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Title: Development of in vitro Lily Scale Bulblets as Related to Virus Elimination

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Lily hybrids vary in their ability to produce virus-free (VF) bulblets when grown from virus-infected scales in tissue culture. Asiatic hybrids typically produce a higher percentage of *in vitro* VF scale bulblets than do *Lilium longiflorum* cultivars. Three hypotheses concerning the cause of this variation are tested on five lily hybrids: an Asiatic hybrid, two *L. longiflorum* cultivars, an Oriental hybrid and *L. candidum*.

The first hypothesis states that VF scale bulblets originate from wound tissue that is naturally low in virus concentration and blocks the passage of virus particles from one cell to the next. The second hypothesis says that scale-to-bulblet vascular connections, which serve as virus pathways, occur in hybrids showing high percentages of

virus-infected scale bulblets, while connections are absent in those hybrids with low numbers of virus-infected bulblets. The third hypothesis concerns the virus concentration in the scale at the site of bulblet origin: bulblets of hybrids producing large numbers of VF bulblets originate from scale tissues low in virus concentration; bulblets of low percentage VF bulblet hybrids originate from scale tissues high in virus concentration.

The first two hypotheses are not supported by the results of this study. First, lily bulblets do not originate from wound tissue. Second, scale-to-bulblet vascular connections consistently occur in 'Enchantment,' an Asiatic hybrid, and occasionally occur in L. candidum. Vascular connections are not detected in the low VF bulblet producers, L. longiflorum cultivars 'Ace' and 'Nellie White,' nor are they seen in the Oriental hybrid 'Stargazer.'

Speculative support exists for the third hypothesis concerning uneven virus concentration in the scale. Distinct virus particles are observed with the electron microscope in the double virus-infected L. longiflorum cultivars and not in the other singly-infected lilies. The doubly-infected lilies produce a continuous layer of divided cells in the adaxial subepidermis of the scale where bulblets originate, whereas the singly-infected

lilies produce cell division masses in the same area but only beneath forming bulblets.

This study suggests that virus particles in L. longiflorum cultivars are more uniformly distributed than particles in the other lilies examined. This occurs not only at the site of bulblet origin but also throughout the scale mesophyll. Whether this is due to concurrent viral infection or to hybrid variation is unknown.

Development of *in vitro* Lily Scale Bulblets as Related to Virus Elimination

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LIST OF ABBREVIATIONS

BYMV	=	bean yellow mosaic virus
CMV	=	cucumber mosaic virus
DAE	=	days after explanting
ELISA	=	enzyme-linked immunosorbent assay
ISEM	=	immunosorbent electron microscope
ISMV	=	iris severe mosaic virus
LSV	=	lily symptomless virus
TBV	=	tulip breaking virus
TBV-L	=	tulip breaking virus longiflorum strain
TEM	=	transmission electron microscope
TMV	=	tobacco mosaic virus
VF	=	virus free
VI	=	virus inclusion
VP	=	virus particle

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INTRODUCTION

Tissue culture methods for producing virus-free (VF)[1] lilies have been known and applied for over twenty years. *In vitro* procedures and virus assay techniques have been modified and perfected during this period and are now available to commercial bulb growers worldwide.

One of the first research teams to develop a reliable *in vitro* method of producing VF lilies was Allen and Fernald (1972). They diced lily bulb scales and incubated the small pieces on nutrient medium in culture tubes. Some of the bulblets, produced on the surface of the virus-infected scale pieces, were free of virus.

The virus elimination procedure developed by Allen and Fernald has been refined to increase the production of VF plants for many lily varieties. Modifications include the early bulblet excision method, where bulblets are excised from the scale piece at an early developmental stage and are cultured separately, (O'Conner, personal communication; Blom-Barnhoorn and van Aartrijk, 1985), addition of heat treatment or chemotherapy (Cohen *et al.*, 1985; Blom-Barnhoorn and van Aartrijk, 1985), and

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[1] A plant is considered VF only for the specific and identifiable viruses for which it has been tested.

establishment of optimum environmental and nutrient conditions (van Aartrijk and van der Linde, 1986). Future advances in lily bulb scale tissue culture methods may include the use of varieties resistant to certain viruses (Derks, 1985).

Despite recent advances in tissue culture techniques, the degree to which each lily variety can produce VF bulblets *in vitro*, as compared to other varieties, has not changed substantially during the last two decades. In 1972, Allen and Fernald cultured virus-infected lily scales of an Aurelian and several Asiatic hybrids and found that 14 percent to 89 percent of the plants produced were VF depending upon the hybrid. Linderman *et al*. (1976) attempted to produce VF plants from two *L. longiflorum* cultivars, 'Ace' and 'Nellie White,' using Allen and Fernald's procedure, however, none of the resulting plants was VF.

Allen *et al*. (1980) eventually produced 'Ace' and 'Nellie White' VF plants through meristem culture, and van Aartrijk (personal communication) succeeded in producing *in vitro L. longiflorum* VF plants with the early bulblet excision method. Yet hybrid variation still exists even with these methods. *L. longiflorum* cultivars continue to produce proportionably fewer VF bulblets per explant than other lily hybrids, such as the Asiatic 'Enchantment,' when identical tissue culture procedures are used.

Developmental variation of scale bulblets has also been reported among lily hybrids when using scaling or *in vitro* propagation methods. Walker (1940), Robb (1957) and Godden and Watson (1962) separately demonstrated that lily hybrids vary in the origin of their scale bulblet and root meristems and in their ability to produce scale-to-bulblet vascular connections. *L. candidum*, *L. longiflorum* and *L. speciosum* bulblets originate solely from subepidermal scale cells. Root initials of these lilies develop from cells within the scale mesophyll, and no scale-to-bulblet vascular connections are produced. In contrast, bulblets of an Aurelian, an Asiatic and an American hybrid arise from both epidermal and subepidermal scale cells. In these three hybrids, root meristems initiate from bulblet cells, and vascular connections develop between the scale piece and the bulblet.

This study searches for evidence of a correlation between lily hybrid variability in bulblet development and variability in the production of VF plants *in vitro*. Three hypotheses related to developmental differences are proposed and tested on five lily hybrids: 1) wound tissue, which could inhibit virus movement, develops between the scale explant and the forming bulblet only in hybrids with a high percentage of VF bulblets; 2) vascular connections, which could provide a pathway for rapid virus movement, occur between the scale vascular tissues and the bulblet

procambium in hybrids with low numbers of VF bulblets; and 3) VF bulblets form from scale tissues that are characteristically low in virus concentration. Such low, virus-concentrated, parent tissues are more abundant in some hybrids than in others.

Support for one or more of the proposed hypotheses could lead to further enhancement of the tissue culture procedure, which would ultimately be beneficial to commercial lily bulb growers.

REVIEW OF LITERATURE

Tissue Culture and Virus Elimination

Plant Regeneration through Tissue Culture

In the early 1900's, Haberlandt (1902) reported growing isolated plant tissues in simple nutrient solutions. His procedure is referred to as plant tissue culture. Although Haberlandt never successfully produced plantlets, which are small *in vitro* plants, his ideas on the subject were visionary. Research in plant tissue culture lay fallow for many years until the 1920's and 1930's when several researchers brought the subject back to life (Robbins and Maneval, 1924; Laibach, 1929; White, 1934; Gautheret, 1939). Development of tissue culture methods have led to callus culture, embryo and ovule culture, micropropagation and protoplast culture.

Micropropagation, the asexual or vegetative propagation of plants *in vitro*, was first reported by Ball (1946) for dicots and by Loo (1945) for monocots. Both researchers used excised shoot tips for explants. Other researchers have since discovered that many parts of higher plants may be used as explants and that the explant choice and success of plantlet production depends upon the species being cultured. For example, shoot tips are used for strawberry explants (Boxus *et al.*, 1977), floral buds are

used for broccoli (Anderson and Carstens, 1977) and leaf petioles work well for begonia (Fonnesbech, 1974).

Many parts of the lily have regenerative capabilities, including stems or shoot tips (Sheridan, 1968; Bigot, 1974), leaves (Niimi and Onozawa, 1979), petals (Takayama and Misawa, 1979) and bulb scales (Robb, 1957; Hackett, 1969; Allen and Fernald, 1973; Stimart and Ascher, 1978). The most productive explants are bulb scales. Aseptically cut into small pieces, the bulb scale explants are placed abaxial surface down on nutrient medium in a culture tube. Bulblets arise from adventitious meristems on the adaxial surface of the explants and grow into plantlets with roots and leaves that can be excised from the scale pieces, transferred to soil, and grown.

Allen and Fernald (1973) used this bulb scale technique to demonstrate that a single lily bulb can generate over 1,000 plantlets. Stimart and Ascher (1978) speculated that 8,000 or more bulbs could be produced from 100 Easter lily scales with a slightly different bulb scale method, and Takayama and Misawa (1979) theorized that approximately 12.5 billion L. speciosum or 3 trillion L. auratum plants could be obtained in one year from a medium-sized bulb using their method.

Virus Elimination with Meristems

In addition to generating a large quantity of plants from a small amount of parent material, tissue culture techniques can also enhance the quality of plant material by eliminating virus. The discovery that all cells of an infected plant do not contain virus particles led researchers to use meristem-tip, callus and protoplast culture to produce VF plants. Morel and Martin (1952) pioneered *in vitro* virus elimination procedures with a micropropagation technique called meristem-tip culture. They used the apical shoot meristem, which is naturally low in or void of virus particles, to free dahlias of dahlia mosaic virus. Svobodová (1965), Hansen and Hildebrandt (1966) and Mori and Hosokawa (1977) eliminated tobacco mosaic virus (TMV) from tobacco plants with callus culture. Shepard (1975) used protoplast culture to free tobacco plants of potato virus X.

The use of callus and protoplast culture can be problematic due to genetic instability of the cells from which the VF plants are produced. The resulting VF plants may not be true to type. Consequently, the preferred *in vitro* virus elimination method is meristem-tip culture. This culture method ensures that the resulting plantlets are genetically identical to the parent tissue as they are derived from pre-existing meristems.

The meristem tip method introduced by Morel and Martin (1952) has become the most important and effective method of producing VF plants *in vitro*. It was initially called shoot-tip culture because the explant was excised from the terminal end of the stem and was relatively large. For example, Sheridan (1968) used the terminal 2 cm of the shoot apex as the explant in his procedure for culturing *L. longiflorum in vitro*. Shoot-tip culture became known as meristem-tip culture as techniques were revised and the explant was reduced in size to include only the meristematic dome and one or two leaf primordia. Nishizawa and Nishi (1966), Mori (1971) and Asjes *et al*. (1974) successfully used meristem-tip culture to produce lily plantlets free of cucumber mosaic virus (CMV) and lily symptomless virus (LSV).

Virus Elimination in Lilies by Adventitious Bud Formation

Other tissue culture procedures have also been used to eliminate viruses from lilies. Allen and Fernald (1972) discovered that VF lilies could be produced from certain hybrids through bulb scale micropropagation. Instead of the shoot meristems producing VF plants as in meristem-tip culture, VF plantlets arise from the adventitious meristematic regions on the adaxial surface of the virus-infected scale explant. However, not all of the plantlets formed are VF and each hybrid varies in its

ability to produce VF plantlets. For example, in Allen and Fernald's study, more than 85 percent of the plants produced by 'Enchantment' and 'Joan Evans' hybrids were VF while 'Cinnabar' and 'Valencia' hybrids produced fewer than 20 percent VF plants.

Allen and Fernald (1972) allowed the bulblets in their study to develop leaves and roots while still attached to the scale explant and assayed these in vitro plantlets for virus only after they had been excised from the scale explant and established in soil in the greenhouse. Similarly, Linderman et al. (1976) assayed for virus at this late stage of plantlet development in their unsuccessful attempt to free L. longiflorum cultivars 'Ace' and 'Nellie White' from LSV.

Allen et al. (1980) succeeded in eliminating LSV from the lily hybrid 'Red Carpet' by modifying the method used by Allen and Fernald (1972). Bulb scales are cultured only until bulblet meristems or young bulblets are formed. These are then excised from the scale explant and further cultured alone on new medium. This procedure is known as the early excision method. The percentage of LSV-free plants generated usually increases when bulblet meristems are excised as early as possible from the explant, although variation among hybrids is still present. In fact, Allen et al. found that VF plants of L. longiflorum cultivars 'Ace' and 'Nellie White' could not be produced by their

early bulblet excision method. These cultivars were freed of LSV only through meristem-tip culture.

Blom-Barnhoorn and van Aartrijk (1985) produced L. longiflorum 'Arai' plants free of LSV and tulip breaking virus (TBV) through an early bulblet excision method similar to that used by Allen et al. in 1980. Blom-Barnhoorn and van Aartrijk attribute their success to either the difference in cultivars or excising the bulblet meristems from the scale explant at an earlier stage.

Using a more precise early bulblet excision method, van Aartrijk (personal communication) produces VF plants from any lily hybrid by removing the forming bulblet from the scale when it consists of the meristematic dome and one or two leaf primordia. However, he concedes that he is more successful with some hybrids than with others.

O'Conner (personal communication) can produce VF plants from any of the lilies grown by Oregon Bulb Farms, but these do not include L. longiflorum cultivars. Additionally, the survival rate of the excised bulblet meristems in O'Conner's procedure is approximately 50 percent of the total number of meristems excised, varying from hybrid to hybrid, thereby making the net VF bulblet yield less than 100 percent of all potential bulblets produced by the scale explants.

Consequently, even though it appears that all lilies can be freed of virus with the early bulblet

excision method, the percentage of VF plants produced from each hybrid still varies.

Virus Elimination through Heat Treatment and Chemotherapy

Other techniques such as heat treatment and chemotherapy can be used with meristem-tip culture to increase the incidence of VF plants (Hollings, 1965; Walkey 1985). Heat treatment is applied either by water or air. The hot air treatment appears to be more successful in eliminating virus than the hot water method and causes less damage to the plant tissue. The hot air treatment involves subjecting either the parent plant or the *in vitro* explants, or both, to an increased air temperature of 30-40C for two to 12 weeks. It is believed that at higher than normal temperatures virus degradation occurs while virus synthesis ceases within the plant cells. As every plant species has a specific heat tolerance, individual tests must be performed for each to ensure successful treatment.

Chemotherapy uses compounds such as ribavirin (Virazole) or vidarabine (Vira A) in the nutrient medium to block virus replication. The principle of chemotherapy is similar to that of heat treatment in that the compound allows the virus to degrade but does not allow it to multiply. *In vitro* chemotherapy has not been as broadly successful in reducing virus concentrations as heat

treatment, but it has been effective in eliminating apple chlorotic leaf spot virus from Chenopodium quinoa (Hansen, 1979), chrysanthemum stunt virus from chrysanthemum (Horst and Cohen, 1980) and necrotic ringspot virus from Prunus species (Hansen, 1984).

Both heat treatment and chemotherapy have been used with some success to inhibit or eliminate virus from lilies cultured in vitro. One of the first reports of virus elimination in lilies involved heat treatment coupled with meristem-tip culture (Kohl and Nelson, 1966). More recently, Cohen et al. (1985) found that LSV and TBV are markedly reduced in scale bulblets of Lilium x parkmanii hybrids when cultured at 30C. They also found that Virazole inhibited LSV in the same hybrids but was ineffective against TBV. Limited success with Virazole was also reported by Blom-Barnhoorn and van Aartrijk (1985) with the early bulblet excision method in L. longiflorum 'Arai' and the Asiatic hybrid 'Enchantment.' It was reported that adding Virazole to the growth medium increased the incidence of VF plants in 'Arai' but was ineffective in 'Enchantment.' However, the percentage of infected 'Enchantment' plants was consistently lower than that of 'Arai' plants with or without the addition of Virazole.

Virus Assay Techniques and Lily Viruses

This study used three virus assay techniques: serology, electron microscopy and cytopathology. Other techniques such as bioassay and molecular hybridization were not used and will not be discussed (except external symptomatology). Walkey (1985) and Horst (1988) should be consulted for a more complete review of available virus assay techniques.

External Symptomatology

Generally, the first assay technique employed when looking for viral diseases is external symptomatology: plants are examined for external symptoms caused by the virus in the plant tissues. This assay method many times forms a basis for the correct identification of the infectious virus for a particular plant species, but the reliability of this assay is limited. Symptoms caused by a single virus may differ due to the strain of virus, the host cultivar or environmental conditions. One virus may cause a single symptom, multiple symptoms or no symptoms at all. Separate, unrelated viruses may cause the same symptoms, such as mosaic, chlorosis or necrosis, in one plant species, or they may simultaneously infect a single host and cause different symptoms. Such symptom

variability requires the use of multiple virus assay techniques to ensure accurate diagnoses.

Serology

The most widely practiced virus assay used today, other than external symptomatology, is serology. Serological tests visualize the binding of antibodies to specific antigens. The plant virus, as the antigen, is injected into an animal, usually a rabbit, where it stimulates the production of antibodies specific to that antigen. The serum containing the antibodies is separated from the other components of the animal's blood, and the resulting antiserum is used for serological tests. Over the past twenty years, many serological tests have been developed to detect viruses in plants, including electron microscope serology, enzyme-linked immounosorbent assay (ELISA), and precipitin, immunodiffusion and agglutination tests.

ELISA, developed by Voller and his co-workers in the mid-1970's (Voller et al., 1976, Voller and Bidwell, 1977), is presently the most popular serological method used because of its high sensitivity and its ability to test large numbers of samples simultaneously. The procedure combines virus particles, either purified or in crude plant sap, with their specific antiserum in the wells of a polystyrene microplate. An enzyme is attached to

either of the reactants (the antigen or the antibody), added to the wells, and a color change occurs if the reactants combine. The plate is read for positive reactions either visually by the color change or quantitatively with a photometer. Several methods of combining the reactants and labelling them with the enzyme exist, but the 'double antibody sandwich' method developed by Clark and Adams (1977) is most commonly used. With this method, the reactants are added to the wells so that the antigen is sandwiched between two layers of antibody. The top antibody layer is labelled with the enzyme.

Electron Microscope Assay

Although not as specific as ELISA, electron microscope assay methods are also valuable in detecting virus in plants at the cellular level. Brandes (1957) first reported a simple 'leaf-dip' method of visualizing plant viruses in crude sap samples. With his method, sap from a freshly cut leaf (usually one with symptoms) is dipped into water on a transmission electron microscope (TEM) grid and allowed to dry. An electron-dense negative stain is then added to the grid. When the sample is viewed with the TEM, the virus particles are seen in negative contrast to the background stain. The 'epidermal-strip' method (Hitchborn and Hills, 1965) is similar, but usually results in cleaner preparations. A strip of the lower

epidermis of a leaf (again, preferably with symptoms) is touched directly to a drop of negative stain on a grid.

Ball and Brakke (1968) combined electron microscope assay and serological techniques for virus indexing. Their procedure incorporates virus-specific antiserum into the drop in which the leaf is dipped. Derrick's method (1973) is similar as it 'traps' virus particles from a plant extract onto a grid coated with a specific virus antibody. The results of this assay method show a higher concentration of particles attached to the grid than with the previous methods. Still another electron microscope assay method is called 'decoration' (Milne and Luisoni, 1975). Here, virus particles are attached to an antiserum-coated grid after which additional antiserum is added. Virus particles become coated or 'decorated' with the antibodies.

Viruses can also be detected in situ in embedded plant tissue samples. Normal TEM fixation, dehydration and embedding techniques are used on tissues followed by thin-sectioning and the application of additional negative stains that surround the virus particles. Rod-shaped virus particles and aggregates of virus particles can be easily seen with this procedure.

Lily Viruses

Symptoms. Several viruses can be infectious in lilies, including arabis mosaic virus, CMV, LSV, TMV, tobacco rattle virus, tobacco ringspot virus and TBV. Only CMV, LSV and TBV are problematic in North America; these three viruses, separately or in combination, can cause a range of symptoms. CMV produces mild mosaic symptoms in Easter lilies and causes necrotic fleck disease if associated with LSV. LSV is present in many lily hybrids. Normally this virus does not cause symptoms when alone in the plant, but it can cause curl-stripe disease in lilies under cool shady conditions. When associated with TBV, LSV can produce brown rings in Lilium Mid-century hybrid bulbs and streak mottle on leaves of L. speciosum varieties. TBV can cause mild to moderate leaf mottling in lily species when not associated with LSV.

Identification. CMV, LSV and TBV, can be seen with the TEM. CMV is best viewed with one of the immunosorbent electron microscope (ISEM) methods (i.e. leaf-dip, decoration, etc.) because individual CMV particles, which are isometric and measure about 30 nm in diameter, can be confused with cytoplasmic organelles such as ribosomes when viewed in situ. When aggregated, CMV particles can be detected in embedded, thin-sectioned tissues.

LSV and TBV are both rod-shaped particles and can be easily detected either with ISEM methods or in thin sections. LSV, a member of the carlavirus group, is slightly flexuous and measures about 640 nm in length and 17-18 nm in diameter. TBV particles are more flexuous than LSV particles, and are slightly longer and thinner. TBV belongs to the potyvirus group and measures about 740 nm in length and 14 nm in diameter. Both viruses often aggregate in the cytoplasm. Such bundles of LSV particles give a 'fingerprint' appearance when viewed in cross-section.

Detection with ELISA. The double antibody sandwich ELISA method is regularly used for the detection of LSV and TBV in lilies because these viruses can be present without causing noticeable symptoms. Initially only mature leaf tissue was tested, but the advent of VF tissue culture programs led to a search for reliable ELISA tests for virus detection in in vitro plantlet leaf tissue and mature bulb tissue.

Testing is done at two stages in the VF lily program: 1) after in vitro plantlets are produced; and 2) after field bulbs are harvested. The first test is necessary to select VF in vitro plantlets. Virus assay of these tissues is customarily performed after the in vitro plantlets are established in the greenhouse.

Blom-Barnhoorn and van Aartrijk (1985) demonstrated that LSV was easily detected in plantlet leaf tissue directly after culturing, but not all of the TBV-infected plantlets showed positive results at this stage.

Cohen et al. (1985) attempted to find a reliable virus detection point during, not after, the in vitro stage by searching for the optimum temperature where virus synthesis is at its greatest. They found that bulblets incubated at temperatures between 20C and 10C show a higher titre than bulblets incubated at higher temperatures. When bulblets, incubated at 30C and showing low titres, are transferred to 21C, a resurgence of virus replication occurs. Cohen et al. suggest that bulblets incubated at lower temperatures will have a higher virus titre and can be used for accurate testing during the in vitro stage.

A second ELISA test, used in VF lily programs, assays bulb tissue to assure that field plants have not been infected with virus by aphids. Bulb testing is especially valuable when done after storage because primary infections with LSV are detected more reliably in stored bulb material than in leaves from the preceding growing season (Derks and Vink-van den Abeele, 1980).

Although recent reports regarding the use of ELISA on postharvest lily bulbs are encouraging, problems continue to be encountered. For example, the original double antibody sandwich ELISA procedure readily detects

LSV in bulb extracts of some lilies such as 'Enchantment' but is unreliable in other hybrids. Beijersbergen and van der Hulst (1980) found it necessary to add the enzyme hemicellulase to tissue extracts of four cultivars to obtain reliable detection of LSV with ELISA.

Reports of a new strain of TBV occurring in L. longiflorum cultivars in the last several years also produced alterations in the ELISA testing procedure (Alper et al., 1982; Derks et al., 1982; Derks and Hollinger, 1984). This new strain is currently called the longiflorum TBV strain (TBV-L) and is not reliably detected with TBV-ELISA tests. As many lily cultivars may be infected with TBV-L and show no symptoms, the Dutch have added TBV-L-ELISA testing to their regular LSV and TBV assay programs (van Schadewijk, 1986).

Primary infection with LSV, TBV and TBV-L is barely detected with ELISA when stored lily bulbs are assayed soon after harvest, but the detectability of the virus increases as the postharvest storage time is lengthened (Derks and Vink-van den Abeele, 1982; van Schadewijk and Eggink, 1982; van Schadewijk, 1986).

Wounding helped to detect bean yellow mosaic virus (BYMV) in gladioli corms (Stein et al., 1988) and iris severe mosaic virus (ISMV) in iris bulbs (van der Vlugt et al., 1988) just after harvesting. When ELISA tests for BYMV and ISMV are performed on gladioli corms and iris

bulbs, respectively, at the time of harvest, virus detection is low. However, if the corms and bulbs are cut after harvest, stored for a period of time and then tested for the presence of virus, detectability is higher, especially in tissue close to the cut surface.

Van Schadewijk (1986) reported that TBV is not as evenly distributed as LSV in bulb scales of the lily cultivar 'Orchard Beauty.' Consequently, sampling only one scale can give a false negative result for TBV-ELISA tests.

Cytological Changes Due to Virus Infection

Virus particles, consisting of nucleic acid surrounded by a coat protein, were viewed for the first time in plant cells with the TEM. This microscope helped researchers understand some of the biology of viruses such as the movement of particles from cell to cell and the effects of viruses on cell organelles.

Movement of Virus Particles within Plants

Viruses move from cell to cell through plasmodesmata, which are strands of cytoplasm that pass through the cell wall connecting the protoplasts of adjacent cells. These cytoplasmic strands provide a passageway for the movement of materials and for communication between cells. They are 50-80 nm in diameter

and sometimes contain a central core that may inhibit the passage of viruses. However, virus particles, both isometric and elongate, have been viewed within plasmodesmata (Esau et al., 1967; Kitajima and Lauritis, 1969; Hearon and Lawson, 1980).

Another pathway for the movement of viruses in plants is through the host's vascular tissues. This movement covers a much greater distance and is more rapid than through plasmodesmata. It is often referred to as 'long-distance' transport. Virus particles have been found in both xylem and phloem cells of vascular tissue (Esau, 1968) and are assumed to move with water and assimilates throughout the plant.

A third way viruses move from one cell to another is by cell division. The dividing, infected mother cell may create one VF daughter cell and one virus-infected daughter cell or two infected daughter cells. Chandra and Hildebrandt (1967) found that both possibilities occur. With callus culture, they produced VF and virus-infected tobacco plants from TMV-infected single cells of tobacco. Esau (1968) found that cell division is not only uninhibited by virus infection but can occur amidst virus aggregates. She found that a TMV aggregate, lying in the plane of a dividing tobacco cell, divided so that it was split between daughter cells creating two virus-infected daughter cells. These studies show that cell division can

produce cells, and eventually whole plants, free of virus, or it can cause the movement of virus particles from one cell to another.

Virus Particle Movement within Lily Tissues

In their attempt to pinpoint the causal viruses of fleck disease in L. longiflorum, Allen and Lyons (1969) found CMV and LSV particle aggregates in root phloem cells. This occurrence suggests that these viruses may move throughout the plant via the vascular tissue. Similarly, it is assumed that CMV, LSV and TBV particles can move from cell to cell through plasmodesmata as do other viruses, but this has not been documented.

Virus Effects on Plant Cells

Each virus causes specific cytological changes in host cells and nearly every cell organelle may become distorted. It was originally thought that all organelle irregularities were degenerative effects of the virus. It is, however, becoming apparent with the advent of autoradiography and an increased knowledge of viruses that many of the cytological changes represent sites of viral synthesis.

Organelle Degeneration. The cause and function of some protoplasmic irregularities found in virus-infected

plants may indeed be due to the degenerative effects of the virus. Two such irregularities include the increase in the number and aggregation of ribosomes (Hiruki et al., 1980) and the occurrence of nonmembrane-bound, intra- and extraprotoplasmic osmiophilic globules (Kim et al., 1974; McMullen et al., 1977). Such globules may consist of the lipid component of degenerated cell membranes caused by viral infection (McMullen et al., 1977; Matthews, 1981). Kim et al. hypothesized that these intraprotoplasmic osmiophilic globules are transformed into myelinic bodies, which are also associated with virus-infected cells (Matthews, 1981), after the osmiophilic globules become extraprotoplasmic. The extraprotoplasmic sacs found by McMullen et al. in virus-infected barley leaf cells contain electron-dense material similar to the intraprotoplasmic osmiophilic globules. However, McMullen et al. did not confirm the hypothesis made by Kim et al. concerning intraprotoplasmic globules becoming extraprotoplasmic myelinic bodies. To complicate matters further, Esau (1968) reported finding similar osmiophilic globules in healthy cells.

Viral Synthesis. Nuclear, chloroplast and mitochondrial abnormalities due to viral infection may be related to virus synthesis; the changes in these organelles are generally found before virus particle production. Such

changes include nucleolar enlargement (Kim et al., 1978), peripheral vesiculation of the outer chloroplast membrane, and swelling and clumping of chloroplasts (Hatta and Matthews, 1974), swelling of chloroplasts containing abnormally large starch grains (Hiruki et al., 1980), vesiculation of the outer mitochondrial membrane (Hatta and Ushiyama, 1973) and clustering of mitochondria (Kitajima and Costa, 1969).

Unknown Effects. Some cell irregularities due to viral infection receive varied interpretations. For instance, Matthews (1981) placed cell wall abnormalities of virus-infected cells in three groups: 1) abnormal thickenings; 2) protrusions associated with plasmodesmata; and 3) paramural bodies. However, the function of each abnormality remains speculative.

Tu and Hiruki (1971) considered the abnormal secondary cell wall thickenings (Matthews' first group) found in potato virus M-infected bean leaf cells to be an indirect host reaction to the virus infection. They hypothesized that the plasmodesmata would be blocked if the thickenings developed quickly enough, which in turn, would block cell-to-cell translocation of the virus particles.

Supporting Matthews' second group, several reports exist of cell wall protrusions associated with plasmodesmata occurring in virus-infected cells

(Kitajima and Lauritis, 1969; Murant et al., 1973; Allison and Shalla, 1974; Hearon and Lawson, 1980). Most of these reports identify virus particles occurring within the unusual plasmodesmata. Matthews (1981) suggested that the new cell wall material is induced by the virus. Kitajima and Lauritis (1969) speculated that such plasmodesmata are modified or extended to accommodate the passage of virus particles from one cell to the next.

Matthews' third group of cell wall abnormalities, paramural bodies, which are membranous, vesicular or fluid-filled structures associated with the plasmalemma, has been documented by Kim and Fulton (1973), Gill (1974) and McMullen et al. (1977). Kim and Fulton postulated three possibilities for such occurrences: 1) the structures act as physical barriers to virus cell-to-cell movement; 2) they function like transfer cells, transporting virus particles from cell to cell instead of solutes; and 3) they are sites of virus particle assembly.

The abnormalities of a single cell, therefore, may be variable, enhancing or inhibiting virus multiplication or intercellular movement of particles.

Virus Inclusions

Virus-infected cells may also contain viral inclusion bodies, which are virus-induced structures occurring in the cytoplasm or nucleus. Their functions are

unknown. Inclusion bodies have been observed in nearly all plant virus groups and vary greatly in size, shape and location in the infected cell. Some inclusion bodies are crystalline or paracrystalline forms, comprising many virus particles. Others, called proteinaceous inclusion bodies, consist of proteins produced by the genome of the virus that differ from the virus coat protein. Some inclusion bodies are characteristic of infection by specific viruses and can be used as a diagnostic tool for virus identification. Extensive reviews of inclusion bodies that can be viewed with the light microscope (Christie and Edwardson, 1977) and with the TEM (Edwardson and Christie, 1978) have been published.

Virus Effects on Lilies

Organelle Abnormalities. Limited information on cytological abnormalities in lily cells can be found from studies on the identification of infectious lily viruses and the symptoms they cause. Allen and Lyons (1969), as a result of identifying fleck disease in L. longiflorum, found abnormal swelling of mitochondria and CMV association with mitochondria in fleck diseased cells.

Virus Inclusions. The virus aggregates observed by Allen and Lyons (1969) are typical inclusion bodies of CMV and LSV. The crystalline formation of CMV particles

and the paracrystalline formation of LSV particles often can be found in the cytoplasm of infected cells. Similarly, TBV particles readily form cytoplasmic, paracrystalline inclusions, and produce proteinaceous, cylindrical inclusion bodies in the cytoplasm. These cylindrical inclusions are called pinwheels because of their configuration in cross-section. Pinwheel inclusions were initially reported by Cremer et al. in 1961 and by Rubio-Huertos and Garcia-Hidalgo in 1964. They were correctly analyzed by Edwardson in 1966 and associated with plasmodesmata by Lawson and Hearon in 1971. Considered characteristic of the potyvirus group, pinwheels were found, without exception, in TBV-infected lily tissue (Allen, 1971).

Cytology of Lily Scale Bulblet Formation

Scale Bulblet Formation by Scaling

Early work with lilies centered on propagation by scaling, a method still widely used today. In this procedure, scales are detached from the bulb and placed in moist peat. One or more bulblets with roots and leaves will form at the basal end of the scale near the point of detachment. These bulblets can be removed from the scale and grown to adult plants.

In 1940, Walker studied the development of bulblets by scaling on L. candidum and L. longiflorum. Godden and Watson (1962) later performed similar studies on Olympic (Aurelian), Fiesta (Asiatic) and Bellingham (American) hybrids. Both studies found that bulblet regeneration was associated with scale wounding (i.e., cutting or breaking the scale from the bulb stem). Walker observed that a callus layer first forms over the cut surface of the scale that is soon followed by the collapse of the wounded scale cells. The parenchymatous cells immediately adjacent to the wounded cells then become embryonic and cell divisions occur in a plane parallel to the cut surface, forming a periderm. Godden and Watson similarly reported that the cut surface of the scale is blocked, followed by meristematic activity that produces a callus tissue. They found that bulblets develop adjacent to the wound area, not within it, in all of the cultivars they examined.

Walker (1940) and Godden and Watson (1962) described the origin of bulblet tissue, vascular connections between the scale and bulblet, and the origin of root tissue. The various hybrids differed on each point. Walker found that bulblets of L. candidum and L. longiflorum originate from subepidermal cells. These cells first divide periclinally and then in any plane, forming a compact mass of meristematic cells. The root primordium develops after the bulblet has several leaf

primordia, and it generally originates in a group of cells adjacent to a scale vascular bundle and just below and lateral to the bulblet primordium. As development continues, the parenchyma cells between the root and bulblet primordia become meristematic and differentiate into vascular elements, eventually forming a connection between the two. However, at no time is a vascular connection observed between the scale vascular bundle and the root and bulblet primordia. Walker also observed that the parent scale tissue degenerates as the bulblet develops.

In contrast, Godden and Watson found in Olympic, Fiesta and Bellingham hybrids that the bulblet arises from simultaneous divisions of the epidermal and subepidermal cells of the scale, making its origin not entirely endogenous as Walker had reported. In these hybrids, vascular connections between the scale and the bulblet are present and occur before root initiation, which develops from bulblet tissue and not from parent scale tissue. They additionally found that the parent scale tissue remains firm and fleshy throughout the incubation period.

In vitro Scale Bulblet Formation

Robb (1957) observed the development of lily bulblets from scale tissue in a successful attempt to culture monocotyledonous tissue *in vitro*. This appears to be the first report of lily bulblets produced from scale

pieces with tissue culture. She examined bulblet regeneration from bulb scale disks of L. speciosum. The first external sign of bulblet formation was the appearance of a swollen ring on the adaxial surface of the scale disk. The ring arises from cell divisions occurring in subepidermal parenchyma cell layers. Bulblets eventually develop from the swollen ring and roots appear later, originating from regions deeper within the scale piece. The vascular system of the roots and bulblets was distinct from the parent scale tissue, similar to observations by Walker.

MATERIALS AND METHODS

Lily Selection

Five lily varieties were examined in this study: an Asiatic hybrid, two L. longiflorum cultivars (Easter lilies), an Oriental hybrid and a L. candidum (Madonna lily). 'Enchantment,' the Asiatic hybrid, was selected because of the high percentage of VF scale bulblets it forms in vitro (Allen and Fernald, 1972; Blom-Barnhoorn and van Aartrijk, 1985). 'Ace' and 'Nellie White,' L. longiflorum cultivars, were chosen because they do not readily produce VF plants from bulb scales in vitro (Linderman et al., 1976; Allen et al., 1980). The Oriental hybrid, 'Stargazer,' and the Madonna lily were unknowns.

Tissue Culture

Lily bulb scale pieces were used as explant material for tissue culture. Four to five lots of explants for each lily variety were cultured from October 1987 to June 1988. Each lot consisted of 20 to 50 explants.

One or more bulbs, either fresh or stored at 4C, were used for each culture group. Damaged or decayed outer scales were discarded from each bulb. The first five to ten healthy scales (depending on the size and number of

bulbs) were removed, surface sterilized in 70% ethanol for 90 sec. and 1% sodium hypochlorite (20% Clorox) for 10 min., and rinsed in sterile distilled water. Strips 2-3 mm wide were aseptically cut from the basal portion of each scale, yielding two to four explants per scale (Figure 1). Each explant was placed abaxial surface down on nutrient medium in a culture tube. The medium contained Sheridan's (1968) macro-, micro- and iron-nutrient solutions, 30 g/l sucrose, 100 mg/l myo-inositol, 0.03 mg/l 1-naphthylacetic acid, 0.01 mg/l thiamine HCl and 1.6 g/l gelrite. The pH was 6.2. The cultures were incubated at room temperature and a photoperiod of 16 hr at 88 lux.

Preparation for Light Microscope Examination

In vitro scale pieces, both with and without bulblet initials, bulblets, and plantlets (bulblets with roots and/or leaves), were used for examination with the light microscope. Tissues 9 to 44 days after explanting (DAE) were fixed at intervals of two to three days. Tissues fixed on the day of explanting (0 DAE) served as controls. Johansen's (1940) standard embedding procedure was used. Tissues were fixed in formalin-acetic-alcohol, embedded in paraffin, sectioned transversely and longitudinally to 6-10 μm, and stained with safranin and fast green.

Virus Assay

Samples of each lily variety were assayed for virus by ELISA and with the electron microscope.

ELISA

The standard double sandwich antibody ELISA procedure (Clark and Adams, 1977) was used on mother plant leaf tissue. Leaf tissue was used for comparison of virus concentrations because some confusion exists regarding the validity of ELISA testing for TBV in bulb tissue. A random sampling of basal leaves was taken from each lily variety. Samples were assayed for LSV, TBV and CMV. Bottom layer antiserum dilutions were 1:800 for LSV, 1:400 for TBV and 1:1000 for CMV. The top layer antisera were conjugated with phosphatase and dilutions for these conjugates were 1:800 for CMV and 1:400 for LSV and TBV. All sample extracts were 1:10 dilutions. Microplate wells were read with a BIOTEK EL-308 EIA photometer at a wavelength of 405 nm. Absorbance readings above 0.2 were considered positive.

Electron Microscope Assay

In vitro scale and bulblet tissues were assayed for virus with the electron microscope. All samples, except for 'Stargazer,' were fixed at 9 DAE, 26 DAE and 40 DAE.

'Stargazer' samples were fixed at 9 DAE, 26 DAE and 37 DAE, instead of 40 DAE, due to lack of 40 DAE tissue. Tissues were fixed in Karnovsky's (1965) fixative, washed in 0.2 M cacodylate buffer, stained with 1% buffered osmium tetroxide, dehydrated in acetone (containing uranyl acetate) and embedded in Spurr's (1969) plastic. Sections were cut to 500-900 Å on a Servall Porter-Blum MT-2 ultramicrotome with a diamond knife and further stained with Reynold's (1963) lead citrate. Thin sections were examined with a Philips EM 300 transmission electron microscope.

Samples were sectioned in three areas (Figure 2): 1) adaxial epidermal cells of the scale adjacent to or beneath the bulblet initial, bulblet or plantlet, if present (designated epidermis area); 2) random scale mesophyll cells (designated mesophyll area); and 3) random bulblet initial, bulblet or plantlet cells, when present (designated bulblet area).

RESULTS

Tissue Culture

General Plantlet Formation

In general, all cultures developed plantlets in a similar sequence (Figure 3). Little change occurred in the explants during the first three to four days in culture. Fungal or bacterial infection often began to show by 5 DAE, and approximately 50 percent of the total number of cultures were eventually lost to contamination. The sides of the explants (those resulting from the original transverse cuts across the scale) began to tan by 5 DAE and darkened as the bulblets developed. By the end of the first week in culture, the explant tops (adaxial surface) began to change from the original ivory color to gradations of tan, yellow and red. This color usually changed to or included some green as bulblets began to form during the second week of incubation. Tiny bumps on the adaxial surface of the explant, measuring 0.2-0.5 mm in diameter, were the first external signs of bulblet formation. These bumps, referred to as bulblet initials, developed into bulblets within seven to ten days. The bulblets often had roots, one or two leaves, and were considered plantlets with the bulblet portion measuring 2-3 mm in diameter.

General Variation among Cultures

Variation in the tissue culture procedure produced variation in the growth and development of the cultures. Fluctuation in the culture room temperature caused variation in the number of bulblets formed on each explant. Warmer room temperatures in the spring and summer months of incubation produced more bulblets per explant than during the cooler fall and winter months.

The length of time the scales were sterilized in Clorox also affected bulblet production. The usual time for this step in the sterilization process was 10 minutes. When the period was extended to 13 or 14 minutes, fewer bulblets were produced on each explant and bulblets took longer to form.

The last batch of cultures for each lily type was initiated in June 1988. These cultures were started from fresh bulb scales, as opposed to stored scales, and yielded the highest number of bulblets per explant. Whether this increase in bulblet number is due to the fresh material, to the warmer culture room temperatures or to both factors is unknown.

Specific Variation among Lily Varieties

Certain differences were observed among the lilies, including: explant side, upper edge and top coloration;

number of bulblets and roots per explant; bulblet and root coloration; and location of bulblets on the explant top. The explant sides of those hybrids that normally produced more bulblets per explant, such as 'Enchantment' and 'Stargazer,' were darker tan than the explant sides of the other lilies. The Madonna lily explants generally had the lightest tan sides. The explant sides of 'Ace' and 'Nellie White' were darker tan in these batches that produced more bulblets. The explant sides of 'Stargazer' began to tan the earliest, sometimes changing within an hour of being cut. The Madonna lily upper explant edges, between the top and the sides of the explant (Figure 3), turned darker tan than the explant sides. The same upper edge tanning was occasionally observed in 'Stargazer.'

The bulb scales of all of the hybrids, except 'Stargazer,' were an ivory color before they were cultured. 'Stargazer' scales were initially slightly pink. During incubation, the explant tops of 'Enchantment' usually turned dark red with small areas of green before bulblets formed. The Madonna lily tops generally remained ivory with small areas of red and tan during incubation. 'Stargazer,' 'Ace' and 'Nellie White' explant tops changed from ivory to yellow and then developed areas of red.

'Enchantment' consistently had the most bulblets per explant, and the Madonna lily had the least. The Madonna lily produced the only bulblets that were

customarily ivory or yellow instead of green. The L. longiflorum cultivars had the most and the longest roots (Figure 4a), and were the only lilies whose roots were occasionally green with white hairs instead of completely white. 'Enchantment' had fewer and shorter roots (Figure 4b). Exact numbers of bulblets and roots were not recorded.

Bulblets of the Madonna lily were normally located at the ends of the explants (Figure 3). The other four hybrids formed bulblets along the top edges of the explant from one end to the other. Some 'Stargazer' explants showed evidence of basal polarity with bulblets forming only on one edge of the explant top surface. 'Enchantment' bulblets also formed in the center of the explant top between the two sides. 'Ace' and 'Nellie White' bulblets sometimes formed only toward the scale ends.

Anatomical Examination

The anatomy of all mature lily bulb scales is similar when viewed in transverse section with the light microscope. All have square adaxial epidermal cells (ends of rectangular cells), isodiametric parenchymatous mesophyll and five to seven vascular bundles spaced fairly evenly across the mesophyll in the middle or upper third of the scale (Figure 3). All of the lilies, except 'Enchantment,' have circularly configured vascular bundles

with the xylem cells lying adjacent and adaxial to the phloem cells (Figure 5a). The vascular cells of 'Enchantment' bundles are often, but not always, in an elongated configuration with the xylem cells stacked perpendicular and adaxial to the phloem cells (Figure 5b).

Scale anatomy does not noticeably change during the first week after explanting. However, the outer cells along the original cut surfaces eventually collapse, and the cells one to two layers deep develop a secondary cell wall (Figure 6). These cell wall thickenings accompany tanning of the scale explants observed in the cultures. Although random secondary cell wall thickenings occasionally occur throughout the scale piece, no distinct wound zones were observed.

Bulblets of each variety originate from cells in the first adaxial subepidermal cell layer. These cells first divide periclinally, followed by anticlinal divisions (Figure 7). Bulblet initial cell division begins about 11-15 DAE and continues throughout the incubation period. The second to possibly fourth adaxial subepidermal cell layers also divide in both periclinal and anticlinal planes as the bulblet develops. The adaxial epidermal cells generally divide only anticlinally in the area of the developing bulblet to accommodate the growth of the subepidermal cells. Bulblets generally develop directly adaxial or slightly adjacent to scale vascular bundles

(Figure 8). Parenchymatous mesophyll cells around the scale vascular bundles also occasionally divide.

Differences among the lily varieties become evident as the bulblets develop. In general, cell division occurs at the bulblet site, both in the subepidermal cell layers and in the mesophyll cells adaxial to the scale vascular bundles, to a greater extent in 'Enchantment' explants than in explants of the other lilies (Figures 8 and 9). In contrast, the adaxial subepidermal cell layers in the Easter lilies divide not only at the sites of developing bulblets but across the entire explant (Figure 10). Subepidermal cell division occurs only at the origin of bulblets in the other hybrids (Figure 11).

In all of the lilies, some cells in the bulblet leaf initials differentiate into procambium, and some cells at the base of the bulblets develop secondary cell wall thickenings typical of xylem tracheids (Figures 10 and 12). However, in 'Enchantment,' and occasionally in the Madonna lily, cells between the bulblet and the scale vascular bundle also differentiate into vascular cells (Figure 12). A vascular connection eventually forms between the scale and the bulblet tissue when adjacent mesophyll and bulblet cells differentiate from the scale vascular bundle through the bulblet body, to the leaf initials. These connections occur frequently in 'Enchantment' and were observed as early as 18 DAE. In the Easter lilies and 'Stargazer,'

cells between the scale vascular bundles and the divided adaxial subepidermal cell layers do not divide or differentiate into vascular cells (Figure 13). Consequently, vascular connections were not detected in these hybrids.

Although bulblet shoot meristems begin in the same scale tissue in all of the lilies, bulblet root meristems do not (Figure 14). The roots of 'Stargazer' and 'Enchantment' bulblets initiate from cells at the base of the bulblet. 'Nellie White' bulblet root initials originate from cells within the scale mesophyll adjacent to scale vascular bundles. Insufficient data were collected to determine bulblet root origins in 'Ace' and the Madonna lily.

Differences were also observed in the firmness of the scale tissue during incubation. Scale tissue of 'Enchantment,' 'Stargazer' and the Madonna lily remains firm throughout the incubation period. The scale tissue of both L. longiflorum cultivars degenerates as bulblets develop.

Virus Assay

ELISA on Mother Plant Tissue

The ELISA tests run on the lily mother plant leaf tissue revealed that none of the five lilies contained CMV,

the Easter lilies and the Madonna lily contained TBV, and LSV was present in all of the lilies except the Madonna lily (Table 1).

Electron Microscope Assay of in vitro Tissues

Electron microscope assay of *in vitro* tissues confirmed the positive leaf tissue ELISA results in the doubly-infected *L. longiflorum* cultivars but did not confirm positive ELISA results in the other lilies that had single virus infections (Table 2).

LSV and TBV virus particles were present in 'Ace' and 'Nellie White' cells in aggregates, loose arrangements and scattered about the cytoplasm (Figure 15).

TBV pinwheel inclusions frequently occurred in cells that contained TBV particles (Figure 16). Sometimes the inclusions were found in large numbers. Occasionally they were adjoined to virus particles, and once the inclusions were found associated with the plasmalemma.

Of the 633 *in vitro* cell sections examined in this study, only 30 contained virus particles and 8 contained TBV pinwheel inclusions (Table 4). The inclusions were present in the epidermis and mesophyll areas of 9 DAE tissues. Virus particles were found in 9 DAE, 26 DAE, and 40 DAE tissues but only in the epidermis and mesophyll areas and never in the bulblet area. Distinct virus particles and inclusions were observed only in the

doubly-infected L. longiflorum cultivars (Tables 2 and 3). It was not always possible to identify the observed rod-shaped particles as either LSV particles or TBV particles. Hence, counts of cells containing the specific viruses were not made.

Ultrastructural Abnormalities

Some ultrastructural abnormalities, previously found to be associated with viral infection, were observed while assaying the in vitro tissues.

Mitochondrial aggregates occurred in Madonna lily 40 DAE mesophyll and bulblet cells (Figure 17).

Swollen, aggregated chloroplasts, both with and without large starch grains, were seen in subepidermal and mesophyll cells of 'Stargazer' and the Easter lilies (Figure 18). In 'Ace' and 'Nellie White' bulblet cells, several chloroplasts were found with vesiculated outer membranes (Figure 19). Similar chloroplasts were observed in 'Enchantment' subepidermal cells in 40 DAE tissue. Madonna lily and 'Enchantment' cells generally contained smaller starch grains than were found in the other hybrids. The starch was found in leucoplasts in the Madonna lily instead of in chloroplasts. Lack of chloroplasts in this hybrid accounts for the observed absence of green coloration in the explant tops and bulblets.

Cell wall thickenings and deformations were found in 'Stargazer' bulblet, 'Enchantment' mesophyll and 'Ace' mesophyll cells (Figure 20).

All hybrids exhibited vesiculation of the cytoplasm with aggregated ribosomes in subepidermal and mesophyll cells (Figure 21). These traits occurred mainly in 9 DAE cells, but were also observed in 'Stargazer,' in 'Nellie White' and most often in 'Enchantment' cells that had been cultured for a longer period.

Mesophyll area sections of 26, 37 and 40 DAE of 'Stargazer' and the Easter lilies had advanced degenerate cells covered with masses of dark, osmiophilic material (Figure 22). Likewise, but to a lesser degree, 'Stargazer,' Madonna lily and 'Enchantment' cells in all three sectioned areas (epidermis, mesophyll and bulblet areas) and at all three section intervals (9 DAE, 26 DAE and 37/40 DAE) had intra- and extraprotoplasmic osmiophilic globules (Figure 23). These globules occurred within plasmodesmata in subepidermal, mesophyll and bulblet cells (Figure 24a). In one 'Stargazer' 26 DAE bulblet cell, this material appeared to have been continuous from one cell to the next transversing a plasmodesmatum (Figure 24b).

DISCUSSION

Only one VF plant is theoretically required to begin a VF program for any lily hybrid. However, greenhouse and field grown lilies may easily become reinfected with LSV, CMV and TBV via aphid transmission. Therefore, VF mother stock is necessary to sustain a VF plant population. Tissue culture procedures, such as adventitious bulblet production from scale piece explants, are an excellent means of establishing and perpetuating mother stock for various lily hybrids. Although only one VF bulblet for each hybrid is necessary to begin such a stock program, using many VF bulblets is economically more efficient. However, not all lily hybrids produce a high percentage of VF bulblets with micropropagation methods.

Three hypotheses related to variation of in vitro VF bulblet production among lily hybrids were proposed and tested in this study.

Hypothesis 1: Wound Tissue

The first hypothesis suggested that VF bulblets produced from virus-infected lily scale tissue originated from wound tissue, which resulted from cutting the scale into pieces. The wound area was thought to inhibit the movement of virus particles from the scale to the newly formed bulblet. Although scale wounding appears to be

essential for the production of bulblets (Walker, 1940; Godden and Watson, 1962), the wound area resulting from cutting the bulb scale into pieces did not produce bulblets in the lilies tested in this study. Bulblets typically form on the adaxial surface of the scale explant along the original transverse cut surfaces (Figure 3). However, these formations arise from normal subepidermal cells, three to four cell layers from the cut surface, and not from wound tissue. Some 'Enchantment' bulblets even develop in the center of the explant top between the two cut surfaces (Figure 3). Consequently, this study did not find that in vitro scale bulblets form from wound tissue that might inhibit virus particle movement from the lily scale to the developing bulblet.

Hypothesis 2: Vascular Connections

The second hypothesis was that a vascular connection, which could provide an easy pathway for virus movement into the bulblet, occurred between the scale and the bulblet. This connection was expected to occur in the Easter lilies, which typically produce a low percentage of in vitro VF scale bulblets, and not in the Asiatic hybrid, 'Enchantment,' which produces a high percentage of in vitro VF scale bulblets. However, the opposite was observed. Vascular connections were never seen in the L. longiflorum

cultivars but were often observed in 'Enchantment' (Figures 12 and 13).

Bulblets of all varieties usually form above scale vascular bundles. Cells within the bulblet leaf initials and at the base of each bulblet develop secondary cell wall thickenings characteristic of xylem tracheids (Figures 10 and 12). Otherwise, the lilies studied vary in bulblet development. In 'Enchantment' explants, cells between the developing bulblet and the scale vascular bundle simultaneously develop secondary cell wall thickenings (Figure 8). As the bulblet continues to develop, mesophyll cells continue to differentiate into tracheids in the direction of the bulblet. A vascular connection eventually forms between the scale and the bulblet in this hybrid (Figure 12).

Similar vascular connections were occasionally observed in the Madonna lily. In 'Ace,' 'Nellie White' and the Oriental hybrid, 'Stargazer,' cells at the base of the forming bulblet and in the leaf primordia differentiate into vascular cells, but cells in the adaxial subepidermal cell layers of the scale do not differentiate (Figure 13). Therefore, no vascular connections form between the scale and the bulblet. The failure to observe vascular connections in the L. longiflorum cultivars was consistent with Walker's (1940) findings whereas the connections observed in the Madonna lily contradict his findings.

It is possible that the elongated configuration of the cells in the vascular bundles of 'Enchantment' scales plays a role in this hybrid's ability to form scale-to-bulblet vascular connections (Figure 5b). The scale xylem of 'Enchantment' is closer to the forming bulblet than in the other hybrids as there are fewer mesophyll cells between the scale xylem and the epidermis. Also, the scale xylem cells are naturally aligned in a plane perpendicular to the epidermis, which appears to be conducive to the addition of xylem cells toward the forming bulblet. The scale xylem cells in the other four lilies lie adjacent to the scale phloem cells and parallel to the epidermis forming a circular configuration (Figure 5a).

The results of this study indicate that vascular connections are not pathways for rapid movement of virus particles from the infected scale to the forming bulblet. If they were, 'Enchantment' would be expected to have a much lower percentage of <u>in vitro</u> VF bulblets than is typically found (Allen and Fernald, 1972; Blom-Barnhoorn and van Aartrijk, 1985) because this hybrid readily forms vascular connections between the infected explant and the new bulblet.

Hypothesis 3: Virus Concentration

The third hypothesis tested in this study regarded virus concentrations in the scales. It was proposed that VF bulblets originated from scale tissues low in virus concentration while the infected bulblets began from scale tissues high in virus concentration. The data produced in this study unfortunately neither confirm nor disprove this hypothesis. Several explanations may exist.

ELISA results of mother plant leaf tissue indicated the presence of LSV and TBV, alone or together, in each of the lily types (Table 1). Virus particles, however, were observed only in 'Ace' and 'Nellie White' tissues when in vitro tissues of all lilies were examined with the TEM (Table 2). Virus particles also should have been found in the other three lilies to support the ELISA results. Ultrastructural abnormalities characteristic of virus infection, however, were observed in these singly-infected hybrids, which suggests that virus infection was present despite the absence of visible virus particles. These abnormalities included: clumped mitochondria (Figure 17); vesiculated outer chloroplast membranes (Figure 19); cell wall deformations (Figure 20); vesiculated cytoplasms with aggregated ribosomes (Figure 21); and intra- and extra-protoplasmic osmiophilic globules (Figures 22, 23 and 24).

The presence of these cytoplasmic abnormalities suggests that the ELISA results for the lily varieties with single virus infections were accurate and that the virus concentration in these lilies was lower than that in the Easter lilies. In fact, as the ELISA results indicated, the LSV or TBV concentrations found in the singly-infected hybrids were equivalent to the LSV concentrations in the L. longiflorum cultivars (Table 1). These ELISA readings ranged from 1.986 in 'Nellie White' for LSV to 2.448 in the Madonna lily for TBV. 'Ace' and 'Nellie White,' however, had additional ELISA TBV readings of 0.941 and 0.926, respectively. Thus, the total virus concentration should have been higher in the double virus-infected L. longiflorum cultivars than in the singly-infected hybrids.

Perhaps virus particles were observed only in the L. longiflorum cultivars because of the higher virus concentration due to double infection. It is also possible that double virus infection characteristically occurs more often in these lilies than in others and is the cause of low production of in vitro VF bulblets normally observed in Easter lilies (Linderman et al., 1976; Allen et al., 1980). Similar results were found by Asjes (1974) in 'Enchantment' lilies concurrently infected with LSV and TBV. In his study, the percentage of VF plants of this lily declined from 70 percent in LSV-infected explants to 25 percent in LSV- and TBV-infected explants.

The extensive adaxial subepidermal cell divisions observed in the L. longiflorum cultivars may also play a part in the low VF bulblet production of these lilies. If the virus particles within the adaxial subepidermal cells are at a high concentration or are evenly scattered within each cell, multiple cell divisions may increase the number of infected cells. Thus, an infected mother cell with many virus particles or one with an even distribution of virus particles will likely produce two infected daughter cells.

Support for this theory can be found in the observations that 'Ace' and 'Nellie White:' 1) were doubly-infected, which may have generated a higher virus concentration throughout the scale tissues than in lilies with single virus infections, and 2) had extensive cell divisions in the area of bulblet origination, which may have created new cells with virus particles. These data suggest that the difference in hybrid variation in VF bulblet production is due to differences in virus concentrations at the site of bulblet origination.

However, questions in this area remain unanswered. Is double infection characteristic of these L. longiflorum cultivars and not characteristic in other Lilium hybrids, or was double infection coincidental in the lilies tested in this study? Do the continuous subepidermal cell divisions, found only in the L. longiflorum cultivars, increase the number of infected cells as proposed here, or

do these cells decrease the virus concentration by dividing faster than the virus particles can multiply, thus creating VF cells?

The third hypothesis may be related to the first hypothesis. Is the apparent increased virus concentration in the L. longiflorum cultivars in this study due to a specific hybrid response to wounding? Some evidence exists that the wounding process may stimulate virus multiplication. Detection of virus with ELISA has been greatly enhanced by pre-wounding iris bulbs (van der Vlugt et al., 1988) and gladiola corms (Stein et al., 1988). Perhaps a similar phenomenon occurs with some lily hybrids such as L. longiflorum, which typically have a lower incidence of VF scale bulblets than other hybrids.

Answers to these questions must be found before a definitive conclusion can be made on the validity of the third hypothesis.

The author also believes that the degree of cell wall deterioration during incubation may play a role in VF bulblet production. The cell walls of the 'Enchantment' lily scale cells remain intact throughout the incubation period whereas those of the L. longiflorum cultivar scale cells disintegrate during this time. It seems likely that intact cells walls would inhibit, or at least slow, the movement of virus particles from infected cells to non-infected cells and that the disintegration of cell

walls would allow virus particles to move freely to intact cells.

Therefore, in the Easter lilies, where only the upper and lower subepidermal scale cells remain intact, virus particles originally located within interior mesophyll cells could migrate to the intact subepidermal cells where bulblets are formed. The virus concentration of these subepidermal cells would consequently increase dramatically. Similar observations of scale cell conditions have been reported by Walker (1940) for L. candidum and L. longiflorum and by Godden and Watson (1962) for an Aurelian, an American and an Asiatic lily, but connections to VF bulblet production have not been recorded.

Likewise, the use of heat and chemical treatments should be explored to determine their effects on the presence of the adaxial subepidermal scale cell layering that occurs in the L. longiflorum cultivars. If heat or chemical treatment reduced the subepidermal layering found in these cultivars, perhaps the percentage of in vitro VF bulblets would increase. In fact, Blom-Barnhoorn and van Aartrijk (1985) found that when Virazole was added to the nutrient medium at a concentration of 40.0 μM, the total percentage of LSV and/or TBV infected L. longiflorum 'Arai' plants was reduced from 61.4 percent to 35.4 percent. Perhaps this is due to a decrease in the cell

divisions occurring in the subepidermal layers where bulblets originate.

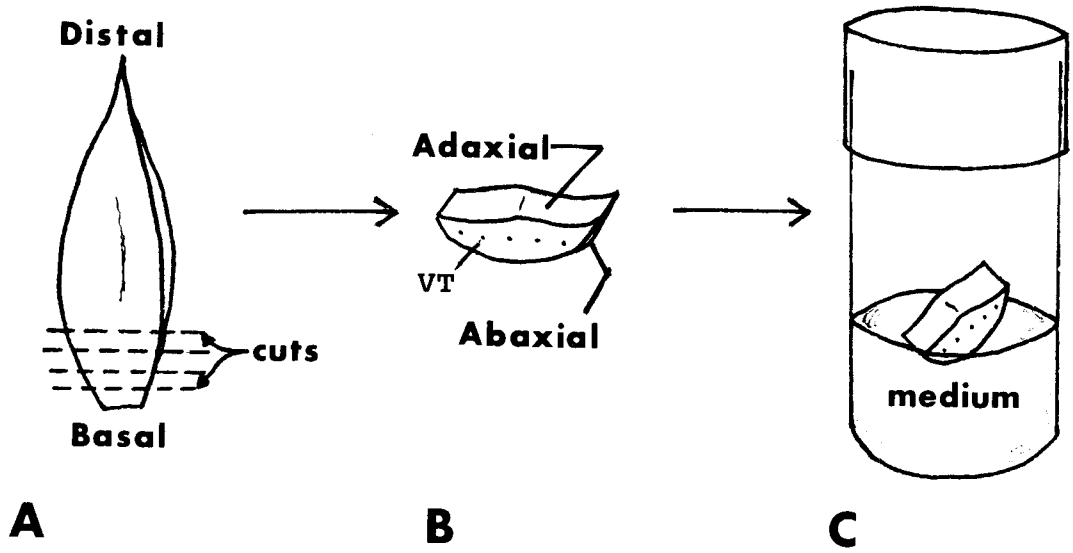

Figure 1. Diagram of tissue culture initiation procedure. (A) Basal end of lily bulb scale cut transversely into 2-3 mm wide strips to produce explants; (B) transverse view of one explant shows exposed vascular tissues (VT), adaxial surface (inner scale surface; top of explant) and abaxial surface (outer scale surface; bottom of explant); and (C) explant placed with abaxial surface in contact with semi-solid nutrient medium in sterile, capped test tube.

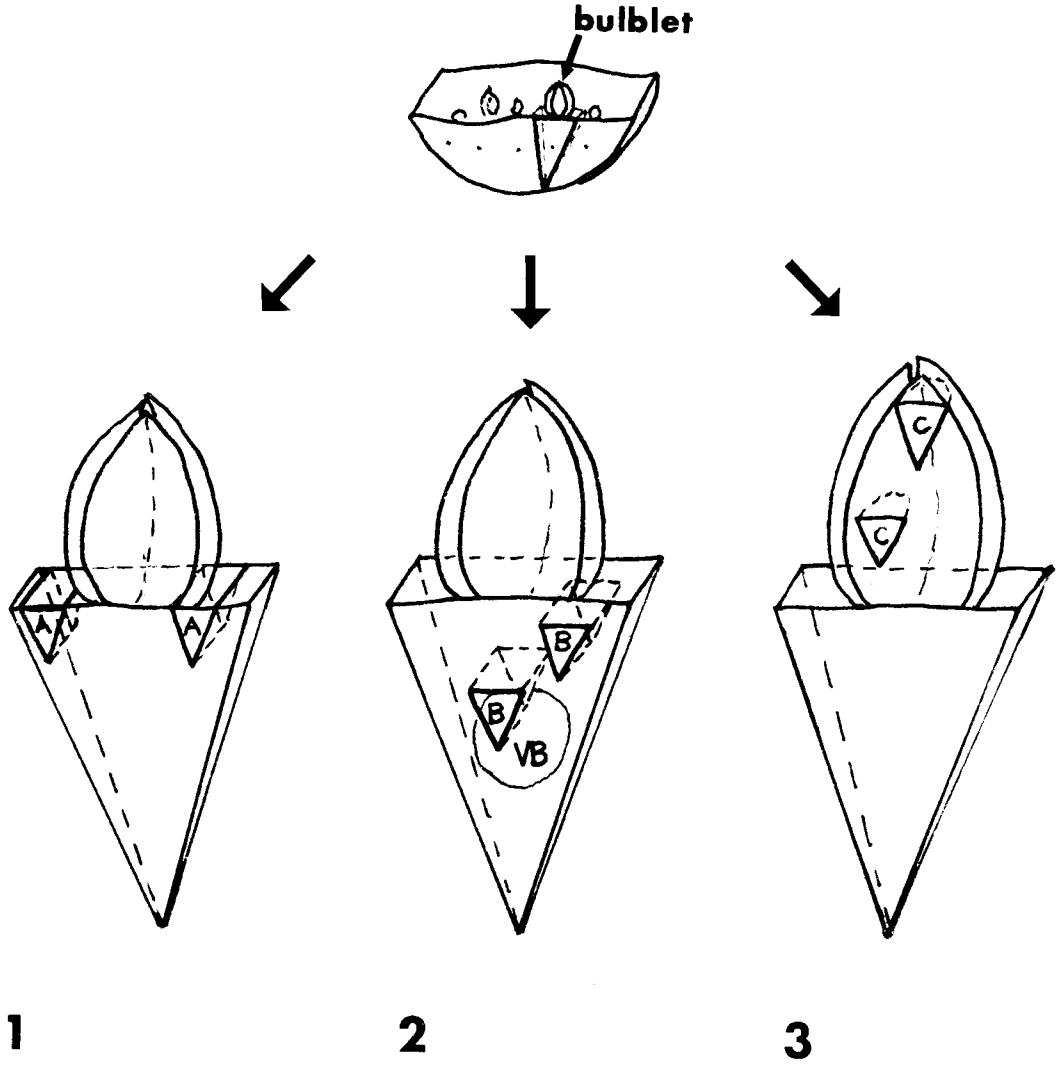

Figure 2. Diagram of in vitro scale explants shows the three areas samples were sectioned for TEM examination. (1) Adaxial epidermal and subepidermal cells of scale adjacent to or beneath bulblet initial, bulblet or plantlet, if present, designated A or epidermis area; (2) random scale mesophyll cells, sometimes containing part of the vascular bundle (VB), designated B or mesophyll area; and (3) random bulblet initial, bulblet or plantlet cells, designated C or bulblet area.

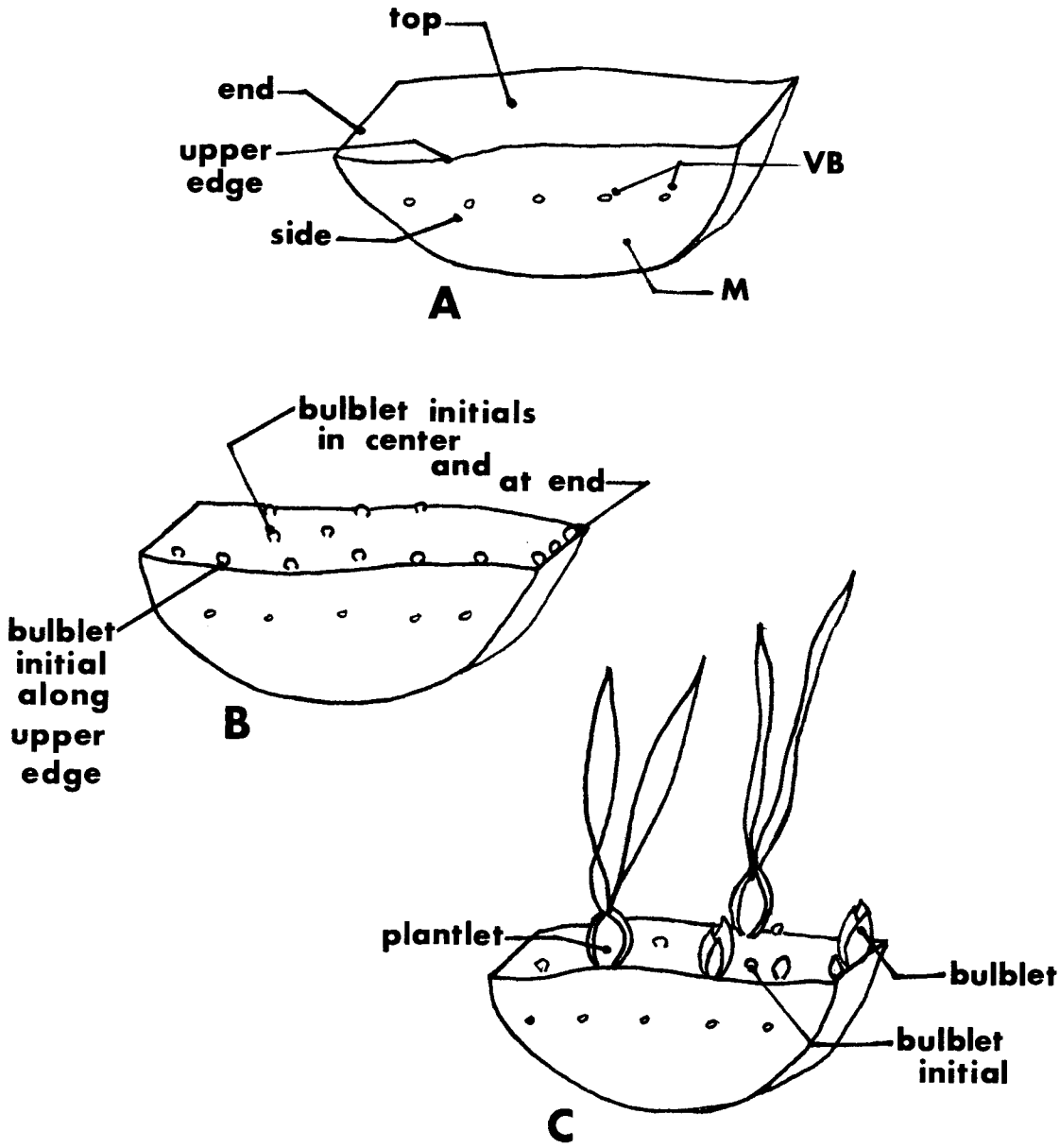

Figure 3. Diagram of in vitro bulblet development and location on explant. (A) 0 DAE, bulb scale cut transversely at initiation exposes mesophyll (M) and vascular bundles (VB) with no bulblets present; (B) 7-10 DAE, bulblet initials (tiny bumps) begin to show on the top (adaxial) surface, usually along the upper edge but also at the ends and in the center, of the scale explant depending upon the lily type; and (C) 28 DAE, all stages of bulblets present simultaneously on the top surface of the scale explant.

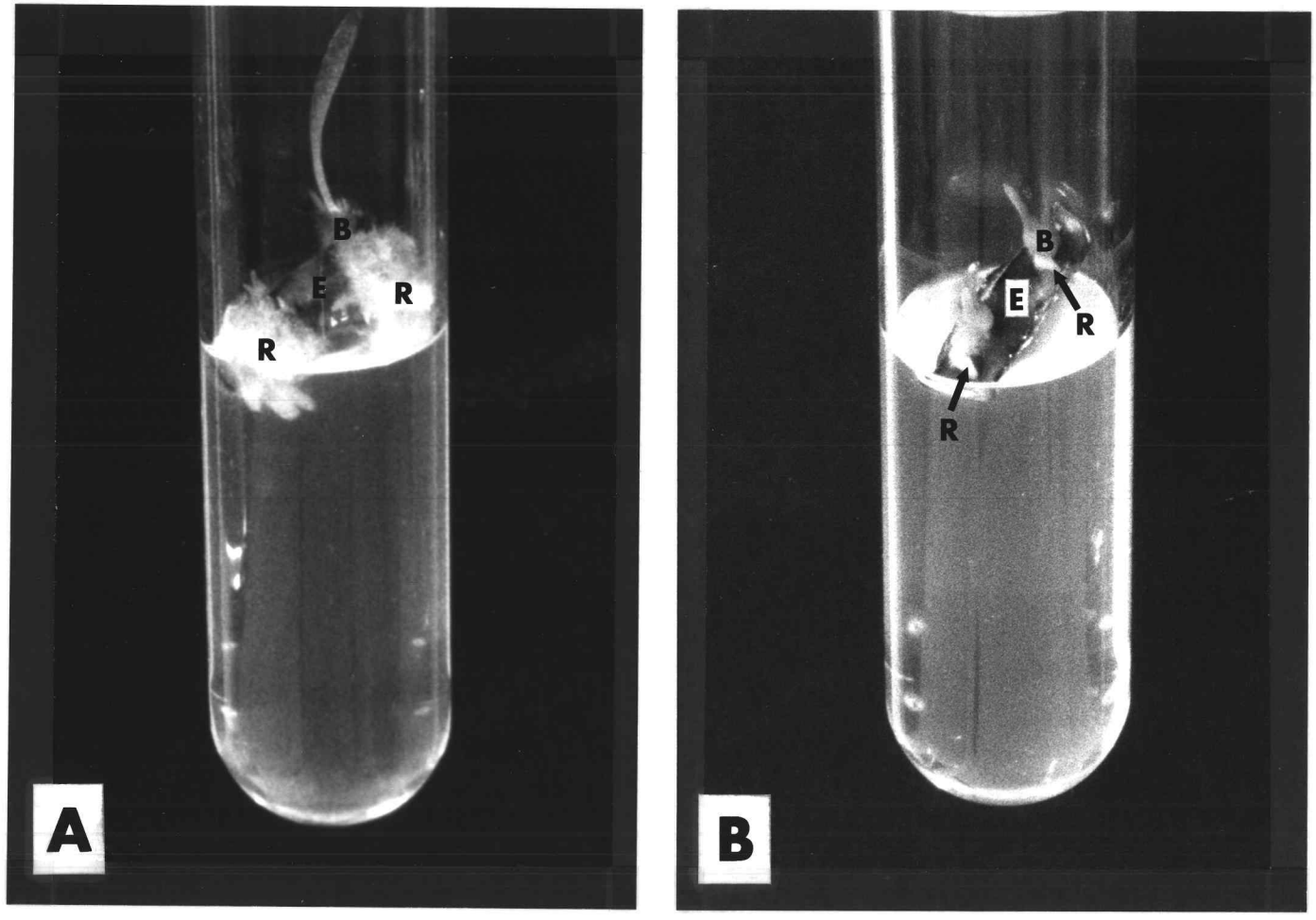

Figure 4. Comparison of bulblet root development. (A) Longest and most roots per bulblet found in Easter lilies. 'Nellie White,' 28 DAE; and (B) shortest and fewest roots occur on 'Enchantment' bulblets. 28 DAE. B = bulblet; R = root; E = explant.

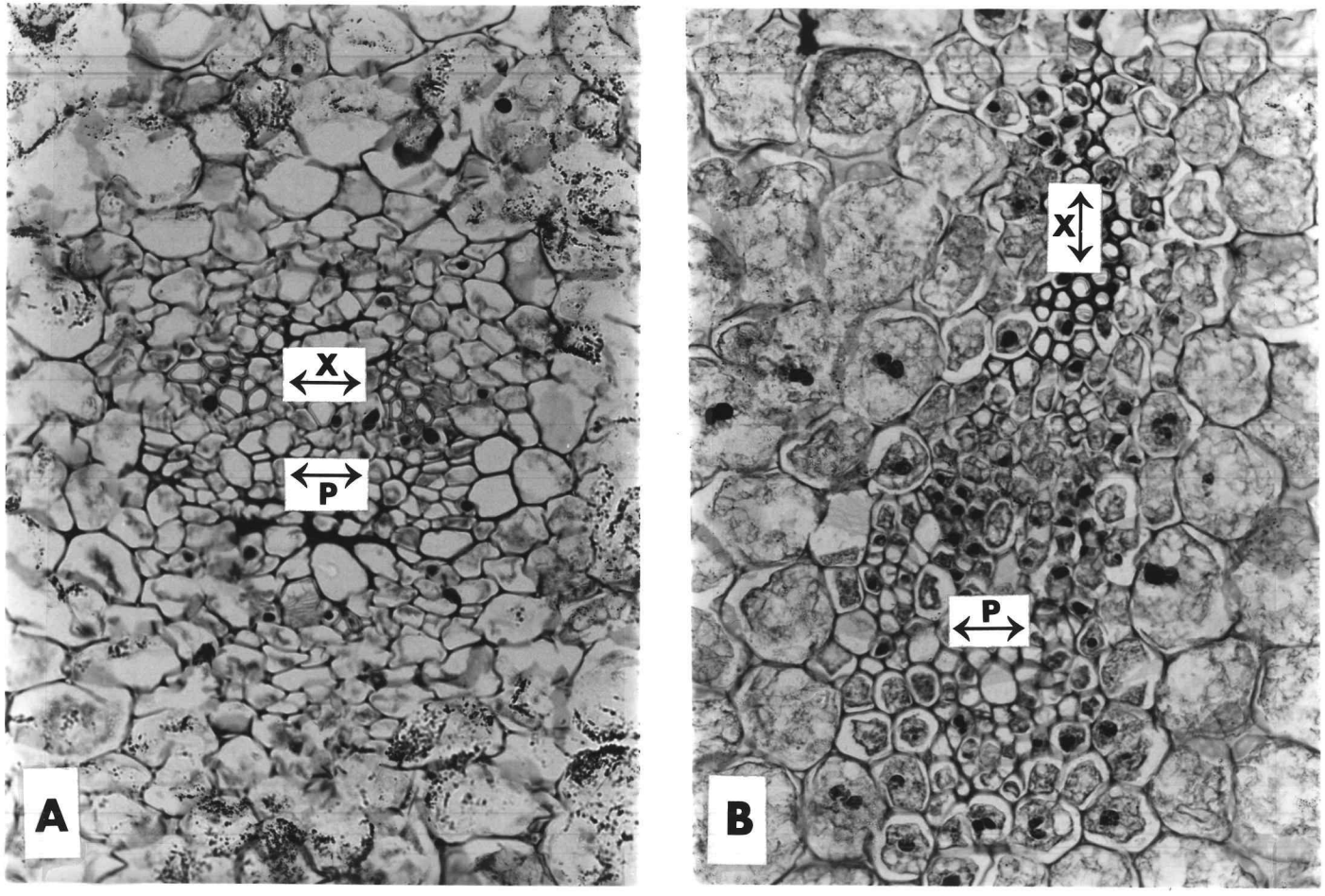

Figure 5. Comparison of scale vascular bundles. (A) 'Ace' scale section shows circular configuration of vascular bundle; and (B) 'Enchantment' scale vascular bundle arranged in an elongated configuration. Both sections at 0 DAE, transverse section, X 190. P = phloem; X = xylem.

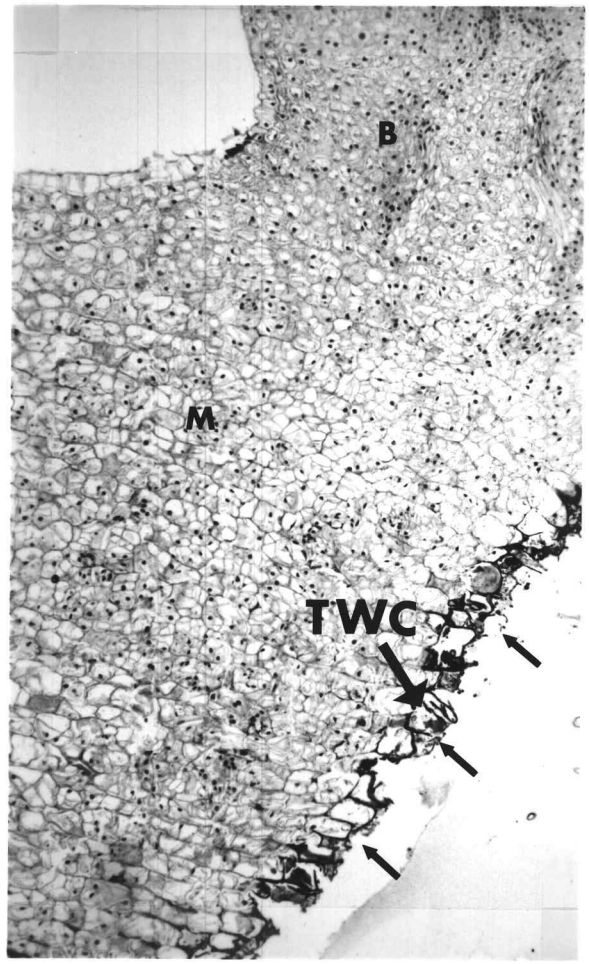

Figure 6. Collapse and cell wall thickening of cells at edge of original transverse cut (small arrows) of scale explant. 'Stargazer,' 26 DAE, longitudinal section, X 47. TWC = thick-walled cells; B = bulblet; M = mesophyll.

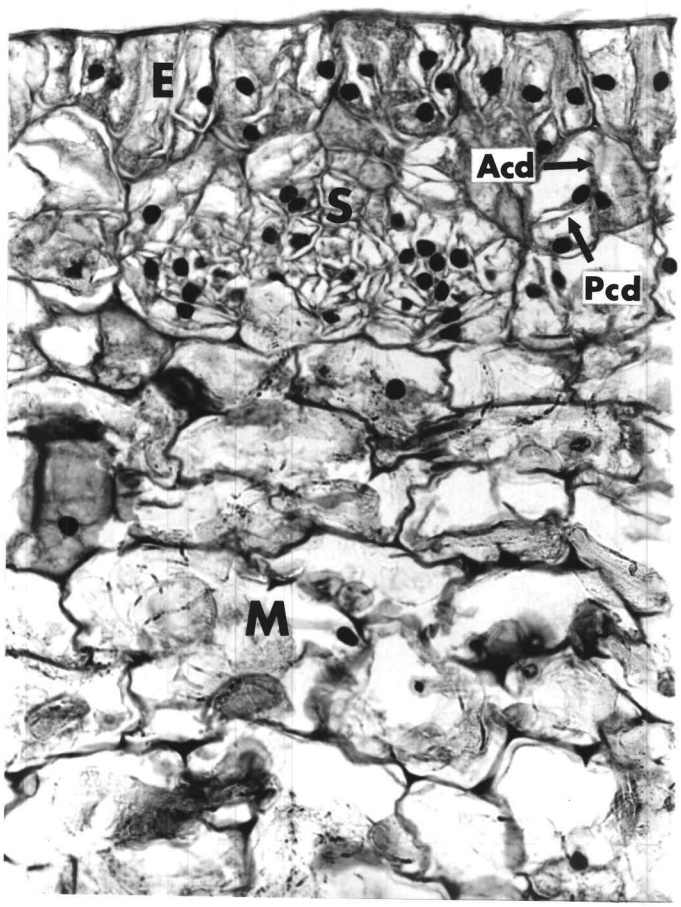

Figure 7. Cell division in epidermal and subepidermal scale layers initiate bulblet development. 'Ace,' adaxial scale surface, 32 DAE, transverse section, X 190. Acd = anticlinal cell division; Pcd = periclinal cell division; E = epidermis; S = subepidermis; M = mesophyll.

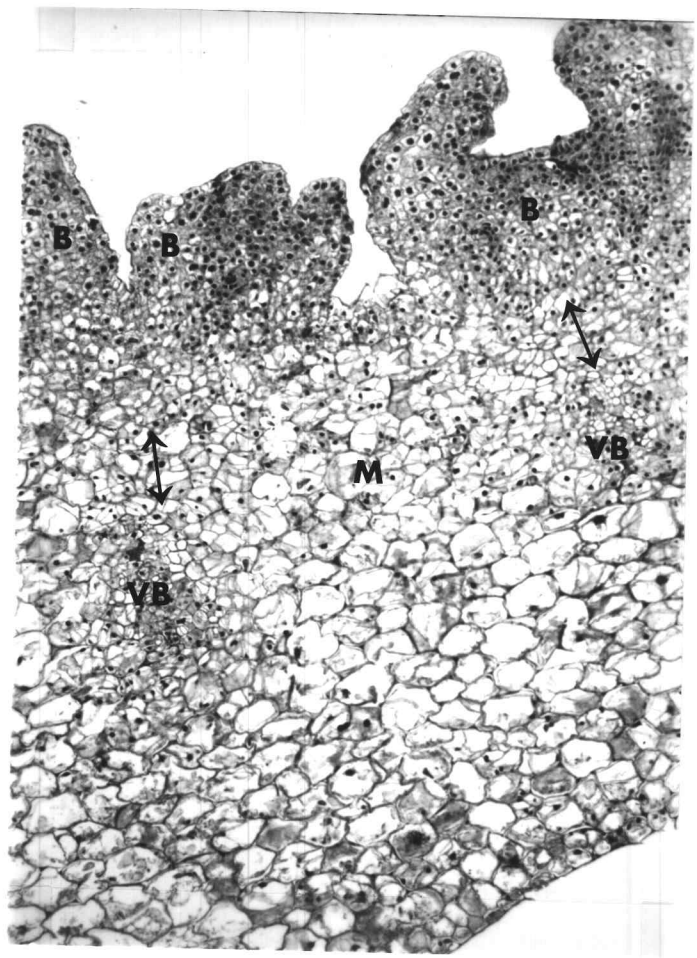

Figure 8. 'Enchantment' bulblets develop above scale vascular bundles. Cell division (arrows) between bulblet and vascular bundle in mesophyll is characteristic of this lily. 23 DAE, transverse section, X 50.
B = bulblet; VB = vascular bundle; M = mesophyll.

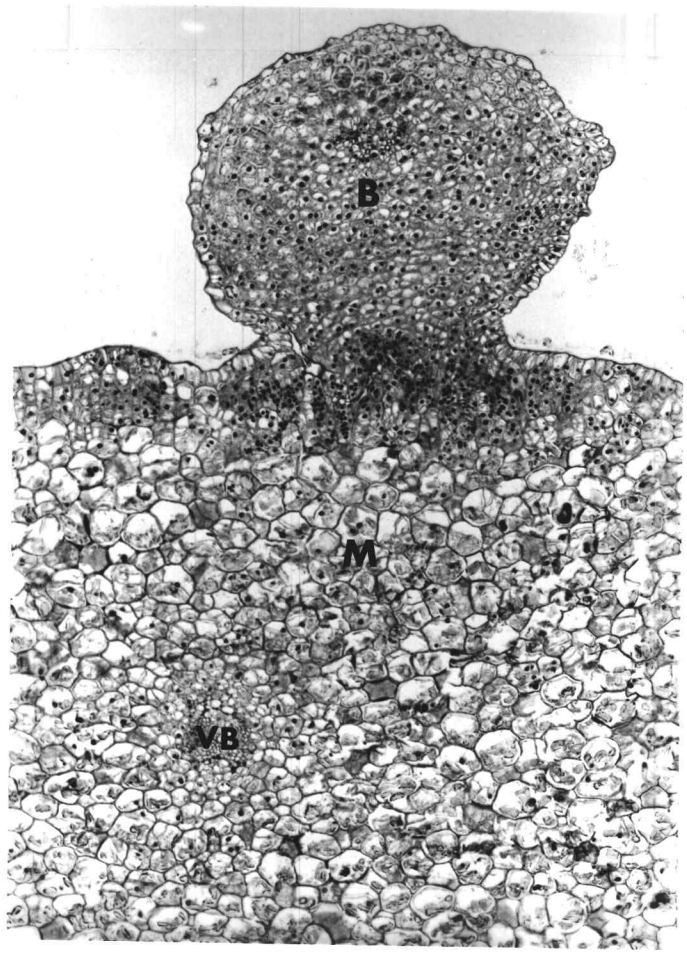

Figure 9. Easter lily bulblet positioned above scale vascular bundle with little cell division in mesophyll between bulblet and vascular bundle. 'Ace,' 30 DAE, transverse section, X 38. B = bulblet; VB = vascular bundle; M = mesophyll.

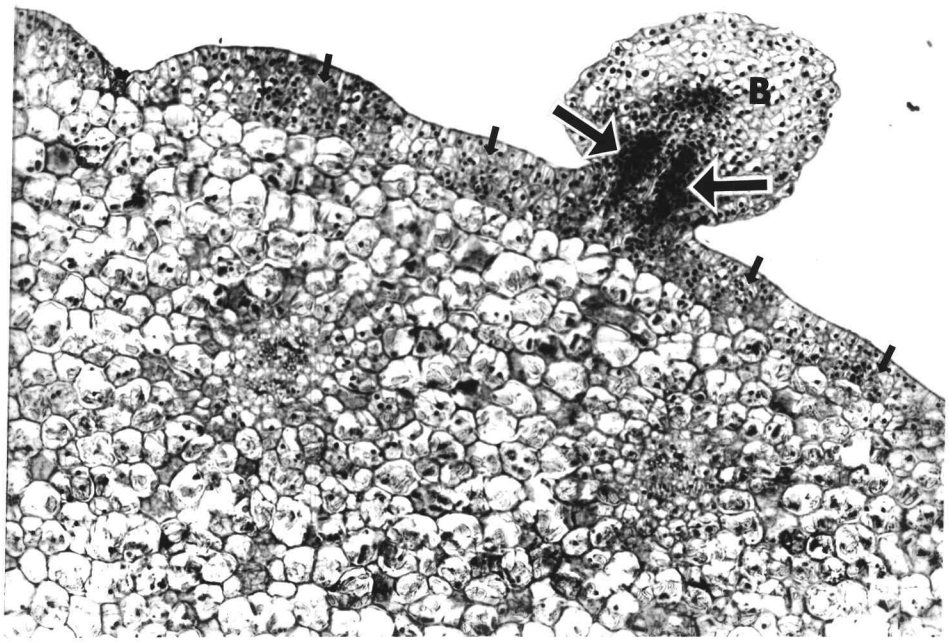

Figure 10. Easter lily section with cell division (small arrows) across entire top (adaxial) surface of scale explant. Bulblet base shows cells with secondary thickening of walls (large arrows). 'Ace,' 30 DAE, transverse section, X 38. B = bulblet.

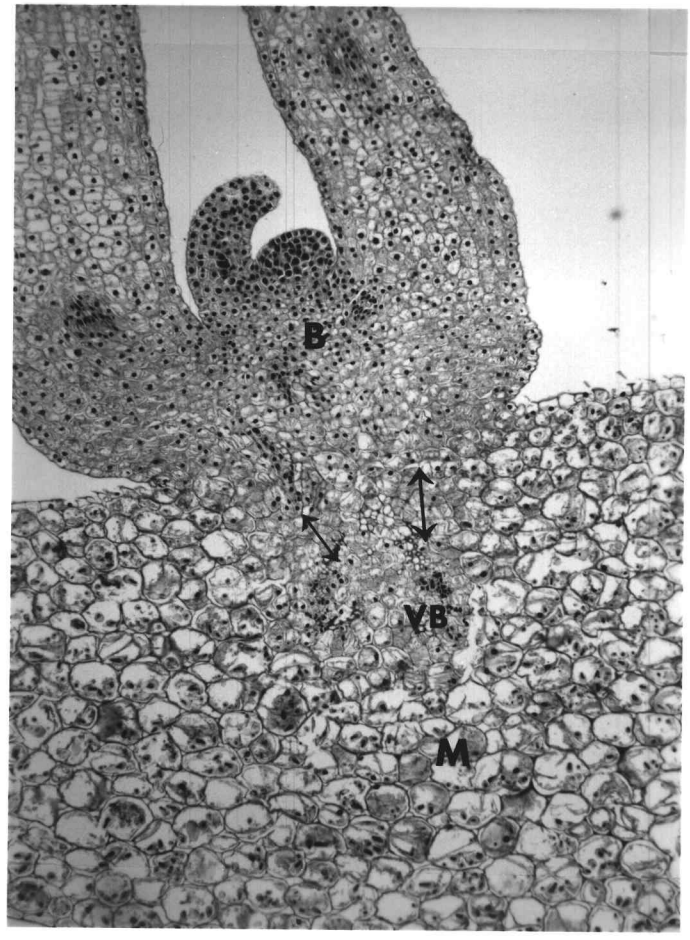

Figure 11. 'Enchantment' section with subepidermal cell division (arrows) only at origin of bulblet. 30 DAE, transverse section, X 50. B = bulblet; VB = vascular bundle; M = mesophyll.

Figure 12. Vascular connections between bulblet procambium and scale vascular bundle occur in 'Enchantment.' 18 DAE, longitudinal section, X 56. B = bulblet; VB = vascular bundle; P = procambium; VC = vascular connection.

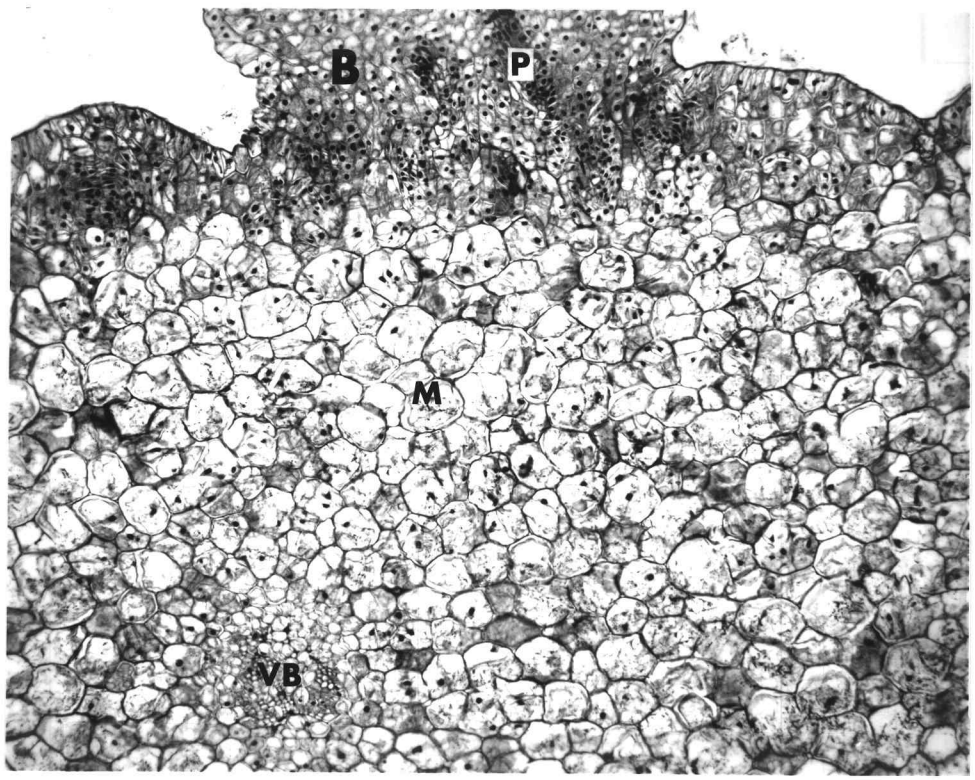

Figure 13. 'Ace' scale section without vascular connection between bulblet procambium and scale vascular bundle. 30 DAE, transverse section, X 56. B = bulblet; VB = vascular bundle; M = mesophyll; P = procambium.

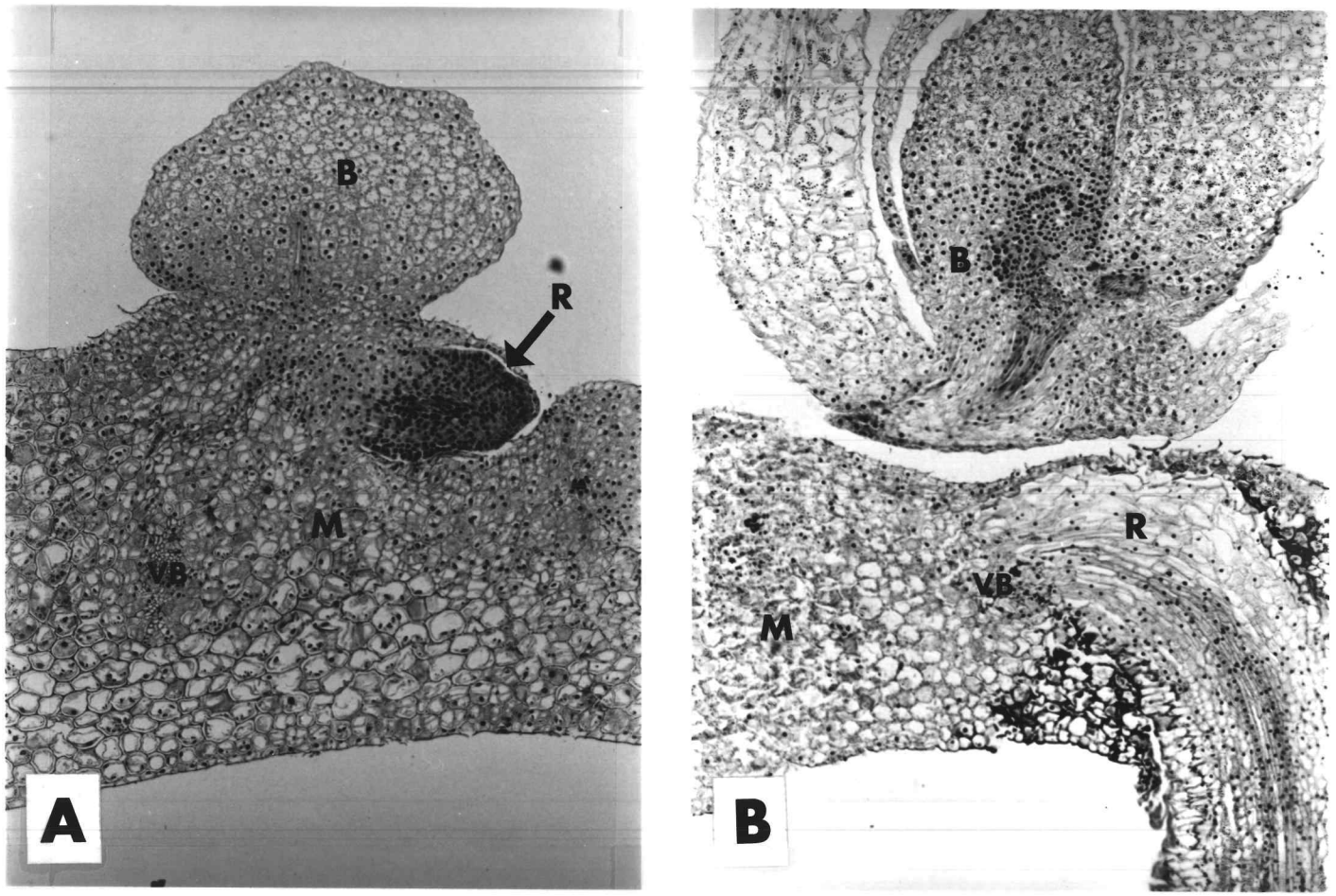

Figure 14. Comparison of scale bulblet root origin. (A) Roots of 'Enchantment' bulblet initiate from cells at base of bulblet. 32 DAE, transverse section, X 38. (B) 'Nellie White' bulblet roots initiate from scale mesophyll cells. 44 DAE, transverse section, X 38. B = bulblet; R = root; M = mesophyll; VB = scale vascular bundle.

Figure 15. LSV and TBV particles present in Easter lily cells. (A) Aggregated virus particles; (B) loose arrangement of virus particles; and (C) virus particles scattered about the cytoplasm.

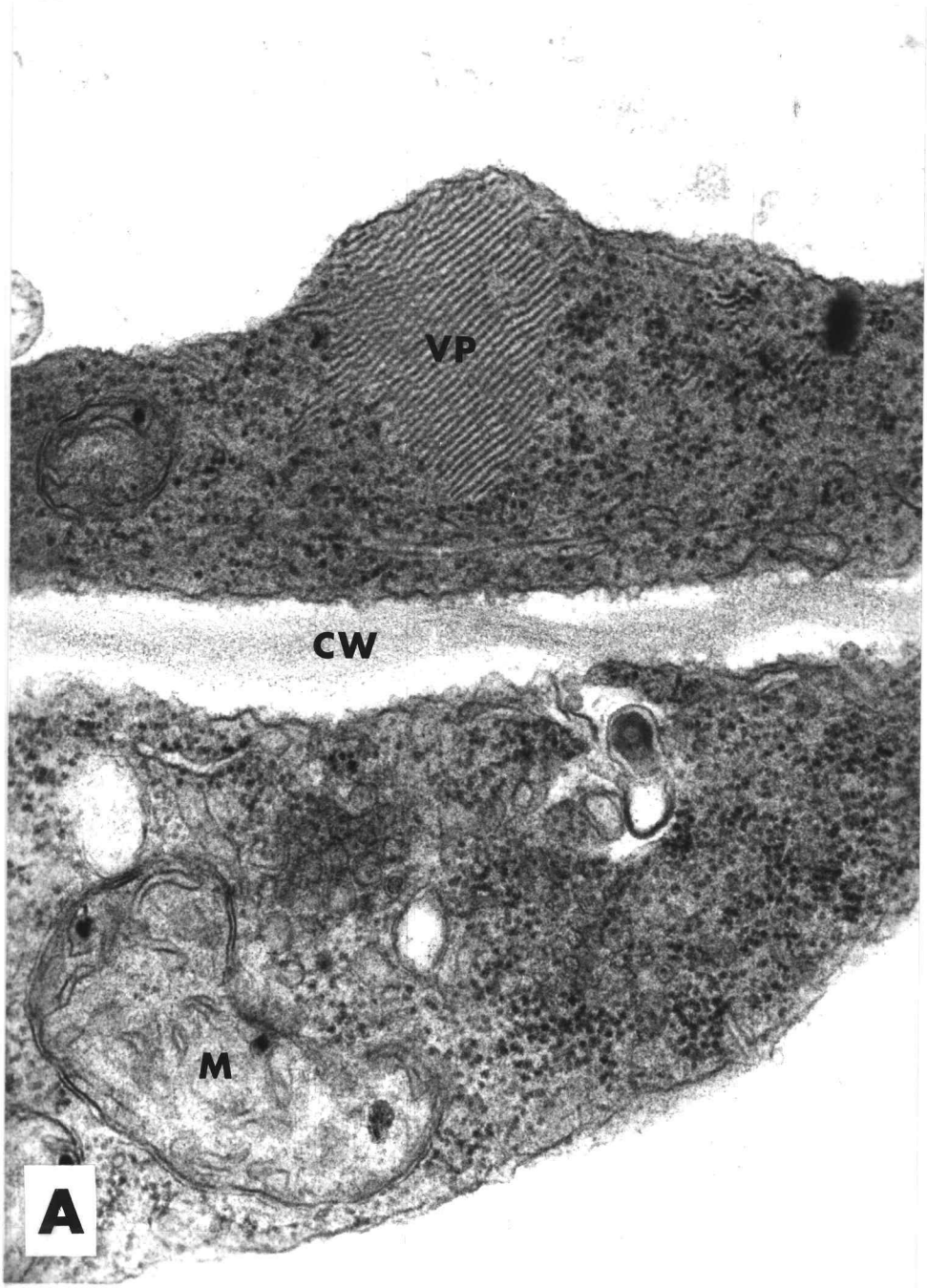

Figure 15(A). Aggregated virus particles. 'Nellie White,' subepidermal cell, 26 DAE, longitudinal section, X 54,000. VP = virus particles; M = mitochondria; CW = cell wall.

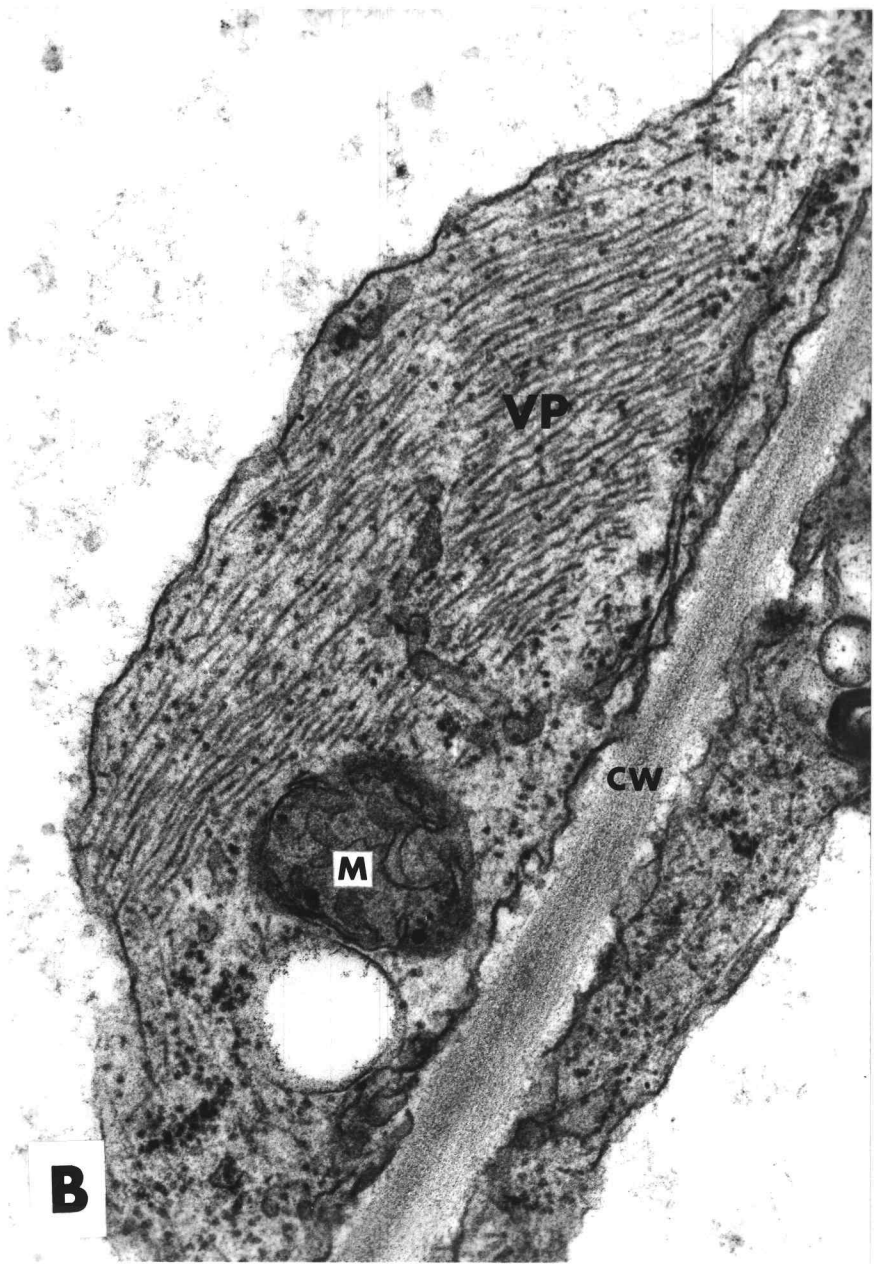

Figure 15(B). Loose arrangement of virus particles. 'Nellie White,' subepidermal cell, 26 DAE, longitudinal section, X 52,000. VP = virus particles; M = mitochondria; CW = cell wall.

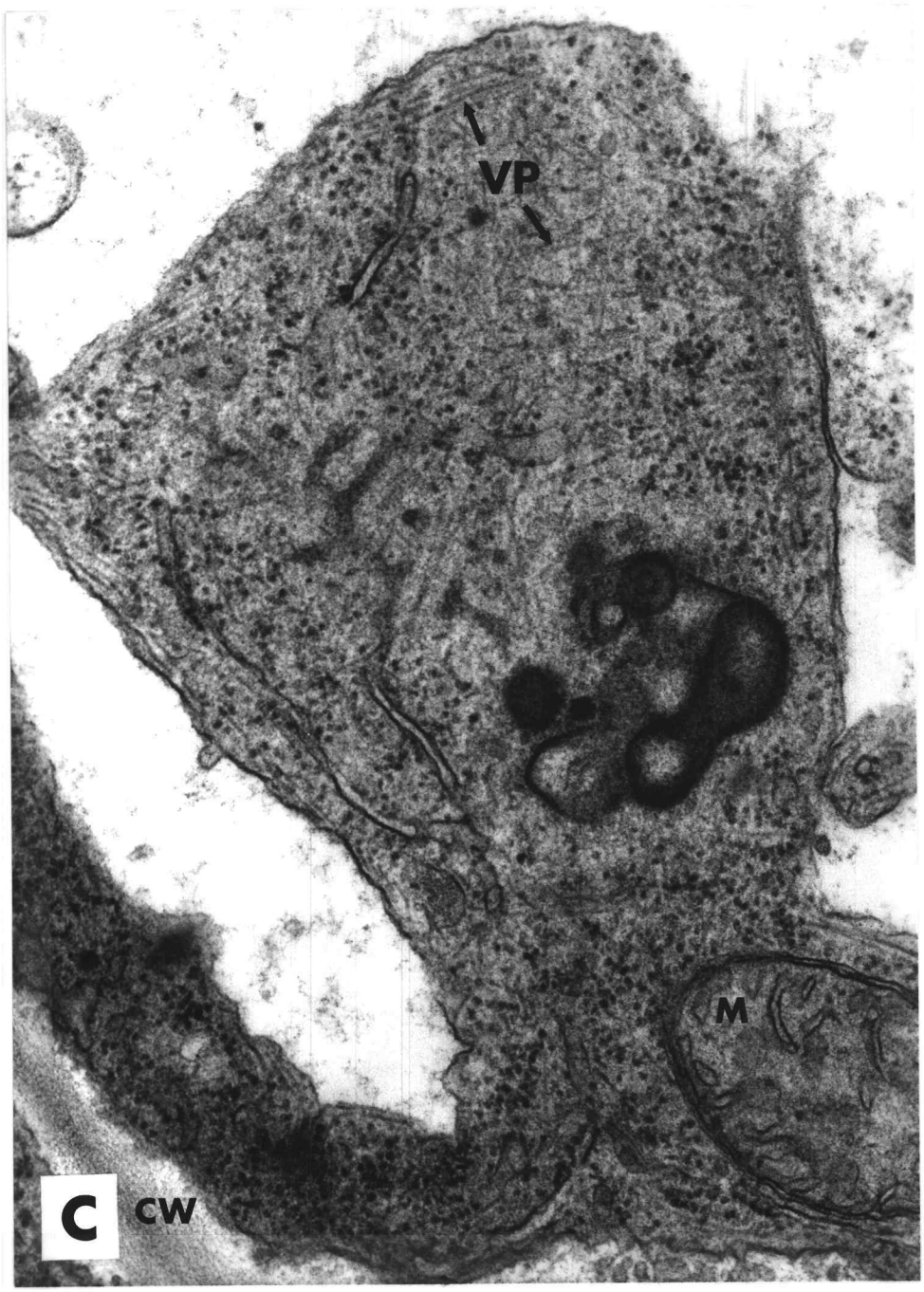

Figure 15(C). Virus particles scattered about the cytoplasm. 'Nellie White,' subepidermal cell, 26 DAE, longitudinal section, X 54,000. VP = virus particles; M = mitochondria; CW = cell wall.

Figure 16. TBV pinwheel inclusions present in Easter lily cells. (A) Pinwheel inclusions found in large numbers and associated with TBV particles; and (B) pinwheel inclusions associated with the plasmamembrane.

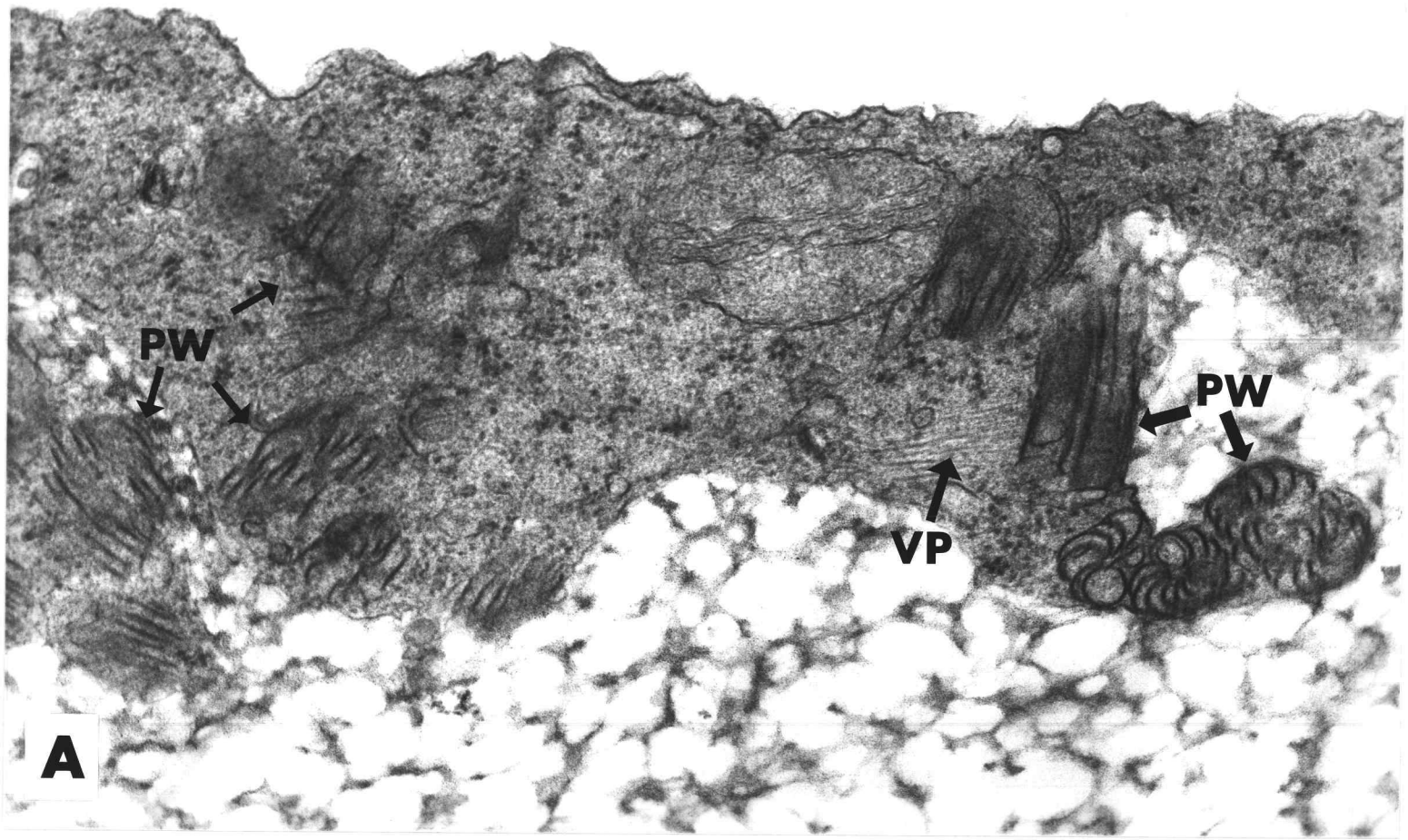

Figure 16(A). Pinwheel inclusions found in large numbers and associated with TBV particles. 'Ace,' mesophyll cell, 9 DAE, transverse section, X 62,400. PW = pinwheel inclusion; VP = virus particles.

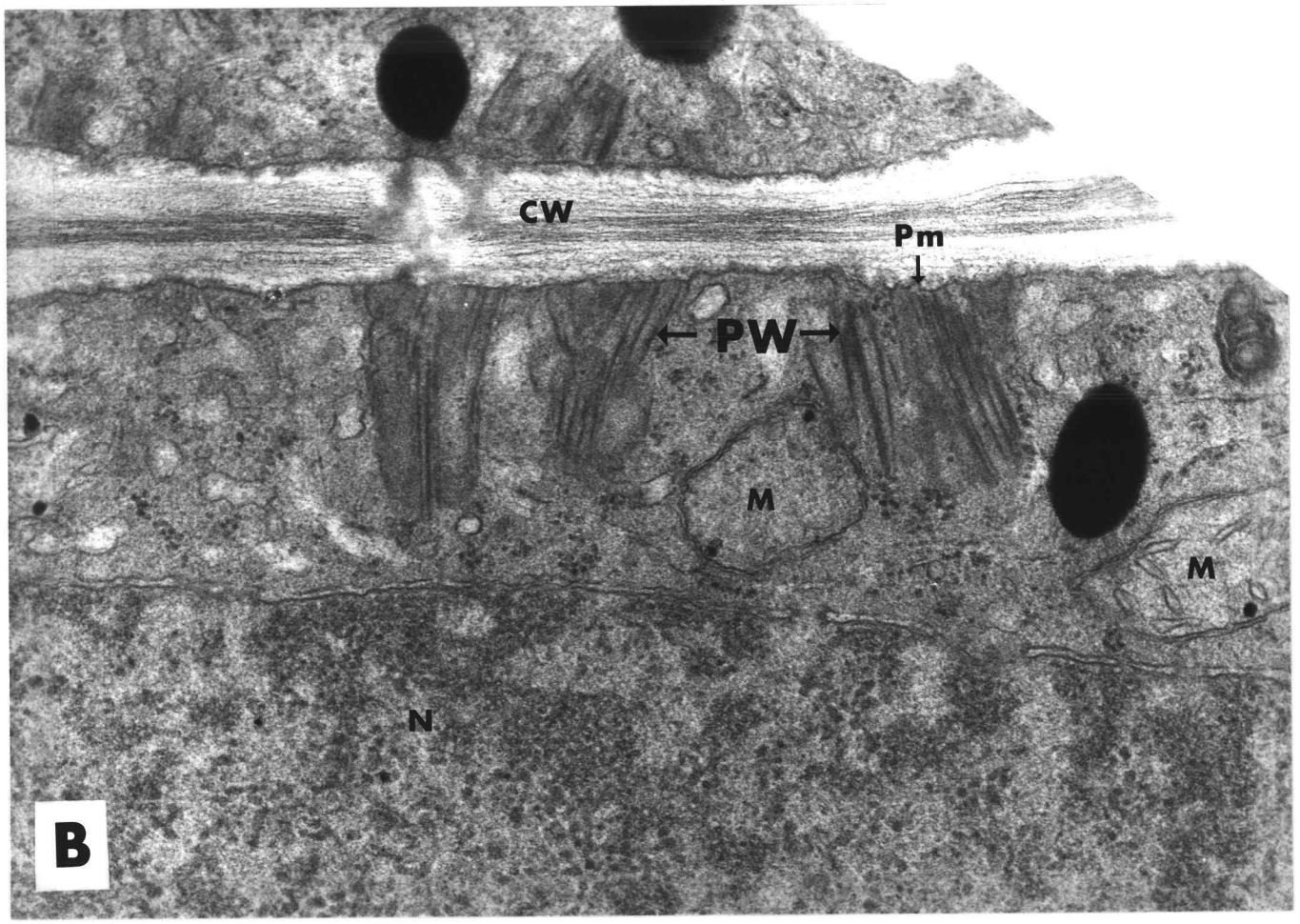

Figure 16(B). Pinwheel inclusions associated with the plasmamembrane. 'Nellie White,' subepidermal cell, 9 DAE, transverse section, X 54,000. PW = pinwheel inclusion; Pm = plasmamembrane; CW = cell wall; M = mitochondria; N = nucleus.

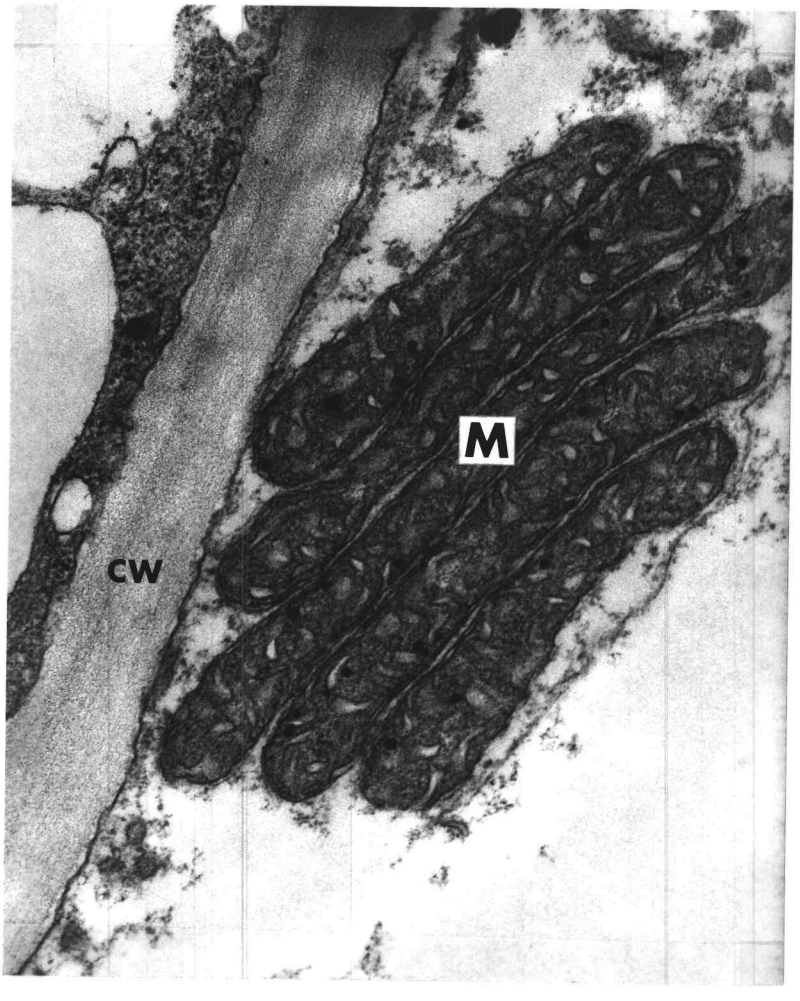

Figure 17. Aggregated mitochondria found in Madonna bulblet cell. 40 DAE, longitudinal section, X 43,200. M = mitochondria; CW = cell wall.

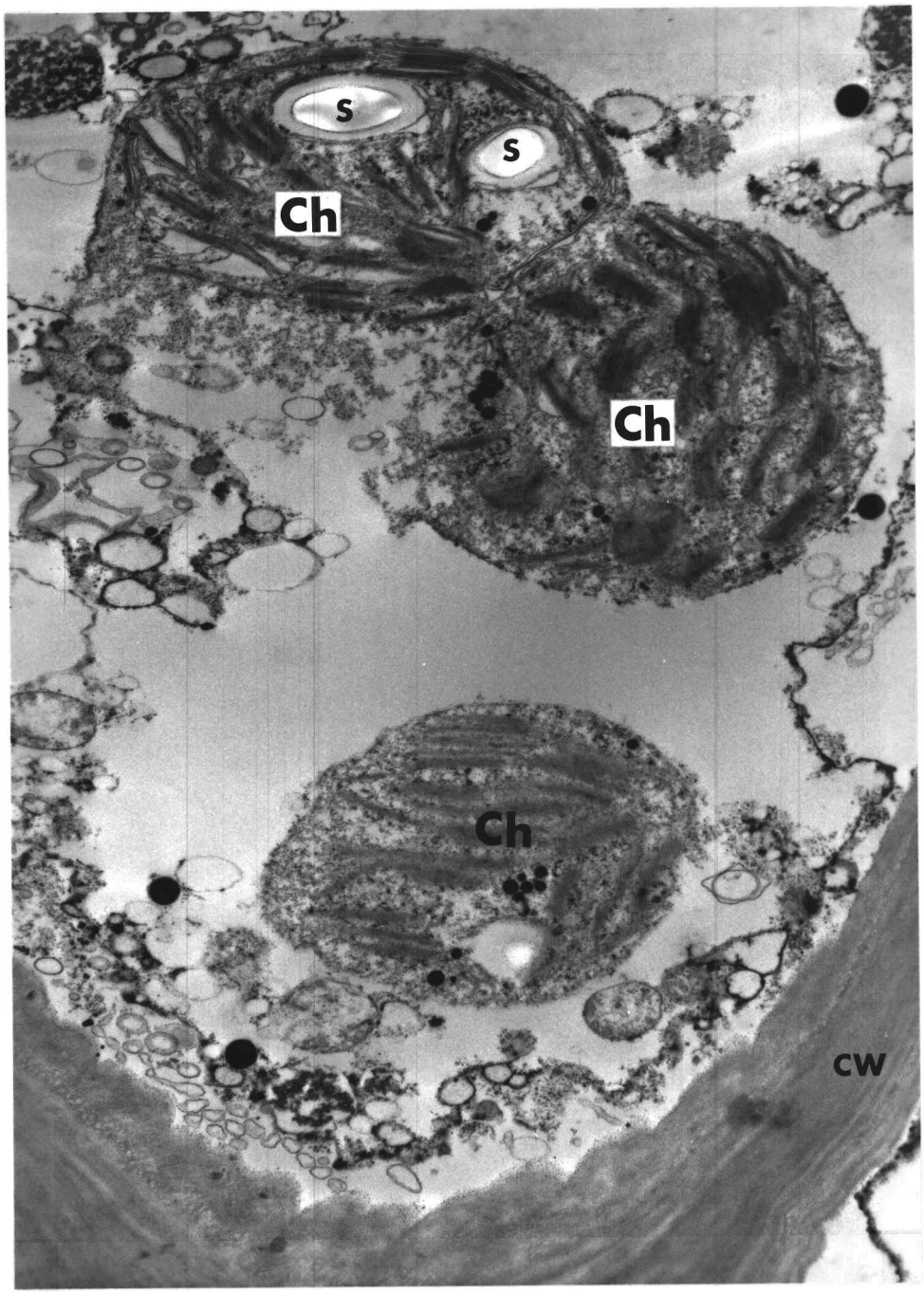

Figure 18. Chloroplasts -- swollen, aggregated, and with or without starch grains -- found in 'Stargazer.' Subepidermal cell, 37 DAE, longitudinal section, X 12,420. Ch = chloroplast; S = starch grain; CW = cell wall.

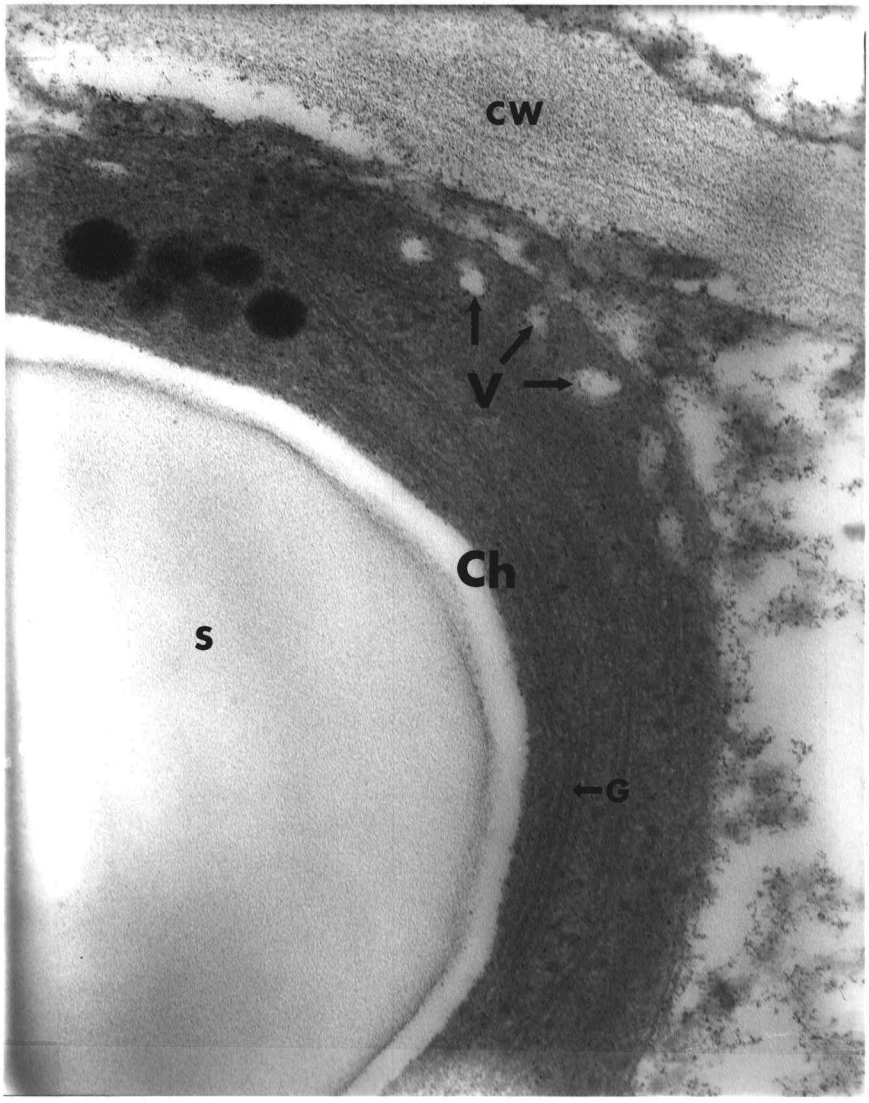

Figure 19. Chloroplast with vesiculated outer membrane found in Easter lily bulblet cell. 'Nellie White,' 26 DAE, transverse section, X 83,200. V = vesicle; S = starch grain; Ch = chloroplast; CW = cell wall; G = grana.

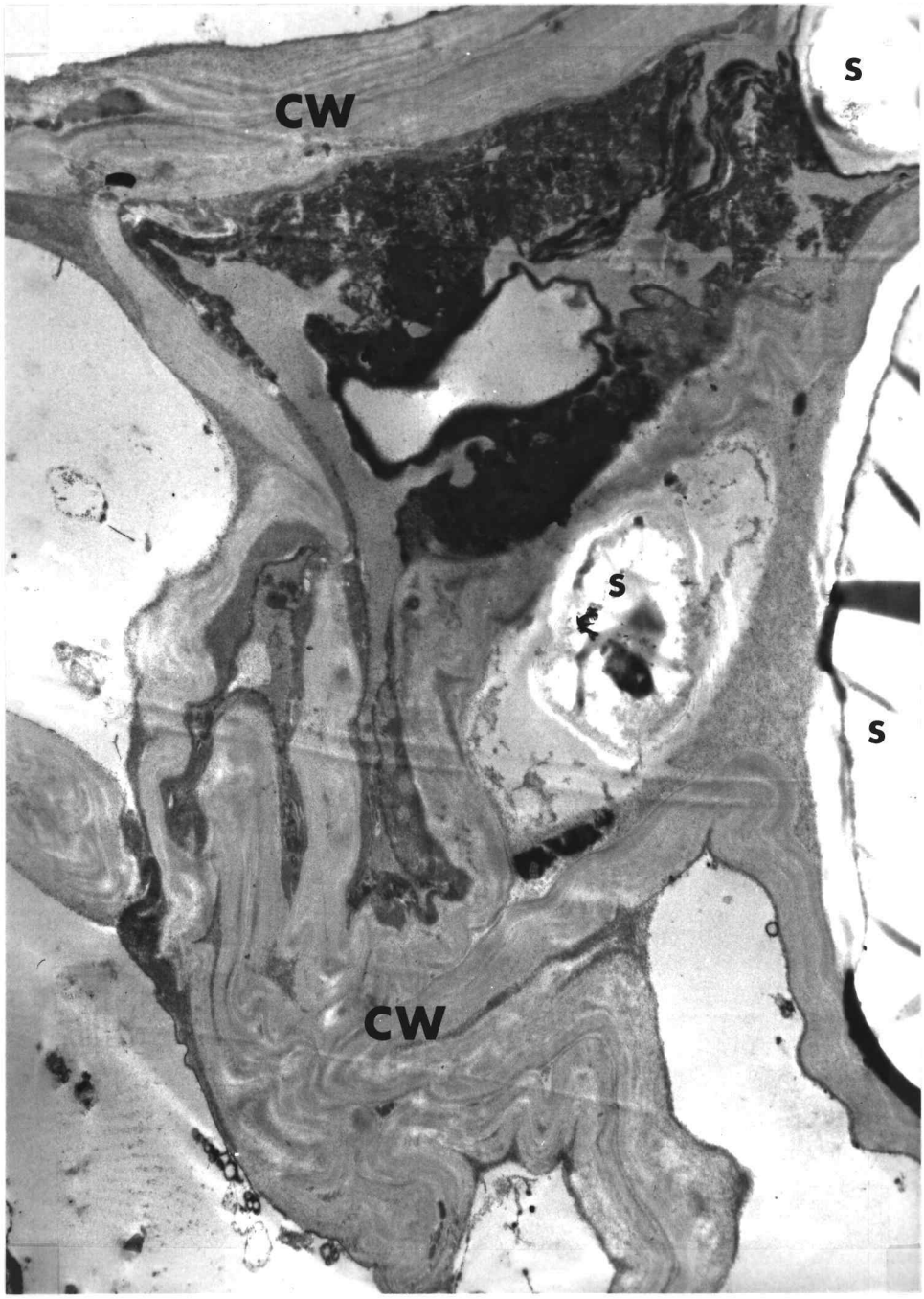

Figure 20. Cell wall deformations present in Easter lily cells. 'Ace,' mesophyll cells, 26 DAE, transverse section, X 8,640. CW = cell wall; S = starch grain.

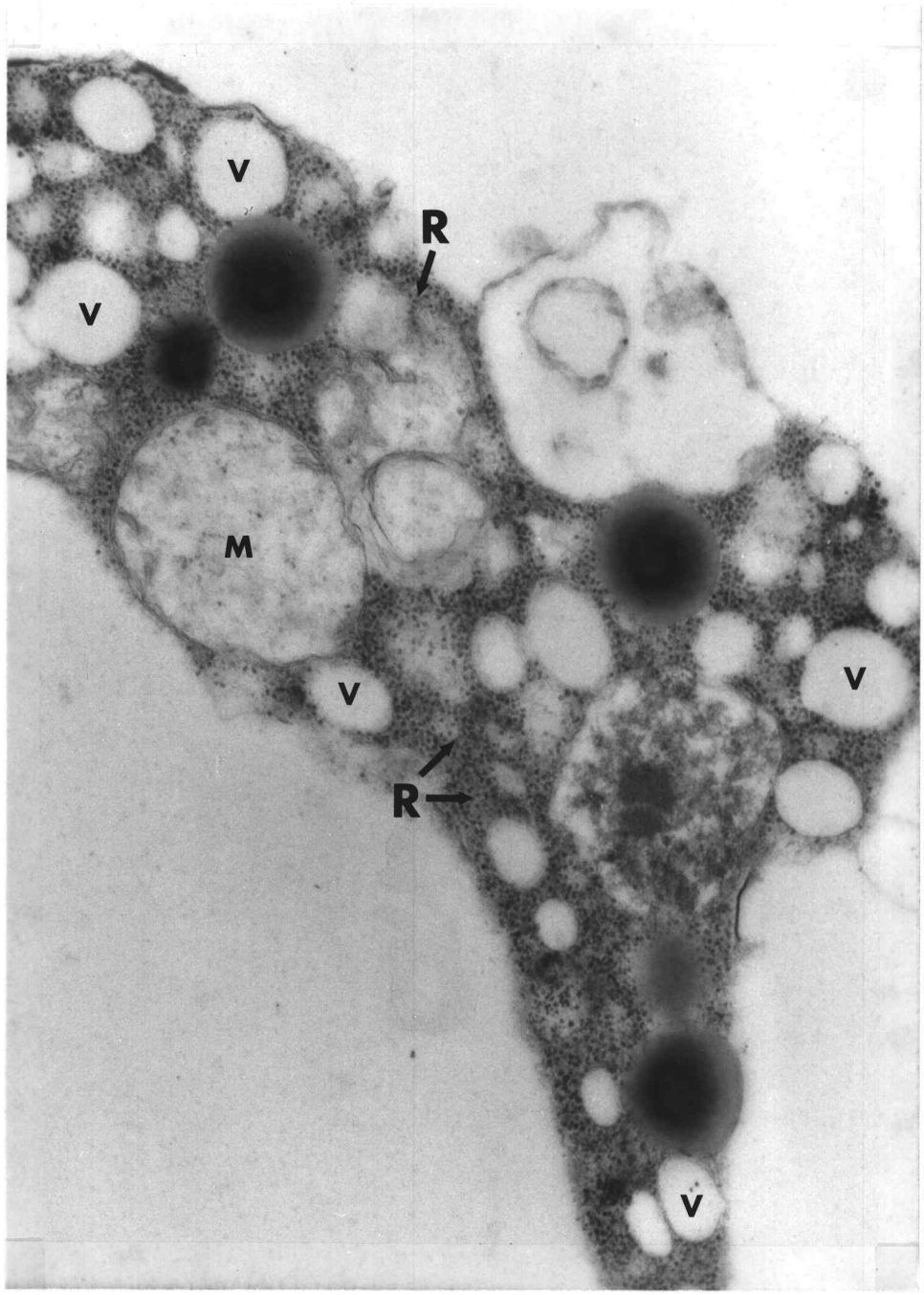

Figure 21. Vesiculated cytoplasm with many, aggregated ribosomes as seen in 'Ace' mesophyll cell. 9 DAE, longitudinal section, X 35,100. R = ribosomes; V = vesicle; M = mitochondria.

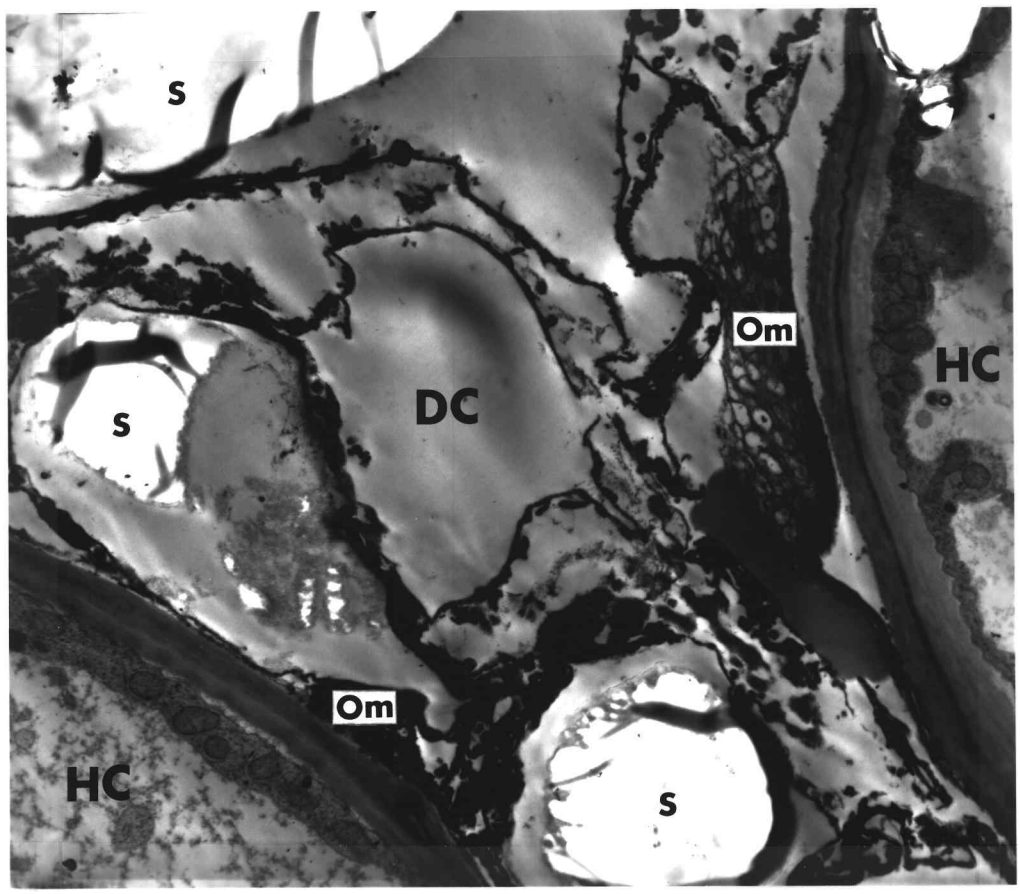

Figure 22. Advanced degenerate cell, covered with dark osmiophilic material, present in 'Nellie White' section. Subepidermal cells, 26 DAE, transverse section, X 5,120. HC = healthy cell; DC = degenerate cell; Om = osmiophilic material; S = starch grain.

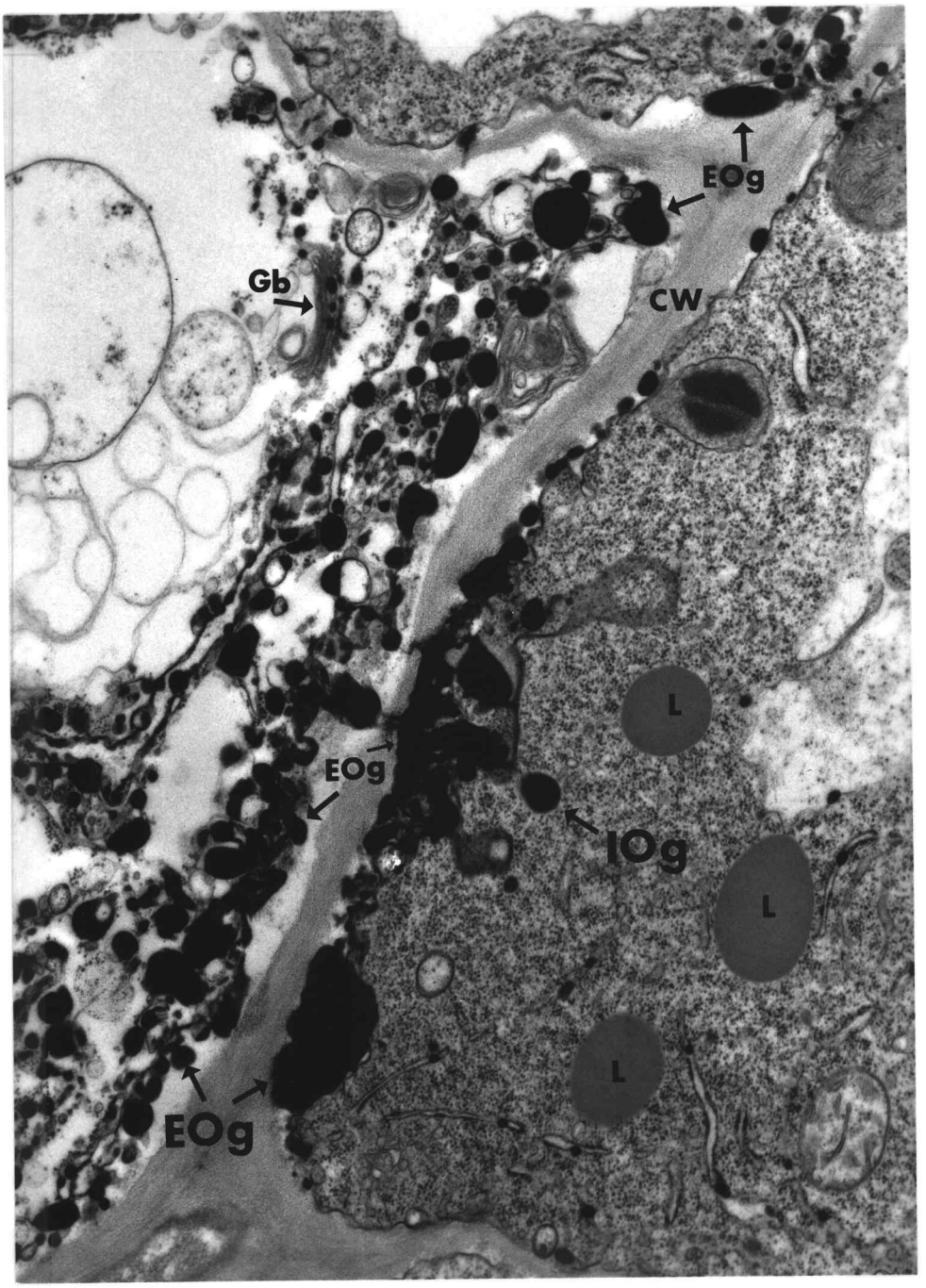

Figure 23. Osmiophilic globules present in 'Enchantment' cells. Bulblet cells, 26 DAE, transverse section, X 21,870. IOg = intraprotoplasmic osmiophilic globule; EOg = extraprotoplasmic osmiophilic globule; CW = cell wall; L = lipid; Gb = Golgi body.

Figure 24. Osmiophilic globules present within plasmodesmata in 'Enchantment' and 'Stargazer' sections. (A) Osmiophilic globules with plasmodesmata; and (B) osmiophilic globules possibly passing through plasmodesmata.

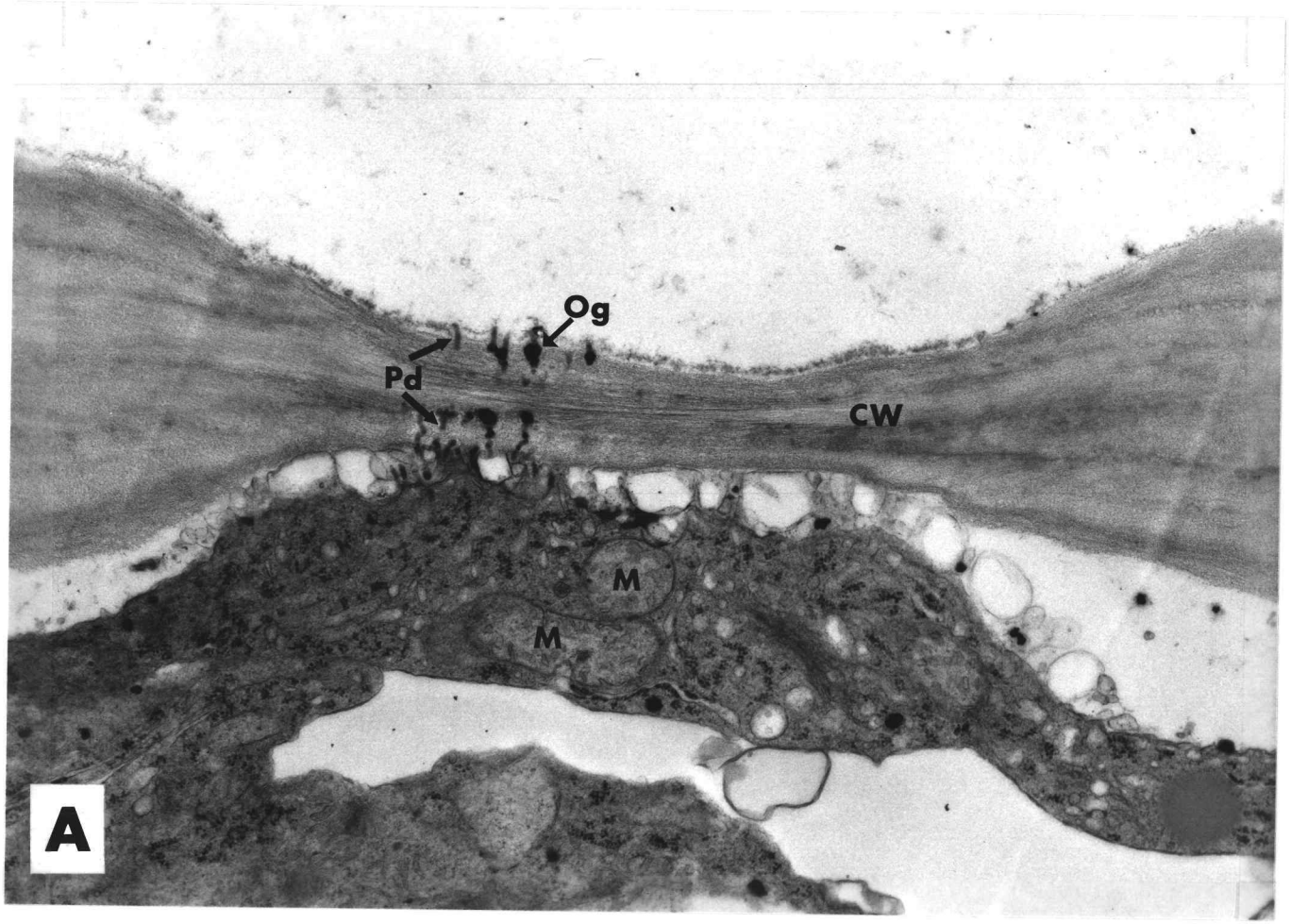

Figure 24(A). Osmiophilic globules within plasmodesmata. 'Enchantment,' mesophyll cells, 26 DAE, transverse section, X 21,870. Og = osmiophilic globule; Pd = plasmodesmata; CW = cell wall; M = mitochondria.

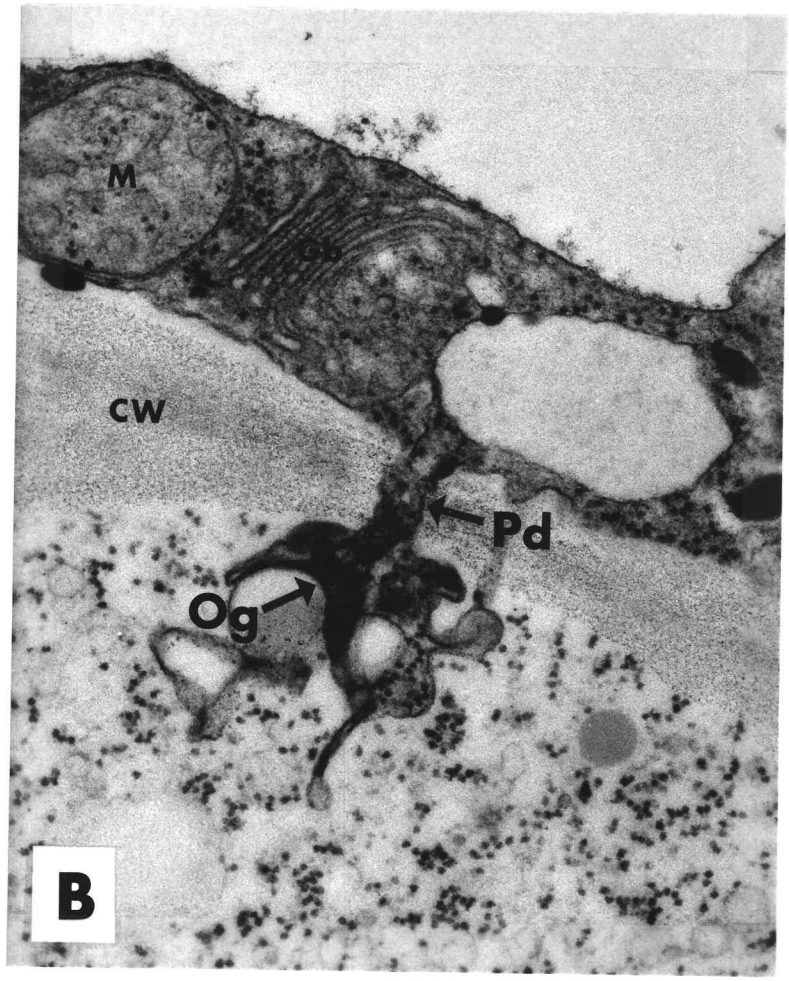

Figure 24(B). Osmiophilic globules possibly passing through plasmodesmata. 'Stargazer,' bulblet cells, 26 DAE, transverse section, X 54,000. Og = osmiophilic globule; Pd = plasmodesmata; CW = cell wall; M = mitochondria; Gb = Golgi body.

TABLE 1 Absorbance for cucumber mosaic virus, tulip breaking virus and lily symptomless virus on lily leaf tissue

Lily	CMV[1]	TBV[2]	LSV[3]
Healthy Control	0.000*	0.029	0.000
Infected Control	1.546	0.685	1.627
'Stargazer'	0.046	0.016	2.153
'Ace'	0.013	0.941	2.036
'Nellie White'	0.037	0.926	1.986
Madonna	0.078	2.448	0.051
'Enchantment'	0.002	0.001	2.246

[1] Plate CMV1 (IgG 1:1000, conjugate 1:800, sample 1:10; read 60 min. after substrate added)

[2] Plate TBV2 (IgG 1:400, conjugate 1:400, sample 1:10; read 93 min. after substrate added)

[3] Plate LSV5 (IgG 1:800, conjugate 1:400, sample 1:10; read 11 min. after substrate added)

* Figures in absorbance units.

TABLE 2 Number of cell sections per lily type containing virus particles (VP) by DAE and tissue area

Lily	9 DAE		26 DAE			40 DAE			TOTAL
	A*	B	A	B	C	A	B	C	
'Stargazer'	0+/12	0/12	0/10	0/11	0/18	0/10	0/12	0/20	0/105
'Ace'	**4**/12	**3**/13	0/13	0/21	0/10	**2**/27	**4**/16	0/27	**13**/139
'Nellie White'	0/17	0/10	**11**/19	0/10	0/16	0/17	**6**/15	0/16	**17**/120
Madonna	0/16	0/17	0/16	0/10	0/20	0/11	0/21	0/16	0/127
'Enchantment'	0/19	0/22	0/16	0/9	0/17	0/19	0/15	0/25	0/142
TOTAL	**4**/76	**3**/74	**11**/74	0/61	0/81	**2**/84	**10**/79	0/104	**30**/633

* A - upper epidermis and first and second sub-epidermal layer cells; B - random mesophyll cells; C - random bulblet cells.

+ Number of cell sections containing VP/number of cell sections viewed.

TABLE 3 Number of cell sections per lily type containing virus inclusions (VI) by DAE and tissue area

Lily	9 DAE		26 DAE			40 DAE			TOTAL
	A*	B	A	B	C	A	B	C	
'Stargazer'	0+/12	0/12	0/10	0/11	0/18	0/10	0/12	0/20	0/105
'Ace'	**1/12**	**5/13**	0/13	0/21	0/10	0/27	0/16	0/27	**6/139**
'Nellie White'	**2/17**	0/10	0/19	0/10	0/16	0/17	0/15	0/16	**2/120**
Madonna	0/16	0/17	0/16	0/10	0/20	0/11	0/21	0/16	0/127
'Enchantment'	0/19	0/22	0/16	0/9	0/17	0/19	0/15	0/25	0/142
TOTAL	**3/76**	**5/74**	0/74	0/61	0/81	0/84	0/79	0/104	**8/633**

* A - upper epidermis and first and second sub-epidermal layer cells; B - random mesophyll cells; C - random bulblet cells.
+ Number of cell sections containing VI/number of cell sections viewed.

TABLE 4 Total number of lily cell sections containing virus particles and virus inclusions by DAE and tissue area

	9 DAE		26 DAE			40 DAE			
	A*	B	A	B	C	A	B	C	TOTAL
Particles	**4**	**3**	**11**	0	0	**2**	**10**	0	**30**
Inclusions	**3**	**5**	0	0	0	0	0	0	**8**
Total No. of Cells	76	74	74	61	81	84	79	104	633

* A - upper epidermis and first and second sub-epidermal layer cells; B - random mesophyll cells; C - random bulblet cells.

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