclei of both cells are visible.

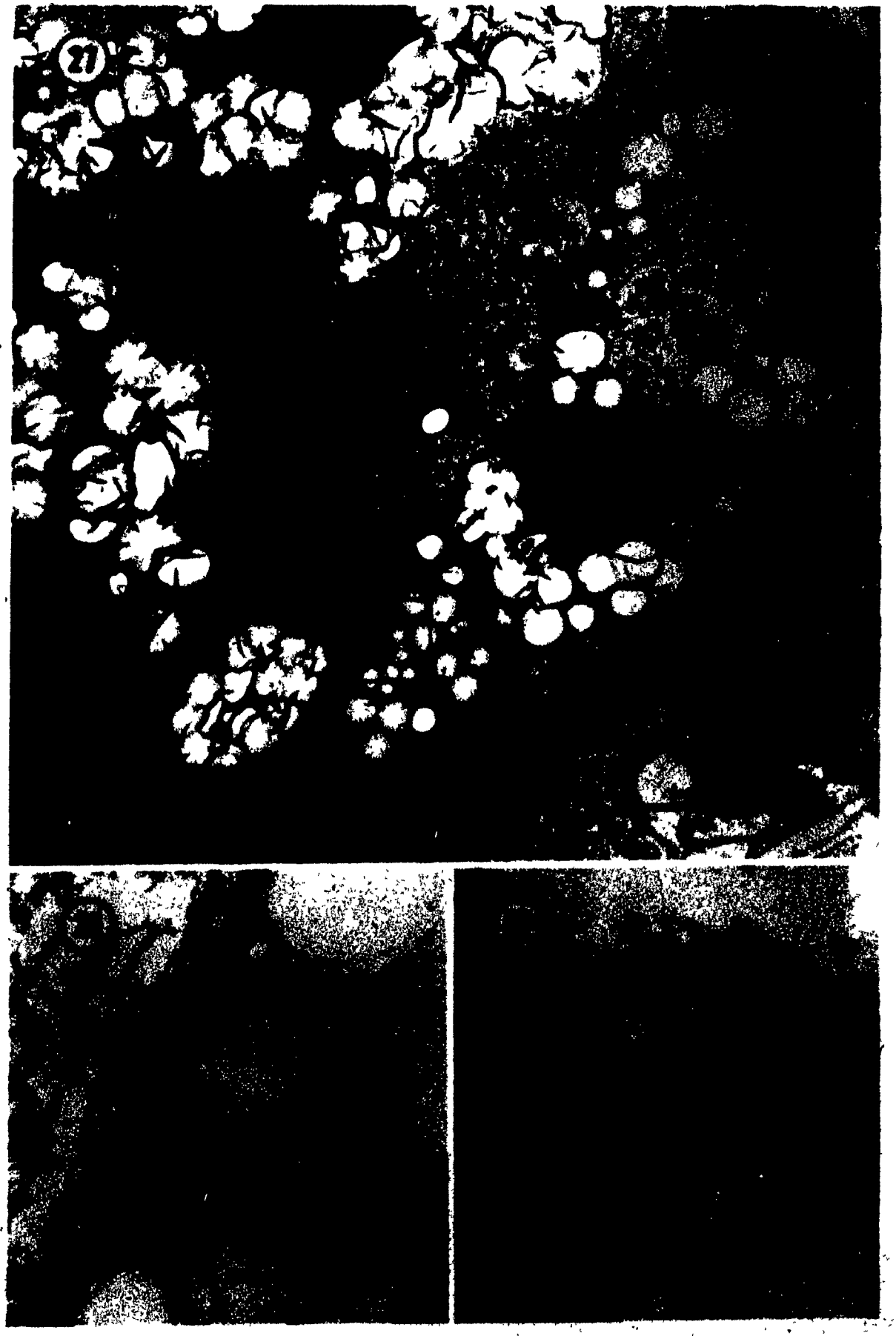
X 7,300

Fig. 28. Higher magnification of area "a" of Fig. 27, showing two groups of virus-like particles

X 35,000

Fig. 29. Higher magnification of area "b" of Fig. 27, showing three virus-like particles in a plasmodesma.

X 105,500



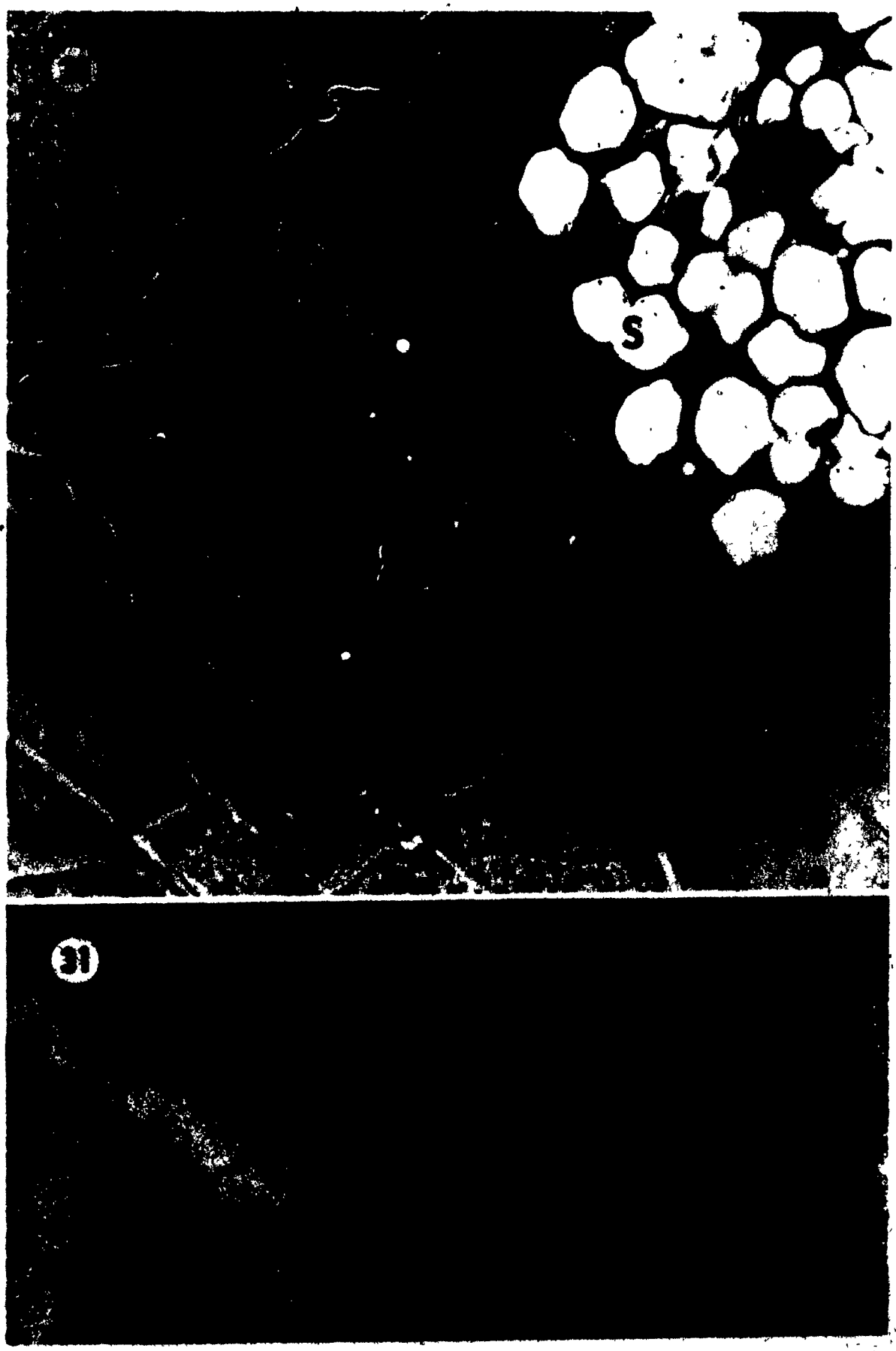
Figs. 30-31. A megagametophyte in an ovule of TRSV-infected soybean.

Fig. 30. Virus-like crystals are located in the antipodal cell (area "a"). The central cell contains a large number of starch granules.

X 8,200

Fig. 31. Higher magnification of area "a" of Fig. 30 showing hexagonally arranged virus-like crystals.

X 61,000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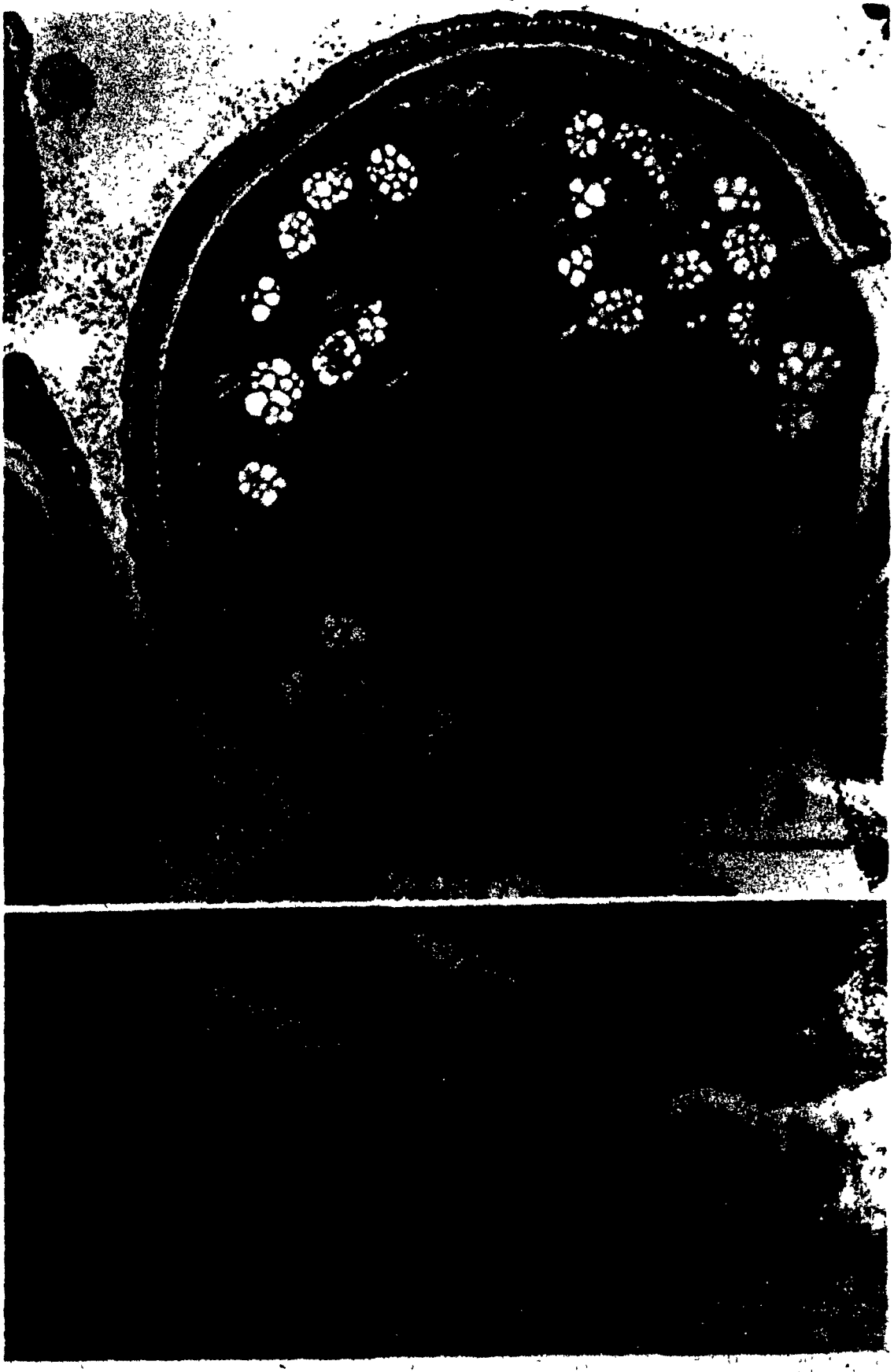
Figs. 32-33. Pollen from healthy soybean.

Fig. 32. A pollen grain showing a prominent vegetative nucleus, starch granules, and grey bodies. The generative cell is visible. The intine is electron-transparent and has a relatively smooth inner surface.

X 5,600

Fig. 33. A portion of the wall of a pollen grain shown in higher magnification. It shows some trapped membrane-bounded amorphous matrix (arrow).

X 35,500



Figs. 34-36. TRSV-infected pollen grain.

Fig. 34. Virus crystals (arrows and squares) are embedded in the intine. Grey bodies and starch granules are visible in the cytoplasm.

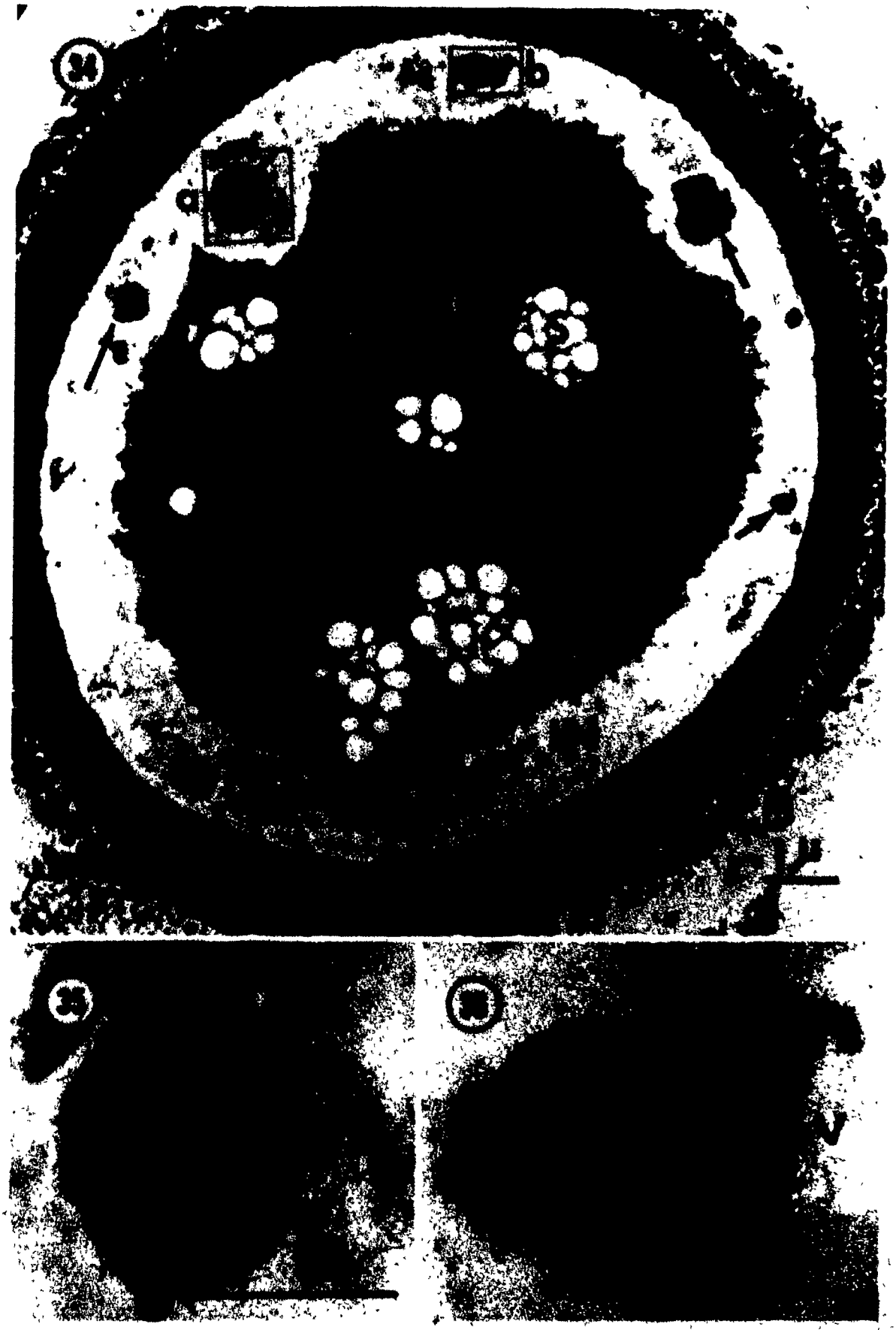
X 16,000

Fig. 35. Higher magnification of area "a" of Fig. 34, showing membrane-bounded virus-like crystals.

X 68,000

Fig. 36. Higher magnification of area "b" of Fig. 34, showing membrane-bounded virus-like crystals.

X 87,500



Figs. 37-38. TRSV-infected pollen grain.

Fig. 37. Virus-like crystal located in the cytoplasmic protrusion in the intine of an infected pollen grain.

X 136,500

Fig. 38. Virus-like crystal embedded in the generative cell wall.

X 38,500

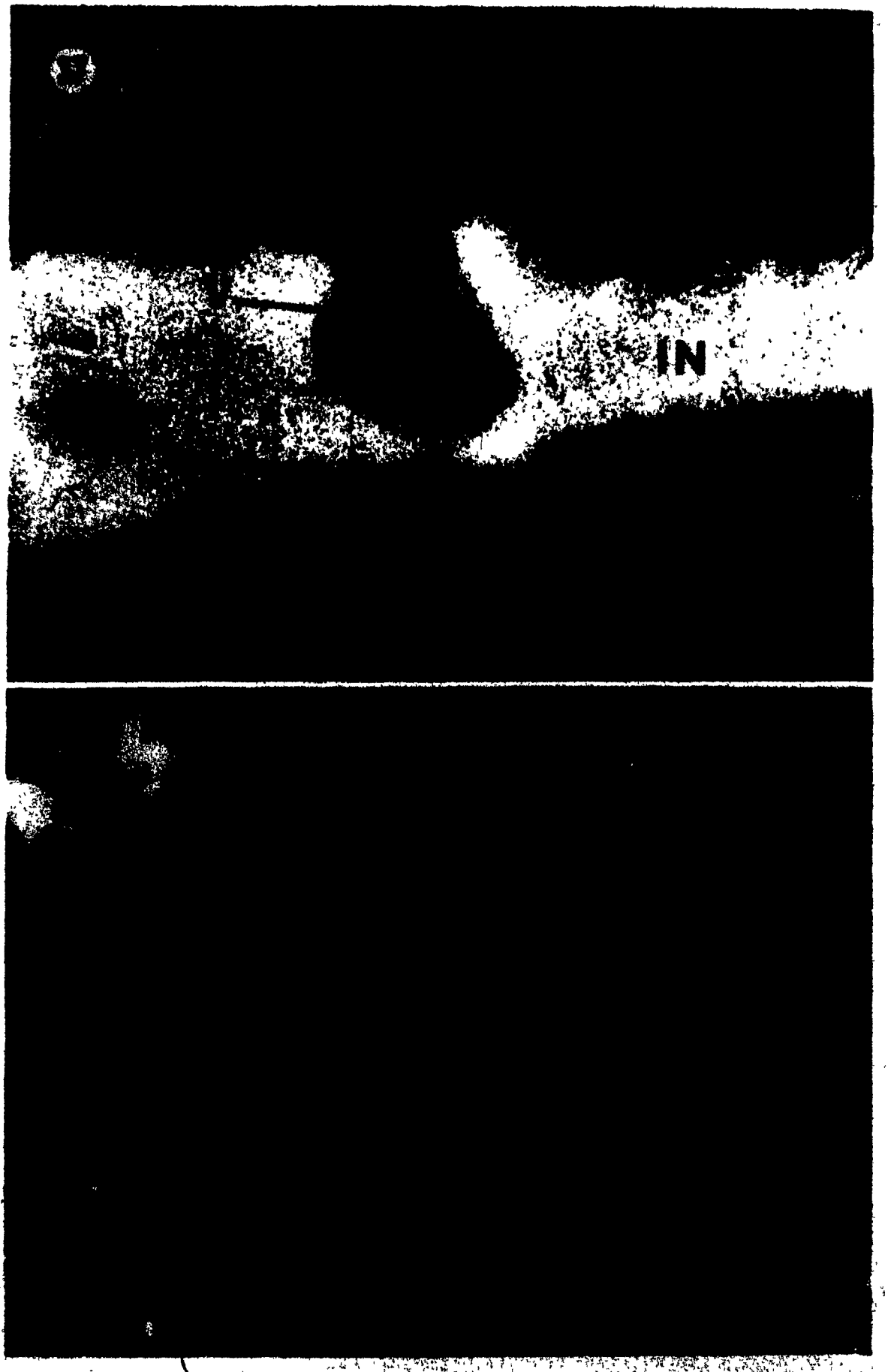

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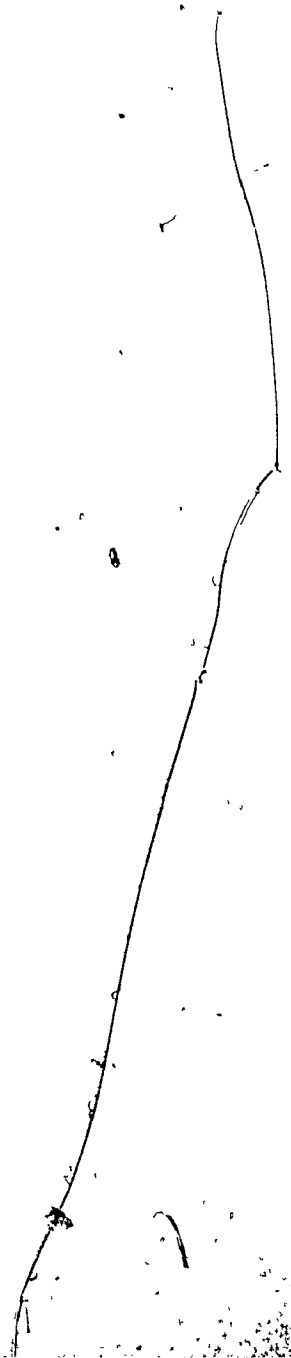
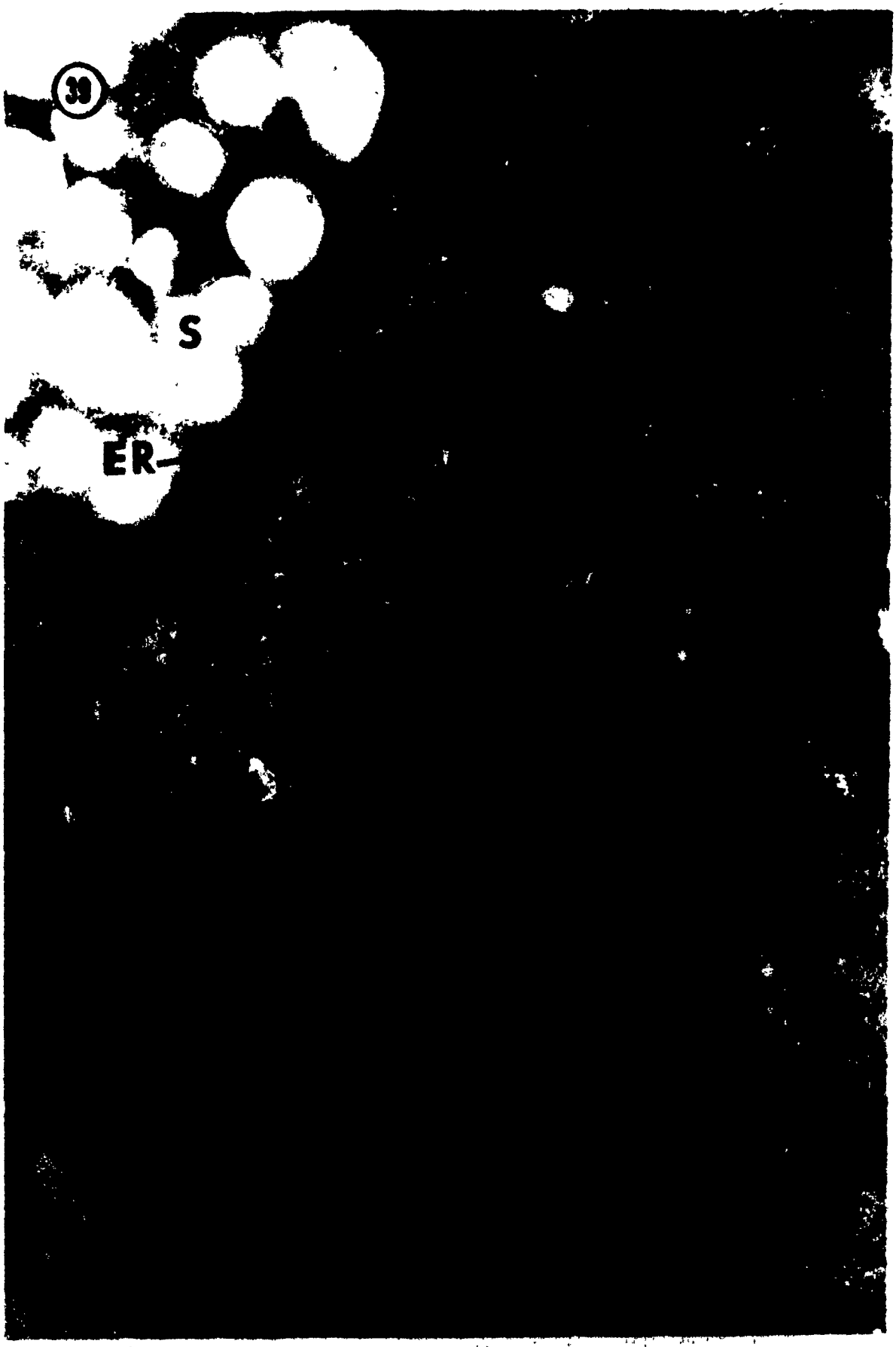
Fig. 39. TRSV-infected pollen grain.

A membrane-bounded virus-like crystal is located in the generative cell of an immature infected pollen grain. Patches of electron transparent wall materials (arrows) are deposited around the generative cell.

X 48,500

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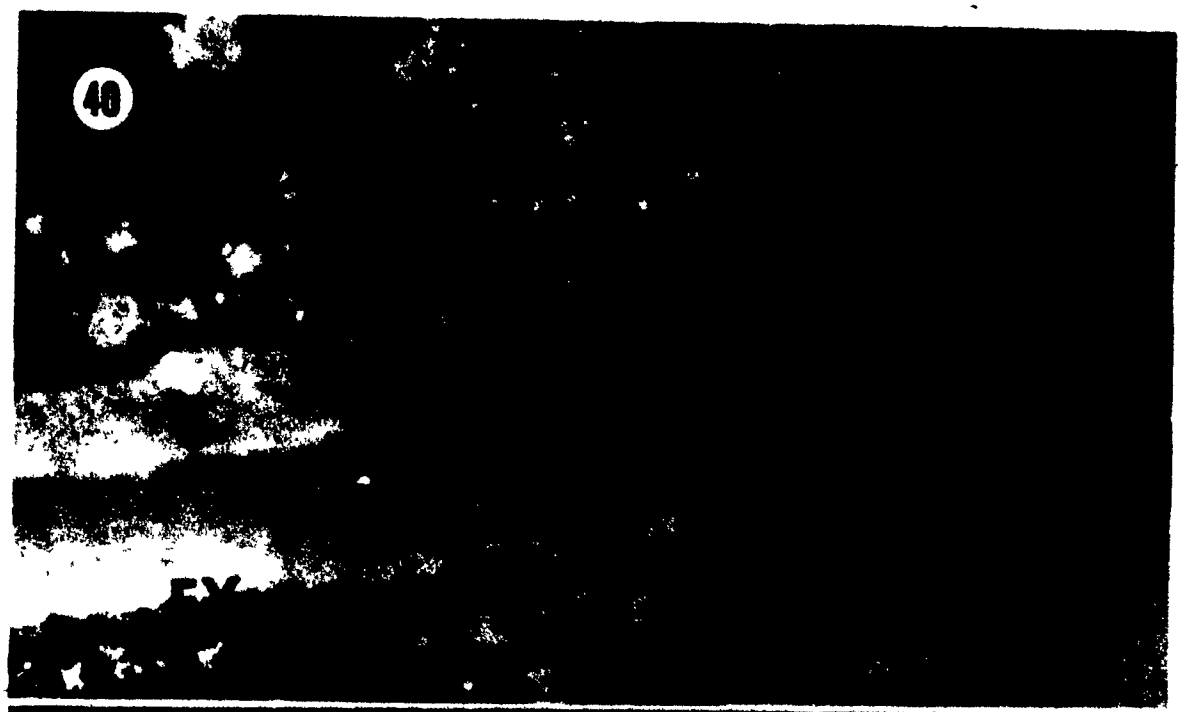
Figs. 40-41. TRSV-infected pollen grain.

Fig. 40. Virus-like crystals are embedded in the intine of an infected pollen grain. The section was stained with protein stain.

X 35,500

Fig. 41. A germ tube originating from TRSV-infected pollen grain showing virus-like crystals and a nucleus located in a thick electron-transparent band (arrow). The electron-transparent band is thought to be the transformed generative cell wall before the onset of nuclear division in the generative cell. The section was stained with protein stain.

X 20,500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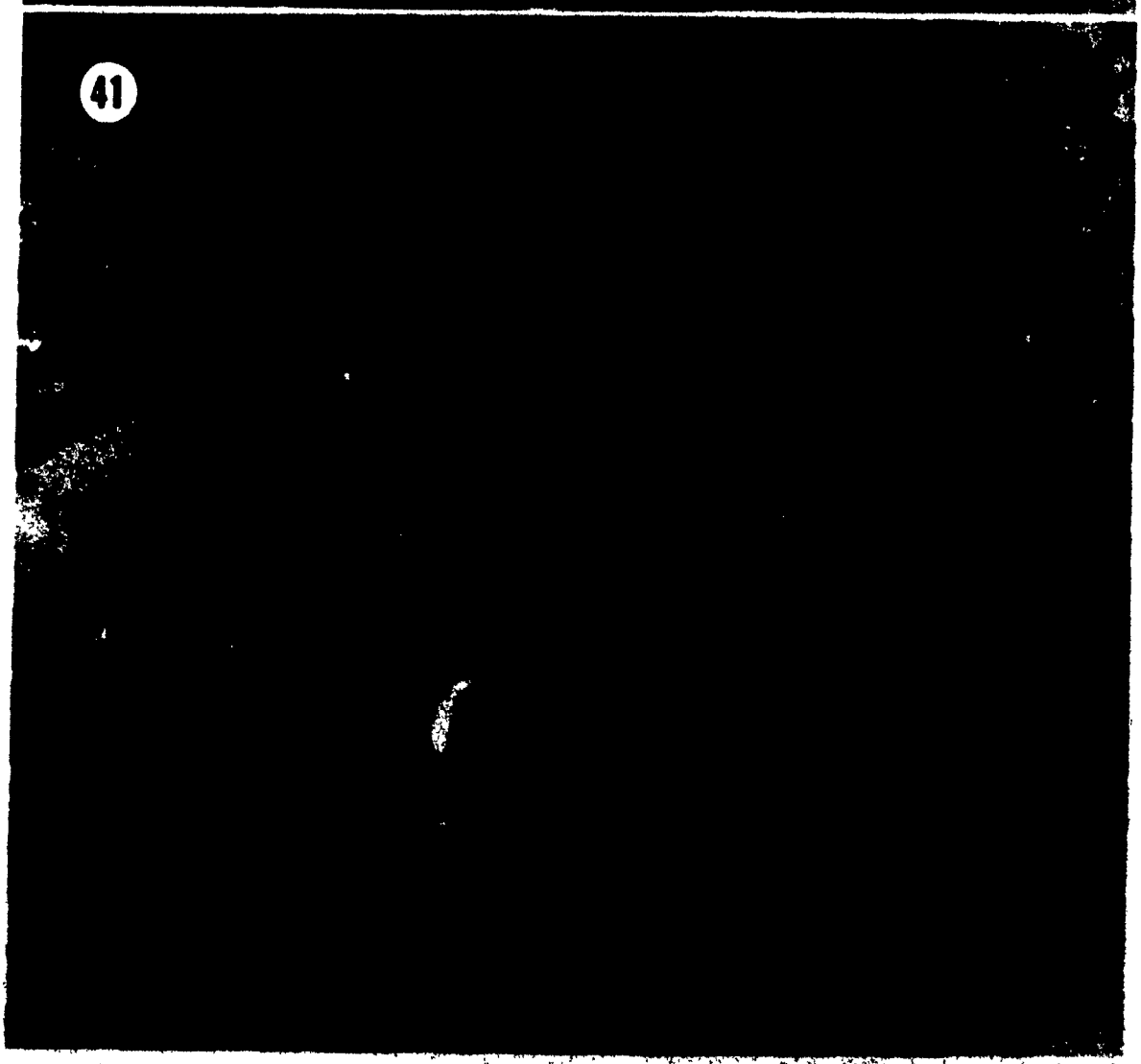
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## 4.4. DISCUSSION.

Various degrees of seed yield reduction caused by TRSV have been reported in the literature (Athow and Bancroft, 1959; Athow and Laviolette, 1961; Crittenden et al., 1966). Such losses could be due to a) reduction in number of ovules per ovary and b) reduction in pollen number, viability, and germination capacity.

The data obtained has shown no evidence of decreasing number of ovules per ovary in TRSV-infected soybean. These ovules were capable of forming seeds if the eggs were fertilized. This was supported by the fact that more multiple-seeded pods were produced when infected flowers were crossed with healthy pollen than when infected flowers were self-pollinated. The majority of the self-pollinated pods produced in the infected plants were single-seeded, with the seed being located at the apical end of the pod. These results are in agreement with the report of exceptional high incidence of outcrossing between TRSV-infected soybean and pollen from neighbouring healthy soybeans under field conditions and the negligible outcrossing in the reverse direction (Brim et al., 1964; Athow, personal communication). The results reported here suggest that the reduction of pollen number, viability and germination capacity is most likely the cause of poor seed set in TRSV-infected soybean. Valleau (1932, 1939, and 1941) reported that TRSV infection caused pollen abortion in tobacco. Some strains could cause complete sterility of pollen and seeds were produced only when infected flowers were crossed with healthy pollen. A similar observation was reported in Datura stramonium infected with Datura quercina virus.

The germination tests and pollen counts reported in this section show that TRSV caused abortion of pollen grain development. Thus infected flowers produced fewer pollen grains and the resulting pollen germinated poorly. Although germination of pollen under artificial conditions is not as good as *in vivo*, the observed germination and lengths of germ tubes *in vitro* are good indications of pollen viability (Rosen, 1968).

The majority of the self-pollinated flowers produced single-seeded pods in infected plants suggesting that inferior pollen grains from TRSV-infected anthers were unable to produce germ tubes of sufficient length to reach those ovules beyond the apical ovules within the critical period of time. A few multiple-seeded pods were produced by the infected plants and this, perhaps, was an indication of an erratic distribution of the virus in the pollen, some of which could have escaped infection. Thus it is possible that the number of ovules that could develop into seeds in each ovary would depend upon the proportion of pollen grains that had escaped infection in the flower. The number of such pollen grains in infected flowers that produced single-seeded pods would be less than those in flowers that produced multiple-seeded pods. Flowers that produced no viable pollen would naturally be shedded. On the other hand, these infected flowers would be likely to set seeds if they were accessible to healthy pollen. Multiple-seeded pods were produced in cross-pollination experiments and a high incidence of outcrossing occurred from healthy to infected plants in nature (Brim *et al.*, 1964). The poor quality of pollen from TRSV-infected plants has been implicated in the unsuccessful crossing of healthy plants with infected pollen (Athow, personal communication).

Virus-like particles were found in several locations within pollen from infected flowers. The most common location was in the intine but they were also detected in crystalline arrays in the cytoplasm of the generative cell and in the wall of the generative cell. No tubules containing virus-like particles were seen in such pollen.

Virus-like particles have been observed both in aggregates and in tubules in every part of the ovule, namely, the integuments, the nucellus and the embryo sac. The tubules were similar to those reported in bean (Davision, 1969; Crowley et al., 1969) and in tobacco (Roberts et al., 1970) infected with TRSV. No such tubules were observed in thin sections from healthy plants. Since both ends of the tubules were found in the cytoplasm of the neighbouring cells, it was taken as evidence of cell to cell movement of the virus (Davison, 1969). In addition to those in plasmodesmata, tubules are often found in cell wall protrusions. The significance of the protrusions containing virus is not known. They would be ineffective in "walling off" the virus because virus-like particles were found in the vacuoles and in plasmodesmata between cells. Other virus diseases known to produce tubules and cause protrusions of cell walls are cherry leaf roll (Jones et al., 1973), bean pod mottle (Kim and Fulton, 1971), cowpea mosaic (Van der Scheer and Groenewegen, 1971), maize rough dwarf (Gerola and Bassi, 1966), and cauliflower mosaic (Conti et al., 1972).

Virus-like crystals were found in the vacuoles of a megagametophytic cell, a nucellar cell and in sieve tubes in the integument of infected ovules. They were also found embedded in the megagametophytic cell walls. This is the first observation of virus-like particles in the embryo sac of a plant.

The significance of virus crystals embedded in the walls of both micro- and mega-gametophytic cells is not known. It is interesting that this kind of virus-host relationship was not found in the mother plant tissue. It would be unique if it is not present in the young sporophytic cell walls.

The results of cross-pollination experiments indicate that the seed transmission rate is determined by the infection of ovules. Although infected plants produced more seeds when crossed with healthy pollen than when self-pollinated, the percentage of seed transmission was as high as in seeds of the latter. The fact that seed transmission of TRSV in soybean was not related to the position of the pods on the stem and the position of seeds in the pods (Athow and Laviolette, 1962) also suggested that megagametophyte infection was the factor contributing to seed transmission. This was confirmed by the presence of infectious virus and virus-like particles in infected ovules, indicating the likelihood of a high frequency of megagametophyte infection.

The role of TRSV-infected pollen in the seed transmission of TRSV is not important because of its poor germination capacity and slow germ tube elongation. Although virus-like crystals are found in the pollen grains, most of them are embedded in the intines. These virus crystals are likely to be left behind when the cytoplasm migrates into the germ tube during germination. The virus-like particles that are found in the generative cells and possibly those in the vegetative cell cytoplasm may be liberated together with the sperm nuclei, thus causing embryo infection, provided that the infected germ tube enters the embryo sac before germ tubes from non-infected pollen. This could be the mechanism of pollen transmission

of those viruses reported to be pollen-transmitted, although virus particles in situ in pollen have been reported only in barley infected with BSMV (Gardner, 1967). However, it is unlikely that seed-borne TRSV in self-pollinated seeds originated from infected pollen grains since the cross-pollination experiments reported here showed that such pollen grains had lost considerable viability.

The fact that TRSV can be found in the gametes of the infected host is most likely associated with its capacity to invade meristematic tissue (Crowley et al., 1969; Roberts et al., 1970). TRSV would thus be able to infect both microspore and megaspore mother cells before the cytoplasmic connections are sealed off during meiosis (Rodiewicz, 1970; Schwab, 1971; Heslop-Harrison, 1966; Echlin and Gowin, 1968). The viability of the pollen is adversely affected by TRSV infection probably because of the shortage of nutrients; whereas the continuous supply of nutrients from the mother plant would allow the infected megagametophytes to survive.

The high rate of megagametophyte transmission of TRSV is most likely related to the high rate of infection of megaspore mother cells. It would be difficult for the virus to infect a megaspore mother cell in each ovule, unless it invades indiscriminately all meristematic cells or some mechanism exists to direct the virus towards the megaspore mother cell. A specific directional movement of TRSV towards the megaspore mother cell is unlikely to occur because not all the seeds were infected. Mass invasion of the meristematic cells by TRSV is more likely. Thus, a high rate of seed transmission could occur and yet some of the seeds could escape infection. Virions that were blocked from entering the megaspore mother

cell would remain outside and would probably be inactivated, although not necessarily. Erratic distribution of other viruses in meristematic cells may explain various rates of seed transmission which have been reported for various host-virus combinations. Presumably, slow meristem invaders enter ovules after the plasmodesmata of the megaspore mother cells are sealed off. Unless those viruses can survive throughout seed formation, maturation and storage in or on the seed coats, as is the case with TMV, they are not likely to be seed transmitted. Inactivation of virus in seed coats during maturation has been reported for cowpea chlorotic mottle virus (Gay, 1969), pea streak virus (Ford, 1966) and for southern bean mosaic virus (McDonald and Hamilton, 1972).

As mentioned in the literature review, BSMV, SBMV, and TRSV could apparently be seed-transmitted even if the infection occurred after flowering (Eslick and Afanasiev, 1955; Crowley, 1959). The fact that BSMV was only found in the seeds from late tillers (Timian, 1967; Carroll, 1972), and that infectious SBMV could not be recovered from embryos (McDonald and Hamilton, 1972) implies that the earlier reports of embryo invasion may be incorrect.

Experiments on embryo invasion by TRSV (Crowley, 1959) have not been repeated. If the report is correct, then the virus would have to be transported into the nucellus from the point of infection and through the thick embryo sac wall in order to invade the embryo; it would also mean that essentially 100 percent seed transmission should have occurred in plants infected at a very young stage, since those virus particles located in the degenerating cells neighbouring the embryo sacs would thus pass through the barrier to infect the embryos. However, this did not occur

because many seeds did not contain the virus. Probably, the infectivity detected by Crowley (1959) was from late flowers of the racemes. TRSV is able to invade bean root meristems 3 days after inoculation of primary leaves (Atchinson and Franki, 1972). Rapid invasion of TRSV into meristematic tissue would possibly lead to infection of megaspore mother cells in late flowers of the racemes, which were not developed when the first flower flowered.

## 5. SUMMARY

1. The ratio of bottom component (B) to middle component (M) of TRSV in cotyledons was relatively stable in TRSV-infected soybean seeds and seedlings germinated from them, whereas the ratio in leaves of mechanically inoculated seedlings increased and approached infinity with age of infection.

2. TRSV-like particles were detected in the intine of the pollen wall, the cytoplasm and wall of the generative cell, the integument, the nucellus, the embryo sac wall and in megagametophytic cells.

3. TRSV infectivity could be detected in the embryo and testa of young seed, but only in the embryo of mature seed.

4. Anthers from TRSV-infected soybean produced less pollen than anthers from virus-free soybean ; pollen from TRSV-infected soybean was poor in germination capacity and its germ tubes elongated slowly compared to pollen from virus-free soybean.

5. The number of ovules per ovary was not reduced by TRSV infection. Crossing TRSV-infected flowers with healthy pollen resulted in the production of more multiple-seeded pods than self-pollinated flowers of the same plants. The percentages of TRSV infection in seeds from cross-pollinated and self-pollinated flowers were essentially equal.

6. Most flowers of healthy plants failed to set seeds when crossed with pollen from infected plants. There was no evidence of TRSV infection in seeds either from self-pollinated or from cross-pollinated flowers of healthy plants.

7. It is concluded that infection of megagametophytes is the main factor contributing to seed transmission of TRSV in soybean.

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