STUDIES ON AN EASTERN ONTARIO ISOLATE OF WHITE CLOVER MOSAIC VIRUS

by

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Short title

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ABSTRACT

Ph.D. A. HAMEED K. KHADHAIR Plant Science (Plant Pathology)

STUDIES ON AN EASTERN ONTARIO ISOLATE OF WHITE CLOVER MOSAIC VIRUS

Characterization of the most frequently isolated virus found during a survey of eastern Ontario red clover fields, which was designated as the Ottawa isolate, was undertaken. Determination of the biological and physico-chemical properties of the virus showed that the virus studied differed in some respects from other WCMV isolates. An ultrastructural examination of virus localization and cytopathological changes in infected red clover and pea plants revealed various types of viral inclusions, including a membrane-bound mass of tubules, not usually associated with infection by potexviruses, in red clover leaves, and severe organelle disorganization within pea leaf tissues. WCMV infection significantly reduced several processes relevant to symbiotic nitrogen fixation, including plant growth, nodulation, nitrogenase activity, leghemoglobin content, and Rhizobium population, but nitrate reductase and acid phosphatase specific activities were increased. The correlation between leghemoglobin content and nitrogenase activity shown at ten-leaf and pre-blooming stages was absent during the flowering and senescence stages. Infectivity assays, immunosorbent electron microscopy, and an ultrastructural examination showed the presence of the virus in nodular tissues; the cytopathological changes seen in bacteroids suggested that virus infection accelerated nodule senescence. Application of a cytochemical technique using diaminobenzidine showed that the leghemoglobin was located in the peribacteroidal space.

RESUME

Ph.D. A. HAMEED K. KHADHAIR Phytotechnie (Phytopathologie)

ETUDES SUR UNE SOUCHE DE VIRUS DE L'EST DE L'ONTARIO CAUSANT LA MOSAIQUE DU TREFLE BLANC

La caractérisation du virus, dénommé souche Ottawa, le plus fréquemment isolé des champs de trèfle rouge dans l'est de l'Ontario est amorcée. Ce virus se distingue des autres souches du virus de la mosaïque du trèfle blanc (WCMV). Une étude ultrastructurelle de la localisation du virus et des altérations cytologiques chez le trèfle rouge et le pois infectés révèle la présence de divers types d'inclusions virales dans les feuilles du trèfle, particulièrement celle d'une masse de tubules renfermée dans une membrane, phénomène inusité pour les infections par les potexvirus (virus X de la pomme de terre), et d'une sévère désorganisation des organelles cellulaires des feuilles du pois. L'infection par WCMV diminue considérablement plusieurs processus reliés à la fixation symbiotique de l'azote, tels que la croissance de la plante, la production de nodosités, l'activité relative de la nitrogénase, la teneur en leghémoglobine et la population de Rhizobium, cependant l'activité relative de la nitrate-reductase et de la phosphatase-acide est augmentée. La corrélation entre la teneur en leghémoglobine et l'activité relative de la nitrogénase décelée au stade dix-feuilles et pré-floraison, est absente au stade floraison et sénescence des fleurs. Les tests du pouvoir infectieux, les examens ultrastructurels et immunoélectromicroscopiques révèlent la présence du virus dans les tissus des nodosités. Les changements cytopathologiques dans les bactéroides indiquent que l'infection virale précipite le vieillissement des nodosités. Un test cytochimique utilisant le diaminobenzidine montre que la leghémoglobine se trouve dans la région péribactéroïdale.

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AMV	:	Alfalfa mosaic virus
BPMV	:	Bean pod mottle virus
BYMV	:	Bean yellow mosaic virus
CYMV	:	Clover yellow mosaic virus
DEMV	:	Dolichos enation mosaic virus
PMV	:	Peanut mottle virus
PSV	:	Pea streak virus
PVX	:	Potato virus X
RCVMV	:	Red clover vein mosaic virus
SBMV	:	Southern bean mosaic virus
SMV	:	Soybean mosaic virus
TMV	:	Tobacco mosaic virus
TRSV	:	Tobacco ringspot virus
WCMV	:	White clover mosaic virus

GENERAL ABBREVIATIONS

C:N	:	Carbon-nitrogen ratio
DAB	:	Diaminobenzidine
EDTA	:	Ethylene-diamine-tetraacetic acid
ISEM	:	Immunosorbent electron microscopy
LHG	:	Leghemoglobin
O.D.	:	Optical density
PBS	:	Phosphate-buffered saline
PEG	:	Polyethylene glycol-6000
RNA	:	Ribonucleic acid
SDS	:	Sodium dodecyl sulfate
UV	:	Ultraviolet

INTRODUCTION

Today's world population explosion has generated a massive hunger problem. Fertile soil ranks first among any nation's resources. Nitrogen is an essential element of all living organisms, but in many areas inorganic N fertilizers are unavailable. Legumes are valuable nitrogen sources for livestock, soil conservation and soil improvement. The association of rhizobia with leguminous plants is unique, and the symbiotic nitrogen fixation mechanism which results from this association is a beneficial as well as an inexpensive phenomenon. Thus, any dereliction in making this mechanism available in leguminous plant culture is not in keeping with efficient use of available resources.

Red clover (Trifolium pratense L.) has a long and significant history among farm crops, and was considered the cornerstone in the cropping systems. It has a wide range of adaptability in Canada. Crop losses due to virus infections were reported as early as 1917 in the United States (U.S.A.), and the destructive nature of virus induced legume diseases has become increasingly apparent since that time. Several viruses have been reported to infect red clover, particularly alfalfa mosaic virus (AMV), bean yellow mosaic virus (BYMV), clover yellow mosaic virus (CYMV), pea streak virus (PSV),

peanut stunt virus (PeSV), red clover vein mosaic virus (RCVMV), and white clover mosaic virus (WCMV).

During the summer of 1979, red clover fields in the Ottawa area were surveyed and 28 plants showing virus-like symptoms were collected. Extracts from 20 of these were found to contain filamentous particles when examined in the electron microscope, and produced local lesions when used to inoculate Gomphrena globosa L. The studied virus isolate originated from a single lesion on G. globosa, which was propagated in red clover and other host plants.

The mechanisms controlling nodule retardation, nodule loss and nodule regeneration after harvest are not understood for any forage legume except alfalfa. Since perennial forage legumes are frequently subjected to virus infections and removal by animal or by mechanical harvesting, and since the rate of symbiotic nitrogen fixation is of great interest, it is important to understand the effect of harvest time as well as the virus infection on nitrogen fixation and other characteristics in red clover nodules.

The present study was conducted to achieve the following objectives:

1. Identification and characterization of the selected virus isolate and comparison with other reported isolates.
2. Survey of the incidence of clover viruses in six fields of red clover in eastern Ontario.
3. To study the cytopathological changes and the distribution of the described virus isolate in various tissues of two legumes, red clover and pea.

4. Determination of the effects of this virus isolate on the patterns of nitrogenase activity and related aspects in the nodules of red clover plants.
5. Investigation of the behavior and localization of the isolated virus in the nodular tissue.

SECTION 1
LITERATURE REVIEW

1.1 General incidence and significance of legume viruses

The diverse plant family, Leguminosae, includes at least 35 species cultivated for protein-rich food, feed and forage, and as green manure crops. Early reports showed that viruses could cause severe losses to legume plants (Stewart and Reddick 1917), and many viruses have been found in various forage crop plants in different areas. Some of the most commonly encountered viruses and the principal crops in which surveys have indicated their presence, are listed below.

Virus	Crops	Area	Reference
pea common mosaic virus (PCMV)	red clover, pea	Idaho, USA	Pierce 1935
bean yellow mosaic virus (BYMV)	sweet clover	Idaho, USA	Pierce 1935
red clover vein mosaic virus (RCVMV), PCMV and BYMV	red clover	Wisconsin, USA	Hanson and Hagedorn 1952
RCVMV	red, alsike and sweet clover	Wisconsin, USA	Graves and Hagedorn 1956
RCVMV, PCMV, BYMV, pea streak virus (PSV), alfalfa mosaic virus (AMV), alsike clover mosaic virus (ACMV)	red clover	Wisconsin, USA	Hanson and Hagedorn 1961

Virus	Crop	Area	Reference
AMV, BYMV and white clover mosaic virus (WCMV)	alfalfa	Rhode Island, USA	Muller 1965
AMV, BYMV, PCMV, RCVMV, WCMV and clover yellow mosaic virus (CYMV)	red clover	Ontario, Quebec, Canada	Pratt 1968
AMV, PSV and clover yellow vein virus (CYVV)	ladino clover	North Carolina, USA	Lucas and Harper 1972
AMV, BYMV, PSV, CVMV and WCMV	ladino clover	Ontario, Canada	Gates and Bronskill 1974

It has been reported that older stands of forage legumes often have a high percentage of virus-infected plants which may be symptomless (Ostazeski et al. 1970), and provide a reservoir from which other plants can be infected. During a survey on 3-year-old plots of 12 alfalfa varieties in central Washington, many were found to contain viruses which were infectious to beans, and the range of symptoms produced in beans and broad bean indicated the presence of 15 to 20 virus strains or combinations in alfalfa plants assayed (Burke 1963).

Although legume viruses are commonly transmitted by insects in the field, seed transmission is fairly frequent in some legume plants. Hampton (1962) reported that three latent viruses were transmitted through bean seeds. In a subsequent report (Hampton 1963), CYMV and WCMV were found transmissible in red clover seeds. Later, Hampton (1967) confirmed the presence of the above-mentioned viruses (i.e., CYMV and WCMV) as well as two other viruses in red clover seeds. They were described as an isometric virus, serologically related to tomato

ring spot virus (TmRSV), and a filamentous one with 760 nm length. Hampton and Hanson (1968) demonstrated seed transmission of unnamed viruses in approximately 1800 red clover seedlings from six lots of seeds assayed on broad bean. Frosheiser (1970) used Bountiful bean as an assay host to detect AMV in uninoculated alfalfa seedlings.

Various effects can result from virus infection in forage legumes. Kreitlow et al. (1957) reported that AMV and BYMV, in single and mixed infection, affected the forage yield and chemical composition of ladino white clover. Roberts (1956) showed that ladino clover plants infected with AMV were more susceptible to cold damage than virus-free plants. In field experiments, Goth and Wilcoxson (1962) found that infection by BYMV reduced stands of red clover as much as 100% under seasonal temperature variation in Minnesota. Significant reduction in the yield of alfalfa hay has resulted from infection by AMV (Crill et al. 1970). In birdsfoot trefoil, it was reported that tobacco ring spot virus (TRSV) affected the yield, persistence, flowering and seed set of the infected plants (Ostazeski et al. 1970).

The majority of forage legumes are cross-pollinated plants, and red clover, which has the most adaptable genotype, is a typical example of this group (Fergus and Hollowell 1960). Since red clover was found to be susceptible to different plant viruses, as described earlier, breeding resistant varieties seems to be the most promising approach toward controlling virus diseases in this crop. Several cultivars of this plant species have been developed. Some of these cultivars were used to control northern and southern anthracnose, as

well as powdery mildew, but only the cultivar Kenstar was found resistant to BYMV (Taylor 1973).

1.2 Distribution of clover viruses

As indicated earlier, red clover can be infected by several viruses, and the following are considered important: alfalfa mosaic virus (AMV), bean yellow mosaic virus (BYMV), clover yellow mosaic virus (CYMV), pea streak virus (PSV), red clover vein mosaic virus (RCVMV), and white clover mosaic virus (WCMV) (Diachun and Henson 1956; Graves 1953; Hagedorn and Hanson 1963; Stuteville and Hanson 1965). It is apparent from this listing that a given virus, e.g., AMV, can be important in more than one crop, and this has led to some confusion in the nomenclature of some of these viruses, such as WCMV.

Early reports by Zaumeyer and Wade (1935, 1936) suggested that the mosaic of white clover (Trifolium repens L.) might be caused by two distinctive viruses. In 1942, Johnson reported that white clover mosaic virus was caused by a complex of two viruses. One, which he named pea mottle virus, infected plants in the Leguminosae, Caryophyllaceae, Cucurbitaceae, and Scrophulariaceae, while the other, which he called pea wilt virus, only infected plants in the Leguminosae.

Matsulevich (1956), who reported a mosaic disease of red clover as a widespread disease in the Ukraine, concluded that pea mottle and pea wilt viruses were present in his samples. Leaf symptoms in clover were distortion, chlorotic flecks, and necrotic stripes. His

conclusion that pea mottle and pea wilt viruses were present was based on the transmissibility of one of the viruses to pea and bean. Later, the presence of these two distinctive viruses was confirmed in two different localities in the United States (Agrawal et al. 1962). Thus, Pratt (1961) suggested the names white clover mosaic virus and clover yellow mosaic virus for pea wilt and pea mottle, respectively. Some isolates of this virus complex have been reported on the basis of host reactions and physical properties, and resemble one, or even both of Johnson's viruses. A German isolate (Brandes and Quantz 1957; Quantz 1956) appeared to be related to pea wilt virus. Another isolate in the Netherlands resembled pea mottle virus by systemically infecting cucumber and in being transmissible by dodder, but resembled pea wilt virus by infecting cowpea (Bos et al. 1959). A California isolate which infected cowpea and snapdragon, but not cucumber, was concluded to be similar to Johnson's pea mottle-pea wilt complex (Scott and Gold 1959). An Indiana isolate resembled pea wilt virus in some of its host reactions, but multiplied in several non-leguminous hosts, including cucumber (Bancroft et al. 1960).

1.3 White clover mosaic virus

White clover mosaic virus (Cryptogram: R/1:2.4/(b):E/E:S/(Ap)) was first isolated from white clover in the U.S.A. by Pierce (1935). Exchange of antisera has shown that WCMV is present in several countries (Bos et al. 1960). This virus was found to cause mosaic and mottle of different degrees of severity in various species of

clover, and vein-clearing, light green mottle with chlorosis, in pea (Bercks 1971). It is considered one of the flexuous viruses of the potex-virus group, with a probable length of about 480 nm and diameter 14 nm (Bancroft et al. 1960; Berks 1971; Blaszezak et al. 1977; Blaszezak and Micinski 1977; Fry et al. 1960; Varma et al. 1970).

Although the virus is easily transmitted mechanically, a report indicated the transmission of a strain of WCMV by dodder (Bos et al. 1959). Scott and Gold (1959) reported no dodder transmission with another isolate (AC-181, ATCC ed.2) in the same serogroup. Unsuccessful seed transmission with Medicago luplina L. could be demonstrated (Bancroft et al. 1960), but it was recorded as 6% in Trifolium pratense L. seeds (Hampton 1963). It was reported that a low percentage of transmission of one strain of WCMV was effected by Acrythosiphon pisum to a clonal line of ladino clover (Goth 1962). Other authors had failed to transmit the virus using various insects, including A. pisum, Aphis gossypii, Myzus persica, Anuraphis bakeri (Bancroft et al. 1960; Pratt 1961; Scott and Gold 1959).

Two procedures have been reported for purification of WCMV. The first method included grinding infected bean leaves with 0.2% ascorbic acid and 0.2% sodium sulphite, and the extracted sap was mixed with an equal volume of ether. After low speed centrifugation, the aqueous phase was added to an equal volume of carbon tetrachloride. Three cycles of low speed centrifugation were followed by two cycles of differential centrifugation. The final high speed pellets were suspended in 0.1 M phosphate buffer (Wetter 1960).

The other method used to purify WCMV included grinding the frozen infected pea plants after thawing, and blending the expressed sap with an equal volume of 0.1 M K_2HPO_4. Three cycles of low and high speed centrifugation were used, and the high speed pellets were resuspended in distilled water. Further purification was done by precipitating the virus with a half volume of saturated ammonium sulphate or by adding drops of 1 N acetic acid to obtain pH 4.5 (Fry et al. 1960).

The widely used PEG «precipitation» method has not been used for purification of WCMV (Wetter 1960; Fry et al. 1960), but Veerisetty and Brakke (1978) have shown its applicability to other legume viruses. The procedure involved precipitation of the virus from the sap of infected peas after clarification with calcium phosphate by addition of 6% polyethylene glycol (PEG-MW 6000) and concentration by two cycles of differential centrifugation. The virus suspension was layered on a pad of 20% and 30% sucrose, respectively, and the final pellets were suspended in 0.0165 M disodium phosphate, 0.0018 M trisodium citrate, pH 9.0 (Veerisetty and Brakke 1978).

The purified virus suspension has UV absorbance 260/280 of 1.0 to 1.8 (Fry et al. 1960; Bancroft et al. 1960), with an extinction coefficient at 260 nm of 3.6 (Fry et al. 1960) for a 1 mg/ml suspension. The virus sediments as a single component in the analytical ultracentrifuge, and has a sedimentation coefficient ($S_{20,w}$) of 112 S to 119 S (Fry et al. 1960; Pratt 1961; Blaszezak et al. 1977; Varma et al. 1971). The isoelectric point of the virus is about pH 4.5 to pH 5.0 (Fry et al. 1960; Bancroft et al. 1960) and it has an electrophoretic mobility

of -5 to -7 x 10^{-5} cm^2 Sec^{-1} $Volt^{-1}$ in phosphate buffer pH 7.0, ionic strength 0.1 (Pratt 1961; Fry et al. 1960).

WCMV particles are flexuous helically constructed filaments containing about 7.2% RNA (Fry et al. 1960; Varma et al. 1970; Bancroft et al. 1960; Miki and Knight 1967). The molar percentage of the nucleotides is: G15.5, A31.8, C26.9, U25.7 (Miki and Knight 1967).

The molecular weight of WCMV coat protein was reported as 1.4 x 10^4 by Miki and Knight (1967) using chemical analysis. However, by using SDS-polyacrylamide gel electrophoresis, values were determined as 2.02 x 10^4 (Koenig et al. 1970), 2.25 x 10^4 (Lesnaw and Reichman1970), and 2.35 x 10^4 (Tung and Knight 1972). The amino acid composition of the protein (mole percent) is: alanine 14.4, arginine 4.0, aspartic acid 9.3, cystine 1.3, glutamic acid 6.9, glycine 5.5, histidine 1.6, isoleucine 6.1, leucine 7.5, lysine 5.8, methionine 1.5, phenylalanine 4.2, proline 6.1, serine 7.6, threonine 8.4, tryptophan 1.6, tyrosine 1.9, valine 5.4 (Bercks 1971).

1.4 Microscopic studies of inclusion bodies

Certain structures produced in virus-infected plant cells have been known since 1903, when Iwanowski observed them in tobacco cells infected with TMV (Iwanowski 1903). As the study of plant viruses expanded, it was found that other viruses can induce similar or comparable structures, which were later termed vacuolate bodies, amoeboid bodies, plasmodium-like masses, spherules, or more commonly as X-bodies (Mckinney 1923; Goldstein 1924).

Several studies directed their efforts towards describing and identifying the inclusions and their nature. Sheffield (1931) followed the in vivo development of X-bodies of the aucuba strain of TMV by observing living infected cells under the light microscope. Considerable advances were made in the development of suitable fixation techniques (Bald 1949) and staining procedures which enabled better detection of inclusion bodies, and differentiation from ordinary cell constituents (McWhorter 1941; Rich 1948; Bald 1949). The extensive work by Christie and Edwardson (1977) has become the focus for rapid diagnosis of the inclusion bodies of different plant virus groups, by light microscopy.

Electron microscopic investigations have greatly expanded knowledge of the various types of inclusion bodies formed within virus-infected cells. In a recent review, Martelli and Russo (1977) classified inclusions by their location and nature as follows:

a - Nuclear : 1 - Nucleolar inclusions
2 - Nucleoplasmic
3 - Perinuclear

b - Cytoplasmic : 1 - Crystalline inclusions:
- Fibrous
- Paracrystalline
- Crystalline
- Laminated
- Small spheres

2 - Proteinaceous

- Amorphous inclusions

- Pinwheels

There are two kinds of nucleolar inclusions. The first is called the satellite body induced by beet mosaic virus (BMV) infecting G. globosa and Chenopodium quinoa (Martelli and Russo 1969). The «sprouting» condition of nucleoli of BMV-infected pea cells can be considered similar to these bodies (Bos 1969a). The second kind of nucleolar inclusions is the crystalline form induced in many hosts by BYMV and related viruses (McWhorter 1941). However, analogous nuclear crystalline inclusions induced by two potyviruses (Edwardson 1974; Kitajima et al. 1968) were found not to differentiate them from crystals of tubular elements with hexagonal packing associated with the nucleolus of tobacco leaf cells infected with caldy strain of AMV (Hull et al. 1970).

Nucleoplasmic inclusions are another type of nuclear inclusion, occurring in the karyoplasm of infected cells, such as those induced by beet curly top virus (Esau 1968), and celery mosaic virus (Christie and Edwardson 1977). Other workers reported that elongated tubular units composed of aggregates of virus particles can be detected in the nucleus. This kind of inclusion was noted only in the cells infected with filamentous viruses, such as beet yellow virus (Esau 1960), beet yellow stunt virus (Hoefert et al. 1970), narcissus mosaic virus (Turner 1971), and a strain of tobacco etch virus (Purcifull and Edwardson 1968).

The last type of nuclear inclusion, the perinuclear inclusion, which can be found between the two lamellae of the nuclear envelope, may appear only in certain plants and at certain stages of the virus infection (Martelli and Russo 1977). Resulting accumulations of virus, which may be large enough to be recognized with light microscope, are caused by the bacilliform virus of sowthistle and Bidens pilosa (Christie et al. 1974), broccoli necrotic virus (Tomlinson et al. 1972) and beet western yellow virus (Esau and Hoefert 1972).

Cytoplasmic inclusions, the second category of inclusion bodies, have been associated with infection by several flexuous viruses. They differ in size, shape, location, composition and ultrastructural arrangement. According to their appearance, they can be divided into crystalline and proteinous types. McWhorter (1965) used the term X-bodies for structures of unknown function, and he recommended use of terms like amorphous and crystalline for inclusions associated with virus formation. The cytoplasmic inclusion (crystalline and proteinous) may be produced by one virus and may occur in parallel bands in the same cell, and different strains of the same virus may produce either crystalline or proteinous inclusions or both (Martelli and Russo 1977).

Aggregated virus particles, in the form of banded bodies occurring mainly in the epidermal and mesophyll cells, have become one of the diagnostic characteristics of infection by the carlavirus group (Edwardson and Christie 1978). However, several reports have indicated that cytoplasmic paracrystalline inclusions consisting of virus particles developed singly and in groups, as in potato virus M (PVM).

PVS, and pea streak infected cells (Christie and Edwardson 1977). Other workers noticed banded bodies occurring in the plant tissues infected with passiflora latent virus (Bos and Rubio-Huertos 1971), PVM (Tu and Hiruki 1970), and PVS (Hiruki and Shukla 1973).

Closterovirus particles aggregated into banded bodies have been observed in cells infected with beet yellow (Esau 1966), beet yellow stunt (Hoefert et al. 1970), carnation necrotic fleck (Inouye and Mitsuhata 1973), carnation yellow fleck (Bar-Joseph et al. 1977), and citrus tristeza (Christie and Edwardson 1977) viruses. Warmke and Edwardson (1966) suggested that paracrystalline bodies formed by members of the tobamovirus group are a secondary aggregation product of the breakdown of pre-existing crystalline structures in TMV-infected plants. This kind of virus aggregate also is of common occurrence in plant cells infected with rhabdoviruses (Edwardson and Christie 1978).

The hexagonally packed monolayers of short bacilliform particles are proposed as a remarkable feature for infection by alfalfa mosaic virus group (Martelli and Russo 1977). However, paracrystalline aggregation forms of isometric viruses also can be recognized in particle-containing tubes, like those present in cells infected by reovirus-like plant viruses, comoviruses, nepoviruses and ilarviruses (Gerola et al. 1969). Cytoplasmic crystalline aggregates, composed of arrays of virus particles, were seen in plant tissues infected with brome-grass mosaic virus (Paliwal 1970), bean pod mottle virus (Kim and Fulton 1973), peanut stunt virus (Kraev et al. 1975), barley yellow dwarf virus (Jensen 1969), arabis mosaic virus (Martelli and Russo 1977), and maize rough dwarf virus (Milne and Lovisolo 1977).

The other major type of cytoplasmic inclusion is the proteinous body which may differ in shape and its cellular location. The final appearance might be attributed to the interaction between the host and the pathogen (Martelli and Russo 1977). Two types of proteinceous inclusions were reported by several workers: the amorphous granular bodies, which are composed of a protein as a major component, associated with viruses such as watermelon mosaic virus (Martelli and Russo 1977), and those induced by papaya ringspot virus (Edwardson 1974). The second type is represented by the pinwheel inclusions, which are induced mainly by the potyvirus group and are regarded as a diagnostic feature for infection by them (Edwardson 1966). The first electron microscopic record of pinwheels was observed in PVY-infected cells (David-Ferria and Borges 1958). The serological behaviour of cylindrical inclusions induced by TEV and PVY showed that they were immunologically unrelated (Hiebert et al. 1971; Shepard and Shalla 1969).

Among viruses with anisometric particles that are not included in any of the above-mentioned groups, soil-borne wheat mosaic virus (SBWMV) induced inclusions in the infected cells. These were first described by McKinney et al. (1923), and further information on their ultrastructure has been reported by other workers (Peterson 1970; Tsuchizaki et al. 1973).

1.5 The inclusion bodies induced by the potexvirus group

It was reported that the potexvirus group consists of 14 viruses, and 10 of them have been examined cytologically (Edwardson and Christie 1978). The studied viruses of this group were found to induce inclusions that were distributed in the host tissues. Banded inclusions of aggregated virus particles have been observed in epidermal, mesophyll or apical meristem cells of tissues infected with the following viruses: CYMV (Bercks and Brandes 1963; Smith and Schlegel 1964; Purcifull et al. 1966; Schlegal and Delisle 1971; Hiruki et al. 1976; Rao et al. 1978; Tu 1979), papaya mosaic (PaMV, Zettler et al. 1968), cymbidium mosaic (CyMV, Christie and Edwardson 1977; Doraiswamy and Lesemann 1974), cactus virus X (CVX, Doraiswamy and Lesemann 1974), narcissus mosaic (NMV, Turner 1971), potato virus X (PVX, Kikumoto and Matsui 1961; Hooker 1964; Kozar and Sheludko 1969; Stols et al. 1970; Shalla and Shephard 1972; Shalla and Petersen 1973; Pennazio and Appiano 1975), cassava common mosaic (CCMV, Kitajima and Costa 1966), potato aucuba mosaic (PAMV, Kassanis and Govier 1972), hydrangea ringspot (HRV, Zeyen and Stienstra 1973) and WCMV (Iizuka and Yunoke 1975). Only banded inclusions of virus particles were reported in the mesophyll cells of WCMV-infected white clover leaves. There was no further indication on the presence or the kind of host inclusions induced by WCMV infection.

Various isolates of PVX, the type member of this group, were found to induce structures known as laminate inclusion components that are apparently unique to this virus (Doraiswamy and Lesemann 1974; Shalla and Shepard 1972). Spindle-shaped inclusions, thought to consist of virus aggregates, have been found in the nuclei of tissues infected by NMV (Turner 1971) and CVX (Milicic 1954). Meanwhile, it has been found that cells infected with PaMV contained fibrous and thin crystalline proteinaceous inclusions closely associated with nuclei (Christie and Edwardson 1977).

1.6 Clover nodulation and nodule contents

Nodules are not found on all of the genera and species of Leguminosae. Most of the plant legumes producing nodules are members of the subfamily Papilionoideae, such as red clover (Trifolium pratense L.). Nodulation occurs as a result of the infection of the clover roots by Rhizobium trifolii (Alexander 1977). This Rhizobium consists of motile gram-negative rods, approximately 2 μm x 1 μm which characteristically stain unevenly with usual basic dyes, and possess more sudanphilic granules than most gram-negative genera (Burdon 1946; Vincent et al. 1962).

Establishment of the symbiotic relationship involves host specificity in initiation of rhizobial infections of root hairs (Li and Hubell 1969). The relationship between Rhizobium trifolii and red clover has been described in several reports. Some

authors suggested that carbohydrate-binding proteins (lectins) might be involved in the process of specific recognition of rhizobia by plants for compatible reproductive and vegetative structures (Crandall and Broke 1968). The specificity in the Rhizobium-clover symbiosis includes the attachment of infective rhizobia to the cell wall of clover root hairs through a 2-deoxy-D-glucose-sensitive receptor site (Dazzo et al. 1976). The exposed surface of clover root walls and the capsular polysaccharide of R. trifolii were found to contain immunochemically unique cross-reactive antigenic determinants called agglutinins. (Dazzo and Hubell 1975). In a subsequent report, Dazzo et al. (1978) indicated that the agglutinins (trifoliin) were anchored on the root hair surface to achieve the recognition of R. trifolii by the clover root.

The nodulation process, after attachment of the rhizobia to the root hair, begins when an infective strain of R. trifolii enters the host through the root hairs (Fahraeus 1957). The bacteria are then enclosed in a tubular structure, the infection thread, and the formation of the latter is considered as a sign of successful infection (Li and Hubell, 1969). In another report, it was noted that the infection thread is initiated by an invagination process when the root hair walls are redirected at a localized point, resulting in the formation of open pores which continue to develop into tube-like structures (Napoli et al. 1975).

Several levels of ultrastructure studies have been carried out mainly on the nodules of subterranean clover (T. subterraneum L.) and white clover. Bergersen (1955) used the light microscope to study

the nodule development and the transformation of the bacterial cell from a rod-like shape to the enlarged spherical bacteroid in subterranean clover. Dart and Mercer (1963) followed the bacteroidal development in the sections of subterranean nodules and noticed an intracytoplasmic membrane system in the bacteroids from the red zone of the nodular tissues. In a subsequent report (Mosse 1963), it was suggested that four zones can be differentiated in nitrogen-fixing nodules of T. parviflorum. The meristematic zone contained dividing cells and infection threads, but the second zone contained numerous rod-shaped bacteria and proliferating infection threads. The bacteroids were located in a third pink-coloured zone and the fourth zone contained disintegrated cells. Tu (1974a, 1975) studied the relationship between the bacteroid envelope and the host plasma membrane in alsike clover nodules. He pointed out the similarity of the membranes and the presence of electron-dense deposits on the host-plasma membrane surface and on the internal side of the bacteroid envelope.

The emergence of the bacteria from the infection thread was found to occur at different times and in different ways in clover nodules (Mosse 1964; Dixon 1967). In other leguminous nodules, the release mechanism is thought to involve pectolytic and cellulytic enzymes (Dart 1977; Vance 1978). After bacteria multiply and fill the cells, nodules synthesize a red protein which is called leghemoglobin. This hemoprotein has an important role in regulating oxygen tension in the nodules (Bergersen 1980), and can be detected with

diaminobenzidine (Gourret and Fernandez-Arias 1974) or by a fluorometric assay (La Rue and Child 1979). The bacteroid is the functional form of the bacterium, and it is believed to be responsible for the nitrogenase activity, which can be measured by gas chromatography (Hardy 1968). The host cell containing the bacteroids has been the subject of differing terminology. It was called bacteroidal cell (Tu 1977), bacteroid-filled cell (Dart 1977), and bacteroid-containing cell (Truchet 1980).

Nitrate reductase is an enzyme which can be detected in various tissues of the higher plants (Beevers and Hageman 1969). The occurrence and some of the properties of this enzyme were described in association with the rhizobia from nodules of soybean (Bergersen 1961b; Rajoud et al. 1973; Chenia and Evans 1960), peas (Chen and Philip 1977), siratro (Macroptilium atropurpureum L.) (Gibson and Pagan 1977), and alfalfa (Vance et al. 1979). Contradictory reports exist concerning the relationship between the nitrate reductase and nitrogenase activities; a positive correlation was observed in soybean (Cheniae and Evans 1960; Rigaud et al. 1973), but in alfalfa the opposite occurred (Vance et al 1979). There are no reports concerning the presence and/or activity of nitrate reductase within clover nodules.

Lysosomes are spherical organelles, formed by Golgi bodies, containing a number of hydrolytic enzymes (Dingle 1973; Wilson 1973). They are about 0.4 μm in diameter, and have a single unit membrane surrounding dense granular stroma which sometimes contains a large vacuole (Clowes and Juniper 1968). Lysosomes contain large amounts

of acid phosphatase; this property has been used in attempts to identify them in root tips of broad bean (Gahan and Maple 1966) and in the leaves and nodules of white clover (Tu 1976). This enzyme was also found associated with the membranes that formed provacuoles (Berjak 1972). The lysosomal hydrolytic enzymes are responsible for the cellular digestion during catabolism, autophagic activities in senescent tissues, and mobilization of reserve nutrients in the cell (Wilson 1973). A higher activity of acid phosphatase was detected in the leaves and nodules of white clover infected with CYMV than in the healthy control (Tu 1976).

1.7 Effects of virus infection on nodulation and N_2-fixation

In general, relatively little research has been done on the effects of virus infection on nitrogen fixation by legumes. One of the earlier studies was carried out by Johnson and Jones (1943), who reported that a mosaic virus complex (Johnson 1942) of pea could be transmitted by Rhizobium leguminosarum. Later, wound tumor virus (WTV) was found to induce overgrowths in the pericycle beneath the newly-formed nodular tissue in Melilotus alba, which led to the premature disintegration of the nodules (Lee 1955). Although Smith and Gibson (1960) did not study the direct effect of bean yellow mosaic virus (BYMV) on nodulation, they found that this virus reduced both vegetative and root yields of white clover. As a result, BYMV caused a marked change by decreasing the plant growth.

Several reports have been published concerning the effect of what was called clover phyllody virus (CPV) on the nodulation of white clover (Trifolium repens). In the first attempt, it was found that infection with a CPV induced a relatively high number of small ineffective nodules on white clover roots (Vanderveken 1964). Another report confirmed the previous results on several Trifolium species infected with CPV compared with healthy controls under the same conditions (Vanderveken 1966). Similar findings were reported by Joshi and Carr (1967) and Joshi et al. (1967), who noticed a striking change in nodulation of white clover grown in both agar and soil. They concluded that CPV infection had a direct effect on the growth of clover plants and the soil Rhizobium population. What was named CPV in the foregoing reports has been identified, since, as the clover phyllody mycoplasma (Sinha and Paliwal 1969).

Some of the extensive studies of the effects of virus infection on nodulation and nodule function have been reported by Tu and coworkers, who have investigated the effects of various viruses in soybeans. Soybean mosaic virus (SMV) and bean pod mottle virus (BPMV), in single and mixed infections, were found to affect nodulation in soybean. The study reported a remarkable reduction in number, size and fresh weight of nodules as a result of infection by each or both viruses. Soybean varietal differences resulted in greater differences in response than those noted after infection by various isolates of SMV. Varying the time of virus inoculation revealed noticeable reductions in nodulation in earlier infections (Tu et al. 1970a). In

a subsequent report, SMV infections of soybean nodules were studied in relation to nodulation and nodular efficiency, in which the latter was measured by the acetylene reduction test under various growth conditions, i.e., temperature and daylength (Tu et al. 1970b). There was a marked difference in the number and weight of nodules between SMV-infected and healthy soybeans, and both were increased by an increase in temperature (up to 26.5°) or daylength (up to 14 hours per day). The virus infection reduced the leghemoglobin content, but increased the total nodular nitrogen. It was suggested that the increase of nitrogen occurred as a consequence of the inefficient use of nitrogenous compounds. The effect of SMV infection was tested again on another soybean cultivar (Bragg). It was recorded that the average nodulation, number and dry weight of nodules were reduced remarkably in virus-infected plants (Dhingra and Chenulu 1980).

In a later report by Tu and Tse (1976), a remarkable reduction in the number of nodules in soybean plants infected with alfalfa mosaic virus (AMV) was noted. According to published results, the number of nodules in both AMV-infected and healthy soybean plants was increased in response to increased rhizobial populations within the soil. It was suggested that the increase in the latter could prolong the age of the older leaves. Tu and Holmes (1980) recently reported that AMV not only reduced the foliage yield, crude protein content and degree of nodulation in alfalfa, but also the winter hardiness, which depends heavily on food reserves accumulated during the preceding season, for which nodule activity is partly responsible.

The decreased nodulation resulting from AMV infection was suggested as the cause of reduced winterhardiness.

Rajagopalan and Raju (1972) studied the effect of dolichos enation mosaic virus (DEMV) on nodulation and nitrogen fixation in the field bean (Dolichos lablab L.) grown in sand culture. They reported that this virus seemed to contribute a beneficial effect to the plants by increasing the number and fresh weight of nodules as well as the nitrogen content in DEMV-infected nodules. Under soil culture, on the contrary, the number of nodules decreased significantly, although their efficiency and both fresh and dry weight were not altered. Similar results were obtained by Raju (1974), who reported that DEMV infection increased the total nitrogen contents in the field bean nodules. Soluble proteins, total soluble nitrogen constituents, and the total insoluble nitrogen increased in the DEMV-infected nodules, but the levels of carbohydrates and sugars decreased in both infected root and nodules.

Venkataraman and Rao (1974) found that bean yellow mosaic virus did not alter the number of lateral roots, while the number of leaves and nodules increased in infected Phaseolus aureus plants. Virus infection gave similar effects regardless of strain of Rhizobium used.

The effect of arhar mosaic virus strains (ASM and AMM) on nodulation and nitrogen-fixing capacity of cowpea (Vigna sinensis Savi), mung bean (Phaseolus aureus Roxb.) and urd bean (P. mungo L.) has been studied by Singh and Mall (1974a). Both virus strains reduced

the growth, fresh weight and «nitrogen-fixing efficiency» of the three infected species. They decreased the number, weight and size of nodules only in cowpea and mung bean. The total nitrogen was lower in the infected nodules of cowpea and mung bean but it was higher in diseased urd bean. In a similar study on sann hemp (Crotalaria juncea L.), both strains of arhar mosaic were found to reduce the nodule number and dry weight, but they increased the total nitrogen contents in shoot, root and nodules (Singh and Mall 1974b). In another report, urd bean and bean mosaic virus were used as the host and virus, respectively, to study their relationship to the nodulation by adding different levels of nitrogen (21, 70, 210, 420 and 630 ppm). Growth, nodulation and nitrogen content of plants showed a gradual increase at 21 to 210 ppm, above which there was a decrease in the same values. The growth response of diseased plants was similar to that of the healthy ones, but the total nitrogen content was higher in the former than in the control.

Reports on the effect of virus infection on N_2 fixation involving other viruses have also been published. Orellana et al. (1978) noticed a remarkable reduction in the top, root, and nodule growth of soybeans infected by tobacco ring spot virus (TRSV). The virus infection caused a delay in the nodulation until the plants were about 40 days old, and maximum nitrogenase activity indicated by acetylene reduction, was similarly delayed. The TRSV infection decreased the nitrogen fixing rate in the last three growth stages. Within the same year, Orellana and Fan (1978) determined the extent to which BYMV infection

affects nodulation, leghemoglobin content, and N_2 fixation efficiency in the bean nodules. The data they presented showed a noticeable reduction in the values of the foregoing parameters as a consequence of the virus infection. In a subsequent report, Orellana et al. (1980) indicated considerable variation in the effect of TRSV upon the nodulation of soybeans, which was interpreted as a result of differences in the Rhizobium strains, and time of virus inoculation.

The effect of an aphid-borne cowpea tobacco mosaic virus (TMV-Cs) infection on growth, nodulation, nitrogen fixation in cowpea was studied by Mali et al. (1979). A considerable reduction in plant growth was caused by the virus infection, which, on the other hand, increased the nitrogen content in the diseased plants. Ohair and Miller (1979) found that nitrogen fixation and crop productivity in cowpea plants were affected by three viruses, southern bean mosaic (SBMV), cucumber mosaic (CMV) and tobacco mosaic virus (TMV). The effects included changes in nitrogenase activity, nodule mass, nodule number and growth rate. A major alteration in the diurnal patterns of nitrogenase activity was observed in the SBMV or CMB-infected cowpea. In another study, four varieties of soybean were inoculated with three strains of SBMV (Almeida et al. 1970). It was concluded that in all the varieties highly significant correlation coefficients were determined between plant dry weight and chlorophyll content.

Two reports have been published on the effect of peanut mottle virus (PMV) on groundnut (Arachis hypogaea L.). Mall et al. (1980) found that PMV infection did not have any adverse effect on nodulation,

fresh and dry weight of nodules and nitrogen content. On the contrary, Wongkaew (1981) assessed the effect of a mild strain of this virus on Early Prolific peanut, inoculated with either effective or ineffective Rhizobium, at four different stages. The PMV-infected plants with effective Rhizobium showed reduction in growth, yield, nodulation, Rhizobium population, nitrogenase activity and leghemoglobin contents. There was no correlation between leghemoglobin content and nitrogenase (C_2H_2) specific activity of nodules from PMV-infected plants but a positive correlation was noted in the healthy control.

Clover yellow vein virus (CYVV), peanut stunt virus (PeSV) and AMV were found to reduce leaf dry weight, nodulation and seven other components of growth in 10 clones of white clover (Gibson et al. 1981). Poor nodulation at 15°C was noticed on four cultivars of bean infected with PeSV. Serological assay (ELISA) confirmed the presence of the virus in the functional nodules of diseased plants (Stavely et al. 1981).

1.8 Nodule cytology and virus infection

The ultrastructure of legume root nodules has been investigated by several researchers (Bergersen and Nutman 1957; Dart and Mercer 1963; Moss 1964; Tu 1977b). Until recently, little attention has been paid to the detection of virus particles or inclusions induced by virus infection in thin sections of nodular tissues. Most of the reported studies have involved some members of the potyvirus group,

which became the focus for this kind of investigation. Vela (1971) studied the possible association of BYMV with nodule-inducing bacteria (Rhizobium phaseoli) and noticed typical inclusions of this virus in ultrathin sections of the diseased bean nodules. Tu (1973) found, during an in vitro comparison study on the dilution end point of SMV in infected root and soybean nodules, that SMV infectivity in the latter was higher and lasted for a longer period than that of the root tissues. The results indicated that the SMV could increase in the nodules regardless of the presence of rhizobia. Electron microscopy confirmed the presence of SMV aggregates and pinwheel inclusions in the young cells of the infected nodules. Tu (1974b) noted two kinds of pinwheel inclusions were associated with SMV-infected nodules of soybean plants. The particulate lamellar type was detected only in the young cells, while the smooth lamellar inclusions were observed in the older cells. The former was not seen in the leaf tissue cells and root nodules containing bacteroids.

In other reports, four major differences between the bacteroids of SMV-infected plants and the counterparts of healthy plants were recognized by using thin sectioning and freeze-fracturing techniques (Tu 1977a). These differences included: (1) the space between the bacteroidal wall and the membrane envelope was wider in the healthy bacteroid. Consequently, it was suggested that a higher concentration of leghemoglobin resulted in an increase of nitrogen-fixing efficiency. (2) The presence of vesicles in the peribacteroidal space of healthy bacteroids indicated a higher movement of material between the bacteroid

and the host cytoplasm. (3) The healthy bacteroid had a much larger amount of an unevenly thickened amorphous layer of electron opaque material lining the inner side of the membrane envelope. (4) In the SMV-infected nodules the bacteroid contained less vesicles outside the plasma membrane than those of healthy controls, suggesting their lower rate of material exchange.

Recently, the effect of another member of the potyvirus group on the nodular ultrastructure of peanut plants was investigated by Wongkaew (1981), who confirmed that there was no infection thread during peanut nodule development. Earlier workers have thus classified them as endogenous, while nodules which arise from an infection thread have been termed exogenous (Allen 1973). Wongkaew's work was divided according to the growth stages of peanuts from which the nodules were collected and the results showed a drastic alteration in the nodular tissues of PMV-infected plants. At the early blooming stage, the absence of bacteroids in the nodular tissues of PMV-infected plants was interpreted as a result of the retardation of nodule development by the virus infection. In the late blooming stage, four major abnormalities were noted in the nodular tissues of PMV-infected plants: (1) The vascular matrix of cortical cells bordering the bacteroidal tissue appeared less granular than that of the normal cell. (2) The bacteroidal tissue in infected nodules appeared less homogeneous. (3) Various kinds of dead cells were found mixed with normal cells in the bacteroidal tissue. (4) The accumulation of amyloplasts was greater in the PMV-infected nodular tissues. At the late blooming stages, three types of

abnormal cells were also recognized and designated as vesiculated, autolysed and autophagic bacteroidal cells. At the pegging and pod-filling stages, it was difficult to recognize the cellular changes in the PMV-infected nodules due to their senescence. Cylindrical and pinwheel inclusions, typical of those induced by the potyvirus group, were abundant in the early blooming stage, and they were occasionally seen in the second stage but not in the last.

Although only two rod-shaped viruses have been examined at the nodular ultrastructure level, a bacilliform virus was reported to infect Trifolium incarnatum (Vela and Rubio-Huertos 1974). Bullet-shaped virus particles and excessive lipid droplets were found in perinuclear spaces of the infected cells in the nodular tissue. The phyllody mycoplasma organisms were found in the sieve tubes of infected nodular tissue of white clover. Seven months after inoculation, numerous bacteroids or bacteroid-containing unusual structures were noticed in the parenchyma cells of the nodules of infected plants (Smets et al. 1977).

SECTION 2

IDENTIFICATION AND INCIDENCE OF RED CLOVER VIRUSES

2.1 Materials and methods

2.1.1 Virus isolation and the growth conditions

During the summer of 1979, red clover fields in the Ottawa area were surveyed and 28 plants showing virus-like symptoms were collected. Twenty-two virus isolates from these samples were established in the greenhouse in red clover plants through sap inoculation. Preliminary examination of leaves from infected plants by the leaf-dip method showed filamentous virus particles in 20 isolates. Several of these virus isolates produced local lesions on Gomphrena globosa L. and one of them, hereafter referred to as the Ottawa isolate, was propagated in red clover from a single lesion and was later identified as WCMV, a potex virus.

All plants were grown in the greenhouse, maintained at about 22-24°C, with supplemental light from fluorescent lamps to give a minimal photoperiod of 16 hours. Excessive temperature fluctuations during the summer months were reduced by using air coolers and shades. The potting medium consisted of two parts pasteurized soil, one part peat moss, two parts mushroom compost and one-half part turface (Division of International Minerals and Chem. Corp., Illinois, U.S.A.).

The plants were grown in clay and plastic pots (10 cm diameter). NPK fertilizer (20-20-20) was added to each plant weekly, and insects were controlled through weekly greenhouse fumigation with recommended insecticides.

2.1.2 Host range, assay hosts and inoculum preparation

The host plants were grown from seeds, and were inoculated at the 4- to 8-leaf stage, depending on plant species. At least five plants were used for each inoculation. To determine the host range, various plant species or cultivars were chosen among different families (i.e., Leguminosae, Amaranthaceae, Solanaceae, Chenopodiaceae, Cucurbitaceae). The virus inoculum was prepared by grinding infected red clover leaves with an equal volume (w/v) of 0.01 M potassium phosphate buffer, pH 7.0, and passing the crude extract through a double layer of cheesecloth. Inoculations were made by gently rubbing the upper surface of plant leaves with the forefinger wet with the inoculum mixed with either carborundum (600-mesh) or celite (diatomaceous earth, Johns-Manville) to facilitate virus infection. The inoculated plants were kept in a greenhouse under the described conditions. Gomphrena globosa L. was used as a local lesion assay host, and systemically infected Pisum sativum, cv. Lincoln, was used as a source for virus purification.

2.1.3 Virus stability in sap

About 50 g of infected red clover leaves were ground with an equal weight of 0.01 M phosphate buffer, pH 7.0, and the extracted juice was divided into three parts to determine the virus stability *in vitro*. To determine the thermal inactivation point (TIP), one ml of the sap was distributed into each of 9 glass tubes which were left at the required temperature in the water bath. The samples were exposed to temperatures from 40 to 80°C, at 5°C increments, for 10 min, after which the tubes were cooled immediately in running water, and then the samples were inoculated onto *G. globosa* leaves.

For determination of the dilution end point (DEP), tenfold dilutions were prepared from the crude sap ranging from 10^{-1} to 10^{-8}, and each dilution was rubbed onto six leaves of *G. globosa*. The stability *in vitro* of the virus was determined by placing the extract samples into tubes which were kept at room temperature for more than 40 days. At various intervals they were inoculated onto 6 leaves of *G. globosa*.

2.1.4 Particle morphology

Virus particle length measurements were made on the negative plates obtained from examination of leaf-dip preparations, which were negatively stained with 2% phosphotungstic acid, pH 7.0, and examined in a Philips 300 electron microscope. Similarly stained purified virus preparations were also examined, and at least 100 virus particles were measured to determine their modal length.

2.1.5 Purification

The virus was purified by using the procedure reported by Veerisetty and Brakke (1978) with some modifications. Frozen infected pea tissue, 8 days after virus inoculation, was homogenized with two parts (w/v) of 0.0165 M phosphate and 0.018 M trisodium citrate (pH 9.0) and one volume of chloroform. After low speed centrifugation of the homogenate at 10000 rpm (12060 g) for 10 minutes in a Sorvall SS34 rotor, the supernatant was clarified by formation of calcium phosphate in situ (one-twentieth volume of 0.2 M Na_2HPO_4 and one-hundredth volume of 1 M $CaCl_2$ were added while extract was stirred), and after centrifugation for 10 min at 10000 rpm (12062 g), the virus was precipitated from the supernatant by adding 6% (w/v) solid polyethylene glycol 6000 (PEG). The PEG-precipitated virus was collected by centrifugation at 10000 rpm (12062 g) for 15 min and resuspended in the extraction buffer containing 1% Triton X-100 (alkylaryl polyether alcohol). The resultant virus suspension, after centrifuging for 10 min at 10000 rpm (12062 g), was layered on a pad of 10 ml of 25% sucrose and centrifuged in rotor no.30 at 27000 rpm (65000 g) for 150 min. Pellets were resuspended in the extraction buffer (one-tenth the original molarity) and centrifuged for 10 min at 8000 rpm (7729 g). The supernatant was designated as «partially purified virus.»

2.1.6 Density gradient centrifugation

For rate zonal centrifugation, sucrose density gradient columns were made by layering 7, 8, 8, 7 ml, of 10, 20, 30 and 40% sucrose,

respectively, dissolved in 0.0165 M Na-phosphate and 0.0018 M Na-citrate, pH 8.9, which were allowed to diffuse overnight to form a smooth gradient. About 0.5 ml of partially purified virus preparation was then floated on the gradients and centrifuged for 90 min, at 5°C, at 23000 rpm (55000 g) in the SW 25.1 rotor, using a Beckman L8-55 ultracentrifuge. The virus zones were removed through the tops of the tubes with a hypodermic needle, pooled and dialyzed overnight against Na-citrate phosphate buffer. Sucrose density gradient columns containing the virus were also analyzed with an ISCO model 185 fractionator coupled to UA-2 absorbance monitor and a recorder. The absorbance at 254 nm was recorded graphically, resolving the ultraviolet absorbing material from any point within the gradient column. The collected fractions containing the virus from gradient zones were then pelleted by centrifugation at 34000 rpm (approx. 105,000 g) for 2 hours in a no.40 rotor. Suspension of the pellets in the same buffer or double-distilled water, and a low speed centrifugation, completed the purification. The purified virus was then used for further characterization.

2.1.7 Determinations of physical-chemical properties

To calculate the virus extinction coefficient, the purified virus pellet was suspended in double-distilled water and the U.V. absorption spectrum was recorded. The weight of the virus was then determined by placing 0.4 ml of virus suspension on a microscope coverglass and drying it at 105°C for 1 hour.

The sedimentation coefficient of the virus was determined in a Beckman Model E Analytical Ultracentrifuge using Schlieren optics. The virus (0.2-1.9 mg/ml) in phosphate-citrate buffer was centrifuged at 28000 rpm using an An-D rotor at 20°C, and photographs were taken every 4 min. The graphical method of Markham (1960) was used to calculate the sedimentation coefficient.

The buoyant density (g/cm³) of the virus was determined by equilibrium banding in cesium chloride (CsCl) in the preparative ultracentrifuge (Brunck and Leick 1969). About 2.5 ml of CsCl ρ = 1.5 (0.6736 g/ml) was introduced into 9/16 in x 3-3/4 in tubes, and an equal volume of CsCl solution ρ = 1.252 (0.3376 g/ml) was carefully layered on the denser CsCl. A 0.2 ml aliquot of the purified virus preparation at 2.5 mg/ml was floated on the step gradient, and the tubes were filled with mineral oil and immediately centrifuged at 30000 rpm (125000 g) for 24 h at 15°C using a SW40 rotor in the L8-55 ultracentrifuge. Southern bean mosaic virus (SBMV) was used as a reference. At the end of the centrifugation period, the rotor was allowed to stop by coasting. The visible zones were removed by hypodermic syringe from the side of the centrifuge tube, and the refractive indices of the various fractions were determined in an Abbe-3L refractometer at 25°C and converted into density values (Sehgal et al. 1970).

2.1.8 Amino acid analysis

The amino acid composition was determined by degrading purified freeze-dried virus in 1 N HCl for 24 hours at room temperature. The precipitated protein was washed twice with 1 N HCl. The samples were hydrolyzed in evacuated, sealed tubes in 6 N HCl at 110°C for 24 and 72 hours, respectively. The hydrolyzates were evaporated to dryness *in vacuo*, taken up in citrate buffer at pH 2.2, and the concentration of amino acids was determined on a Beckman Model 120 B amino acid analyser.

2.1.9 SDS - Polyacrylamide gel electrophoresis of viral protein

Gel electrophoresis of proteins was performed in 2.5, 5.0, and 10% polyacrylamide gels containing SDS. Virus protein and standards were boiled for 1 min in 0.1 M sodium phosphate buffer, pH 7.2, containing 4 M urea, 1% SDS, and 1% mercaptoethanol. The protein markers were bovine serum albumin (BSA), ovalbumin, lysozyme, pepsin, and lactoglobulin (Appendix I). The markers were used separately and in various combinations with the virus protein at a concentration of about 10 µg per gel. Electrophoresis was carried out at 7 mA/gel for 3 hours after which the gels were fixed and stained with Coomassie Blue in trichloroacetic acid by the method of Chrambach et al. (1967).

2.1.10 Insect transmission

Attempts were made to transmit the virus by means of the pea aphid Acrythosiphon pisum (Harris) from red clover to red clover and pea, and from pea to pea and red clover. Diseased source plants were obtained through mechanical inoculation with purified virus. Virus-free aphids, reared on peas, were starved for 2 hours before the acquisition access period of 15 minutes on an infected source plant, and then transferred in groups of 10 to healthy plants for a test feed of 2 hours. The inoculated plants were held in the greenhouse for one month and observed for symptom development.

2.1.11 Antiserum preparation and serological tests

One ml of freshly purified preparation containing 2-2.5 mg virus was emulsified with an equal volume of Freund's incomplete adjuvant and administered intramuscularly into the hind leg of a rabbit. Ten such injections were given at weekly intervals. Twelve days after the last injection, one additional injection of the virus suspension was given intravenously. Five days later the rabbit was bled through the marginal ear vein. The serum was separated by low speed centrifugation (after clotting overnight) and stored in small lots at -20°C, after addition of 0.01% NaN_3 as preservative. Preimmune serum was obtained from the rabbit before giving the injections.

The titer of the antiserum was measured by microprecipitin and ring tests against purified virus (1 mg/ml), and dilutions of both

virus and antiserum were made in 0.85% NaCl (saline). The serological relationships among these dilutions were examined by the Ouchterlony double-diffusion technique using 0.75% Noble agar in 0.02 M phosphate buffer, pH 7.0, containing 0.01% NaN_3. The wells were made with a no.3 cork borer. Center wells were charged with the purified virus (1 mg/ml), and outer wells were charged with the prepared antiserum and four other antisera, and vice versa.

2.1.12 Virus incidence

Six red clover fields located within 50 km around Ottawa were surveyed. The number of healthy plants and those showing virus-like symptoms in each field were recorded by walking in a W-pattern. Plants showing virus-like symptoms were collected randomly from each field and an extract of each sample was tested against the antisera to WCMV (Ottawa isolate), BYMV, RCVMV, PSV, and CYMV (these antisera were obtained from the bank of antisera, Agriculture Canada, Vancouver) by the micro-precipitin test. The extracts were prepared by grinding leaf samples with an equal volume (w/v) of 0.05 M potassium phosphate buffer saline (pH 7.0, 0.85% NaCl) containing 0.5% mercaptoethanol, and the crude extract was passed through a double layer of cheesecloth. An equal volume of chloroform was then added to the filtrate and stirred well using a vortex shaker. The mixture was centrifuged at 12000 rpm (14700 g) for 10 min and the supernatant was used for serological tests.

2.2 Results

2.2.1 Host range and symptomatology

Nineteen plant species belonging to five different families were inoculated with the virus to determine its host range (Table 1). Only two plant species outside the family Leguminoseae, Chenopodium amaranticolor and G. globosa, were found susceptible to the virus. Both produced local lesions while other susceptible plants developed systemic symptoms. The plants which did not show symptoms were checked for symptomless infection by assaying them on G. globosa. None of these assays showed local lesions on the test plants. Infected red clover plants showed vein clearing at early stages of infection and later developed mosaic symptoms. Some of the plants with disease symptoms are shown in Figure 1.

2.2.2 Particle morphology

Electron micrographs of both purified virus preparations and leaf-dip preparations (Figure 2) showed flexuous filamentous particles. Some end-to-end aggregation and a few broken virus particles were observed in the purified preparations. The modal length of the particles, calculated from leaf-dip preparations, was 450-500 nm.

2.2.3 Homogeneity of purified virus

Examination of the virus suspension obtained after using the purification procedure recommended for carlaviruses (Veerisetty and Brakke 1978) indicated that it contained some host plant materials.

TABLE 1. Host range of WCMV isolate

Susceptible hosts Test plants	First appearance of symptoms after virus inoculation (days)	Non-susceptible hosts Test plants
Leguminoseae		Leguminoseae
Trifolium pratense L.	13	Phaseolus vulgaris
cv. Kenland		cv. Early Blue
T. repens L.	13	Henderson
cv. New Zealand		Kentucky
Melilotus alba Desr.	15	Pinto
Medicago sativa L.	13	Glycine max (L.) Merr.
cv. DuPuits		cv. Bragg
Vicia faba L.	6	Davis
cv. Bell Bean		Solanaceae
Vigna sinensis (Torner) Savi		Datura stramonium L.
cv. Blackeye	4	cv. R. Fulton
Pisum sativum L.		Petunia hybrida Hort. Vilm-Andr.
cv. Alaska	8	cv. King Henry
Progress #9	6	Lycopersicon esculentum Mill.
Thomas Laxton	6	cv. Marglobe
Perfected Wales	6	
Dark Skin Perfection	6	
Lincoln	8	Chenopodiaceae
Phaseolus vulgaris L.		Chenopodium quinoa
cv. Burpee's Brittle	7	C. album
Black Turtle Soup	7	Spinacea oleracea L.
Bountiful	7	cv. Bloomsdale Long
Contender	7	
Great Northern	7	
Tendergreen	7	

(table continued)

TABLE 1 (continued)

Susceptible hosts Test plants	First appearance of symptoms after virus inoculation (days)	Non-susceptible hosts Test plants
Amaranthaceae		Cucurbitaceae
Gomphrena globosa L.	2	Cucumis sativus L. cv. Chicago pickling
Chenopodiaceae		
Chenopodium amaranticolor Coste and Reyn.	3	

Figure 1. Leaf symptoms on some susceptible hosts of WCMV. Left: leaves from healthy plants. Right: leaves from infected plants.

A. Infected broad bean leaves showing severe chlorotic symptoms.

B. Infected Gomphrena globosa L. leaves showing the local lesions.

C. Infected bean leaves showing mottling symptoms.

D. Infected Ottawa red clover leaves showing mosaic and vein banding.

E. Infected Perfected Wales pea showing severe wilting.

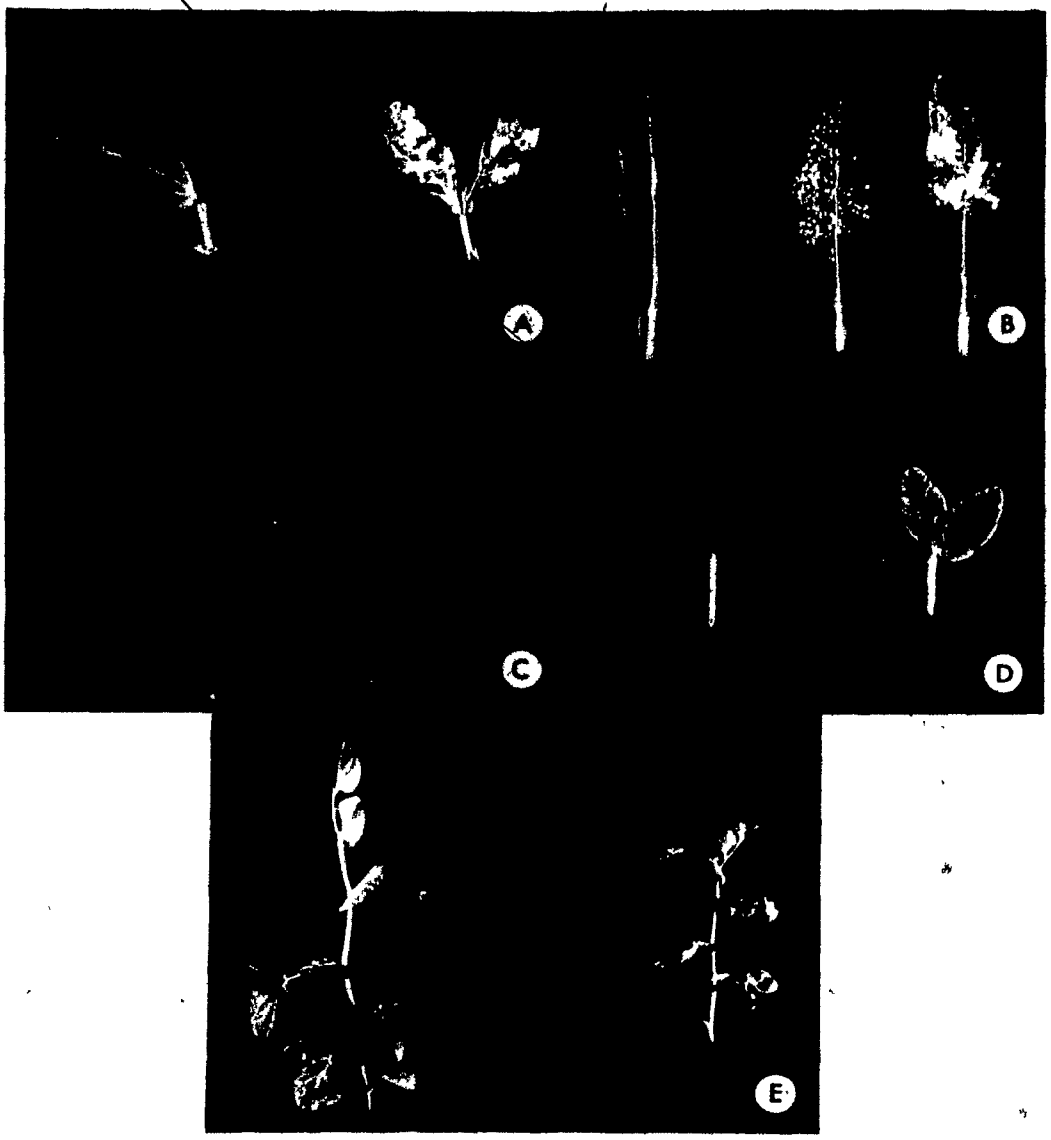

A
B
C
D
E

Figure 2. Electron micrographs of WCMV preparations negatively stained with 2% phosphotungstic acid.

A. WCMV particles in a leaf-dip preparation.

B. WCMV particles in a purified preparation.


0.5 μ
A
0.5 μ
B

Chloroform clarification and using one pad of 25% sucrose were the main procedural changes. The modifications incorporated in this method provided purer virus preparations in a shorter period. The degree of homogeneity of the virus was examined by electron microscopy, analytical ultracentrifugation, rate-zonal density gradient centrifugation, polyacrylamide gel electrophoresis and CsCl density gradient.

Density gradient centrifugation yielded a single sedimenting band, as observed visually and revealed in optical density tracings of fractionated gradients. The band interface toward the meniscus was sharp and the one toward the bottom of the tube more diffuse. In CsCl density gradients a sharp band was observed as an indication of the presence of one virus in the purified preparation. Also one band was noticed on polyacrylamide gel electrophoresis. The UV absorption of the purified virus is typical of filamentous viruses with a maximum at 258-259. The A260/A280 and A259/A247 ratios were more than 1.25 and 1.10, respectively (Figure 3).

2.2.4 Virus properties

The *in vitro* properties of this virus in crude sap showed that the dilution end point was 10^{-5}, longevity *in vitro* at room temperature was 2 weeks, and the thermal inactivation point was about 70°C.

The purified virus in phosphate-citrate buffer showed a single peak upon analytical centrifugation (Figure 4). The sedimentation coefficient ($S_{20,w}$) of the virus, in four suspensions containing 0.2 to 1.9 mg/ml virus, increased with dilution from 105 S to 115 S. The

Figure 3. UV absorbance spectrum of a purified WCMV preparation.

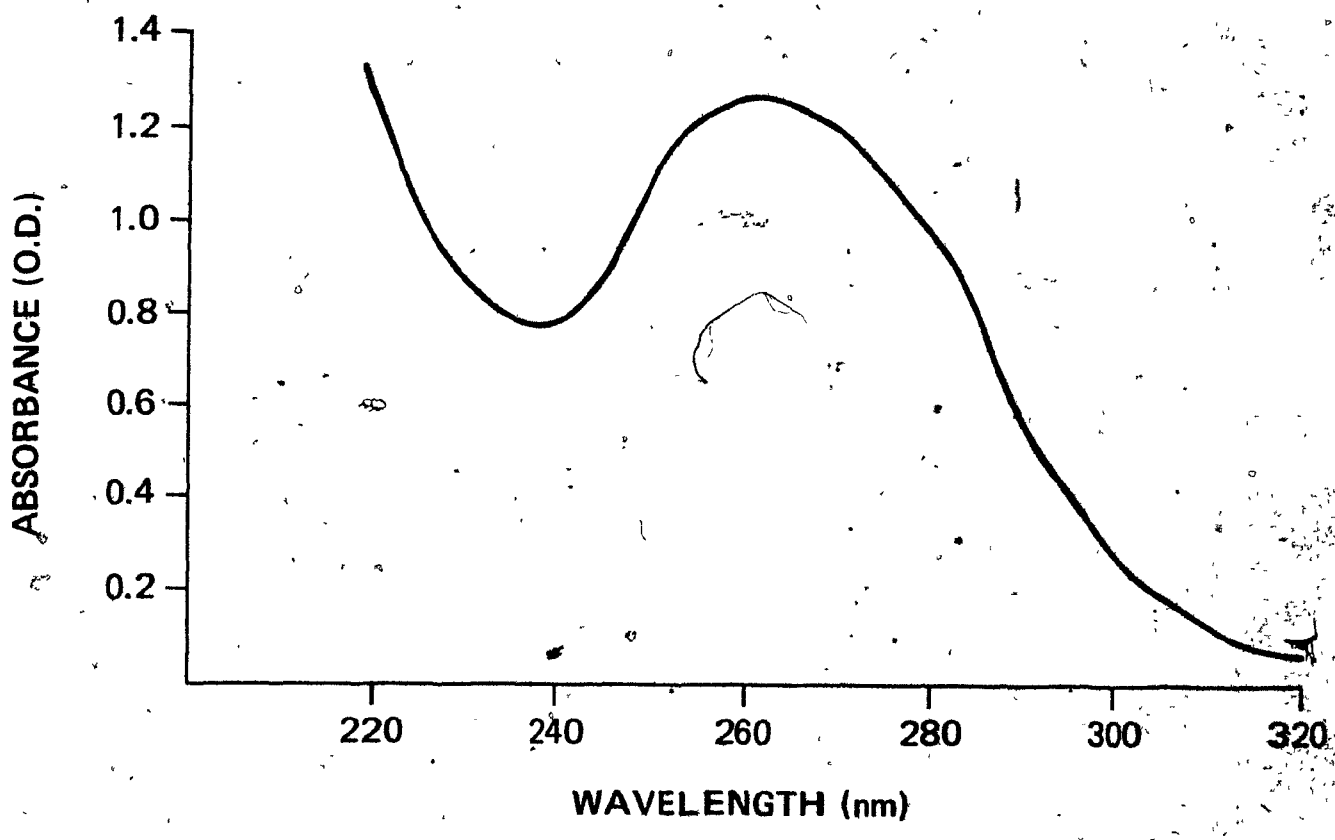
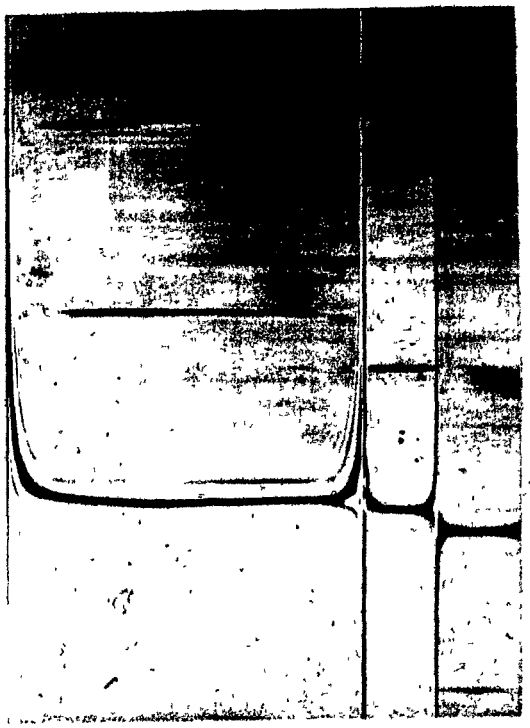

1.4
1.2
1.0
0.8
0.6
0.4
0.2
ABSORBANCE (O.D.)
220
240
260
280
300
320
WAVELENGTH (nm)

Figure 4. The single sedimenting peak of WCMV during analytical ultracentrifugation, shown by Schlieren optics.



$S_{20,w}$ of the virus, extrapolated to infinite dilution, was 117 (Figure 5).

The buoyant density (ρ) of the virus was calculated to be 1.298 g/cm³ in all three experiments. The ρ of SBMV, centrifuged with WCMV isolate in the same tube, was determined as 1.359 g/cm³, which agrees with the reported value (Sehgal et al. 1975). Virus samples removed from the bands obtained after the different centrifugations mentioned above were infectious and the particles retained their normal morphology. The absorbance of 1 mg/ml of purified virus at 260 nm, determined by weighing the virus at different concentrations, was 3.1 ± 0.05 O.D. units (not corrected for light scattering).

The molecular weight of the virus protein, determined by polyacrylamide gel electrophorosis, was 2.4×10^4 (Table 2). The amino acid composition of the virus protein was determined using three virus preparations purified from different batches of infected plants. Results of analysis of all preparations were within 5% of the average values of individual amino acids. Molar percentage of various amino acids is shown in Table 3. The determined biophysical properties of Ottawa isolates of WCMV compared to the other known isolates are summarized in Table 4.

Several attempts to isolate RNA by various methods (Brakke and Van Pelt 1969; Miki and Knight 1967; Reichmann and Stace-Smith 1959), and to transmit the virus by means of pea aphids, were unsuccessful.

Figure 5. Sedimentation coefficient ($S_{20,w}$) of WCMV. particles determined by analytical ultracentrifugation.

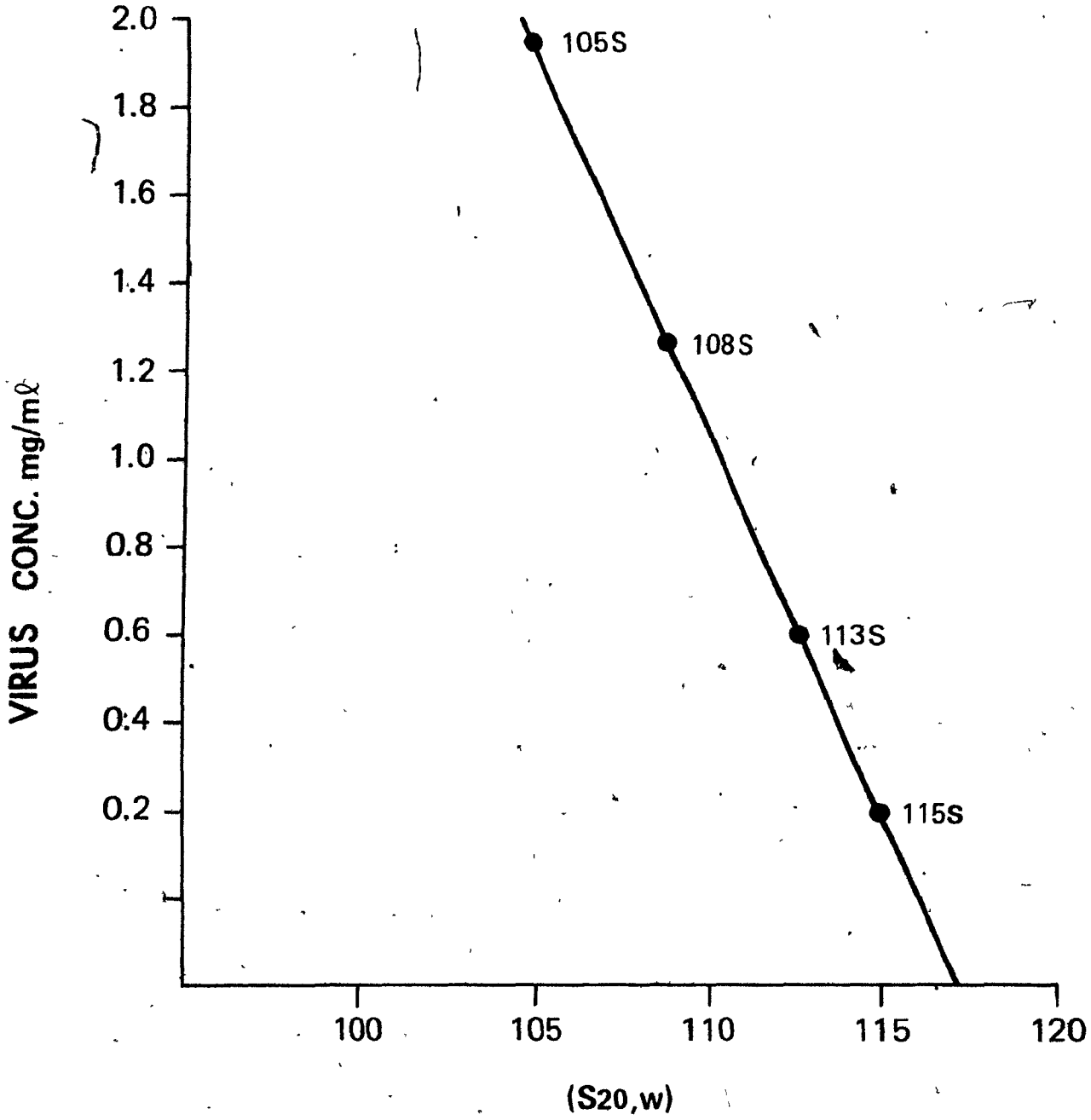

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
VIRUS CONC. mg/mℓ
105S
108S
113S
115S
100
105
110
115
120
(S20,w)

TABLE 2. Determination of the molecular weight of WCMV protein on 10% polyacrylamide gel electrophoresis

Sample	Molecular weight	Dye mobility (cm)	Protein mobility (cm)	Relative mobility
Lysozyme	14,300	7.0	6.4	0.91
		7.1	6.65	0.93
Lactoglobulin	18,300	7.0	5.55	0.79
Pepsin	34,700	7.5	3.4	0.45
		7.3	3.3	0.45
Ovalbumin	43,000	7.3	2.5	0.34
		7.1	2.4	0.33
BSA	67,000	7.2	1.55	0.21
		7.2	1.60	0.22
WCMV (Ottawa isolate)	24,000	7.0	4.5	0.64
		7.1	4.6	0.64

TABLE 3. Amino acid composition of WCMV protein

Amino acid	Molar percentage of hydrolyzate		
	Ottawa isolate*		Maximum value
	24 h	72 h	
Alanine	12.26	12.29	12.29
Arginine	3.90	3.80	3.90
Aspartic acid	7.94	8.01	8.01
Glutamic acid	6.66	6.77	6.77
Glycine	5.60	5.70	5.70
Histidine	1.77	1.78	1.78
Isoleucine	4.76	5.92	5.92
Lucine	6.93	7.12	7.12
Lysine	5.37	5.72	5.72
Methionine	1.30	1.26	1.30
Phenylalanine	2.62	3.88	3.88
Proline	5.78	5.99	5.99
Serine	4.83	6.36	6.36
Threonine	6.88	7.90	7.90
Tyrosine	1.48	0.99	1.48
Valine	4.92	4.87	4.92
Tryptophan	n.d.	n.d.	n.d.
Cysteine	n.d.	n.d.	n.d.

*The data represent average of three experiments for the isolate under investigation.

n.d. not determined.

TABLE 4. Biophysical properties of WCMV

Property	Ottawa isolate	Other isolates
Thermal inactivation point	70-75°C	60°-80°C
Dilution-end point	10^{-5}-10^{-6}	10^{-5}-10^{-6}
Longevity *in vitro*	14-16 days	10-99 days
Sedimentation coefficient ($S_{20,w}$)	117 S	112-119 S
Extinction coefficient (for 1 mg/ml)	3.1	3.6
Buoyant density	1.298	not reported
Particle modal length	450-500 nm	480 nm
A260/A280	1.24	1.20
Molecular weight (protein)	2.4×10^4	1.40×10^4 - 2.35×10^4

2.2.5 Serology

The titer of the antiserum, using 1 mg/ml of purified virus, was 1024 and 512 as determined by microprecipitin and precipitin ring tests, respectively. The minimum concentration of purified virus detectable by both tests was 0.4 mg/ml. Clarified extracts of the infected clover plants also gave positive reactions in both tests. To obtain a positive reaction in the immunodiffusion test, virus particles in the purified preparations as well as in the clarified extracts were disrupted by 3 cycles of freezing and thawing. Such virus preparations produced a thick precipitin band, midway between the antiserum and antigen wells. To check for nonspecific reactions, concurrently with each experiment antiserum and preimmune serum were tested against clarified extracts of healthy clover plants, and purified virus was also examined against the preimmune serum. No positive reactions were observed in any of these control tests.

To confirm the identity of the studied isolate, purified virus preparations at 1 mg/ml were tested against undiluted antisera of WCMV (obtained from two sources), BYMV, CYMV, PSV and RCVMV. Positive reactions were observed only with WCMV-antiserum in all three types of serological tests (i.e., microprecipitin, ring and double diffusion tests).

2.2.6 Virus incidence

Virus-like symptoms were observed on 17% (88/504) of red clover plants during the survey of six fields. The incidence of such plants

in different fields varied from 16.6 to 23.3%. The clarified extracts from 346 randomly collected plant samples showing virus-like symptoms were tested serologically for the presence of certain filamentous viruses. WCMV was most prevalent, followed by BYMV, CYMV, PSV and RCVMV (Table 5). About 60% of the test plants were found to be infected with more than one virus.

TABLE 5. Incidence of some filamentous viruses infecting red clover in eastern Ontario

Field	No. of plant samples tested serologically[a]	Number of plant samples reacted positively against the antisera of [b]					No. of samples reacted negatively
		WCMV	BYMV	CYMV	PSV	RCVMV	
1	60	48	18	4	3	3	8
2	46	13	8	3	2	1	26
3	48	25	23	19	16	15	3
4	67	13	8	8	7	6	43
5	70	40	4	3	3	2	22
6	55	16	6	6	5	7	34
Total	346	155	67	43	36	34	136
Per cent		44	19	12	10	9	39

[a]Clarified extracts of plants showing virus-like symptoms were tested by microprecipitin test.

[b]WCMV: white clover mosaic virus; BYMV: bean yellow mosaic virus; CYMV: clover yellow mosaic virus; PSV - pea streak virus; RCVMV: red clover vein mosaic virus.

2.3 Discussion and conclusion

The virus in this investigation undoubtedly belongs to the potexvirus group and is designated as WCMV (Ottawa isolate) on the basis of host range, particle length and shape, and some chemical and physical properties. The virus showed positive reaction with WCMV antiserum obtained from American Type Culture Collection and the bank of antisera in British Columbia, but there was no reaction with antisera against BYMV, CYMV, PSV, RCVMV by microprecipitin and double diffusion tests.

During the study of the host-range and symptomatology, various plant species were chosen among different families as recommended by the International Working Group on legume viruses (Hampton et al. 1978). The virus produced typical local and systemic symptoms of WCMV on pea, and produced local lesions on Gomphrena globosa and Chenopodium amaranticolor. One strain of WCMV has been reported to produce similar symptoms of G. globosa (Bercks 1971).

Electron microscopy showed flexuous filamentous virus particles with modal length approximately within the range of the reported values (Pratt 1961; Blaszezak et al. 1977; Bancroft et al. 1960; Varma et al. 1970; Fry et al. 1960; Bercks 1971). Purification of the virus by a procedure recommended for carlaviruses (Veerisetty and Brakke 1978) provided purer virus preparations in a shorter period. The modification introduced to this procedure by addition of the chloroform to the leaf tissues and the buffer extract can be considered a positive element in such improvement. The length of the procedure was reduced through combining the steps of using 20% and 30% sucrose, into one step

of 25% sucrose pad. Pea plants served as a good source of the virus due to their tender and soft tissue. Also, a higher yield of purified virus was obtained per kilogram of fresh leaf tissue after density gradient and overnight dialysis. However, such results may depend on the right choice of source of virus (i.e., young plant, concentrated inoculum) and favourable conditions for virus multiplication. The date of harvesting infected leaves was an important factor for successful purification as well. The degree of the purity of this virus was confirmed by electron microscopy, which showed few broken virus particles, and relatively little contaminating materials or debris. A single peak of sedimenting material was observed by analytical ultracentrifugation. Also, only one visible band was obtained by rate-zonal density gradient centrifugation and SDS-polyacrylamide gel electrophoresis.

Sedimentation coefficient ($S_{20,w}$) of WCMV calculated in this investigation is relatively close to some of the values reported, e.g., 119S (Varma et al. 1970), 112S (Fry et al. 1960; Pratt 1961), 114S (Blaszezak et al. 1977). The average value of the potexvirus group is 110-125S. The extinction coefficient (mg/ml) of the virus at 260 nm seems to be less than the reported value (Fry et al. 1960). The buoyant density was calculated in this study and apparently it is not reported yet for the known isolates of WCMV (Bercks 1971). Southern bean mosaic virus, which was used as a reference, had a buoyant density similar to the published value (Sehgal et al. 1970).

The protein of WCMV (Ottawa isolate), when examined by SDS-polyacrylamide gel electrophoresis, migrated as a single component with a mobility indicating a molecular weight different from those reported for other isolates as 1.4×10^4 (Miki and Knight 1967), 2.02×10^4 (Koenig et al. 1970), 2.25×10^4 (Lesnaw and Reichman 1970), and 2.35×10^4 (Tung and Knight 1972). The composition molar percentage calculated from amino acid analyses of 24 and 72-hour hydrolyzates of virus preparations presented in Table 3 are lower than the reported values (Miki and Knight 1967).

The serological study showed that the virus was highly immunogenic, which might be due to the stability of the capsid structure of the virus particle (Koenig 1964). Although specific precipitates were formed in agar gel-diffusion tests with disrupted virus, such tests are less sensitive than the precipitin test with intact virus particles. The titer determined by microprecipitin test was higher than the reported value of 16 (Blaszezak et al. 1977), and of 256 (Bos et al. 1960), but close to that reported by Pratt (1961) as 1280.

The manner in which red clover in the field is infected by WCMV still remains undetermined. Plants showing symptoms of virus infection were observed relatively early in the growing season during the second half of May. Although Goth (1962) reported that the pea aphid Acrythosiphon pisum (Harris), acted as a vector of WCMV, experiments during the current study under greenhouse conditions did not show any evidence of transmission by this insect. Other workers (Bancroft et al. 1960; Pratt 1961; Scott and Gold 1959) have reported

similar findings in studies with other isolates of WCMV and several other kinds of aphids, including pea aphid. It is known that most potexvirus members are readily transmitted mechanically, but have few or no known insect vectors (Matthews 1982). Therefore, insect transmission as a manner of field spread seems unlikely. Hampton (1963) reported that WCMV could be seed-borne, and it is possible that the plants observed had grown from infected seeds. Red clover is widely grown, both because of its wide adaptability and because the seed is not particularly expensive; it is unlikely that seed lots are examined or inspected for freedom from virus contamination.

Previous reports indicated that the incidence of some viruses infecting red clover in Ontario, namely, AMV, BYMV, RCVMV and PSV, occurred at low levels, without giving an estimated figure for the incidence of WCMV (Pratt 1968; Gates and Bronskill 1974). In this study, surveying of six red clover fields in the Ottawa region indicated the presence of five legume viruses including WCMV, RCVMV, CYMV, PSV and BYMV. Virus-like symptoms were observed on 17% of red clover plants, and WCMV was the most prevalent, followed by BYMV, CYMV, PSV and RCVMV. About 60% of the test plants were found to be infected with more than one virus. Therefore, it is most likely that these viruses are transmitted to red clover plants, either through seeds or by unknown vectors from nearby fields, where virus-infected red clover and/or other legumes are growing.

SECTION 3
CYTOLOGICAL STUDIES ON WCMV IN RED CLOVER AND PEA

3.1 Materials and methods

3.1.1 Light microscopy of inclusion bodies

A dye solution was made by dissolving 0.1 gm of Azure A stain in 100 ml of 2-methoxyethanol. Just prior to staining, 18 parts of the dye solution were mixed with two parts of 0.2 M disodium phosphate in distilled water. The dye and the phosphate solutions were stored separately until ready to use. For application, the solutions were mixed drop-wise in the proper proportions on glass slides which were tilted to allow accumulation of the liquid at one end (Christie and Edwardson, 1977).

Epidermal strips from lower surface of leaves of WCMV-infected pea and red clover were removed and floated on the stain for 15 minutes, so that the torn surface was in contact with the stain. After staining, the tissues were rinsed briefly in 95% ethanol for 30 seconds, placed in 2-methoxyethyl acetate for 15 minutes, and mounted directly in Euparal between a slide and cover slip. A compound microscope with bright field and phase contrast optics was used to view the stained tissue, and Kodak Ektachrome 160 film was used for photography. Epidermal strips from leaves of noninoculated plants served as controls.

3.1.2 Immunofluorescence microscopy

The indirect fluorescent antibody technique used by Sinha (1974) was employed. Epidermal strips from WCMV-infected leaves of peas and red clover were cut into 2-mm squares and placed on glass slides smeared with Haupt's adhesive. These slides were then immersed in 95% ethanol for 15 minutes and dried at 37°C for 5 min. The epidermal tissues covered with the antiserum were kept in a moist chamber at 37°C for 90 minutes. The slides were then rinsed with four changes of phosphate buffered saline (PBS), and then placed in a jar containing PBS for 10 min, then washed with double distilled water. The epidermal strips were then stained with sheep anti-rabbit gamma globulins labelled with fluorescein isothiocyanate (FITC) for 30 minutes at room temperature. The slides were rinsed again with two changes of PBS at 37°C for 10 minutes, with each change, and then mounted in PBS-glycerine (one part glycerine and 9 parts PBS). A set of control specimens was also prepared to verify the specificity of the reaction, including the use of normal serum and a heterologous antiserum (anti-CYMV) on infected tissues or «staining» healthy epidermal tissues with WCMV-antiserum.

The stained sections were examined with a Zeiss microscope equipped with a transfluorescence system. The exciter filters II and BG 38 were used, along with 50/44 barrier filters, to view the fluorescence of the tissues. Photographs were taken by using Kodak Tri-X film, with an exposure time of 25 minutes.

3.1.3 Electron microscopy

Samples (leaves, petioles, stems, roots) from systemically infected pea (Pisum sativum cv. Lincoln) and red clover (Trifolium pratense L. cv. Ottawa) were cut into 1 x 10 mm lengths at 8 and 13 days, respectively, after inoculation with WCMV. The sample tissues were fixed in 6% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, at 4°C overnight. The fixed materials were then washed 3-4 times with 0.1 M phosphate buffer, pH 7.0 for 15-20-minute intervals at the same temperature, and postfixed in 2% osmium tetroxide (OSO_4) for 4 hours at room temperature.

Dehydration of the samples was done through a graded ethanol series. Sample tissues were then washed with a mixture of equal parts of ethanol-propylene oxide, followed by propylene oxide for 30 minutes, respectively, at room temperature. The dehydrated materials were infiltrated in Araldite-Epon mixture (Mollenhauer 1964) with propylene oxide in proportions, 1:1, 2:1, 3:1, for 1 hour in each of the first and second mixtures, and overnight for the third mixture. Subsequently, the materials were infiltrated in resin mixture (without propylene oxide) for 4 hours, embedded in Beem capsules filled with resin mixture, and cured in the oven at 65°C for 48 hours.

Sections were cut on a Reichert ultramicrotome using a diamond knife and picked up with formvar-film coated and carbon stabilized grids (200-mesh, Cu). The gold-silver sections were stained with 2% uranyl acetate for 90 minutes at 37°C, washed in 50% ethanol, and stained with lead citrate (Reynolds 1963) for 5 minutes. Each grid

was washed in 0.1 N NaOH, then with filtered double distilled water, and viewed in a Philips 300 electron microscope at 60 KV. The magnification of the instrument was periodically calibrated by examination of a replica of a diffraction grating having a spacing of 54,800 lines per inch.

3.2 Results

3.2.1 General remarks, definitions, abbreviations

Two legume species, namely pea and red clover, infected with WCMV were studied to examine the pattern of virus and ultrastructural changes due to virus infection in different parts of the plants. Previous investigations involving the intracellular location of WCMV have not included these important plant species.

Different kinds of inclusion bodies induced by virus infections have been reported by various workers (Christie and Edwardson 1977), and some of these inclusions described in this investigation are defined as follows:

Inclusion bodies: microscopic bodies induced by virus infection of the plant cells, which are different from other cellular contents.

Complex inclusions: inclusions consisting of combinations of virus particles, or aggregates and host organelles, which can be round or ellipsoid.

Crystalline inclusions: inclusions having some or all the properties of a crystal, which may be composed of virus particles.

Banded inclusions: bundles of parallel aggregated virus particles.

Abbreviations used in micrograph labelling:

AP	Amyloplast
CI	Crystal
Ch	Chloroplasts
CW	Cell wall
CV	Crystalline virus inclusion
D	Electron dense deposit
ER	Endoplasmic reticulum
G	Grana
GB	Golgi body
IS	Intercellular space
M	Mitochondria
N	Nucleus
Nℓ	Nucleolus
NM	Nuclear membrane
OG	Osmiophilic globules
P	Proplastid-like organelle
PB	Paramural body
Pl	Plasmalemma
R	Ribosomes

St	Starch
T	Tonoplast
TC	Transfer cell
V	Virus aggregate
Va	Vacuole
VC	Vascular cell
Ve	Vesicle
VI	Inclusion induced by virus infection

3.2.2 Immunofluorescence and light microscopy

Immunofluorescence and conventional light microscopy of WCMV-infected leaf tissues revealed large amorphous cytoplasmic inclusions in both legumes examined. These inclusions were first detected in epidermal strips 8 days after inoculation of pea and 13 days after inoculation of red clover with WCMV. These times corresponded to the appearance of the first visible symptoms. The inclusions were situated in the corners against the cell wall (Figure 6A) but no inclusions were detected in the corresponding healthy tissues (Figure 6B).

The results of immunofluorescence microscopy confirmed the presence of WCMV-antigens in the epidermal cells of leaf tissue. The application of the indirect method showed the antigenic nature of the amorphous inclusions which reacted positively with WCMV-antiserum, and retained the shape of the inclusions seen by light microscopy (Figure

Figure 6. The epidermal tissue of red clover leaves stained with Azure A.

A. Amorphous cytoplasmic inclusions (indicated with arrows) in the WCMV-infected tissue.

B. Healthy tissue.

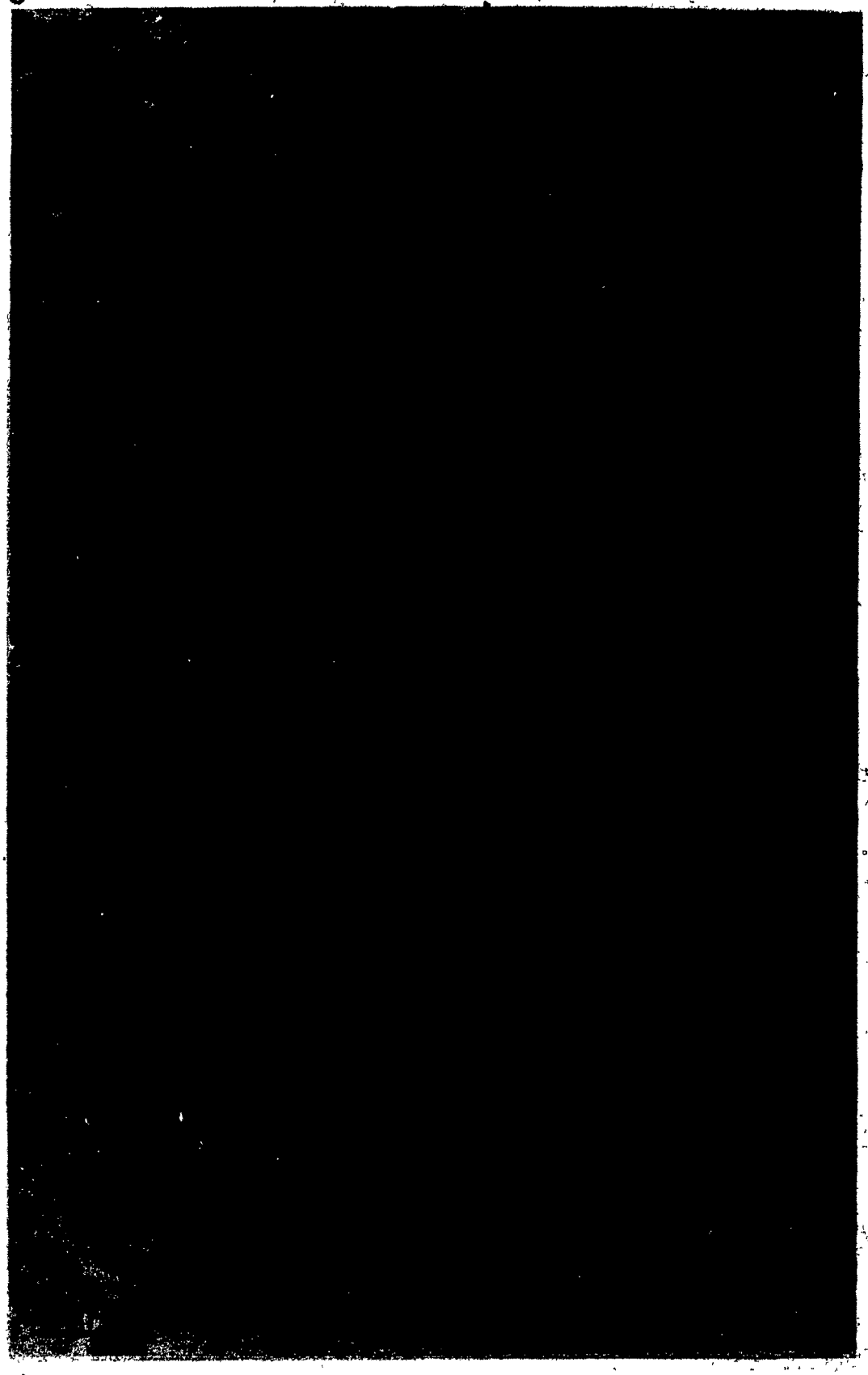
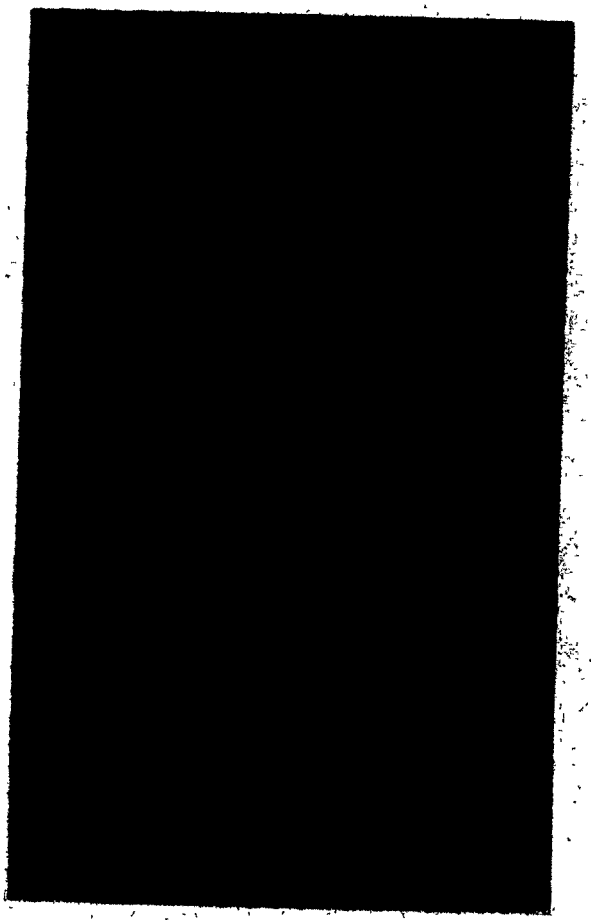


Figure 7. Photomicrographs of the epidermal tissue stained by the indirect immunofluorescence method, showing:

A. Specific reaction in the WCMV-infected tissue.

B. Very slight autofluorescence in the healthy tissue.



7A). Although some nonspecific fluorescence was seen in both healthy and diseased samples, it was reduced by diluting the FITC stain with normal saline (Figure 7B).

3.2.3 Evaluation of cellular preservation by examination of healthy tissue

Sections of healthy tissues of both pea and red clover were examined in order to assess the fixation methods used. In general, the appearance of cellular organelles was typical, and seemed representative of adequate fixation and preservation. Each mesophyll cell contained a large central vacuole, and plasma membranes were noticed at the periphery along the cell wall (Figure 8A, C). A conspicuous nucleus with a prominent nucleolus was observed in the various cells (Figure 8A). Sometimes the chromatin material was organized in discrete areas, in which electron opacity provided a sharp contrast to the remaining electron transparent portions.

The mitochondria were scattered in the cytoplasm and cristae were easily recognized as fingerlike projections into the mitochondrial matrix (Figure 8B). Typical vascular cells were found in various tissues (Figure 8D). The living cells were interconnected by plasmodesmata, which were clearly seen in, or extending through, the cell wall (Figure 8B).

Figure 8. Electron micrographs showing the appearance of various organelles of healthy red clover and pea tissues.

A. Chloroplasts in red clover leaf tissue, the inset showing prominent nucleolus in the nucleus.

B. Mitochondria and cell wall containing plasmodesmata in red clover stem tissue.

C. Chloroplasts in pea leaf tissue.

D. Vascular conducting cells of pea leaf tissue.


2μ
0.5μ
2μ
C
D

3.2.4 Electron microscopy of diseased red clover tissues

3.2.4.1 Localization and forms of inclusion bodies

Ultrathin sections of red clover tissues fixed 13 days after virus inoculation revealed two major kinds of cytoplasmic inclusions: virus particle aggregates and membrane-bound bodies. In the leaf tissues, different formations of WCMV particle aggregates were scattered in the cytoplasm of parenchyma cells. Generally, all the forms of WCMV aggregates were localized at the periphery of the cell or between the chloroplasts in the ground cytoplasm (Figure 9A), and most often appeared as spiral aggregates (Figure 9B). Cells of comparable uninoculated plants were free of virus particles or such inclusions.

WCMV aggregates similar to those observed in the leaf tissues were also found in the infected cells of petiole and stem tissues. Sometimes, bands of aggregated virus particles were arranged as arms of a pinwheel in the last two tissues (Figure 9C).

In the root tissue, concentration of WCMV appeared to be less than in the cells of the infected leaf, stem and petiole tissues. The virus particles were localized mainly in the parenchyma cells, and sometimes distributed randomly as single masses of virus aggregates (Figure 9D). Occasionally, dispersed virus particles were recognized in the conducting cells of the stem tissue (Figure 10A). and/or were associated with ribosomes and degenerated mitochondria in a sphere of condensed cytoplasm (Figure 10B). Groups or scattered WCMV aggregates

Figure 9. Different arrangements of WCMV aggregates in various tissues of red clover.

A. WCMV aggregates localized near the chloroplasts in the leaf tissue.

B. Spiral forms of WCMV aggregates in the leaf tissue.

C. Bands of WCMV aggregates which appeared as arms of a pinwheel in the petiole and stem tissues.

D. Single masses of aggregated WCMV in the root tissue.

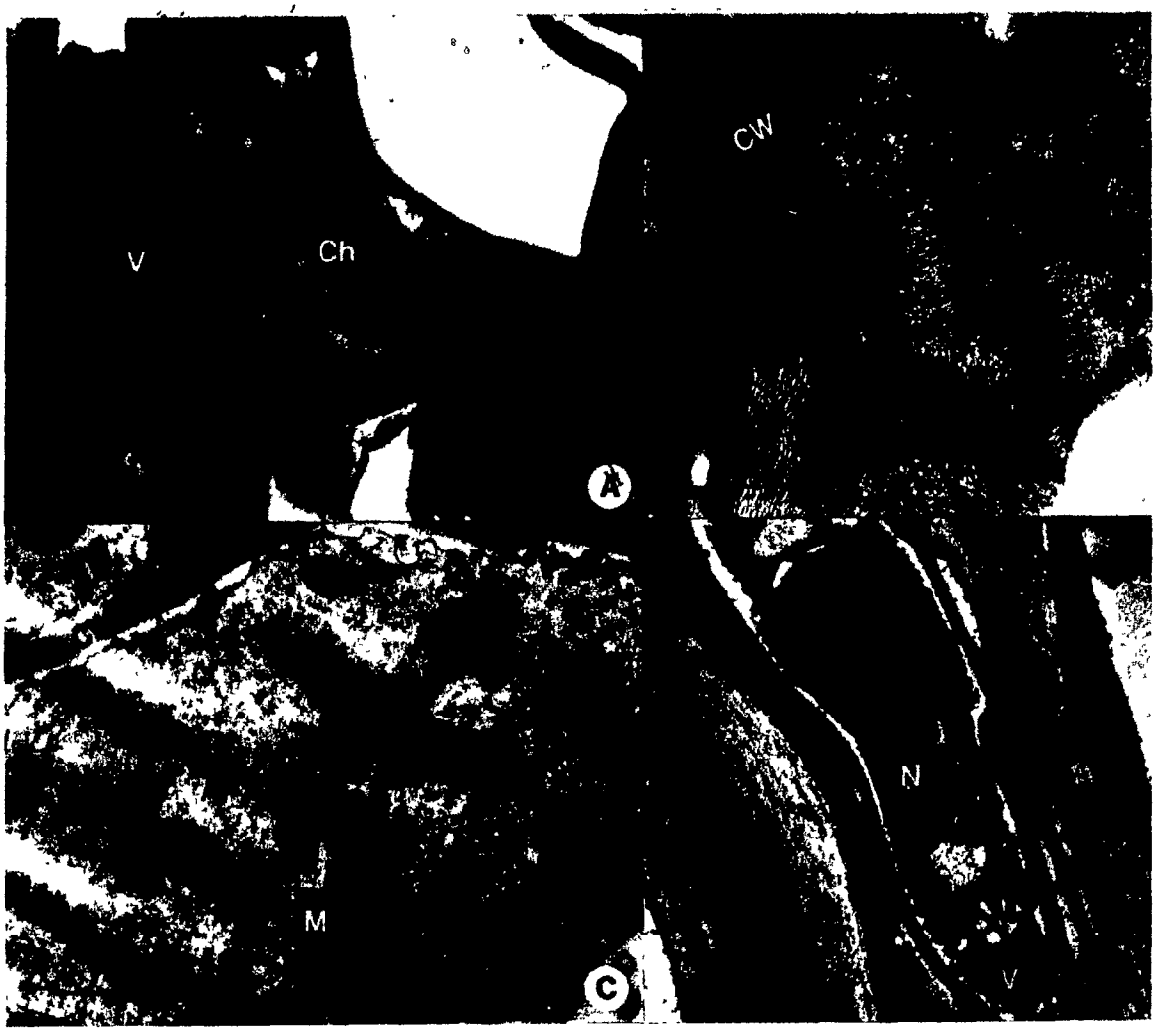
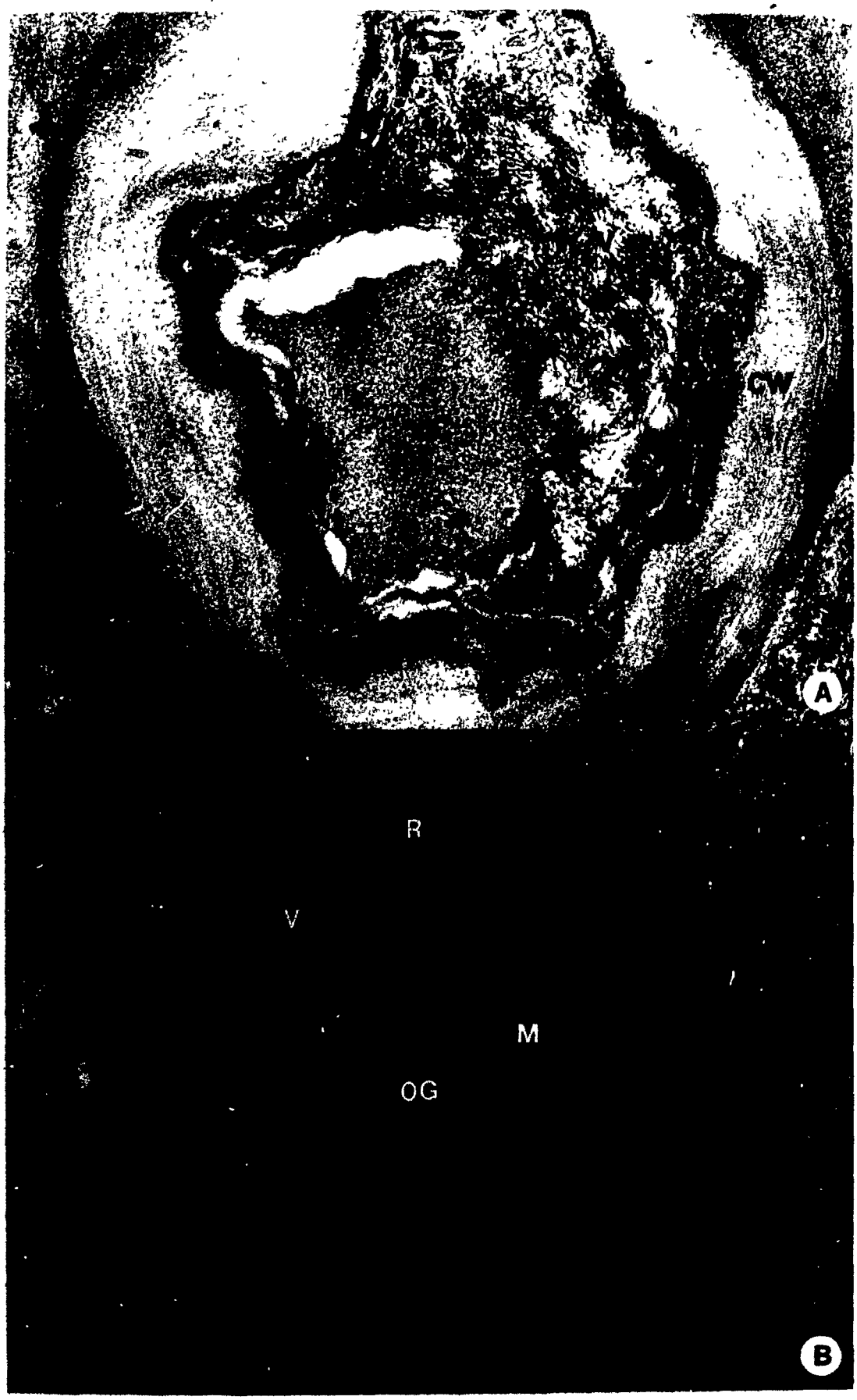

CW
V
Ch
A
N
M
C
V

Figure 10. Photomicrographs showing WCMV aggregate in the stem tissue associated with:

A. Vascular conducting cells.

B. Degenerated mitochondria.


CW
A
R
V
M
OG
B

were found sometimes in other cells of the vascular system in stem and petiole tissues (Figure 11A, B).

In the infected leaf tissue, interesting membrane-bound bodies were observed in the mesophyll cells. The bodies were elliptical or round in shape and were often found abutted to or within the proximity of chloroplasts (Figure 12A, B). They seemed to consist of two kinds of tubular masses which appeared either as convoluted tubules as in Figure 12A, B, or fairly straight tubules (Figure 12C). These inclusion bodies were rarely seen in the infected cells of petioles or stem, and were not encountered in diseased root tissues, or healthy control tissues.

3.2.4.2 Cytopathological changes

The virus infection with WCMV generally did not induce drastic changes in the shape or the size of the chloroplasts in the infected leaf tissue (Figure 13B), but in the WCMV-infected cells of petiole and stem tissues, an earlier deterioration of many chloroplasts was noticed, which was associated with the presence of numerous vesicles (Figure 13E). Although most of the mitochondria appeared normal in the infected cells (Figure 13A), some of them seemed to have been affected in that the limiting membrane disintegrated and the cristae were disorganized and distorted. An increase in the frequency of osmiophilic globules in the mitochondrial matrix was also observed (Figure 10B). In some infected cells, particularly in stem and petiole tissues, the plasmalemma was withdrawn partially or completely away

Figure 11. Some cytopathological changes induced by WCMV infection in the vascular cells of some tissues of red clover.

A. Showing the plasmalemma partially withdrawn from the cell wall in the stem tissue.

B. Showing prominent vacuolation, lower cytoplasmic contents, in some conducting cells in the petiole tissue.


M
Va
VC
V
GB
CW
PL
ER
A
M
Va
Ch
V
Va
B

Figure 12. Membrane-bound inclusion bodies in the WCMV-infected red clover leaf tissue containing:

A and B. Convoluted tubles.

C. Straight tubules.

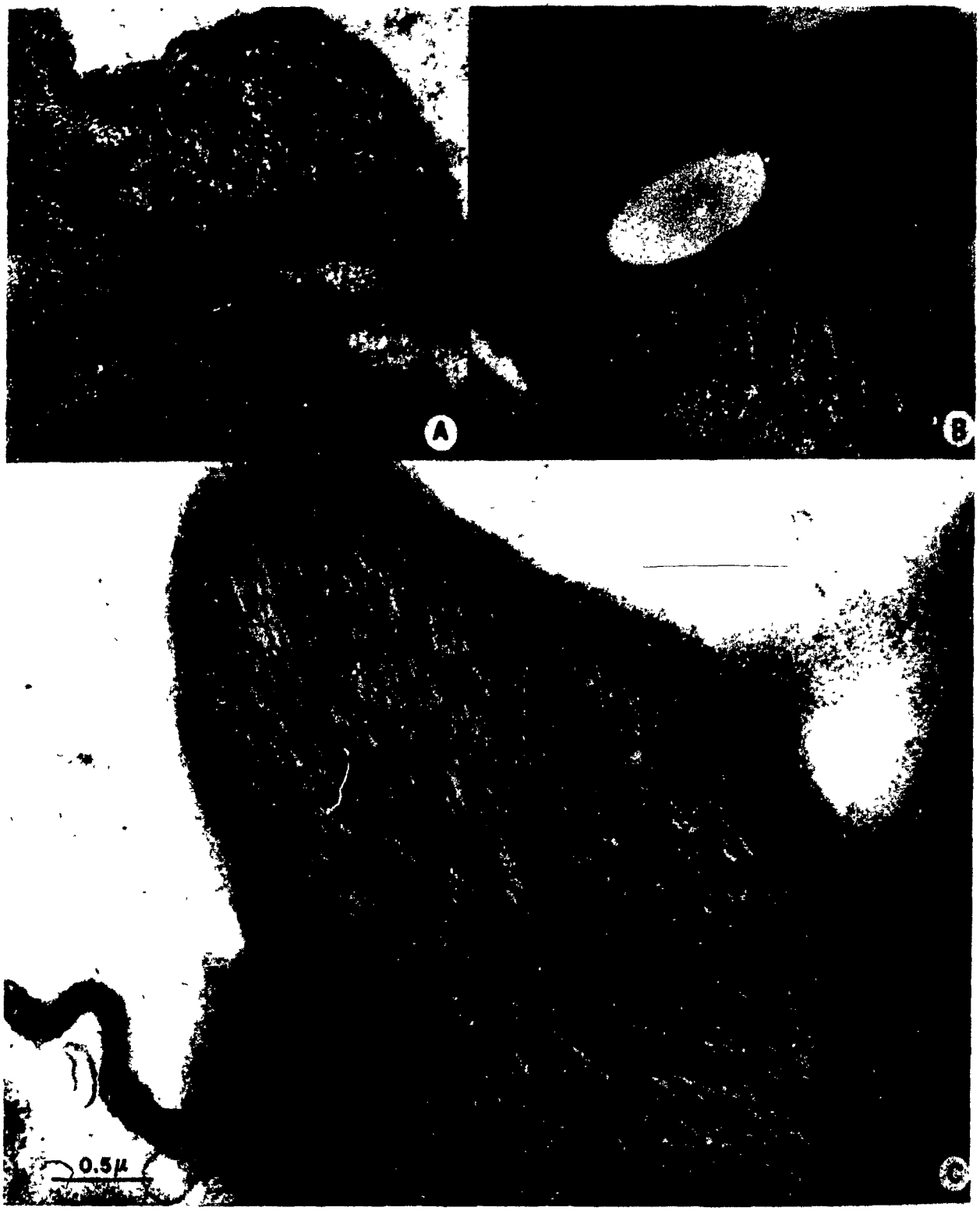

A
B
C
0.5μ

Figure 13. Various cytopathological changes in the infected red clover tissues.

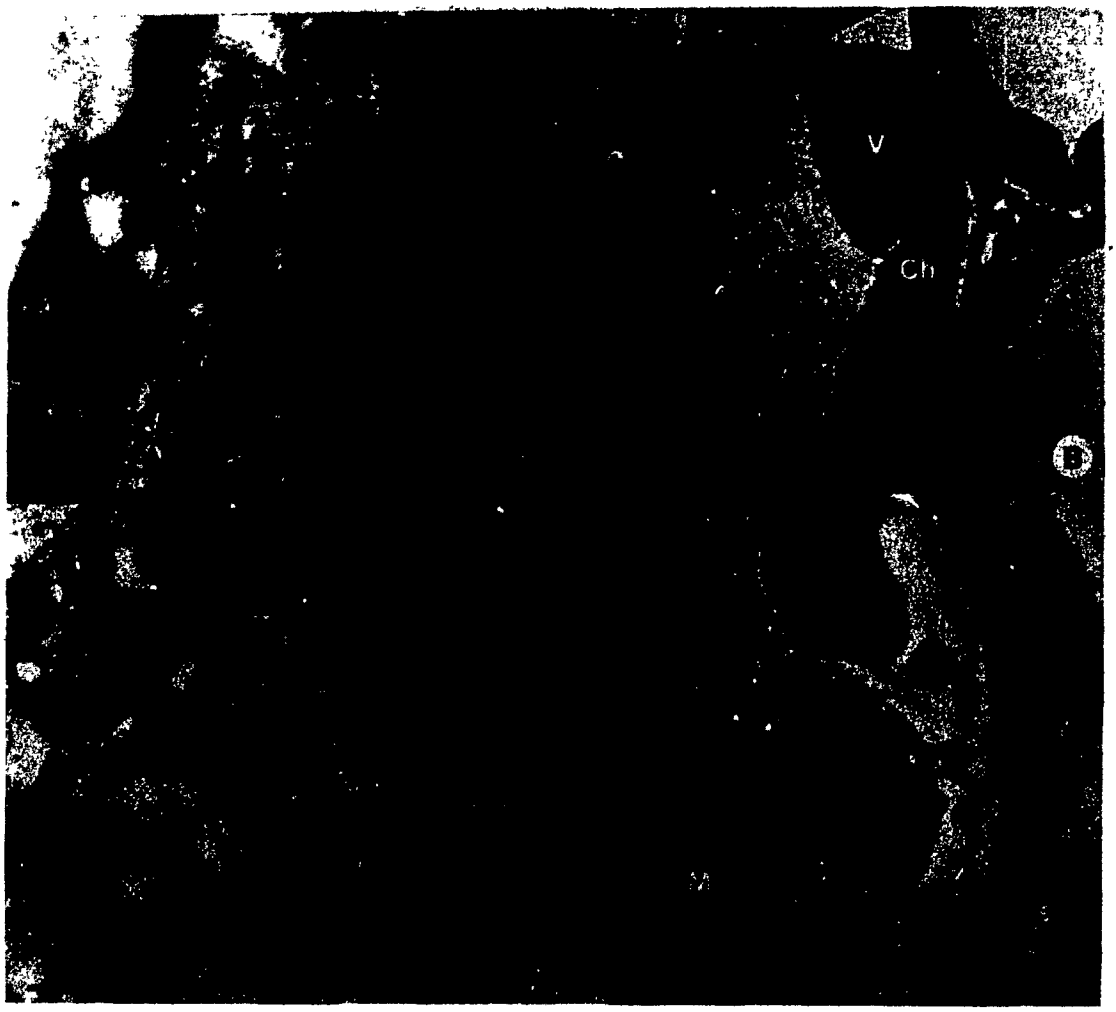
A. Showing the normal appearance of the mitochondria in the presence of WCMV aggregates.

B. Apparently normal chloroplasts in the cells of infected leaf tissue.

C. Disruption of the intracellular components in some cells of infected root tissue.

D. Accumulation of vesicles between the plasmalemma and the cell wall.

E. Deteriorated chloroplasts associated with numerous vesicles.


V
Ch
B

from the cell wall, and an accumulation of vesicles was noted between them (Figures 11A, 13D).

In the root tissues, several WCMV-infected parenchyma cells showed various cytological changes similar to those described in stem, petioles and leaves. As disease progressed, some WCMV-infected cells showed a disruption of the intracellular components, especially those cells which were rich with starch grains (Figure 13C).

3.2.5 Electron microscopy of infected pea tissues

3.2.5.1 Localization and forms of viral inclusions

Chlorotic symptoms appeared on the WCMV-infected leaves 7-8 days after virus inoculation, which was followed by severe wilting 3 days later. Thin sections of WCMV-infected pea tissues processed at 8 days after inoculation were examined. As in red clover plant tissues, large masses of WCMV aggregates occurred in the pea leaf mesophyll cells. Some infected cells showed lesser accumulation of WCMV particles, presumably as a result of the early disintegration of their contents, caused by or associated with the virus infection (Figure 14A, D).

In the infected cells of stem and petiole, the WCMV particles were organized in the same manner as in the leaf tissue. The most common viral aggregate consisted of bands of elongated virus particles with irregular spacing between these bands (Figure 15 B1). The WCMV

Figure 14. Electron micrographs showing the severe effects of WCMV infection in green tissues of pea plants, which include:

A. Deteriorated chloroplasts in the leaf tissues.

B. Complete disintegration of the chloroplasts, associated with accumulation of osmiophilic globules, leaving the thylakoids as the only recognizable remnants in the leaf tissue.

C. Rupture of the chloroplast membrane, leaving the thylakoid and the grana as the remnants. Electron dense deposits were found on the mitochondrial membrane (indicated with small arrow heads) in the stem tissue.

D. Thylakoid and the grana associated with dispersed WCMV particles in the petiole tissue.

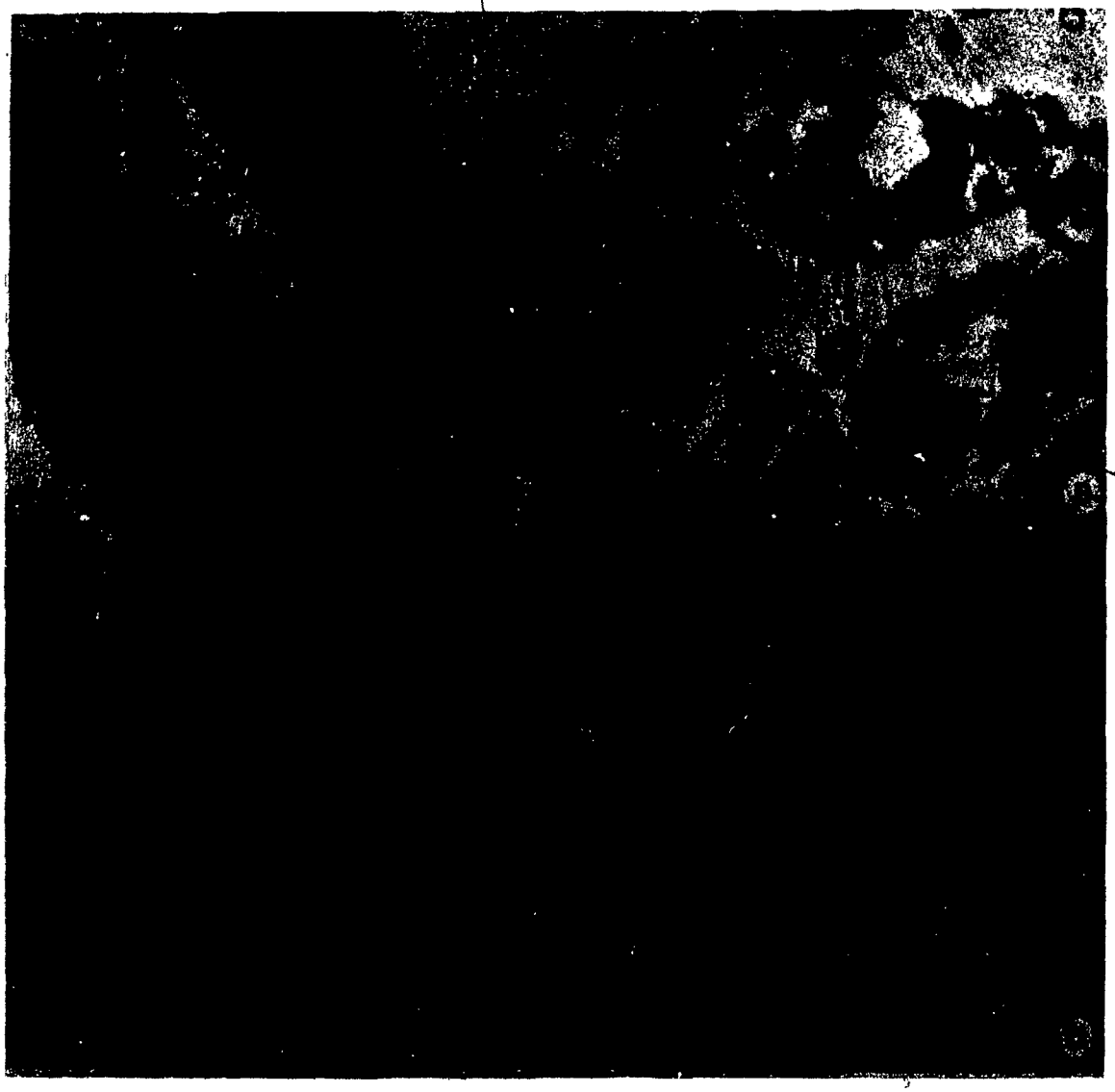


Figure 15. Electron micrographs showing some striking cytopathological changes induced by WCMV infection in pea tissues.

A. Vascular conducting cells, with apparent deterioration of cytoplasmic contents and prominent peripheral vesiculation. Small globular electron dense deposits (arrows) occur frequently in the plasmalemma of adjacent cells.

B. Malformation of the chloroplasts associated with (B1) appendages; (B2) invagination.


GB
M
N
V
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Va
A
M
Ch
Cy
μ
Ch
V
B2
B1

particles were found only in the cell cytoplasm and the organelles were devoid of them. No virus-like particles were seen in healthy plant tissues.

In the root tissue, crystalline virus inclusions were the most frequent form of WCMV aggregates observed in the cytoplasm of parenchyma cells (Figure 16A). These inclusions appeared to be three-dimensional and were relatively uniform in shape and size (Figure 16B). The length of the virus particles was approximately the same as that of the particles measured in leaf dip preparations. WCMV aggregates were found in the transfer cells also. These masses of virus particles were abutted against the cell wall and its protrusions (Figure 17A). Some proplastid-like organelles were occupied by WCMV aggregates, and surrounded by dispersed virus particles in the ground cytoplasm (Figure 17B). Infrequently, virus particles were noted in the structure resembling intercellular space (Figure 17C).

3.2.5.2 Cytopathological changes

In leaf tissue, chloroplast deterioration was characterized by compaction of thylakoids in several chloroplasts (Figure 14A). In other cells, apparently in more advanced stages of deterioration, chloroplasts were completely disintegrated and fragments of thylakoids were the only remnants of chloroplasts left in the cell cytoplasm. Many spherical deposits were characteristically associated with these chloroplast remnants (Figure 14B). The mitochondria were either malformed, with swollen cristae (Figure 14A), or unrecognizable in the disintegrated cellular contents (Figure 14B).

Figure 16. Electron micrographs of infected pea root tissue showing:

A-B. Crystalline virus inclusions (CV) in the transfer cells.

C. Large deposits on the tonoplast in the infected cell.

D. Some disintegrated cellular components associated with numerous vesicles, crystalline virus inclusions and electron dense deposits on the mitochondrial membrane (arrowhead).

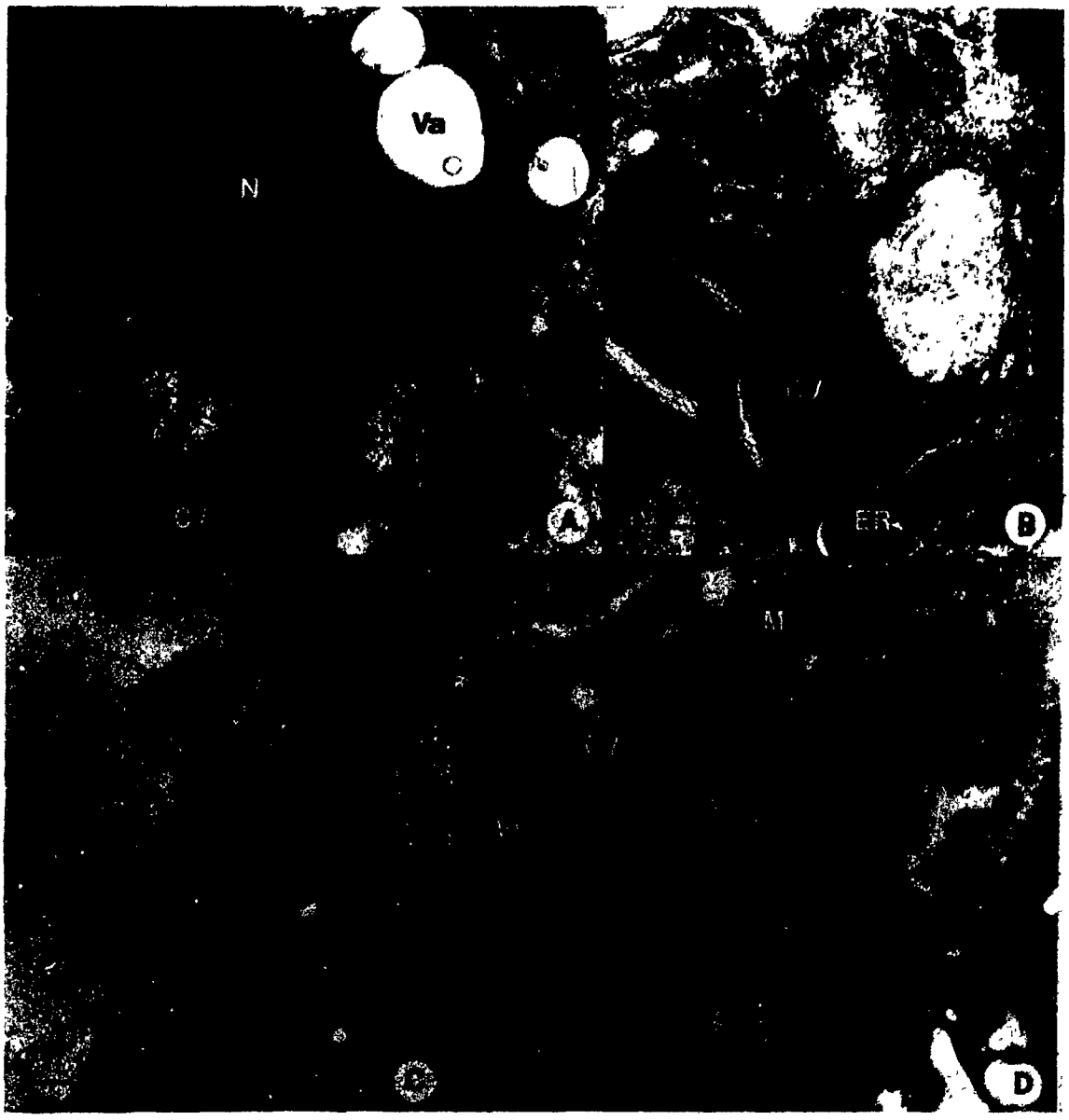
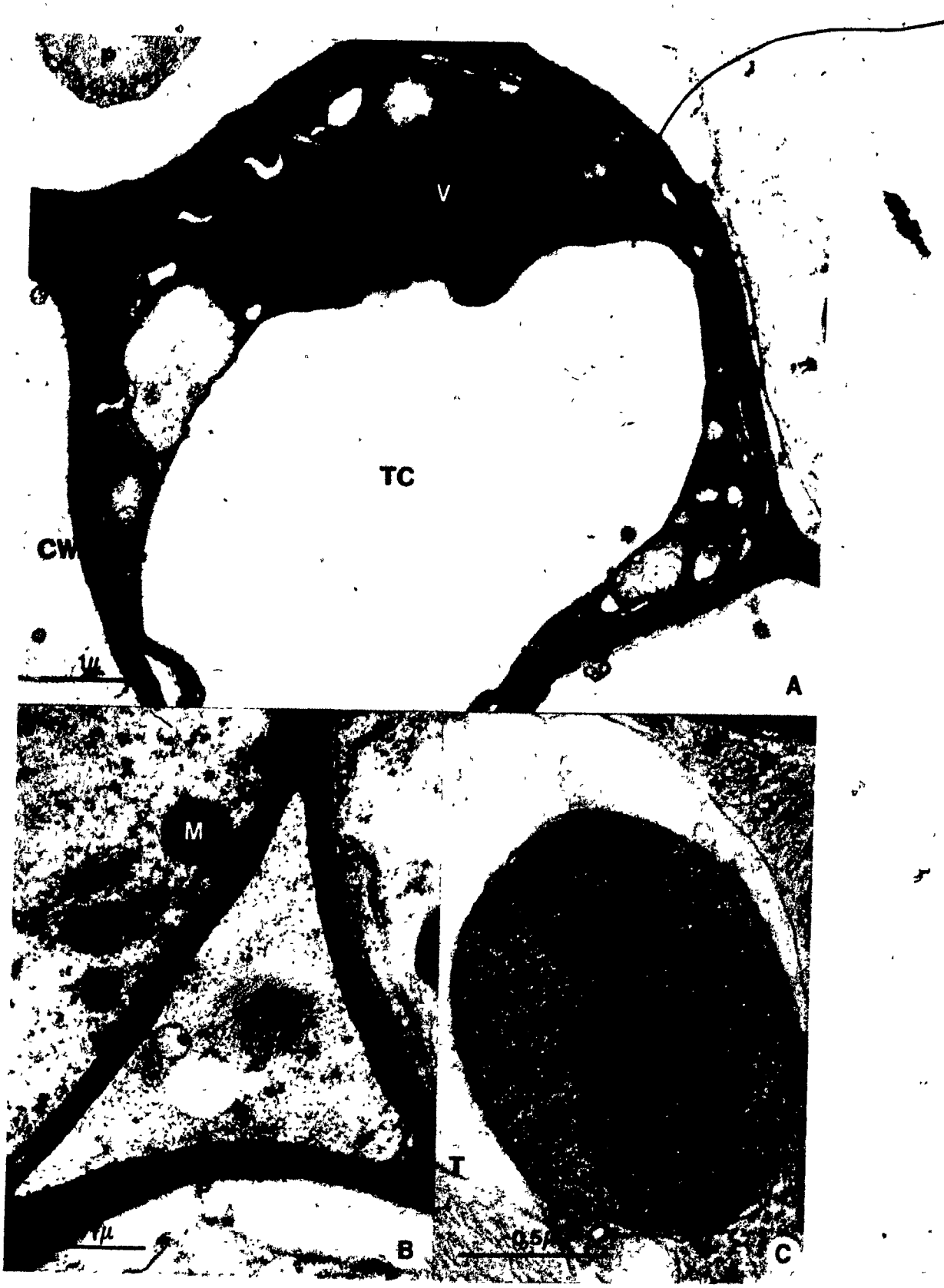

Va
N
ER
B
M
D

Figure 17. WCMV aggregates found in various arrangements in infected pea root tissue.

A. Aggregates of WCMV abutted against the cell wall of the transfer cell.

B. Virus aggregates localized in a structure resembling an intercellular space.

C. Proplastid-like organelles containing WCMV aggregates.


V
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CW
1μ
A
M
1μ
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T
0.5μ
C

Generally, similar changes, apparently not as severe as in leaves, occurred in stem and petiole tissues. The most striking changes in some severely affected cells included the rupture of the chloroplast membrane, so that the thylakoid and the grana were the only remnants (Figure 14C, D). Invaginations and appendages in chloroplasts were common in other infected cells (Figure 15B). Small, globular, electron-dense deposits along the plasmalemma were observed in vascular conducting cells (Figure 15A). Since these generally occurred in cells which had dense cytoplasm and organelles with a normal appearance, the small deposits may represent one of the early responses to virus infection. In infected cells of both petiole and stem tissues, some mitochondria became conspicuous due to electron dense deposits around their limiting membrane (Figure 14C).

The changes in the root tissue of WCMV-infected pea plants consisted of rounded deposits (as seen in 2 dimensions) on the intracellular organelles and cytoplasmic membranes. Some of these deposits were noticed on the tonoplasts (Figure 16C), as well as on proplastid-like organelles (Figure 18C). Similar deposits were also observed on the mitochondria, which resembled those found in the infected petiole and stem tissues (Figure 16D). In the healthy tissue, some proplastid-like structures contained crystals (Figure 18B), but packed rods and crystals (with a finer spacing) were also seen in WCMV-infected roots (Figure 18D). Meanwhile, irregularly shaped, distorted organelles, which may have been deteriorated proplastids, were observed in this tissue (Figure 18A).

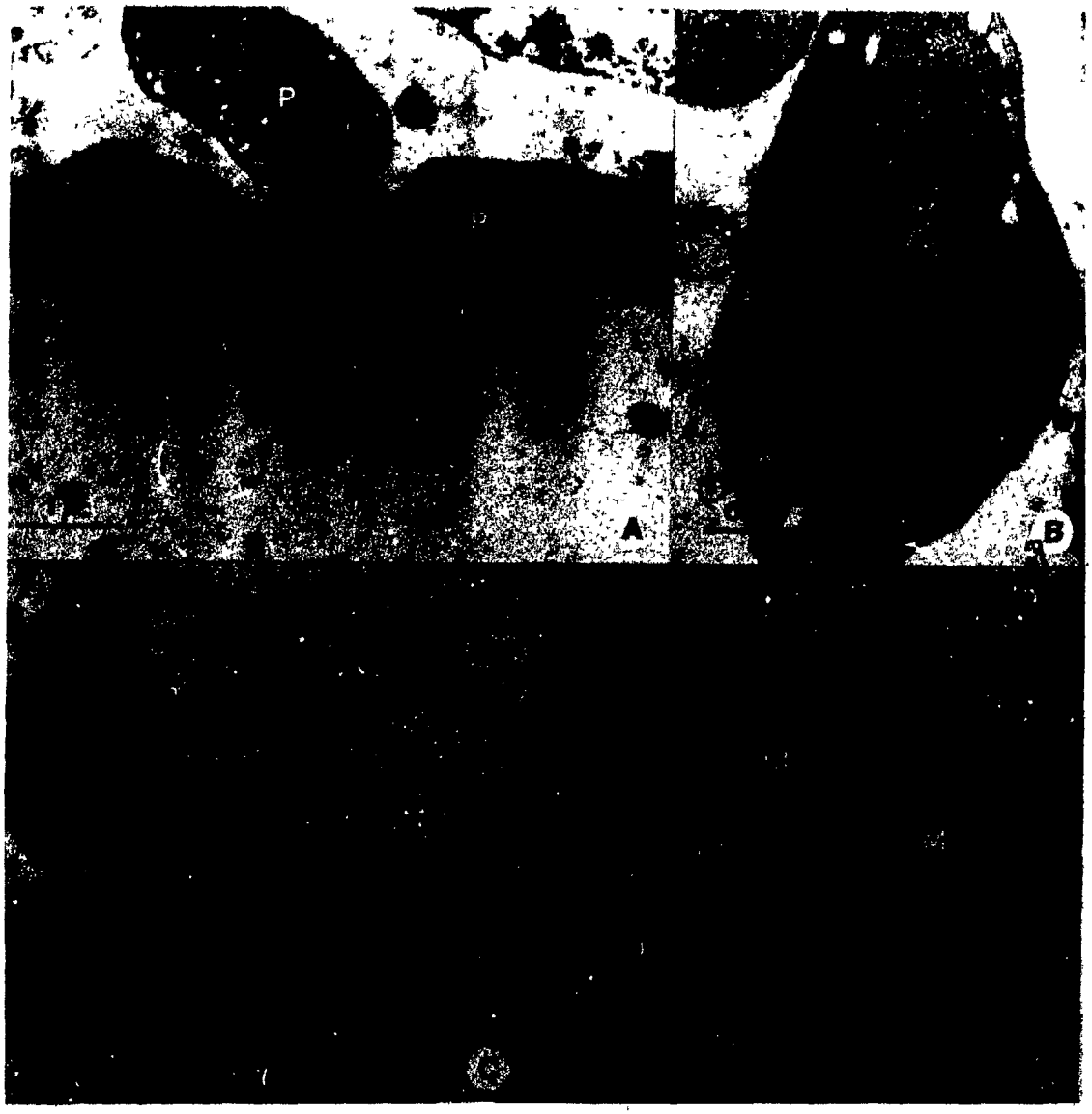
Figure 18. Electron micrographs showing the cytopathological changes induced by WCMV infection on some organelles in the infected pea root tissue.

A. Deteriorated proplastids containing osmiophilic globules.

B. Proplastid-like structure, containing crystal.

C. Degenerated cellular components associated with numerous vesiculated bodies, and electron dense deposits on the membrane of proplastid-like organelles (arrowheads).

D. Intramitochondrial crystals in diseased tissue, which appeared as packed rods.


P
A
B

3.3 Discussion and conclusions

Light microscopy revealed amorphous inclusions induced by WCMV in the epidermal cells of pea and red clover leaves. These inclusions, which might be aggregates of virus particles, or combination of viral and host cell materials, generally resemble those formed during infections by some other potexviruses, such as CYMV in pea (Christie and Edwardson 1977; Hiruki et al. 1976) and a different isolate of WCMV (Iizuka and Yaroki 1975) in white clover leaves. The presence of virus particles, or at least of polymerized virus coat protein, in these inclusions was indicated by their detection by indirect immunofluorescence, a technique which has not been applied for the detection of other potexvirus inclusions. The immunofluorescence microscopy results resemble those reported by workers who have used direct immunofluorescence for detection of CYMV viral inclusions in pea leaves (Rao et al. 1978).

The electron microscopic examination elucidated patterns of virus localization and aggregation, and also showed that several types of inclusions and cytopathological alterations were associated with WCMV infection of pea and red clover plants. Many of the observations made in the present study are generally similar to those described in studies of other members of the potexvirus group, e.g., PVX, CYMV, PaMV, CyMV, CVX, NMV, CCMV, PAMV, and HRV (Christie and Edwardson 1977).

The WCMV particles were found in several configurations in cells of the infected tissues, the most frequent being the banded virus aggregates. These banded virus aggregates have been suggested as a

main diagnostic characteristic of infection by the potexvirus group (Doraiswamy and Lesemann 1974; Christie and Edwardson 1977). Spiral forms of WCMV particle aggregates were observed in the petiole tissues of both legumes, and only in the stem tissue of pea. This type of virus inclusion is similar to that induced by CYMV in the pea leaf tissue (Purcifull et al. 1966; Schlegel and Delisle 1971; Hiruki et al. 1976).

Certain arrangements of WCMV particles and/or their inclusions were seen only in specific cells or tissues. It is perhaps noteworthy that the crystalline pattern of WCMV particles occurred only in the parenchyma cells of the infected pea root tissues. Since the crystals were absent in other tissues, it can be suggested that only the root cells provide the conditions suitable for crystallization of virus particles. Such conditions could include the alteration of water content in the roots after virus infection (Martelli and Russo 1977). Although Hiruki et al. (1976) noted spiral forms of CYMV particles in the transfer cells of infected pea stem tissues, they did not consider them as crystalline inclusions.

Only a few cases of nonfilamentous viruses in the xylem are known (Schnieder and Worley 1959; Chambers and Francki 1966). Therefore, it is difficult to explain the occasional occurrence of WCMV in conducting cells, some of which are probably xylem cells. Since the virus was not found in these cells consistently and only low concentrations occurred, this vascular tissue cannot be considered as the route of virus translocation in the plants. However, a likely

explanation may be that virus particles seen in the conducting cells are vestiges of the virus that may have been synthesized earlier in these cells before they differentiated as xylem and matured.

Presence of WCMV particles in the proplastid-like organelles of infected pea root tissue is noteworthy. The plastids have been described as an adequate nutritional source in the plants (Menke 1962), and were postulated as possible sites of TMV multiplication in tobacco leaves (Zaitlin and Boardman 1958). The gradual wilting of infected pea leaf tissue due to WCMV infection probably created a physiological condition that affected the virus survival in the infected cells of root tissue. Therefore, the virus particles might have compartmentalized in a membrane bound body (i.e., proplastid-like organelles) to keep them separate from other cellular contents, which may be a manifestation of a host cell defense reaction.

Since the bulk of virus particles was found in the cytoplasm of WCMV-infected parenchyma cells of various tissues, it seems logical to assume that virus particles found in the mature phloem and transfer cells, were synthesized while those cells were immature. It would appear that virus particles could assemble in the cytoplasm only by active movement from living cells, probably through plasmodesmata. These observations are identical to the reported information of the assembly of TMV (Shalla 1964; Milne 1966), and on replication of ryegrass mosaic virus (RGMV) in the cytoplasm of the infected cells of ryegrass leaves (Paliwal 1975).

The membrane-bound inclusions of tubules, which were either straight or convoluted, were seen most frequently in infected red clover leaf tissue. The tubules in these inclusions were distinct from virus particles and were clearly differentiated from them when seen in cross section. This type of inclusion has not been reported previously in descriptions of cytological changes associated with potexvirus infections, but similar masses of convoluted tubules were observed in wheat leaf cells infected by soil-borne wheat mosaic virus (Peterson 1970). As Diener (1963) has pointed out, pathogensis during virus infection is essentially a genetic phenomenon. The formation of types of inclusions atypical for a particular virus could result from incorporation of virus genetic determinants with the corresponding ones of the host cell. The tubules could represent host cell material, or alternatively, they could represent some precursor polymerization state of excess virus protein.

The effects of WCMV infection on the normal cellular organelles were of considerable interest in both host legumes. It seems logical to assume that the early breakdown of the chloroplasts in the WCMV-infected leaf tissue of pea was consistent with the severe symptoms which developed in this tissue. Less damage was seen to this organelle in the infected green tissues of red clover. Degeneration of chloroplasts occurs in many systemic plant virus infections, e.g., TMV (Esau and Cornshaw 1967), CMV (Porter 1954) and RGMV (Paliwal 1975). The extrusions and protrusions on the chloroplasts of WCMV-infected pea petioles and stem tissues observed in this study are

similar to those observed in TMV-infected tomato cells (Shalla 1964) and in BYMV-infected broad bean tissue (Weintraub and Ragetli 1966).

The other marked effects of WCMV infection on the cell organelles also included alterations in mitochondria. Degeneration of the mitochondrial limiting membrane, disorganization and proliferation of cristae and an abundance of osmiophilic deposits in the mitochondrial matrix and membranes constituted the major changes, suggesting that virus infection would have affected the respiration and energy metabolism of infected plants. Such metabolic effects are known to be associated with other plant virus infections (Diener 1963). The presence of amorphous deposits on the plasmalemma in the WCMV-infected petiole tissue of pea may reflect an increase in hydrolytic enzyme activity, as suggested earlier for CYMV (Tu 1976).

The immediate cause of degeneration of cells in a virus-infected plant is still not clear, especially in view of the observation that virus particles are not always present in the degenerating cells. Moreover, cells containing virus, sometimes large amounts of it, may show apparently normal host cytoplasm (Esau 1968). However, the presence of the virus infection in one cell may affect the metabolism in other noninvaded adjacent cells.

SECTION 4

EFFECT OF WCMV ON N_2 FIXATION AND OTHER CHARACTERISTICS IN RED CLOVER

4.1 Materials and methods

4.1.1 Isolation of Rhizobium trifolii

Rhizobium trifolii was isolated from nodules of healthy red clover plants at the experimental farm in Ottawa. A number of nodules were excised and rinsed in running tap water while wrapped in cheese-cloth to remove gross surface contamination. Subsequently, these nodules were immersed 3 or 4 times in 0.1% $HgCl_2$ to achieve surface sterilization. The nodules were then thoroughly washed in at least 6 changes of sterile distilled water. Each of several nodules was crushed in a drop of sterile water between two sterile slides. A wire loop was dipped into the milky nodular fluid and streaked on the surface of a plate of yeast-extract-mannitol agar (Appendix 2). The plates were incubated at 25°C and checked for colony development conforming with that expected of Rhizobium along the streaking pattern. Each isolated single colony was increased on a slant of yeast-extract-mannitol agar, which was incubated at 25°C for 3 days, and then used directly as an inoculant.

4.1.2 Plant culture

Red clover seeds (cv. Ottawa) were surface sterilized by immersion in 0.5% sodium hypochlorite (Javex) for 10-15 minutes under vacuum After thorough rinsing with at least 8-10 changes of sterile water, or until the Javex smell disappeared, the seeds were distributed on plates containing sterile moist filter papers, and incubated at 25°C for 48 hours.

Plastic growth pouches (Figure 19A) were prepared by cutting 5 equidistant 2 mm holes in the bottom of the «V» of a paper wick and inserting two 6-inch applicator sticks to provide support and facilitate aeration of the contents. To each pouch 60 ml of N_2-free nutrient solution (Appendix 3) was added. Twenty-five such pouches were placed in a 6-inch wire basket, which was covered with aluminium foil and autoclaved.

Seedlings with 1.0-1.5 cm radicles were selected and transferred aseptically into the «V» of the paper wick at the rate of 5 seedlings per pouch. The seedlings were inoculated by pipetting 2 ml of rhizobial suspension (approx. 1.0×10^8 cells/ml) on the radicles. Each container basket was covered with a sterile transparent plastic bag to reduce evaporation and eliminate cross contamination. Pouches containing uninoculated plants served as the control in each experiment.

The plants were grown in a controlled environment room maintained at 24 ± 1°C under a 16-hour light intensity of 200-220 $\mu E\ m^{-2}\ s^{-1}$ at the leaf surface, provided by cool white fluorescent tubes supplemented

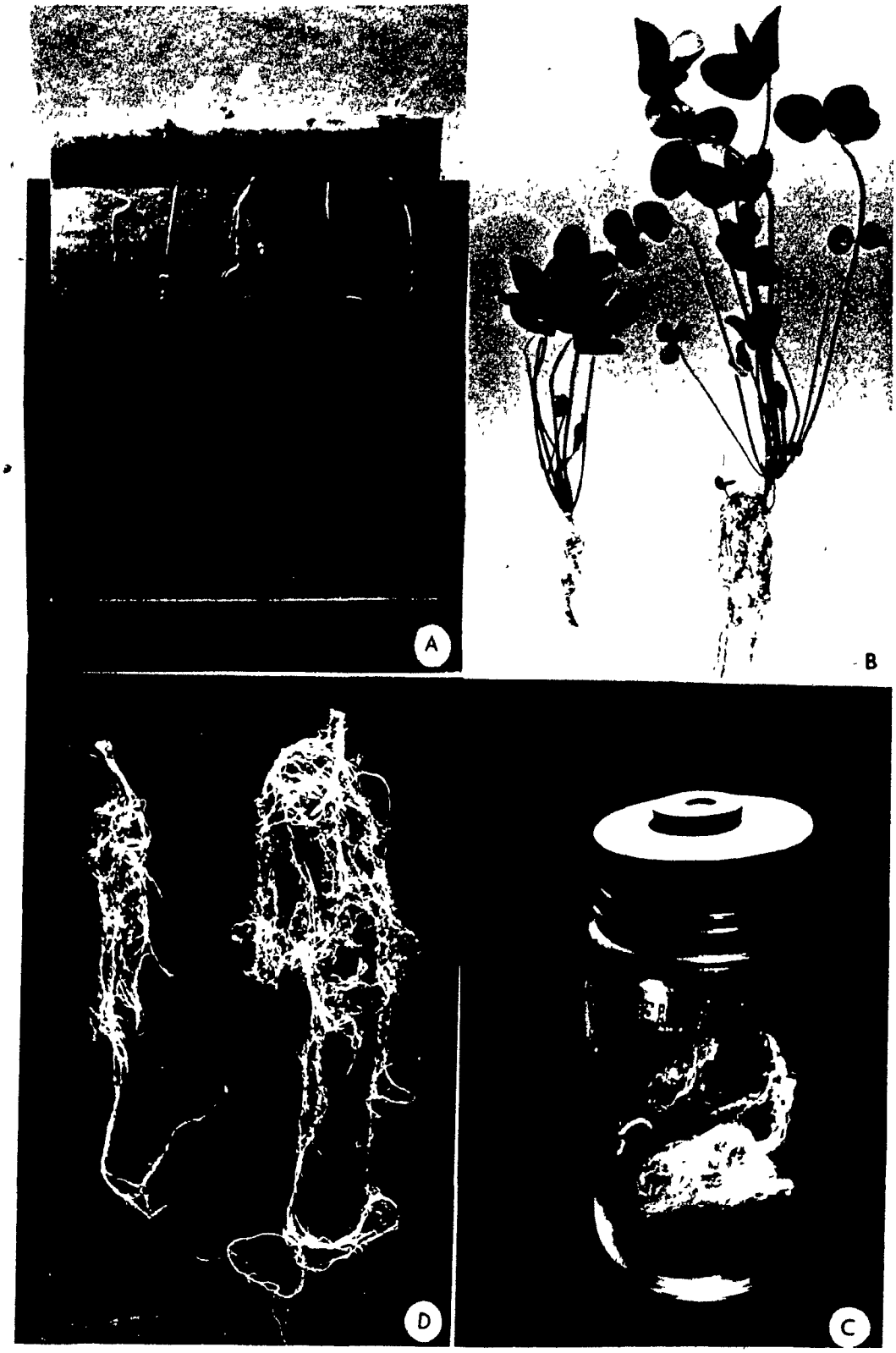
Figure 19. Some materials used in this investigation.

A. Plastic pouch used for holding five red clover seedlings after inoculation with Rhizobium suspension.

B. Red clover plants, after washing the roots. The plant on the left is infected and the right one is healthy.

C. A 210 ml jar, capped with plastic stopper, used for determination of the nitrogenase activity per plant.

D. Comparison of WCMV infected (left) and healthy (right) red clover root systems.


A
B
D
C

with incandescent bulbs. Two days prior to transplanting, the plastic covered baskets were removed to permit maximum illumination. The seedlings were transplanted two weeks after inoculation, into autoclaved sand in 4-inch sterilized plastic pots (one plant/pot). A total (Kjeldahl) nitrogen content of 0.032% was found in the sand before transplanting (Chapman and Pratt 1961). After transplanting, the plants were kept in two growth chambers (cabinets) under controlled conditions of 16 hours light per day with 24°C/19°C day/night temperature and a relative humidity of 75-80%. Deionized water was added twice daily, and nitrogen-free nutrient medium was added weekly.

4.1.3 Experimental design and statistical analysis

A factorial experiment was conducted using two growth cabinets (i.e., two replicates), and repeated in order to obtain four replicates. In each replicate (cabinet) 40 red clover plants, inoculated with WCMV by the procedure described earlier, were distributed randomly among an equal number (40) of virus-free plants, which were considered as healthy controls. The day of inoculation of red clover with WCMV, subsequently designated as infected plants, was considered the zero-time index, after which the plants were sampled for observations at four harvest times, i.e., 3, 6, 9 and 12 weeks. These times corresponded sequentially to the ten-leaf stage, preblooming, blooming and flower senescence. An analysis of variance was carried out on all characteristics measured, assuming a design with 4 replicates, and 8

treatment combinations which resulted from two factors, the first with 2 levels (healthy vs. infected) and the second with 4 levels (four harvest times).

4.1.4 Yield assessment

At each harvesting time, an equal number (10) of healthy (virus-free) and infected plants (Figure 19B) were collected randomly from each cabinet. The sand was washed off all plant roots with slowly running water, which was passed through a sieve to collect all of the root fragments and detached nodules. The roots were dried by blotting with paper towels and were subsequently detached from the shoots. Root and shoot fresh weights were recorded separately. The data are presented as means of 4 replicates of 40 plants in each treatment.

Nodules were detached from roots with tweezers under a table-top magnifier-illuminator after they had been processed for nitrogenase (C_2H_2) activity assay. Subsequently, they were counted and weighed. The nodules used for other physiological determination were kept in capped vials in an ice bath.

4.1.5 Nitrogenase (C_2H_2) activity assay

The acetylene reduction test was used to determine the nitrogenase activity of each plant. The assay was done by placing the excised roots (Figure 19D) in incubation chambers (Figure 19C). The incubation chambers were air-tight glass jars, 210 ml capacity, with a rubber seal in the lid. Hypodermic syringes were used for injection or removal of

gas samples through the rubber seal. One-tenth of the volume of the air present in each jar was withdrawn with a syringe and replaced with an equivalent volume of C_2H_2. Acetylene was generated in situ by the reaction of some calcium carbide with tap water, as described by Sirois and Peterson (1982). The intact nodules were incubated for 45-60 min at room temperature, and 0.5 ml of the gas was then withdrawn from each jar and injected into a Carle 9700 gas chromotograph with a column filled with 80-100 mesh Porapak N.

The amount of ethylene produced in each sample was determined by comparing the measured peak height to a standard C_2H_4 concentration curve constructed (Appendix 4) as described by Burris (1974). The nitrogenase activity was expressed in nmoles of ethylene (C_2H_4) produced per plant per hour. The data are presented as means of activities averaged from 4 replicates of 40 nodulated roots for each treatment.

4.1.6 Leghemoglobin determination

4.1.6.1 The fluorometric method

A fluorometric method, described by La Rue and Child (1979), was applied to measure the leghemoglobin content of the nodules. Ten mg nodules from each plant were ground with a pestle and mortar in 10 ml of an extracting solution containing 0.02% potassium ferricyanide and 0.1% sodium bicarbonate. The homogenates were subsequently centrifuged at 9,000 rpm (9,750 g) to remove debris, using a SS-34 rotor in a Sorval RC-2B refrigerated centrifuge for 10 minutes, after which 0.5 ml of clear supernatant was drawn off and mixed with 4.5 ml saturated

oxalic acid solution in a 5 ml capped hexagonal-base microflask (Fisher Scientific, Ltd., No.5630). The mixture was autoclaved at 120°C for 30 minutes and then cooled to room temperature. The fluorescence of the sample from each plant was measured with a Hitachi MPF-2A fluorescence spectrophotometer. The excitation wavelength was 405 nm and the excitation slit was set at 32 nm, while the emission wavelength and slit were 595 nm and 10 nm, respectively. A similarly heated mixture of extracting medium and oxalic acid was used as a blank. The fluorescence readings were converted into leghemoglobin content by comparing them with a standard concentration curve of beef blood hemoglobin (BDH Ltd., product No.26111) shown in Appendix 5. The data are presented as means of µg hemoglobin per mg nodule fresh weight, averaged from 4 replicates each of forty single plants per treatment.

4.1.6.2 Cytochemical detection of leghemoglobin

The ultrastructural-cytochemical procedure reported by Graham and Karnovsky (1966), which was later applied by De Zoeten et al (1973) and Gourret (1975), was used to detect leghemoglobin in the bacteroids in both healthy (virus-free) and WCMV-infected nodules of red clover. Small pieces of nodules corresponding to regions 2 and 3 (Mosse, 1963) were fixed in 3% glutaraldehyde in 0.1 M Na-cacodylate buffer, pH 7.2 for 45 minutes at 4°C. Subsequently, the fixative was removed and the nodules were rinsed with cacodylate buffer for 3 changes, 10 minutes each. The tissue pieces were then rinsed vigorously for 40 minutes

with 0.05 M propandiol buffer (2-amino-2-methyl-1,3-propanediol) at pH 9. The nodular pieces were then incubated in the standard medium described by Novikoff (1970), containing diaminobenzidine (DAB), for 60 minutes in a water bath at 37°C, with occasional swirling to prevent tissue pieces from floating to the top. After incubation, the tissues were rinsed thoroughly with 3 changes, 5 minutes each, of cacodylate buffer, and then immediately fixed for 2 hours in 1% OsO_4 in cacodylate at room temperature. The nodules were then washed with cacodylate buffer for 20 min, followed by rinsing with distilled water for 3 changes, 20 minutes each. The dehydration, infiltration, and embedding were carried out as previously described for other tissues of red clover and pea. No further staining was done after ultrathin sectioning of the embedded nodular tissue; grids were washed with double distilled water.

4.1.7 Nitrate reductase assay

The procedure applied for the determination of this enzyme's activity, in both healthy and WCMV-infected nodules of red clover, was similar to that reported by Jaworski (1971) and used by Vance et al (1979). Approximately 200 mg of fresh intact nodules per replicate were rinsed with cold tap water, followed by cold deionized water, and then transferred to capped vials containing 5 ml of incubation medium (0.1 M phosphate buffer, pH 7.0; 0.1 M KNO_3 and 0.07% tergitol, BDH Chemical Ltd.). The nodules were then vacuum infiltrated 3 times, 2 minutes each. The vials were then kept in an incubator at 30°C, and

the nitrite released into the medium was measured at zero time, and at various intervals (4 days apart) after each harvest. Five samples from each treatment were prepared by adding 0.1 ml of the suspension medium containing the nodules to 0.45 ml each of 1% sulfanilamide in 3 N HCl and 0.2% N-1-naphthyl-ethylenediamine-HCl, and all the samples were incubated for 20 min at room temperature. Subsequently, the O.D. at 540 nm was recorded with a Spectronic-70 colorimeter. The readings were converted into nmole nitrite per hr.gm. fresh weight by comparing them with a standard concentration graph of sodium nitrite, shown in Appendix 7.

4.1.8 Determination of acid phosphatase activity

4.1.8.1 Colorimetric method

The acid phosphatase specific activity in two samples was determined in nodules of healthy and WCMV-infected red clover plants using the procedure applied by Tu (1976). The extracts were prepared by grinding 0.2 g of each tissue sample in 5 ml of distilled water in precooled pestles and mortars. The homogenate was diluted 10-fold with water and filtered through double layers of cheesecloth. About 0.5 ml of each crude extract was mixed with 5-8 ml of 0.1 N NaOH in separate tubes as crude sap controls. Two sets of five tubes, each containing 1.0 ml of incubation medium, were used for assaying each sample. The incubation medium was prepared by addition of 0.5 ml of 0.012 M p-nitrophenyl phosphate to 0.5 ml of citric acid, pH 4.8. A

series of healthy or diseased crude saps of 0, 0.05, 0.1, 0.2 and 0.4 ml were added sequentially to the five tubes. The first tube with no crude sap was considered as a blank control for the test. The tubes were then placed in a rack and immersed in a water-bath at 37°C for 30 minutes, and then 4.8 ml of 0.1 N NaOH was added to each tube to stop the reaction. A Bausch and Lomb Spectronic-70 colorimeter was used to read the O.D. of each solution at 405 nm. The data obtained were converted into µg of p-nitrophenol released by comparison with a standard concentration curve (Appendix 6) of p-nitrophenol (99.8% purity, Sigma Chemical Co.). The results are averaged from 2 series of 5 samples per replicate.

4.1.8.2 Cytochemical method

Acid phosphatase activity was detected quantitatively in ultrathin sections of pelleted lysosomes, extracted and treated as described by Tu (1976). Approximately 25 g of healthy and WCMV-infected red clover leaves were ground in 50 ml of an extraction medium, consisting of 0.44 M sucrose, 0.003 M EDTA, 0.05 M Tris-HCl, and 0.1% bovine serum albumin, pH 7.2. The suspension was filtered through double layers of cheese-cloth and the filtrates were clarified by two cycles of low speed centrifugation at 2000 rpm (150 g) and 8000 rpm (7719 g) for 10 minutes each, respectively. Pellets were obtained from the final supernatant by centrifugation at 10,000 rpm (12,062 g) for 10 minutes, and each pellet of partially purified lysosomes was homogenized in 0.5 ml of the extraction medium.

For cytochemical localization of acid phosphatase, the suspension was mixed with an equal volume of 2% Noble agar (w/v) at 45°C, and the mixtures were allowed to gel at room temperature. Small pieces of the agar blocks were then fixed for 10 minutes in 1% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0. The blocks were then washed twice for 10 minutes each in the same buffer, and further fixed for 5 minutes in 1% OsO_4 in cacodylate buffer. They were then washed for an hour in three changes of Tyrode's solution (0.15 M NaCl, 0.003 M KCl, 0.002 M $CaCl_2$, 0.001 M $MgCl_2$, 0.006 M glucose, 0.012 M $NaHCO_3$, and 0.0004 M NaH_2PO_4). The healthy and infected samples were then each divided into two groups. One group was incubated overnight (14 hours) in Gomori's acid phosphatase substrate mixture, i.e., 13.6% acetate buffer (pH 4.7–5.3), 5% lead nitrate, 2% sodium alphaglycerophosphate (Gomori 1941) in a water bath at 37°C, while the other (control) group was similarly incubated in the same mixture to which 0.01 M sodium fluoride, an inhibitor of acid phosphatase, had been added.

The samples were then washed in Tyrode's solution and rinsed for 2 minutes in 2% acetic acid. Lead sulfide, an electron dense material, was then formed in the tissues by covering them with 2% ammonium sulfite for 1 minute. The agar blocks containing the tissues were then rinsed with Tyrode's solution for 2 minutes, and later they were dehydrated, infiltrated, and embedded in resin for sectioning and electron microscopy, as described earlier.

4.1.9 Rhizobium population evaluation

The plate counting of Rhizobium colonies was done on both healthy (virus-free) and WCMV-infected red clover nodules. Approximately 200 mg of nodules from each replicate were surface sterilized with 0.5% sodium hypochlorite for 10 minutes, followed by rinsing with sterile distilled water for 5 changes of 2 minutes each. The disinfested nodules were then aseptically transferred under a laminar flow chamber into 200 ml of autoclaved 1% mannitol solution (w/v) and comminuted for 5 minutes with a Heidolph homogenizer at 2000 rpm to release rhizobia from the nodular tissue. Four ten-fold serial dilutions with 1% mannitol solution were made from this stock. Subsequently, 0.1 ml of each dilution was pipetted onto the surface of a hardened congo-red yeast-extract mannitol agar plate (Appendix 2). The inoculum was spread with a sterile glass triangle to cover the entire surface of the medium. Six plates were used for each dilution of each replicate. The plates were incubated at 27 ± 1°C for 4-5 days to count the visible colonies. The observation on dilution 10^{-3} was finally used for comparison of the healthy and WCMV-infected nodules. The results represent the mean of colony number per mg nodular tissue averaged from 2 series of 6 plates per replicate.

4.1.10 Assay of WCMV in nodules

4.1.10.1 Infectivity assay

To determine the dilution end point of WCMV in the nodular tissue of red clover, about 100 mg of freshly harvested nodules from healthy and infected plants were used for preparation of inoculum as described in section 2.1.3. After each harvest, four ten-fold dilutions (10^{-1}-10^{-4}) were made of each inoculum stock, and manually inoculated on half leaves of *G. globosa*. The inoculum residue was washed off with distilled water, and the plants were kept in the same growth chambers used for growing red clover. The infectivity assessment was done after 48 hours when necrotic local lesions were apparent. Data are presented as lesion number per leaf averaged from ten half leaves which were used for each inoculum.

4.1.10.2 Immunosorbent electron microscopy (IEM)

The procedure of Derrick and Brlansky (1976) was used for detecting WCMV in crude sap of infected red clover nodules. Antiserum against this virus, which had been prepared earlier, was diluted 10^{-1}-10^{-3} in 0.05 M Tris-HCl, pH 7.2, containing 0.15 M NaCl (Tris-NaCl). Nickel grids (400-mesh), with diamond shaped openings, were coated with film (0.5% solution of parlodion in amyl acetate) and stabilized with carbon. The grids were floated on drops of the diluted antiserum for 30 minutes, followed by washing them in a stream of Tris-NaCl buffer to remove nonadsorbed serum protein and then with double distilled water.

Subsequently, the grids were not allowed to dry, but immediately were placed on drops of homogenate saps for 1 hour. The homogenates were prepared by grinding 50 mg of fresh nodules in 5 ml of Tris-NaCl buffer, and clarification by low speed centrifugation at 5000 rpm (3015 g) for 5 minutes, after which the supernatant was collected. The grids then were washed thoroughly in a stream of double distilled water, floated on drops of 1% uranyl acetate in 50% ethanol for 10 minutes, rinsed with 50% ethanol and then dried. They were examined with a Philips E.M.300 electron microscope. Control grids were prepared using normal serum and antisera against pea streak virus (PSV) and clover yellow mosaic virus (CYMV).

4.2 Results

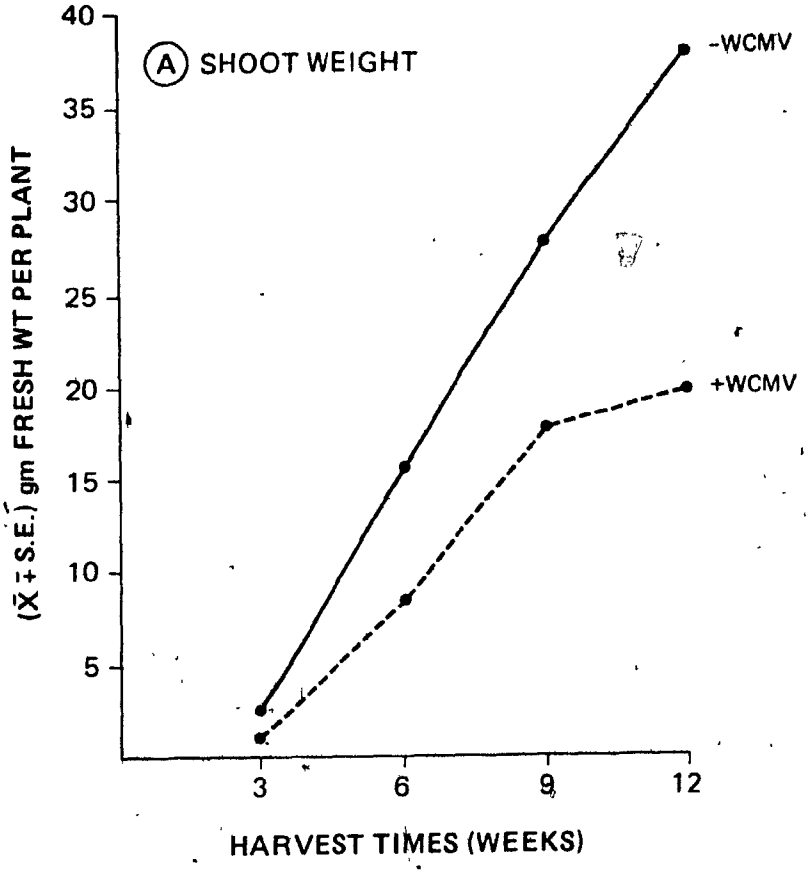
4.2.1 Plant growth

The fresh weight of the forage (top) and the roots of red clover plants increased with age of both healthy and WCMV-infected plants (Figure 20). However, the WCMV infection reduced the shoot growth significantly ($P \leq 0.01$, Appendix 8B). The highest response of red clover plants to infection by WCMV appeared 3 weeks after virus inoculation, when yield was reduced about 50% in the diseased plants compared with the healthy control. This difference gradually narrowed as the plants became older at 6, 9 and 12 weeks after virus infection, respectively (Table 6A). A significant reduction in the root weight (Appendix 8A), which reached its maximum of 46% at 12 weeks after virus inoculation (Table 6B), was shown by the WCMV-infected plants. There were obvious

Figure 20. Growth of healthy (- WCMV) and infected (+ WCMV) red clover plants.

A. The mean shoot weight per plant ± 2.3.

B. The mean root weight per plant ± 0.95.


A SHOOT WEIGHT
-WCMV
+WCMV
40
35
30
25
20
15
10
5
3
6
9
12
(X̄ + S.E.) gm FRESH WT PER PLANT
HARVEST TIMES (WEEKS)

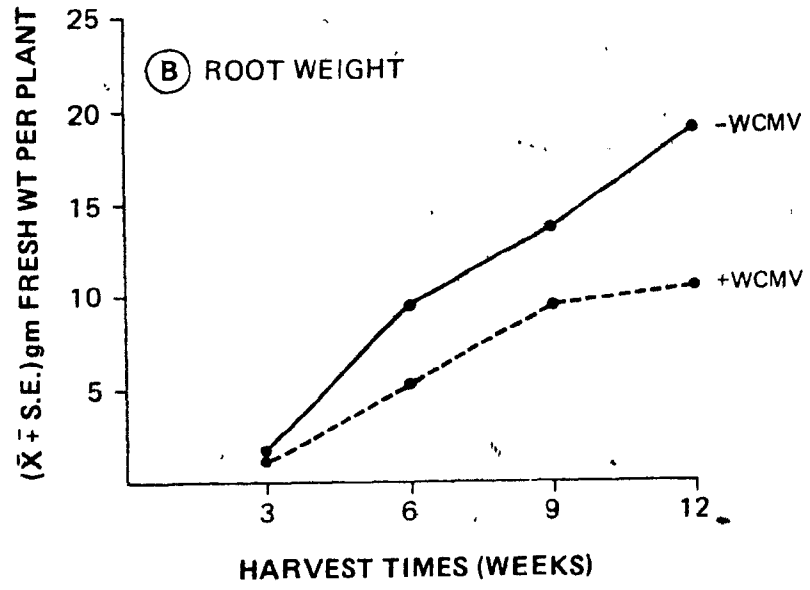

B ROOT WEIGHT
-WCMV
+WCMV
25
20
15
10
5
3
6
9
12
(X̄ + S.E.)gm FRESH WT PER PLANT
HARVEST TIMES (WEEKS)

TABLE 6A. Effect of WCMV infection on shoot fresh weight yield of red clover

Treatment[1]	Gm. fresh weight per plant at various times after virus inoculation (weeks)[2]				Mean[3]
	3	6	9	12	
Healthy	2.23	15.63	27.32	32.79	19.49
WCMV-infected	1.12	8.48	18.10	19.79	11.87
Mean	1.67	12.06	22.71	26.29	15.68
% reduction in infected plants	50	46	34	40	39

[1]S.E. for treatments (T) = ± 1.19

[2]S.E. for harvest times (D) = ± 1.68

[3]S.E. for (D) x (T) = ± 2.3

TABLE 6B. Effect of WCMV infection on root fresh weight of red clover

Treatment[1]	Gm. fresh weight per plant at various times after virus inoculation (weeks)[2]				Mean[3]
	3	6	9	12	
Healthy	1.88	9.50	13.63	19.07	11.02
WCMV-infected	1.18	5.30	9.59	10.33	6.60
Mean	1.53	7.40	11.61	14.70	8.81
% reduction in infected plants	37	44	30	46	40.16

[1]S.E. for treatment (T) = ± 0.47

[2]S.E. for harvest times (D) = ± 0.67

[3]S.E. for (T) x (D) = ± 0.95

differences in growth between healthy and WCMV-infected red clover plants. The healthy control with its dark green leaves showed earlier flowering than the pale diseased plants.

4.2.2 Nodulation

Infection by WCMV caused considerable reduction ($P \leq 0.01$) in the number and fresh weight of nodules (Appendix 8C,D). From Figure 21A it can be seen that nodule numbers of diseased plants were less than those of healthy controls at the various harvest times. The maximum reduction in nodule number, 37%, occurred 12 weeks after virus inoculation (Table 7A). The nodule fresh weight also decreased in the diseased plants and showed the highest reduction as 59% in the last stage (Table 7B). Both nodule fresh weight (Figure 21A) and number of nodules (Figure 21B) per plant were also higher in the healthy red clover than in the WCMV-infected plants when they were measured at 3, 6, 9 and 12 weeks after virus inoculation. Visual observation showed that the nodules on the WCMV-infected plants were smaller than those on healthy plants. A few white nodules were occasionally seen on the healthy and diseased red clover roots.

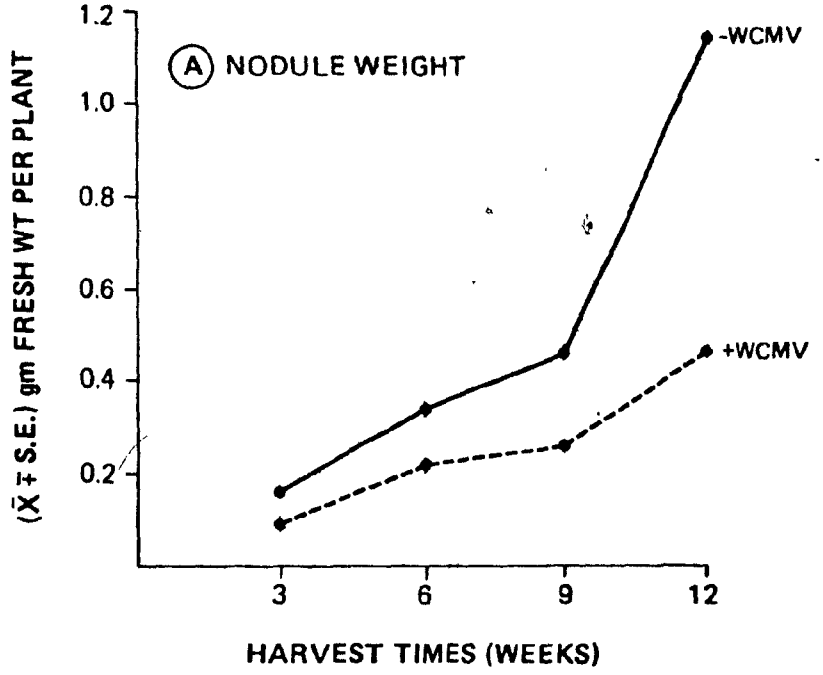
4.2.3 Nitrogenase (C_2H_2) activity

The nitrogenase (C_2H_2) activity of red clover nodules was calculated on an individual plant basis. In the WCMV-infected nodules this activity was significantly lower ($P \leq 0.01$; Appendix 8E) than that of the healthy control (i.e., 26.61, 57.39, 86.13 and 25.62

Figure 21. Nodule growth of healthy (- WCMV) and infected (+ WCMV) red clover.

A. The mean nodule weight per plant ± 0.04.

B. The mean nodule number per plant ± 37.86.


A NODULE WEIGHT
-WCMV
+WCMV
(X̄ ∓ S.E.) gm FRESH WT PER PLANT
1.2
1.0
0.8
0.6
0.4
0.2
3
6
9
12
HARVEST TIMES (WEEKS)

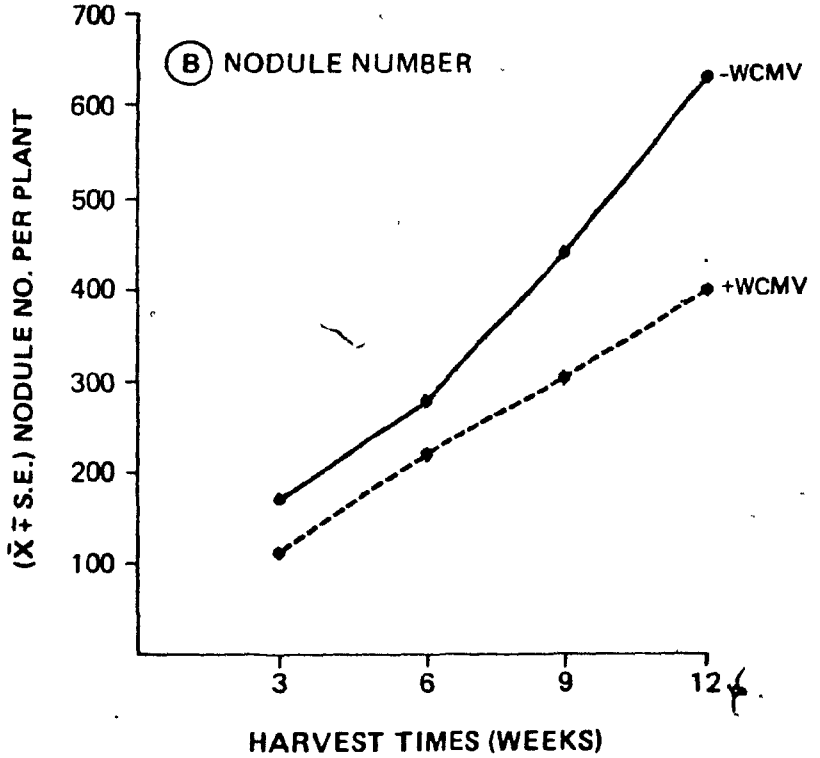

B NODULE NUMBER
-WCMV
+WCMV
(X̄ ∓ S.E.) NODULE NO. PER PLANT
700
600
500
400
300
200
100
3
6
9
12
HARVEST TIMES (WEEKS)

TABLE 7A. Effect of WCMV infection on nodulation of red clover number of nodules per plant

Treatments[1]	No. of nodules per plant at various times after virus inoculation (weeks)[2]				Mean[3]
	3	6	9	12	
Healthy	172.05	281.90	440.02	635.38	382.34
WCMV-infected	114.07	225.25	307.45	401.43	262.05
Mean	143.06	253.57	373.74	518.40	322.19
% reduction in infected plants	34	20	30	37	31.5

[1]S.E. for treatments (T) = ± 18.93

[2]S.E. for harvest times (D) = ± 26.77

[3]S.E. for (T) x (D) = ± 37.86

TABLE 7B. Effect of WCMV infection on nodulation of red clover nodule fresh weight gm. per plant

Treatments[1]	Nodule fresh weight (gm) per plant at various times after virus inoculation (weeks)[2]				Mean[3]
	3	6	9	12	
Healthy	0.16	0.34	0.46	1.13	0.52
WCMV-infected	0.09	0.22	0.26	0.46	0.26
Mean	0.12	0.28	0.36	0.80	0.39
% reduction in infected plants	44	35	43	59	50

[1]S.E. for treatments (T) = ± 0.02

[2]S.E. for harvest times (D) = ± 0.03

[3]S.E. for (T) x (D) = ± 0.04

nmoles of C_2H_4.plant^{-1}.hr^{-1} for WCMV-infected compared with 54.35, 122.95, 155.70 and 64.40 nmoles, respectively, for healthy nodules at 3, 6, 9, and 12 weeks, with S.E. equal to ± 12.27.

The activity of nitrogenase enzyme reached its maximum in both healthy and diseased nodules 9 weeks after WCMV inoculation, with a reduction of 45% in the diseased nodules (Figure 22). The lowest nitrogenase (C_2H_2) activity of WCMV-infected plants was about 60% less than that of healthy ones when measured at 12 weeks (Table 8).

4.2.4 Leghemoglobin (LH) content

The leghemoglobin content, determined on a fresh weight basis, in healthy (virus-free) and WCMV-infected nodules, increased steadily in the latter with age, but declined slightly in the former at 12 weeks. In the healthy nodules the content was significantly higher ($P \leq 0.01$) than that in the diseased nodules (Appendix 8F). The leghemoglobin content reached its peak in the noninfected nodules of 1.35 ± 0.2 μg.mg.nodule^{-1} at 9 weeks, and the similar value for WCMV-infected nodules was 0.58 ± 0.2 μg.mg.nodule^{-1} at 12 weeks (Figure 23). The greatest reduction in the leghemoglobin content in the diseased nodules was exhibited 6 weeks after virus inoculation, when the LH content was 62% less than the healthy control (Table 9).

In the cytochemical study, the leghemoglobin areas in ultrathin sections of the nodular tissue were identified as electron dense spots attached to the inner surface of the peribacteroidal membrane (Figure 24). These areas were recognized in the bacteroids of both healthy

Figure 22. Profiles of nitrogenase (C_2H_2) activity of healthy (- WCMV) and infected (+ WCMV) red clover expressed as the mean per plant ± 12.27.

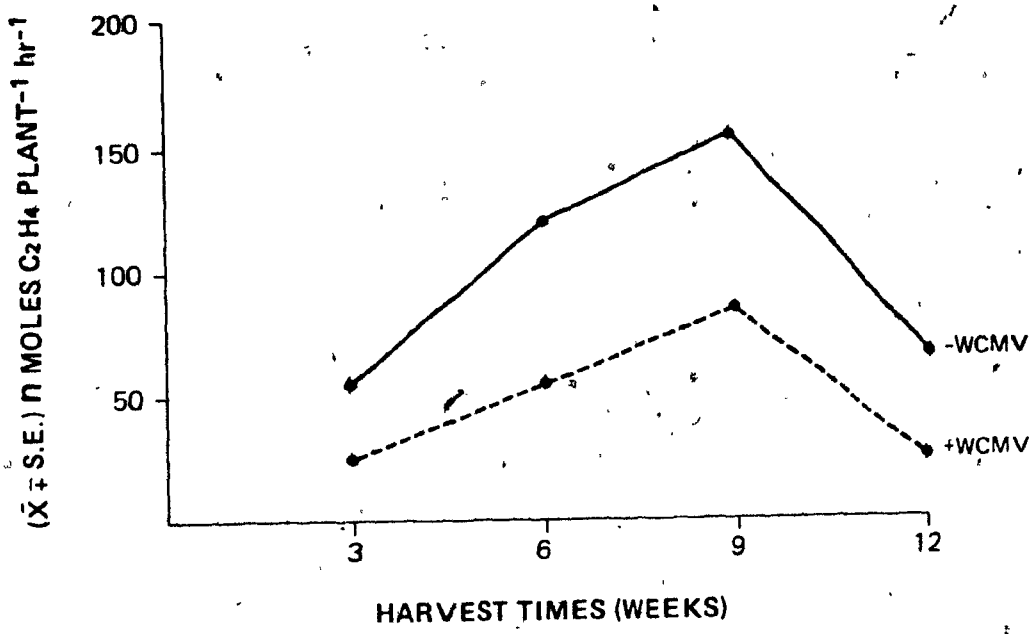

200
150
100
50
3
6
9
12
-WCMV
+WCMV
HARVEST TIMES (WEEKS)
(X̄ ± S.E.) n MOLES C2H4 PLANT-1 hr-1

TABLE 8. Effect of WCMV on nitrogenase (C_2H_2) activity in red clover nodules

Treatments[1]	Nitrogenase activity (nmoles C_2H_4.plant^{-1}.hour^{-1}) at various times after virus inoculation (weeks)[2]				Mean[3]
	3	6	9	12	
Healthy	54.35	122.95	155.70	64.40	99.35
WCMV-infected	26.61	57.39	86.13	25.62	48.94
Mean	40.48	90.17	120.91	45.01	74.14
% reduction in infected plants	51	53	45	60	50.7

[1]S.E. for treatments (T) = ± 6.13

[2]S.E. for harvest times (D) = ± 8.67

[3]S.E. for (T) x (D) = ± 12.27

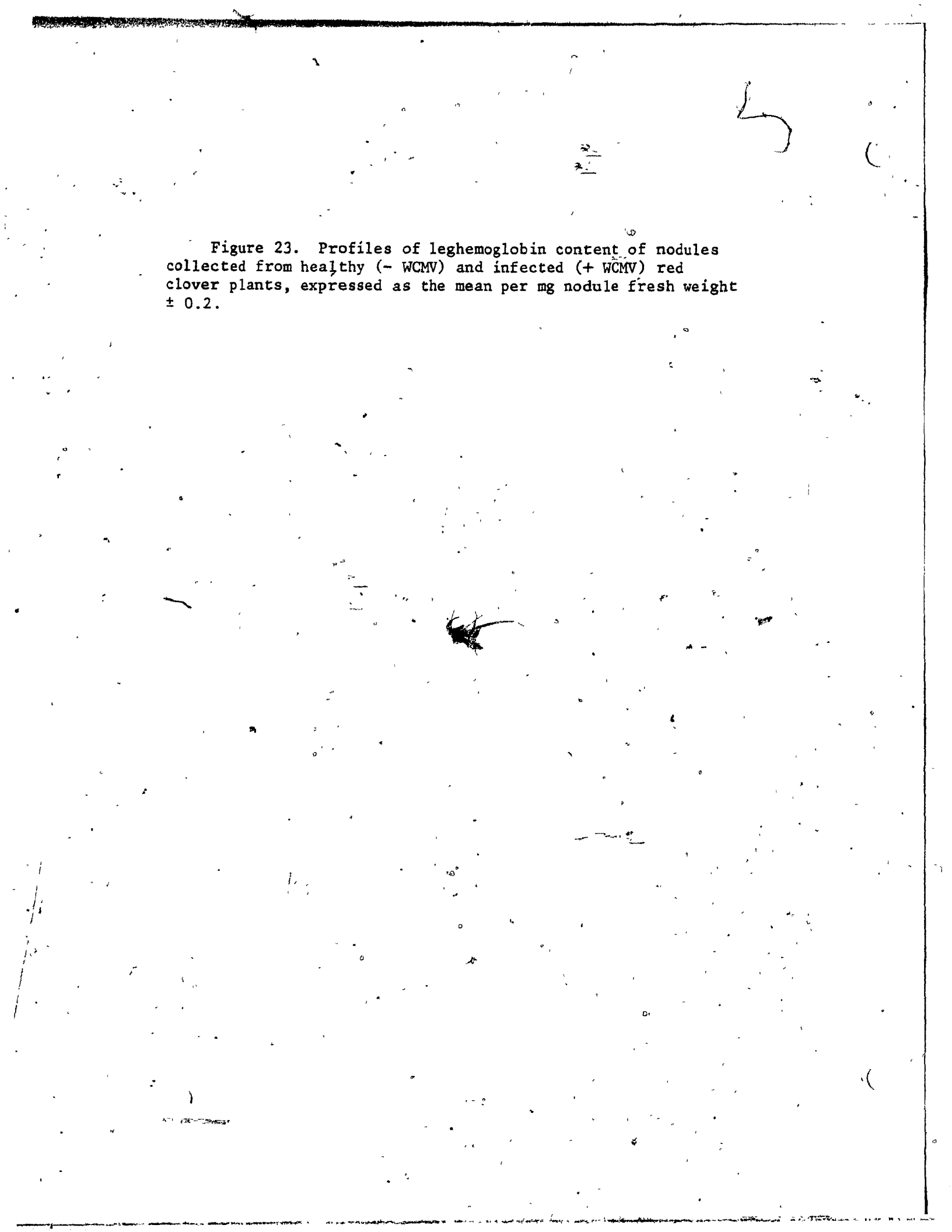


Figure 23. Profiles of leghemoglobin content of nodules collected from healthy (- WCMV) and infected (+ WCMV) red clover plants, expressed as the mean per mg nodule fresh weight ± 0.2.

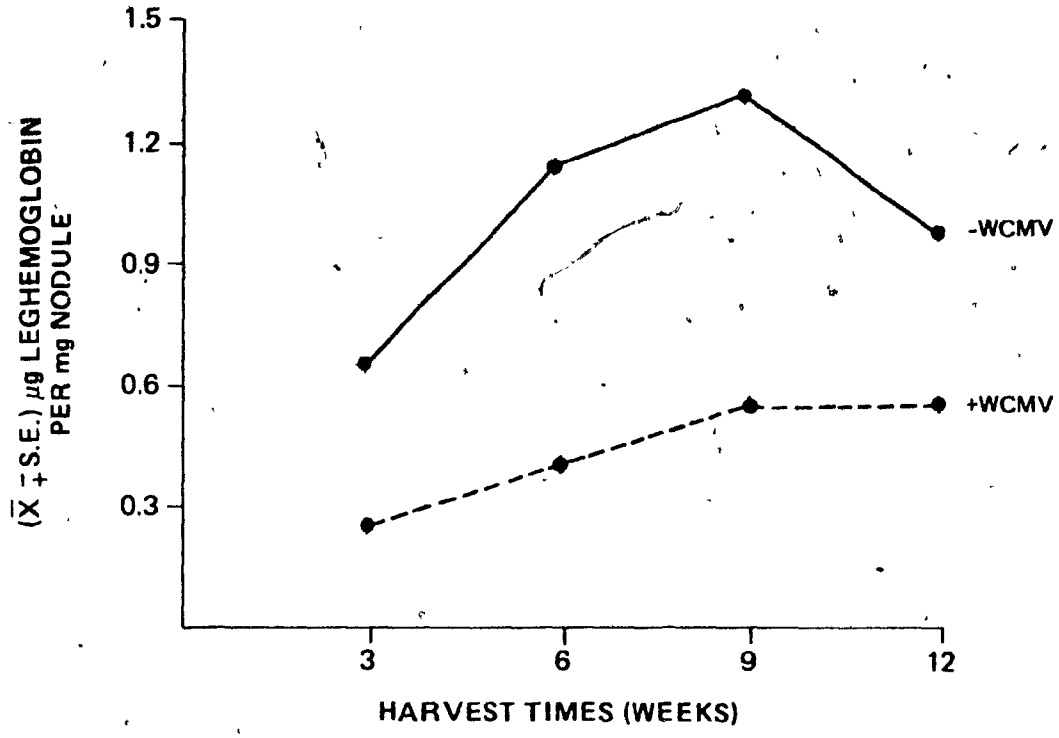

1.5
1.2
0.9
0.6
0.3
(X̄ ± S.E.) µg LEGHEMOGLOBIN PER mg NODULE
-WCMV
+WCMV
3
6
9
12
HARVEST TIMES (WEEKS)

TABLE 9. Effect of WCMV infection on leghemoglobin content in red clover nodules

Treatments[1]	Leghemoglobin content (µg per mg of nodule) at various times after virus inoculation (weeks)[2]				Mean[3]
	3	6	9	12	
Healthy	0.64	1.13	1.35	1.09	1.05
WCMV-infected	0.28	0.43	0.57	0.58	0.46
Mean	0.46	0.78	0.96	0.83	0.76
% reduction in infected plants	56	62	58	47	56.2

[1]S.E. for treatment (T) = ± 0.1

[2]S.E. for harvest times (D) = ± 0.45

[3]S.E. for (T) x (D) = ± 0.2

Figure 24. A thin section of the nodular tissue treated with DAB, showing electron dense deposits, representing the leghemoglobin, localized between the bacteroidal membranes (indicated with arrow).

and WCMV-infected nodular tissue. The visual observation showed that the number of leghemoglobin spots was less in the diseased samples as compared with the healthy control.

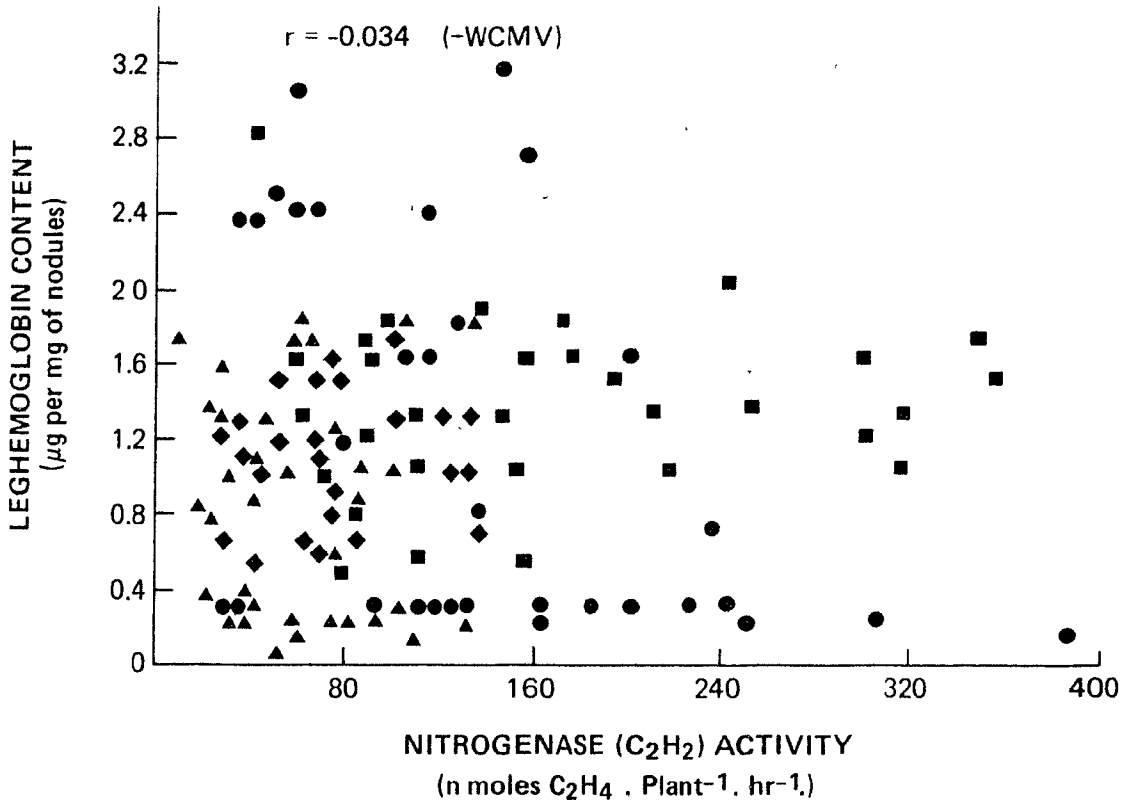
4.2.5 The correlation between leghemoglobin content and nitrogenase activity

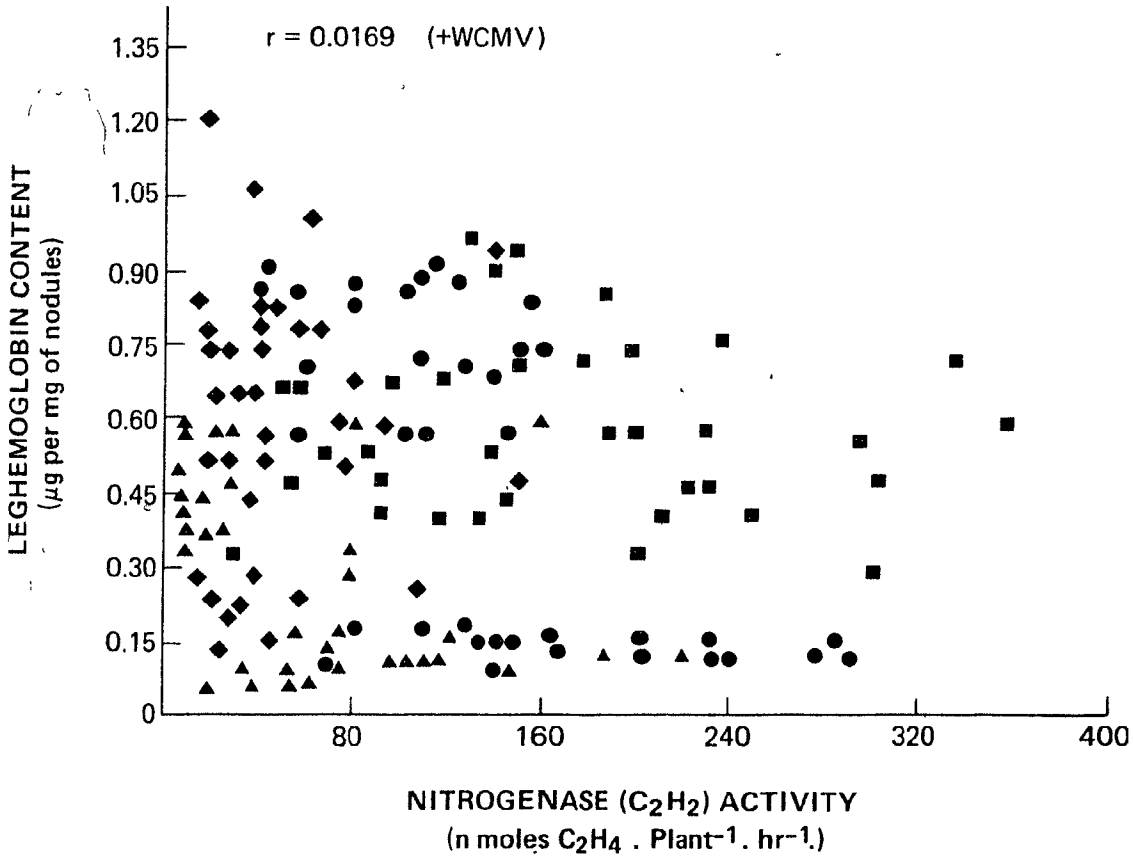
The regression analysis showed a highly significant correlation between LH and nitrogenase activity at 3 and 6 weeks after virus inoculation within the nodules of either healthy or WCMV-infected plants. At these two sampling times, the correlation coefficients in the healthy nodules were $r = -0.469$ and -0.453, respectively, while in the infected ones they were $r = -0.523$ and -0.327, respectively. No significant correlations were found between these two characteristics at 9 and 12 weeks. The overall interaction (Figure 25) between LH and nitrogenase activity at all sampling times indicated that there was no significant correlation within the nodules of healthy ($r = -0.034$) or infected plants ($r = -0.017$).

4.2.6 Nitrate reductase activity

The specific activity of the nitrate reductase enzyme was determined by measuring nitrite released into the assay medium at four days incubation intervals after each harvest of nodules. When the nodules were collected at 3 and 6 weeks, the maximum activity was recorded when 8-day assay incubation periods were used, but in nodules harvested at 6 and 12 weeks, maximum activity was detected with 4-day

Figure 25. Plots of data relating nitrogenase activity (C_2H_2 reduction) and leghemoglobin content of healthy (- WCMV) and infected (+ WCMV) red clover nodules. The symbols are: 3 weeks (●), 6 weeks (▲), 9 weeks (■), and 12 weeks (◆).


r = -0.034 (-WCMV)
LEGHEMOGLOBIN CONTENT
(μg per mg of nodules)
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
0
80
160
240
320
400
NITROGENASE (C_2H_2) ACTIVITY
(n moles C_2H_4 . $Plant^{-1}$. hr^{-1}.)


r = 0.0169 (+WCMV)
LEGHEMOGLOBIN CONTENT
(μg per mg of nodules)
1.35
1.20
1.05
0.90
0.75
0.60
0.45
0.30
0.15
0
80
160
240
320
400
NITROGENASE (C_2H_2) ACTIVITY
(n moles C_2H_4 . $Plant^{-1}$. hr^{-1}.)

assay incubation periods. The data obtained from 4-day incubation assays (Table 10) were used to compare the enzyme activity in virus-free and virus-infected samples at each harvest time (Figure 26). The nitrite released into the medium containing nodules from infected plants was significantly ($P \leq 0.01$) higher than that from healthy control plants at various incubation times after each harvest (Appendix 8G). The maximum nitrate reductase specific activity of healthy nodules (i.e., 322.70 nmoles nitrite.hour^{-1}.gm nodule^{-1}) was shown at the 6-week harvest. The corresponding value for nodules from infected plants was 375.20 nmoles nitrite.hour^{-1}.gm nodule^{-1}. The greatest increase in activity shown by nodules from infected plants was 25.6% which occurred in nodules harvested 3 weeks after virus inoculation.

4.2.7 Acid phosphatase specific activity

4.2.7.1 Colorimetric determination

The «acid phosphatase specific activity», expressed in terms of the rate of conversion of p-nitrophenyl phosphate into p-nitrophenol, increased gradually with age in both healthy (virus-free) and infected nodules, and reached its peak at 9 weeks, but decreased sharply 12 weeks after virus inoculation (Figure 27). However, the virus infection significantly increased ($P \leq 0.01$) the enzyme activity in the WCMV-infected nodules compared with virus-free ones (Appendix 8H). The «acid phosphatase activity» of diseased plants (i.e., 174.2 µg/mg nodule) was about 41.9% higher than that of the healthy control

TABLE 10. Effect of WCMV infection on nitrate specific activity in red clover nodules

Treatments[1]	Nitrate reductase specific activity at various times after virus inoculation (weeks)[2]				Mean[2]
	3	6	9	12	
Healthy	252.50	322.70	175.40	145.55	224.04
WCMV-infected	339.35	375.20	223.30	194.60	283.12
Mean	295.92	348.95	199.35	170.08	253.58
% increase in infected plants	25.6	13.9	21.45	25.2	

[1]S.E. for treatments (T) = ± 1.71

[2]S.E. for harvest times (D) = ± 2.41

[3]S.E. for (T) x (D) = ± 3.41

Figure 26. Profile of nitrate reductase specific activity within healthy (- WCMV) and infected (+ WCMV) red clover nodules ± 3.41.

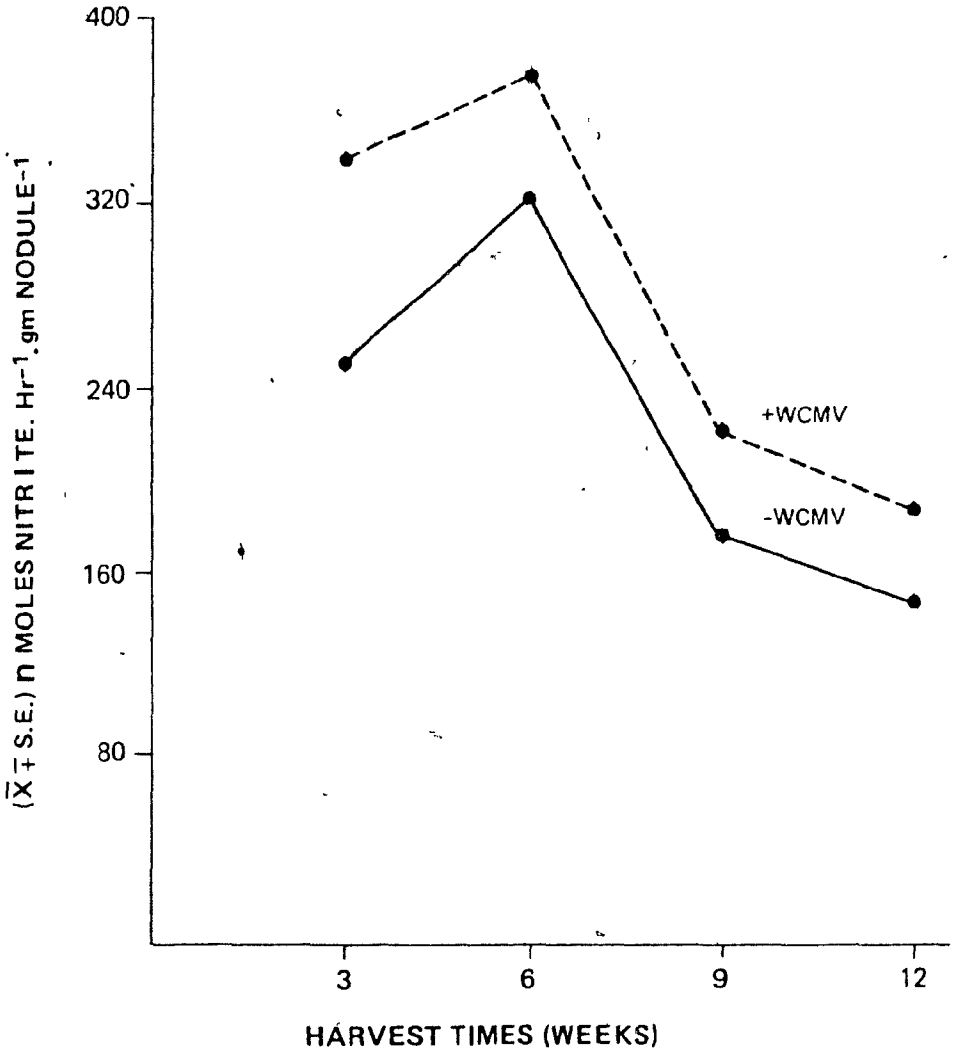

400
320
240
160
80
(X̄ ∓ S.E.) n MOLES NITRITE. Hr⁻¹.gm NODULE⁻¹
+WCMV
-WCMV
3
6
9
12
HARVEST TIMES (WEEKS)

Figure 27. Profile of acid phosphatase specific activity of healthy (- WCMV) and infected (+ WCMV) red clover expressed per mg nodule fresh weight ± 2.687.

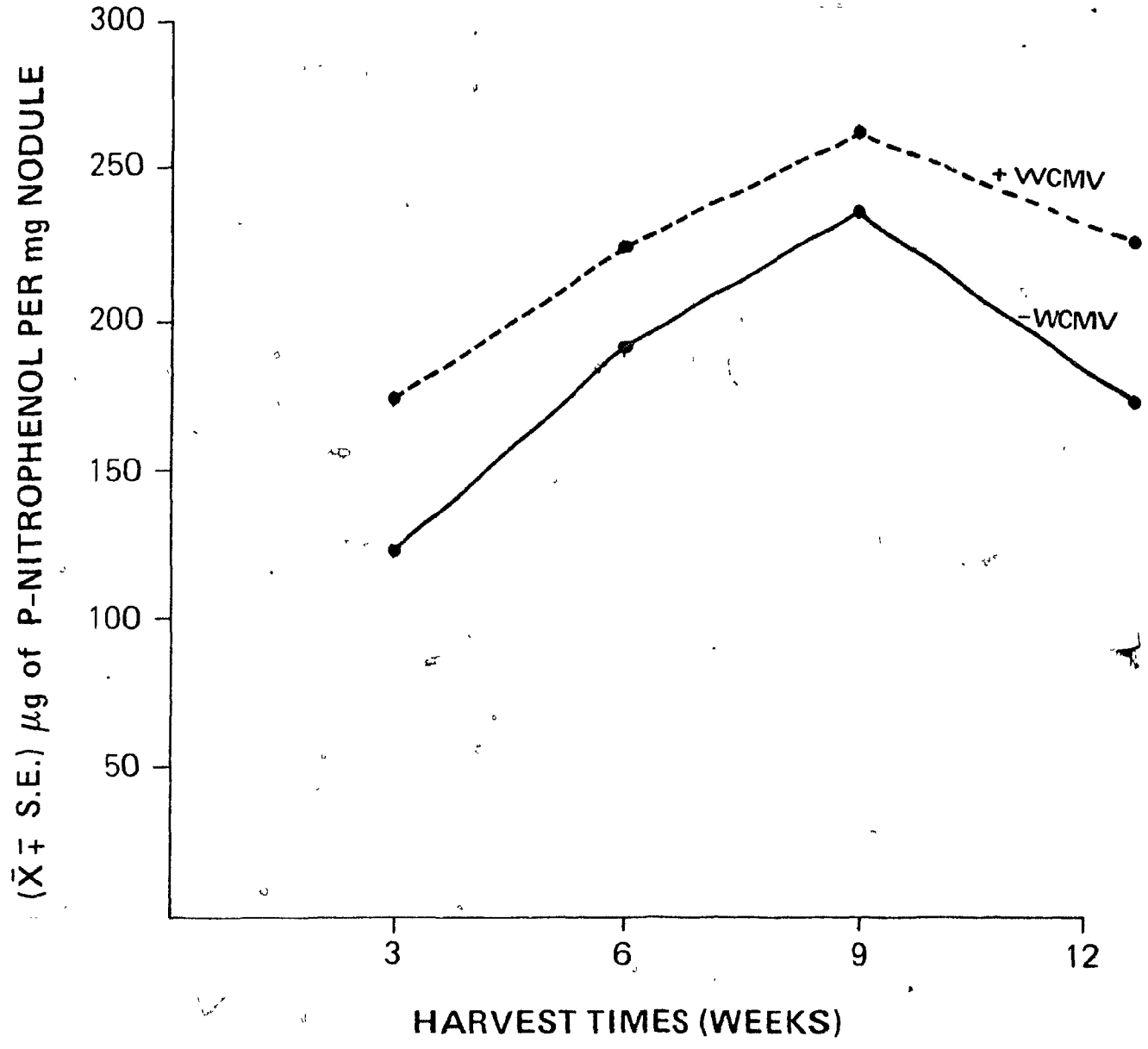

300
250
200
150
100
50
(X̄ ± S.E.) µg of P-NITROPHENOL PER mg NODULE
+ WCMV
-WCMV
3
6
9
12
HARVEST TIMES (WEEKS)

TABLE 11. Effect of WCMV infection on acid phosphatase specific activity in the nodules of red clover

Treatments[1]	Acid phosphatase specific activity (μg p-nitrophenol.min^{-1}.mg nodule^{-1}) at various times after virus inoculation (weeks)[2]				Mean[3]
	3	6	9	12	
Healthy	122.6	182.9	229.6	172.6	176.9
WCMV-infected	174.2	226.1	263.6	224.4	222.1
Mean	148.4	204.4	246.6	198.5	199.5
% increase in infected nodules	41.9	23.6	14.8	30.0	25.6

[1] S.E. for treatments (T) = ± 2.687

[2] S.E. for harvest times (D) = ± 1.900

[3] S.E. for (T) x (D) = ± 3.800

(i.e., 122.6 µg/mg nodule) when measured at 3 weeks after virus inoculation (Table 11).

4.2.7.2 Cytochemical localization of acid phosphatase

In the pellet sections of red clover leaves, obtained from the same plant as the nodules, semi-quantitation of electron-dense deposits on the single bound membranes of lysosome or vacuole-like bodies (Figure 28) showed a gradual increase in the number of these deposits in both treatments at 3, 6, 9 and 12 weeks. The average number per micrograph at each period was 5, 8, 14, 19 in WCMV-infected samples, corresponding to 2, 5, 8, 13 in the healthy leaves. The micrographs of sections of pellets treated with sodium fluoride, an acid phosphatase inhibitor, showed a negligible number of similar electron dense deposits.

4.2.8 Rhizobium population in the nodule

As shown in Figure 29, the nodules of healthy plants had a slightly higher bacterial population than those of WCMV-infected plants at the first (3 weeks), second (6 weeks) and fourth harvest (12 weeks), but not at the third harvest (9 weeks). The analysis of variance showed a significant difference between healthy and diseased nodules at $P \leq 0.01$ (Appendix 8J). The greatest difference in the number of bacterial colonies per mg of nodules between the treatments was 26.4% at 12 weeks after virus inoculation (Table 12). However,

Figure 28A. Electron dense deposits (arrows) attributable to acid phosphatase activity, along the membranes of vacuole-like bodies.

Figure 28B. Control preparation, treated with NaF to inhibit acid phosphatase activity.

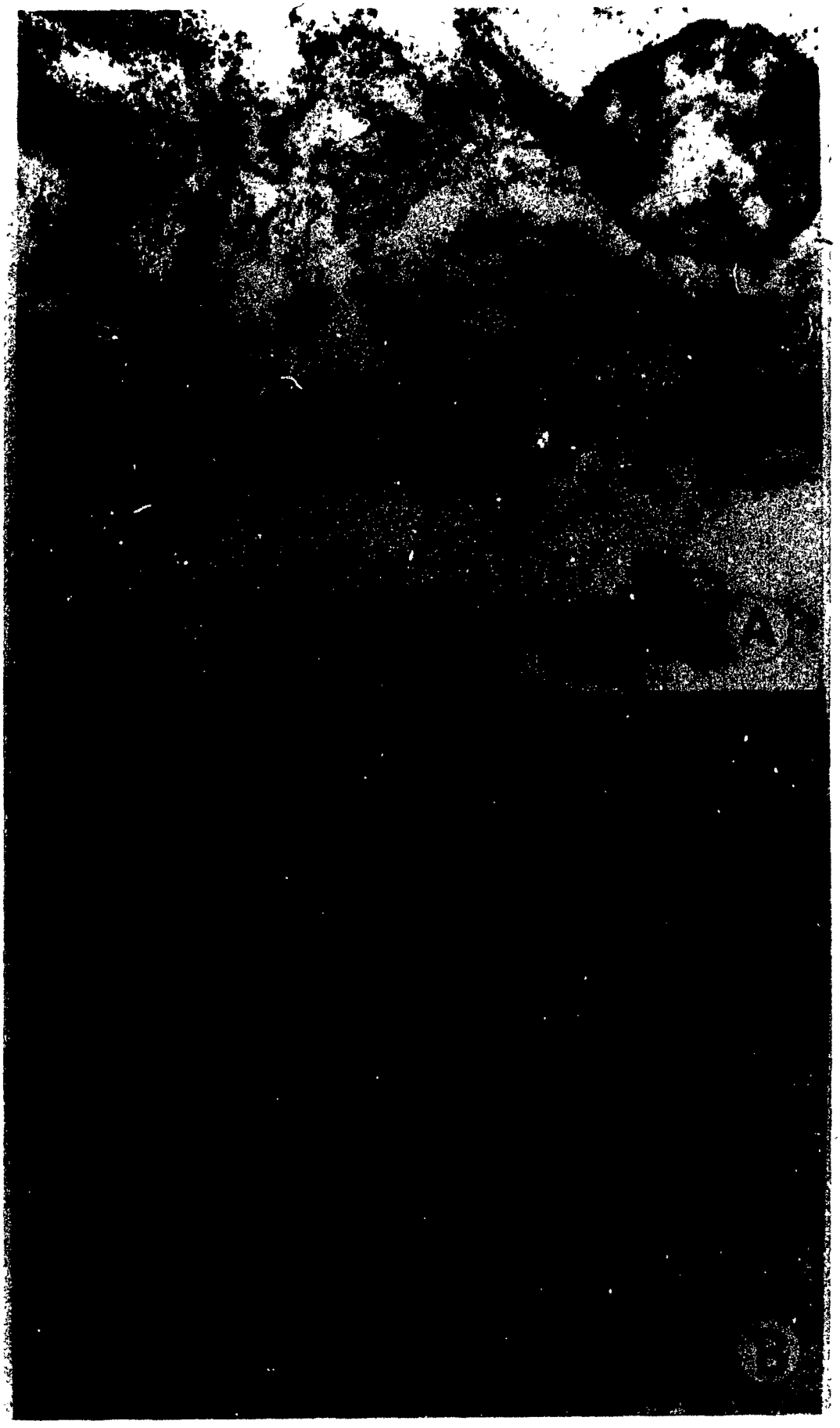

A
B

Figure 29. Profile of viable Rhizobium population in healthy (- WCMV) and infected (+ WCMV) nodules expressed as the mean number of colonies per mg of nodules ± 2.20.

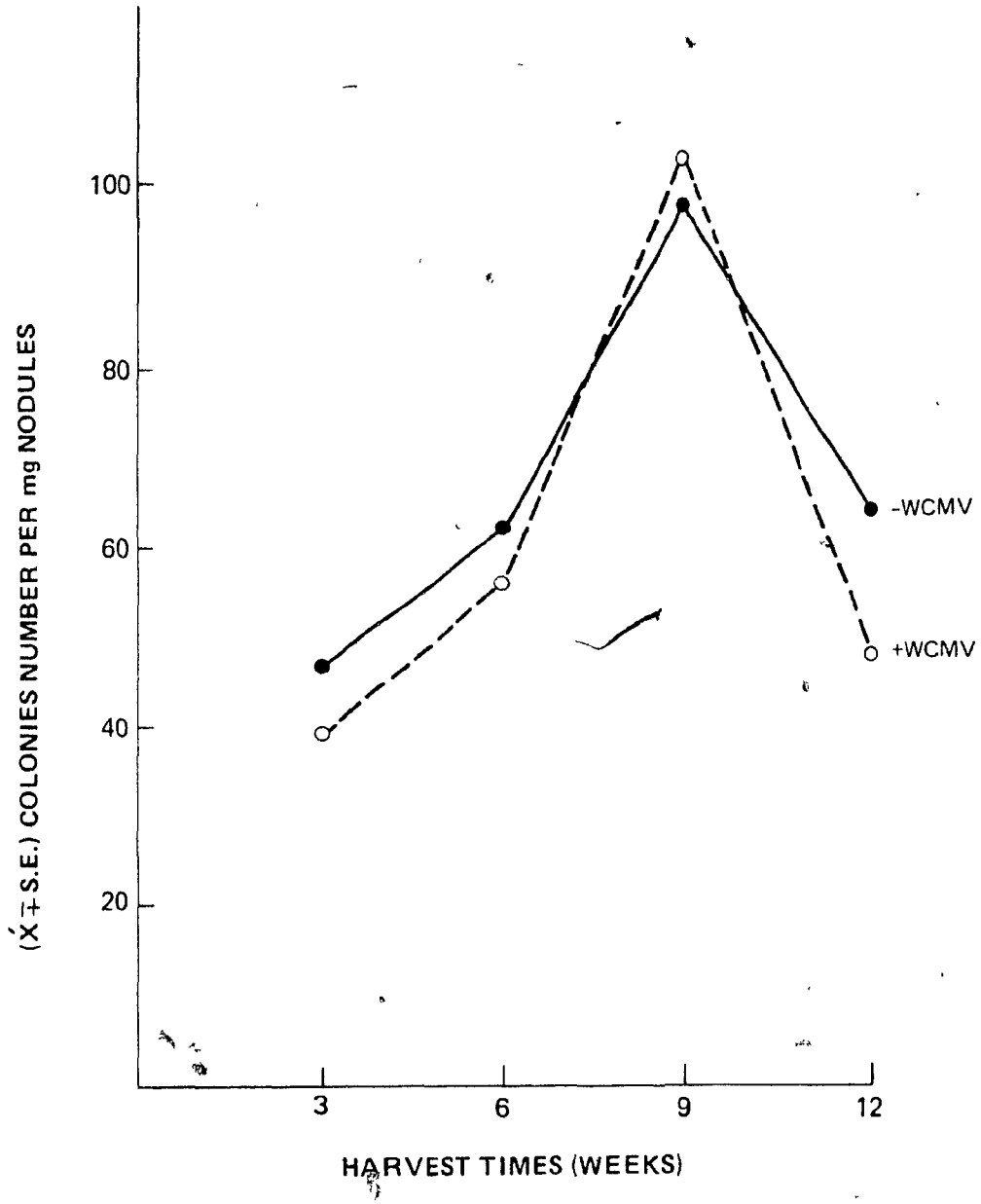

100
80
60
40
20
(X̄ ± S.E.) COLONIES NUMBER PER mg NODULES
3
6
9
12
HARVEST TIMES (WEEKS)
-WCMV
+WCMV

TABLE 12. Effect of WCMV infection on viable rhizobial population in red clover nodules

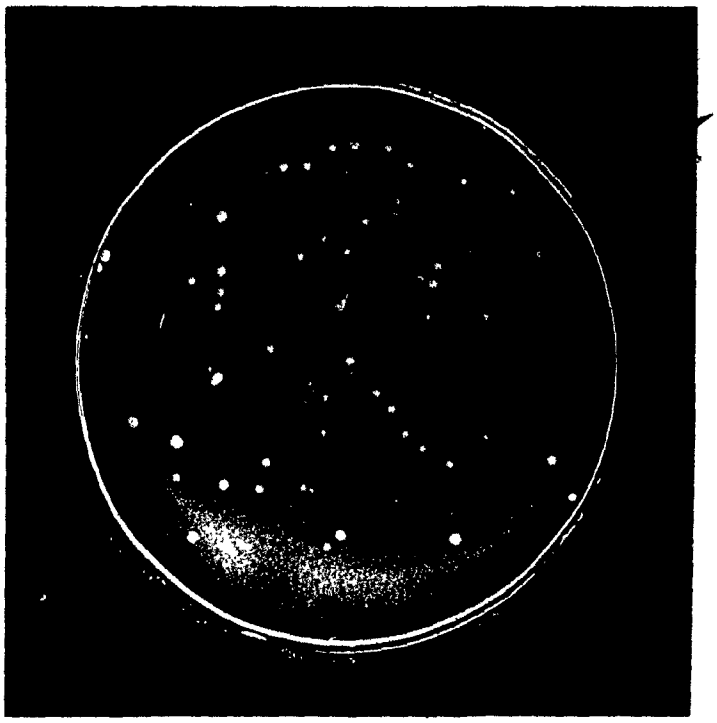
Treatments[1]	No. of colonies in dilution 10^{-3}/mg nodules at various times after virus inoculation (weeks)[2]				Mean[3]
	3	6	9	12	
Healthy	47.5	62.1	98.3	64.7	68.1
WCMV-infected	39.9	56.0	103.6	47.6	61.8
Mean	43.7	59.1	100.9	56.1	64.95
% reduction in infected nodules	16.0	9.8	- 5.4	26.4	9.25

[1]S.E. for treatments (T) = ± 2.20

[2]S.E. for harvest times (D) = ± 1.56

[3]S.E. for (T) x (D) = ± 1.10

Figure 30. Colonies of clover Rhizobium grown on congo-red yeast-extract manitol agar.



at 9 weeks, this trend was reversed and the WCMV-infected nodules had 5.4% more viable Rhizobium cells than the healthy control. Isolated colonies (Figure 30) obtained from each dilution of healthy and diseased nodule suspensions were grown again on agar slants. The bacterial preparations made from each one (as described earlier) were inoculated back onto red clover seedlings to see whether virus transmission took place. The bacteria produced numerous nodules. There were no virus symptoms on the leaves of red clover plants inoculated originally with bacteria isolated from WCMV-infected nodules.

4.2.9 Assay of WCMV in the nodules

Extracts of nodules obtained at 3, 6, 9 and 12 weeks after virus inoculation of source plants produced 26, 31, 21, and 16 lesions, respectively, when assayed on Gomphrena globosa L. (Figure 31A). There were a few scattered individual lesions on a few healthy leaves of G. globosa, which might have resulted from the damage by mechanical inoculation. The number of local lesions reached a peak at 6 weeks after virus inoculation (Figure 32B).

The immunosorbent electron microscopy (IEM) technique was used to record number of WCMV particles bound by homologous antiserum (Figure 31B). The detection was performed by counting the number of these particles in 100 micrographs. The average numbers were 48, 55, 59, and 53 virus particles per micrograph at various times after virus inoculation, i.e., 3, 6, 9, 12 weeks (Figure 32A). No virus particles were observed on the grids bearing extracts from healthy nodules or other controls (Figure 31C).

Figure 31. The results of the assay of WCMV in the infected nodular tissue determined by:

A. Infectivity assay on Gomphrena globosa L.
Right: healthy; Left: WCMV-infected.

B. Micrograph taken during immunosorbent electron microscopy (IEM), showing WCMV particles (arrows).

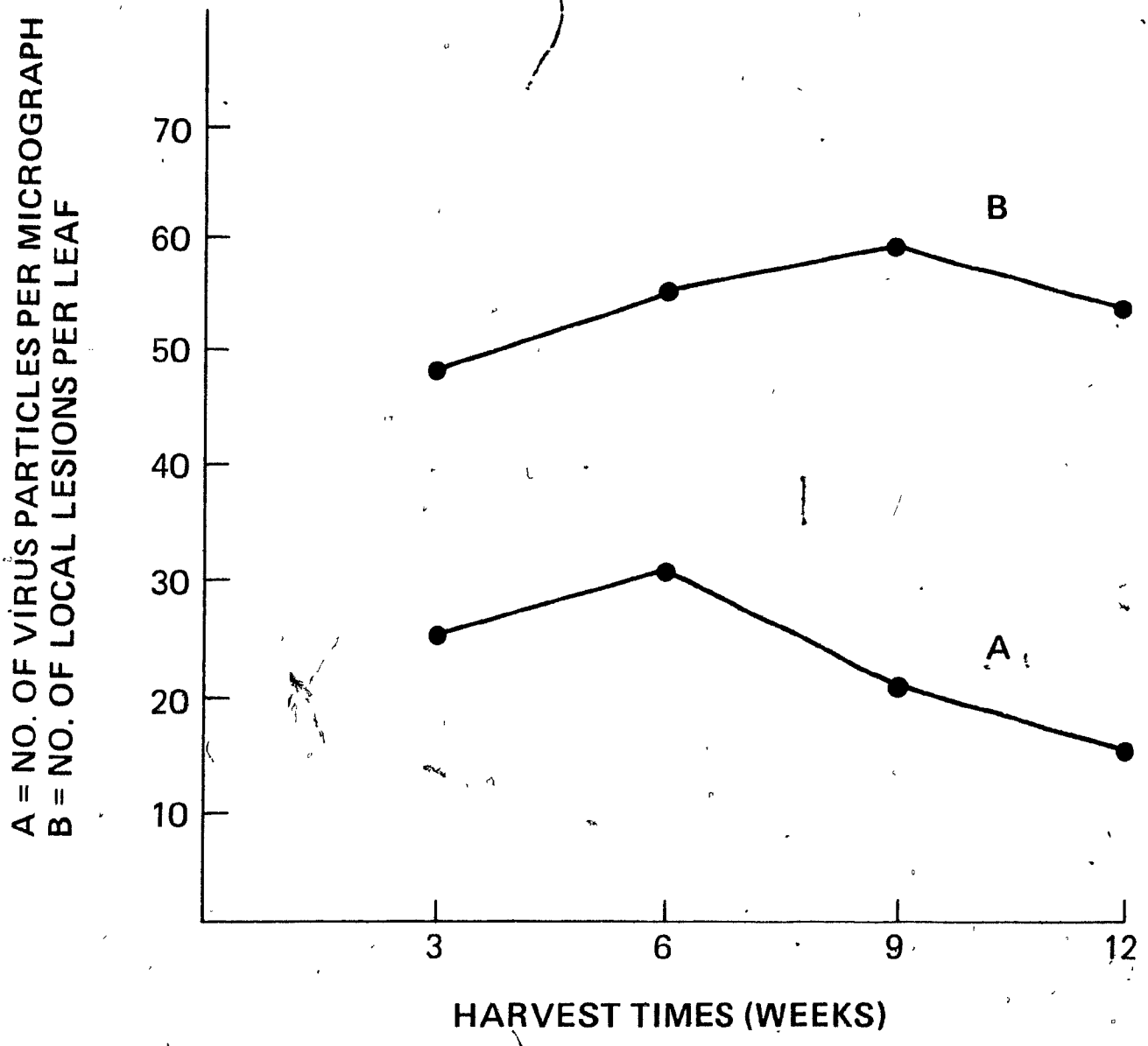
C. Similar photograph of an extract from healthy nodules.


a
b
A
1 μ
B
C

Figure 32. WCMV assay using:

A. Immunosorbent electron microscopy.

B. Infectivity on G. globosa leaves.


A = NO. OF VIRUS PARTICLES PER MICROGRAPH
B = NO. OF LOCAL LESIONS PER LEAF
70
60
50
40
30
20
10
B
A
3
6
9
12
HARVEST TIMES (WEEKS)

4.3 Discussion and conclusions

The analysis of variance showed that the healthy and infected plants differed significantly in forage and root fresh weights per plant at the various harvest times. As mosaic diseases generally reduce photosynthetic activity and increase respiration rates, the virus infection probably exerted an impact on plant growth at all stages of development. The greatest reduction in WCMV-infected forage weight, at 3 weeks after virus inoculation, may be attributed to the wounding damage caused during the inoculation process, coupled with the general effect of virus infection. Similarly, the 46% decrease in the root weight at the last harvest (12 weeks) probably resulted from the cumulative effects of virus infection and senescence.

WCMV-infected plants showed reductions of 20-37% and 35-59% in number and fresh weight of nodules, respectively, when averaged for all the harvest times. Both effects became more pronounced as plants aged. Smaller nodules were frequently observed on the infected plants, which would have contributed the decrease in average nodule weight. Similar decreases in nodule weight have been reported in tobacco ringspot virus infected soybeans (Orellana and Fan 1978), and in peanut mottle virus infected peanuts (Wongkaew and Peterson 1983). The growth and development of legume nodules requires both auxins and cytokinins (Dart 1975; Syono et al. 1976). Auxin imbalances are sometimes associated with virus infections (Diener 1963), which can also impair synthesis and/or translocation of growth regulators (Matthews 1982). It has been reported that leguminous plant nodules are themselves auxin producers

(Kefford et al. 1960), and that growth regulating substances may be released by nodules of virus-infected plants (Lee 1955). Thus it can be concluded that WCMV infection suppressed nodule growth, possibly by reducing photosynthetic activity and/or the availability of photosynthate, or by causing unfavorable shifts in the levels of available growth regulating substances.

Detached nodules have been found to lose nitrogenase activity when kept at room temperature (Aprison and Burris 1952) or at low temperatures (Mustafa 1969). Thus the use of intact nodules on the roots in the acetylene (C_2H_2) reduction assay provides a more stable sample. Although the acetylene reduction assay provides a direct method of measuring the activity of the nitrogenase system (Hardy et al. 1968), the relationship between acetylene reducing ability and N_2 reduction is variable (Sprent 1979).

The nitrogenase (C_2H_2) activity of red clover began to increase before virus inoculation, and continued to increase up to 9 weeks after virus infection. However, a sharp decline was observed when measured 12 weeks after virus inoculation. The continued increase in activity was probably associated with a general increase in plant growth, appearance of new leaves, and nodule development. The reduction at 12 weeks could be related to senescence of most of the active nodules. It has been generally considered that the nitrogenase activity of rhizobia is associated only with their condition as bacteroids within nodules (Bergersen 1974). Also, it has been reported that nodules of perennial legumes remain functional for only one growth season (Dart 1977). The

lowest reduction of nitrogenase (C_2H_2) activity in virus infected plants, 45%, occurred at the blooming stage, 9 weeks after virus inoculation. At this time, highest number of viable rhizobia were recoverable from nodules also. The greatest reduction of nitrogenase activity in WCMV-infected plants, 60%, was recorded at 12 weeks after virus inoculation, which probably resulted from the combined effects of virus infection and senescence of most functional nodules.

The reduction in plant growth (top and root weight), as well as nodulation in WCMV-infected red clover may suggest that the virus infection has affected the photosynthetic activity and/or availability of photosynthate. However, it is evident that the amount of photosynthate available was sufficient for maintenance of a certain level of nitrogenase activity. Bean plants infected with BYMV (Orellana and Fan 1978), various varieties of soybean infected with TRSV (Orellana et al. 1979, 1980), and peanut infected with PMV (Wongkaew and Peterson 1983) are similar examples with respect to the effect of virus infection on nitrogen fixation.

Various methods of determination of leghemoglobin content in nodular tissue have been described (Bergersen and Goodchild 1973; Wilson and Reisenauer 1963; Johnson and Hume 1973), but most of them required a large amount of nodular tissue and were not readily applicable to small samples of red clover nodules. The sensitive method described by La Rue and Child (1979) was more practical for this study because fewer nodules were required.

A significant reduction of leghemoglobin content was observed in nodules of WCMV-infected plants, which suggests that virus infection somehow suppressed the production of leghemoglobin. Decreases in leghemoglobin content have also been reported in nodules of peanuts infected by PMV (Wongkaew and Peterson 1983), and in nodules of soybeans infected by SMV (Tu et al. 1970b).

A consistent and highly significant negative correlation between nitrogenase (C_2H_2) activity and leghemoglobin content was detected in both healthy and infected nodules at 3 and 6 weeks after virus inoculation, but not at 9 and 12 weeks. This may indicate that leghemoglobin level is a determinant factor involved in initiation of nitrogenase activity in nodular tissue. However, the overall interaction between these two characteristics at all sampling times indicated no significant correlation in nodules of either healthy or infected plants. This might have resulted from aging of some of the nodules, or from plant blooming, which may represent a stage of N_2 consumption.

The relationship between nitrogen fixation and leghemoglobin content is still a controversial subject. Bergersen (1961a) considered leghemoglobin content to be an index of the volume of active nodular tissue fixing nitrogen, while Gibson (1969) found that rates of N_2 fixation per unit of leghemoglobin are Rhizobium strain dependent. In other reports dealing with virus infections, Orellana et al. (1978) observed a negative correlation between leghemoglobin accumulation and nitrogenase activity in TRSV-infected soybean nodules, while Wongkaew (1981) found a positive correlation between them in healthy peanut nodules, but not in those of plants infected with peanut mottle virus.

The positive relationship between DAB and heme protein has been exploited in cytochemical studies of virus-infected leaves (De Zoeten et al. 1973) and healthy nodules (Gourret and Fernand-Arias 1974). Although attempts to quantitate the number of electron-dense deposits gave a less reliable estimate of leghemoglobin concentration than the fluorometric assay, the location of these deposits indicated that leghemoglobin is probably synthesized or accumulated in the peribacteroidal space. This agrees with findings reported by other investigators (Bergersen and Goodchild 1973; Dilworth and Kidby 1968) who have studied nodules of soybean and serradella plants.

It is generally accepted that nitrate is reduced to ammonia to accomplish the incorporation of nitrogen into plant nitrogenous compounds (Devlin 1975). This process requires the energy provided by respiration, which usually increases with virus infection (Matthews 1982), and also makes use of the carbohydrate produced by photosynthesis, which usually decreases with virus infection (Matthews 1982). Nitrate reductase, an enzyme which catalyzes the reduction of nitrate to nitrite, has been purified from soybean leaves and has been characterized as an inducible enzyme (Devlin 1975).

Instability is one of the main characteristics of nitrate reductase activity, both in vitro (Anacker and Stoy 1958) and in vivo (Candela et al. 1975; Hageman and Flesher 1960; Hewitt 1951). Several internal and external factors may determine or influence nitrate

reductase activity in nodules, including plant species, strain of Rhizobium, nodule morphology, rate of shoot growth, amount of nitrogen fixed, nutrient availability, C:N ratio, and competition between plant parts for energy. The interrelationships among some of these factors have been reported for several annual grain legumes (Gibson 1977; Hardy and Havelka 1975) and alfalfa (Vance et al. 1979). However, little information is available concerning the activity of nitrate reductase activity in red clover nodules, and the effect of virus infection on nitrate reductase activity has not been investigated previously.

Both healthy and infected nodules showed a rapid increase in the specific activity of nitrate reductase at 3 and 6 weeks after virus infection, which was probably related to the physiological state of the nodular tissue at early stages of development. The sharp decline observed at 9 and 12 weeks may be a reflection of competition of other plant parts with the nodules for energy and carbohydrate during flowering.

The significant increase of nitrate reductase specific activity of nodules from WCMV infected plants is of particular interest, and suggests that decreased carbohydrate or photosynthate, caused by virus infection, was not a limiting factor governing the enzyme's activity. Nitrite, the product of nitrate reductase activity, strongly inhibits nodular nitrogenase activity in several leguminous plants (Chen and Phillips 1977; Kennedy et al. 1975; Gibson and Pagan 1977). Therefore, the observed increase in nitrate reductase activity probably contributed to the reduced nitrogenase activity in nodules of WCMV infected plants.

According to Wilson (1973), various cellular structures and organelles, including the lysosomes, lomasomes, spherosomes, pinocytic vacuoles, provacuoles, Golgi vesicles and excretory vesicles, collectively can be regarded as parts of the «lysosomal system.» These membrane-bound structures may serve as compartmentalized loci for hydrolytic enzymes involved in the most vital plant processes, such as seed germination, storage, responses to injury or disease organisms and senescence (De Duve and Wattiaux 1966).

Acid phosphatase, one of the best known hydrolytic enzymes, occurs in the nucleus and spherosomes (Devlin 1975), and catalyzes many reactions in plant cells. Relatively little is known of the luence of virus infection on acid phosphatase activity. Sommer (1957; c.f. Matthews 1982). reported that a decrease in the enzyme's activity was associated with infection of sugar beets by the beet yellows virus (BYV), but Tu (1976) reported significant increases in acid phosphatase activity in leaves and nodules of CYMV-infected white clover, when measured a few days after virus inoculation.

The nodular acid phosphatase specific activity in infected samples rose to a maximum at 9 weeks after virus inoculation, followed by a decline in activity, which may reflect the pattern of hydrolysis of materials within the nodules for transport or use, or simply the involvement of this enzyme in digestion of cellular components as nodules aged and became senescent. The decline observed at 12 weeks could have resulted from inhibition of the enzyme by accumulated products, or a retardation of enzyme synthesis within the nodules.

No decline in acid phosphatase activity of the leaf tissues was observed when a cytochemical determination was used. Although this may have resulted from the use of a different detection method, it could also indicate differences in the metabolic functions of the aerial plant parts where the only senescent tissues were floral parts. The latter were not included in the extract used for cytochemical determination of acid phosphatase activity.

Since acid phosphatase activity was increased in both nodular and leaf tissues of infected plants, it seems logical to conclude that virus infection was directly or indirectly responsible for the increase. Because the cellular reactions in which phosphatases are involved are coupled to other reactions, it is difficult to identify any virus-determined mechanism for the increase in phosphatase activity. Tu (1976), who detected similar increases in acid phosphatase activity and lysosome content in virus infected leaves, suggested that the increased acid phosphatase activity could be a reflection of increased autophagic activity.

It is well known that rhizobial bacteria can multiply only before transformation to bacteroids. Because of the inevitable bacterial multiplication during the process of preparation of serial dilutions, the accuracy of estimating the viable Rhizobium population in nodules is limited. To overcome this obstacle, to a certain extent, the isolation technique recommended by Brockwell (1963) was used in this study, because the minimal generation time for R. trifolii, growing in the exponential phase in synthetic medium, is 5 hours (Bergersen 1961a).

The continuous increase in the viable bacterial population in nodules at the first three harvests (3, 6, 9 weeks after virus inoculation) probably indicates the continued multiplication of the rhizobia as the nodules developed. The decline of the population at 12 weeks probably indicates the onset of nodule senescence.

Generally, fewer colonies were obtained from nodules of infected plants, although there was little difference in number of colonies of viable rhizobia from healthy and infected plants at 9 weeks. The presence of WCMV particles in the nodules of infected plants may have affected the multiplication of rhizobia, as well as their subsequent transformation into fully developed bacteroids. At 9 weeks after virus inoculation, nitrogenase activity was reduced by about 45% in nodules of infected plants. This was also the smallest reduction in nitrogenase activity observed, i.e., it coincided with the least observed difference in rhizobial population between healthy and infected nodules.

Transmission of a WCMV-complex by R. leguminosarum was reported by early investigators (Johnson and Jones 1943), who used a homogenate of infected nodular tissue (as inoculum). There was no evidence for transmission of WCMV by R. trifolii isolated from infected nodules, cultured, and inoculated back to red clover seedlings. There have been no reports of infection of bacterial cells by plant viruses, and, in other phases of this study, virus particles were not observed within rhizobial cells or bacteroids by electron microscopy. It seems likely that the earlier report of rhizobial transmission was based on inadvertant carryover of virus particles into the inoculum.

The pattern of virus infectivity indicated that the relative concentration of WCMV reached its maximum at 9 weeks after virus inoculation. This could be related to a higher rate of WCMV replication in the non-nodular tissue, followed by transport to, and accumulation within the nodules. Alternatively, the virus may have been synthesized directly within nodular tissue. The peak in virus infectivity coincided with the maximum nitrogenase activity. Therefore, a possible photosynthate movement from the areial to the underground tissues might have enhanced the virus accumulation in the nodules. The decline in the virus infectivity at 12 weeks can be attributed either to disintegration of some of the virus particles (Krass and Ford 1969) or to the presence of inhibitory materials in the extracts prepared from older nodules. The presence of inhibitors to the virus infections in senesced plant tissue has been reported (Bawden 1964).

Several serological methods, based on specificity of the antigen-antibody reaction, have been used extensively for detection of viruses and determining their relationships (Bos et al. 1960). Although agar double diffusion and microprecipitin tests require only simple procedures, they are not always sensitive enough to detect plant viruses at low concentration in crude sap. The application of IEM provides a rapid and sensitive procedure to identify virus particles (Derrick and Brlansky 1976). The maximum relative concentration of WCMV particles in nodular extracts was detected at 6 weeks by this technique. Since maximum virus infectivity was detected at 9 weeks, it is possible that some of the WCMV particles detected earlier by IEM may have been

non-infectious, because the antibody coating of the grid could have bound empty virus protein capsids, or virus fragments. An alternative explanation for the discrepancy seen between maximum particle numbers and maximum infectivity could be that nodule extracts had a varying interference effect on either type of assay.

SECTION 5

EFFECTS OF WCMV INFECTION ON NODULAR CYTOLOGY

5.1 Materials and methods

5.1.1 Histological and ultrastructural studies

At each harvest, 2 nodules were randomly excised from the roots of each plant. A total of 40 nodules from healthy and infected plants were collected and divided into two groups. The 25 nodules making up the first group were cut into halves longitudinally and some of them were cut vertically into four quarters representing regions 1, 2, 3 and 4 (Mosse 1964). This group was used for light and electron microscopy, while the second group was used for immunofluorescence microscopy.

5.1.2 Light and electron microscopy

The sliced nodular tissues were fixed in 3% glutaraldehyde in 0.1 M Na-cacodylate, pH 7.2 (Gourret 1975) overnight at 5°C. After removal of the fixative, the tissues were washed thoroughly with 4 changes of cacodylate buffer during 80 minutes, and postfixed in 2% OsO_4 in cacodylate buffer for 2 hours at room temperature. The fixed nodular tissues were rinsed again, and left overnight in the same buffer at 5°C in uncapped vials. They were then washed with 3 changes of cold

distilled water, 20 minutes each, followed by staining with 1% uranyl acetate in double-distilled water (w/v) for 4 hours at 5°C, and rinsed with distilled water for 15 minutes. Subsequently, the nodular samples were dehydrated, infiltrated and embedded, as described earlier.

Thin sections were cut using glass knives mounted on a Reichert OM-U2 ultramicrotome and mounted on copper grids (200 mesh) coated with Formvar and stabilized with carbon. They were poststained with 2% uranyl acetate for 90 minutes, and lead citrate for 5 minutes. Sections were examined with a Philips 300 electron microscope.

Nodule development and differentiation into zones was examined by light microscopy. Relatively thick sections (2 μ) were cut and transferred to glass slides and stained with toluidine blue on a warm plate. The sections were rinsed with distilled water and examined with a Ziess universal microscope, using either phase contrast or bright field illumination. Micrographs were taken on Kodak Ektachrome-160 film.

5.1.3 Immunofluorescence microscopy

5.1.3.1 Tissue preparation

Halves and quarters of the remaining 15 nodules were fixed in 3% glutaraldehyde in cacodylate buffer for 4 hours at 5°C. After tissues were washed with 6 changes of distilled water, for 10 minutes each, they were dehydrated with a cold graded ethanol-tertiary butanol series and infiltrated with paraffin oil plus butyl alcohol (Johansen 1940).

Subsequently, the tissues were embedded in a paraffin polymer with a melting point of 56.5°C («Tissue Prep,» Fisher Scientific Co., Ltd.). An AO Spencer No.820 rotary microtome was used to cut the embedded tissues into 8-10 μ thick sections. The sections were affixed to slides with Mayer's adhesive. The embedded nodules were either stored as paraffin blocks or sections, at 5°C.

5.1.3.2 Isolation and conjugation of anti-WCMV globulin

The immunoglobulin from WCM-antiserum was precipitated with saturated ammonium sulfate using a procedure similar to that described by Sinha (1974). The suspension was centrifuged at 12000 rpm (14,400 g) for 20 minutes and precipitated antibodies were washed with a 50% saturated solution of ammonium sulfate, recentrifuged, and resuspended in phosphate buffered saline (PBS). The suspension was dialyzed against several changes of PBS for 48 hours with continuous stirring using a magnetic stirrer. After measuring the O.D. at 280 nm, the protein concentration was adjusted to 1% in 0.25 M sodium carbonate buffer (pH 9.8), placed in a dialysis tube, and dialyzed against 10 volumes of the same buffer containing 0.1 mg/ml fluorescein isothiocyanate (FITC, Sylvana Co., Milburn, N.J.) for 24 hours. The labelled globulin was then dialyzed against PBS to eliminate excess FITC. The conjugates were then absorbed for 1 hour with 50 mg of healthy pea leaf acetone powder per 2 milliliters of conjugated preparation (Coons 1958), and after centrifugation at 3000 rpm for 15 minutes, the supernatant

containing FITC-labelled antibodies, was collected. Conjugated normal serum was prepared as described for anti-WCMV globulin, and used as a control.

5.1.3.3 Staining

Sections were deparaffinized by placing the slides in a jar containing xylol which was changed twice, at 10-minute intervals. Subsequently, they were washed with two changes of 100% ethanol for 10 minutes and then dried for 10 minutes at 37°C. The direct staining method was carried out by covering the sections with drops of the FITC-labelled antibody. Sections of healthy nodules, stained with FITC-labelled anti-WCMV globulin, and sections of infected nodules stained with FITC-labelled globulin from normal serum, were prepared as controls. The slides were incubated in petri dishes on a rack standing in a water bath, for 2 hours at 37°C. The sections were then washed with 2 changes of PBS, 10 minutes each at 37°C, followed by mounting them with equal parts of PBS and glycerol. The observations were made through a Zieiss universal microscope equipped with a transfluorescence system. The exciter filter IC (combination of BG 38 and BG 3/4) was used, along with a 50/44 barrier filter to view the fluorescence of the tissues. Photomicrographs were taken by using Kodak Tri-X film, with exposures of 20-25 minutes.

5.2 Results

5.2.1 General remarks, definitions and abbreviations

The present study will discuss in detail the major tissues in red clover nodules relative to those published on the nodules of other clover species, particularly T. subterraneum, T. parviflorim and T. repens L. (Dart and Mercer 1963; Moss 1964; Gourret 1975). Cytological changes in nodular tissues of virus-infected plants were investigated in conjunction with determination of nitrogenase activity, leghemoglobin content, and other characteristics by harvesting nodules at various plant growth stages. Hence, the approximate age of the nodules at each harvest would be equivalent to the time of sampling (number of weeks after virus inoculation) plus 4 weeks to represent the period between inoculation of red clover seedlings with Rhizobium suspension and the time of inoculation with WCMV.

This investigation was carried out to study the nodule ontogeny of red clover plants, as well as to determine the cytopathological changes and the localization of WCMV in the various types of cells in the nodular tissue. Although the gross structure either of healthy or WCMV-infected nodules was similar, at the ultrastructural level, many differences were observed and will be described also.

Apparently, several concepts have been used in naming the various structures and cellular organelles in the nodular tissues. To avoid confusion, the most common terms will be used for describing the

cellular contents of the healthy and diseased nodules. Because they have been used somewhat ambiguously by previous workers, it is necessary to define the most important ones.

Bacteroid: the final form of the differentiated Rhizobium, often spherical, and presumably the active agent in the N_2-fixation process.

Bacteroid-filled cell: the host cell containing bacteroids. The term «bacteroidal cell» has been used by some workers.

Infection thread: a finger-like invagination passing through the host cell wall and containing bacteria embedded in its matrix.

Peribacteroidal membrane: a membrane surrounding the bacteroid.

Peribacteroidal space: the area between the bacteroid wall and the peribacteroidal membrane.

Rhizobium: the bacterium, which still retains its rigid rod shape and may be enclosed within a membrane which probably originated from the host cell membranes.

The abbreviations used earlier for the various structures and organelles in red clover and pea tissues (see page 69) will be used here. In addition, the following abbreviations for the structures found in the nodular tissue of red clover are used in the electron micrographs.

B bacterium

Bd bacteroid

BM bacteroid membrane

BW bacterial cell wall

BZ	bacteroid zone
CB	cells containing bacteroids
CC	companion cell
CP	carbohydrate platelet
CT	cortex tissue
DB	deteriorated bacteroid
DC	dead cell
Gr	granular bodies
GG	glycogen granule
IC	interstitial cell
IM	infection thread matrix
IT	infection thread
ME	membrane envelope
MS	multivesicular structure
Pd	plasmodesmata
PHB	poly-B-hydroxybutyrate
PM	peribacteroidal membrane
PS	peribacteroidal space
Rh	Rhizobium
S	spheroplast
SE	sieve element
TW	thread wall
VB	vascular bundle
VL	vesicle-like structure

5.2.2 Nodule development and Rhizobium ontogeny

5.2.2.1 Nodule morphology and gross structure

The nodules started to form laterally on red clover roots 8 days after inoculation with Rhizobium trifolii. They had a hemispherical shape at the early stage of development, and they became fully elongated 14 days after inoculation. The nodule color was frequently pinkish to reddish-brown, due to the leghemoglobin in the bacteroids, but aged nodules became more brownish. They were individually distributed, mainly on the root hairs, and their basal ends were attached to the roots.

Light and electron microscopy of various sections of red clover nodules revealed that the tissue is composed of three major parts:

1. Apical area.--This is represented by several layers of meristematic cells distributed at the distal end of the nodule, which produces files of cells towards the bacterial zone. These layers of cells can be divided into two regions: Region 1, which includes actively dividing cells, which stained irregularly with toludine blue, confined to the tip portion of the nodules, and usually associated with bacterial infection threads and/or a few rhizobia (Figure 33A); Region 2, characterized by the enlargement of meristematic cells, which were evenly stained with toludine blue and contained many rhizobia (Figures 33B, 34B). The cells in this area of the young nodules showed an indication of the normal meristematic pattern and contained large

Figure 33. Light micrographs of various areas in the healthy nodular tissue, stained with toluidine blue.

A. Region 1, consisting of meristematic cells containing few rhizobia.

B. Region 2, which contains numerous rhizobia. Some cells having large central vacuoles (Va) remain.

C. Bacteroid zone (region 3), composed of cells containing bacteroids (CB).

D. In the bacteroid zone there are cells without bacteroids called interstitial cells (IC).

E. The vascular bundles are adjacent to the bacteroid zone.

F. Region 4 contains cells with degenerated contents (DC).

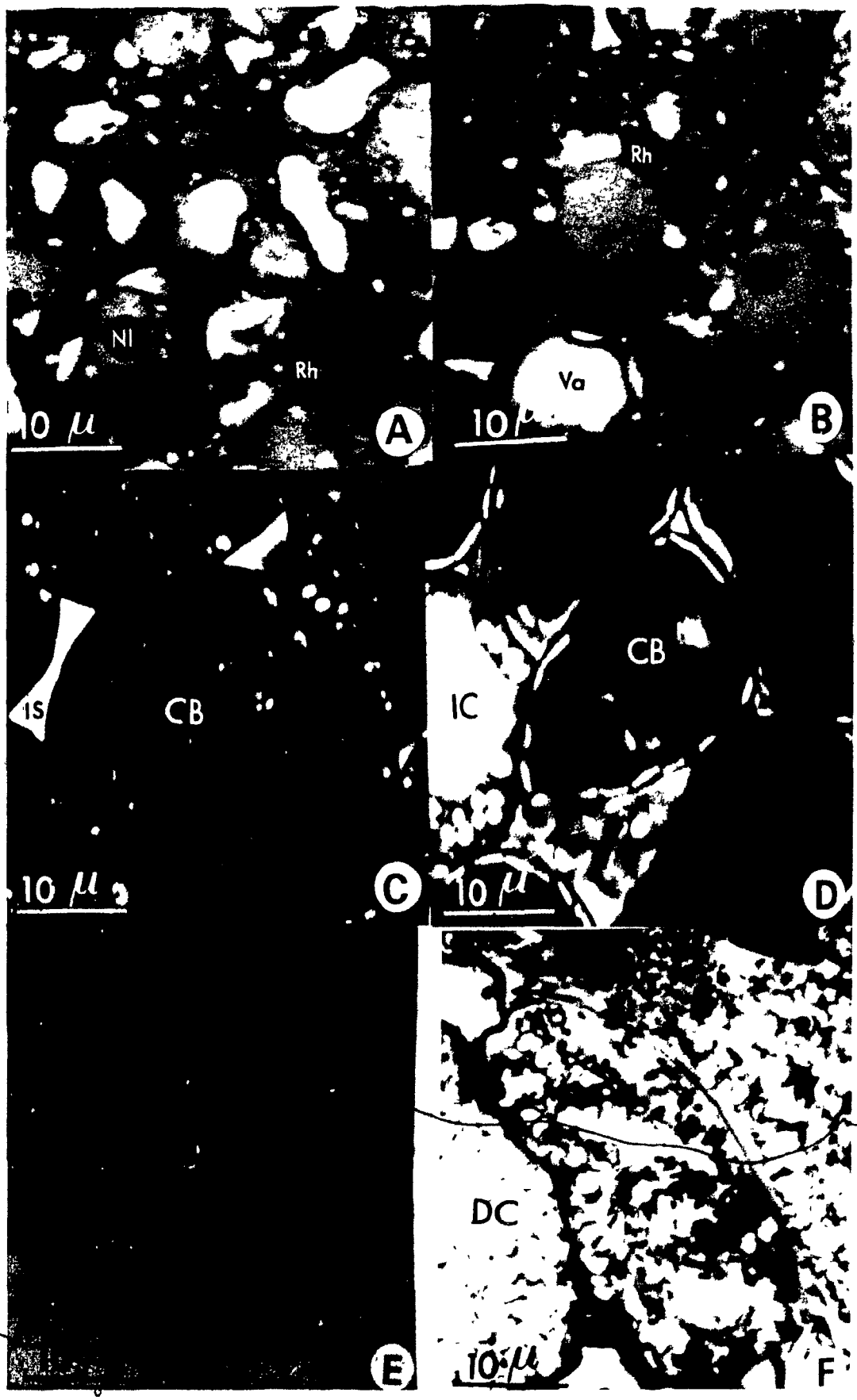

Nl
Rh
10 μ
A
Rh
Va
10 μ
B
IS
CB
10 μ
C
CB
IC
10 μ
D
DC
10 μ
E
F

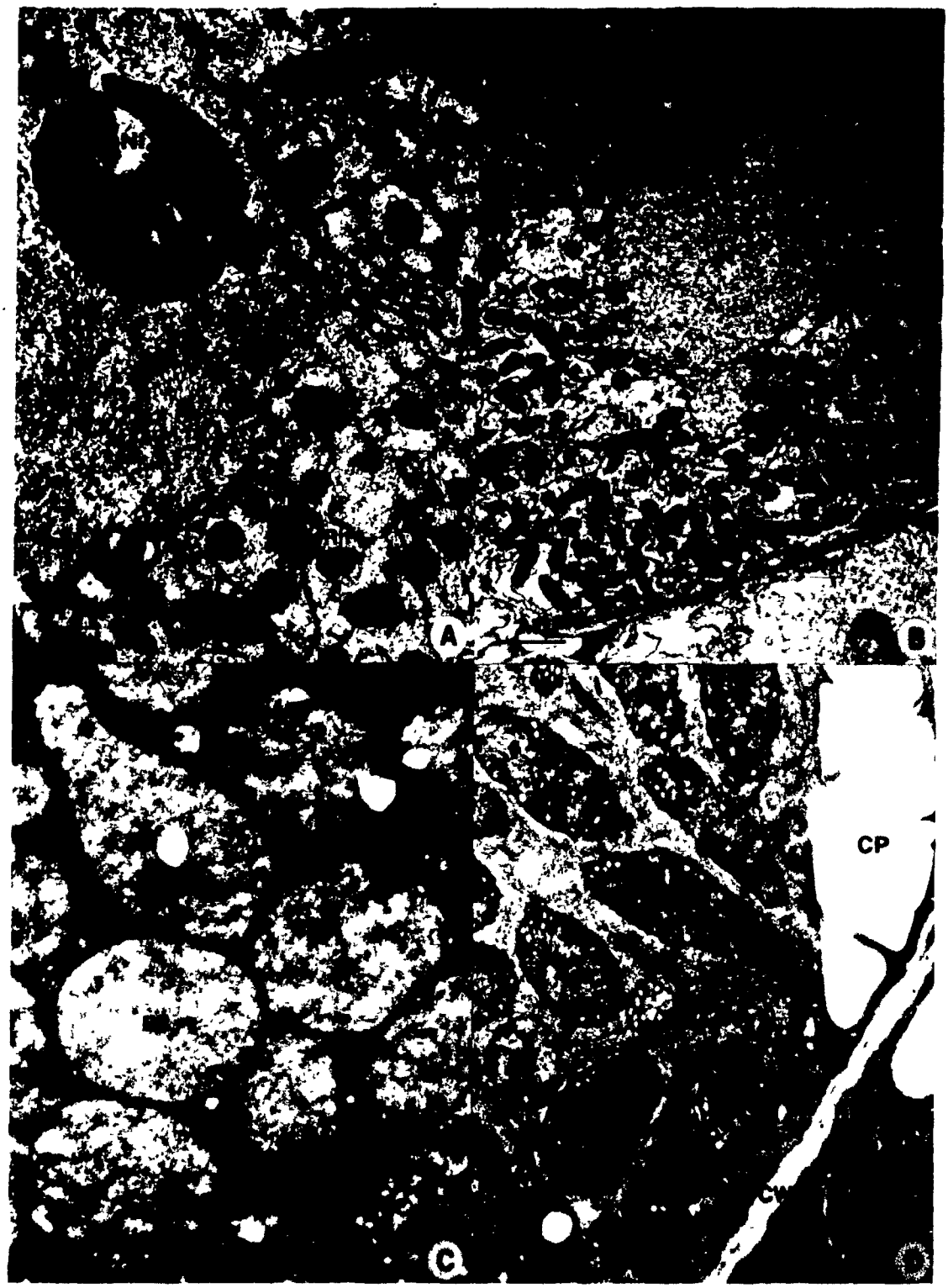
Figure 34. Electron micrographs showing the cells of regions 1, 2 and 3 in healthy nodular tissue.

A. Meristematic cells, in region 1, with large nucleus and prominent nucleolus (NI). Note the presence of a few rhizobia, associated with extensive endoplasmic reticulum (ER).

B. Meristematic cells of region 2, having less densely stained nucleus (N), in which discrete nucleoli were not seen. Numerous rhizobia are present in the cytoplasm.

C. The early stage of Rhizobium transformation into bacteroids (Bd).

D. Fully developed bacteroids in a cell in region 3. Endoplasmic reticulum (ER) and Golgi bodies (GB) are interspersed with bacteroids.


N
A
B
CP
C

nuclei, defined nucleoli, extensive endoplasmic reticulum (ER), mitochondria. ribosomes and Golgi bodies (Figure 34A).

2. Bacteroid zone.--This occupied a large area of the nodular tissue. To keep the sequences of the Rhizobium transformation in order, it can be divided into two successive bacteroidal regions (i.e., 3 and 4). In region 3, the host cells are filled with the symbiotic form of bacteroids (Figures 33C,D; 34C,D); central vacuoles or nuclei could be recognized in these cells. Occasionally, uninfected cells (no bacteroids), called interstitial cells, were found in this zone (Figure 33D). These cells typically had a prominent nucleus and nucleolus, a large central vacuole and numerous starch granules (Figure 33A). Intercellular spaces were observed often among the cells in this zone, and carbohydrate platelets were also frequently seen scattered or forming a belt surrounding the bacteroids and parallel to the host cell wall (Figure 35B). Region 4, located in the basal part of the nodule, showed a gradual increase in the extent of degradation of host cells (Figure 33F) and contained some free bacteria (Figure 35C). Typical bacteroids could be found easily in the transitional zone between regions 3 and 4 (Figure 35D).

3. Vascular bundles and cortex tissue.--Vascular bundles anastomosed with the steles of the roots were noted within the cortex tissue (Figure 33E) and were distributed around the bacteroidal zone of the nodule. Each bundle consisted typically of xylem elements and phloem cells. The xylem elements were adjacent to a highly specialized cell with protruding wall ingrowths called transfer cells. The latter contained

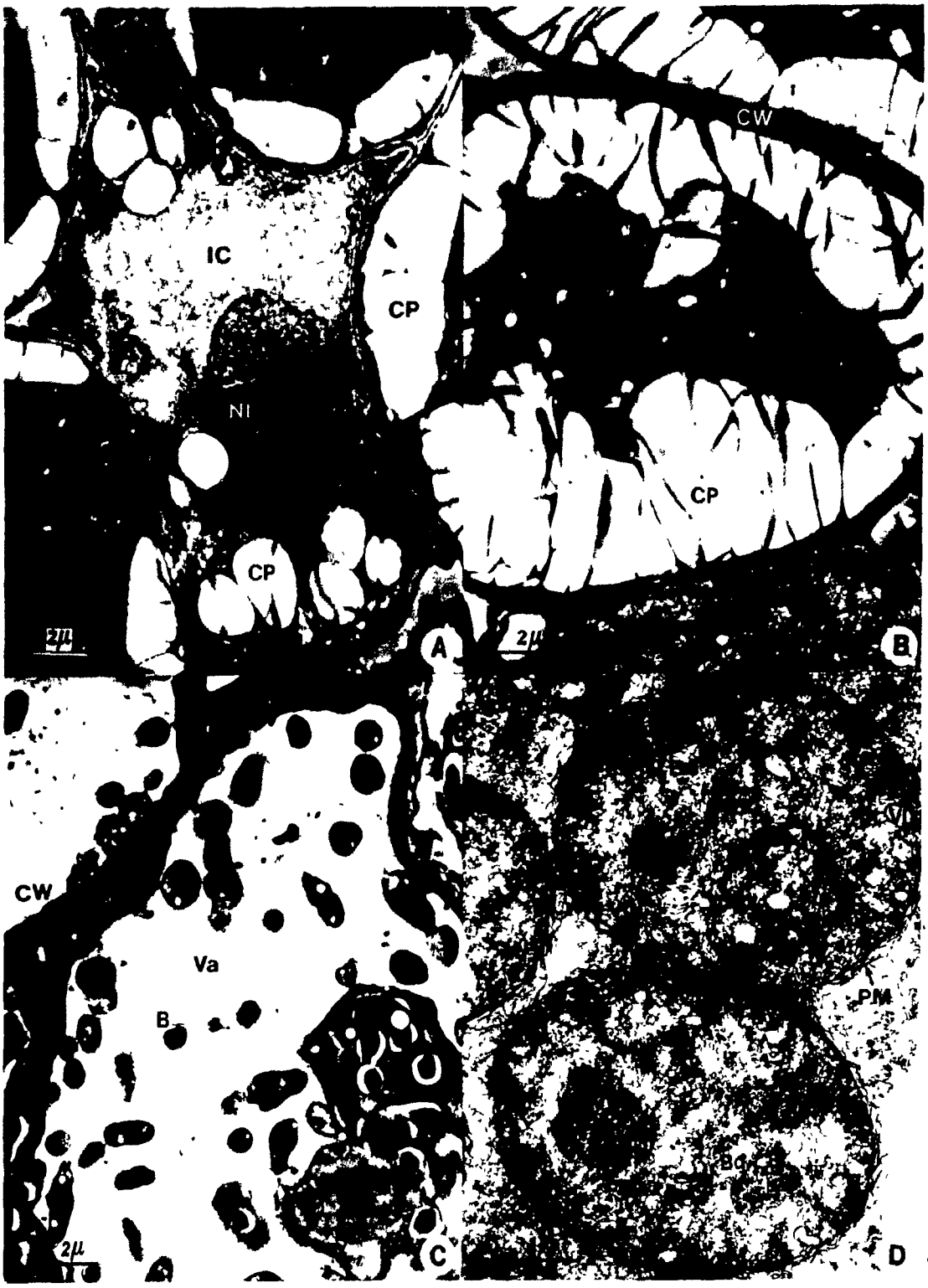
Figure 35. Ultrastructural details of the cells of the bacteroid zone in healthy nodules.

A. Note that the interstitial cell is adjacent to the cells containing bacteroids.

B. Cell containing bacteroids (Bd), surrounded by a belt of carbohydrate platelets (CP) lining the cell wall.

C. Cell in region 4, containing less cytoplasm, and both free bacteria (B) and bacteria embedded in the infection thread (IM).

D. Typical bacteroids in the transformation area between regions 3 and 4.


CW
IC
CP
NI
CP
CP
2μ
A
2μ
B
CW
Va
B
PM
2μ
C
D

normal cellular contents, and presumably are part of the pericycle, which consists of 1-5 cell layers. The cortex tissue was composed of highly vacuolated cells, which were not infected with the Rhizobium. These separated the vascular bundles from the bacteroidal zone, with a layer of three or more cells, which are probably surrounded by sclerenchyma tissue.

5.2.2.2 Infection thread and emergence of bacteria

The sequence of events which led to nodule formation was initiated with the development of an infection thread in the root hair (Figure 36). Observations showed that infection threads can be found frequently as projections terminating in the cytoplasm of the meristematic cells, i.e., regions 1 and 2 (Figure 37A), but it was found occasionally in the bacteroid zone (Figure 37B). The infection thread was surrounded by a thick wall and a membrane which appeared to be a continuation of the cell wall and the plasmalemma of the host cell (Figure 37A). Each thread possessed electron-dense matrix in which the bacteria were embedded (Figure 37C). Also, it was noted that the embedded bacteria were arranged in single file or a group in each thread. Every bacterium was about 0.7-1.0 μ long and the bacterial cytoplasm contained prominent electron transparent bodies which many workers have assumed represent poly-B-hydroxybutyrate (PHB) (Figure 37A).

Unwalled infection threads were noticed frequently in the host cytoplasm, in which the bacteria lie singly and near the vesiculated

Figure 36. Diagram showing the Rhizobium ontogeny and nodule development on clover species. The fully developed nodule (at the bottom) can be divided into four regions. (Copied from Gourret 1975.)

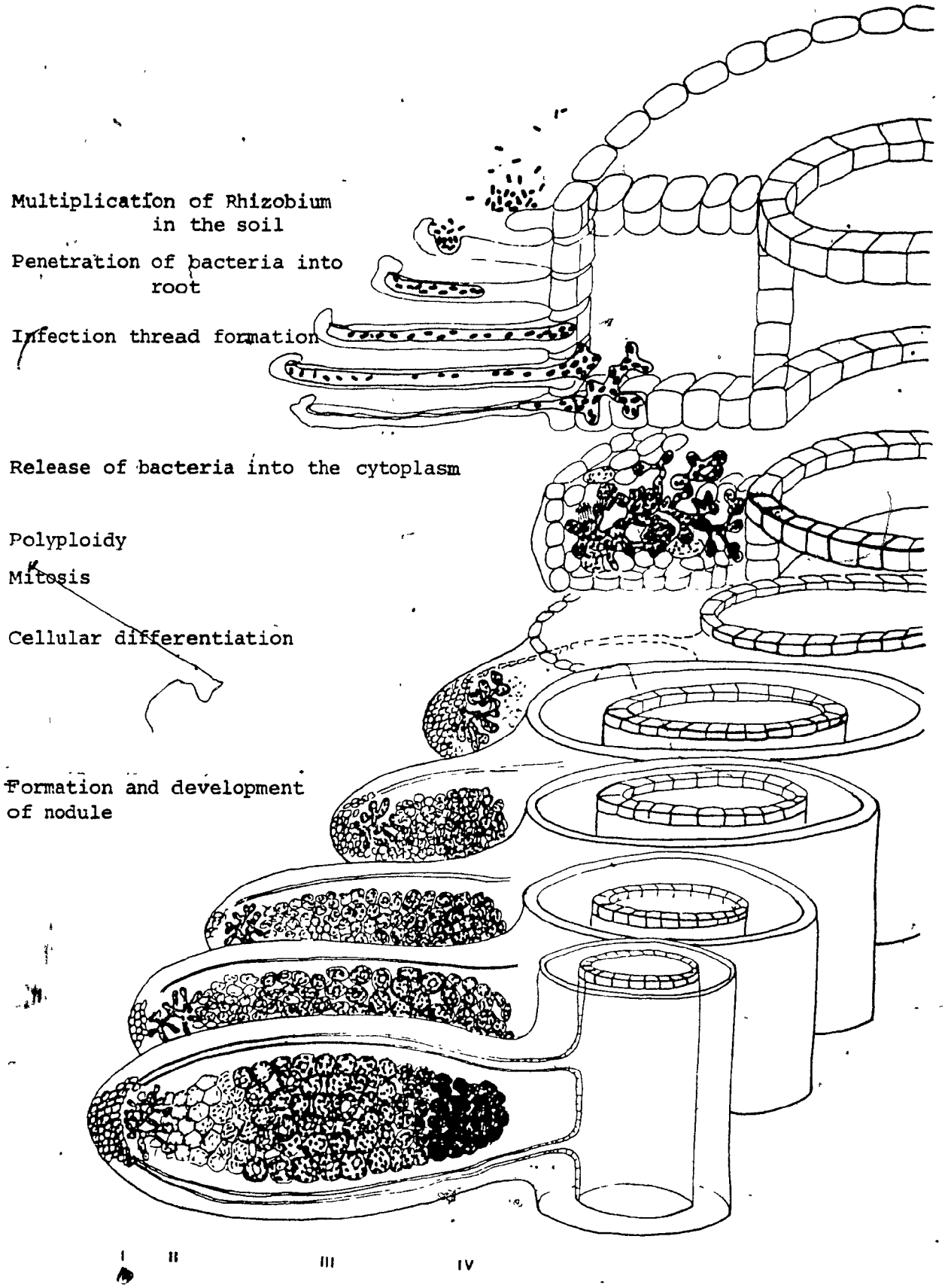

Multiplication of Rhizobium in the soil
Penetration of bacteria into root
Infection thread formation
Release of bacteria into the cytoplasm
Polyploidy
Mitosis
Cellular differentiation
Formation and development of nodule
I
II
III
IV

Figure 37. Electron micrographs showing the infection threads in which the bacteria are embedded, and the rhizobia in the meristematic cells of healthy nodules.

A. Longitudinal section of the infection thread (IT), which appeared as projection in the cytoplasm surrounded by the continuation of the host cell wall and the plasmalemma. The bacteria embedded in the matrix of the thread contained electron transparent areas, which probably represent poly-B-hydroxybutyrate (PHB).

B. Bacteria arranged in a single file, which seemed to be in the process of release from the infection thread.

C. Cross section of the infection thread which is surrounded by a thick wall.

D. Cross section of infection thread, in which matrix (IM) is not surrounded by heavy wall, and the bacteria are arranged at the periphery of the thread.

E. The bacterial rhizobia after emergence, surrounded by a membrane envelope (ME) and containing glycogen granules (GG).

A
B
C
D
ME

borders of the thread matrix (Figure 37D). The released bacteria (rhizobia) seemed to contain granular bodies, probably glycogen, and were surrounded by cytoplasmic membranes or membrane envelopes, which may have been of host cell origin. Each membrane was parallel to the bacterial cell wall, and a defined space was noticeable between them. Meanwhile, extensive endoplasmic reticulum, ribosomes, and several mitochondria were associated with the rhizobia in the cytoplasm of the host cell (Figure 37E).

5.2.2.3 Rhizobium ontogeny and bacteroid differentiation

As described earlier, several rhizobia were noticed in region 1 of the meristematic cells (Figure 34A), while many of them were observed in region 2 (Figure 34B). At this phase, each rhizobium seemed to have lost its PHB, which was associated with other cytological changes in the cytoplasm of the host cells containing rhizobia. These changes involved mainly the nucleus which increased in size, and then became amoeboid, and the nucleolus which lost its identity and was replaced by scattered chromatin materials (Figure 34B). Most frequently, the nucleus deformed and converted into a prominent vacuole. Other organelles, such as mitochondria and proplastids, were seen at the periphery of the cells, while the Golgi bodies and ER were dispersed among the rhizobia. The cell walls were thinner between the invaded cells, and were thicker adjacent to the intercellular spaces.

At the early phase of region 3, considerable enlargement of the rhizobia was observed. They were changed from rod-shaped into short-club, pear-shaped, ellipsoid and round shapes (Figure 34C). Spherical or oval forms of the transformed rhizobia, which constituted the so-called bacteroid, considered to be the active agent in symbiotic N_2-fixation, were frequent in this region. Each bacteroid was surrounded by two membranes, the cytoplasmic membrane and peribacteroidal membrane, which were parallel to the bacterial cell wall. The space formed between the two outer membranes, or peribacteroidal space, may be considered the site for leghemoglobin synthesis or accumulation, as was shown by the cytochemical reaction with DAB (Figure 24). In this region, the mature host cells were often occupied by bacteroids which contained crystalline granules and numerous vesicle-like structures as well (Figure 35D). At the end of this region, the senesced bacteroids and the degenerated organelles of the host formed the basal region of the nodule (i.e., region 4) which contained some free bacteria, disintegrated infection thread and degenerated cells (Figure 35C).

5.2.3 Effect of WCMV infection on the nodule cytology

5.2.3.1 Distribution of WCMV particles in the nodular tissue

At each harvest, ultrathin sections of WCMV-infected red clover nodules were prepared and examined by electron microscopy, to investigate the distribution of WCMV particles and other cytological changes within the nodular tissue. The observations revealed that

aggregates of virus were the most common form encountered in the cytoplasm of the infected cells (Figure 38A,B,C). In addition, similar virus inclusions were noticed in the nuclei of some meristematic cells (Figure 38A), and transfer cells (Figure 40C) as well.

Occasionally, in nodules harvested 9 weeks after virus inoculation, WCMV aggregates were found in banded form associated with the infection thread (Figure 39A) and/or situated between the thread and the wall of some host cells (Figure 39B). Frequently, aggregates of virus particles were noted among the immature and mature bacteroids in the bacteroid-filled cells (Figure 39C,D), but they were not detected inside the bacteroids, or in any cells of the healthy nodular tissue. Mature bacteroids in cells in which virus was also found appeared to be less densely packed.

5.2.3.2 Cytopathological changes

The observations in this section concern the cytological changes observed in nodules taken at various growth stages of red clover. However, only the significant alterations in the cells of WCMV-infected nodular tissue, which were infrequently seen in the corresponding cells of healthy nodules, will be detailed.

Figure 38. Distribution of WCMV aggregates in various areas in the infected nodular tissue.

A. Viral inclusions in the meristematic cells. The smaller (lower) aggregate is apparently within the nucleus.

B,C. A WCMV aggregate in the cytoplasm of the companion and parenchyma cells.

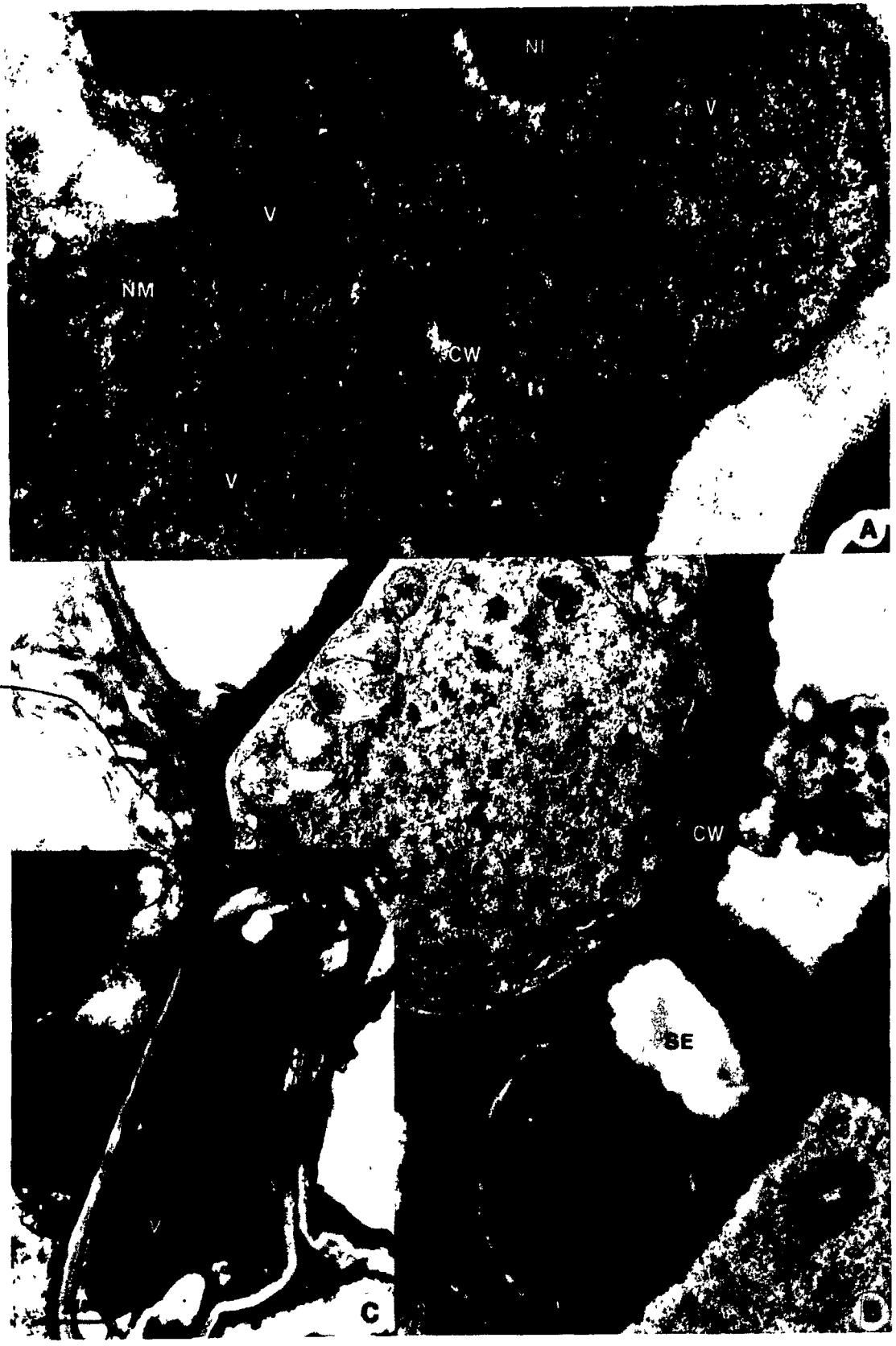

NI
V
V
NM
CW
V
A
CW
SE
C
D

Figure 39. Electron micrographs showing WCMV aggregates in nodules harvested 9 weeks after virus inoculation.

A. Banded virus aggregates (V) associated with the infection thread, and in cell below it.

B. WCMV aggregates (V) near the bacteria and the cell wall.

C,D. WCMV aggregates (V) distributed among the immature (39C) and mature (39D) bacteroids. In this region, the bacteroids apparently are not filling the cells completely.


B
IT
V
Rh
Rh
Rh
St
A
B
PS
Bd
PS
C
D

5.2.3.1 Ten-leaf stage (3 weeks after virus inoculation)

Some of the cytopathological changes in the different zones of WCMV-infected nodules harvested at 3 weeks after virus inoculation are shown in Figure 40A,B. Some of the most apparent changes at this stage were noted in the meristematic cells (Figure 40B), which were characterized by (1) having electron-dense cytoplasm, apparently due to the high concentration of the free ribosomes, (2) presence of numerous vesicles, distributed randomly in the cytoplasm, (3) relative enlargement of the space between the membrane envelope and the bacterial cell wall, (4) invaginations of the plasma membrane and formation of vesiculated structures, (5) an apparently dilated endoplasmic reticulum, with distinct lumena in these cells, (6) large starch grains which were found occasionally in disintegrated proplastids. The mitochondria in these cells did not reveal any peculiar changes that could be attributed to the virus infection. Similarly, normal mitochondria were found in the parenchyma cells containing WCMV aggregates and associated multivesiculated bodies (Figure 40A).

At this stage (3 weeks) the bacteroids in WCMV-infected nodules (Figure 39C,D) did not completely fill the host cells (i.e., in region 3), and retained the normal shape shown by their counterparts in healthy nodular tissue (Figures 34C, 35D). Less than 6% of these bacteroids appeared deteriorated or contained unusual bodies, such as those shown in Figure 41B in bacteroids of diseased nodules. Frequently, the endoplasmic reticulum was associated with the immature bacteroids in this

Figure 40. Various changes in the different cells of WCMV-infected nodules.

A,B. Cytopathological changes in nodular meristematic tissue detected 3 weeks after virus inoculation.

A. WCMV aggregates (V) and multivesicular structure (MS) in cytoplasm.

B. Numerous vesicles (Ve) and rhizobia (Rh) having enlarged space between membrane envelope and bacterial cell wall, or apparently dilated membrane envelopes.

C,D. Cytopathological changes observed 6 weeks after virus inoculation.

C. Transfer cell, with typical wall ingrowths (lower right), and numerous vacuoles (Va), some of which are fused with apparently deteriorated mitochondria (M).

D. Interstitial cell, with several peripheral vacuoles (Va). Arrows indicate thinner areas of cell wall.

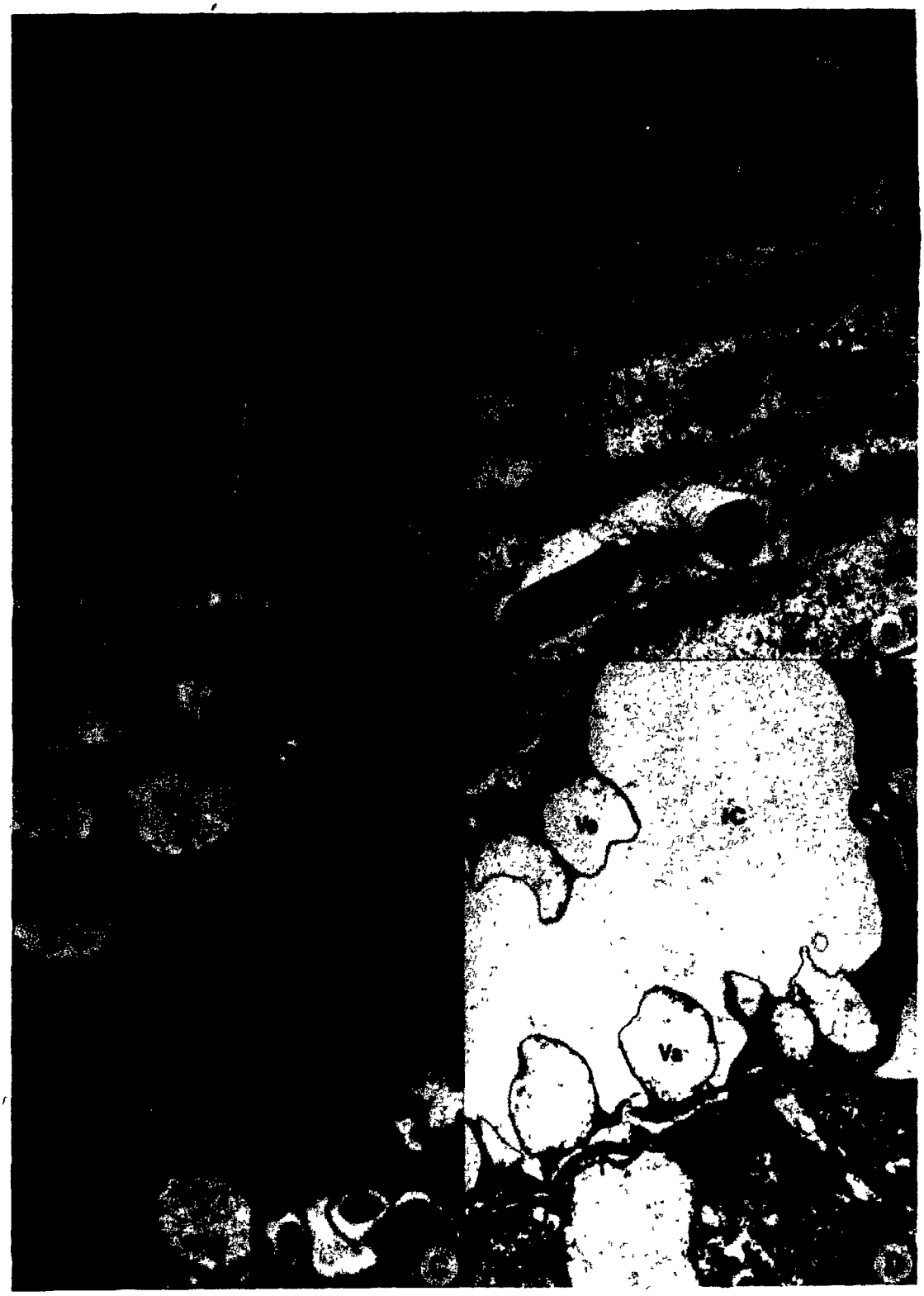
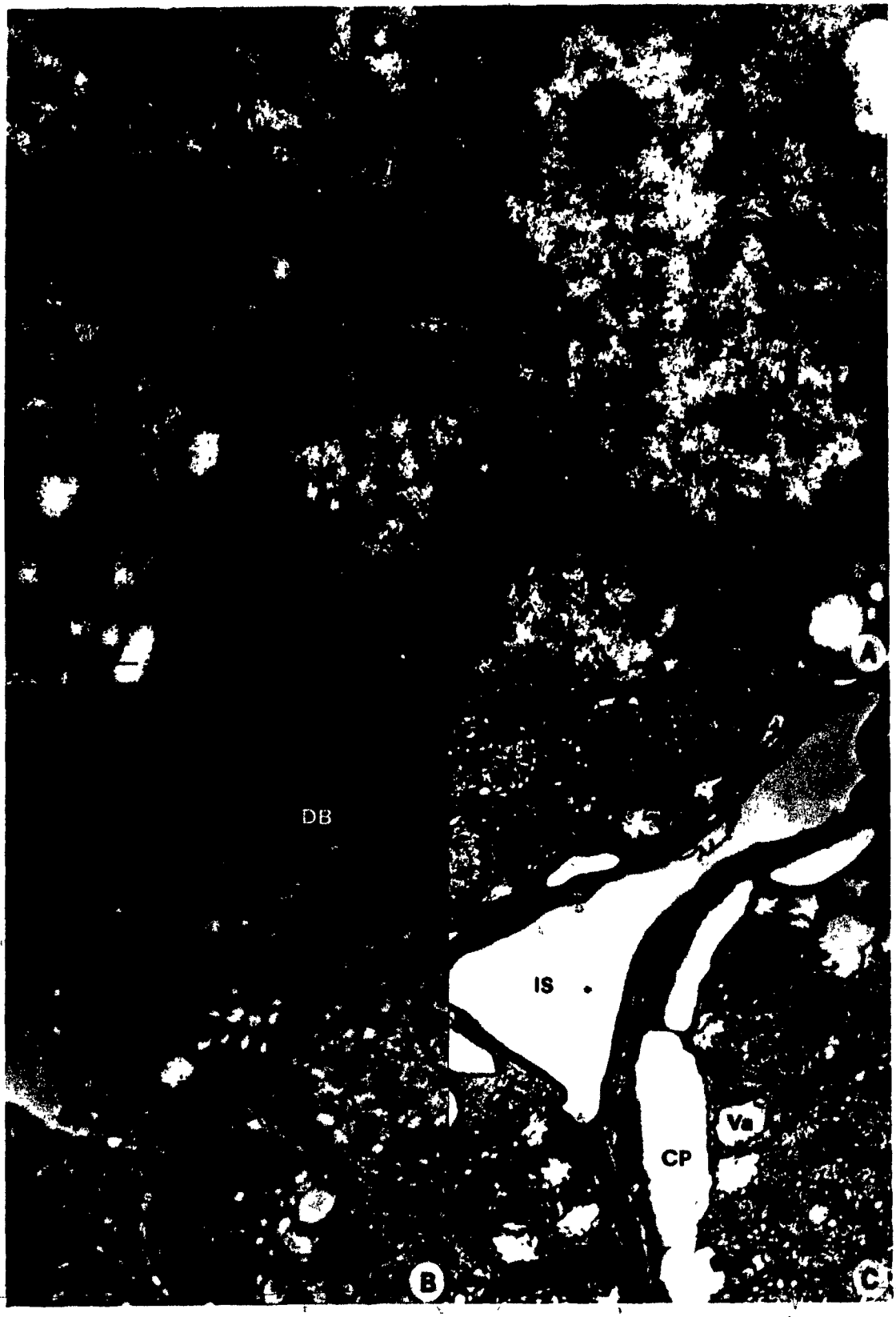

Va

Figure 41. Additional changes noticed in the bacteroid zone in nodules harvested 6 weeks after virus inoculation.

A. Some bacteroids (Bd) with a medial constriction (small arrow head). and crystalline granules near one end.

B. Apparently deteriorated bacteroids (DB) containing many vesicles were found in some cells.

C. Large intercellular space (IS), and deteriorated structures, possibly remnants of proplastids, at the periphery of cells containing bacteroids.


DB
IS
CP
Va
A
B
C

tissue (Figure 39C). No significant changes in the cells of the vascular tissue of infected nodules were noted except the presence of WCMV particles in the cytoplasm and/or the nuclei as was described earlier (Figure 38).

5.2.3.2.2 Preblooming stage (6 weeks after virus inoculation)

At this stage, more advanced cytopathological changes were recognized in the WCMV-infected nodules. The most remarkable alteration was noticed in some of the mitochondria, which showed disintegrated cristae (Figure 40C). This was detected mainly in the transfer cells and the bacteroid zone, including the interstitial cells, in which vacuolated structures, possibly the remnants of the degenerated mitochondria, appeared (Figure 40D). In the transfer cells, variable thickness of the cell wall and its internal protrusions was noted, the nucleolus seemed to be granulated, and numerous vesicles were seen between the plasma membrane and the cell wall (Figure 40C).

Observations showed no further differences between meristematic cells of healthy and WCMV-infected nodules except that wider spaces were noticed between the bacterial walls and their membrane envelopes (Figure 40B). Structures which may have been disintegrated proplastids, associated with degenerated mitochondria, were noticeable at the periphery of the cells containing bacteroids, and enlarged intercellular spaces were found occasionally among those cells (Figure 41C). Some

bacteroids exhibited medial constriction and their membranes appeared invaginated. In these bacteroids the crystalline granules moved to one end, and electron-dense deposits were found along the periphery of each bacteroid. Also, round vesicles were observed between the membranes at the constriction area (Figure 41A). Deteriorated bacteroids, resembling those described at ten-leaf stage (Figure 41B) were seen, and represented approximately 15% of those present in the WCMV-infected nodular tissue.

5.2.3.2.3 Full blooming stage (9 weeks after virus inoculation)

At this stage, several kinds of intracellular changes were noted in the various zones of WCMV-infected nodular tissue. Some of the most striking changes were observed in the meristematic cells (i.e., regions 1 and 2) of which there were two types of cells: (1) cells not invaded by the infection thread or rhizobial bacteria; (2) invaded cells containing rhizobia. The first type was frequently seen in region 1, and was characterized by the absence of the rhizobia (Figure 42A) and the presence of large and small vacuoles as well. The vacuolar matrix contained or was surrounded by numerous electron-dense deposits along the inner surface of membranes. In some of these cells proplastids were occupied by starch grains, and pleomorphic nuclei with distinct nucleoli were observed. In the second type (Figure 42C), the rhizobia seemed to have a hazy shape, and PHB accumulations were not frequently recognized. Abnormal mitochondria and remnants of disintegrated cellular organelles, as well as a less electron-dense cytoplasm, were often seen in these cells. Occasionally, structures resembling proplastids were filled with WCMV particles, and associated with numerous microbodies, probably spherosomes (Figure 42D).

Figure 42. Cytopathological changes in nodules harvested 9 weeks after virus inoculation.

A. Meristematic cells not invaded by bacterial rhizobia contained small and large vacuoles. Note numerous large electron-dense deposits (arrows) distributed in and around the vacuolar matrix.

B. Vesiculated bacteroids, with associated WCMV aggregates, in the infected cells containing bacteroids.

C. Meristematic cell, containing rhizobia, with abnormal mitochondrion (M), and multivesiculated structure.

D. Disintegrated meristematic cell in which hazy rhizobia can be seen. Note WCMV aggregates in proplastid-like organelle surrounded by numerous microbodies (arrow heads).


St
A
B
P
V
S
Rh
0.5μ
C
0.5μ
D

Although many of the bacteroids appeared normal in the cells containing them, about 30% of them seemed to be deteriorated. These bacteroids were vesiculated, with larger spaces between the bacteroidal walls and the peribacteroid membranes (Figure 42B). Also, there was an observable reduction in the carbohydrate platelets which usually lined the cell walls, and aggregates of WCMV particles were occasionally seen distributed among the bacteroids. Similarly, the vascular pattern showed significant alteration in the cells of WCMV-infected nodules. In some cells, particularly transfer cells, degradation of the cell wall ingrowths and the presence of numerous vesicles between the plasma membrane and the cell wall were frequently observed (Figure 43A), while these protrusions were not affected in some other cells (Figure 43B). Also, in other cells, some organelles exhibited abnormalities, such as deformation of mitochondria and an abnormal abundance of osmiophilic globules in the proplastids. Small vacuoles, probably the remnants of disintegrated organelles, and multivesicular bodies were noted in some phloem parenchyma cells.

5.2:3.2.4 Senescence stage (12 weeks after virus inoculation)

The flowers senesced at this stage, and various cytopathological changes were detected in various cells of different zones in the diseased nodular tissue. Cytological changes associated with maturity and/or senescence were also seen in healthy nodules, but were more evident in infected nodules. Highly vacuolated cells were seen frequently in the meristematic regions, and a few cells contained

Figure 43. Vascular conducting cells in the WCMV-infected nodular tissue at 9 weeks after virus inoculation.

A. Transfer cells in which degraded cell wall ingrowths (asterisk) are obvious. Note numerous vesicles between plasmalemma and the cell wall, also several vacuoles (Va) are noticeable. The large nucleus (N) appears less dense than those of healthy cells.

B. Although the wall ingrowths in the transfer cell are not disintegrated, some degenerated mitochondria (M), multivesicular structures (MS), and associated WCMV aggregates (V) are recognizable.

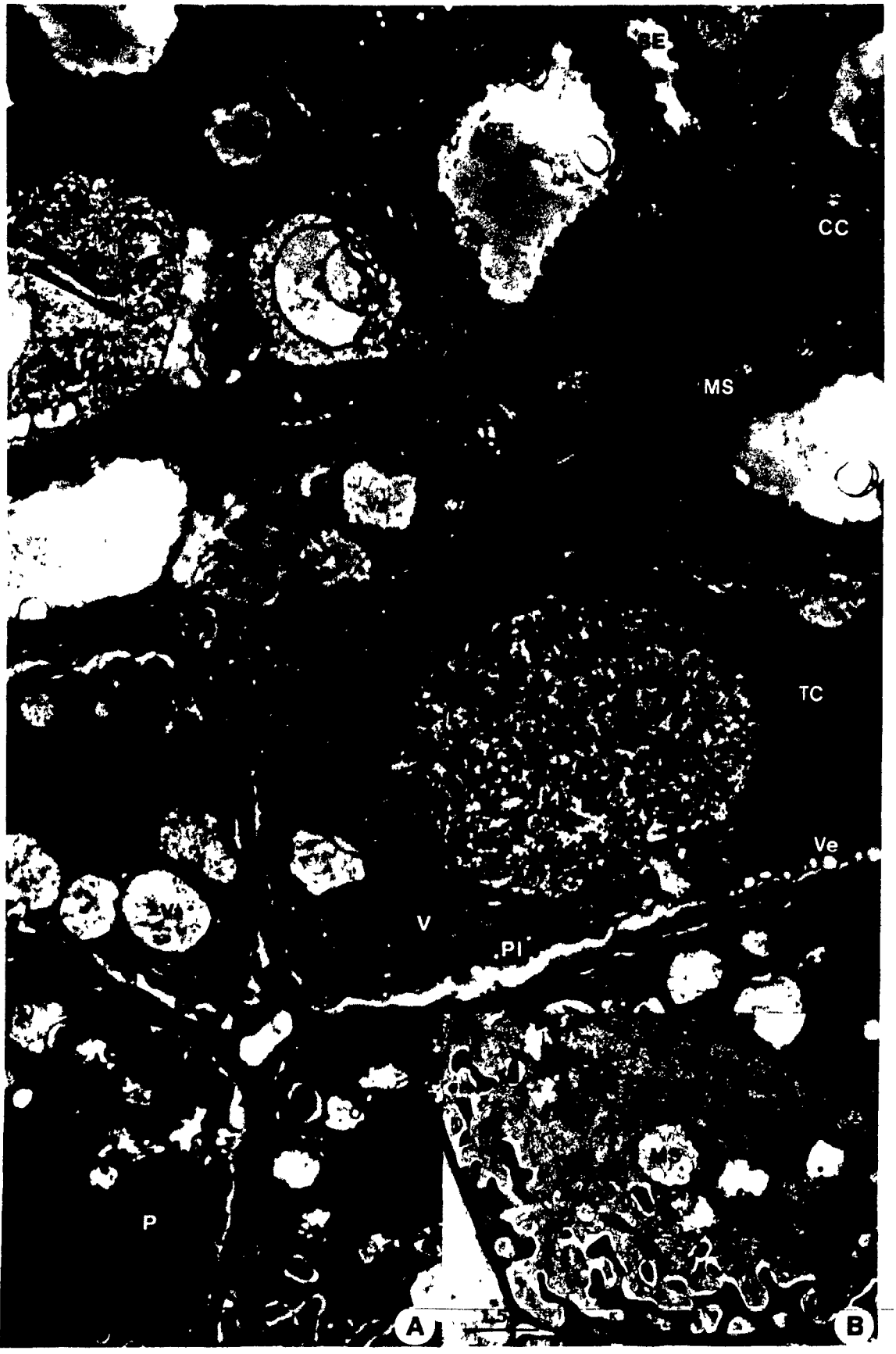

CC
MS
TC
Ve
V
Pl
P
A
B

Figure 44. Electron micrographs showing the most prominent changes in WCMV-infected nodular tissue at 12 weeks after virus inoculation.

A. Meristematic cell containing a nucleus (N) in which nucleolus is not discernible. Note the presence of infection threads (IT) as well as WCMV aggregates (V).

B. Highly vacuolated meristematic cell not invaded by rhizobial bacteria. Numerous large electron-dense deposits (arrows) are located on the tonoplast.

C. Aggregates of WCMV (V) associated with the bacteria (B) in the infection thread in disintegrated cells.

D. Round bacteroids (Bd) in the nodular tissue of virus-free plant.

E. Heterogeneous bacteroids (Bd) in the cells of diseased nodules, some of which have an enlarged peribacteroidal space (PS).

nuclei, which lacked distinct nucleoli (Figure 44A). Some cells were characterized by the presence of a higher number of electron-dense deposits on the tonoplast than those seen at blooming stage (Figure 44B). Large aggregates of WCMV particles were found, associated with the rhizobia in the infection thread, or free in the cytoplasm of the host cells (Figure 44C).

In the bacteroid zone, deteriorated bacteroids were observed, often in the diseased nodular tissues. Three general types of bacteroid containing cells could be distinguished according to their appearance: (1) vesiculated, (2) autolysed, and (3) autophagic. About 27% of the bacteroid containing cells observed contained vesiculated bacteroids which looked more heterogeneous than those in the healthy nodules (Figure 44D,E). The autolysed bacteroids showed a highly disorganized pattern in which the nucleoid accumulated as amorphous granular materials. Approximately 18% of the bacteroid-filled cells contained autolysed bacteroids, and WCMV particles were noted among them (Figure 45A,B). The host cells containing autophagic bacteroids (nearly 12%) were less prevalent than those containing vesiculated and autolysed bacteroids. Their cytoplasmic matrix appeared very thin and debris and remnants constituted the major contents of these cells (Figure 45C). Occasionally, dilated peribacteroid membranes were recognized around the autophagic bacteroids, also the cell wall was less electron-dense (Figure 45C). In region 4, a higher autophagic activity seemed to have occurred in the host cells; only infection threads or some bacteria could be recognized, and other cellular


Vm

Figure 45. Various appearances of distorted cellular contents in WCMV-infected nodular tissue harvested 12 weeks after virus inoculation.

A,B. Deteriorated bacteroids (DB) associated with WCMV aggregates. Note the accumulation of electron-dense materials (arrows) in the bacteroids in (A).

C. Cell with a thin cytoplasmic matrix, and autophagic deteriorated bacteroids.

D. An apparently dead cell, containing various types of debris, such as the remnants of disintegrated bacteroids. An infection thread (IT) and bacteria (B) are also visible.

E. Aggregates of WCMV intermixed with the debris in diseased nodular tissue.


DB
A
V
1.5μ
B
B
IT
DC
DC
1μ
B
D
DB
B
DB
CW
B
V
V
DC
1.5μ
C
0.5μ
E

contents were confused (Figure 45D). Also, electron-dense deposits and WCMV particles, intermixed with the remnants of disintegrated cellular organelles were noticeable (Figure 45E). Ultimately, in the WCMV-infected nodular tissue, the majority of the bacteroids apparently lost their identity and appeared as a clump of dark granules earlier than in the healthy control.

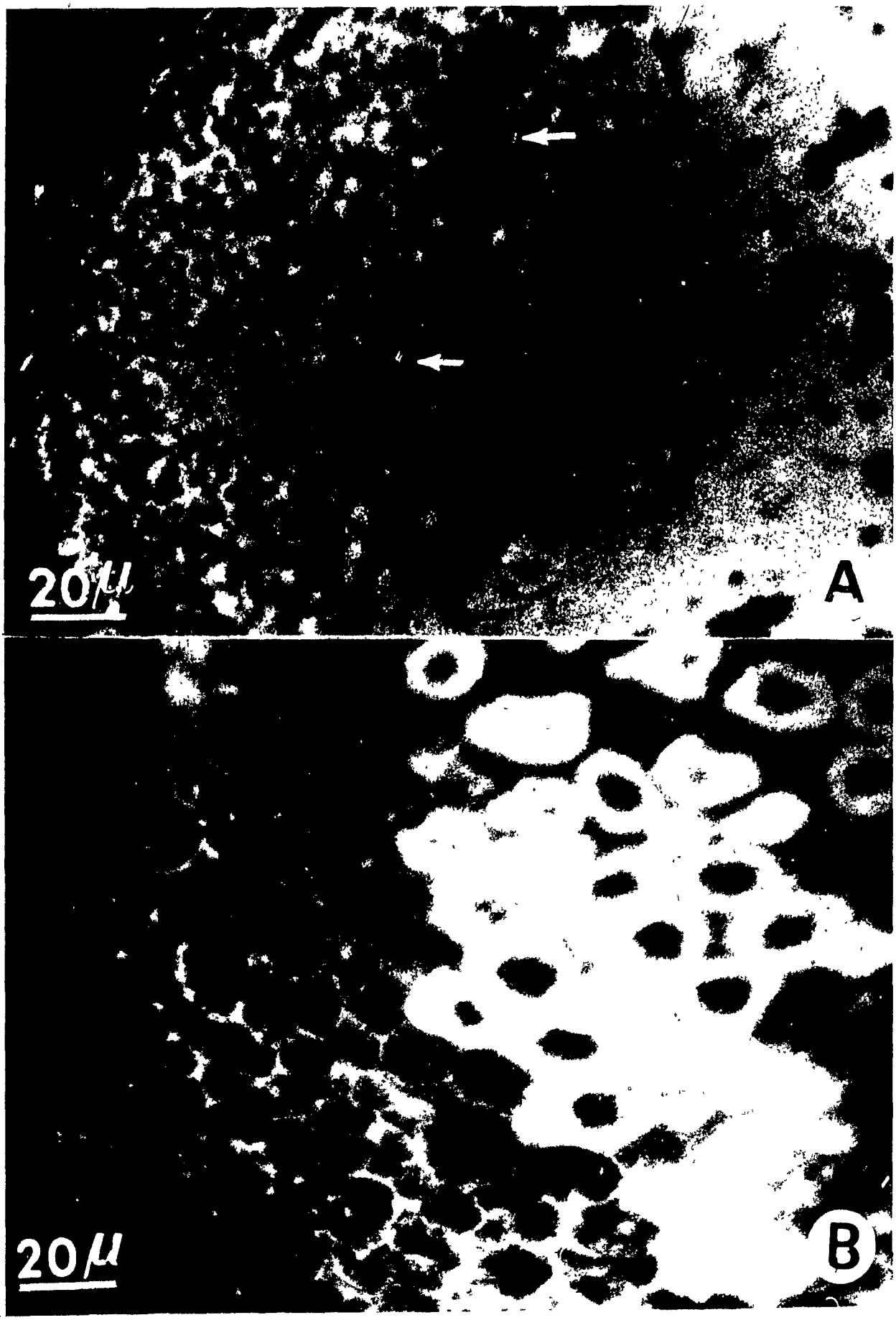
5.2.4 Immunofluorescence microscopy

The observations made on thick sections (8-10 μ) of nodular tissue showed the presence of both auto- and specific fluorescence in the WCMV-infected cells (Figure 46). Only autofluorescence was noticed in the various control combinations (Figure 46B). Occasionally, WCMV antigens, represented by the specific fluorescence in some areas of the diseased nodules were observed (Figure 46A). Fluorescence of the vascular bundle and the bacteroidal zone were seen frequently in both healthy and WCMV-infected nodules. This probably resulted from a combination of autofluorescence and nonspecific fluorescence of the added FITC conjugated antiserum. Although the sections treated with FITC-conjugated gamma-globulin were washed several times with PBS to eliminate excess fluorescein, it is most likely that some was not completely removed. Hence, unwanted fluorescence was noticeable in the sections of both healthy and diseased nodules. Generally, however, WCMV antigens trapped by the FITC-conjugated gamma-globulin analogous antiserum were recognized as specific fluorescence (Figure 46A).

Figure 46. Photomicrographs of nodular tissue prepared and stained by the direct immunofluorescence technique.

A. Nodular tissue from WCMV-infected plant. Small areas of specific fluorescence (arrows) are visible, as well as larger areas of auto- and/or nonspecific fluorescences.

B. Healthy nodular tissue. Large areas of autofluorescence are present, but smaller areas of fluorescence are less frequent.


20μ
A
20μ
B

5.3 Discussion and conclusions

The micrographs in Figures 33-37 represent a sampling of virus-free tissues during the sequence of changes involved in nodule initiation and formation. The observations generally agree with those reported by other investigators. It is well known that the nodule formation is initiated by the recognition of R. trifolii through a binding with lectins (trifolin) on the root hair membranes of red clover (Dazzo et al. 1978). This specific reaction results in the formation of an infection thread, which in turn stimulates meristematic activity in the roots and subsequent nodule development (Dart 1977). The nodules examined during this study were distributed somewhat unevenly on root hairs, which previous workers have attributed to the endogenous hormone levels required to induce the formation of root hairs and nodules (Dullart 1970). The pinkish color of the nodules indicated that they were effective.

The ultrastructure of the examined nodular tissue of red clover was basically identical to the structural organization described for other clover species (Dart and Mercer 1963; Mosse 1964; Gourret 1975). One of the interesting observations made during this study concerns the apparent emergence or release of bacteria from the infection thread, which was surrounded by a membrane. The bacteria were apparently released or extruded from the mature infection thread either by a pinching off or vesiculation mechanism which occurred on the surface of the infection thread, which lacked a thick wall where this occurred (e.g., Figure 37D). The bacteria probably entered the host cytoplasm

by a process resembling endocytosis. A similar manner of rhizobial release has been described for some other clover species (Dixon 1964, 1967), pea (Dixon 1964; Newcomb 1967), soybean (Goodchild and Bergersen 1966), alfalfa (Vance et al. 1980; Patel and Yang 1981), and broad bean (Newcomb 1981).

The micrographs suggest two possible sources for the membrane envelope surrounding the bacteria after emergence. One source could have involved fragmentation of the host cell plasmalemma, as the infection thread was enclosed by a continuation of this membrane. This contention would support the findings published by Tu (1974a, 1975), who examined the identity of the membrane by histological and freeze-fracturing techniques. In addition, membrane synthesis may have occurred in the host cell cytoplasm, as a second source, to complete the formation of the envelope which enclosed the released bacteria. This conclusion is in agreement with the speculation of Mosse (1964).

The membrane envelope surrounding the released bacteria seemed irregular, and apparently enlarged and maintained itself as the bacteria divided (e.g., Figure 34B, in which the membrane encloses more than bacterial cell). Therefore, because the membrane ultimately enclosed the spherical, non-dividing bacteroid, it seems preferable to refer to it as the «peribacteroidal membrane» to avoid the confusion in terminology used by different investigators (Newcomb 1967, 1981; Dixon 1967).

The bacteroid zone may be considered the cornerstone in the symbiotic strategy of the legume nodular tissue. Two kinds of living cells were found in this zone, particularly in region 3, i.e., one containing bacteroids and the other without bacteroids. The cells lacking bacteroids, or interstitial cells, were less prevalent. The functions of these cells may be related to the communications among the cells containing bacteroids (Tu, personal communication). Similar observations were made in white clover nodules by and Braybrook (1981), who noticed large central cavities in these cells. He also found central vacuoles in the bacteroid-filled cells, which were not seen very often in the corresponding cells in this investigation. Such vacuolation phenomena probably depend on the internal environment in the nodule (i.e., the meristematic activity, Rhizobium differentiation, biochemical properties), clover species and the age of the plant. The presence of the carbohydrate platelets in the bacteroid-filled cells, analogous to those reported by Mosse (1964) may suggest that when the symbiotic Rhizobium-plant relationship is effective, it consumes these carbohydrates during the active N_2 fixation period.

Degenerated bacteroid-filled cells were frequently observed in the basal part of the nodule (region 4). The observed disintegration of these cellular contents is generally similar to that reported in white clover (Mosse 1964; Gourret 1975). The occasional presence of numerous bacteria in the vacuolar areas of the collapsed cells in this region could be attributed to a delay in the bacterial release from immature infection threads. Possibly, the lysis of such threads became the only

route to release the bacteria which in turn became lysed due to the low symbiotic activity in this region.

The observation of some cellular organelles such as mitochondria and plastids lined up at the periphery of the cells containing bacteroids resembles reports by previous workers (Bergersen and Goodchild 1973; Dart 1977). It has been suggested that such organelles moved there to have sufficient oxygen for their activity, mainly when they are accumulated near the intercellular spaces (Dart 1975). The function of the latter as aeration pathways mediating gaseous exchange between the nodular tissue and its surroundings has been well described (Bergersen and Goodchild 1973). Transfer cells, with cell wall ingrowths, which were observed associated with the xylem cells, have also been noticed in pea and white clover (Pate et al. 1969).

The presence of WCMV particles in various cell types of the infected nodular tissue, which was detected by both electron microscopy and immunofluorescence, may suggest that this virus has the ability to multiply within these cells (Krass and Ford 1969). The data obtained from the infectivity assay on G. globosa would also support this conclusion. The absence of WCMV particles in the bacteroids may reflect a fact that the virus can multiply only in the living plant host cells. The frequent association of WCMV aggregates with vital sites such as phloem or bacteroid containing cells may be connected with the photosynthate current coming from the green tissues.

Since nuclear inclusions have not been generally observed during potexvirus infection, their occurrence in meristematic and transfer

cells of WCMV-infected nodules is rather interesting. This may indicate that the nuclei are favorable sites for WCMV replication and accumulation. As was described earlier, this kind of inclusion was not found in other tissues of diseased red clover and pea plants. Therefore, the different structural organization of the nodular tissue may enhance the penetration of the nuclear membrane by WCMV particles. Also, the growth conditions (using sand and fertilizer without nitrogen) under which the nodulated red clover plants were grown probably had a certain effect on the physiological state of the plant because the virus was originally propagated in non-nodulated red clover.

The most prominent cytopathological changes were noted in nodules taken 12 weeks after virus inoculation. This suggests that the nodular tissues responded more slowly to virus infection than leaves, which showed mosaic symptoms 13 days after virus inoculation. Consequently, the slow reaction at the earlier stages probably indicates the great adaptability of nodular tissue towards the virus infection. However, the different kinds of intracellular changes in WCMV-infected nodular tissue at all growth stages coincided with the reduction in the nitrogen fixing activity as nodules aged, which was comparable with healthy controls. Despite all the abnormalities induced by the virus infection, the nodules still retained their activity at the fourth harvest, as they showed considerable nitrogenase activity at this time. Therefore, it is most likely that the vesiculated bacteroids contributed positively by maintaining the N_2-fixing activity, which may have been inhibited in autolysed and autophagic bacteroids. These results are in agreement

with those reported by Wongkaew (1981), and the speculation made by Tu (1977), who theorized that the relationship between N_2-fixing activity and bacteroid morphology involved the peribacteroidal space.

The infection by WCMV, along with the aging of the diseased nodules, may have incited intracellular alterations in the vascular activity. Deterioration of the mitochondria and the presence of osmiophilic globules in the proplastids are probably consequences of the earlier senescence in the infected nodular tissue. Degradation of the cell wall ingrowths in the transfer cells might have led to an interruption or decrease in assimilate movement, since it is known that these cells are a part of the transportation system in the nodular tissue (Pate et al. 1969). Similarly, the formation of excessive vesicles or multivesicular bodies in the WCMV-infected cells is analogous to those described by Fowke and Setterfield (1969), who attributed their presence to the physiological state of the plant.

It is surprising that WCMV particles were associated with the infection thread at the senescence stage, although various explanations are possible. First, a convenient atmosphere for WCMV multiplication probably was not available in the cells containing retarded bacteroids. Second, the presence of certain lysed areas on the walls of the infection thread might have made it easier for WCMV particles to penetrate them.

Matile (1974) pointed out that autolysis and autophagic processes seemed to occur in the plant tissue as a result of an adverse environmental condition. Judging from the bacteroids' morphology, these two

phenomena apparently occurred first in the cytoplasm of the cells containing bacteroids. Such lethal activities probably affected the functions and longevity of the nodular tissue. Somewhat similar observations were described in the nodular tissue of peanuts infected with PMV (Wongkaew and Peterson 1980), and after defoliation of alfalfa (Vance et al. 1980). Formation of electron-dense deposits occupying small and large vacuoles in the meristematic and transfer cells has been attributed to digestive enzyme activity (Matile 1974).

SECTION 6
GENERAL DISCUSSION AND CONCLUSIONS

Legume viruses have a world-wide distribution and are of great importance in agriculture. Since much confusion exists as to their identity and names, reliable identification of the viruses involved is a «corner-stone of efficient disease control» (Bos et al. 1969b). Extensive studies were carried out in this investigation on what was designated as an Ottawa isolate of WCMV, which was the most frequently isolated virus found in the samples of infected red clover collected from six fields in eastern Ontario. Several filamentous viruses (i.e., BYMV, CYMV, PSV, RCVMV) were also detected in these samples. The viruses were identified on the basis of serological reactions, as these are the most informative methods to determine the virus relationships (Bos et al. 1960), because electron microscopy does not name the virus particles it reveals. PSV and BYMV were frequently found during previous surveys of clover fields in eastern Canada (Gates and Bronskill 1974; Pratt 1968), but the incidence of WCMV, the most prevalent virus encountered during this study, was not indicated in the earlier reports. Regional differences may lead to such variation in distribution of virus disease. This is probably related to the geographical location of the forage legume fields and the presence of other crops adjacent to them, the plant cultivars, environmental

conditions, prevalence of insect vectors, the time of surveying and age of forage stand.

Various biological and physico-chemical properties of the Ottawa isolate of WCMV were determined, including host range and symptomatology, particle morphology, sedimentation coefficient, buoyant density, UV absorbance characteristics, protein molecular weight and composition, and serological reactions. The Ottawa isolate differed from some of the other described WCMV strains in some of these characteristics, particularly in UV extinction coefficient and protein composition, indicating that it may be a different or distinct strain. However, this can be established only through exchange of different virus strains and antisera to determine the quantitative serological relationships among them (Bos et al. 1960), and by observing the host reactions to various virus strains under similar conditions (Hampton et al. 1978).

The ultrastructural examination of infected leaves was undertaken in order to gain familiarity with patterns of virus localization before examining nodular tissues similarly. Since pea leaves rapidly developed symptoms of virus infection which were more severe than those in red clover, it was not surprising to find more drastic cellular cytopathological changes at the cellular level in the former. However, infected red clover tissues harbored a wide variety of types of WCMV aggregates. This could be a reflection of clover's less drastic response, or tolerance, to virus infection.

The structural organization of red clover nodules generally resembled that described in other clover species (Moss 1964; Gourret 1975). The cytological observations suggested relatively little response of nodular tissue to WCMV infection at the first two sampling times (3 and 6 weeks). This is somewhat similar to the behaviour of leaf tissue, despite the ultrastructural differences between leaves and nodules. There were various indications of an earlier senescence in infected nodules than in healthy nodules. Nodule senescence was quite obvious at 12 weeks after virus inoculation, and was associated with a sharp decline in nitrogenase activity. The probable increase in autolytic and autophagic activities, along with the cytopathological alterations in other tissues, might have contributed to such an impairment. However, the nitrogenase system remained functional, probably in non-senesced and vesiculated bacteroids. It is known that the nodule life span in perennial legumes is only one season (Dart 1977).

Nuclear viral inclusions, which are not regarded as a common characteristic of infection by potexvirus (Christie and Edwardson 1977), were observed within infected clover nodules, but were not seen in any non-nodular tissues. This may indicate that the nuclei within nodules are suitable sites for accumulation and/or replication of WCMV. However, the pea and red clover plants in which non-nodular tissues were examined were grown under different conditions (i.e., in soil mixture, with NPK fertilizer), which might also be related to their lack of nuclear inclusions.

Red clover nodules are of the exogenous kind, in which the rhizobial bacteria are released from an infection thread (Allen 1973). Effective and viable bacteria are required for initiation and development of the nodules, which are considered the central system for N_2 fixation process. In this process, a series of enzymes collectively termed «nitrogenase» plays an active role in converting the atmospheric N_2 into a usable form for plant growth. Numerous factors may affect the Rhizobium-legume relationship, such as Rhizobium strain, plant species, environmental conditions, plant disease, etc. The pink color of effective nodules is caused by the presence of leghemoglobin, a haemoprotein which regulates oxygen tension within nodular tissue (Bergersen 1980).

It is known that the nitrogenase enzyme is located within the bacteroid (Vance and Johnson 1981), but the precise location of leghemoglobin is still a controversial subject. The cytochemical test using DAB as a stain in this study indicated that the haemoprotein is concentrated in the peribacteroidal space. These observations are similar to those reported by other workers (Dilworth and Kidby 1968; Bergersen and Goodchild 1973; Gourret and Fernandez-Arias 1974). There was no evidence for the presence of leghemoglobin outside the peribacteroidal membrane, in the cytoplasm of the cells containing bacteroids, as was reported by Verma et al. (1978), who studied soybean nodules. Adequate resolution of this controversy will require more extensive use of the same DAB-cytochemical technique, or other suitable tests on nodular samples from various leguminous species. Since the

reports on different locations of leghemoglobin have involved various host plants, it is possible that the host species influences the localization of leghemoglobin.

The relationship between leghemoglobin concentration and nitrogenase activity in nodules is still obscure. However, nitrogen fixation does not occur in nodules lacking leghemoglobin, and various investigators have reported a correlation between leghemoglobin concentration and rate of nitrogen fixation (Virtaneu and Miettineu 1963). In the nodules of the clover plants studied, leghemoglobin concentration and nitrogenase activity were both reduced by WCMV infection, but there was a significant correlation between them at the 10-leaf and preblooming stages only (i.e., 3 and 6 weeks after virus inoculation), in either healthy or infected plants.

The results in this study suggest various possible ways in which WCMV infection could affect some of the processes involved in biological N_2-fixation. In the first place, it is known that the biochemical pathway of N_2-fixation starts with the breakdown of the triple bond of molecular nitrogen, and ultimately results in the formation of amino acids. In one of the final steps of this process, ammonia reacts with glutamate to form glutamine; this reaction is mediated by the enzyme glutamine synthetase. Thus, the inhibition of this enzyme by an excess of amino acids would suppress the N_2-fixation process (Brill 1977). Protein synthesis in various tissues of WCMV-infected plants is probably subjected to two demands, one imposed by virus encapsidation and the other by normal plant growth processes. Since WCMV reaches a high

concentration in infected plants, and since the forage yield of infected plants was reduced considerably, it can be suggested that virus protein synthesis predominated, and that the resulting excess of amino acids contributed to an inhibition of glutamine synthetase and nitrogen fixation.

Another effect of virus infection on N_2-fixation may have been caused by the general decline in the viable rhizobial population. If fewer rhizobia were transformed into active bacteroids in infected plants, lower nitrogenase activity would probably result.

Virus infection usually causes a reduction in the amount of carbon incorporated into sugars during photosynthesis (Matthews 1982). The lowered availability of photosynthates to the nodules of infected plants presumably would have restricted the energy required for nodular functions, which could have reduced either the breakdown of the triple bond of molecular N_2 or the synthesis of nitrogenase. The reduction of photosynthesis, together with a probable increase in growth inhibitors and/or an imbalance of auxin levels (Lee 1955; Diener 1963) could have contributed to the suppression of nodulation in the WCMV-infected plants. However, this effect may have been counteracted by the increased respiration rate often shown by mosaic infected plants (Matthews 1982).

Various investigators have reported that an excess of nitrite, resulting from nitrate reductase activity (Rigaud et al. 1973; Kennedy et al. 1975; Chen and Phillips 1977; Gibson and Raju 1977), can repress

nitrogenase activity. Thus, the increased nitrate reductase activity observed in WCMV-infected nodules probably also contributed to a reduction of nitrogenase activity. Generally, at least some, and possibly all, of the aforementioned mechanisms may have been responsible for the reduction of nitrogen fixation in WCMV-infected plants.

The nodular tissue, as any other plant tissue, undergoes senescence, which is commonly associated with an increase in the activity of hydrolytic enzymes (Wilson 1973). Acid phosphatase, the best known of the hydrolytic enzymes, is supposedly involved with the lysosomal systems in various plant tissues (Wilson 1973). Little information is available concerning the effect of virus infection on acid phosphatase activity, but Tu (1976) reported that significant increase in this activity occurred in leaves and nodules of CYMV-infected white clover a few days after inoculation with virus. The results obtained in this study generally resemble Tu's findings. Acid phosphatase specific activity increased with plant age/nodule development when determined at the first three sampling times, and then sharply declined at 12 weeks after virus inoculation. The decline suggests a possible inhibition of the lysosomal function, which may have been caused by an excess of inhibitory materials. Since this decline coincided with the lowest nitrogenase activity recorded, it may be a reflection of a general retardation of bacteroid function. The application of a cytochemical method for determination of acid phosphatase activity in leaf tissue did not indicate a similar decline, which probably results from the continuation of normal metabolic activities in the green tissues of the perennial legume.

However, it is difficult to generalize the effects of WCMV infection on other leguminous plant species, because of the variation in the host susceptibility to virus infection. Also, several other factors may be involved, such as plant cultivar, virus isolate (Tu et al. 1970a), concentration of Rhizobium inoculum (Tu and Tse 1976), Rhizobium isolate and time of virus inoculation (Orellana et al. 1980). There are two groups of investigators who have described the effect of virus infection on N_2-fixation. The first reported that the virus infection generally had adverse effects on this process (Tu 1970a, 1970b; Orellana et al. 1978, 1980; Wongkaew and Peterson 1983), while the other claimed the opposite (Venkataraman and Rao 1974; Rajagopalan and Raju 1972). The results in this study support the first group.

Generally, several conclusions can be made based on the present study. The field survey indicated that several filamentous viruses frequently infecting red clover plants are mostly found in mixed infections. Various kinds of inclusions made up of virus aggregates or host inclusions induced by the virus infection were found in the WCMV-infected tissues but not in the healthy ones. The cytoplasm of mesophyll parenchyma cells seems to be the primary site of accumulation and assembly of WCMV particles. The translocation and distribution of WCMV particles probably occurs intercellularly through plasmodesmata, and to other organs of the plant via vascular conducting cells. Most of the degenerative changes which occurred in the sampled tissues are not a consequence of normal senescence, but do simulate those associated with naturally aging tissue. Significant suppression of the plant

growth, nodulation, nitrogenase activity, leghemoglobin content, and Rhizobium population was observed in WCMV-infected red clover plants. This was associated with an increase in the nitrate reductase and acid phosphatase activities. Since red clover is a perennial legume, the reduction of nitrogenase activity by WCMV infection probably will cause a shortage in the stored reserves which may adversely affect the winter hardiness, as in alfalfa infected with AMV (Tu 1980). Also, because leguminous crops are often grown in rotation with nonleguminous plants, the reduction in the nitrogenase activity, caused by virus infection, ultimately will decrease the amount of nitrogenous material available from one season's legume crop to fertilize the next season's grain crop. Similarly, the suppression in the nodular rhizobial population of the WCMV-infected plants will in turn affect the rhizobia released into the soil after degeneration of nodules. Red clover may become a problematic WCMV-reservoir, from which spread of WCMV to other neighbouring economical crops can occur, since the annual legumes of the host range were more susceptible to this virus than other perennial plant species. Because of these factors, and because WCMV is generally transmitted through seeds (Hampton 1967), effective methods are needed to eliminate or minimize the overall consequences resulting from infection of economic crop plants by WCMV. Such effects of WCMV could be offset by screening for resistant varieties of clover and more efficient strains of Rhizobium. Heat therapy of red clover seeds from unknown sources might help to provide virus-free seed lots and serological or other testing methods could be initiated to determine present levels of seed infection and to certify that future seed lots are virus-free.

SECTION 7
SUMMARY

1. Several filamentous viruses were detected in 17% of red clover samples showing virus-like symptoms during a survey of six fields in eastern Ontario. The incidence of these viruses varied from 16.6 to 23.3%. WCMV was most prevalent, followed by BYMV, CYMV, PSV, RCVMV. About 60% of the test samples were found to be infected with more than one virus.

2. An isolate of WCMV was designated as the «Ottawa isolate» on the basis of host range and symptomatology, particle length (500 nm), sedimentation coefficient ($S_{20,w}$ = 117 S), buoyant density (1.298 g/cm^3), extinction coefficient at 260 nm for mg/ml (3.1), A260/A280 (1.24), molecular weight of viral protein (2.4×10^4), amino acid analysis, physical properties in crude sap and serological tests.

3. Cytoplasmic inclusions were detected by light and immunofluorescence microscopy in the leaf epidermis. Ultrathin sections of various tissues of red clover and pea plants infected by WCMV were examined by electron microscope to study the distribution of virus particles and the cytopathological changes induced by virus infection. In the infected leaf tissue of the former plant species, interesting membrane-bound bodies containing masses of convoluted and/or fairly

straight tubules, which were often against or near chloroplasts. In the various pea tissues infected by WCMV, the cellular organelles showed considerable disorganization and degeneration due to the virus infection.

4. WCMV infection caused a significant reduction ($p \leq 0.01$) in the forage yield and root weight, and suppressed the number and weight of nodules in the diseased red clover plants. The greatest reductions in these four characteristics were 50%, 46%, 37% and 59%, respectively in the infected plants.

5. The nitrogenase enzyme activity, expressed in nmoles C_2H_4.plant^{-1}.hour^{-1}, in red clover nodules reached its peak 9 weeks after virus inoculation. The WCMV infection decreased the activity significantly; the greatest reduction, 60%, occurred in the diseased nodules at 12 weeks.

6. The leghemoglobin content was determined as µg of leghemoglobin per mg of nodule. It reached a maximum concentration at 9 weeks after virus inoculation. Infection by WCMV decreased this content considerably and the greatest reduction was 62% in the infected nodules sampled 6 weeks after virus inoculation. There was a significant correlation between leghemoglobin content and nitrogenase activity at 3 and 6 weeks, but not at 9 and 12 weeks, in both healthy and diseased nodules.

7. The viable Rhizobium population was determined on the basis of number of colonies in a 10^{-3} dilution per mg of nodule. A

significant suppression in the population was caused by the WCMV infection at 3, 6 and 12 weeks, but not at 9 weeks after virus inoculation. The greatest reduction in the rhizobial population in the extracts of diseased nodular tissues was 26.4%. There was no indication that R. trifolii can transmit WCMV to healthy red clover plants.

8. The acid phosphatase specific activity was studied in nodular tissues of red clover plants and expressed as μg p-nitrophenol. $minute^{-1}.mg\ nodule^{-1}$. This activity increased with age in both healthy and diseased samples, and reached a peak 9 weeks after virus inoculation. In the leaf tissue, the enzyme activity was determined by a cytochemical method, which showed no decline at any sampling time after virus inoculation. The WCMV infection increased the activity significantly ($p \leq 0.01$) in the diseased tissues compared with healthy control. The greatest increase in the former due to the WCMV infection was 41.9% at 3 weeks after virus inoculation.

9. Nitrate reductase specific activity was determined on the basis of nmoles $nitrite.hr^{-1}.gm\ nodule^{-1}$. The maximum enzyme specific activity was detected at 6 weeks, followed by a sharp decline at 9 and 12 weeks after virus inoculation. Within each harvest, the enzyme activity decreased gradually during the incubation of the nodular tissue. The WCMV infection increased the specific activity of the enzyme at all harvest times in the infected nodules. In the latter, the highest increase was 25.6% at 3 weeks after virus inoculation.

10. WCMV in the nodular tissue was detected by infectivity assay, immunosorbent electron microscopy and immunofluorescence microscopy. The highest number of local lesions on G. globosa L. leaves was observed at 9 weeks after virus inoculation, while the greatest number of WCMV particles trapped by their homologous antiserum was detected at 6 weeks. Immunofluorescence microscopy confirmed the presence of WCMV antigens in the nodular tissue.

11. The ultrastructural findings in the red clover nodules suggested a slow response of this tissue toward the WCMV infection at 3 and 6 weeks after virus infection. The most remarkable alteration due to WCMV infection was noticed at 12 weeks. Three kinds of deteriorated bacteroids were found in the diseased tissues: vesiculated, autolysed and autophagic bacteroid. Interesting nuclear viral inclusions were seen in the nuclei of infected meristematic and transfer cells at all harvest times.

SECTION 8
CLAIMS OF ORIGINAL WORK

1. The first report indicating that the most prevalent WCMV isolate in red clover in eastern Ontario differs from most other characterized strains.

2. The first study to compare the virus distribution and the cyto-pathological changes induced by WCMV in various tissues of an annual (i.e., pea) and a perennial (i.e., red clover) legume.

3. The first investigation in which the indirect immunofluorescence technique was used to detect the cytoplasmic inclusions in the WCMV-infected leaf epidermis.

4. The first report to describe inclusions consisting of membrane-bound masses of straight and convoluted tubules associated with infection by a potexvirus member.

5. The first study in which nuclear viral inclusions were found in the cellular nuclei of nodular tissue infected by a potexvirus.

6. The first attempt to study the effect of infection by a potexvirus on plant growth, nodulation, nitrogenase activity, leghemoglobin content and Rhizobium population.

7. The first attempt to report the localization of leghemoglobin in the peribacteroidal space of red clover nodules by a cytochemical technique using DAB.

8. The first attempt to determine the nitrate reductase specific activity in red clover nodules, and the effect of the virus infection on this activity.

9. The first investigation in which the virus distribution and the cytopathological changes induced by a potexvirus infection were studied at various times on exogenous nodules of perennial legume in parallel with measurement of other characteristics related to N_2 fixation.

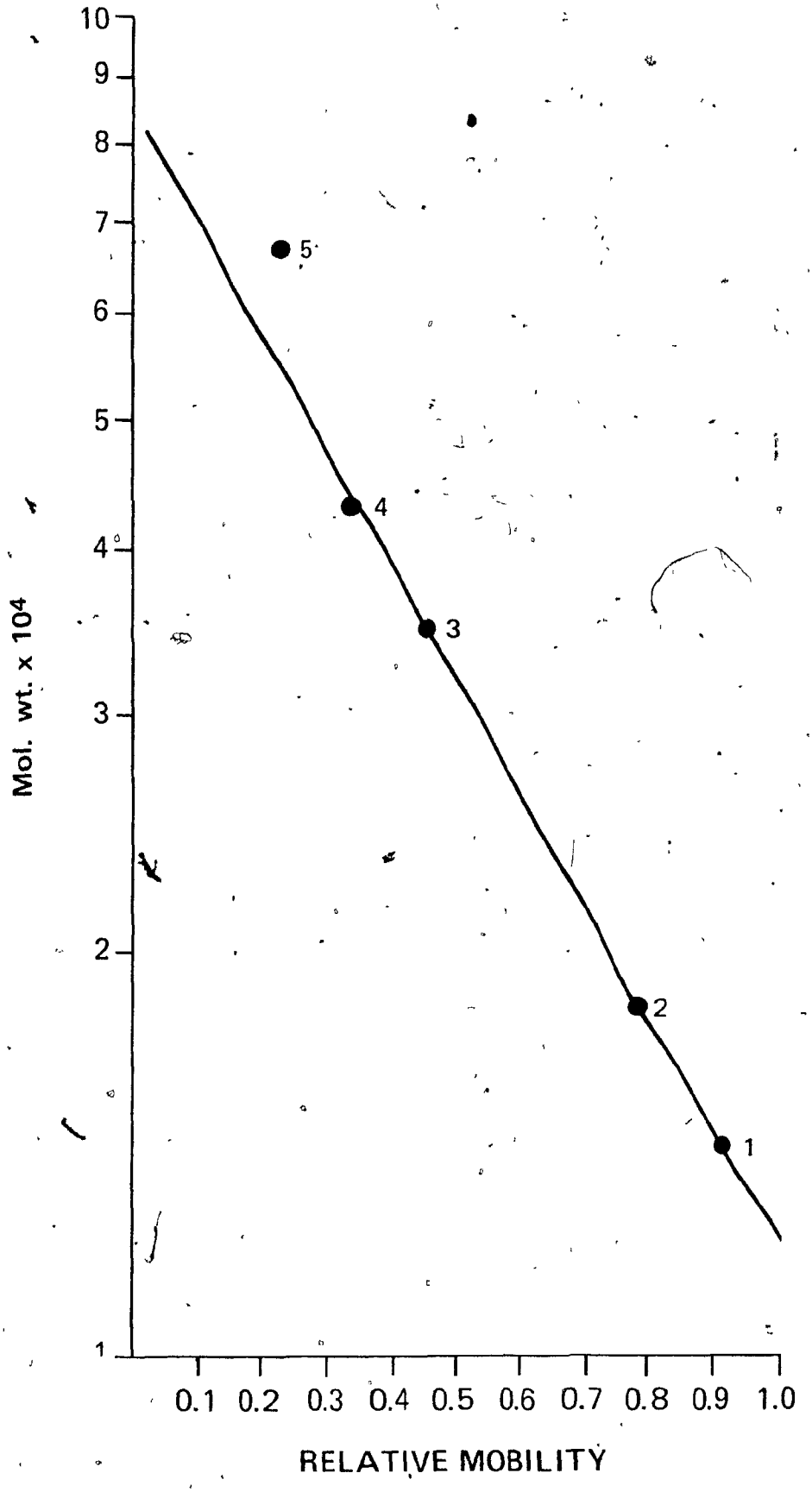
10. The first report which indicates that the virus studied in this investigation is not transmissible by rhizobial bacteria, contrary to a previous finding.

11. The first attempt in which direct immunofluorescence and immunosorbent electron microscopy techniques were used to detect the virus antigen in the nodular tissue.

SECTION 9
APPENDICES

Appendix 1. Standard graph to determine the molecular weight of WCMV protein, using five protein markers.

1. Lysozyme
2. Lactoglobulin
3. Pepsin
4. Ovalbumin
5. Bovine serum albumin


10
9
8
7
6
5
4
3
2
1
Mol. wt. x 104
5
4
3
2
1
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
RELATIVE MOBILITY

APPENDIX 2. Yeast-extract mannitol medium composition (after Wangnai 1975)

Mannitol	10.0 g
Yeast extract	0.5 g
K_2HPO_4	0.5 g
$MgSO_4.7\ H_2O$	0.2 g
NaCl	0.1 g
$CaCO_3$	3.0 g
Agar	15.0 g
Distilled water	1000.0 ml

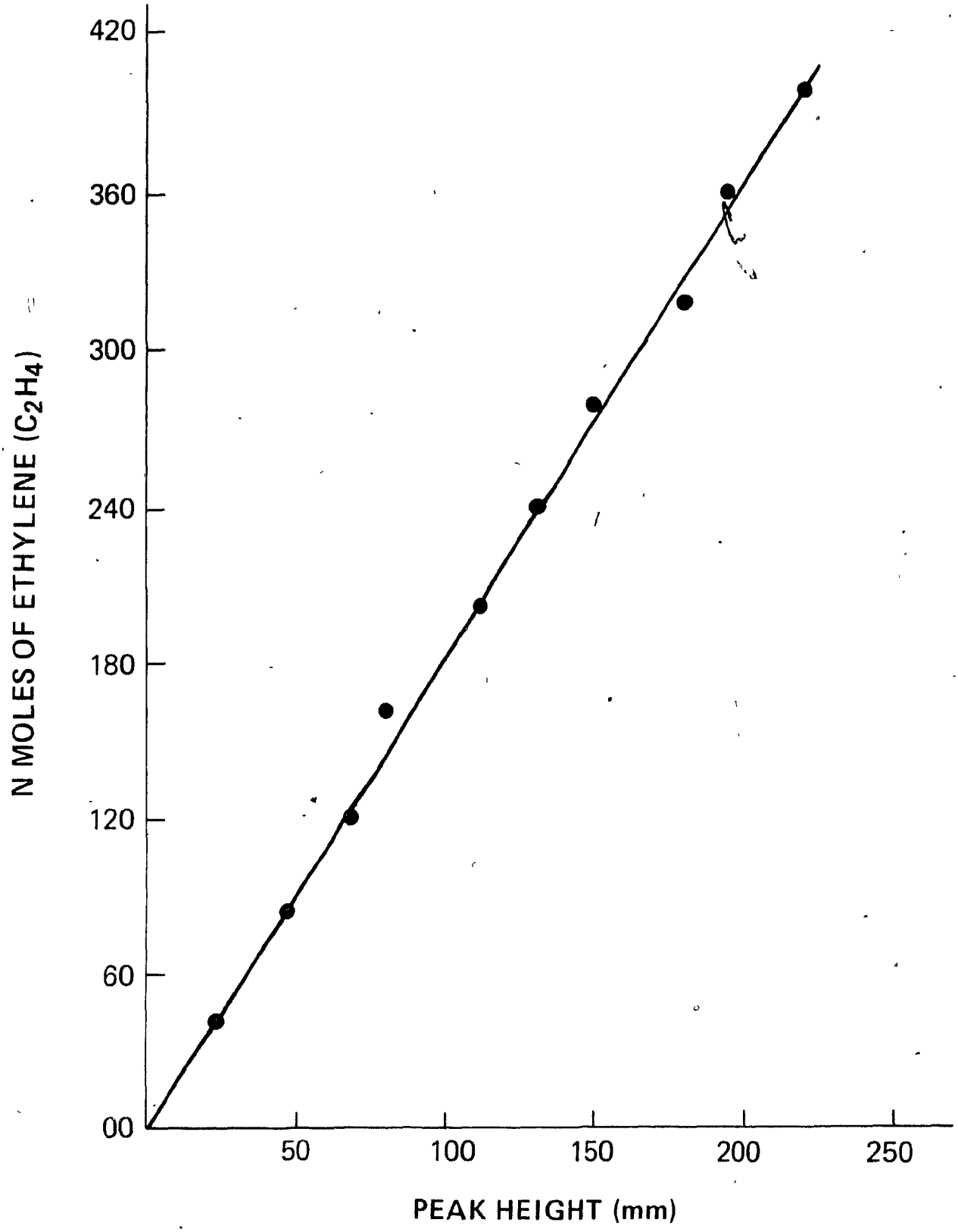
$CaCO_3$ and agar were omitted from the liquid medium. 2.5 ml of 1% Congo red solution was added to the sterilized medium to make congo-red yeast-extract mannitol agar used as a differentiation medium for Rhizobium spp.

APPENDIX 3. Formulation of modified nitrogen-free planting medium (after Sirois and Peterson 1982)

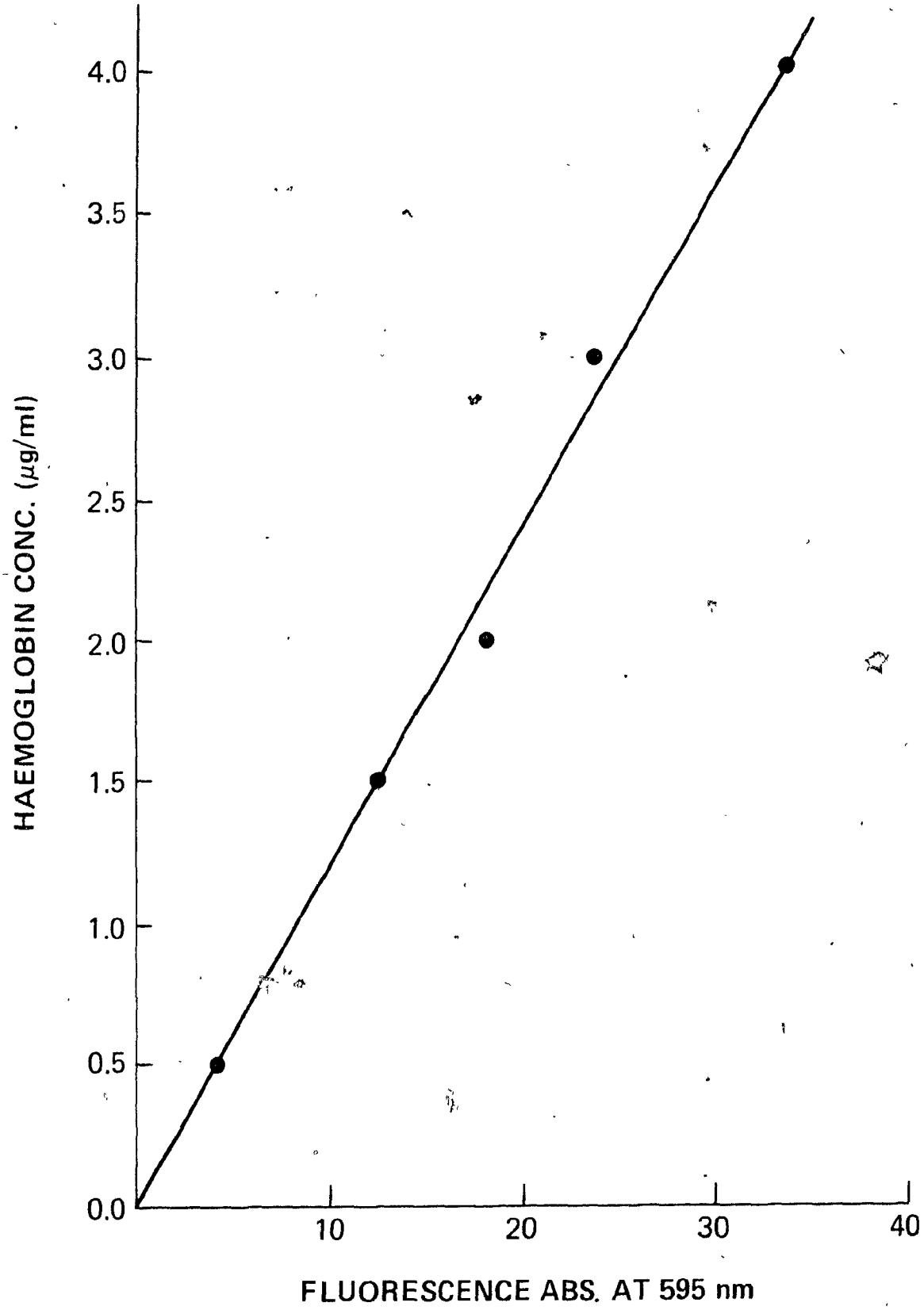
Macroelements:	
$CaCl_2.2\ H_2O$	0.12 g
KH_2PO_4	0.14 g
$MgSO_4.7\ H_2O$	0.25 g
K_2HPO_4	0.01 g
Water	1000.00 ml
Trace element stock solution:	
$MnSO_4.4\ H_2O$	0.85 g
$CuSO_4.5\ H_2O$	0.16 g
$ZnSO_4.7\ H_2O$	0.58 g
H_3BO_3	1.55 g
$Na_2MoO_4.2\ H_2O$	0.121 g
$CoCl_2.6\ H_2O$	0.125 g
Seq. 330 Fe	2.80 g
Water	1000.00 ml

One ml of trace element solution was added to every 30 ml of the macroelement solution before use. The final pH of the medium was about 6.5.

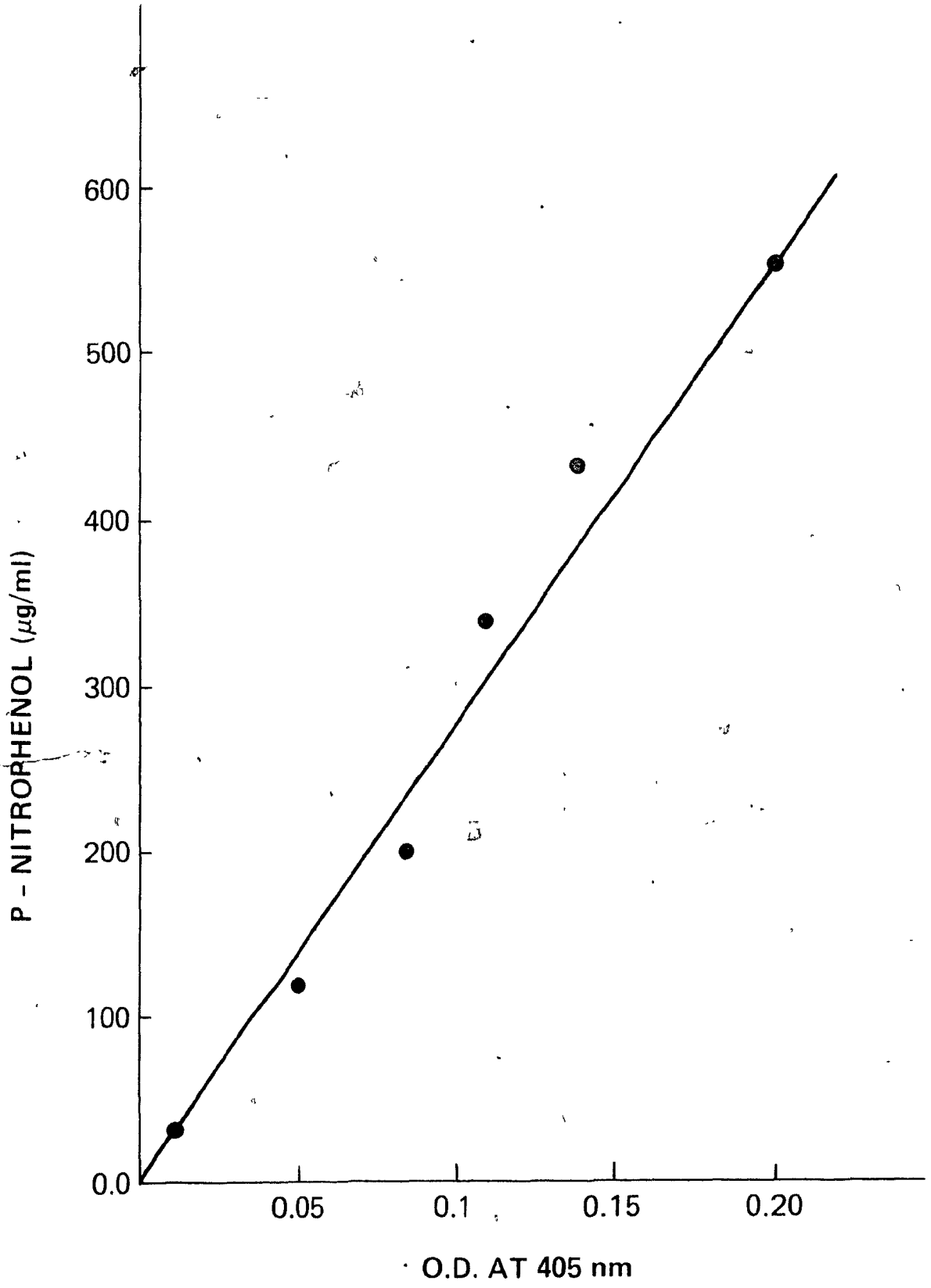
Appendix 4. A standard curve constructed from the readings of ten serial dilutions of a known concentration ethylene (C_2H_4) and the peak heights formed after injecting each dilution in the gas chromatography machine, as described in the nitrogenase activity assay.


420
360
300
240
180
120
60
00
N MOLES OF ETHYLENE (C_2H_4)
50
100
150
200
250
PEAK HEIGHT (mm)

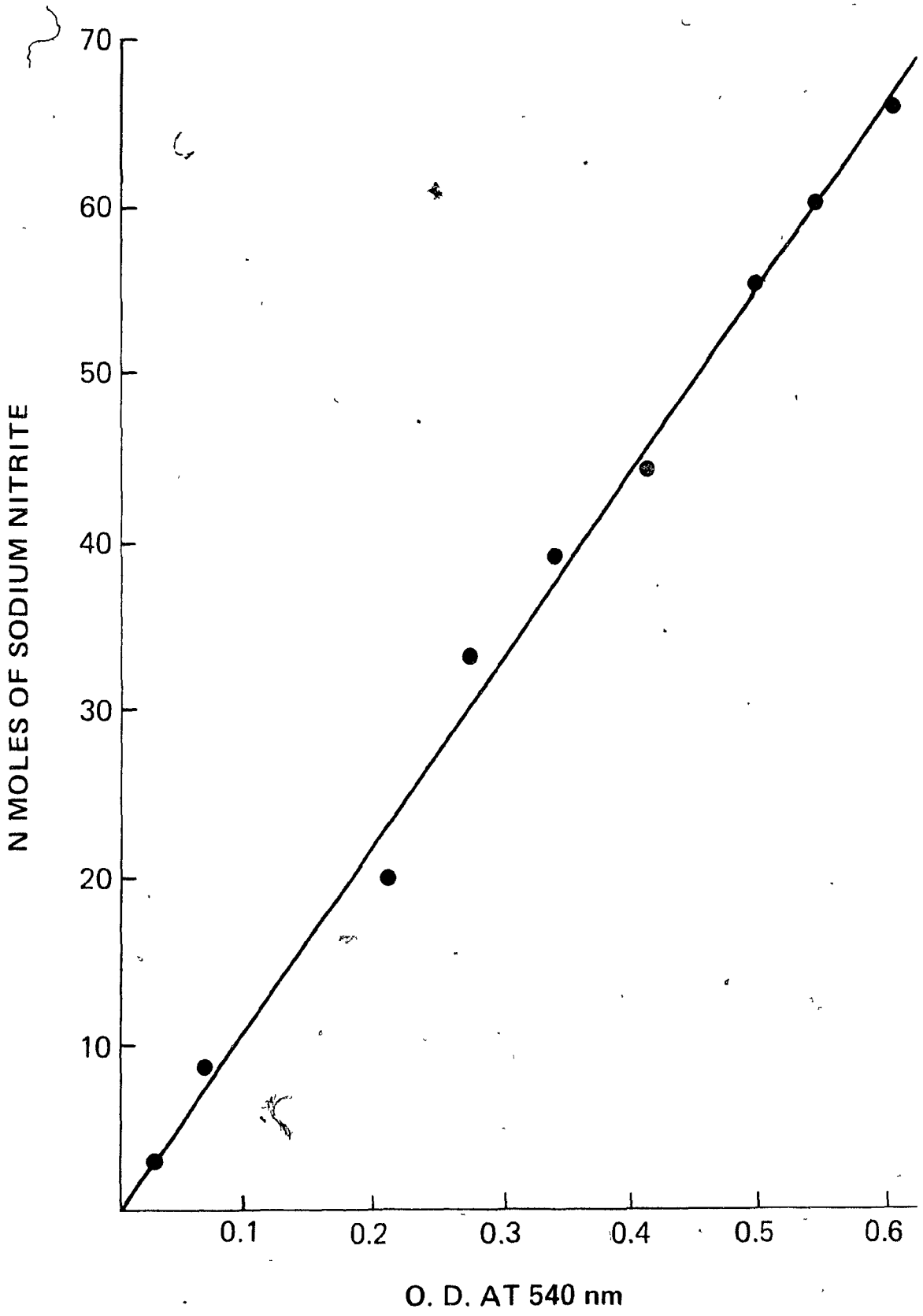
Appendix 5. A standard curve constructed from the fluorescence readings at 595 nm and the known concentrations of five serial dilutions prepared from purified beef hemoglobin.


HAEMOGLOBIN CONC. (μg/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
10
20
30
40
FLUORESCENCE ABS. AT 595 nm

Appendix 6. A standard curve constructed from six dilutions of known concentrations of p-nitrophenol and the absorbancy readings at 405 nm.


600
500
400
300
200
100
0.0
P - NITROPHENOL (μg/ml)
0.05
0.1
0.15
0.20
O.D. AT 405 nm

Appendix 7. A standard curve constructed from nine serial dilutions of sodium nitrite and the absorbancy readings at 540 nm.


70
60
50
40
30
20
10
N MOLES OF SODIUM NITRITE
0.1
0.2
0.3
0.4
0.5
0.6
O. D. AT 540 nm

APPENDIX 8A. Analysis of variance of root fresh weight

Source of variation	D.F.	Sum of squares	Mean square	Cal.F.	Level of significance
Replicates (R)	3	0.14	0.05		
Date (D)	3	26.75	8.92	159.23	**
Treatment (T)	1	2.23	2.23	39.86	**
D x T	3	0.12	0.04	0.70	NS
Error	21	1.18	0.06		
Total	31	30.42			

APPENDIX 8B. Analysis of variance of shoot fresh weight

Source of variation	D.F.	Sum of squares	Mean square	Cal.F.	Level of significance
Replicates (R)	3	0.17	0.06		
Date (D)	3	41.55	13.85	128.25	**
Treatment (T)	1	3.21	3.21	29.69	**
D x T	3	0.19	0.06	0.58	NS
Error	21	2.27	0.11		
Total	31	47.39			

** = Significant at $P \leq 0.01$
NS = Non-significant

APPENDIX 8C. Analysis of variance of number of nodules

Source of variation	D.F.	Sum of squares	Mean square	Cal.F.	Level of significance
Replicates (R)	3	1.93	0.64		
Date (D)	3	523.63	174.54	42.28	**
Treatment (T)	1	85.53	85.53	20.27	**
D x T	3	11.93	3.98	0.96	NS
Error	21	86.70	4.13		
Total	31	709.72			

APPENDIX 8D. Analysis of variance of nodules fresh weight

Source of variation	D.F.	Sum of squares	Mean square	Cal.F.	Level of significance
Replicates (R)	3	0.28	0.09		
Date (D)	3	14.65	4.88	88.78	**
Treatment (T)	1	3.40	3.40	61.73	**
D x T	3	0.20	0.07	1.22	NS
Error	21	1.15	0.06		
Total	31	19.68			

** = Significant at $P \leq 0.01$
NS = Non-significant

APPENDIX 8E. Analysis of variance of nitrogenase activity

Source of variation	D.F.	Sum of squares	Mean square	Cal.F.	Level of significance
Replicates (R)	3	2.39	0.80		
Date (D)	3	9.66	3.22	14.64	**
Treatment (T)	1	5.00	5.00	22.72	**
D x T	3	0.33	0.11	0.50	NS
Error	21	4.62	0.22		
Total	31	22.00			

APPENDIX 8F. Analysis of variance of leghemoglobin content

Source of variation	D.F.	Sum of squares	Mean square	Cal.F.	Level of significance
Replicates (R)	3	7.02	2.34		
Date (D)	3	6.15	2.05	4.88	**
Treatment (T)	1	4.50	4.50	10.71	**
D x T	3	0.07	0.03	0.06	NS
Error	21	8.81	0.42		
Total	31	26.55			

** = Significant at $P \leq 0.01$
NS = Non-significant

APPENDIX 8G. Analysis of variance of nitrate reductase activity

Source of variation	D.F.	Sum of squares	Mean square	Cal.F.	Level significance
Replicates (R)	3	0.92	0.31		
Harvest times (H)	3	28.36	9.45	42.95	**
Treatment (T)	1	8.89	8.89	40.40	**
H x T	3	0.80	0.27	1.20	NS
Error A	21	2.89	0.14		
Interval (I)	5	387.97	77.59	352.60	**
T x I	5	1.56	0.31	1.40	NS
H x I	15	64.54	4.30	19.54	**
H x T x I	15	3.76	0.25	1.14	NS
Error B	120	26.45	0.22		
Total	191	526.14			

** = Significant at $P \leq 0.01$
NS = Non-significant

APPENDIX 8H. Analysis of variance of acid phosphatase activity in the nodules

Source of variation	D.F.	Sum of squares	Mean square	Cal.F.	Level of significance
Replicates (R)	3	234.33	78.11		
Date (D)	3	38832.74	12944.25	224.09	**
Treatment (T)	1	16330.76	16330.76	282.73	**
D x T	3	427.65	142.55	2.47	NS
Error	21	1213.03	57.76		
Total	31	57038.52			

APPENDIX 8J. Analysis of variance of the Rhizobium population

Source of variation	D.F.	Sum of squares	Mean square	Cal.F.	Level of significance
Replicates (R)	3	75.35	25.12		
Date (D)	3	14844.88	4948.29	255.76	**
Treatment (T)	1	325.13	325.13	16.80	**
D x T	3	502.68	167.56	8.66	**
Error	21	406.30	19.35		
Total	31	16154.34			

** = Significant at $P \leq 0.01$
NS = Non-significant

SECTION 10

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