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AN ULTRASTRUCTURAL STUDY OF OLPIDIUM BRASSICAE AND ITS TRANSMISSION OF TOBACCO NECROSIS VIRUS

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INTRODUCTION

In 1958 GROGAN et al. and FRY showed an association between *Olpidium brassicae* (Wor.) Dang. and the big-vein disease of lettuce. In the following years other data were published to prove that the big-vein disease is caused by a graft-transmissible agent, the lettuce big-vein virus (BVV), and that the association could be interpreted as a virus – vector relationship (CAMPBELL et al., 1961; CAMPBELL, 1962; CAMPBELL et al., 1962; TOMLINSON and GARRETT, 1962; CAMPBELL and GROGAN, 1963). BVV has still not been characterized morphologically (LIN et al., 1970). During the same period *O. brassicae* was found to be the vector for two other plant viruses, tobacco necrosis virus (TNV) (TEAKLE, 1960; TEAKLE, 1962) and tobacco stunt virus (HIDAKA, 1960; HIDAKA and TAGAWA, 1962; HIRUKI, 1965).

Much research has been carried out to establish the nature of the relationship between *Olpidium* and the above plant viruses and the general aspects of this relationship have been elucidated. Thus it is known which viruses are carried inside the zoospores and which outside, which viruses are acquired by the fungus inside the host plant and which outside, and how specific the vector role is with regard to host plant and fungus isolate (TEAKLE and GOLD, 1963; CAMPBELL and GROGAN, 1964; KASSANIS and MACFARLANE, 1964a; CAMPBELL and FRY, 1966; FRY and CAMPBELL, 1966; HIRUKI, 1967; SMITH et al., 1969). However, the exact mode of virus transmission is not known and no satisfactory explanation has been offered for the vector specificity. This is particularly true for the *Olpidium* – TNV relationship (TEAKLE and HIRUKI, 1964; KASSANIS and MACFARLANE, 1965; MACFARLANE, 1968b).

Since TNV transmission and vector specificity were expected to have ultrastructural aspects, an electron microscope investigation was undertaken. The results are laid down in this publication, although parts have been published previously elsewhere (TEMMINK and CAMPBELL, 1968; TEMMINK and CAMPBELL, 1969a, b, c; TEMMINK et al., 1970). In order to study the nature of an interaction between different organisms (including viruses) with the electron microscope, it is a prerequisite to have or to obtain information on the ultrastructure of each of the separate organisms involved. Only on that basis can it be decided whether any interactions occur between the organisms and what the nature of these interactions is.

The first part of this publication will, therefore, consist of a compilation of ultrastructural data on each of the organisms involved in the relationship under investigation. The second part of the thesis will describe results of the study of the interaction between host plant, fungus vector, and transmitted virus and compare the data and conclusions with those of other investigators.

PART I

1. LITERATURE REVIEW

1.1. OLPIDIUM BRASSICAE AND ULTRASTRUCTURE OF FUNGI

Olpidium brassicae (Wor.) Dang. is a chytrid fungus described by WORONIN (1878) and assigned to the genus *Olpidium* by DANGEARD (1886). Although some controversy exists concerning the taxonomy of *Olpidium brassicae* (GROGAN and CAMPBELL, 1966; MACFARLANE, 1968a), we have chosen to maintain this name. Its morphology, life cycle, and taxonomy have been studied extensively (SAMPSON, 1939; JACOBSEN, 1943; KOLE, 1954; SAHTIYANCI et al., 1960; SAHTIYANCI, 1962). As a summary, the life cycle in its conventional form is represented in Plate 1-1 with light micrographs illustrating some of the stages. Question marks indicate where observations by us and other workers cast some doubt on the correctness of the life cycle in its present form.

The publication by SAHTIYANCI (1962) has most often served as our reference. Although she classified the lettuce isolate of *O. brassicae* as *Pleotrachelus virulentus* on taxonomically doubtful grounds, her description of the developmental stages in the life cycle generally was substantiated by our observations. Nevertheless there are some differences between her observations and ours, or between the interpretations of them. Probably the most important difference is concerned with whether the resting spore represents the sexual part of the life cycle. We did not find any indications for a sexual phase in the life cycle of our lettuce isolate (TEMMINK and CAMPBELL, 1969a). Fusion of zoospores has not been seen by us, but zoospores with two flagella and approximately twice the body volume occur occasionally. The number of these 'double' zoospores, however, does not seem to increase with time after sporulation. Some investigators have expressed the same view (TOMLINSON and GARRETT, 1964; GARRETT and TOMLINSON, 1967), whereas others report data in support of a sexual phase in *O. brassicae* (KOLE, 1954; SAHTIYANCI, 1962) and in other *Olpidium* species (KUSANO, 1912; BARR, 1968).

In many light microscopic studies of *Olpidium* some attention has been given to pathogenicity (VAN DER MEER, 1926; JACOBSEN, 1943; SAHTIYANCI, 1962; BRITTON and ROGERS, 1963). The lettuce isolate of *O. brassicae* penetrates the root hairs and epidermis cells, preferentially in the zone of elongation, but also in older parts of the root at higher inoculum densities. Cortex cells are seldom infected. Callus tissue, on the other hand, is readily infected by the lettuce isolate (KASSANIS and MACFARLANE, 1964b). Host roots as well as some non-host roots seem to have an attractive, or at least a trapping effect on zoospores in suspension. Infection of the root has no clear pathogenic effect on the host plants, although infected cells may be more susceptible to other pathogens and heavy infection may cause a slight decrease in overall growth of the plant (GROGAN

and CAMPBELL, 1966). The lettuce isolate of *O. brassicae* has a wide host range (JACOBSEN, 1943; TOMLINSON and GARRETT, 1964; HIRUKI, 1967), including some monocotyledons and many genera of dicotyledons. A mustard isolate of *O. brassicae* in Germany has, according to SAHTIYANCI (1962), a narrower host range than the lettuce isolate and seems able to penetrate into the first four layers of the root cortex. Results of work at this laboratory (Department of Plant Pathology, U.C.D.), however, suggest a wider host range for a mustard isolate from California than was reported for the German isolate (TEMMINK et al., 1970). Whether the two mustard isolates were also different in other respects, was not determined.

Studies on other root-infecting Phycomycetes indicate that the attraction and trapping by exudates and the preference for the elongation zone of the root are very common characteristics of these fungi (ZENTMEYER, 1961; CUNNINGHAM and HAGEDORN, 1962; ROYLE and HICKMAN, 1964; ROVIRA, 1965; HICKMAN and HO, 1966; RAI and STROBEL, 1966; HO and HICKMAN, 1967b). A possible exception to this rule may be the infection of monocotyledon roots by *Pythium* (KRAFT et al., 1967). In addition, there is evidence that in some cases exudates act as inhibitors of encystment (TURNER, 1963) and that exudates of some non-hosts attract zoospores as much or more than those of susceptible hosts (GOODE, 1956; TURNER, 1963).

Until recently (TEMMINK and CAMPBELL, 1968 and 1969a, b; LESEMANN and FUCHS, 1970a, b), little was known about the ultrastructure of *Olpidium*, except that the shadowed flagellum had the usual 9 + 2 fibrillar structure (MANTON et al., 1952). Although both the zoospores and the thalli had been studied in the light microscope (JACOBSEN, 1943; KOLE, 1954; SAHTIYANCI, 1962), phase contrast as well as staining techniques led to conflicting interpretations of the organelles that were seen. On the other hand, a number of Phycomycetes had been studied with the electron microscope (MOORE, 1965; FULLER, 1966; BRACKER, 1967). Some of the information obtained in these studies seems to apply generally to all or most lower fungi, like the presence of mitochondria, glycogen, endoplasmic reticulum, vacuoles, and lipid bodies, the nature of the mitotic division, and the relation between the kinetosome and centriole. In other respects the different genera proved to be quite different, e.g. the structure of the rhizoplast and other parts of the kinetid (CANTINO et al., 1963; FULLER and REICHLE, 1965; REICHLE and FULLER, 1967; FULLER and REICHLE, 1968; REICHLE, 1969; TEMMINK and CAMPBELL, 1969a), presence or absence of intranuclear microtubules (BERLIN and BOWEN, 1965; SHATLA et al., 1966; HEATH and GREENWOOD, 1968; ICHIDA and FULLER, 1968; LESSIE and LOVETT, 1968, TEMMINK and CAMPBELL, 1968), nature of zoospore cleavage (CHAMBERS and WILLOUGHBY, 1964; RENAUD and SWIFT, 1964; BRACKER, 1966; COLHOUN, 1966; GAY and GREENWOOD, 1966; HOHL and HAMAMOTO, 1967, BRACKER, 1968; LESSIE and LOVETT, 1968; TEMMINK and CAMPBELL, 1968), and the mode of infection (BERLIN and BOWEN, 1964; TEMMINK and CAMPBELL, 1969b; LESEMANN and FUCHS, 1970a, b).

A matter of special interest is the mode of withdrawal of the axoneme and this

seems to defy generalization even within the series of the Chytridiomycetes, which are posteriorly uniflagellate Phycomycetes (KOCH, 1961; KOLE, 1965; CRUMP and BRANTON, 1966; HO and HICKMAN, 1967a; UMPHLETT and OLSON, 1967; KOCH, 1968). Different modes have been described or suggested (KOCH, 1968). They belong basically to the 'wrap-around' or the 'reeling-in' mode, both of which have been reported in *Blastocladiella* (CANTINO et al., 1963; FULLER, 1966). In the 'wrap-around' mode the axoneme is coiled around the body of the zoospore in such a way that subsequent uptake of the axoneme happens by fusion of axonemal sheath and zoospore body plasmalemma. In the 'reeling-in' mode of axonemal retraction the axoneme is pulled inside the body of the zoospore at one point with or without concurrent formation of a flagellar vesicle. The differences in axonemal withdrawal within the Chytridiomycetes made it impossible to predict how *Olpidium* zoospores behave in this respect. HIRUKI (pers. comm.) claims to have evidence for the 'wrap-around' mode, whereas our interpretation suggests the 'reeling-in' mode (TEMMINK and CAMPBELL, 1969b).

1.2. ULTRASTRUCTURE OF PLANT ROOTS

This part of the literature review will be limited to data concerning those tissues of the plant that may be affected by infection with *Olpidium* or TNV, that is essentially to the epidermis and the outer cortical layers of the seedling root. Most of the electron microscope studies made on the outer cell layers of roots were either primarily concerned with cell wall structure (DAWES and BOWLER, 1959; SIEVERS, 1963; NEWCOMB and Bonnett, 1965) and growth of root hairs (CORMACK, 1962; NEWCOMB and BONNETT, 1965; BONNETT and NEWCOMB, 1966), or they were aimed at describing the cell structure in the root meristem (WHALEY et al., 1960; PORTER and MACHADO, 1960; LEDBETTER and PORTER, 1963; NEWCOMB, 1967), an area apical to the zone infected by *Olpidium*. Furthermore, studies on a certain plant species, grown under certain conditions (SCOTT et al., 1958; SCOTT, 1963 and 1965) are of relatively little importance for information on other species, grown under different conditions because of the variety of possible structures in different species and the great influence of growth conditions on the same tissues (VON GUTTENBERG, 1968). Only one paper is directly concerned with the ultrastructure of organelles in the epidermis and cortex of the root hair forming zone of radish seedling roots (BONNETT and NEWCOMB, 1965). Of special interest is their description of accumulations in the cisternae of the endoplasmic reticulum (ER) and of the polyribosomes.

The epidermis of the root system of dicotyledons serves primarily as an adsorption layer of water and nutrients; it covers the root over a relatively short distance (VON GUTTENBERG, 1968) and its life span is generally measured in days (ESAU, 1965). When the dead epidermal cells are not sloughed off, their walls may become suberized or even lignified. Root hairs are formed acropetally by some of the epidermal cells in an area just behind the elongation zone of the root. The number of trichomes formed and their length seems to depend on several

factors like the moisture conditions and the rate of growth; their distance from the apex depends on the rate of growth of the root (CORMACK, 1962; VON GUTTENBERG, 1968). They die and slough off with the epidermis. Root hairs grow at the tip, where a concentration of cytoplasm occurs (NEWCOMB and BONNETT, 1965) and the Golgi dictyosome seems to be involved in the process (SIEVERS, 1963; BONNETT and NEWCOMB, 1966). Hairless cells elongate much faster than trichoblasts in a short area immediately behind the meristem (AVERS, 1957; CORMACK, 1962); consequently hair cells are much narrower in the root hair forming zone than hairless cells are. The outer wall of the epidermal cell is hardly ever thicker than the other cell walls and it is similar to the root hair cell wall. Chemical composition and ultrastructure of the epidermal wall of several plant species has been investigated (CORMACK, 1962). An inner cellulose layer is covered by an outer mucilage layer of pectins. The outer part of the cellulose layer may be cutinized, but a real cuticle (SCOTT et al., 1958) does not exist (VON GUTTENBERG, 1968). Death of the epidermal cells is generally concurrent with suberization of the subepidermal exodermis layer or layers (VON GUTTENBERG, 1968). In many plants (e.g. *Lactuca sativa*) all cells of the exodermis are elongated and have a suberin layer in their cell walls; in other plants suberinized cells occur beside unsuberinized ones. Sometimes no suberin lamella is apparent, but fatty materials or lignin may be identifiable (ESAU, 1965). In all cases the exodermis serves as a protective layer together with the dead epidermal cells, if the latter do not slough off. There are no intercellular spaces between the cells of the epidermis and the exodermis and sometimes exodermal cells grow out into the epidermal layer (VON GUTTENVERG, 1968).

The parenchymous outer cortical cells are inside the exodermis and at maturity consist of a thin layer of cytoplasm around a large vacuole. Schizogenous intercellular spaces occur between the cells and, depending on the longevity of the cortex, differentiation of some cortical cells may occur.

Few studies have been made of the damage to and the death of root cells. Since our investigation was concerned with the reaction of host plants to parasites, these publications on damage to cells were considered relevant. Cell disruption was shown to occur well in advance of the penetrating fungus in onion roots infected with *Pyrenochaeta terrestris* and the disruption seemed to result from chemical substances excreted by the fungus (HESS, 1969). There is no reason why the compression and death of cortical cells that is caused by formation of lateral roots (BONNETT, 1969), could not also be brought about by other kinds of pressure from inside or outside. Some of the symptoms caused by the treatment of roots with victorin (a 'pathotoxin' produced by *Helminthosporium victoriae*) can also occur under natural conditions in untreated root cells (HANCHEY et al., 1968; HANCHEY and WHEELER, 1969). Other victorin symptoms, such as wall-lesions, were very reminiscent of those described by others as a result of invasion by pathogenic fungi (MCKEEN et al., 1966; EHRLICH et al., 1968; TEMMINK and CAMPBELL, 1968; MCKEEN et al., 1969).

1.3. Tobacco necrosis virus

Tobacco necrosis virus was found in the roots of normal appearing tobacco seedlings in 1935 (Smith and Bald). Much research has been done on the virus and the results have been summarized (Kassanis, 1964). Although it was known almost from the beginning that TNV was soil-borne, it was not until 1960 that a possible vector was reported (Teakle).

TNV has a wide host range but causes only two economically important diseases, tulip necrosis (Augusta disease) (de Bruyn Ouboter and van Slogteren, 1949) and stipple-streak of bean (Bawden and van der Want, 1949). When mechanically inoculated to leaves, it is generally restricted to the inoculated leaves, but sometimes systemic necrosis occurs (van Koot and van Dorst, 1955; Resconich, 1963). In inoculated leaves of some plants it may reach high concentrations without showing symptoms and naturally infected plants may have leaves that contain virus without showing symptoms (Price, 1940 as cited by Kassanis, 1964).

TNV is known to be associated with a smaller sized particle (Pirie et al., 1938; Bawden and van der Want, 1949; Bawden and Pirie, 1950). This so called satellite virus has a very small particle that needs the presence of another activating virus (i.e. TNV) in the same host plant for its complete reproduction (Kassanis and Nixon, 1960 and 1961; Kassanis, 1962). Since the satellite virus was found, much work has been done on its nature and the serological relationship to the different TNV strains (Reichmann, 1964; Kassanis, 1966; Grogan and Uyemoto, 1967; Kassanis and Woods, 1968; Uyemoto, 1968; Uyemoto et al., 1968; Liu et al., 1969; Uyemoto and Grogan, 1969; Kassanis and Phillips, 1970; Rees et al., 1970).

The physico-chemical properties of TNV have been described extensively (Lesnaw and Reichmann, 1969a and b; Roy et al., 1969; Uyemoto and Grogan, 1969; Rees et al., 1970) and need not be repeated here. It is of interest only that TNV has polyhedral particles with a hexagonal outline in negative stain and a diameter of 26–30 nm, depending on the condition of the virus and the method of preparation, whereas satellite virus is polyhedral and approximately 17 nm in diameter (Kassanis, 1966).

2. MATERIALS AND METHODS

The materials and methods described in this chapter are those that were used routinely throughout this investigation (Part I as well as Part II). A number of more special techniques, that were applied incidentally in order to obtain answers to more specific questions, are described in later chapters with the results of their application.

2.1. GROWTH OF OLPIDIUM AND HOST PLANTS

The four virus free, single sporangium isolates of *O. brassicae* used in this study have been described (TEMMINK et al., 1970). They will hereafter be referred to as the lettuce *Olpidium*, the tomato *Olpidium*, the oat *Olpidium*, and the mustard *Olpidium*. The hosts in which these isolates were grown and their maintenance have been described (TEMMINK et al., 1970). An isolate of *O. cucurbitacearum* obtained from H. F. Dias, was maintained in the same way in roots of cucumber (*Cucumis sativus* L. cv. 'National Pickling'). For studies of *Olpidium* infection, seedlings grown in pasteurized quartz sand to facilitate cleaning of roots prior to processing for microscopy, were germinated in small beakers under continuous artificial light at approximately 30 °C for 3–4 days, except tomatoes that were kept for 5–7 days. After inoculation with zoospore suspensions the seedlings were transferred to growth chambers with 14h artificial light per day and a temperature of 18 °C. Sometimes, when a heavy infection was wanted, a few seedlings were inoculated by placing their roots in a zoospore suspension in a petri dish in the dark at room temperature (23 °C). Two-4th after inoculation these seedlings were fixed and processed for electron microscopy, or transferred into sand and incubated for longer periods before fixing.

2.2. VIRUS PURIFICATION

The TNV strains used in this study are: New Zealand strain (NZ-TNV) (CAMPBELL and FRY, 1966), tomato strain (T-TNV) (SMITH et al., 1968), and strain AC 36 (AC-36) (UYEMOTO et al., 1968). These strains are serologically closely related to each other (UYEMOTO et al., 1968; SMITH et al., 1969) and belong to what has been called the D-serotype (BABOS and KASSANIS, 1963). In some experiments the C-strain of satellite virus (SV-C) (UYEMOTO et al., 1968) and an isolate of cucumber necrosis virus (CNV) (DIAS, 1970a) were used. In transmission experiments purified preparations of TNV were added in vitro to a zoospore suspension 5–10 min before inoculation of the host plants. The final virus concentration in these suspensions was approximately 2 μg/ml. The presence of virus in the inoculated host plants was verified by looking for symptoms

and by electron microscopy of negatively stained dip preparations. The virus concentration in the host plants and in purified preparations was determined by mechanical assay on the primary leaves of bean (*Phaseolus vulgaris* L. cv. 'Bountiful') and expressed as the number of lesions per leaf (CAMPBELL and FRY, 1966).

NZ-TNV and T-TNV were purified in two ways. The infected cotyledons of pumpkin (*Cucurbita pepo* L. cv. 'Small Sugar') were frozen and homogenized in an equal volume of 0.05 M phosphate buffer, pH 7.6 with 0.1% thioglycolic acid. The extract was clarified overnight with 8% butanol, subjected to two cycles of differential centrifugation (CAMPBELL and FRY, 1966), and resuspended in 2 ml of 0.05 M phosphate buffer per 100 g of tissue. The partially purified preparation was sometimes further purified on a sucrose density gradient column (10–40% sucrose in buffer) at 22,500 rpm for 3h in the Spinco 25-1 swinging bucket rotor (UYEMOTO et al., 1968). The virus was collected by means of an Isco model 170 density gradient fractionator, sedimented at 50,000 rpm for 2h in a Spinco 65 rotor, and resuspended in distilled water. The same method of purification was used when TNV-infected tomato seedling roots served as virus source. In a few later experiments mung bean (*Phaseolus aureus* Roxb.) seedlings were inoculated. TNV was purified according to a method described by LIU et al. (1969) with time and *g*-force of the high speed centrifugation (1h at 100,000 *g*) adapted to TNV instead of satellite virus. The final virus concentration was determined by optical density measurements at 260 nm, assuming an extinction coefficient of 5.5 (KASSANIS, 1964). The purity of the preparation was checked by means of spectrophotometry, analytical centrifugation and electron microscopy.

The preparations of AC-36 and SV-C were obtained from J. K. Uyemoto and had been purified by him as described (UYEMOTO et al., 1968). Later it became apparent that the AC-36 preparation was slightly contaminated with SV-C and that the SV-C preparation was slightly contaminated with AC-36 (UYEMOTO and GROGAN, 1969). Inoculum of CNV was kindly supplied by H. F. Dias; it was multiplied and purified by us according to his description (DIAS, 1970a).

2.3. FIXATION AND EMBEDDING

Olpidium zoospores, lettuce roots with encysted zoospores, and lettuce roots with growing or mature thalli were fixed by the modified method of SABATINI et al. (1963) or that of KARNOVSKY (1965), embedded in a mixture of epon and araldite, and stained with uranyl acetate and lead citrate as described previously (TEMMINK and CAMPBELL, 1968; TEMMINK and CAMPBELL, 1969a and b). Non-inoculated host plants, host plants infected with virus free *Olpidium*, host plants infected with TNV-carrying *Olpidium*, and TNV-carrying zoospores were treated in the same manner unless otherwise stated. Some material was embedded in a plastic mixture developed by SPURR (1969). The components of this plastic are much less viscous than those of the epon-araldite mixture. They can therefore be mixed more easily and penetrate the tissues much faster. However,

sufficient contrast was not always obtained, even after extending the fixation in osmic acid to 3–4h and the staining in uranyl acetate to 2–3h, followed by a lead citrate stain of 10–15min. Other material was stained by a faster procedure in which sections were put in a 1:1 mixture of distilled water and a saturated solution of uranyl acetate in 95% ethanol for 10min, rinsed in distilled water, and stained with lead citrate (VENABLE and COGGESHALL, 1965). The purified TNV was prepared for observation in the electron microscope by different techniques. A virus pellet was fixed, embedded, sectioned, and stained in the same way as zoospores. In addition, unfixed virus particles were observed with the electron microscope on grids coated with collodion plus carbon. The virus particles on the grid were either air-dried or dried by the critical point method (ANDERSON, 1951). Contrast was enhanced by shadowing the particles with uranium (BRADLEY, 1965) or by applying 4% uranyl acetate as a negative stain (HORNE, 1965). TNV was also negatively stained with 1% potassium phosphotungstate (KPT) at pH 6.5 after fixation in vapor of osmic acid to prevent disintegration of the virus in the KPT. All pictures of virus particles were taken at the same magnification in an RCA EMU-3H that had been calibrated with a carbon grating.

After a suspension of *O. brassicae* zoospores ($0.5–1.5 \times 10^6$/ml) was mixed with TNV for 10min and washed by two or three centrifugations (10min at 10,000 rpm in a Servall SS 34 rotor) (CAMPBELL and FRY, 1966), small drops were placed on electron microscope grids coated with collodion plus carbon, fixed for 1 min in vapor of osmic acid, and negatively stained with 4% uranyl acetate. Zoospores of *O. cucurbitacearum* mixed with CNV were treated similarly, except that they were washed two times by centrifugation at 5000 rpm for 5min, since these zoospores do not survive the higher centrifugal forces used routinely on zoospores of *O. brassicae* (DIAS, 1970a).

3. ULTRASTRUCTURE OF OLPIDIUM BRASSICAE

The ultrastructure of virus-free lettuce *Olpidium* was studied in the zoospores, in the cysts during the infection of lettuce roots, and in the vegetative thallus in infected roots. The results of this study have been published previously (TEMMINK and CAMPBELL, 1968; TEMMINK and CAMPBELL, 1969a and b) and are summarized here. Since the present publication is concerned with the vector role of *Olpidium*, special emphasis has been placed here on cytological aspects that were considered important for the virus transmission, whereas other strictly mycological aspects have been omitted. In the mean time two other papers on the ultrastructure of infection by *O. brassicae* have been published (LESEMANN and FUCHS, 1970a and b). The data in those papers essentially confirm our own results and reference to these papers will be made only where discrepancies seem to exist.

3.1. ZOOSPORES

The body of lettuce *Olpidium* zoospores is pyriform, approximately $2 \times 3\mu m$, and surrounded by a continuous plasmalemma (Fig. 3-1). It is not symmetrical with respect to the long median axis, since it has a lobe at one side and the nucleus and kinetid are located off center (Fig. 3-1). The nucleus is round to ovoid, approximately 1 μm in diameter and located in the anterior part of the body (Fig. 3-1). It is surrounded by an envelope that has a number of pores and is continuous in some places with the endoplasmic reticulum (TEMMINK and CAMPBELL, 1969a). The mitochondria are variable in shape and size and mainly located around the nucleus (Fig. 3-1). The most posterior mitochondrion is very big and doughnut-shaped; its location between the nucleus and the rhizoplast (Fig. 3-3b) suggests that it may be involved in locomotion.

Osmiophilic globules are found in almost all cells. They occur generally in clusters in an area anterior to the lobe (Fig. 3-1, 3-4a, 3-4b). These globules often show considerable differences in density of stain. Since most of them are thought to be lipid globules, this may be correlated with the degree of saturation of these lipids. (CRIEGEE, 1936 as cited by DE BRUIJN, 1969). Vacuole-like vesicles of different size and shape are present in most zoospores and generally many of them are in close proximity to or connected with the plasmalemma (Fig. 3-2a, 3-2b). Others may be found in contact with or close to what was previously called the multivesicular bodies (Fig. 3-2c, 3-2e) (TEMMINK and CAMPBELL, 1969a). Possibly these vesicles are involved in a process of exocytosis as was described for *Blastocladiella* zoospores (CANTINO et al., 1968) or endocytosis as has been observed in protozoa (DE DUVE and WATTIAUX, 1966). In both cases the close contact of these vesicles with the multivesicular bodies (mvb) suggests that the mvb's have a function similar to lysosomes. Whether they can actually be called lysosomes awaits cytochemical studies of the enzyme systems, but

morphologically they resemble lysosomes. They have a single surrounding membrane, a varying number of small vesicles, and granular contents with different degrees of electron opacity (Fig. 3-2c, 3-2d, 3-2e). Endoplasmic reticulum occurs throughout the zoospore body as short tubules or vesicles many of which seem to consist of smooth membranes without attached ribosomes (Fig. 3-1, 3-2e). Ribosomes are present in great number, however, but mostly free in the structureless ground substance that also surrounds the cytoplasmic organelles. Both the ribosomes and the ground substance look rather electron dense after fixation with glutaraldehyde and osmium and double staining; this is responsible for the relatively low contrast of many cellular membranes.

The kinetid consists of a 21 μm long axoneme, a kinetosome, an extra centriole, and a rhizoplast (Fig. 3-1, 3-4a) The axoneme is 200–250 nm wide and surrounded by a membranous sheath that is continuous with the plasmalemma of the zoospore (Fig. 3-1). The internal structure of the axoneme is the same as in most other flagellate organisms, showing nine double outer fibrils and a pair of inner fibrils, all of which are approximately 20 nm wide (Fig. 3-3a). There is no terminal plate at the transition from axoneme to kinetosome, but the inner fibrils of the axoneme end at the approximate place where the terminal plate occurs in other flagellates (Fig. 3-3a). The outer axonemal fibrils are continuous with those of the kinetosome (Fig. 3-1, 3-3a). The anterior quarter of the kinetosome, which is approximately 165 × 500 nm, is more electron dense and consists of a ring of triplet fibrils (Fig. 3-3a, 3-3c, 3-3e). A centriole lies beside and parallel to the anterior part of the kinetosome and looks exactly like it (Fig. 3-3c, 3-3e), except that it seems posteriorly open (Fig. 3-3c). Between the kinetosome and the nucleus there is a 1 μm long rhizoplast consisting of many electron dense cross-bands separated by more electron transparant areas in a manner that seems characteristic of the species (Fig. 3-3a, 3-3b, 3-3c). The detailed description of this typical structure has been given previously (TEMMINK and CAMPBELL, 1969a).

Many investigators have shown recently that fungal tissues may contain glycogen granules as a form of reserve material (MCKEEN et al., 1967; PEAT and BANBURY, 1967; BRACKER, 1968; EHRLICH et al., 1968; LESSIE and LOVETT, 1968). Glycogen can occur in two different configurations, alpha-particles and beta-particles (DROCHMANS, 1962). The rosette-like alpha-particles are easily recognized when present in ultrathin sections. The beta-particles, however, as seen in the electron microscope after conventional double staining, strongly resemble the cellular ribosomes in size and shape. To determine whether *Olpidium* zoospores have this latter type of glycogen in addition to ribosomes, sections on nickel grids were oxidized with 2% periodic acid for 30 min, rinsed with distilled water, dried, and then stained with lead citrate for 15–30 min (PERRY and WADDINGTON, 1966; PERRY, 1967). This technique gives the glycogen particles a specific image of densely stained subunits (PERRY, 1967). Because the treatment gave negative results (Fig. 3-4a), it was concluded that zoospores of *O. brassicae* do not contain glycogen. To substantiate this negative conclusion, other sections were treated according to STADHOUDERS (1965). They were stained with a half-

saturated aqueous solution of uranyl acetate at pH 5, followed by a half-saturated solution of lead acetate at pH 7.3 for 20–30 min. These sections were compared with similar sections that had been stained the same way and had been stained additionally in REYNOLDS' lead citrate (1963) for 15 min. The lead acetate enhances the electron density of ribosomes, but not of glycogen particles, whereas lead citrate enhances the electron opacity of both. Glycogen is detected by comparing sections of similar areas treated each way. This technique also failed to show the presence of glycogen in the zoospores of *O. brassicae* (compare fig. 3-4b with fig. 3-1), thus substantiating our conclusion that glycogen is not present in these zoospores.

3.2. INFECTION

As zoospores of *Olpidium* swim near host roots, they encyst on the epidermal cells, especially in the zone of elongation (FRY and CAMPBELL, 1966), and infect. Encystment starts with the withdrawal of the axoneme, but the method by which this is done is uncertain. SAHTIYANCI (1962) described it as 'pulled in', whereas HIRUKI (pers. comm.) suggested the 'wrap-around' mode. We were unable to confirm these observations with the light microscope. This is due to the small dimensions of the zoospores, the speed of the withdrawal, and to inhibition or reversal of the first phases of encystment by light (BEEVERS and FRY, 1970) Axonemal withdrawal cannot be studied with the electron microscope because at this stage the cysts are not firmly attached to the root epidermis and are washed off during the processing. After axonemal withdrawal, a cyst wall is secreted around the zoospore body that then remains in situ during processing. Pictures obtained at this stage commonly show cross-sections and longitudinal sections of the axonemal fibrils coiled within the cyst (Fig. 3-5a, 3-5b).

Two pictures provide support for the 'reeling-in' mode of axonemal withdrawal (Fig. 3-6b, 3-6c), possibly with one or more of the variations recently described (KOCH, 1968). Both pictures are median sections through the zoospore body and the posterior part of the kinetosome (Fig. 3-6b) or the rhizoplast (Fig. 3-6c) was sectioned longitudinally. In both cases the intracellular part of the kinetid is oriented so that the anterior portion is located near the plasmalemma instead of being in the center of the zoospore body as in motile zoospores (Fig. 3-5a).

Because the 9 + 2 axonemal fibrils within the cytoplasm are not surrounded by an axonemal sheath, the fate of this sheath upon withdrawal is intriguing. In some young cysts bundles of membranes can be found in contact with or very closely associated with the plasmalemma (Fig. 3-5b, 3-6a). The presence of these structures, resembling a whorl of wound-up membranes, suggests that, upon axonemal withdrawal, the sheath is pulled in and breaks, forming the coiled membranes (TEMMINK and CAMPBELL, 1969b). The close association of these membranes with lomasomes (LESEMANN and FUCHS, 1970a) supports this hypothesis because lomasomes very likely are structures in which a cell stores surplus membranous material (HEATH and GREENWOOD, 1970). The alternative

suggestion that the axonemal sheath fuses with the zoospore plasmalemma (LESEMANN and FUCHS, 1970a) seems highly unlikely because one would expect to find traces of a fusion of approximately 15μm^2 of axonemal sheath into approximately 28μm^2 of zoospore plasmalemma. This has never been reported.

The penetration of the host cell has two separate aspects, namely the reaction of the host cell to the fungal penetration and the actual entrance of the cyst cytoplasm into the host cytoplasm. The host cell may react to the fungal infection by forming a papillum just below the encystment site. This papillum consists of many vesicles embedded in a rather electron transparant matrix that seems to be continuous with the host cell wall (Fig. 3-8a, 3-8b, 3-9). It varies greatly in size, perhaps reflecting cell to cell differences in sensitivity to the parasite (TEMMINK and CAMPBELL, 1969b). The host cell cytoplasm often has an increased number of ER-cisternae (Fig. 3-8b, 3-9) and Golgi dictyosomes around the newly formed papillum. Sometimes the host cytoplasm is necrotic (Fig. 3-8a). It was suggested previously that this is due to heavy infection (TEMMINK and CAMPBELL, 1969b), but it is also possible that in some cases the necrosis is caused by the removal of sand particles from the root or by anoxia if the roots were inoculated by immersion in a zoospore suspension for several hours.

The movement of the fungal protoplasm into the host cell starts approximately 2h after encystment (SAHTIYANCI, 1962). A vacuole develops in the cyst, generally distally from the attachment site, and increases in size while a hole appears in the cyst wall (Fig. 3-7a). Then the cytoplasm, surrounded by a newly formed plasmalemma, moves into the host cell through a hole in the host cell wall (Fig. 3-7b) and in the papillum, if present (Fig. 3-8a). Sometimes wall-like material around part of the penetrating cyst is continuous with the cyst wall (Fig. 3-7b). The cyst plasmalemma, tonoplast, and often some other membranous material remain in the empty cyst (Fig. 3-7b, 3-8a). The cyst protoplast moves through a hole in the host cell plasmalemma into the host cell cytoplasm (Fig. 3-8b). The young thallus is often removed by cyclosis from the site of penetration and settles elsewhere in the host cell to continue its growth (Fig. 3-9).

3.3. VEGETATIVE GROWTH

During the first 24h after penetration, the young, uninucleate thallus grows larger. It is separated from the host protoplasm only by the thallus plasmalemma (Fig. 3-10a, 3-10b). The organelles in these thalli have been described extensively in a previous paper (TEMMINK and CAMPBELL, 1968) and are the same as found in many other fungi.

Between 24h and 48h after infection, the thalli that will develop into zoosporangia become multinucleate (Fig. 3-11a) and start to from a gradually thickening thallus wall outside the plasmalemma while retaining their round to ovoid shape. After approximately 72h they form cleavage vesicles by extruding lipid globules from their multivesicular bodies and develop an exit tube filled with a slime plug (Fig. 3-11b). The cleavage vesicles then fuse to delineate the

zoospores (Fig. 3-12a) and each of the zoospores starts to develop its own axoneme (Fig. 3-12b, 3-13a). The mature zoosporangium has a fully developed exit tube (Fig. 3-13b) and a varying number of fully differentiated zoospores (Fig. 3-14a) that are ready for release shortly after contact with fresh water.

It was suggested that some thalli develop into resting spores (TEMMINK and CAMPBELL, 1968). These thalli do not become multinucleate between 24h and 48h after infection, but they produce great amounts of reserve material and quickly develop a thick, undulating wall (Fig. 3-14b).

To test for glycogen in thalli, sections of material fixed 48–72h after inoculation were treated by the two cytochemical techniques described in section 3-1. The stippled appearance of the granules after treatment with periodic acid (PERRY, 1967) indicates that glycogen is present in these thalli (Fig. 3-15a, 3-15b). The same conclusion can be drawn from the presence of areas with relatively high electron transparency after the treatment described by STADHOUDERS (1965) (Fig. 3-15c) as compared to similar areas in the same material that are more electron opaque after conventional double staining (Fig. 3-15d). The presence of lightly stained particles in Fig. 3-15a and the failure to obtain completely clear areas in Fig. 3-15c indicates, however, that there is a mixture of glycogen particles and ribosomes. This is also suggested by the differences in electron density of the particles in the control (Fig. 3-15d), although the location of the particles relative to the sectioned and stained surface may also affect their apparent density. Glycogen is assumed to be utilized or converted just prior to or during zoospore cleavage because zoospores seem to be free of it (see section 3.1).

3.4. SUMMARY OF ULTRASTRUCTURAL DATA THAT MAY BE IMPORTANT FOR THE UNDERSTANDING OF THE ROLE OF O. BRASSICAE IN THE TRANSMISSION OF TNV

Electron micrographs of the zoospores of *O. brassicae* show that the axonemal sheath is continuous with the body plasmalemma. This suggests that both have the same structure and therefore probably react similarly upon contact with free virus particles. Furthermore, the cytoplasm of these zoospores (and of the other phases of the life cycle) is filled with ribosomes. In the sectioned material they have an apparent size that may make it difficult to differentiate between them and the virus particles if both should occur intermixed in the cytoplasm.

Evidence is presented that during encystment the axoneme is probably retracted by the 'reeling-in' mode and that the axonemal sheath is probably taken inside the cyst cytoplasm. This would have far-reaching consequences for virus particles that might be adsorbed on the axonemal sheath. In addition it is shown that the cyst plasmalemma (originally the zoospore plasmalemma) remains behind in the empty cyst together with the newly-formed cyst tonoplast. This could influence the transmission of virus that might be adsorbed to the zoospore plasmalemma.

Finally, micrographs were presented to indicate that the young fungal thallus in the infected host cell during the first 24 h after infection is separated from the host cytoplasm by a single membrane. An interaction between fungus and host resulting in the release of virus into host cytoplasm would have to take place during this phase before the thallus begins to form a wall.

4. ULTRASTRUCTURE OF HOST ROOT EPIDERMIS AND CORTEX

Many aspects of root anatomy and cytology are important for understanding the invasion by pathogens and the transport of viruses (SCOTT, 1963). For that reason and in order to provide a basis for comparison of pathological changes induced by virus or fungus infection, non-inoculated seedling roots of lettuce and tomato were investigated with the electron microscope.

Zoospores of lettuce *Olpidium* encyst on and infect the epidermal cells of the root. Virus is thus transmitted initially into the epidermal cells, although it may move to cortical cells at a later stage of infection. Therefore, this study of healthy seedling roots was confined to the epidermal and outer cortical cells. Because zoospores encyst in the area of elongation (approximately 1 mm behind the root tip) and thalli are mature after 2–3 days, when, under our growing conditions, the infected area is approximately 15 mm behind the root tip, the ultrastructure of the epidermal and cortical cells in the area between 1 and 15 mm from the tip was investigated.

4.1. LETTUCE ROOT

Epidermal cells of lettuce seedling roots in the apical part of the zone of elongation (1 mm behind tip) are regularly shaped, packed closely together and measure approximately 15 × 25 μm in cross-section (Fig. 4-1a). They contain much cytoplasm with many of the usual organelles, viz. mitochondria, dictyosomes, nuclei, and spherosomes (Fig. 4-1a, 4-1b). Cristae of endoplasmic reticulum run parallel to the cell wall and are dotted with ribosomes (Fig. 4-1b). Several vacuoles of different size are present and sometimes interconnected (Fig. 4-1a). The walls of epidermal cells are moderately electron-dense and approximately 500 nm thick. The outer wall is covered with a mucilaginous layer of 500 nm, but cutin seems to be absent (Fig. 4-1a, 4-2a). Remnants of calyptra cells sticking to the outer epidermal walls are seldom found. The cells of the subepidermal layer differ little from the epidermal cells, but they generally are slightly wider (Fig. 4-1a). No intercellular spaces occur between the cells of the epidermis and of the outer cortical layers. Necrotic cells are sometimes found (Fig. 4-1b); they may have been damaged during harvesting of the roots.

In cells at 1–2 mm from the root tip, the many vacuoles have fused into one big, central vacuole, leaving a band of cytoplasm alongside the cell wall (Fig. 4-2a). With increasing distance from the root tip the epidermal cells increase in over-all size by increasing the volume of the central vacuole and the band of cytoplasm at the same time gets progressively thinner (Fig. 4-2b). Some epidermal cells increase in size primarily in tangential direction, others more in radial direction. The cells of the outer cortical layers, as seen in cross-section, also

enlarge but more isodiametrically. Sometimes the subepidermal cells of the cortex have an electron-dense deposit at the tonoplast (Fig. 4-2b). At this stage in root development the greater size of the cells combined with the decrease in relative amount of cytoplasm causes distortion during the preparation of the material for electron microscopy. During the first steps of plastic impregnation the cell shrinks, giving the epidermal and outer cortical cells the configuration of a jig-saw puzzle with part of the mucous layer sometimes indicating the outline of the epidermis before shrinkage occurred (Fig. 4-2b). The shrinkage does not occur in the inner parts of the root and attempts to impregnate the tissue more slowly with the plastic did not prevent the shrinkage.

At 5–15 mm from the root tip most epidermal and outer cortical cells have very little cytoplasm left. The tonoplast touches the plasmalemma in many places or has disintegrated completely and fixation damage to these membranes is frequent and severe. Only small areas inside the cells seem to have some cytoplasm left and this very often is located around the nucleus. Even in those areas, however, the tonoplast has disintegrated or ruptured during the fixation. The older epidermal and cortical cells of the lettuce seedling root thus contain little or no cytoplasm and they probably deteriorate rapidly and are sloughed off.

4.2. Tomato root

Epidermal cells in the apical part of the zone of elongation of tomato seedling roots (1 mm from root tip) resemble those of lettuce roots in many respects (Fig. 4-3a). They are regularly shaped cells fitting together without intercellular spaces; they contain a number of vacuoles of different size surrounded by a wide peripheral band of cytoplasm. In addition to the long parallel arrays of rough endoplasmic reticulum, the dictyosomes and the mitochondria (Fig. 4-3b), the cytoplasm contains numerous plastids with few tubules but with many starch grains (Fig. 4-3c). In contrast to the epidermis of lettuce roots, the epidermis of the tomato root at this stage is still largely covered by cells of the calyptra (Fig. 4-3a). These cells have a thin layer of cytoplasm surrounding a central vacuole and they fit closely to the underlying epidermal cells.

In epidermal cells at 2 mm from the root tip the central vacuole increases in size by fusion of smaller vacuoles and the band of cytoplasm along the wall becomes narrower (Fig. 4-4a). At this stage the same organelles are still present as in the younger cells, but the calyptra cells have shriveled and the epidermal wall has become exposed (Fig. 4-4a, 4-4b).

After this stage growth in cell size with concomittant decrease in the relative amount of cytoplasm progresses as in the epidermal cells of the lettuce root. The cells cannot be processed for electron microscopy without considerable distortion and often little cytoplasm is found. This final stage of epidermal break-down is reached later than in the case of lettuce, but the difference may be due to the slower over-all development of tomato roots as compared to lettuce roots.

4.3. Summary of ultrastructural data that may be important for the understanding of the role of lettuce- and tomato roots as hosts for O. brassicae and TNV

Although the epidermal cells of lettuce- and tomato roots seem to die at a relatively small distance from the tip (15–25mm), they can successfully be parasitized by *O. brassicae* because the fungus infects them almost exclusively in the elongation zone and because the life cycle of the fungus is completed within three days. Because TNV requires even less time for multiplication (Harrison, 1956a, b), the longevity of the epidermal cells would also be sufficient to sustain the multiplication of transmitted TNV. This virus could easily be released into the soil by the break-down of the epidermal cells at a later stage, thus providing free inoculum for the next generation of *Olpidium* zoospores.

It was not determined whether the presence of calyptra cells on the epidermis of the tomato root makes infection of these roots by *Olpidium* more difficult. Even if this were the case, however, it may be compensated for by the slightly greater longevity of tomato epidermal cells as compared to those of the lettuce root. It is also possible that some calyptra cells are infected by *Olpidium* as was described for a mustard isolate on kohlrabi (*Brassica oleracea L.* Gongyloides group.) (Lesemann and Fuchs, 1970b).

5. ELECTRON MICROSCOPY OF TNV

Purified TNV was prepared for electron microscopy by different methods and studied so that it could be recognized in or on infected host and vector material. It is a polyhedral virus with an apparent diameter varying between 21 and 30 nm, depending on the method of preparation (KASSANIS, 1964; EDWARDSON et al., 1966). Our measurements of TNV fall in the same size range. Air-dried, negatively stained TNV particles had an apparent diameter of 30–32 nm (Fig. 5-1c, 5-1e, 5-1j), and air-dried, shadowed particles were of the same size (Fig. 5-1a). After critical-point-drying the particles had a size of 26–28 nm, whether negatively stained (Fig. 5-1d) or shadowed (Fig. 5-1b). When a pellet of TNV particles was embedded, sectioned, and stained with uranyl acetate plus lead citrate, the particles had an average diameter of 20 nm (Fig. 5-1f). Satellite virus particles in air-dried, negatively-stained preparations had an apparent diameter of 16–17 nm (Fig. 5-1i, 5-1j), and CNV had an apparent diameter of 30–32 nm (Fig. 5-1h).

Several explanations can be given for the apparent size differences between TNV particles in sections of embedded pellets and free virus particles on membrane-coated grids. The air-drying of unembedded virus particles tends to flatten them (ANDERSON, 1951) and this could account for the greater apparent size of the particles prepared this way as compared to the unembedded particles that were dried by the critical point method and to the embedded particles in sections. In addition, negative staining as well as shadowing accentuates the outline of the virus particles, whereas positive staining of sectioned particles tends to enhance contrast to a visible level only through the center of the protein subunits.

The size of TNV particles in a pellet is sufficiently close to that of ribosomes (Fig. 5-1g) to create difficulties in their identification when both are present in the cytoplasm, as was suggested in section 3.4. This problem does not arise, however, if the virus particles should prove to be located in areas where ribosomes do not occur, or if they should cluster in specific patterns. The chance that virus particles can not be identified by their shape and uniformity of size in negatively stained preparations, even if these preparations are contaminated with other particulate material, seems rather small. TNV in shadowed preparations may be more difficult or even impossible to recognize, if contaminating particles are present, because the size of a shadowed particle without any further structure is an insufficient basis for identification.

PART II

6. LITERATURE REVIEW

6.1. FUNGUS VECTOR RELATIONSHIPS

Fungus transmission of plant viruses was reviewed by GROGAN and CAMPBELL (1966). Since then the role of some fungus vectors has been substantiated and additional fungus vectors have been identified (TEAKLE, 1969). The vector role of *O. brassicae* with regard to BVV, TSV, and TNV has been established beyond reasonable doubt (GROGAN and CAMPBELL, 1966). CAMPBELL and FRY (1966) have made a distinction on the basis of the vector relationship between viruses that are externally borne and those that are internally borne. This distinction is roughly analogous to stylet-borne and circulative transmission of viruses by sucking insects (KENNEDY et al., 1962). It is not only of descriptive importance, but points to differences in how the viruses survice in soil under adverse conditions, methods of virus acquisition by the vector (in vitro and in vivo) (CAMPBELL, 1968), and possibly multiplication in the vector.

It has been shown that BVV transmissibility survives in air-dry soil for eight years (PRYOR, 1946) and that *O. brassicae* survives strong acid and base treatments and continues to transmit BVV (CAMPBELL, 1962; CAMPBELL and FRY, 1966). From this it was concluded that BVV is borne internally in resting spores of the fungus. BVV was acquired in vivo during one life cycle of the fungus in roots of a host infected with the virus (CAMPBELL and GROGAN, 1964).

Because soil containing TSV (HIDAKA, 1965) remains infective after many years of air-drying (HIDAKA et al., 1956; HIRUKI, 1965) and because TSV is only acquired in vivo (HIRUKI, 1965), it is assumed that TSV is also borne internally by the fungus. Both BVV and TSV are considered incapable of multiplication in their fungus vector because the fungus can be freed of the virus by serial transfer to plants that are hosts for the fungus but not for the virus (sugarbeet for BVV and cowpea for TSV) (CAMPBELL, 1962; TOMLINSON and GARRETT, 1964; HIRUKI, 1965). However, FUKISHIMA and HIDAKA (1969) have suggested that TSV does multiply in *Olpidium* zoospores because they found cells in a pellet of viruliferous *Olpidium* zoospores with many virus-like particles in the cytoplasm.

Olpidium cucurbitacearum transmits cucumber necrosis virus (CNV) (DIAS, 1970a and b). The virus (MCKEEN, 1959; DIAS and DOANE, 1968) is acquired in vitro and is not within the resting spores. Thus the relationship between *O. cucurbitacearum* and CNV resembles the one between *O. brassicae* and TNV (see section 6.2).

Wheat mosaic virus (WMV) is transmitted by *Polymyxa graminis* (Led.) (BRAKKE et al., 1965; CANOVA, 1966; ESTES and BRAKKE, 1966; BRAKKE and ESTES, 1967; RAO, 1968; RAO and BRAKKE, 1969). Zoospores immersed in antiserum and resting spores treated with strong acid or strong base were infec-

tious and transmitted WMV to healthy wheat seedlings. It was concluded that the virus was either inside the zoospores and resting spores or so tightly adsorbed to the outside that it was protected from inactivation (RAO and BRAKKE, 1969).

Spongospora subterranea (Wallr.) Lagerh. has been shown to transmit potato mop-top virus (PMTV) (CALVERT and HARRISON, 1966; JONES and HARRISON, 1969), based on a correlation between fungus infection and virus transmission. Infective soil or viruliferous fungal spore balls were air-dried and retained their ability to transmit PMTV (JONES and HARRISON, 1969). While this suggests that PMTV is carried internally in the resting spores, it is not proof because TNV survives in air-dried soil containing infected roots even though it is not carried within the resting spores(SMITH et al., 1969).

Pythium ultimum Trow has been suggested as a vector of pea false leafroll virus (THOTTAPILLY and SCHMUTTERER, 1968), but the evidence is not yet satisfactory. In view of the fact that the virus is also said to be mechanically-, seed-, and insect-transmitted, additional experimental results are needed to substantiate the first report. The fungus seems able, however, to support multiplication of TMV (BRANTS, 1969).

Finally, there is a report that *Synchytrium endobioticum* (Schilb.) Perc. is able to transmit potato virus X (PV-X) from virus diseased potato tubers to healthy tubers (NIENHAUS and STILLE, 1965). The virus is acquired in vivo but not in vitro and not all zoospores from PV-X diseased tubers are able to transmit PV-X.

In table 1 a summary is given of the known or suggested fungus – virus vector relationships as described in this section.

TABLE 1. Fungus – virus vector relationships described in the literature. (For references see section 6.1 and 6.2).

Fungus	Virus	Location of virus *in or* on vector	Shape and approximate size of virus
Olpidium brassicae	Lettuce big-vein virus	internal	?
Olpidium brassicae	Tobacco stunt virus	internal	polyhedral; ⌀ 18nm
Olpidium brassicae	Tobacco necrosis virus	external	polyhedral; ⌀ 26nm
Olpidium brassicae	Satellite virus	external	polyhedral; ⌀ 17nm
Olpidium cucurbitacearum	Cucumber necrosis virus	external	polyhedral; ⌀ 30nm
Polymyxa graminis	Wheat mosaic virus	internal	rod; 25 × 160nm
Spongospora subterranea	Potato mop-top virus	internal	rod; 20 × ??nm
Pythium ultimum	Pea false leafroll virus	?	?
Synchytrium endobioticum	Potato virus X	internal	rod; 13 × 515nm

6.2. OLPIDIUM AS A VECTOR OF TNV

The relationship between *O. brassicae* and TNV is external and the virus acquisition occurs only outside the virus infected host plant (KASSANIS and

MACFARLANE, 1964a; CAMPBELL and FRY, 1966; VAN SLOGTEREN and VISSCHER, 1967; SMITH et al., 1969). Experiments using centrifugation and TNV-specific antiserum indicate that TNV is tightly associated with the zoospores (CAMPBELL and FRY, 1966).

Although *Olpidium* isolates from lettuce transmit TNV, isolates from crucifers have failed as vectors. TEAKLE and HIRUKI (1964) showed that a mustard isolate of *Olpidium* did not transmit TNV. From their experiments on release of virus from heat-treated zoospores they hypothesized that their mustard isolate was less effective in acquiring the virus than the lettuce isolate. They ruled out the possibility that the *Olpidium* – host plant relationship affected transmission. The data do not rule out the possibility, however, that virus is irreversibly adsorbed to zoospores of the mustard isolate (GROGAN and CAMPBELL, 1966; MOWAT, 1968). KASSANIS and MACFARLANE (1965) conceded the possibility that transmission might depend on specific relations between zoospores and virus, but favored the hypothesis that infection and virus multiplication depend on the reaction of the host plant to fungal invasion. They showed that TNV multiplication in cress roots could be inhibited by inoculating the roots with the virus-free mustard isolate of *Olpidium* after inoculation with a viruliferous isolate. Recently, results of our own investigations on the acquisition and on the specificity of transmission of virus by *Olpidium* zoospores have been published partially (TEMMINK et al., 1970).

There is evidence that satellite virus (SV) is transmitted by *O. brassicae* and that there is specificity of transmission among SV isolates and *Olpidium* isolates (KASSANIS and MACFARLANE, 1968). Studies on the difference between NZ-TNV and AC-36 with respect to SV-C in the host plant showed that AC-36 was always found naturally associated with SV-C but could be kept permanently free of it during subculturing after thorough purification (compare UYEMOTO et al., 1968 with UYEMOTO and GROGAN, 1969). NZ-TNV, on the other hand, was isolated free of SV-C but it was capable of activating SV-C after inoculation of cowpea with a mixture of the two viruses (UYEMOTO et al., 1968). Since SV was acquired in vitro, it is surmised that the virus – vector relationship is similar to that found with *O. brassicae* and TNV.

7. EXPERIMENTS ON THE MODE OF TRANSMISSION OF TNV

7.1. ADSORPTION OF TNV ON ZOOSPORES OF O. BRASSICAE

If in vitro acquisition of TNV by zoospores consists of a tight surface adsorption of virus onto the zoospores (CAMPBELL and FRY, 1966), it should be possible to detect the acquired virus in the electron microscope. Experiments were done with NZ-TNV or T-TNV and zoospores of lettuce *Olpidium* that transmits six TNV strains (TEMMINK et al., 1970).

7.1.1. *Negative Staining*

When zoospores were mixed with TNV, washed, and processed for electron microscopy, the axonemal sheaths and body plasmalemmas of the washed zoospores were covered with virus particles (Fig. 7-1a, 7-1b). Adsorption of the virus did not seem to occur at random on the membranes; distinct patterns of adsorption were seen on the axonemal sheath and similar patterns occur on the plasmalemma. These patterns may indicate that special chemical components are responsible for adsorption and that these components occur in a somewhat regular manner in the membranes.

Usually there were a few virus particles that were not attached to the zoospores but that were in close proximity to them. This was interpreted to mean that these particles were attached to the zoospores in vivo, but were dislodged from the zoospores due to killing with the concurrent changes in membranes or due to physical forces during the drying of the grid. If unwashed zoospores were observed, many virus particles were on the zoospores, but there were also many particles elsewhere on the grid (Fig. 7-1c). After the usual three washes, this excess virus was not found whereas the adsorbed virus was still present. Thus, the first step in transmission of TNV is in vitro acquisition that consists of a tight binding of virus to the zoospore membrane. The bond between zoospores and adsorbed virus is apparently very strong. This confirms the indirect evidence of CAMPBELL and FRY (1966) and further contradicts the evidence for a loose association (KASSANIS and MACFARLANE, 1964).

In another experiment zoospores of lettuce *Olpidium* were killed with 0.5% glutaraldehyde before exposure to NZ-TNV and washed. After 2 wash cycles many adsorbed particles were present on the zoospores but there were also many detached and/or excess particles. After 3 wash cycles there still was some adsorbed virus but no background or excess virus. Apparently TNV is adsorbed to killed zoospores but the bond is weaker than with living zoospores.

Adsorbed virus was visible either if the amount of stain was enough to positively stain the axoneme and zoospore body so they served as a dark background for the unstained virus particles on top of them, or if the layer of stain close to the zoospore was thin enough not to cover the virus particles completely. Some-

times no adsorption was detected on the zoospores because staining conditions were not favorable: either the carbon coat on the grid was so hydrophobic that all stain was pulled off, or too much stain collected around the axonemes and bodies of the zoospores and submerged them completely in an electron opaque layer. These poor staining conditions rarely affected all the surface area of a grid and with practice some control was possible over the amount of stain removed. Nevertheless, the results are based on observations of more than hundred zoospores on each of two to five grids made in different trials.

7.1.2. *Critical point drying*

Virus particles on a grid are exposed to strong physical forces at the moment of drying. These forces, presumably occurring during the passage of a liquid – gas boundary over the material, cause flattening of the virus particles (ANDERSON, 1951 and 1956) and horizontal dislocations of the particles as seen in shadowed droplet patterns of virus preparations sprayed on a grid coated with carbon and collodion. To avoid the effects that air-drying might have on the association between virus particles and the vector zoospores, the critical point method of drying the material (ANDERSON, 1951) was tested. After applying a drop of viruliferous zoospores to an electron microscope grid covered with a formvar membrane, the grid was transferred to 70% ethanol for 1 min and 95% ethanol for 1 min. After three 10 min rinses in absolute ethanol, the grid was put through three changes each of 10 min of 100% amyl acetate and placed in the pressure vessel of a critical point drying apparatus (American Instrument Co, Inc.). The vessel was flushed with liquid carbon dioxide at 0–4 °C to replace the amyl acetate. After 20 min the inlet- and outlet valves of the vessel were closed and it was heated in water of 60–80 °C. As the critical point was reached, the pressure in the vessel rose from 800–900 psi to 1500–2000 psi; then the outlet valve of the vessel was opened and the carbon dioxide gas released. The dried material on the grids was shadowed with uranium at a low angle and observed with the electron microscope (Fig. 7-2a, 7-2b, 7-2c, 7-2d). Other grids were negatively stained with 2% uranyl acetate prior to the critical point drying and observed without further treatment (Fig. 7-2e).

This technique was effective in preserving the original three-dimensional position of the zoospores on the grid (Fig. 7-2a, 7-2d). However, comparison of zoospores that had been exposed to TNV and not washed (Fig. 7-2a, 7-2b) or washed (Fig. 7-2d) with those that had not been exposed to virus at all (Fig. 7-2c), showed that other particles of varying size were adsorbed and that virus particles could not be recognized with certainty. This is probably due to the fact that in shadowed preparations the outline of particulate contamination is similar to that of the virus particles. Zoospores that had been mixed with TYMV and washed also had the same appearance as those that were not exposed to any virus. Because it was impossible to resolve adsorbed virus, the critical point drying technique was not used further.

7.1.3. *Sectioning*

TNV adsorbed to the plasmalemma and axonemal sheath of the zoospores should be detectable in thin-sections of zoospores. When zoospores were mixed with TNV, washed, fixed, embedded, sectioned and stained, TNV particles were readily found on the plasmalemma (Fig. 7-3a) and the axonemal sheath (Fig. 7-3c, 7-3d, 7-3e). Repeated washing of the zoospores prior to fixation had distorted some of them rather badly (Fig. 7-3b). Similarity of the adsorbed particles with those in a sectioned pellet of TNV (Fig. 5-1f) indicates that the attached particles are probably TNV and not ribosomes that had gotten outside the zoospores. This tentative conclusion is supported by the absence of these particles in sections of zoospores that had not been mixed with TNV prior to fixation (see chapter 3).

7.2. Uptake of TNV in cysts

With the mode of acquisition of TNV established, the next step was to determine whether or not the adsorbed virus is taken inside the zoospore or cyst. If virus remains on the outside of the plasmalemma during and after encystment, it could only infect the root epidermal cells if the fungal protoplast with its surrounding plasmalemma enter the host cell as a unit (Campbell and Fry, 1966). Both assumptions seem unwarranted: No virus particles were found between the cyst wall and the plasmalemma in any of the encysted viruliferous zoospores (Fig. 7-4e, 7-5a), and the plasmalemma remains behind in the cyst when the protoplast infects the plant cell (Temmink and Campbell, 1969b; chapter 3).

If the virus that is adsorbed to the zoospore plasmalemma is pushed away during the formation of the cyst wall and remains attached to the outside of the cyst wall, it might be pushed into the host cell mechanically at the point of penetration as suggested by Teakle and Gold (1963) and Kassanis and Macfarlane (1964). Although we have seen rare cases where virus particles are stuck to the outside of the cyst wall (Fig. 7-4a) and also have found a few examples of virus located between the host cell wall and the cyst wall (Fig. 7-4b, 7-4c), we consider the frequency of occurrence too low to explain the efficiency of *Olpidium* zoospores as vectors of TNV by this push-in mechanism of virus penetration alone. For the efficient transmission of TNV by *Olpidium* zoospores it thus seems likely that some of the adsorbed virus gets into the fungal cytoplasm before or during the encystment.

One possible way for the fungus to take the virus in would be endocytosis, a process by which cells, including protozoa, are known to engulf substances necessary for their survival (de Duve and Wattiaux, 1966). Pictures of cross-sections through a small number of the zoospores that were mixed with TNV at least 10 min before fixation support this contension. Vacuole-like structures without any cytoplasmic content but containing a number of virus-like particles have been observed (Fig. 7-4d). Because all the particles are more regular in

outline than ribosomes, because they closely resemble particles in sections of a TNV pellet, and because they have never been found in similar vacuoles of non-viruliferous zoospores, it is concluded that these particles are probably TNV. If a process like endocytosis occurs in zoospores and is responsible for the uptake of some virus particles, it provides another explanation for some earlier results. KASSANIS and MACFARLANE (1964a) and CAMPBELL and FRY (1966) reported that TNV-specific antiserum usually prevented virus transmission when added to an *Olpidium* – TNV mixture before host plants were inoculated, but occasionally did not completely inhibit transmission if it was added to a zoospore – TNV mixture that had been standing for some time. It was postulated that encystment of some zoospores protected some TNV from inactivation by antiserum (CAMPBELL and FRY, 1966), but this does not explain how that virus got into the host plants because the cysts would not be able to infect the host. It is doubtful, nevertheless, that much virus enters through endocytosis. Because zoospores of *Olpidium* do not survive longer than a few hours under most circumstances, it is unlikely that endocytosis is an important process for them. This may be why it is only encountered in few, and probably older, zoospores.

It seems more likely, therefore, that most of the transmitted virus is taken up by the fungus during axonemal withdrawal, a process that occurs as every zoospore encysts. Although the actual mode of axonemal withdrawal is difficult to study and no definitive proof has been given for either the 'wrap-around' or the 'reeling-in' withdrawal, the latter is preferred (TEMMINK and CAMPBELL, 1969b; chapter 3). Virus adsorption to the axonemal sheath followed by the pulling in of the axonemal fibrils and the concurrent inclusion of the axonemal sheath within the cyst cytoplasm seems to be the most probable way by which the virus enters a cyst. This explanation is supported by pictures that show virus-like particles inside cysts (Fig. 7-5a, 7-5b, 7-5c, 7-5d). These particles are often in close proximity to whorls of membranes and this distinguishes them from particles possibly taken in by endocytosis, although the distinction is not always clear. The whorls of membranes seem to be digested and recycled rather quickly and this may account for the failure to detect virus particles on them. The virus-like particles are considered to be TNV for reasons stated before, but final proof with regard to the nature of these particles will require virus-specific labeling experiments.

7.3. RELEASE OF TNV IN HOST CELLS

Transmission of TNV by zoospores of *O. brassicae* involves acquisition of the virus by the zoospores and probably the movement of the virus particles into the fungus cytoplasm. If TNV is taken into the fungus cytoplasm, the last phase of the transmission is the release of the TNV particles into the host plant cell following infection by the fungus. This was investigated and the results are reported here.

There is some ultrastructural evidence for virus release inside the host cytoplasm. In a number of epidermal cells infected by viruliferous zoospores, vacuole-like structures were detected close to the young thalli that were fixed 4–24 h after inoculation and were not yet surrounded by a thallus wall. These structures sometimes had contents comparable to the vacuolar contents of cysts (Fig. 7-6a, compare with Fig. 8-4c, 8-5b), sometimes they contained virus-like particles (Fig. 7-6c), and sometimes they had broken and released virus-like particles (Fig. 7-6b). For the reasons given before, the virus-like particles are assumed to be TNV. The release mechanism strongly resembles excretion or regurgitation by exocytosis (DE DUVE and WATTIAUX, 1966), and a similar process can be responsible for release of the virus in the host cell cytoplasm. The phenomenon was not observed often enough to be sure that this is actually the case, but the vacuole-like structures were never encountered when many cells infected by virus-free *Olpidium* were studied (see section 3.3). Thus it is necessary to postulate that, if exocytosis is responsible for release of TNV in the plant cell, this process is triggered by the presence of the virus in the young fungal thallus.

Because it has not been proven beyond any doubt that the virus-like particles found in encysting zoospores (section 7.2) or in the host cells (section 7.3) are TNV particles, a staining procedure that can differentiate between ribosomes and virus particles would be useful. Such a technique has been reported (HILLS and PLASKITT, 1968) and was applied to viruliferous and non-viruliferous zoospores encysting upon young lettuce seedling roots and to lettuce root epidermal cells two days after inoculation with *Olpidium* and TNV, or *Olpidium* only. The technique differs considerably from the standard technique as described in section 2.3 and is given here. Tissues were fixed overnight in 2% glutaraldehyde buffered to pH 7.2 with 0.05M cacodylate-HCl. Dehydration was done in five 10 min steps in an increasing ethanol series, each of the ethanol solutions being saturated with uranyl acetate at room temperature. After transfer to 100% acetone saturated with uranyl acetate at room temperature, the tissues were embedded in a mixture of araldite and epon in the usual manner (TEMMINK and CAMPBELL, 1968). After sectioning and mounting on a grid, the material was stained for 1 min according to MILLONIG (1961).

Organelles in healthy cells show a normal profile for glutaraldehyde-fixed material (Fig. 7-7a, 7-7b) and virus particles in infected cells stain very strongly (Fig. 7-7c, 7-7d) as reported by HILLS and PLASKITT (1968), but ribosomes in virus-free fungal cysts (Fig. 7-7a) and in non-necrotic root cells (Fig. 7-7b, 7-7e) stain almost as densely as virus particles. The technique thus was considered inadequate to differentiate between ribosomes and virus particles and was not preferable to the conventional staining techniques. The discrepancy between our results and those reported by HILLS and PLASKITT (1968) may be because they applied stain to cells that had few or no ribosomes, probably as a result of necrosis. Because ribosomes are absent and the virus often forms crystal-like arrays, the virus nature of the particles in such cells is known, and the technique is redundant.

7.4. Summary and discussion

It has been established in section 7.1. that zoospores of lettuce *Olpidium*, that are able to transmit NZ-TNV and T-TNV, adsorb many virus particles when in contact with these viruses in a suspension. It was no surprise that the body plasmalemma and the axonemal sheath of the zoospore act identically in this respect, because the two are continuous and probably similar in structure (see section 3.4.). The adsorption pattern on the zoospores suggests that special adsorption sites occur on the membranes. Since an excess of virus is present in unwashed suspensions, the available sites seems to be fully occupied and it is concluded that the number of sites may be limited.

In section 7.2. arguments were presented for uptake of the adsorbed virus particles by the encysting zoospores. The arguments are all based on the fact that zoospores of lettuce *Olpidium* efficiently transmit NZ-TNV and T-TNV. The circularity of the reasoning, however, makes it necessary to state that uptake seems likely for the virus particles that are transmitted but that a number of other adsorbed particles may undergo a fate that was considered unimportant for explaining transmission. The most likely mechanism of uptake of virus that is adsorbed to the axoneme was based on our study of the encystment of virus-free encysting zoospores (see sections 3.2. and 3.4.). Other possible mechanisms of virus uptake were considered less likely on the same ground as cited above (i.e. efficiency of transmission) and with the same limitation.

If TNV is taken into the cyst cytoplasm transmission requires that this virus be released inside the host cytoplasm. Although not enough evidence could be presented in section 7.3. to prove a regurgitation or excretion process, the available data do suggest such a phenomenon. This process, that would have to be induced by the presence of the virus in the thalli, might be similar to that by which some animal viruses are released from infected cells (Bernard as cited by Fenner, 1968; Grimley et al., 1968).

Finally, it should be pointed out in this connection that it is possible and even likely that some of the virus particles that are taken up by the encysting zoospores, are not released inside the plant cytoplasm. This implies that these particles remain in the maturing thalli and that they may eventually be found in the newly formed zoospores, provided that the virus is not broken down in the mean time. This 'pseudo' – in vivo acquisition is probably not important for the virus transmission because no virus multiplication occurs inside the fungus and therefore the virus is greatly diluted in the next generation of zoospores.

It thus was shown in this chapter that all steps necessary for efficient transmission of TNV by lettuce *Olpidium* occur during infection of host roots by zoospores that had previously been mixed with the virus. The possible occurrence of other processes that prevent virus particles from being transmitted, could not completely be excluded.

8. EXPERIMENTS ON SPECIFICITY OF TRANSMISSION

Transmission of TNV isolates by different *Olpidium* isolates to several host plants provided information for studying specificity of transmission. The data for hosts, fungus isolates, and virus isolates studied herein are condensed in table 2 from TEMMINK et al. (1970). Mustard *Olpidium* does not transmit TNV to plants susceptible to the virus even though the fungus infects these plants, whereas the lettuce and tomato *Olpidium* transmit TNV to all plants infected by the fungus and susceptible to the virus. Such specificity of transmission has been studied by earlier investigators and been ascribed to a difference in the fungus – virus relationship (TEAKLE and HIRUKI, 1964; MOWAT, 1968) or to differences in host – fungus relationship (KASSANIS and MACFARLANE, 1965). Oat *Olpidium* transmits NZ-TNV to some host plants it infects but not others that are susceptible to the virus (TEMMINK et al., 1970). *O.cucurbitacearum* transmits CNV, but not TNV, to a number of host plants (DIAS, 1970a). Specificity of transmission of satellite virus by *O.brassicae* zoospores has been reported (KASSANIS and MACFARLANE, 1968) and virus acquisition apparently occurs in vitro. All these examples of virus transmission by isolates of *Olpidium* have been investigated to determine the causes for the specificity of transmission.

In section 8.1. the fungus – virus relationship is studied in order to determine whether and to what extent this relationship can shed light on some forms of specificity. Section 8.2. evaluates the possible effect of some fungus – host relationships on the transmission of TNV and in section 8.3. the possible role of host – virus interactions in the specificity problem is studied on two host plants infected with TNV. In section 8.4. an attempt is made to integrate the results of the first three sections and to offer an explanation for all cases of specificity of virus transmission mentioned in this chapter.

8.1. FUNGUS – VIRUS RELATIONSHIP

8.1.1. *Olpidium isolates – TNV and CNV*

Differences in adsorption of virus to the fungal zoospores seemed the simplest and most plausible explanation for the differences in fungus – virus relationship and therefore negatively stained zoospores of the different isolates of *O.brassicae* and of *O.cucurbitacearum* were compared after mixing with NZ-TNV, T-TNV, and CNV according to the method described above (section 2.3.).

Whereas the lettuce *Olpidium* showed abundant adsorption of particles of NZ-TNV and T-TNV (Fig. 8-1a, 8-1b), the mustard *Olpidium* did not adsorb either TNV isolate (Fig. 8-1c, 8-1d). These results were interpreted to mean that there is a qualitative difference between these fungus isolates in their ability or inability to adsorb TNV (table 2) and that this difference in virus acquisition is directly correlated with their ability to transmit TNV. This is in partial agree-

TABLE 2. Transmission of 2 TNV isolates by 4 isolates of *O. brassicae*.

Olp. isolate	Lettuce *Olpidium*			Tomato *Olpidium*			Mustard *Olpidium*			Oat *Olpidium*		
Host Plant	Olp.[a]	NZ-TNV[b]	T-TNV[b]	Olp.[a]	NZ-TNV[b]	T-TNV[b]	Olp.[a]	NZ-TNV[b]	T-TNV[b]	Olp.[a]	NZ-TNV[b]	T-TNV[b]
Lettuce	+	122	110	+	150	98	−	0.5	0.4	+	3	0
Mustard	P	2[c]	10[c]	+	22[c]	13[c]	+	0	0	+	0	0.1
Tomato	P	108	149	+	150	150	−	0.1	0	−	2	0
Cowpea	+	150	150	+	150	124	+	0	0	+	101	4

a) Infection and reproduction in host after inoculation with *Olpidium* alone.
+ normal reproduction, zoospores discharged in quantity
P infection but poor reproduction, few or no sporangia produced; zoospores not detected
− no evidence of infection or reproduction.
b) Transmission of TNV-isolates indicated by average number of local lesions per bean leaf per replicate produced when 4 'Bountiful' bean leaves were inoculated with homogenized roots of host plants from *Olpidium* + TNV treatments in 3 or 6 ml of tap water. The average number of local lesions produced by assay plants when inoculated with 'TNV without *Olpidium*' treatments of the same host were subtracted. There were no local lesions in assays of host plants from '*Olpidium* alone' and 'noninoculated' controls.
c) The low number of local lesions in these assays is due to the presence of an inhibitor in the homogenate of mustard roots.

ment with the explanation of TEAKLE and HIRUKI (1964), who suggested quantitative differences in adsorption between the *Olpidium* isolates, but does not support the suggestion that the mustard *Olpidium* adsorbs TNV so tightly that it cannot transmit the virus (GROGAN and CAMPBELL, 1966; MOWAT, 1968).

As a further check on the specificity of TNV adsorption, zoospores of lettuce and mustard *Olpidium* were similarly mixed with solutions of purified turnip yellow mosaic virus (TYMV), cucumber necrosis virus (CNV), or tomato bushy stunt virus (TBSV). No particles of these viruses were found on the zoospores of either *Olpidium* isolate after washing. NZ-TNV was adsorbed to the zoospores of oat *Olpidium*, but there were considerably fewer particles than on the zoospores of lettuce *Olpidium* (Fig. 8-2a, 8-2b). The effect of this on the specificity of transmission will be discussed later. In contrast, T-TNV was not adsorbed to the zoospores of the oat isolate.

When zoospores of *O.cucurbitacearum* were mixed with CNV, few virus particles were found attached to the zoospores, but many particles were found around the zoospores, even after three washings (Fig. 8-2c, 8-2d). Since *O. cucurbitacearum* is vector for CNV (DIAS, 1970a and b), we concluded that adsorption had probably taken place, but that subsequent preparatory treatments had broken the bonds. TNV was not adsorbed by the zoospores of *O.cucurbitacearum*.

A methodological matter should be raised in connection with the detection of virus adsorption to zoospores. If virus particles were found on the zoospores, it was concluded that the zoospores adsorbed the virus particles strongly enough to prevent subsequent dislodging during the killing and processing for electron microscopy. If no virus particles were seen on or near the zoospores, it was concluded that no adsorption had taken place. Because the zoospores had been processed on the electron microscope grids, it was assumed that if part or all of the virus particles had dislodged during preparation, they would be found in the vicinity of the zoospores on the grid. It should be remembered in this connection that all non-adsorbed virus had been removed by washing the zoospores before fixation.

8.1.2. *Lettuce Olpidium – TNV and SV*

Because of the association of SV-C with at least one TNV strain (see 6.2.) it seemed likely that SV-C is adsorbed and transmitted by zoospores of lettuce *Olpidium*. When these zoospores were exposed in vitro with a mixture of AC-36 and SV-C, both viruses were readily adsorbed onto the plasmalemma and the axonemal sheath (Fig. 8-3a, 8-3b, 8-3c). The ratio of the number of adsorbed TNV and SV particles was variable between individual zoospores, but the variation seemed random among the zoospores on the grid. To exclude the possibility that the adsorption of SV-C was dependent on the presence of AC-36, zoospores were also mixed in vitro with SV-C only. SV-C was readily adsorbed to the zoospores (Fig. 8-3d). Furthermore, the possibility was checked that adsorption of SV-C to zoospores is prevented when these zoospores are exposed in vitro to a mixture of SV-C and NZ-TNV (that is not naturally associated

with SV-C). Particles of SV-C as well as NZ-TNV were adsorbed by the zoospores (Fig. 8-3e). Finally, the possibility that TNV and SV might compete for the same adsorption sites on the zoospores was tested by adding one virus 5–10 min before the other. No observable difference in the number of adsorbed TNV and SV particles was found when NZ-TNV was added 5–10 min before SV-C (Fig. 8-3f) or when SV-C was added 5–10 min before NZ-TNV (Fig. 8-3g). This suggests either that the adsorption sites are specific for certain viruses, or else that so many adsorption sites are present on the membranes that saturation does not occur even at the high virus concentrations used in the experiments. The latter possibility seems less likely because much unadsorbed virus is always washed off in these experiments (see section 7.4.).

Zoospores that were mixed in vitro with AC-36 plus SV-C, AC-36 only, SV-C only, or with no virus at all, were inoculated to cowpea and lettuce seedlings. Cowpea roots showed severe necrosis after infection with AC-36 plus SV-C and with AC-36 only, and many particles of both viruses were detected in dip preparations of the necrotic areas. The presence of SV-C particles in the roots infected with AC-36 only, is attributed to the contamination of the AC-36 preparation with a small amount of SV-C (see section 2.2.). No symptoms appeared on roots inoculated with *Olpidium* and SV-C only, or *Olpidium* alone and no virus was found in dip preparations from those roots. Similar results were obtained with the inoculated lettuce roots except that there were fewer local lesions than in cowpea roots and fewer particles were detected on the grids with dip preparations from necrotic areas.

8.2. Fungus – host relationship

The infection of lettuce seedling roots by lettuce *Olpidium* described in chapter 3 indicates a rather well adapted host – fungus combination, which is not surprising considering that it is a naturally occurring one. Many zoospores are attracted to the lettuce seedling root, especially to the elongation zone, and quickly encyst on it. The cysts attempt to infect the host epidermal cells which usually are not able to prevent infection. Thus thalli are established in cells that sustain their growth into mature sporangia.

Since the interaction between the host and different fungus isolates might affect transmission of TNV, the encystment and infection by different isolates was studied. The phase-contrast microscope was used to observe encystment on samples taken 1 h after inoculation. Other samples were taken 1–24 h after inoculation and processed for electron microscopy.

It was assumed that if a host – fungus combination was compatible, the zoospores would encyst, infect, and grow normally as has been described for lettuce *Olpidium* on lettuce. This was found to be the case with oat *Olpidium* on lettuce (Fig. 8-6c) and on cowpea and with tomato *Olpidium* on tomato. Thus, the host – fungus combinations cited as 'normal reproduction' (table 2) were not investigated further.

A less favorable host – fungus compatibility was expected in those combinations designated in table 2 as reproducing poorly or as non-infecting. Two types of reaction were found. In the first the zoospores encysted and infected normally but after approximately 24 h the thalli began to disintegrate. This was observed with lettuce *Olpidium* in the roots of mustard (Fig. 8-4a, 8-4b, 8-4c, 8-4d) and with mustard *Olpidium* in the roots of lettuce (Fig. 8-5a, 8-5b, 8-5c, 8-5d). The second type of reaction was represented by lettuce *Olpidium* on tomato. When tomato seedlings were inoculated with zoospores of this isolate, it was extremely difficult to find any cysts in samples taken one hour later (Fig. 8-6a) and we were unable to find developing thalli in samples taken 24 h later (cf. SMITH et al., 1969). If lettuce and tomato seedlings were simultaneously exposed to a zoospore suspension of lettuce *Olpidium* no encystment occurred on the tomato roots while the lettuce roots were covered with cysts. Environmental factors such as light and temperature were, therefore, not responsible for the failure to encyst on tomato roots. When tomato seedlings were inoculated with mustard *Olpidium*, it was as difficult to find cysts (Fig. 8-6b) as in the case of lettuce *Olpidium*. Oat *Olpidium* likewise encysted so rarely on tomato roots that no electron micrographs were obtained. Thus lettuce induced encystment of many zoospores, irrespective of the fungus isolate, whereas tomato roots induced abundant encystment only when inoculated with zoospores of the tomato isolate of *Olpidium*.

A comparison of cysts of different *Olpidium* isolates on different hosts did not show any morphological differences between those that eventually infected their hosts and those that did not (Fig. 8-4a, 8-5a, 8-6a, 8-6b, 8-6c, 8-6d). Only old cysts that had failed to penetrate after 24 h showed some dark-staining material in big vacuoles (Fig. 8-5b), but this was interpreted as a sign of degeneration, because the same material was found in disintegrating thalli (Fig. 8-4c,8-4d).

The different forms of incompatibility between fungus and host have the same gross effect on the survival of the fungus. They have different effects on the transmission of TNV, however, because transmission can take place when incompatibility occurs after infection of the host plant, whereas transmission is impossible in those cases where fungus infection does not happen.

8.3. HOST – VIRUS RELATIONSHIP

The differences in host reaction to NZ-TNV were investigated at the ultrastructural level on lettuce or tomato roots harvested at intervals after inoculation with NZ-TNV and the lettuce or tomato *Olpidium*, respectively. Because it was shown above that hosts may react specifically to the vector isolates, compatible *Olpidium* isolates were used and it was assumed that this would not substantially influence the host – virus relationship. The differences between NZ-TNV and T-TNV regarding their effect on host plants were not studied microscopically, but the data from previous work (table 2) show negligible differences in assayable amounts of virus in plants infected with NZ-TNV or T-TNV.

8.3.1. *Lettuce*

In epidermal cells of lettuce roots, virus could not be detected with the electron microscope 14h after inoculation, but from 18h onward irregular clusters (Fig. 8-8a, 8-9a) or crystal-like arrays (Fig. 8-7a, 8-7b, 8-7c, 8-9c) of virus particles were present in the cytoplasmic remnants of the necrotic epidermal cells. At 18h after inoculation necrosis seemed to occur only in the fungus-infected cells (Fig. 8-7a). By 24h after inoculation, many adjacent epidermal cells and even cells of the two outermost cortical layers had also become necrotic and at this stage necrosis was macroscopically observable. No virus particles were detected, however, in the cortical cells at any stage. *Olpidium* thalli were not found in necrotic cells and only papilla were seen (Fig. 8-7a). This supports the observation that roots infected with *Olpidium* and TNV released fewer zoospores than roots infected with *Olpidium* only (FRY and CAMPBELL, 1966). If a cell contained growing thalli, it apparently had escaped virus infection because no virus was found in such cells. These observations indicate that little cell-to-cell spread of virus occurred in lettuce. In this host necrosis is probably effective in preventing the virus from moving to neighboring cells. Occasionally non-necrotic epidermal cells containing virus particles were found 24h or more after inoculation of the root. These cells also seemed to have relatively large amounts of cytoplasm (Fig. 8-8b, 8-8c, 8-9b) and thus were thought to be younger at the time of infection. In these cells the first cytopathological effects of TNV infection could still be observed. The number of mitochondria was very high (Fig. 8-9b), probably indicating a high respiration rate, and there seemed to be a more extensive Golgi complex and possibly more endoplasmic reticulum (Fig. 8-8c), than in non-infected cells.

In summary, most cells of lettuce seedling roots react necrotically to TNV infection; this seems to prevent the virus from spreading along the epidermis or into the cortex; the necrotic reaction is not limited to the infected cells.

8.3.2. *Tomato*

When tomato seedling roots were infected with tomato *Olpidium* and NZ-TNV, the infected cells did not react with necrosis, even 72h after inoculation. Thus, *Olpidium* thalli grew well in these cells (Fig. 8-10b, 8-10d, 8-11a). Crystal-like clusters of virus that were so easily recognized in TNV-infected lettuce cells, were not found in the infected tomato cells, perhaps because there was no necrosis or 'decompartmentalization' of the cytoplasm (DE ZOETEN and GAARD, 1969 and 1970). This made it rather difficult to unequivocally identify cells as being TNV-infected during the first 24h after inoculation. But later the cells were so full of virus particles that mistaking virus for ribosomes became less likely (Fig. 8-10a, 8-10c, 8-11a). After 24h it was possible to find cortical cells containing virus particles. Since tomato *Olpidium* only infects epidermal cells, this virus must have gotten into the cortex by cell-to-cell movement. Thus the presence of TNV in the cortex of non-necrotic tomato roots compared to its absence in cortical cells of the necrotically reacting lettuce roots supports the view that cell necrosis prevents cell-to-cell movement of virus particles.

Olpidium thalli grew in virus infected epidermal cells on tomato, but many thalli looked as if they were developing into resting spores (Fig. 8-10d). Perhaps the virus multiplication in these cells depletes them so much that this development is induced. Although special attention was paid to the thalli in TNV-infected cells, we found no virus particles inside these thalli. This indicates that TNV does not multiply in the vector and supports the conclusion that zoospores are free of virus when released from sporangia (CAMPBELL and FRY, 1966; SMITH et al., 1969). Another effect of the virus infection upon the tomato host cells is that the parallel cisternae of the endoplasmic reticulum often lose their ribosomes and the space between many of these cisternae is filled with material that sometimes seems crystalline in nature, sometimes more irregularly granular (Fig. 8-11a, 8-11b, 8-11c). Perhaps this material is connected in some way with the virus multiplication.

Tomato is a better host for TNV than lettuce because no necrotic reaction occurs; therefore many virus particles can be produced in each infected cell and cell-to-cell movement can occur easily. It also is a better host because fewer virus particles seem to be necessary at a given infection site for infection to occur, as will be seen later (see section 8.4.). This explains the difficulty encountered by FRY and GROGAN (1964) in maintaining a TNV culture by zoospore transfers in lettuce.

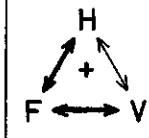
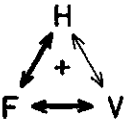
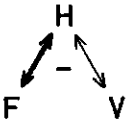
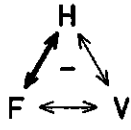
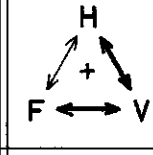
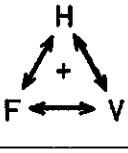
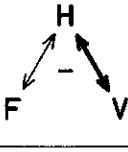
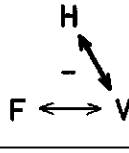
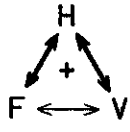
8.4. FUNGUS – HOST – VIRUS RELATIONSHIP

As was described in section 8.1., some isolates of *O. brassicae* adsorb more virus particles than others and this will influence their efficiency as a vector. Some plant species attract more zoospores of one *Olpidium* isolate than other species and consequently may contract more infections. This fact and others discussed in section 8.2. affect the success of TNV transmission to particular host plants. Finally, as was explained in section 8.3., hosts vary in their suitability for virus infection, multiplication, and spread.

In this section we will combine the data that were found and explain specificity of transmission of NZ-TNV by some isolates of *O. brassicae*, with special reference to the cases where lettuce and tomato were host plants. In addition, these data will be summarized in a diagram (Table 3). Even where failure to transmit TNV to a certain host by a certain *Olpidium* isolate seems to be due to one specific property of either the fungus vector or the host plant, it is still better to think in terms of a triangular virus – fungus – host relationship with one of the sides of the triangle assuming the value zero. The advantage of such an approach is that all instances of specificity can be explained with the same scheme.

Transmission in specific host – fungus – virus combinations was assayed (Table 2) and is indicated in table 3 by a + or — sign. All relationships will be symbolized in the diagram by arrows. A thick arrow indicates that the relationship is favorable, a thin arrow means that the relationship is much less favorable,

TABLE 3. Graphical summary of information on the relationships between isolates of *Olpidium brassicae*, host species, and NZ-TNV. (For explanation of symbols see section 8.4).

OLPIDIUM ISOLATE / HOST PLANT	LETTUCE	TOMATO	MUSTARD	OAT
LETTUCE				
TOMATO				
COWPEA				

and absence of an arrow means that the particular relationship does not occur, i.e. the value is near zero. With regard to the fungus – virus relationship the arrows indicate the amount of virus acquired by the *Olpidium* isolate. As far as the host – fungus relationship is concerned, the arrows indicate the frequency of encystment and subsequent penetration. For virus transmission it is not important whether the fungus develops mature sporangia inside a particular host or not. Finally, the arrows for the host – virus relationship show whether susceptibility to the virus is good, poor, or non-existant.

The relationships between lettuce seedling roots, lettuce *Olpidium* and TNV are considered first. TNV is transmitted to the lettuce root and multiplies. This is not surprising considering that the lettuce *Olpidium* readily encysts and penetrates into lettuce roots and that this isolate is able to acquire many virus particles. Although lettuce is not a very good host for virus multiplication because it reacts with local necrotic lesions, a favorable fungus – virus relationship and a favorable host – fungus relationship together compensate for the rather unfavorable host – virus relationship.

The same is also true for the combination lettuce – tomato *Olpidium* – TNV.

Although a favorable host – fungus relationship exists also in the combination lettuce – oat *Olpidium* – TNV, the less favorable fungus – virus relationship

together with the unfavorable host – virus relationship seem to make the establishment of the virus in the host improbable. The failure of oat *Olpidium* with its few adsorbed particles of TNV to infect lettuce with the virus indicates that many particles are probably needed to establish virus infection in lettuce. It is possible, however, that the failure of oat *Olpidium* to effectively transmit TNV to lettuce is due to the fact that the thalli do not release the virus inside the host epidermis cells. Although this seems rather unlikely, we have no data that allow us to exclude this possibility.

The failure of mustard *Olpidium* to transmit TNV to lettuce is due to the fact that it does not adsorb any virus particles. Thus the fungus – host relationship and the host – virus relationship are irrelevant.

Tomato seedling roots allow a good TNV multiplication when inoculated with TNV-carrying lettuce *Olpidium* (table 2). In view of the fact that hardly any zoospores of lettuce *Olpidium* encyst upon tomato seedlings and even fewer seem to infect, it is surprising that the TNV titer can build up to the levels found in assay. The only possible explanation is that the tomato root is an exceptionally good host for the virus. This is in agreement with ultrastructural data on the effect of TNV on epidermal cells of tomato roots.

The tomato – tomato *Olpidium* – TNV combination is probably the most favorable one studied, since all three aspects of the relationship are very favorable. The tomato *Olpidium* zoospores adsorb many virus particles, encyst in great numbers on tomato roots, and carry the virus into very favorable host cells.

It could not be determined if the oat *Olpidium* fails to establish TNV infection in tomato because it carries many fewer particles than necessary to start a succesful infection, or because the isolate does not penetrate the host cells.

Mustard *Olpidium* does not transmit TNV to tomato roots for the same reason as in the case of lettuce roots.

It has been shown that either a poor host – fungus relationship (e.g. tomato – lettuce *Olpidium*) or a poor host – virus relationship (e.g. lettuce – NZ-TNV) can be compensated for by favorable conditions in the other parts of the host – virus – fungus relationship, so that transmission occurs. The infection of cowpea roots with NZ-TNV by oat *Olpidium* shows that an unfavorable fungus – virus relationship can also be overcome if both the host – virus and the host – fungus relationship are good. Apparently only a few TNV particles are necessary to start infection in a root epidermal cell of cowpea.

9. SUMMARY

This thesis concerns transmission of tobacco necrosis virus (TNV) by zoospores of *Olpidium brassicae*. Electron microscopic observations were made on: a. the fungus, the virus, and the outer layers of seedling roots of two host species (part I); b. ultrastructural aspects of the mode of virus transmission and specificity of transmission (part II). Summarized results of part I are:

1. Uniflagellate zoospores of *O. brassicae* have a body plasmalemma that is continuous with the axonemal sheath (section 3.1).
2. Upon encystment of the zoospore, the axoneme is withdrawn inside the body. This probably happens by a 'reeling-in' mode of withdrawal and later a structure that could be the axonemal sheath is present inside the cyst body (section 3.2).
3. After encystment the cyst cytoplasm penetrates into the host cytoplasm through a hole in the host cell wall and through the papillum that develops between the host wall and the host plasmalemma at the attachment site. During the penetration the cyst plasmalemma and tonoplast remain behind in the empty cyst wall, but the young thallus is immediately surrounded by a new outer membrane (section 3.2).
4. Up to 24h after penetration the young thallus is separated from the host cytoplasm by the thallus membrane only. Older thalli develop a wall (section 3.3).
5. The epidermal cells in the zone of elongation of lettuce seedling roots rapidly change with increasing age. The relative amount of cytoplasm decreases and in the region between 5 and 15mm from the root tip very little cytoplasm remains and many cells seem to have died (section 4.1).
6. Epidermal cells of tomato roots undergo deterioration with increasing age, but at a slightly slower pace than lettuce. These cells are often covered with calyptra cells in the zone of elongation (section 4.2).
7. Particles in sections of a TNV pellet have an apparent size of approximately 20nm after conventional double staining. For this reason TNV particles may be difficult to identify if present in cells that also contain ribosomes (chapter 5).

Results published in part II of this thesis are also based partially on data obtained in part I. Summarized results of part II are:

1. Zoospores of the lettuce isolate of *O. brassicae*, that transmit TNV after exposure to the virus, acquire TNV in vitro by a tight adsorption of the virus particles to their body plasmalemma and the axonemal sheath (section 7.1).
2. Part of the adsorbed virus is taken into the encysting zoospore cytoplasm and this virus is eventually transmitted. Uptake of the virus probably takes place when the axoneme with its sheath is withdrawn. The fate of TNV not taken into the cyst by axonemal withdrawal is discussed and considered unimportant for transmission (section 7.2).

3. TNV taken into the infecting zoospores is transmitted into the cytoplasm of the root epidermal cell by release from the young thallus. A possible mechanism for this virus release is proposed (section 7.3).
4. Adsorption of TNV to zoospores of the lettuce isolate of *O. brassicae* is very specific in the sense that particles of other polyhedral viruses like cucumber necrosis virus (CNV), tomato bushy stunt virus (TBSV), and turnip yellow mosaic virus (TYMV) are not adsorbed under similar circumstances (section 8.1.1).
5. Adsorption of TNV is also very specific in the sense that zoospores of the lettuce and tomato *Olpidium* adsorb many TNV particles, those of the oat *Olpidium* adsorb many fewer TNV particles, and those of the mustard *Olpidium* apparently do not adsorb any TNV under similar experimental conditions (section 8.1.1).
6. A form of specificity of adsorption comparable to that between TNV and the lettuce *Olpidium* was encountered in the case of zoospores of *O. cucurbitacearum*. Zoospores of this species adsorb many particles of CNV, but they do not adsorb TNV when mixed in vitro with these viruses (section 8.1.2).
7. Zoospores of lettuce *Olpidium* adsorb particles of satellite virus (SV) when mixed with a suspension of SV alone or in combination with either of two different isolates of TNV (NZ-TNV and AC-36) (section 8.1.2).
8. Incompatibility in the fungus – host relationship is sometimes due to the absence or low incidence of encystment of a particular fungus isolate on that host plant, or to a break-down of the developing fungal thallus after a normal appearing infection process. The first form of incompatibility certainly influences transmission of TNV (section 8.2).
9. Tomato roots seem to be good hosts for TNV inasmuch as they allow the build-up of a high TNV titer after infection and do not react necrotically but lettuce roots react with necrosis 18 h after TNV infection. Also, lettuce roots seem to be less favorable hosts for TNV in the sense that many virus particles have to be present at a given infection site in order for the infection to take place (sections 8.3 and 8.4).
10. In epidermal cells of lettuce roots infected by TNV, thalli of *O. brassicae* do not mature. No virus was detected in lettuce epidermal cells that were not infected by *Olpidium*, nor in the cortical cells. Nevertheless these cells often were necrotic. TNV-infected epidermal cells of tomato roots often contain growing thalli of *O. brassicae* but virus was not found in the thalli. These host cells also sometimes have granular or crystalline material between the cisternae of the endoplasmic reticulum. Virus could be detected in epidermal and cortical cells of tomato that had not been infected by *Olpidium* (section 8.3).
11. It is possible to explain some forms of specificity of TNV transmission just by differences in fungus – virus relationship. Other examples of specificity of TNV transmission, however, can only be explained by taking into account simultaneously the combined effect of differences in fungus – virus, host – fungus, and virus – host relationship (section 8.4).

In conclusion, transmission of TNV by *O. brassicae* is explained by a sequence of events, all of which were shown to take place during exposure of zoospores to TNV and subsequent exposure of host roots to those zoospores. Furthermore, all known forms of specificity of TNV-transmission by different isolates of *O. brassicae* can be understood on the basis of specific fungus – virus, host – fungus, and virus – host relationships.

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In a meeting held in 1966 at the Laboratory of Virology, Wageningen, with Prof. R. N. CAMPBELL and Prof. J. P. H. VAN DER WANT, the author presented an outline for the present study. He then envisaged the study to result in a thesis to be published in partial fulfilment of the requirements for a doctor's degree from the State Agricultural University, Wageningen, The Netherlands. Subsequent meetings in 1968 and 1969 in addition to regular written contact with Prof. VAN DER WANT have offered the benefit of his continuous interest and valuable advice.

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SAMENVATTING

Het mechanisme van de overdracht van tabaksnecrosevirus (TNV) door de schimmel *Olpidium brassicae* (Wor.) Dang. is nog niet opgehelderd. Dit probleem leek een aantal aspecten te hebben die zich goed lenen voor een elektronenmicroscopische benadering. Het hier beschreven onderzoek bestond dan ook uitsluitend uit een submicroscopische studie van de virusoverdracht door de schimmel. Om het in deel II beschreven onderzoek naar de wijze van virusoverdracht en naar de specificiteit in die overdracht mogelijk te maken, werd allereerst een studie gemaakt van de virusvrije schimmel, van de wortelepidermis van twee virusvrije waardplanten en van de virusdeeltjes. De resultaten van deze voorstudie zijn beschreven in deel I van deze publikatie. Na een algemene inleiding op de aard en oorsprong van de aan de orde gestelde vragen, worden beide delen begonnen met een literatuuroverzicht.

Hoofdstuk 1 bespreekt enige literatuurgegevens over Phycomycetes in het algemeen en *O. brassicae* in het bijzonder. Verder zijn in dat hoofdstuk gegevens bijeengebracht over de cytologie van de buitenste cellagen van plantewortels en tenslotte wordt aandacht besteed aan wat bekend is over TNV.

Hoofdstuk 2 behandelt de technieken die regelmatig gedurende het gehele onderzoek toegepast werden: het kweken van waardplanten en van de verschillende schimmelisolaten, het zuiveren van het voor de overdrachtsproeven benodigde virus, en alle fasen in de preparatie van het materiaal voor de elektronenmicroscopie.

Hoofdstuk 3 geeft een beschrijving van de ultrastructuur van virusvrije *O. brassicae*. Hierbij wordt slechts resumerend een aantal reeds elders gepubliceerde, meer mycologische, aspecten behandeld om daarna vooral de aandacht te richten op de resultaten die van belang lijken voor het onderzoek naar de virusoverdracht. In paragraaf 3.1, waarin de zoösporen behandeld worden, komt in dat verband als voornaamste gegeven naar voren dat het plasmolemma van het zoösporelichaam continu is met en misschien van dezelfde structuur als de membraan rondom het axonema. Bovendien blijken de zoösporen veel ribosomen, maar geen glycogeen te bevatten. In paragraaf 3.2, waar het infectieproces van de waardplant aan de orde wordt gesteld, komen als belangrijkste resultaten naar voren dat het axonema van de zoösporen wordt ingetrokken bij de cystevorming, dat een cystewand gevormd wordt, en dat bij de infectie het plasmolemma en de tonoplast van de cyste in de lege cystewand achterblijven. Nadat er in paragraaf 3.3 nog op gewezen is dat de jonge schimmelthallus in het waardcelcytoplasma gedurende de eerste 24 uur van zijn groei geen thalluswand doch uitsluitend een begrenzend plasmolemma heeft, worden in paragraaf 3.4 de gevolgen besproken die de in dit hoofdstuk gevonden resultaten mogelijkerwijs hebben voor het begrijpen van de virusoverdracht en de specificiteit daarbij.

In hoofdstuk 4 wordt een beschrijving gegeven van de ultrastructuur van epidermis- en cortexcellen in dat gedeelte van de wortel waarin zich gewoonlijk

de infectie door en de ontwikkeling van *O. brassicae* voltrekt, d.w.z. in de zone van lengtegroei en wortelhaarvorming. In paragraaf 4.1 wordt erop gewezen dat de cellen van de epidermis en van de buitenste cortexlagen van de slawortel vlak achter de groeitop (1–2 mm) gevuld zijn met cytoplasma en de gebruikelijke celorganellen, maar dat op iets grotere afstand van de top (5–15 mm) de relatieve hoeveelheid cytoplasma sterk is afgenomen en dat deze cellen dan ook betrekkelijk snel sterven en afsloffen. In paragraaf 4.2 wordt eenzelfde ontwikkeling beschreven voor de wortel van zaailingen van tomaat, met dien verstande dat het afstervingsproces hier iets langzamer lijkt te verlopen. Tenslotte wordt in paragraaf 4.3 duidelijk gemaakt dat de korte levensduur van de bestudeerde cellen waarschijnlijk geen nadelige gevolgen heeft voor de groei van de schimmel noch voor de vermeerdering van eventueel overgedragen TNV.

Hoofdstuk 5 geeft een beeld van TNV zoals dat er na een aantal prepareertechnieken uitziet en bespreekt de mogelijke oorzaken van de gevonden verschillen in grootte der deeltjes. Tevens worden ter vergelijking beelden getoond van ribosomen in een zoöspore van *O. brassicae*, van komkommernecrosevirus (CNV), en van satellietvirus (SV) met en zonder bijgemengd TNV.

Hoofdstuk 6 bespreekt ter inleiding van deel II van dit proefschrift de literatuurgegevens die bestaan over de bekende schimmelvectoren (paragraaf 6.1) en meer in het bijzonder de publikaties over de relatie tussen *O. brassicae* en TNV en SV (paragraaf 6.2).

In hoofdstuk 7 wordt een poging ondernomen de overdracht van TNV door zoösporen van *O. brassicae* op de voet te volgen. Paragraaf 7.1 laat zien dat de eerste fase van de overdracht bestaat in de adsorptie van TNV-deeltjes aan het plasmolemma en de flagelmembraan van de zoösporen van *O. brassicae*. De voor het zichtbaar maken gebruikte technieken worden kritisch besproken en tevens wordt gewezen op het feit dat de op zichzelf zeer nuttige 'critical point drying method' geen voordelen biedt voor het onderhavige onderzoek. In paragraaf 7.2 worden argumenten aangevoerd om aannemelijk te maken dat het aan het axonema geadsorbeerde virus bij de cystevorming in het schimmelcytoplasma wordt opgenomen tijdens het intrekken van het axonema. Hoewel geïllustreerd wordt dat mogelijk ook enig virus op andere wijze in het cytoplasma belandt en dat sommige virusdeeltjes zelfs tussen de cystewand en de waardcelwand kunnen geraken, wordt er tevens op gewezen dat dit waarschijnlijk van geen of weinig belang is voor de virusoverdracht. In paragraaf 7.3 worden enige gegevens vermeld die leiden tot de veronderstelling dat het virus dat in het schimmelcytoplasma aanwezig is na infectie van de plantecel door *O. brassicae*, wordt uitgestoten in het waardcelcytoplasma door middel van exocytose na de vorming van vacuole-achtige structuren aan de periferie van de jonge thallus. Een in deze paragraaf beschreven methode om door specifieke kleuring te differentiëren tussen TNV-deeltjes en ribosomen wordt als onvoldoende gediskwalificeerd. In de afsluitende paragraaf 7.4 wordt de op grond van de resultaten voorgestelde wijze van TNV-overdracht door *O. brassicae* kort geresumeerd en van enige kritische kanttekeningen voorzien.

Hoofdstuk 8 beschrijft de experimentele benadering van het probleem der

specificiteit in de virusoverdracht door *O. brassicae*. Nadat alle bekende gevallen van specificiteit in de virusoverdracht zijn genoemd, wordt het probleem op drie verschillende manieren benaderd (paragrafen 8.1, 8.2, en 8.3). Tenslotte wordt gepoogd de resultaten van de benaderingswijzen te integreren om te komen tot een algemene verklaring (paragraaf 8.4). In paragraaf 8.1 wordt de relatie tussen schimmel en virus onder de loupe genomen. Het blijkt dat zoösporen van de isolaten van *O. brassicae* afkomstig van sla en tomaat zeer veel virusdeeltjes adsorberen na menging met een suspensie van gezuiverd TNV, doch dat zoösporen van het isolaat van *O. brassicae* afkomstig van mosterd daartoe niet in staat zijn. Zoösporen van het isolaat van *O. brassicae* afkomstig van haver adsorberen wel TNV-deeltjes uit een suspensie, maar in veel geringere mate dan de zoösporen van de isolaten van sla en tomaat. Zoösporen van geen der onderzochte isolaten zijn in staat CNV, turnip yellow mosaic virus, of tomato bushy stunt virus te adsorberen. Zoösporen van *O. cucurbitacearum* zijn daarentegen wel in staat deeltjes van CNV te adsorberen, doch niet deeltjes van TNV. Tenslotte blijken zoösporen van het isolaat van *O. brassicae* afkomstig van sla goed in staat SV-deeltjes uit een suspensie te adsorberen, alleen of in combinatie met TNV.

Het onderzoek in paragraaf 8.2 naar de relatie tussen schimmel en waardplant wijst uit dat voor een aantal isolaten van *O. brassicae* sla, tomaat of mosterd geen geschikte waardplanten zijn. Dit kan zich manifesteren in het geheel of grotendeels achterwege blijven van cystevorming door de zoösporen of van penetratie van de waardcel door het cystecytoplasma, maar ook in een niet volledig uitgroeien en een desintegreren van de schimmelthallus in de geïnfecteerde waardcel. Er wordt op gewezen dat de ene vorm van incompatibiliteit wèl, de andere geen invloed kan hebben op de virusoverdracht.

In paragraaf 8.3 wordt gekeken naar de relatie tussen virus en waardplant en geconstateerd dat sla een slechte waardplant voor TNV is. Weliswaar zijn 18 uur na inoculatie al kristal-achtige groepen van TNV-deeltjes in het epidermiscytoplasma te onderkennen, maar alle door virus geïnfecteerde cellen reageren met sterke necrose en het virus verplaatst zich niet van primair geïnfecteerde cellen (nl. cellen geïnfecteerd door *O. brassicae* èn TNV) naar ernaast liggende epidermis- of cortexcellen. Bovendien kan uit de resultaten van overdrachtsproeven van TNV naar sla en cowpea door zoösporen van het isolaat van *O. brassicae* afkomstig van haver afgeleid worden dat waarschijnlijk op de slawortel, in tegenstelling tot de cowpeawortel, veel TNV-deeltjes per 'infection site' nodig zijn om een geslaagde infectie te krijgen. De tomatewortel blijkt daarentegen een goede waard te zijn voor TNV. Hoewel virus-specifieke symptomen optreden, reageren epidermiscellen van de tomatewortel niet necrotisch op TNV-infectie en bovendien verbreidt het virus zich van cel tot cel in deze waardplant, zodat uiteindelijk een groot aantal nieuwe virusdeeltjes kunnen resulteren uit iedere geslaagde overdracht.

Paragraaf 8.4, die het laatste hoofdstuk en daarmee de publikatie afsluit, geeft een poging weer om de verschillende vormen van specificiteit van TNV-overdracht door verschillende isolaten van *O. brassicae* te verklaren door de ad-

ditieve invloed van de relaties tussen respectievelijk virus en schimmel, schimmel en waardplant, en waardplant en virus. De samenvatting van deze verklaring, die schematisch is neergelegd in tabel 3, luidt als volgt:

1. TNV wordt door een isolaat van *O. brassicae* naar een bepaalde plant overgedragen als zoösporen van het isolaat veel TNV-deeltjes adsorberen en bovendien de plant door veel zoösporen van het isolaat van *O. brassicae* geïnfecteerd wordt en een goede waard is voor het virus.
2. Geen overdracht van TNV door een isolaat van *O. brassicae* naar een bepaalde plant treedt op als de zoösporen van het isolaat geen TNV-deeltjes adsorberen of als de plant geen waard is voor de schimmel of het virus.
3. Overdracht van TNV door een isolaat van *O. brassicae* naar een bepaalde plant kan plaats vinden ondanks het feit dat de zoösporen van het isolaat maar weinig TNV-deeltjes adsorberen, of dat de plant een slechte waard is voor de schimmel of het virus. Overdracht treedt dan echter alleen op wanneer binnen het complex van betrekkingen tussen schimmel, virus en waard naast een zwakke relatie tussen schimmel en virus, of tussen waard en schimmel, of tussen virus en waard, de twee overige betrekkingen zeer goed zijn.

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LIGHT AND ELECTRON MICROGRAPHS

The plates with photographs in this thesis are numbered in such a way that the first digit indicates the number of the chapter to which the photographs of that plate belong. The second digit indicates the number of the plate belonging to that particular chapter.

All plates have legends to explain in general terms what the plates illustrate.
All separate photographs on a plate are marked as Figure A., Figure B., etc. The more specific information concerning these figures is printed in the legends to the figures.

The symbols used in the legends serve to give summarized data on the mode of preparation of the material and on the final magnification of the photographs. The meaning of the subdivision in the set of symbols is: fixation / embedding / staining / magnification.

All electron micrographs were taken with an RCA EMU-3H electron microscope operating at an accelerating voltage of 50 KV. All magnifications are approximations.

List of abbreviations used in the legends and their meaning:

A = axoneme
AF = axonemal fibrils
AS = axonemal sheath
C = centriole
Ca = calyptra
Co = cortex
CC = cyst cytoplasm
CNV = cucumber necrosis virus
CP = cyst plasmalemma
HP = host plasmalemma
HT = host tonoplast
HW = host wall
K = kinetosome
L = lipid globule
Lo = lomasome
LoB = lomasome-like body
M = mitochondrion
MB = multivesicular body
MT = microtubules
P = plastid
Pa = papillum
CT = cyst tonoplast
CV = cleavage vacuole
CW = cyst wall
D = dictyosome
Ep = epidermis
ER = endoplasmic reticulum
ET = exit tube
G = glycogen
HC = host cytoplasm
PM = perinuclear membrane system
R = rhizoplast
Ri = ribosomes
RV = rhizoplast vesicles
SV = satellite virus
SW = sporangium wall
T = thallus
TNV = tobacco necrosis virus
TP = thallus plasmalemma
TW = thallus wall
V = vacuole
W = wall
ZP = zoospore plasmalemma

Explanation of symbols used to indicate the mode of preparation (For details see section 2.3):

Fixation:	G + O	= fixation according to SABATINI et al. (1963)
	G	= fixation with glutaraldehyde only (HILLS and PLASKITT, 1968)
	FG + O	= fixation according to KARNOVSKY (1965).
	O-vapor	= fixation for 1 min in vapor of 4% osmic acid in water.
	PP	= fixation in 1% unbuffered potassium permanganate.
Embedding:	A + E	= embedding in a mixture of araldite and epon.
	ERL	= embedding in a plastic mixture developed by SPURR (1969).
Staining:	Uac + Lci	= staining in 2% uranyl acetate for 30–60 min, followed by staining in lead citrate according to REYNOLDS (1963).
	Uac(e) + Lci(VC)	= staining in 1:1 mixture of saturated uranyl acetate in ethanol and water for 10 min, followed by staining in lead citrate according to VENABLE and COGGESHALL (1965).

U(dehy) + Lhy	= staining procedure as used by HILLS and PLASKITT (1968).
Uac + Lac	= staining procedure as described by STADHOUDERS (1965).
PI + Lci	= staining procedure according to PERRY (1967).
Uac	= 2% uranyl acetate used as a negative stain.
U(s)	= uranium metal used for shadowing.

The – sign in any part of the set of symbols means that the particular treatment was not given.

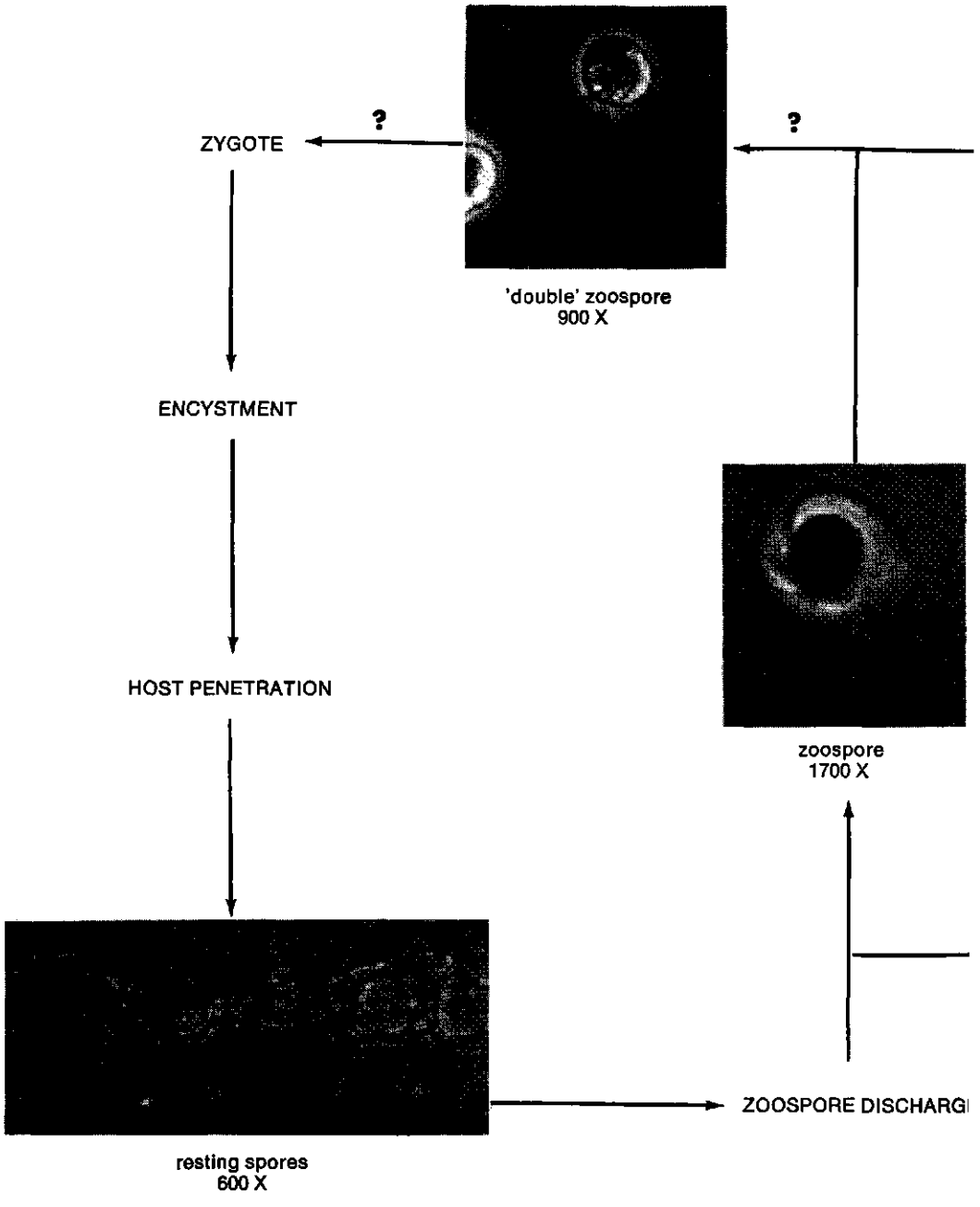
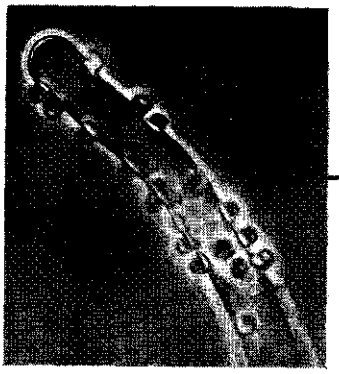
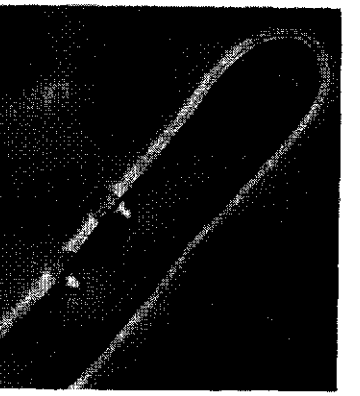


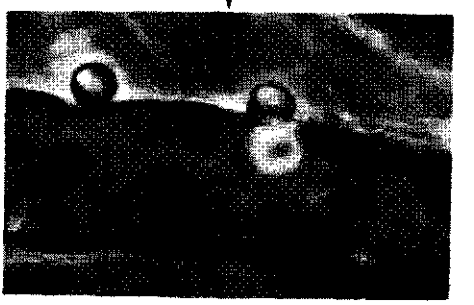
PLATE 1-1. Life cycle of *Olpidium brassicae* with light micrographs illustrating some of the stages.



encystment
500 X



papillum formation
750 X



host penetration
1100 X

mature thalli (zoosporangia)
600 X

zoospore discharge
600 X

PLATE 3-1. Median longitudinal section through zoospore of *O. brassicae*. Note continuity between zoospore plasmalemma (ZP) and axonemal sheath (AS).
G+O/A+E/Uac+Lci/50.000

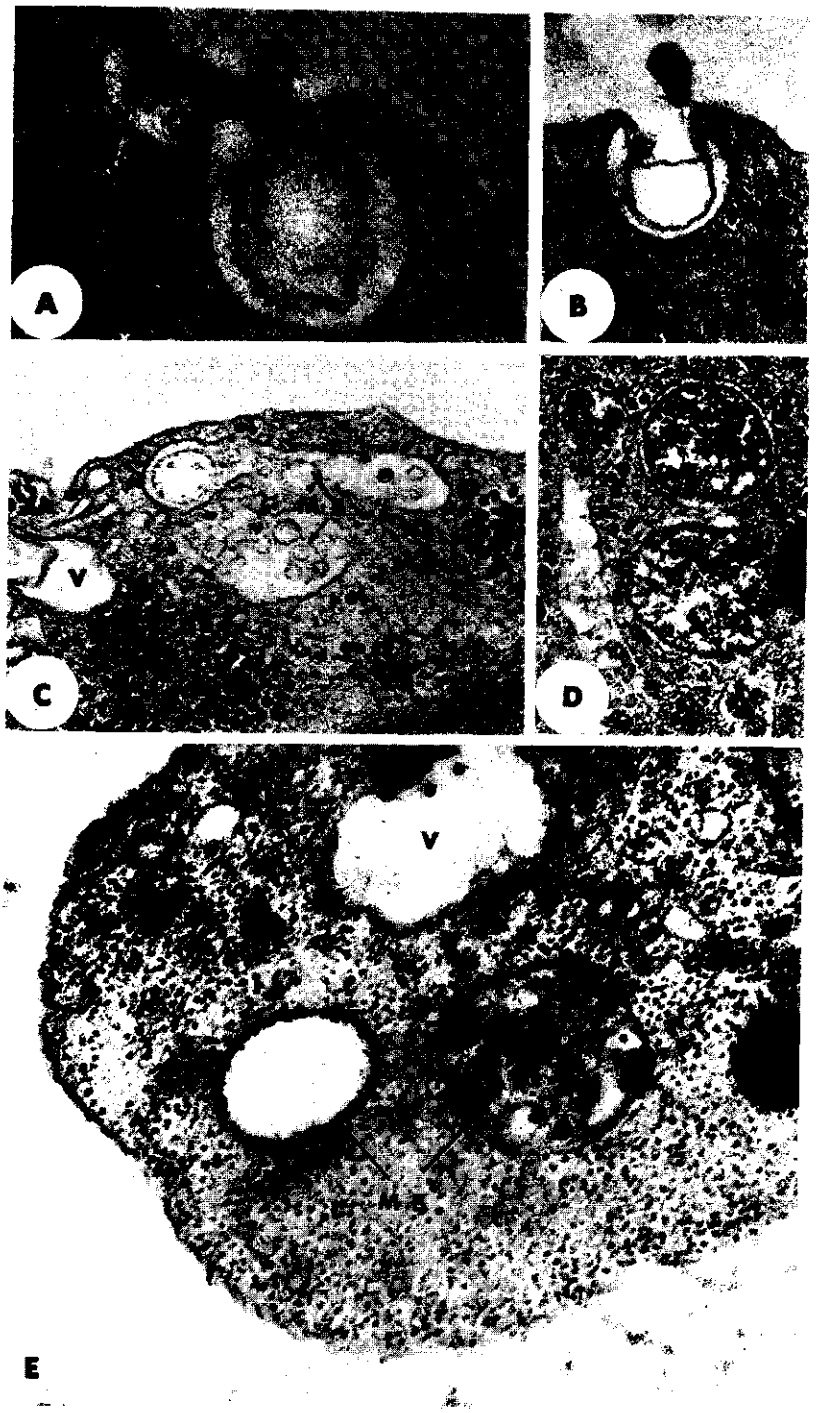
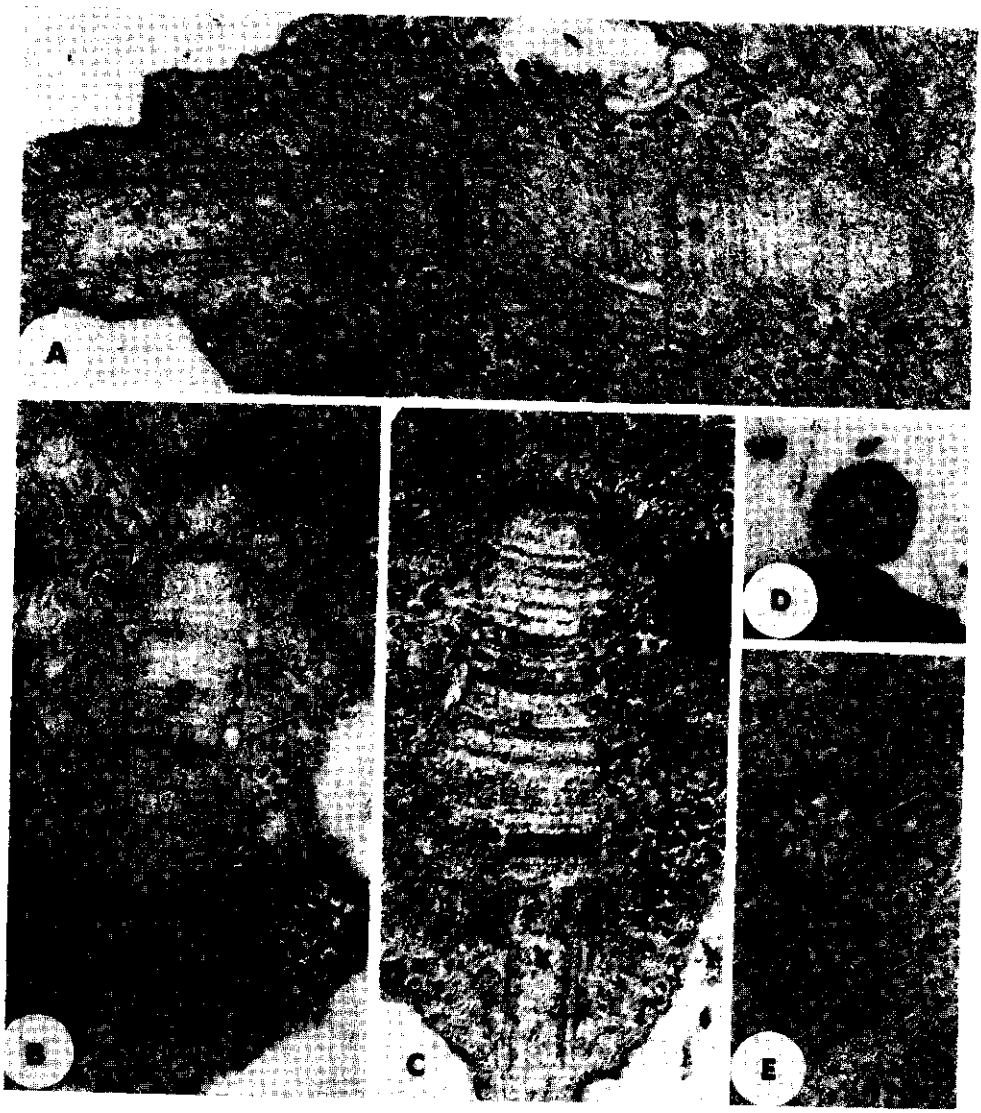

A
B
C
v
D
v
E

PLATE 3-2. Cytoplasmic structures in zoospores of *O. brassicae*.
FIG. A. Vacuole-like structure in close contact with plasmalemma.
G+O/A+E/Uac+Lci/55.000
FIG. B. Vacuole-like structure after fusion with plasmalemma.
G+O/A+E/Uac+Lci/50.000
FIGS. C, D, and E. Different appearances of multivesicular bodies (MB). These structures may function as lysosomes.
FIGS. C and D. G+O/A+E/Uac+Lci/74.000
FIG. E. G+O/A+E/Uac+Lac/64.000

PLATE 3-3. The kinetid in zoospores of *O. brassicae*.
FIG. A. Longitudinal section through rhizoplast (R) and kinetosome (K).
G+O/A+E/Uac+Lci/80.000
FIG. B. Longitudinal section through anterior part of kinetid. Note rhizoplast vesicles (RV) lining the rhizoplast (R) and mitochondrion (M) lying between rhizoplast and nucleus (N).
G+O/A+E/Uac+Lci/69.000
FIG. C. Longitudinal section of same area as in Fig. B., but in plane perpendicular to that. Note centriole (C) lying next to and parallel with anterior part of kinetosome (K).
G+O/A+E/Uac+Lci/72.000
FIG. D. Cross-section of axoneme lying against zoospore body.
G+O/A+E/Uac+Lci/52.000
FIG. E. Cross-section through axonemal kinetosome (K) and centriole (C).
G+O/A+E/Uac+Lci/64.000


A
B
C
D
E


A
B

PLATE 3-4. Zoospores of *O. brassicae* after application of staining techniques specific for glycogen.
FIG. A. No glycogen-specific reaction apparent after treatment according to PERRY (1967).
G+O/A+E/PI+Lci/48.000
FIG. B. No glycogen detectable after treatment according to STADHOUDERS (1965).
G+O/A+E/Uac+Lac/50.000

PLATE 3-5. Encysting zoospores of *O. brassicae* after axonemal withdrawal.
FIG. A. Cyst with axonemal fibrils (AF) visible in cross-section. Axonemal sheath does not surround the fibrils. Note large vesiculate structures designated lomasomes (Lo) or lomasome-like bodies (LoB).
FG+O/A+E/Uac+Lci/43.000
FIG. B. Tangential section through axonemal fibrils (AF) without surrounding sheath in encysting zoospore. Whorl of membranes at bottom of picture is interpreted as pulled-in axonemal sheath (AS).
FG+O/A+E/Uac+Lci/58.000


A F
A
A F
B

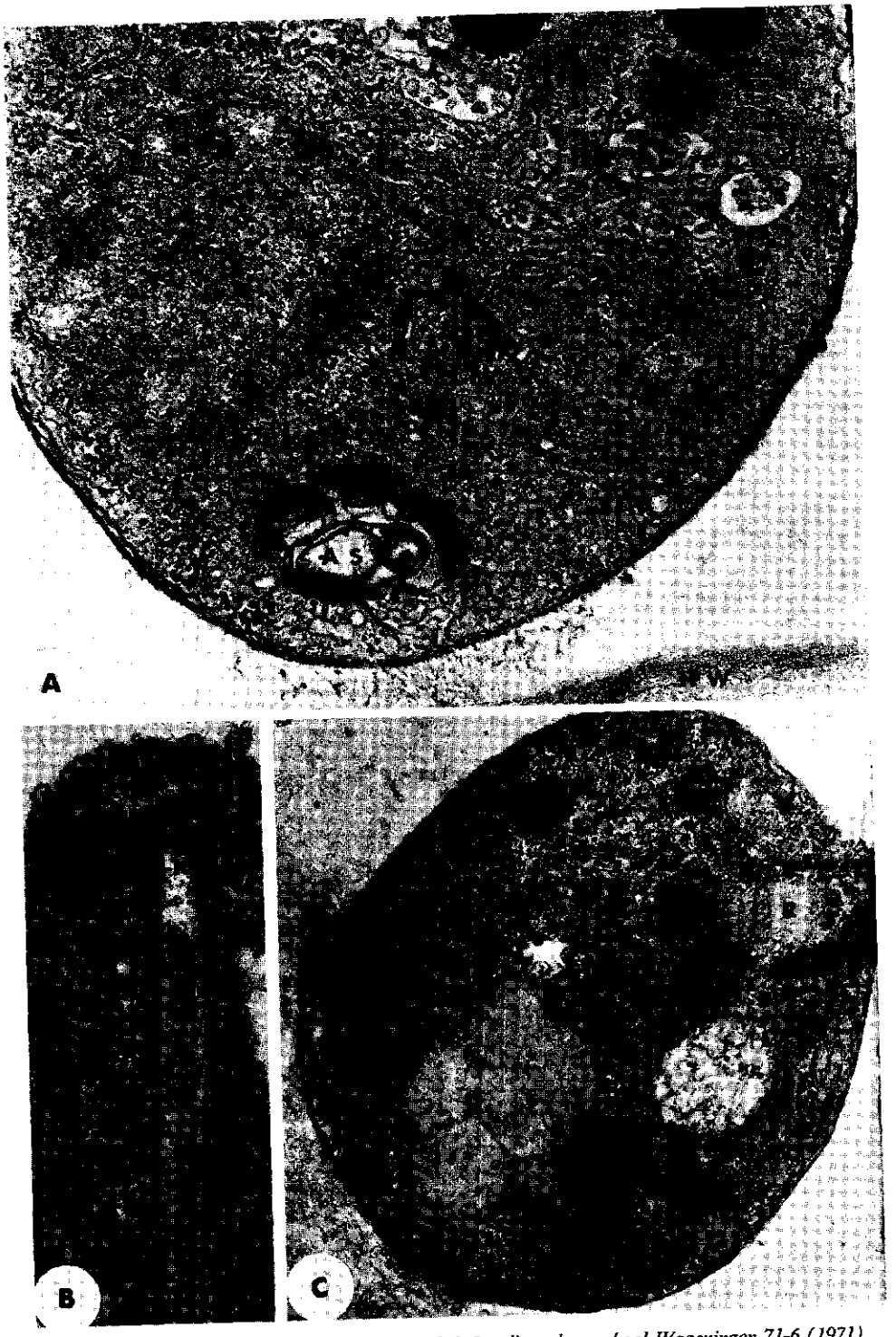
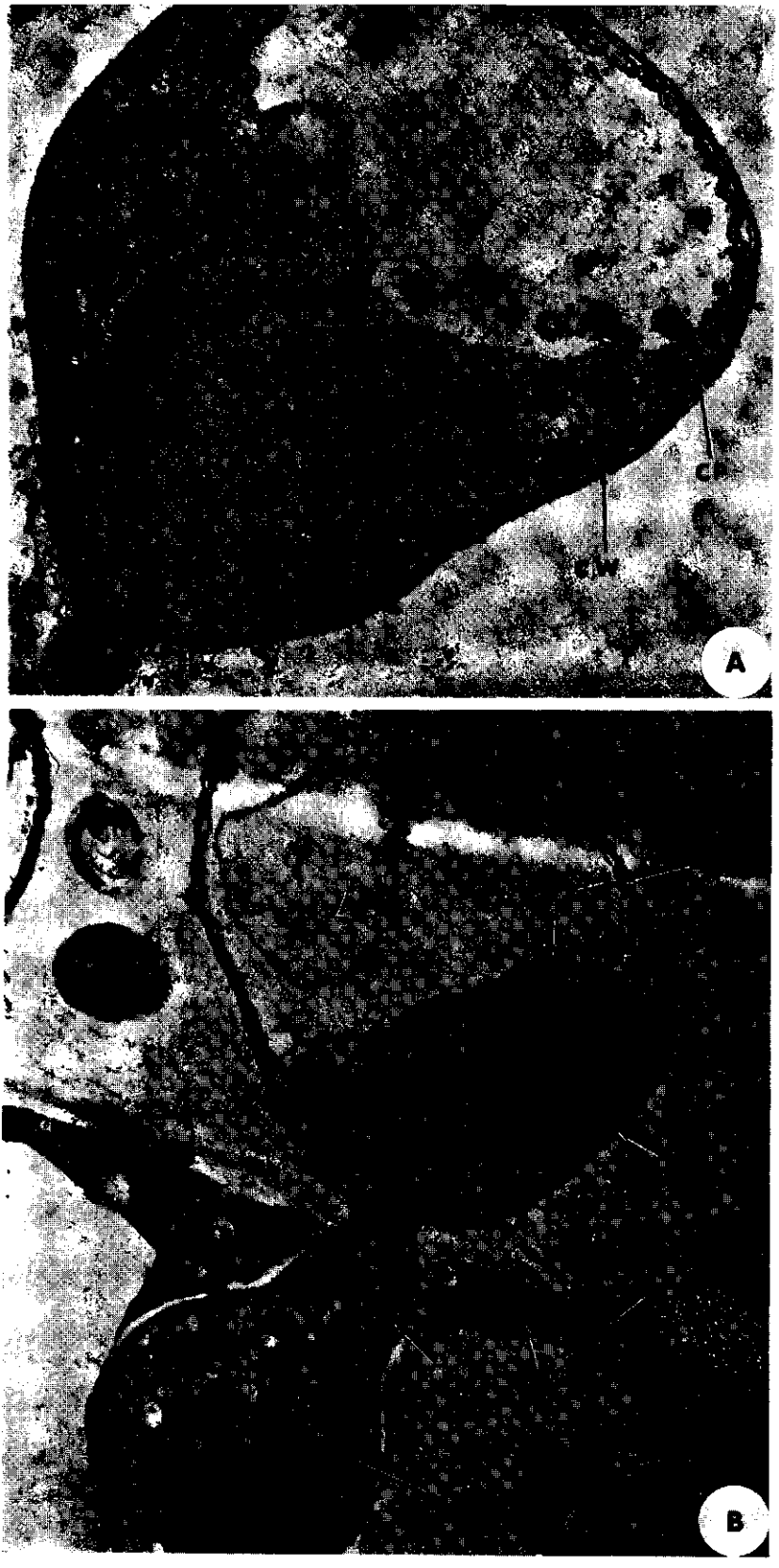

A
B
C

PLATE 3-6. Encysting zoospores of *O. brassicae* after axonemal withdrawal.
FIG. A. Part of cyst with membranes of what is considered axonemal sheath (AS) in close association with a lomasome (Lo).
FG+O/A+E/Uac+Lci/56.000
FIGS. B and C. Kinetids in encysting zoospores oriented in a way that suggests 'reeling-in' withdrawal of axoneme rather than 'wrap-around'.
FIG. B. G+O/ERL/Uac+Lci/50.000
FIG. C. G+O/ERL/Uac+Lci/35.000

PLATE 3-7. Infection of host plant by *O. brassicae*.
FIG. A. Early stage of movement of cyst cytoplasm through hole in cyst wall (CW).
FG+O/A+E/Uac+Lci/35.000
FIG. B. Cyst cytoplasm penetrating into host cell. Note that cyst plasmalemma (CP) and tonoplast (CT) remain behind, but that penetrating cytoplasm (CC) is surrounded by a membrane (unlabeled arrow).
G+O/A+E/Uac+Lci/40.000


CP
CW
A
B

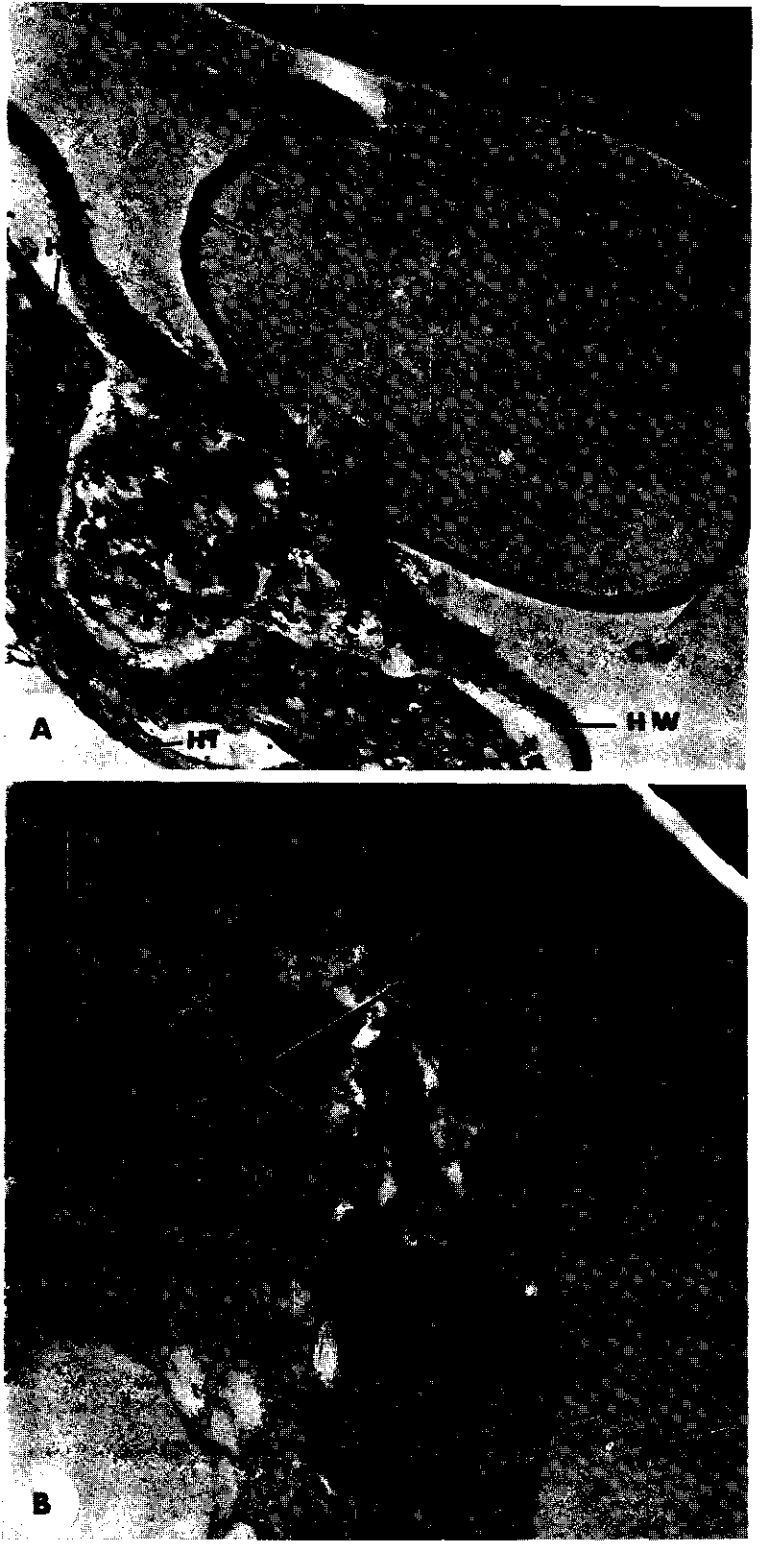

A
HT
HW
B

PLATE 3-8. Completed infection of host cell by *O. brassicae*.
FIG. A. Empty cyst wall (CW) with remaining cyst plasmalemma (CP) and tonoplast (CT). Note well developed papillum (Pa) with infection channel between host wall (HW) and host plasmalemma (HP).
FG+O/A+E/Uac+Lci/29.000
FIG. B. Young thallus (T) in newly infected host cell. Infection channel of papillum (Pa) seems to open into host cytoplasm.
G+O/A+E/Uac+Lci/20.000

PLATE 3-9. Young thalli of *O. brassicae* in newly infected epidermal cell. Thallus is only surrounded by a plasmalemma.
FG+O/A+E/Uac+Lci/27.000

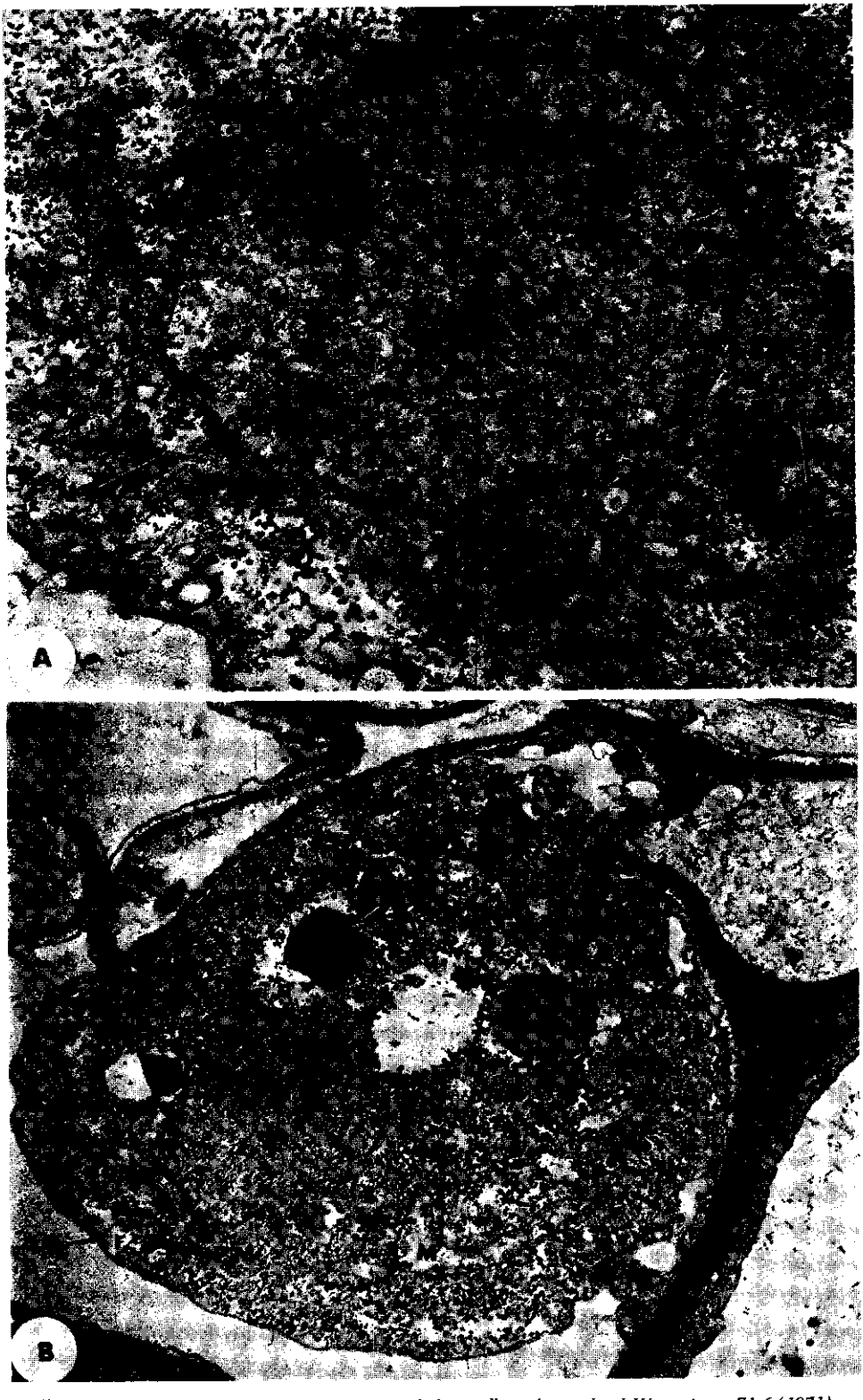

A
B

PLATE 3-10. Thalli of *O. brassicae* in host cell at 24h after inoculation.
FIG. A. Part of young thallus showing nucleus with perinuclear membrane system (PM), centriole (C), and extranuclear microtubules (MT).
G+O/A+E/Uac+Lci/39.000
FIG. B. Uninucleate thallus in epidermal cell of lettuce seedling root. Note that thallus is separated from host cytoplasm by a plasmalemma (TP) only.
G+O/A+E/Uac+Lci/7.000

PLATE 3-11. Older thalli of *O. brassicae*.
FIG. A. Thallus at 48h after infection, with many nuclei (N) and beginning formation of a wall. G+O/A+E/Uac+Lci/4.000
FIG. B. Thallus at 72h after infection with developing exit tube (ET). Cleavage vesicles seem to develop out of multivesicular bodies (MB) that extrude lipid globules (L).
G+O/A+E/Uac+Lci/10.000


A
B
ET

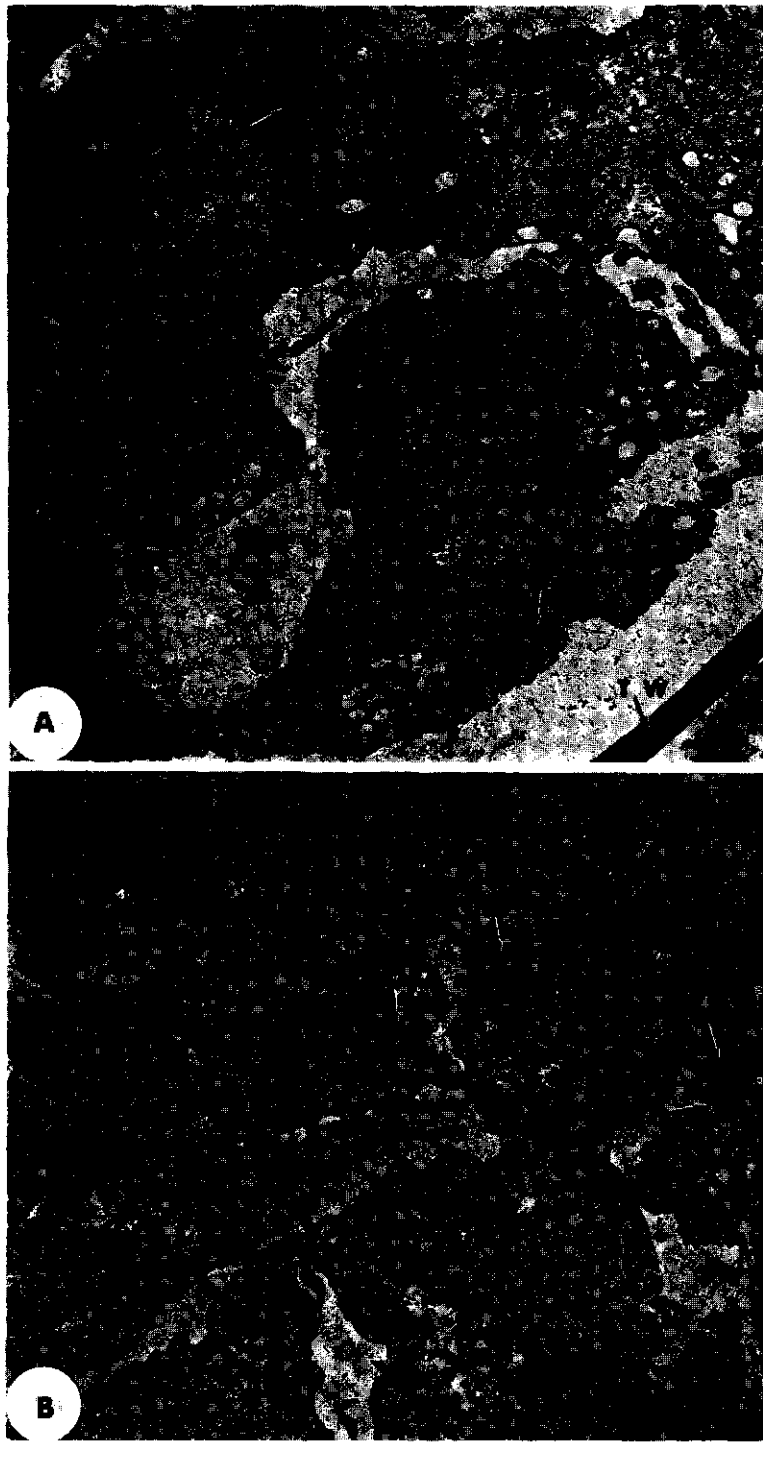

A
B

PLATE 3-12. Zoospore formation in nearly mature sporangia of *O. brassicae*.
FIG. A. Formation of zoospores by fusion of cleavage vesicles (CV)
G+O/A+E/Uac+Lci/14.000
FIG. B. Development of new axonemes on newly-formed zoospores in nearly mature sporangia.
G+O/A+E/Uac+Lci/18.000

PLATE 3-13. Mature sporangia of *O. brassicae*.
FIG. A. New fully-formed zoospores in mature sporangium.
G+O/A+E/Uac+Lci/38.000
FIG. B. Fully developed exit tube (ET) on mature sporangium.
G+O/A+E/Uac+Lci/16.000


A
S W
B

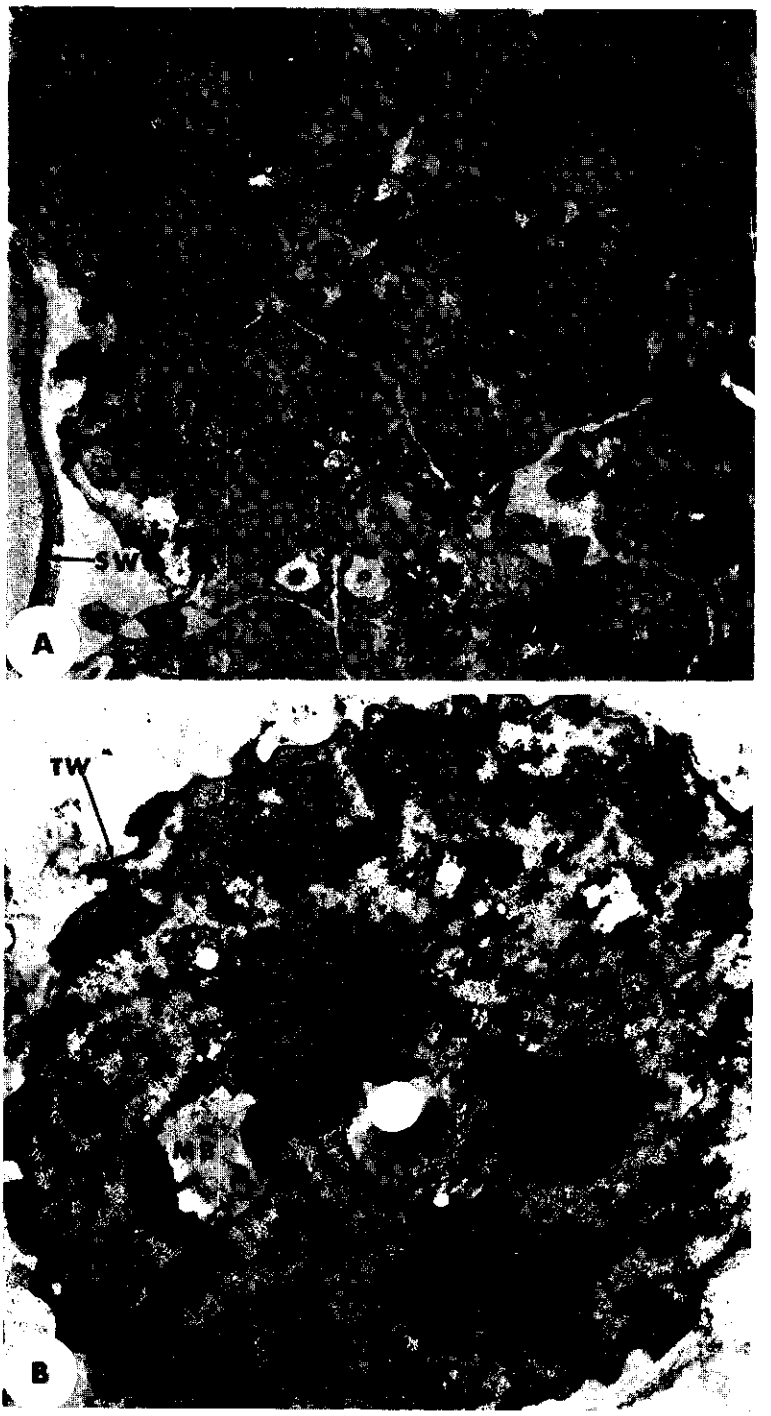

SW
A
TW
B

PLATE 3-14. Sporangia of *O.brassicae* at 72h after infection.
FIG. A. Mature zoosporangium with newly formed zoospores lying ready for discharge.
PP/A+E/–/21.000
FIG. B. Developing resting sporangium with large accumulation of reserve material.
G+O/A+E/Uac+Lci/17.000

PLATE 3-15. Sporangia of *O.brassicae* after application of staining techniques specific for glycogen.

FIG. A. Reaction of ribosomes (Ri) and glycogen (G) to treatment with periodic acid according to PERRY (1967).

G+O/A+E/PI+Lci/56.000

FIG. B. High magnification view of area like in Fig. A.

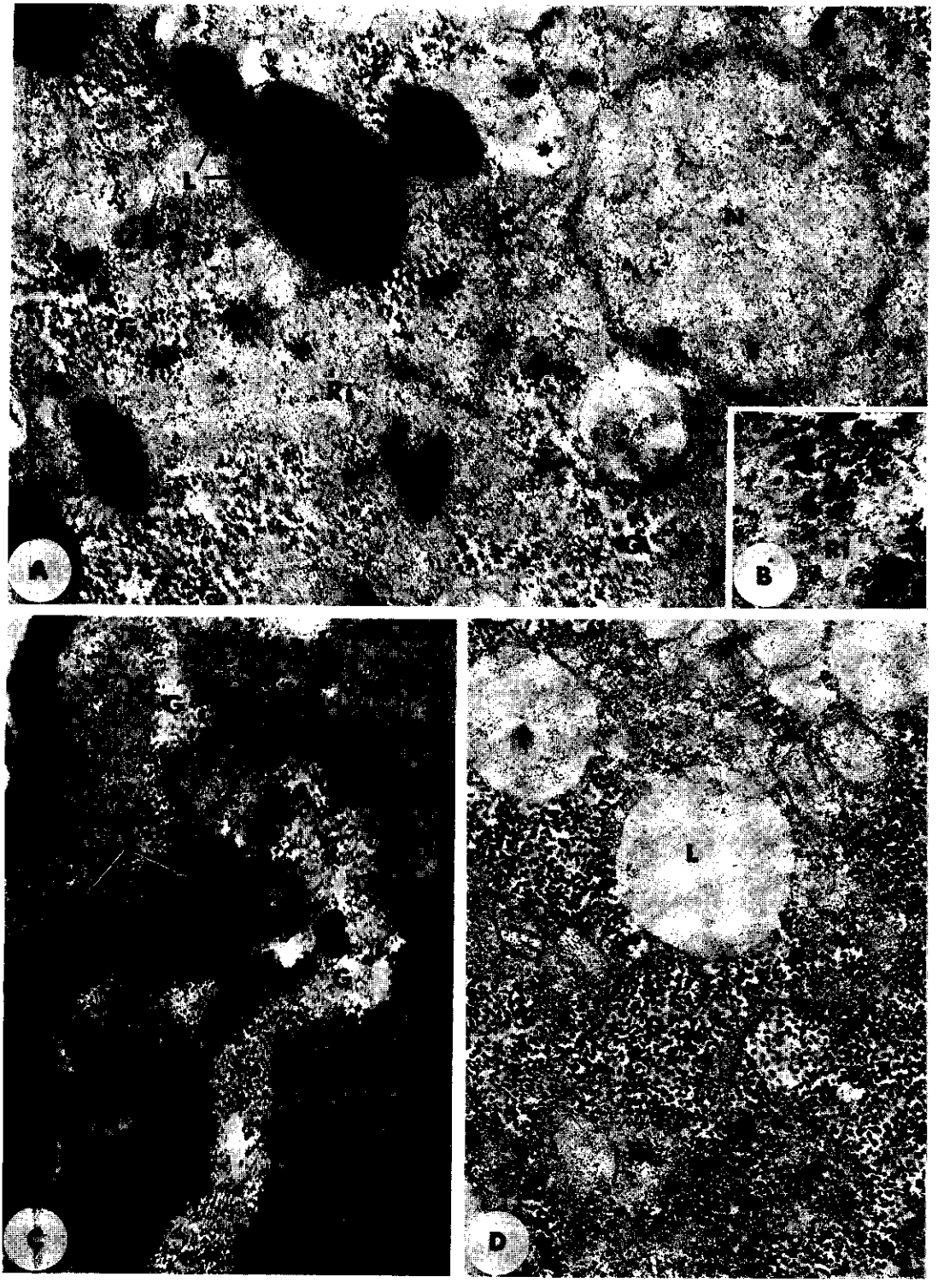
G+O/A+E/PI+Lci/135.000

FIG. C. Low electron density of area with glycogen (G) after staining with lead acetate according to STADHOUDERS (1965).

G+O/A+E/Uac+Lac/44.000

FIG. D. Area comparable to that in Fig. C. but after additional conventional double staining as control for Fig. C.

G+O/A+E/Uac+Lac+Lci/44.000


L
N
M
G
A
B
C
D

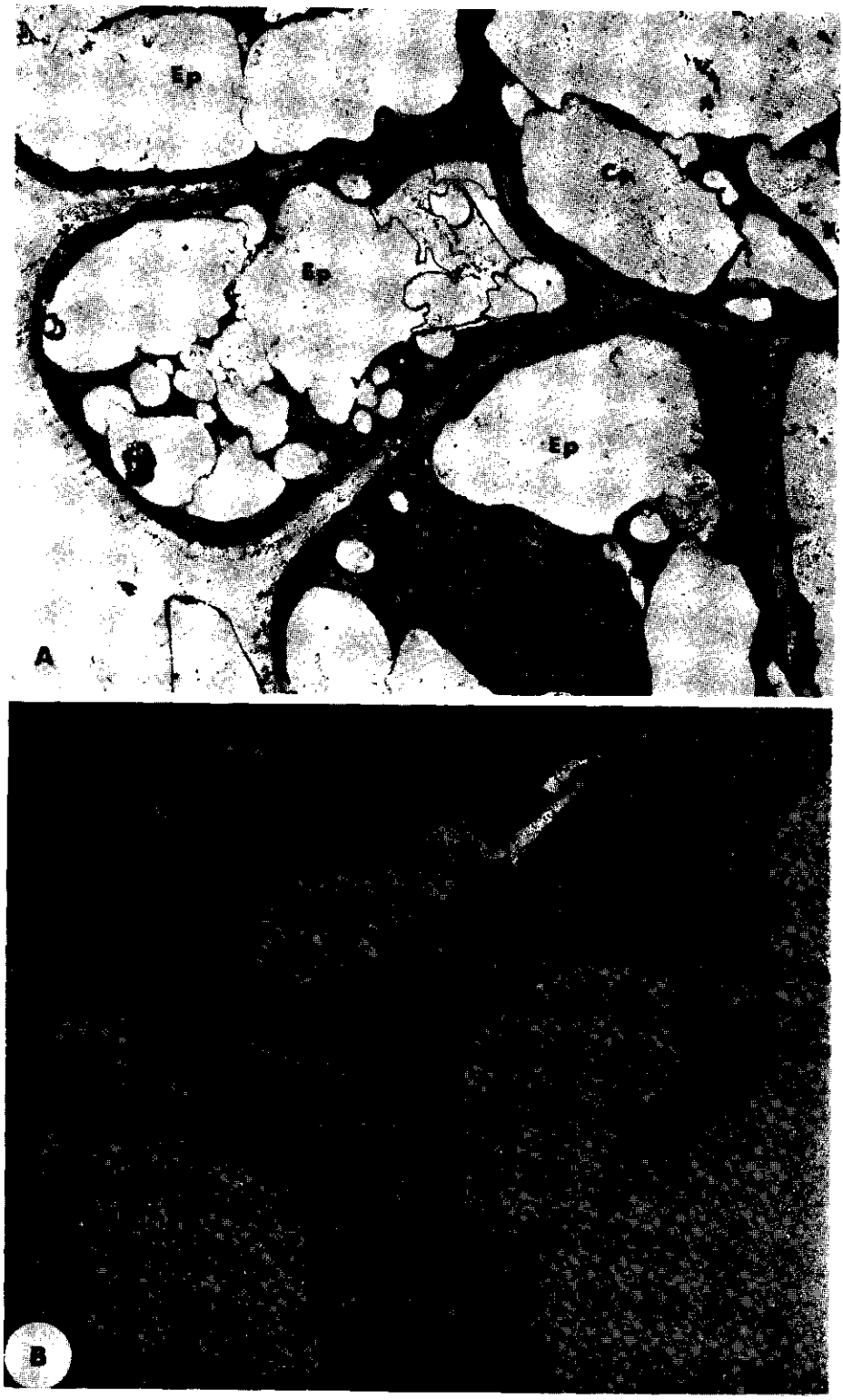
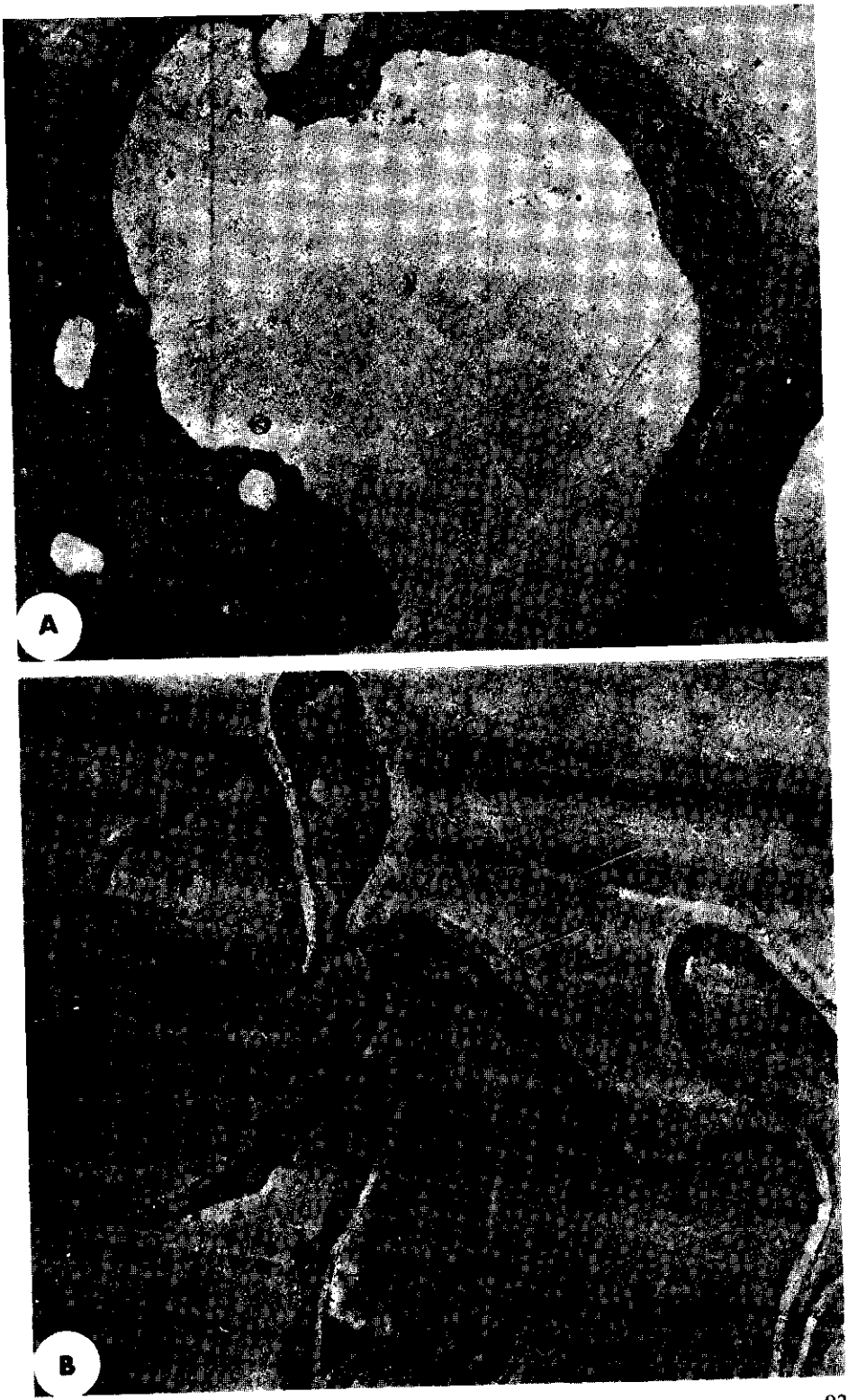

Ep
Ep
Ep
A
B

PLATE 4-1. Epidermal cells in zone of beginning elongation of lettuce seedling roots.
FIG. A. Epidermal cells (Ep) in zone of early elongation (1 mm from root tip) with many small vacuoles.
G+O/ERL/Uac(e)+Lci(VC)/3.500
FIG. B. Two epidermal cells (Ep) and one cortical cell (Co) at 1 mm from root tip. Note necrosis in one cell and good preservation in adjacent cells.
G+O/ERL/Uac(e)+Lci(VC)/16.000

PLATE 4-2. Epidermal cells in zone of advanced elongation of lettuce seedling roots.
FIG. A. Epidermal cell at approximately 2 mm from root tip. One big, central vacuole (V) has been formed but host cell has not collapsed during processing.
G+O/ERL/Uac(e)+Lci(VC)/8.000
FIG. B. Epidermal cells and cortical cells at approximately 4 mm from root tip. Cells have thin layer of cytoplasm surrounding large central vacuole and have collapsed during processing. Note electron dense deposit at tonoplast of cortical cells and rupture of mucilaginous layer of epidermis (unlabeled arrow).
G+O/A+E/Uac+Lci/4.000


A
B

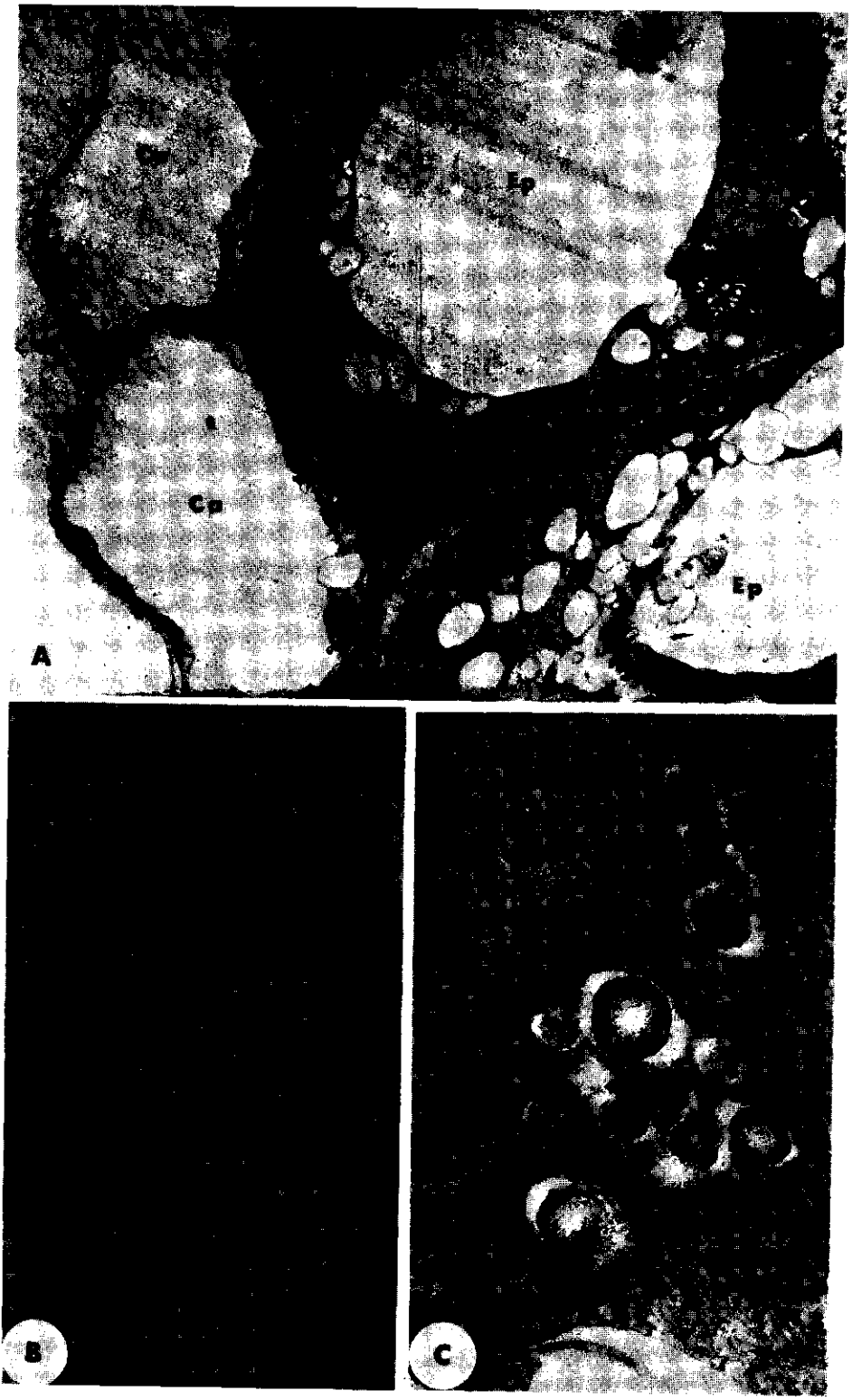
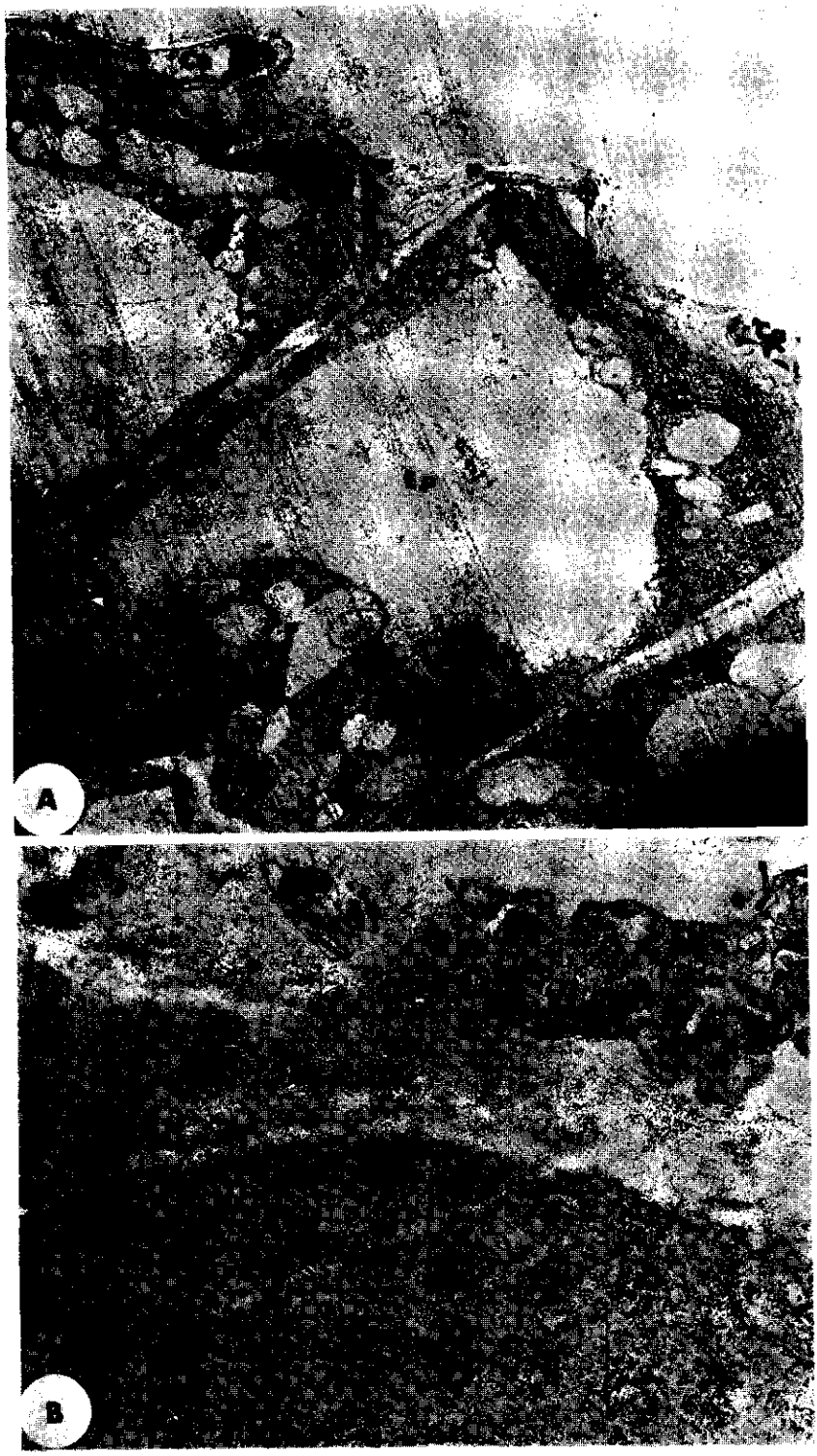

Ep
Cp
Ep
A
B
C

PLATE 4-3. Epidermal cells in zone of beginning elongation of tomato seedling roots.
FIG. A. Epidermal cells (Ep) at approximately 1 mm from root tip with beginning formation of large central vacuole and covered with calyptra cells (Ca).
G+O/A+E/Uac+Lci/6.000
FIG. B. Rough endoplasmic reticulum (ER), mitochondria (M), and a dictyosome (D) in epidermal cell like those shown in Fig. A.
G+O/A+E/Uac+Lci/33.000
FIG. C. Plastids (P) as present in high number in epidermal cells like those in Fig. A.
G+O/A+E/Uac+Lci/33.000

PLATE 4-4. Epidermal cells in zone of advanced elongation of tomato seedling root.
FIG. A. Epidermal cell at approximately 2 mm from root tip. Band of cytoplasm is narrower and calyptra cells (Ca) have shriveled.
G+O/A+E/Uac+Lci/3.000
FIG. B. Higher magnification of similar epidermal cell with shriveled calyptra cells (Ca) attached to outer epidermal wall (W).
G+O/A+E/Uac+Lci/27.000


A
B

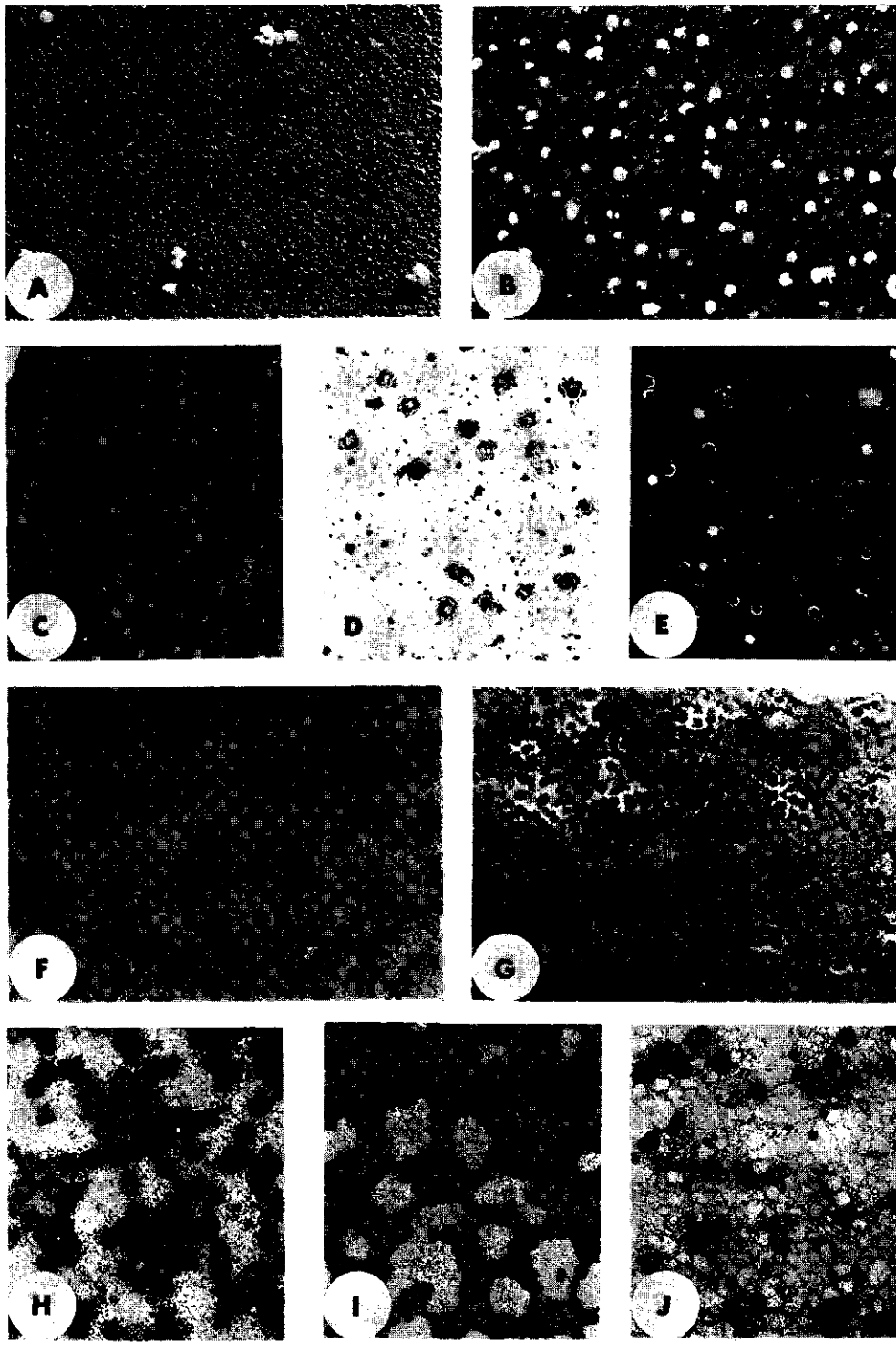

A
B
C
D
E
F
G
H
I
J

PLATE 5-1. Particles of TNV, SV, CNV, and ribosomes after various methods of preparation.
FIG. A. NZ-TNV after air-drying and shadowing with uranium. 70.000
FIG. B. NZ-TNV after critical point drying and shadowing with uranium. 70.000
FIG. C. NZ-TNV after air-drying and negative staining with uranyl acetate. 70.000
FIG. D. NZ-TNV after negative staining with uranyl acetate followed by critical point drying. 70.000
FIG. E. NZ-TNV fixed in vapor of osmic acid, air-dried, and negatively stained with potassium phosphotungstate. 70.000
FIG. F. NZ-TNV in a section through a virus pellet.
G+O/A+E/Uac+Lci/70.000
FIG. G. Ribosomes in a section through a pellet of virus-free zoospores of *O. brassicae*.
G+O/A+E/Uac+Lci/70.000
FIG. H. CNV after air-drying and negative staining with uranyl acetate. 70.000
FIG. I. SV-C after air-drying and negative staining with uranyl acetate. 70.000
FIG. J. Mixture of SV-C and AC-36 after air-drying and negative staining with uranyl acetate. 70.000

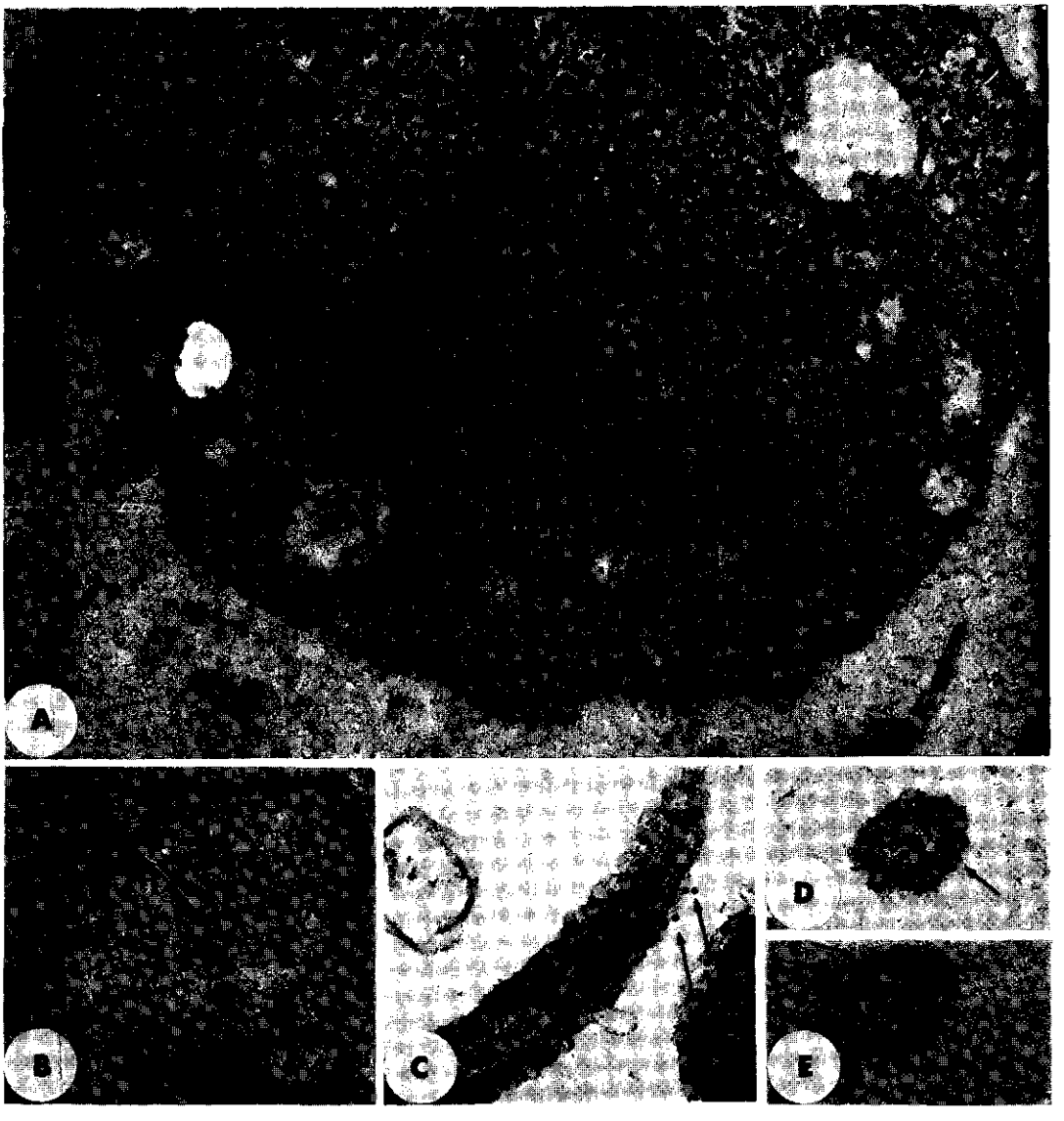
PLATE 7-1. Adsorption of NZ-TNV to zoospores of lettuce *Olpidium*.
O–vapor/–/Uac/35.000
FIG. A. Virus particles adsorbed to axoneme of zoospore after three washes in tap water.
FIG. B. Virus particles adsorbed to body plasmalemma of zoospore after three washes in tap water.
FIG. C. Virus particles adsorbed to zoospore axoneme and excess virus in unwashed preparation.


A
B
C


A
B
C
D
E

PLATE 7-2. Zoospores of lettuce *Olpidium* dried by the critical point method.
FIG. A. Zoospore exposed to TNV and dried without washing.
O-vapor/–/U(s)/3.000
FIG. B. Axoneme of zoospore as in Fig. A.
O-vapor/–/U(s)/28.000
FIG. C. Axoneme of zoospore that has not been exposed to TNV.
O-vapor/–/U(s)/28.000
FIG. D. Zoospore exposed to TNV and dried after three washes in tap water.
O-vapor/–/U(s)/3.000
FIG. E. Axoneme of zoospore that has been exposed to TNV and stained with uranyl acetate prior to drying.
O-vapor/–/Uac/28.000

PLATE 7-3. Adsorption of NZ-TNV to zoospores of lettuce *Olpidium*.
FIG. A. Part of zoospore body with TNV particles adsorbed to outside of plasmalemma.
G+O/A+E/Uac+Lci/48.000
FIG. B. Part of zoospore with distorted plasmalemma and virus particles adsorbed to it (arrow).
G+O/A+E/Uac+Lci/24.000
FIG. C. Longitudinal section through part of axoneme with virus particles attached to outside of sheath (arrows).
G+O/A+E/Uac+Lci/48.000
FIGS. D and E. Cross-sections through axonemes with TNV particles attached to outside of sheath (arrows).
G+O/A+E/Uac+Lci/48.000


A
B
C
D
E

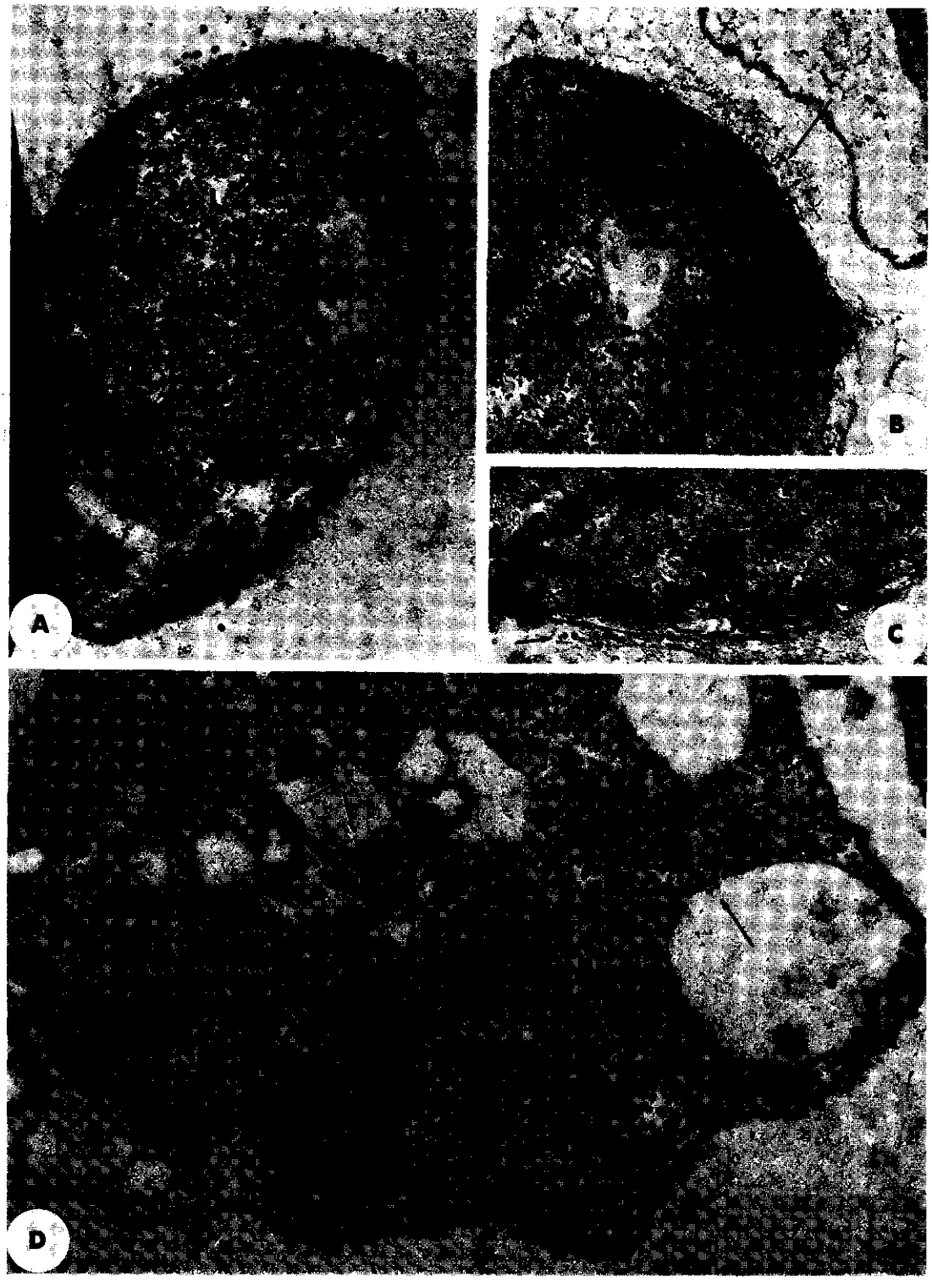
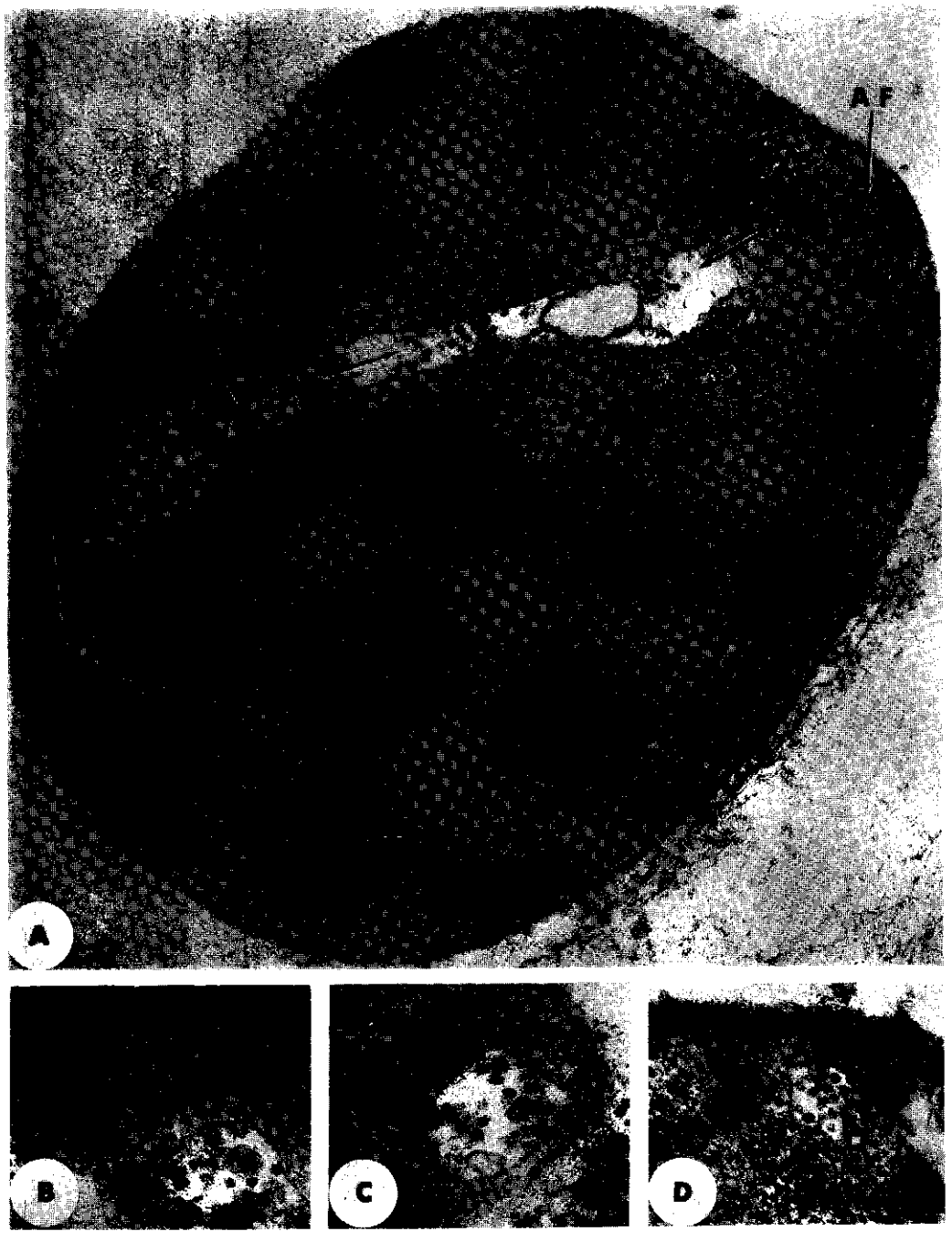

A
B
C
D

PLATE 7-4. The fate of some TNV particles after adsorption to zoospores of lettuce *Olpidium*.
FIG. A. TNV particles attached to outside of cyst wall.
G/A+E/Uac(dehy)+Lhy/48.000
FIGS. B and C. TNV particles trapped between host cell wall and wall of encysted zoospore (arrows).
G+O/ERL/Uac+Lci/56.000
FIG. D. TNV particles present in vacuole-like vesicles inside zoospore body (arrows).
G+O/A+E/Uac+Lci/48.000

PLATE 7-5. TNV particles taken up inside encysted zoospores of lettuce *Olpidium*.
FIG. A. Encysted zoospore with numerous TNV particles (arrows) in vacuole-like structure located between fibrils of withdrawn axoneme (AF).
G+O/ERL/Uac+Lci/64.000
FIGS. B–D. Numerous TNV particles in vacuole-like structures and perhaps also free in cytoplasm of encysted zoospores.
G+O/ERL/Uac+Lci/64.000


A
B
C
D

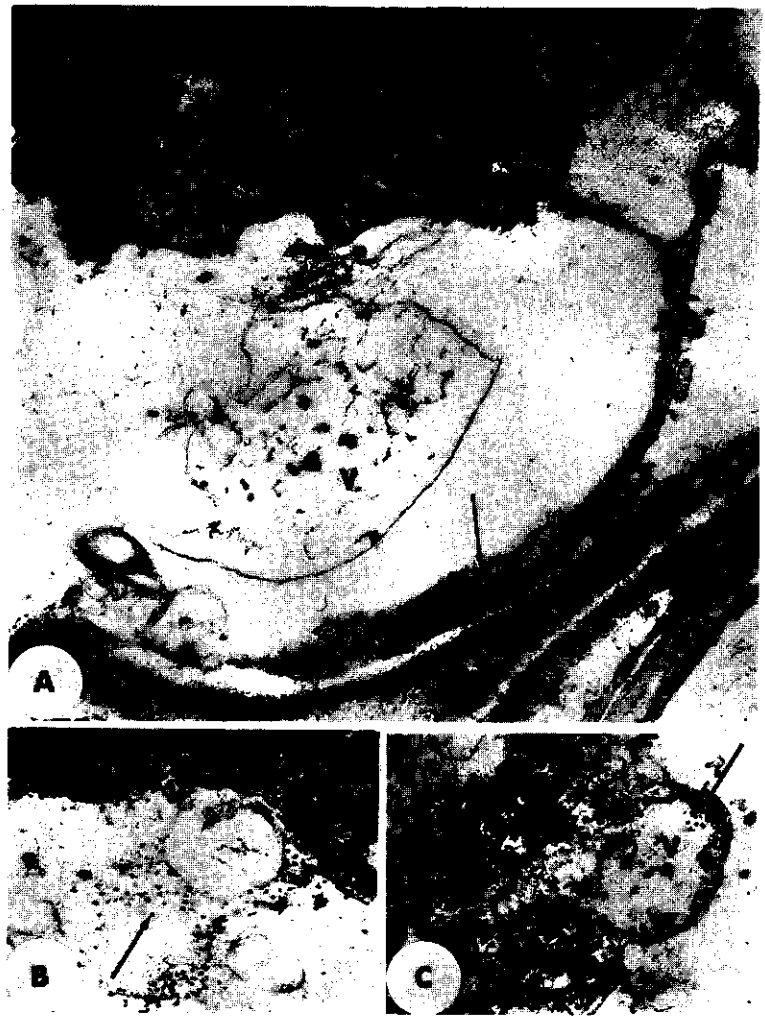

A
B
C

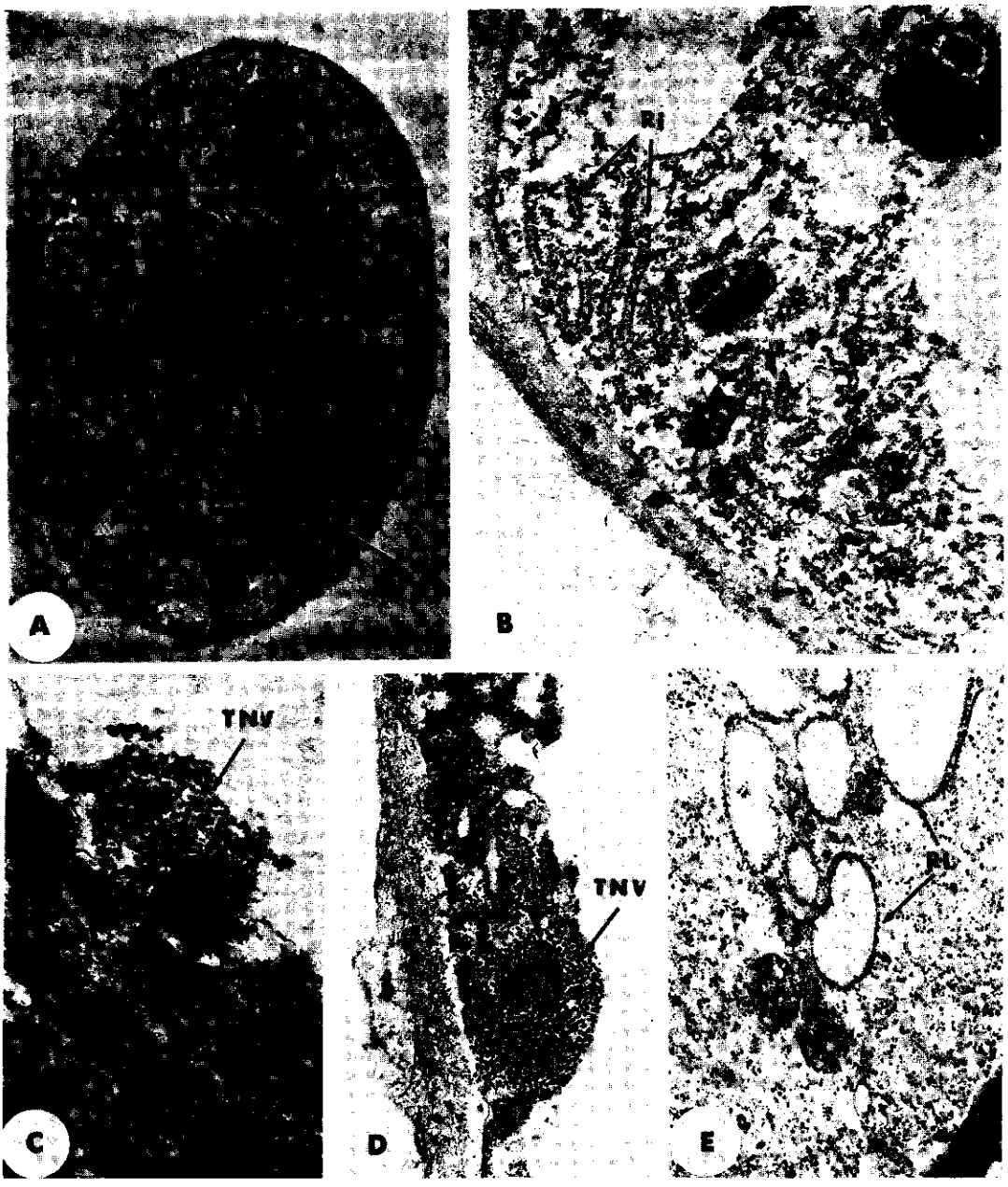
PLATE 7-6. Release of TNV particles from thalli of lettuce *Olpidium* inside infected host epidermal cells at 18h after inoculation.

FIG. A. Fungal vacuole-like structure (V) in host cytoplasm adjacent to young thallus (T). Note presence of virus particles in cell cytoplasm (arrow).
G+O/A+E/Uac+Lci/22.000

FIG. B. Virus particles (arrow) surrounding vacuole-like structures that lie close to fungal thallus from which they may have originated.
G+O/A+E/Uac+Lci/34.000

FIG. C. Virus particles (arrow) inside vacuole-like structure (V) that seems to be extruded by fungal thallus (T).
G+O/A+E/Uac+Lci/34.000

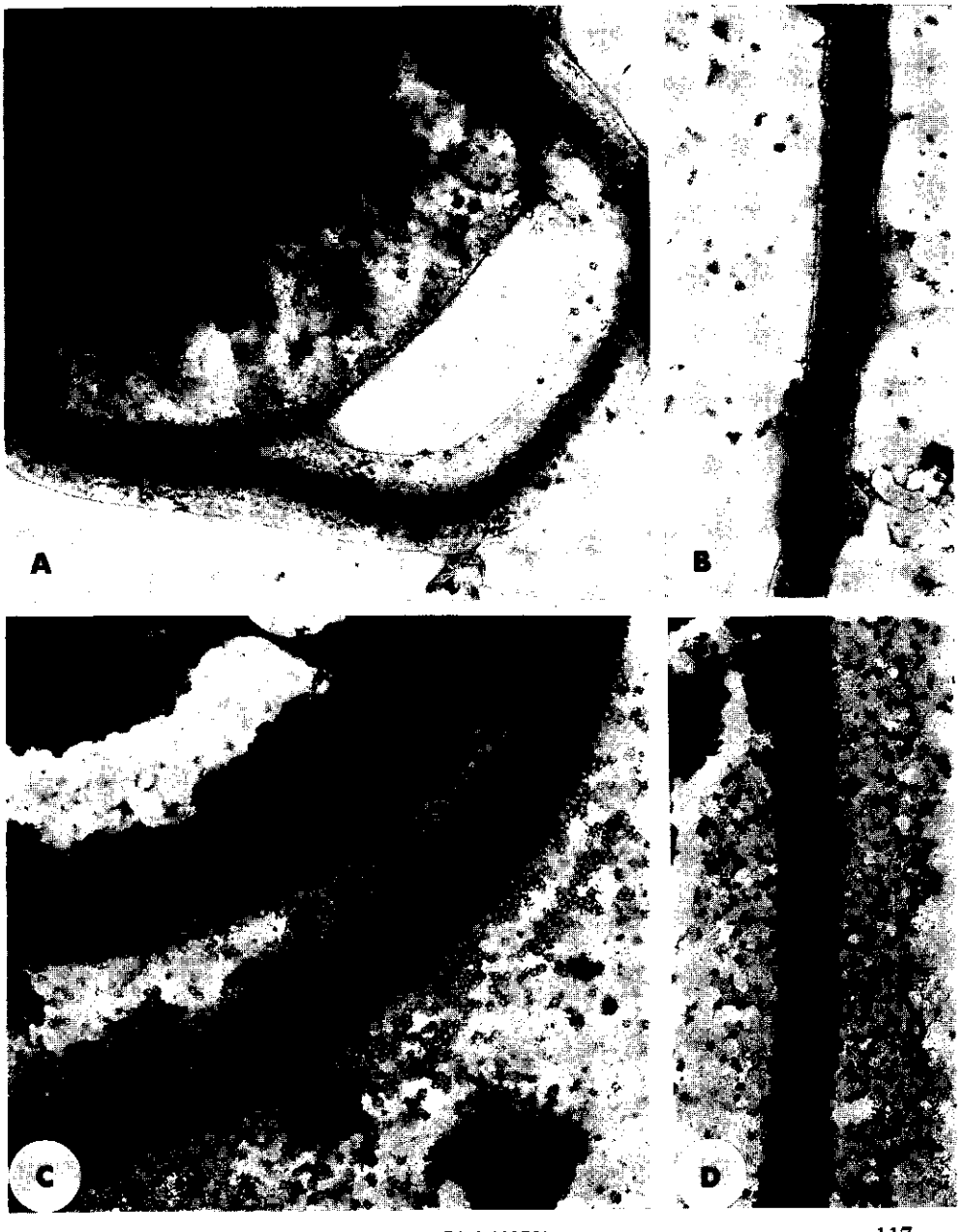
PLATE 7-7. Attempts to use a virus-specific staining technique.
G/A + E/Uac(dehy) + Lhy/32.000
FIG. A. Encysted zoospore of tomato *Olpidium* with well-stained ribosomes (Ri).
FIG. B. Tomato root epidermal cell with ribosomes lining cisternae of endoplasmic reticulum (Ri).
FIGS. C and D. Necrotic epidermal cells of lettuce root with clusters of well-stained TNV particles.
FIG. E. TNV-infected epidermal cell of lettuce root with ribosomes (Ri) around inflated cisternae of endoplasmic reticulum and possibly with virus particles.


A
B
RI
C
TNV
D
TNV
E
RI


A
B
C
D

PLATE 8-1. Difference between zoospores of lettuce *Olpidium* and mustard *Olpidium* in adsorption of NZ-TNV.
O-vapor/-/Uac/28.000
FIGS. A and B. Part of body and axoneme of washed zoospores of lettuce *Olpidium* with adsorbed virus particles after in vitro exposure to TNV.
FIGS. C and D. Part of body and axoneme of washed zoospores of mustard *Olpidium* without any adsorbed virus particles after in vitro exposure to TNV.

PLATE 8-2. Adsorption of virus particles by oat *Olpidium* and *O. cucurbitacearum*.
O-vapor/–/Uac/28.000
FIGS. A and B. Part of body and axoneme of washed zoospores of oat *Olpidium* with few adsorbed virus particles after in vitro exposure to NZ-TNV.
FIGS. C and D. Part of body and axoneme of washed zoospores of *O. cucurbitacearum* with many adsorbed and detached virus particles after in vitro exposure to CNV.


A
B
C
D

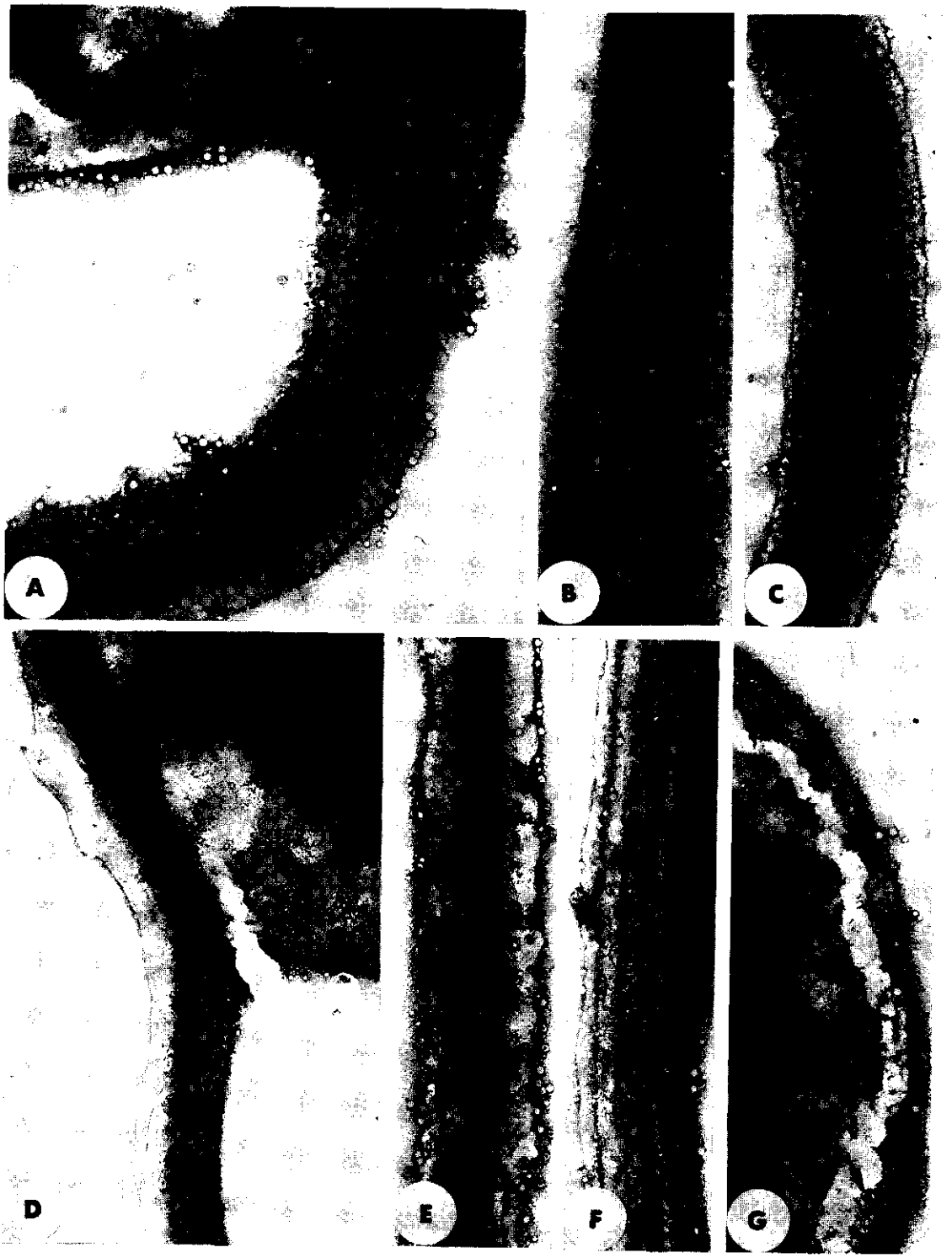
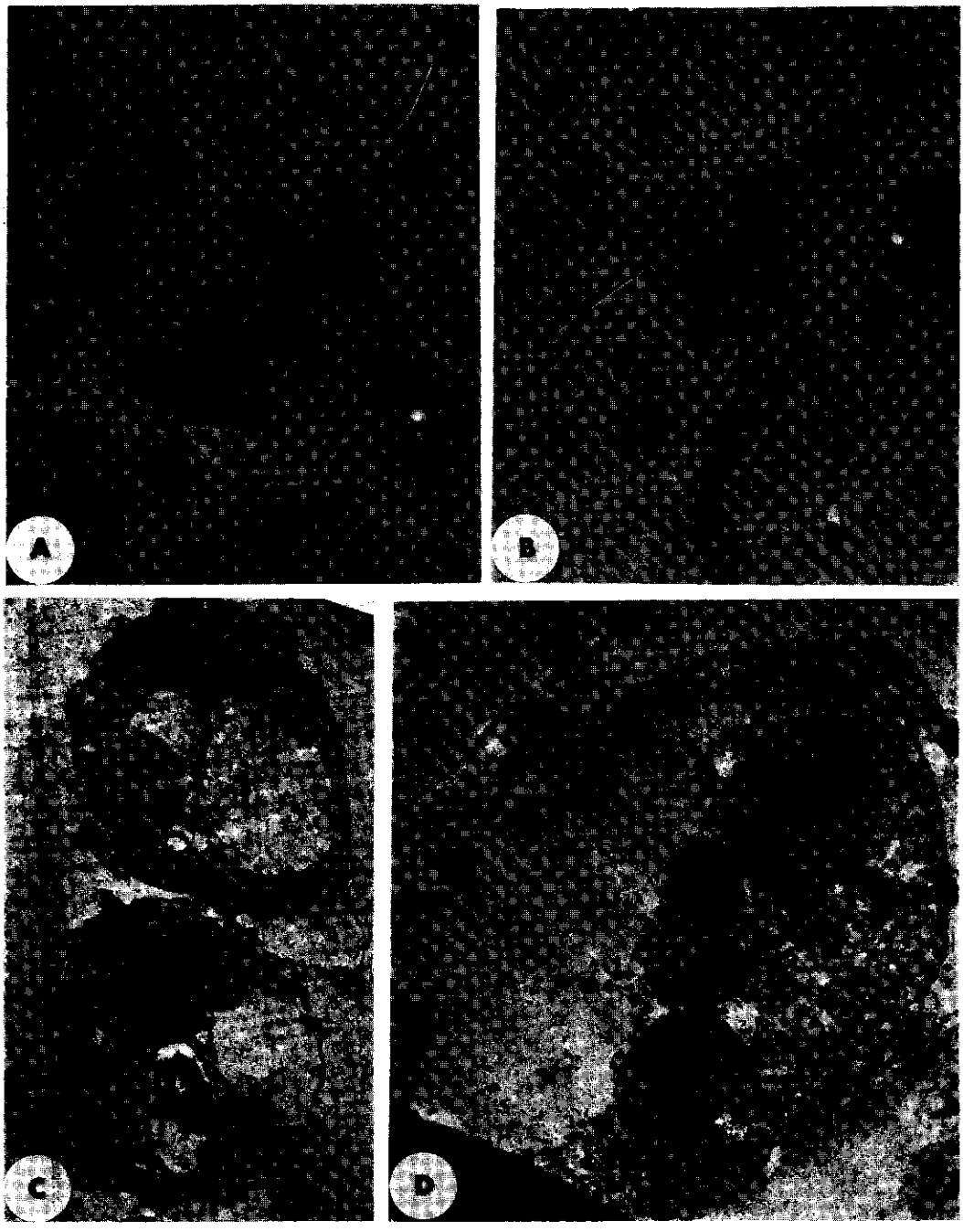

A
B
C
D
E
F
G

PLATE 8-3. Adsorption of TNV and SV to zoospores of lettuce *Olpidium*.
O–vapor/–/Uac/32.000
FIGS. A–C. Adsorption of AC-36 and SV-C to body and axoneme of washed zoospores of lettuce *Olpidium* after in vitro exposure to mixture of both viruses.
FIG. D. Adsorption of SV-C to body and axoneme of washed zoospore of lettuce *Olpidium* after in vitro exposure to SV-C only.
FIG. E. Adsorption of NZ-TNV and SV-C to washed zoospore of lettuce *Olpidium* after in vitro exposure to mixture of both viruses.
FIG. F. Adsorption as in Fig. E., but after in vitro exposure to NZ-TNV alone, followed by exposure to NZ-TNV and SV-C.
FIG. G. Adsorption as in Fig. F., but after in vitro exposure to SV-C alone, followed by exposure to SV-C and NZ-TNV.

PLATE 8-4. Infection of mustard seedling roots by lettuce *Olpidium*.
FIG. A. Normally encysting zoospore on host epidermal wall (HW).
G+O/ERL/Uac+Lci/24.000
FIG. B. Cyst detached from host cell during processing for electron microscopy but otherwise going through normal infection process.
G+O/ERL/Uac+Lci/24.000
FIG. C. Young fungal thalli (T) in host epidermal cytoplasm (HC). Note abnormally large vacuoles containing electron dense material.
G+O/ERL/Uac+Lci/7.000
FIG. D. Fungus thallus (T) in host cell disintegrating at 24h after inoculation.
G+O/ERL/Uac+Lci/14.000


A
B
C
D

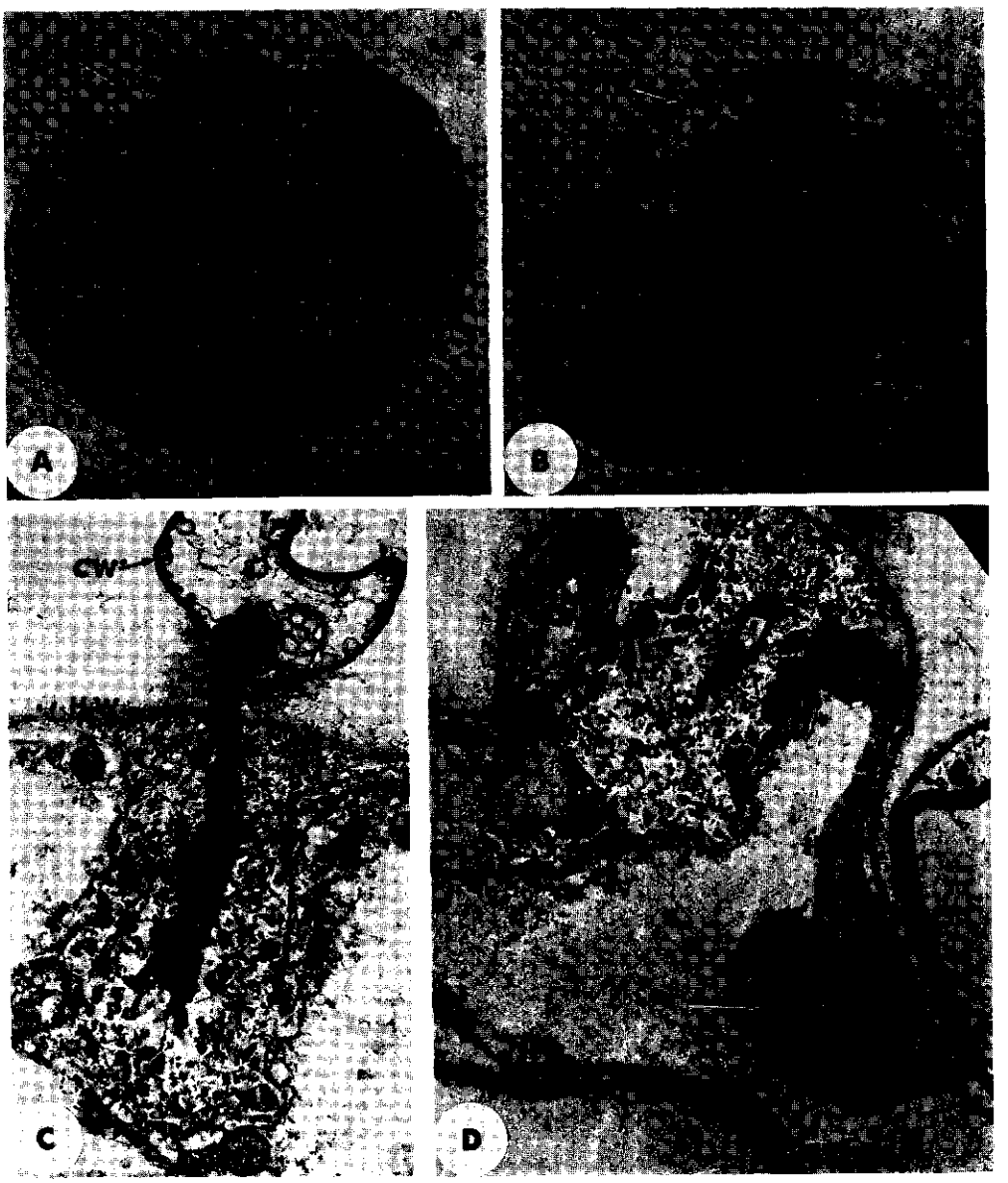
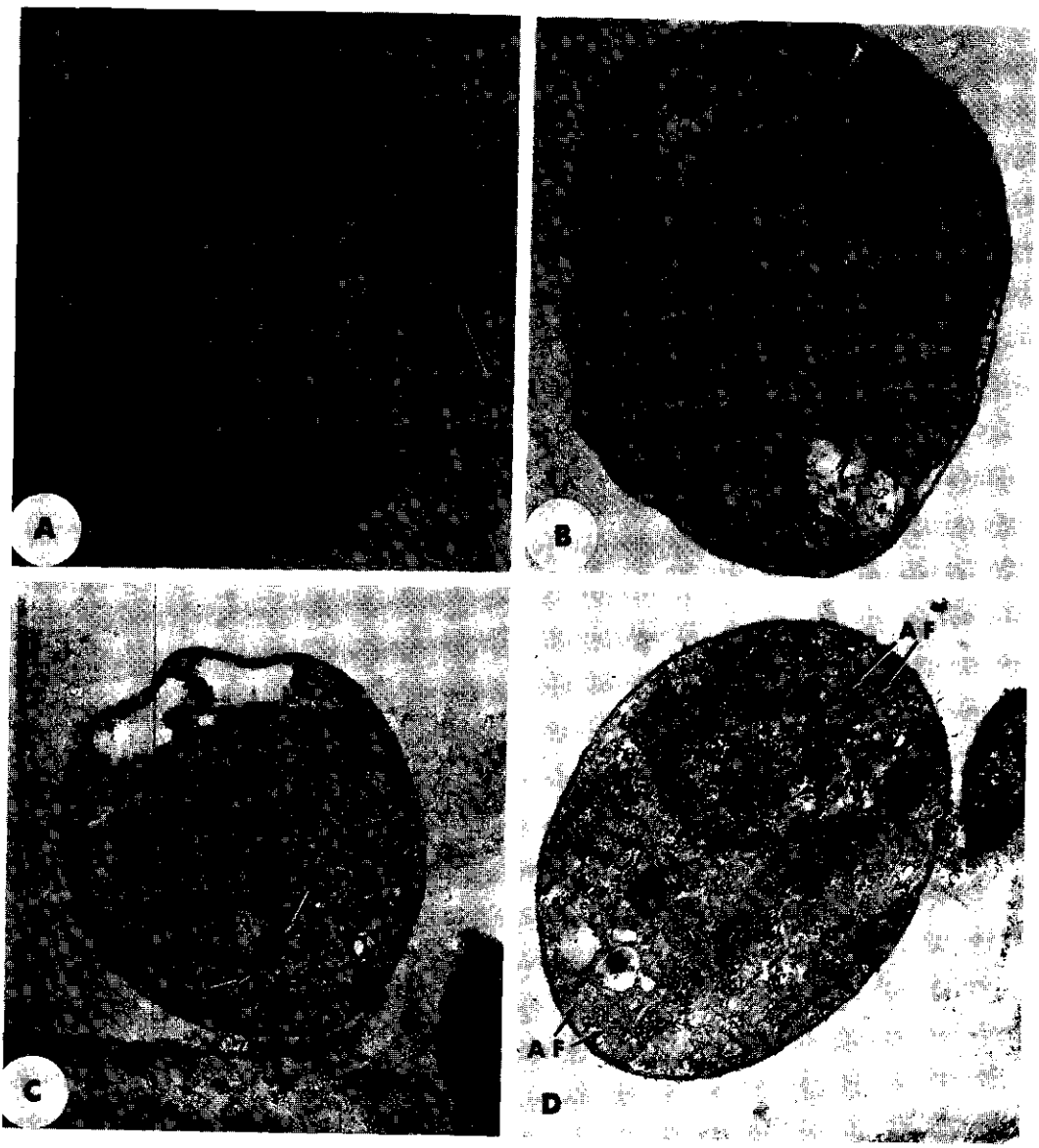

A
B
CW
C
D

PLATE 8-5. Infection of lettuce seedling roots by mustard *Olpidium*.
FIG. A. Normally encysting zoospore on host root epidermis.
G+O/A+E/Uac+Lci/22.000
FIG. B. Cyst that had failed to infect the host cell at 24h after inoculation. Note vacuole-like structures with electron-dense material.
G+O/A+E/Uac+Lci/22.000
FIG. C. Infection papillum (Pa) and empty cyst wall (CW) after a seemingly normal infection process.
G+O/A+E/Uac+Lci/11.000
FIG. D. Infection papillum (Pa) and young thallus (T) in host cell at 8h after inoculation.
G+O/A+E/Uac+Lci/8.000

PLATE 8-6. Cysts of different *Olpidium* isolates.
FIG. A. Zoospore of lettuce *Olpidium* encysting on tomato seedling root.
G+O/ERL/Uac+Lci/27.000
FIG. B. Zoospore of mustard *Olpidium* encysting on tomato seedling root.
G+O/A+E/Uac+Lci/27.000
FIG. C. Zoospore of oat *Olpidium* encysting on lettuce seedling root.
G+O/A+E/Uac+Lci/18.000
FIG. D. Zoospore of tomato *Olpidium* encysting on lettuce seedling root.
G+O/A+E/Uac+Lci/27.000


A
B
C
AF
AF
D

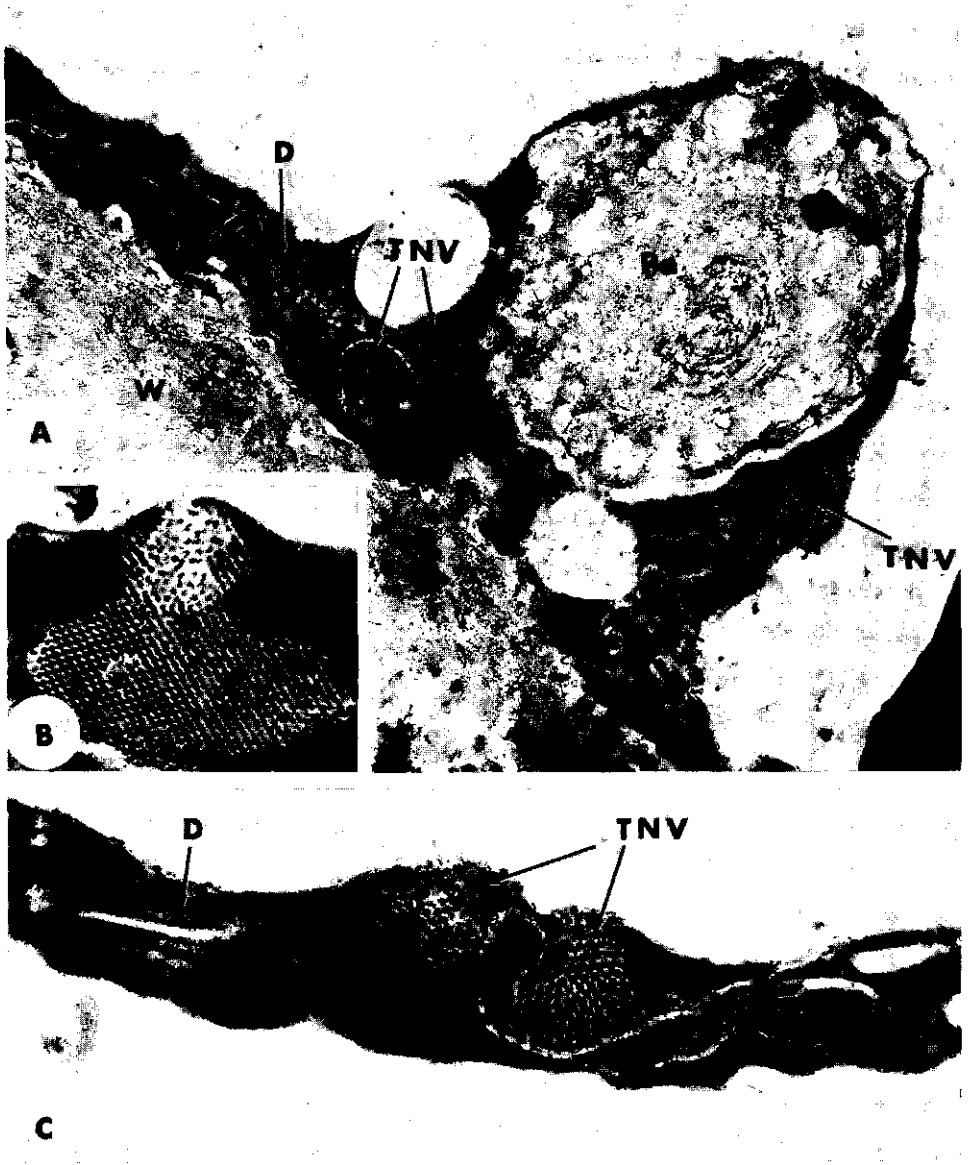

D
TNV
W
A
TNV
B
D
TNV
C

PLATE 8-7. TNV in the epidermal cells of lettuce seedling roots at 18h after inoculation with viruliferous zoospores of lettuce *Olpidium*.
FIG. A. Pockets of TNV particles in necrotic cell cytoplasm closely associated with infection papillum (Pa).
G/A+E/Uac(dehy)+Lhy/34.000
FIG. B. Cluster of TNV particles in necrotic host tissue showing crystal-like array of virus particles.
G/A+E/Uac(dehy)+Lhy/50.000
FIG. C. Crystal-like array of TNV particles in necrotic cytoplasm of host epidermis.
G/A+E/Uac(dehy)+Lhy/67.000

PLATE 8-8. TNV in lettuce root epidermal cells at 24h after inoculation with viruliferous zoospores of lettuce *Olpidium*.
FIG. A. Irregular array of virus particles in necrotic epidermal cytoplasm.
G+O/A+E/Uac+Lci/90.000
FIG. B. Clusters of virus particles (unlabeled arrows) and cristae of rough endoplasmic reticulum (ER) in non-necrotic epidermal cytoplasm between cell wall and plastid (P).
G+O/A+E/Uac+Lci/34.000
FIG. C. Non-necrotic epidermal cell with clusters of virus particles (unlabeled arrows) in large cytoplasmic area of seemingly high metabolic activity.
G+O/A+E/Uac+Lci/34.000


A
B
V
C

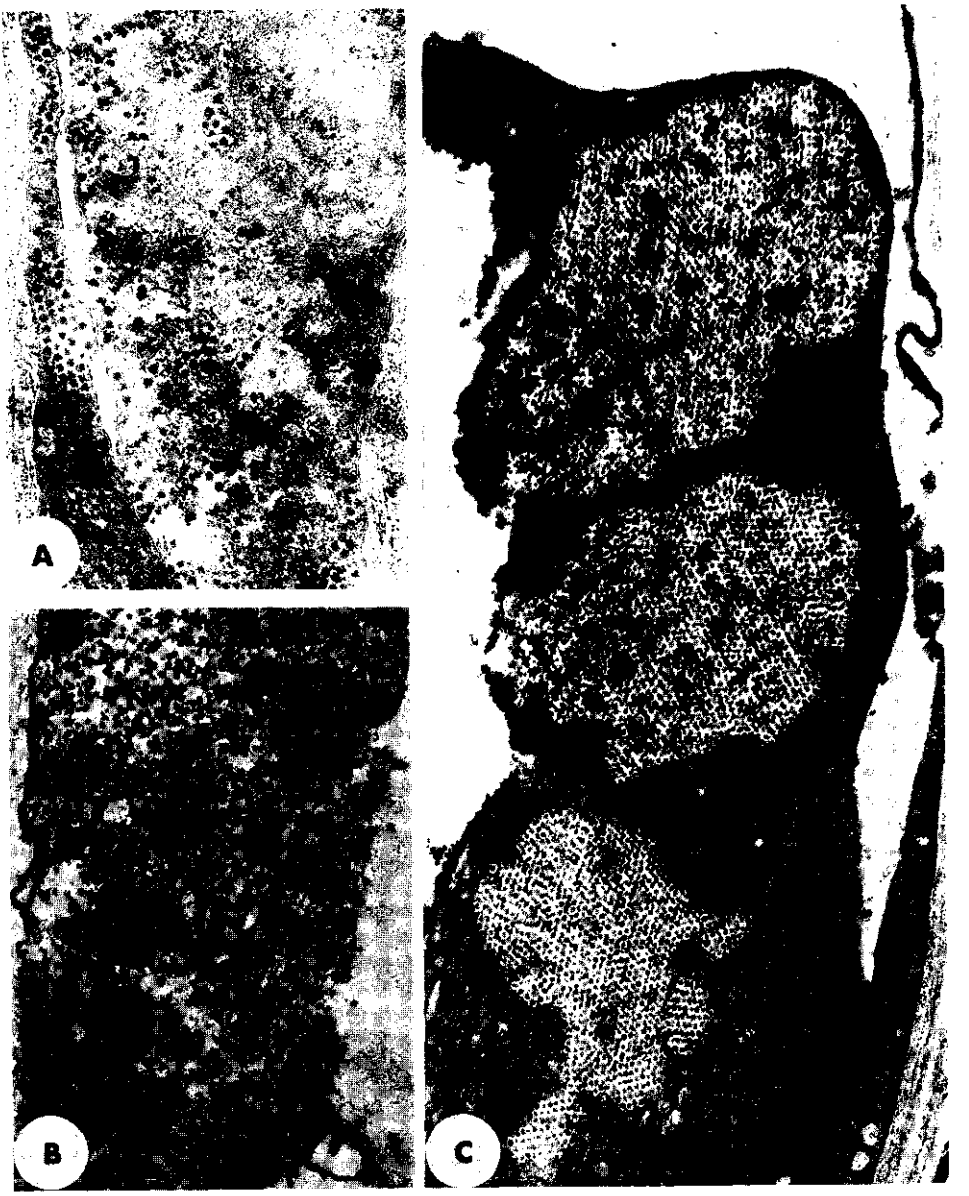

A
B
C

PLATE 8-9. TNV in epidermal cells of lettuce roots more than 24h after inoculation with TNV-carrying zoospores of lettuce *Olpidium*.
FIG. A. Necrotic cell with clustered and loose TNV particles at 36h after inoculation.
G+O/A+E/Uac+Lci/48.000
FIG. B. Non-necrotic cell with loose TNV particles at 60h after inoculation. Note high number of mitochondria (M).
G+O/A+E/Uac+Lci/34.000
FIG. C. Crystal-like array of TNV particles in necrotic cell at 60h after inoculation.
G/A+E/Uac(dehy)+Lhy/34.000

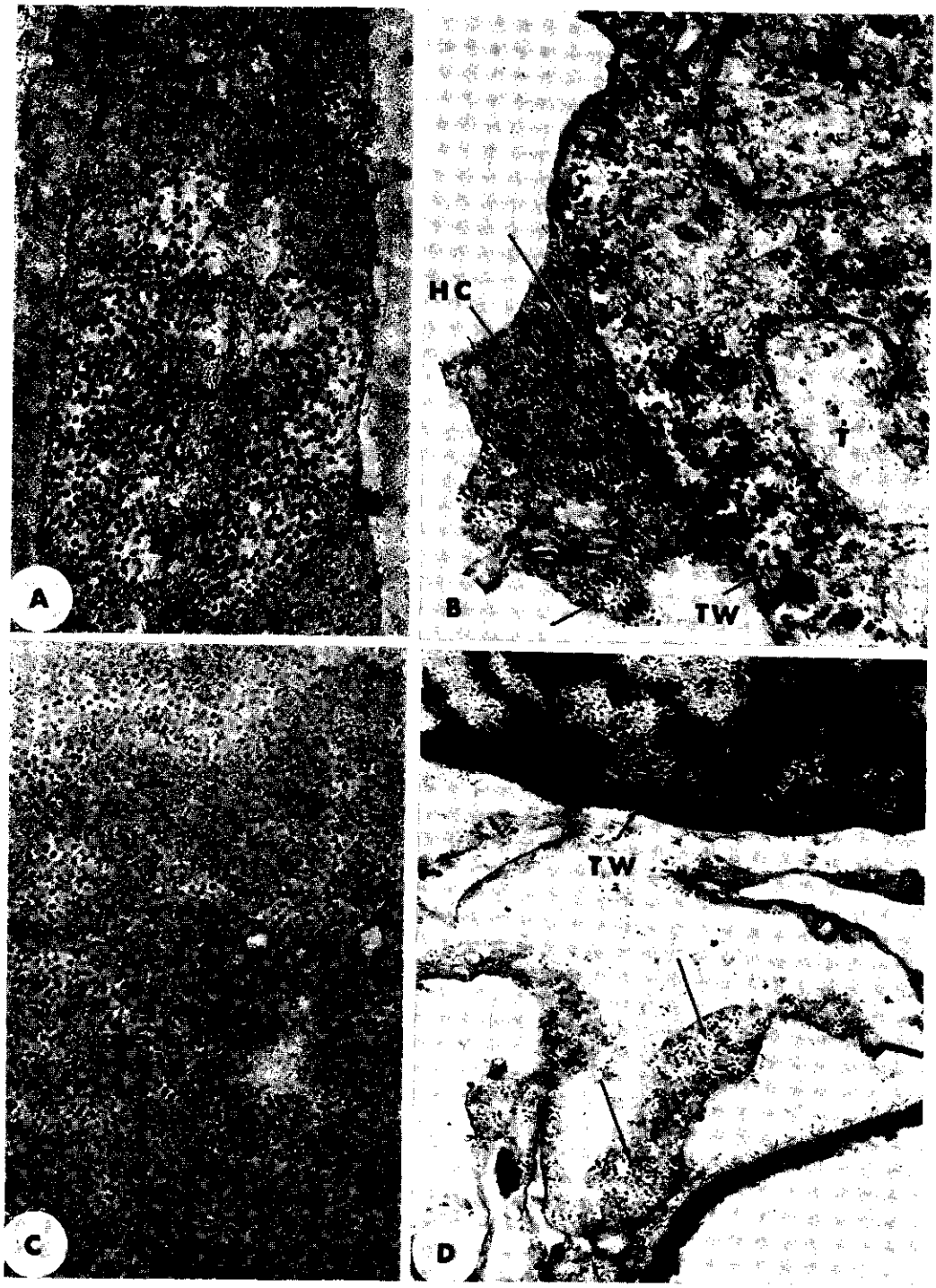
PLATE 8-10. TNV in epidermal cells of tomato seedling roots after infection with viruliferous zoospores of tomato *Olpidium*.

FIG. A. Epidermal cell with many loose TNV particles at 24h after inoculation.
G+O/A+E/Uac+Lci/48.000

FIG. B. Epidermal cell with fungal thallus (T) and TNV particles (unlabeled arrows) in cytoplasm of host cell at 24h after inoculation. Note absence of TNV from thallus cytoplasm.
G+O/A+E/Uac+Lci/34.000

FIG. C. Subepidermal cortical cell with many loose TNV particles in host cytoplasm at 48h after inoculation.
G+O/A+E/Uac+Lci/48.000

FIG. D. Epidermal cell with TNV particles (unlabeled arrows) in host cytoplasm adjacent to fungal thallus (T).
G+O/A+E/Uac+Lci/34.000


A
HC
T
B
TW
C
TW
D


TNV
A
B
C

PLATE 8-11. Epidermal cells of tomato seedling roots 24h after infection with viruliferous zoospores of tomato *Olpidium*.
G+O/A+E/Uac+Lci/34.000
FIG. A. Long cisternae of endoplasmic reticulum in TNV-infected cells. Many of the cisternae seem to have lost their ribosomes. Note crystalline material between the ER cisternae.
FIG. B. Extensive area of long cisternae of endoplasmic reticulum almost completely free of ribosomes. Only little crystalline material present between the ER cisternae.
FIG. C. TNV-infected cell showing crystalline material between cross-sections of ER cisternae (arrows).