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UNIVERSITY OF ALBERTA

STUDIES ON WHEAT SPOT MOSAIC DISEASE AND ASSOCIATED DOUBLE MEMBRANE BODIES

BY

KEVIN STEWART ZAYCHUK

A THESIS

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THE UNDERSIGNED CERTIFY THEY HAVE READ, AND RECOMMEND TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH FOR ACCEPTANCE, A THESIS ENTITLED STUDIES ON WHEAT SPOT MOSAIC DISEASE AND ASSOCIATED DOUBLE MEMBRANE BODIES SUBMITTED BY KEVIN STEWART ZAYCHUK IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PLANT PATHOLOGY.

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ABSTRACT

Wheat spot mosaic (WSpM) agent causes chlorotic spots, stunting, and sometimes kills wheat plants. It frequently occurs in association with wheat streak mosaic virus since they share a common eriophyid mite vector, *Aceria tulipae* Keifer. The examination of the infected plants by electron microscopy has revealed double membrane bodies (DMBs) 0.1-0.2 μm in diameter.

Using differential and sucrose density gradient centrifugation a membrane fraction was isolated from WSpM-affected wheat plants. The presence of endoplasmic reticulum (ER) was confirmed morphologically using a transmission electron microscope and with a differential stain. Electron microscopical examination of the membrane fraction from the WSpM-affected plant material revealed structures which were often electron-dense and enveloped in ER. These were similar to those observed in thin sections of WSpM-affected wheat. Similar structures were not observed in fractions from healthy material.

Double-stranded RNA analysis of 9 infection ages of 3 cultivars of wheat and 2 cultivars of barley ranging from 3 to 35 days after infestation with WSpM-associated mites revealed no WSpM-specific bands. Also, no WSpM-specific components were detected in total leaf proteins analyzed on 7.5-20% polyacrylamide gels whereas the coat protein of brome mosaic virus was readily detected 7 days post-inoculation.

Results using a lactoferrin-colloidal gold conjugated DNA probe indicated that the DMBs do not contain DNA. The probe bound specifically to DNA containing organelles within the cells of healthy and affected tissue as well as to plant mycoplasmas and spiroplasmas. However, no gold particles were observed on the DMBs.

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CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Common wheat (*Triticum aestivum* L.) is the number one food grain consumed by humans. It is grown across a wide range of environments around the world, and its production is greater than any other crop including rice, maize, and potatoes. No other commercial crop has more hectares of land in production than wheat (Briggle and Curtis 1987).

Although wheat is a cool-season crop, with the optimum temperature for growth being about 25°C, it is grown in many different climatic regions. It has been stated that a crop of wheat is harvested somewhere in the world during every month of the year. However, the majority of the harvest occurs between April and September in the Northern Hemisphere, where most of the wheat is grown, and October to January in the Southern Hemisphere (Briggle 1980).

In addition to its use in breads and pasta, wheat is also used as an animal feed grain and to a lesser extent by various industries.

One of the major production hazards of wheat is the constant threat of disease. From work on wheat diseases, many important discoveries have been made in plant pathology, genetics, and breeding as researchers strived to protect this valuable crop.

Improvement in stability and the level of quality and yield have been a result of scientific work directed at the control of wheat diseases. Diseases which were once devastating in many parts of the world have been brought under control with great economic benefits. However, there is a great deal of work

which still can be done since often when one disease is controlled, a new disease may appear which previously was not competitive. Also, there is the constant problem of genetic variability in pathogens and the evolution of new virulent strains or races emerging from the existing pool of known disease agents. Changes in production practices also lead to outbreaks or increased levels of disease. Irrigation, shorter periods of rotation, less tillage, and higher rates of fertilizer all have effects on disease incidence and often on the vectors which are responsible for the transmission of certain pathogens.

WHEAT SPOT MOSAIC

The disease known as wheat spot mosaic (WSpM) in Alberta, was discovered during investigations of wheat streak mosaic virus (WSMV) (Slykhuis 1953, 1955, 1956). WSpM and WSMV are often found together in mixed infections in wheat as they share a common vector, the wheat curl mite, *Aceria tulipae* Keifer (Slykhuis 1955, 1956; Nault and Styer 1970).

WSMV is an important disease of wheat and was probably responsible for the "yellow mosaic" first recognized in 1922 in Nebraska (Hiruki, C., personal communication). It has been reported not only in North America (Slykhuis 1953, 1955; Staples and Allington 1956; Ashworth and Futrell 1961; McKinney *et al.* 1966; Orlob 1966; Nault and Styer 1969) but in many other wheat producing regions of the world as well, including the USSR (Razvyazkina et al 1963; Stein-Margolina 1966), Romania (Pop 1962; Slykhuis and Bell 1963), Bulgaria (Markov *et al.* 1975), Turkey (Bremer 1973), Yugoslavia (Tosic 1971), and Jordan (Slykhuis and Bell 1963). A severe mosaic is produced by WSMV on most cultivars of winter wheat as well as oats, barley, rye and some cultivars of maize (Brakke 1971). WSMV belongs to a group of the putative potyviruses in

that it morphologically resembles the flexous rod-shaped (700 nm in length) particles of that group and induces the characteristic cylindrical or pinwheel-shaped cytoplasmic inclusions, but it is not transmitted by aphids. WSMV is relatively well characterized and much is known about its genomic RNA and the structural and non-structural proteins which it codes for (Brakke 1971; Niblett *et al.* 1988; Brakke *et al.* 1990).

In contrast to WSMV, very little is understood about the causal agent of WSpM. It has not been possible to transmit the disease agent to healthy plants by mechanical inoculation and graft transmission has not been attempted. Therefore, the only means of transmission is by the wheat curl mite. Although this disease was initially believed to be caused by a virus, and has at one time been referred to as wheat spot mosaic virus, no virus or virus-like particles have been found in the infected plants. This is an example of a disease which may become a more severe problem as new cultivars are introduced that are resistant only to WSMV.

The infected plants have been the subject of numerous electron microscopic studies. The most striking ultrastructural characteristic of WSpM-affected plants is the presence of double-membrane bodies 0.1-0.2 μm in diameter, scattered or aggregated, in the epidermal and subepidermal parenchyma cells (Bradfute and Nault 1969; Bradfute *et al.* 1970; Edwards and McMullen 1988; Hiruki 1989). Studies on WSpM-affected wheat using thin sections labelled with a DNA-specific gold probe revealed that although the probe bound specifically to DNA-containing organelles, it did not bind to the DMBs (Zaychuk *et al.* 1990). Low molecular weight double- stranded (ds) RNA analysis revealed several mite-related RNA bands, however no WSpM-specific RNA species could be detected (Ohki *et al.* 1985). Hiruki (1989) found that the

disease could be heat-cured after 2 weeks at 35°C but tetracycline at 100 µg/ml for 3 weeks did not result in symptom remission. With a similar disease of wheat which was found in North Dakota, tests using both ELISA and dsRNA analysis did not implicate any known pathogen (Edwards and McMullen 1988).

Geographical Distribution

In addition to Alberta, WSpM has been reported in North Dakota (Edwards and McMullen 1988), and Saskatchewan (Hiruki 1989). Slykhuis (1961, 1964) also reported a disease in Ontario similar to WSpM but with less severe symptoms. This disorder was termed chlorotic mottle virus, however, it may have been caused by the same agent as WSpM since Slykhuis (1956) reported earlier that the sypmptoms varied widely among isolates of WSpM. A disease with characteristics similar to WSpM was reported in Ohio and termed as the wheat spot chlorosis pathogen (WSCP) (Nault *et al.* 1970). There is increasing evidence that this disease may be widespread across the northern United States and southern Canada and possibly in all of the areas where WSMV is found.

Symptomatology

As indicated by the name, the first symptoms to appear on infected wheat are chlorotic spots approximately 0.5 mm x 1 mm in size (Figure 1). They usually appear on the youngest leaves of wheat in the 1-3 leaf stage, 3-4 days after infestation with infective mites (Slykhuis 1956). Similar symptom development on wheat was reported by Nault *et al.* (1970) with the development of mild chlorotic spotting on all of the 13 varieties tested. The symptoms appear 6-8 days after allowing the mites a 48-96 hour inoculation access period on

plants in the 2-3 leaf stage. Progression of the disease results in enlarged spots and their eventual coalescence causing chlorosis of the entire leaf blade, dieback of leaf tips, necrosis of the entire leaf, and finally death of the entire plant. On Oh28 inbred corn, chlorotic spots appear in 2-3 days, followed by interveinal chlorosis, severe wilting, necrosis of leaf tips and death of the plant (Nault and Styer 1969). Slykhuis (1956) reported symptom differences in various isolates of the disease of wheat ranging from a "vaguely defined mottle" to severe necrosis, stunting, and death of the plants. Some isolates were so pathogenic to wheat that the culture was difficult to maintain and lost within a year of isolation. There are also considerable differences in cultivar reaction to particular isolates of WSpM (Slykhuis 1956; Nault *et al.* 1970; Hiruki, unpublished data).

Host Range

Several studies have been conducted to determine the host range of WSpM and WSCP which in addition to wheat, can affect barley, corn oats, rye, sorghum, and several annual wild grasses (Slykhuis 1956; Nault *et al.* 1970; Hiruki, unpublished data). The range of WSpM and WSCP is limited however, by the range of the eriophyid mite. The mite must be able to colonize the plant to transmit the disease. Therefore, the limiting factor for the disease is the ability of the mite to colonize and feed on the host. Since the disease agent is not mechanically transmissable, workers have been unable to determine if the agent can affect a wider range of plants.

Vector

The eriophyid mites have been the subject of numerous reviews and book chapters regarding their role in the transmission of plant disease agents (Slykhuis

1960, 1962, 1963, 1965, 1969, 1973; Oldfield 1970; Slykhuis 1980; Hiruki, in press). There are over 1,250 species of mites in the superfamily Eriophyoidae and most of these are in the family Eriophyidae (Keifer *et al.* 1982). A general characteristic of most eriophyoids is their narrow host range. Although many eriophyoids cause neither detectable alteration nor damage to their hosts, the eriophyids, commonly known as gall, rust, bud, or blister mites, are of considerable economic importance. The soft bodied worm-like mites are the smallest arthropods known to infest plants and often go unnoticed to the unaided eye since they are approximately 100-250 µm in length. The four developmental stages of the mites are the egg, first nymph, second nymph and adult. Males hatch from unfertilized eggs and females from fertilized eggs. As the female crawls over the spermatophores deposited by the male, sperm is released into her spermatheca.

The eriophyids are exclusively phytophagous and are one of the most specialized groups of plant feeders. Although symptoms from mite feeding ranges from simple russeting to complex gall formation, the host-mite relationships are very specific which reflects their high degree of specialization.

In addition to the malformations caused by the mites, they have also been implicated as the vectors of a number of plant pathogens. Currently there are 16 diseases that are associated with eriophyid mites. These have been grouped into three categories by Hiruki (personal communication) in the most recent review on this subject. The first group includes eight serologically related viruses which have flexuous particles about 700 nm in length. The second group consists of four diseases in which the cytoplasm of the infected cells contains ovoid, double-membrane bodies. This is the category under which WSpM has been classified.

The third group also consists of four diseases where mites are known to be the vector but the exact causal agent is unknown.

One of the most important eriophyid mites inhabiting monocotyledonous crops is *Aceria tulipae*, is the vector of WSMV and WSpM. It is known as the wheat curl mite or dry bulb mite since the first known host for this mite was the bulbs of tulips which are in the Liliaceae family (Keifer 1938). In the same family, onion and garlic are the most commonly infested hosts (Jeppson *et al.* 1975).

Among the monocotyledonous plants however, *A. tulipae* has the widest range of hosts in the Graminae (Slykhuis 1955; Connin 1956a, 1956b). Because of the extremely small size of this mite and its comparatively small mouthparts, it causes very little feeding damage to its host. The feeding and colonization habits of the mite were studied by Orlob (1966). He observed that most mites begin to feed on epidermal cells immediately after transferring them onto a leaf and that the majority of mites are found within the grooved areas of the leaf between the veins. The mites prefer to feed in the tightly rolled sections of the leaves probably because of the high relative humidity prevailing there since they are susceptible to drying. The mouthparts consist of a two-lobed rostrum and a pair of stylets. The structure of the stylets is such that only about one-third of the total stylet length (5μm) is involved in actual penetration of a plant cell. Therefore, the mites feed on the epidermis and do not penetrate the subepidermal tissues (Orlob 1966).

A mite infested monocotyledonous host such as wheat exhibits rolling of the leaves and looping of the leaves which become entrapped (Figure 2). Typical feeding mites as they are observed in a partially unrolled leaf are shown in Figure 3. In some cases, mites which are not carrying WSpM ("healthy mites")

may cause a pronounced chlorosis on the leaves of wheat if the population is very heavy (Slykhuis 1956). The feeding of *A. tulipae* on corn causes a disorder known as kernel red streak (Slykhuis *et al.* 1968). The mites cause typical twisting and looping of the leaves as well as discoloration of the grains. The kernels are discolored near the tips of the ears where the mites gain entry. It is believed that this disorder is not caused by a pathogen and that it is probably the result of injected mite salivary toxins. The mite also damages garlic in California (Lange 1955) and has been reported to cause virus-like symptoms in this crop (Smalley 1956).

Electron microscopical examination of mites fed on WSMV-infected plants resulted in the observation of large concentrations of flexous rod-shaped particles which were considered to be WSMV (Paliwal and Slykhuis 1967; Takahashi and Orlob 1969). The particles were mainly observed in the hindgut and the posterior part of the midgut whereas only a few aggregates were found in the middle part of the midgut. In addition to WSMV, brome mosaic virus (BMV) has also been observed in thin sections of mites fed on BMV-infected plants (Paliwal 1972; Stein-Margolina 1973). Although *A. tulipae* is not a vector of this virus, Paliwal (1972) noted that BMV appeared to multiply locally in the gut tissue of the mites. A thorough transmission and scanning electron microscope study of the fine structure of the mite was carried out by Whitmoyer *et al.* (1972) with the intent to further examine mites carrying WSCP for the presence of the DMBs which were observed in the affected plants. However, since this initial publication, no further reports have been published. In the case of fig mosaic and WSpM-carrying mites, a preliminary TEM ultrastructural examination revealed no virus or virus-like particles or abnormal ultrastructural changes within the mites (Hiruki, C., personal communication).

Transmission Characteristics

Slykhuis (1955) found that eggs from mites on WSpM-affected plants produced mites that did not induce symptoms on healthy plants. However, all stages of the mite which had been reared on diseased plants could transmit the disease agent. Therefore, although infectivity was retained during molting, transovariate transmission did not occur. Similar results were obtained by Nault and Styer (1970) working with WSCP in respect to transmission of the agent. However, acquisition tests revealed that adult mites could not transmit the agent unless they had acquired it previous to their final molt. By maintaining mites on an artificial medium, the longevity of the agent in the mite was determined to be 8 days. There was a decline in transmission of the pathogen by mites, with an increase in time after removal from the infected source plants. This was also the case with WSpM in that transmission was about 98% at day 0 and declined to 50% at day 3 and 0% at day 10 (Hiruki 1989).

Control

Achieving control of the mites is important in controlling WSpM and WSMV since hosts resistant to WSMV have been found to be susceptible to WSpM. Since the mites cannot survive without the host plant, cultural control methods have been somewhat effective. Slykhuis (1955) recommended the control of volunteer wheat on summerfallow to ensure elimination of the mites as well as to avoid early seeding of winter wheat, thus preventing an overlap period with spring wheat which has not yet matured. The control of perennial hosts surrounding fields is also necessary since the mite is dispersed by wind and can easily move long distances to infest a crop.

Attempts to control the mite using seed treatment or foliar applied systemic acaricides did not significantly reduce the mite population or reduce the incidence of WSMV (Kantack and Knutson 1958). However, Harvey *et al.* (1979) found that a systemic insecticide applied to the soil during the fall planting period proved to be an effective control method for the mite and reduced the incidence of WSMV, hence increasing yields.

Mite populations are also affected by leaf pubescence since a pubescent leaf presents a more favorable landing site for an airborne mite than a glabrous leaf (Harvey and Martin 1980).

More recently, attempts at controlling the mite have focused on developing wheat varieties that are resistant to leaf curling and thus eliminating the environment required for its survivial (Thomas and Conner 1986; Whelan 1988; Whelan and Hart 1988; Whelan and Conner 1989). Therefore the inability of the mite to colonize the host would appear to be an effective means of eliminating the spread of WSMV and WSpM.

OBJECTIVES

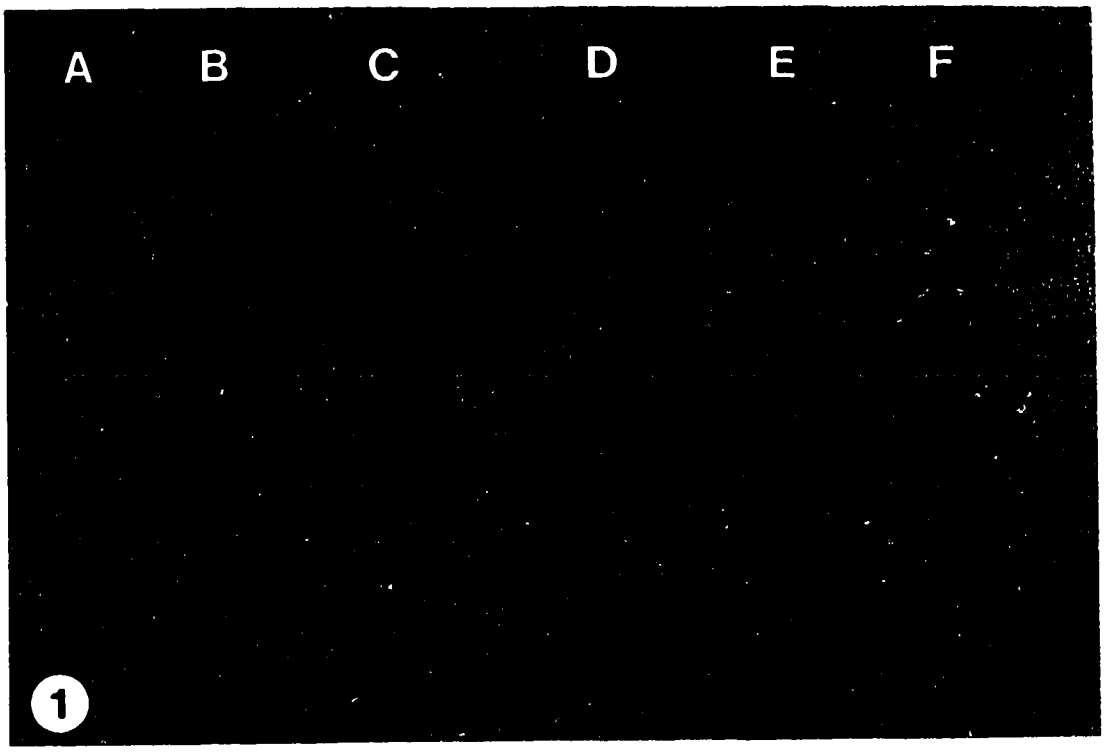
The objectives of this study were to: **1)** develop a technique to isolate the DMBs and to examine their structural characteristics by transmission electron microscopy **2)** analyze WSpM-affected wheat for the presence of double-stranded RNA and WSpM-specific proteins and **3)** probe the DMBs *in vivo* for the presence of nucleic acids.

Figure I-1. Symptoms of WSpM on wheat.

A - Healthy leaf.
B - Partially rolled leaf caused by *Aceria tulipae.*
(C-F) - Stages of symptom development ranging from slight chlorosis (F) to small chlorotic spots (E) which coalesce to cause general chlorosis and some necrosis (C).

Figure I-2. Leaf rolling of wheat caused by the wheat curl mite (*Aceria tulipae*)

A - 2 week old TAi wheat 1 week after introduction of mites
B - 3 week old TAi wheat 2 weeks after introduction of mites
C - 4 week old TAi wheat 3 weeks after introduction of mites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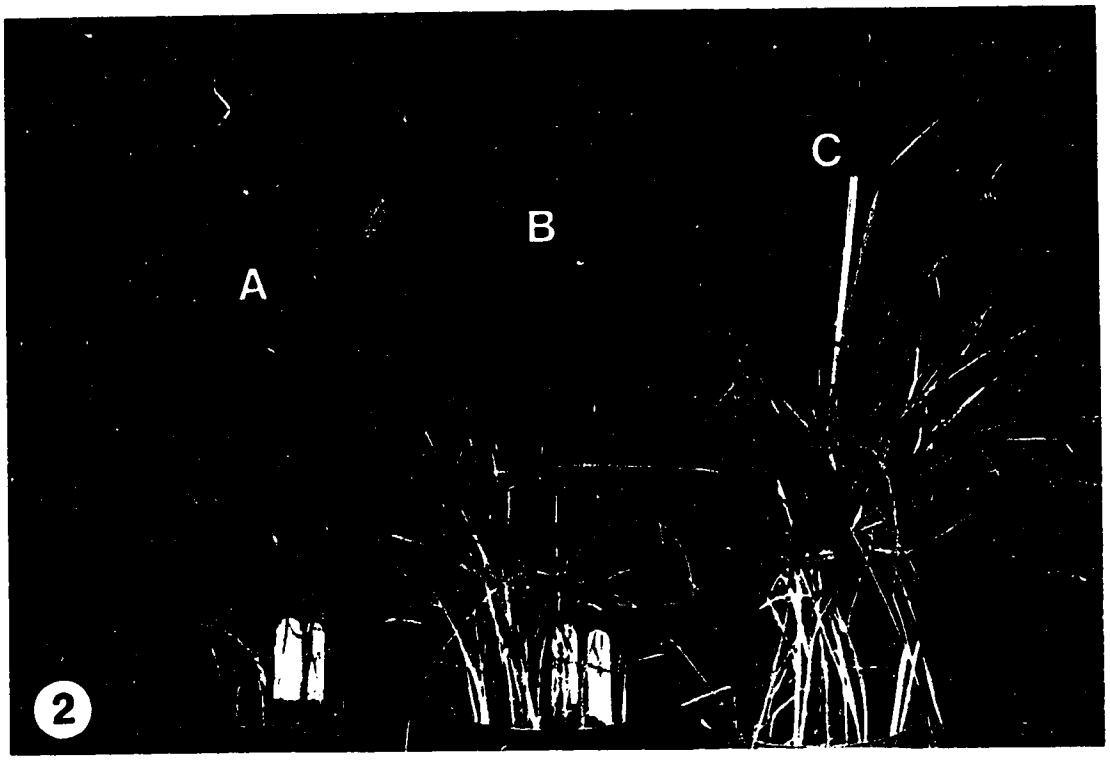
A
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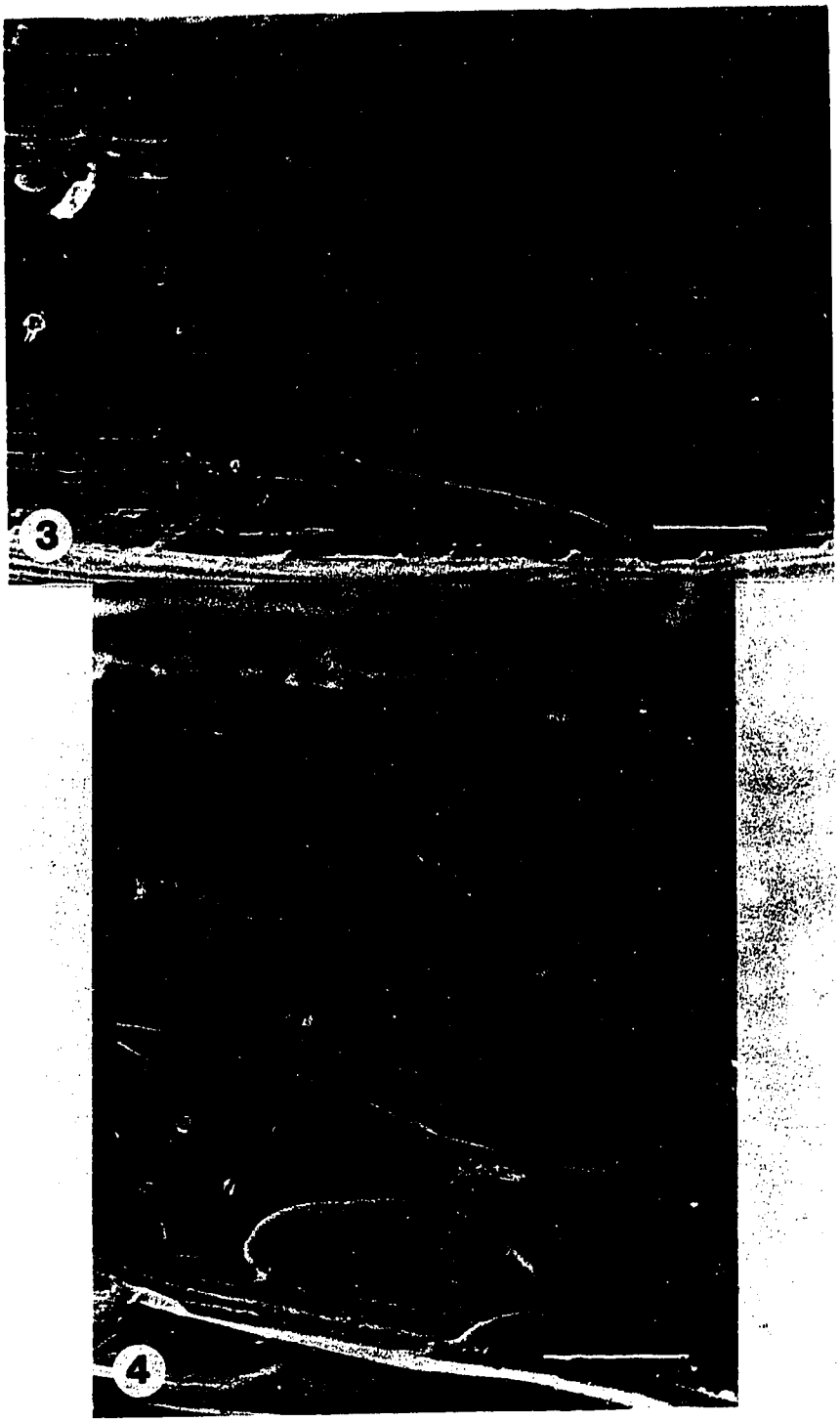
2

Figure I-3. Scanning electron micrograph of a partially unrolled wheat leaf infested with the wheat curl mite (*Aceria tulipae).*
Bar = 0.2 mm

Figure I-4. Enlarged area of figure 3 showing detail of *Aceria tulipae.* Note the spermatophore being deposited on the leaf and the numerous spermatophores on the leaf surface.
Bar = 0.1 mm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CHAPTER II

PARTIAL PURIFICATION OF THE ENDOPLASMIC RETICULUM AND THE ASSOCIATED DOUBLE MEMBRANE BODIES IN WHEAT SPOT MOSAIC-AFFECTED WHEAT

INTRODUCTION

The disease of wheat known as wheat spot mosaic (WSpM) was first recognized in Alberta in 1952 (Slykhuis 1956). It is often found in southern Alberta and in many regions wherever wheat is grown (Slykhuis 1964; Nault *et al.* 1970; Edwards and McMullen 1988; Hiruki 1989). The wheat curl mite, a small (100-200 μm), worm-like, soft-bodied arthropod, (*Aceria tulipae* Keifer) is the vector of wheat streak mosaic virus and has also been proven to transmit WSpM (Slykhuis 1956). The relationship of WSpM with its vector is similar to that of WSMV. It can be transmitted by all stages of the mite, however, it must be acquired before the adult stage. Infectivity is retained during molting but there is no transovarial passage. WSpM can not be transmitted by mechanical inoculation.

Although the causal agent of WSpM has not been identified, electron microscopy of leaf and root tissue of the infected plants has revealed the presence of double membrane bodies (DMBs) that range in size from 100-200 nm (Bradfute and Nault 1969; Bradfute *et al.* 1970; Hiruki *et al.* 1988; Hiruki 1989). The DMBs are found scattered, or form large aggregates, in the cytoplasm of epidermal and subepidermal parenchyma cells as well as in phloem elements. Similiar DMBs have also been found to be associated with other eriophyid mite transmitted disorders such as fig mosaic in California (Bradfute *et al.* 1970), and in Italy (Plavsic and

Milicic 1980; Appiano 1982; Appiano *et al.* 1986; Lovisolo *et al.* 1986), rose rosette in Arkansas (Gergerich *et al.* 1983), and pigeon pea sterility (Hiruki, C., personal communication). In the case of rose rosette, Gergerich and Kim (1983) termed the DMBs "virus-like particles" and believed that the agent belongs to a group of poorly characterized plant pathogens that are transmitted by eriophyid mites. Bradfute *et al.* (1970) indicated that the DMBs were observed in "regions of elaborated endoplasmic reticulum" (ER) and that the outer membrane of the DMB is acquired by budding from the lumen of the ER to the cytoplasm. Chen and Hiruki (1990) demonstrated the presence of a physical connection between the ER and DMB's using the serial section technique. When this technique was employed, almost 67% of the DMBs were physically connected to the ER suggesting that they were originally formed from this membrane structure. Three forms of the DMBs were noted in thin sections of infected tissue. DMBs which originated from the cisternae or central lumen of the ER were classified as a budding form, whereas a phagocytosis form occurred when the ER engulfed the matrix of cytoplasm so that the two cytoplasmic side membranes formed the double membrane of the DMB. The third form of the DMBs observed was a cocci form. In this case they formed a chain which could be seen only through serial sections or longitudinal sections.

In this study, isolation and purification of the DMBs from the WSpM-affected wheat leaf and root tissues was attempted to further examine the presence of these DMBs in the affected tissue, their association with the ER, and possibly their role in the etiology of the disease. Because of the association of the DMBs with the ER, a method that was developed for the isolation of ER was employed, utilizing differential and sucrose density gradient centrifugation. The membrane fractions were examined for purity by transmission electron microscopy. Isolated membranes

were identified on the basis of morphology and their differential staining characteristics using a chromic acid/phosphotungstic acid stain.

MATERIALS AND METHODS

Membrane Isolation

Isolation of the endoplasmic reticulum was carried out according to a modified protocol of Butcher and Evans (1986). Healthy or WSpM-affected leaves with a fresh weight of 25g respectively, were homogenized in a Waring blender in 100 ml of homogenization buffer (0.25 M sucrose, 3 mM EDTA, and 25 mM Tris adjusted to pH 7.2 with 1 M Mes). The extracts were filtered through four layers of cheesecloth and centrifuged at 13,000*g* for 15 minutes (Sorvall RC5B, SS34 rotor). The supernatants were then centrifuged at 80,000*g* for 30 minutes in a Beckman L5-75 ultracentrifuge with a Type 30 rotor. The pellets were resuspended in homogenization buffer and the centrifugation repeated. Each pellet was then resuspended overnight in 2 ml of suspension buffer (18%, w/v, sucrose, 1 mM dithiothreitol, 1 mM $MgSO_4$, and 1 mM Tris adjusted to pH 7.2 with 1 M Mes). The samples were then applied to a linear sucrose density gradient (20-45%, w/v, sucrose in suspension buffer). and centrifuged at 80,000*g* for 40 minutes using a Beckman VTi 65.1 vertical rotor. The gradient was divided by puncturing the tubes with a syringe and the fraction with a density of 1.13-1.18 g/cm^3, which is the reported sedimentation density for rough and smooth ER (Jones 1980), was drawn off from each sample.

Electron Microscopy

The fractions were prepared for electron microscopy by first pelleting the solutions at 80,000*g* for 90 minutes. The pellets were transferred to 1.5 ml microcentrifuge tubes to which 500 µl of 2.0% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) was added. The pellets were left overnight at 4°C, rinsed twice in phosphate buffer, and post-fixed for 2 hours in 2% OsO_4 in phosphate buffer. Dehydration of the pellets was carried out in an ethanol series (70%, 90%, 95%, 100%). They were then rinsed three times in propylene oxide and left for two hours in a 50/50 propylene oxide/Araldite resin mixture in stoppered glass vials. The corks on the vials were removed and the propylene oxide was allowed to evaporate overnight. After resin infiltration, the pellets were embedded in Araldite and polymerized in a J.B.S. No. 306 flat mold (J.B. EM Inc., Montreal) for 20 hours at 65°C. Sections were cut with glass knives using a Reichert OmU2 ultramicrotome and collected on 200 mesh Formvar-coated nickel grids. The sections were stained with uranyl acetate for 30 minutes, rinsed with distilled water for five minutes, stained five minutes in lead citrate and rinsed once again with distilled water for five minutes. All staining was carried out using the apparatus of Chen (1973).

Differential Staining

The differential staining procedure of Roland *et al.* (1972) was utilized to stain thin sections of the membrane fractions. Sections of WSpM-affected wheat were included as controls.

Sections were collected on 300 mesh nickel grids which had been dipped in 0.03% Formvar in chloroform to aid in section adhesion. The grids were incubated section side down in 1% periodic acid for 30 minutes at 36°C after a 3 minute pretreatment in distilled water. The acid was thoroughly rinsed from the grids with a

2 minute jet wash of distilled water and the grids were then transferred to a solution of 1% phosphotungstic acid in 10% chromic acid. The grids were washed once again after an incubation period of 10 minutes at 21°C. This staining procedure was carried out using 20 µl drops of the respective solutions in the wells of a Falcon 30340 microtest plate (Falcon Plastics, Oxnard, CA).

The specimens were examined at 75 kv on a Hitachi H7000 (Nissei Sangyo, Mountain View, CA) electron microscope.

RESULTS

Differential Staining

The specificity of the phosphotungstic acid-chromic acid (PACA) stain is shown in Figures 1 and 2. The specimen in Figure 1 was pretreated in periodic acid to remove osmium from the tissue. Although the chloroplast and cell wall are discernible in this micrograph, the contrast is limited and fine membrane structures are not readily observed. In the case of the thin sections which were pretreated in periodic acid and subsequently stained with PACA, both the cell wall and the plasma membrane were intensely stained in relation to the other cellular components and organelles.

Differentially stained thin sections of the membrane fractions revealed relatively few positively stained membranes (Figures 3,4). The sections of the membrane fraction isolated from healthy plant material were generally observed to contain more contaminating plasma membrane (Figure 3) than the fraction obtained from the WSpM-affected material (Figure 4). The thickness of the heavily stained membrane (approx. 10 nm) further identified it as plasma membrane since ER is a

much thinner membrane (approx. 6 nm). The fraction from the WSpM-affected wheat is not stained with PACA indicating that there is no detectable contamination with plasma membrane (Figure 4). Both rough and smooth ER could be observed in the fraction whereas the ER in the healthy fraction was mainly of the smooth type.

Membrane Isolation

Examination of the double-stained membrane fractions from healthy (Figure 5) and WSpM-affected wheat (Figure 6) revealed marked differences in the morphology of the ER. The healthy membrane fraction revealed the presence of ER and ER derived vesicles which are relatively electron luscent (Figure 5). The fraction appears to be composed of mainly smooth ER as no ribosomes attached to membrane can be observed. The fraction obtained from the WSpM-affected wheat, however, consists of ER in addition to numerous electron dense bodies which were often observed to be physically connected to the ER (Figure 6). The enlarged photograph of Figure 6 clearly shows the circular or spherical bodies of varying electron densities (Figure 7). Figures 8 and 9 further illustrate the membrane fractions obtained from the WSpM-affected plant material. These structures or WSpM agent-like bodies (WSpMBs) were consistently found interspersed within the membrane fraction. The WSpMBs are unlike the osmiophilic lipid bodies which are frequently observed in chloroplasts and are also found in both the healthy and WSpM-affected membrane fraction (Figures 5, 6). The membrane or ER found in conjunction with the WSpMBs appears to partially envelop or surround the electron dense sphere. In some cases a relatively large section of membrane is seen attached to or engulfing the WSpMB whereas other electron-dense bodies are either partially bound or completely free of membranous material. The majority of the WSpMBs observed in the fractions fall within the size range of 100-200 nm. The smaller

WSpMBs are more electron dense than the larger bodies indicating that the section was cut through a membrane surrounding the sphere. A section through the middle of WSpMB reveals a membrane surrounding a less electron dense core (Figure 8).

DISCUSSION

The purity of the endoplasmic reticulum (ER) fraction isolated from healthy wheat is comparable to fractions obtained by more time consuming techniques such as those developed by Jones (1980). The results of the phosphotungstic/chromic acid (PACA) differential staining are in agreement with those of Roland *et al.* (1972) who successfully used this technique on the plasma membrane of maize and oats in addition to a number of other organisms, and Nagahashi *et al.* (1978) who used the technique to identify plasma membranes of barley roots. The positive staining of the plasma membrane on the thin sections of the intact starting material further demonstrates that the staining procedure is carried out correctly (Morre 1990). Since the plasma membrane is one of the main cellular components which may contaminate ER fractions due to its similar sedimentation coefficient (Robinson 1985), the differential stain was valuable in determining the purity of the fractions. In addition, measurement of the thickness of various membranes in the fractions which permits identification upon isolation (Morre *et al.* 1987), revealed that the ER fraction was relatively free of other membranous components. Therefore, as indicated by the purity of the fractions, or the lack of PACA stained membranes, the technique of Butcher and Evans (1986) was useful in obtaining a relatively pure fraction of ER in a short time.

The ER isolated from the healthy wheat was morphologically similar to the smooth ER isolated by Jones (1980) from barley aleurone layers. However, the membrane fraction isolated from the WSpM-affected wheat contained numerous electron dense ovoid bodies. These bodies which will be referred to as wheat spot mosaic agent-like bodies (WSpMBs), are not unlike the structures observed in thin sections of tissue affected with WSpM (Hiruki 1989). The size of the WSpMBs (100-200 nm) corresponds to the size of the double membrane bodies which have been reported in the three other eriophyid mite associated disorders; fig mosaic (Bradfute *et al.* 1970), rose rosette (Gergerich *et al.* 1983), and pigeon pea sterility (Hiruki, C., personal communication). The appearance of some of the WSpMBs with fragments of attached membrane or ER demonstrates their association with this cellular component. In some cases two seperate membranes can be observed in conjunction with an electron dense body. It is these two membranes which probably compose the double membrane system when the DMBs are observed *in vivo*. This relationship was suggested by Bradfute *et al.* (1970) and was demonstrated recently by Chen and Hiruki (1990) using the serial section technique. However, this is the first report of the isolation of these structures from WSpM-affected tissue or any of the plants affected with the DMB-producing disorder.

The isolated WSpMBs clearly show their spherical nature and the association with ER. This type of structure is unique to the three disorders previously mentioned which are associated with eriophyid mites. Similar, but not identical structures have been observed upon ultrastructural examination of various other diseased tissues. In one case, observation of tomato spotted wilt virus (TSWV) infected watermelon revealed the presence of flexous tube-like structures with two layers of membrane (Honda and Iwaki 1984). When viewed in cross-section, the structures with an internal diameter of 80-90 nm and an external diameter of 100-120

nm, resembled the DMBs observed in thin sections of WSpM-affected wheat. However, the tube-like nature of the TSWV-associated structures is not observed in the DMBs. Tubular structures have also been observed in *Diodia virginiana* infected with a whitefly transmitted closterovirus (Larsen *et al.* 1991) and in a human T lymphotrophic virus infected Epstein-Barr virus-producing B-cell line (Ohtsuki *et al.* 1990). Distinct cytoplasmic changes involving the ER have also been reported in association with chimpanzee and human hepatocytes infected with non-A, non-B hepatitis (Pfeifer *et al.* 1980; Busachi *et al.* 1981; Gravelle *et al.* 1982). Busachi and co-workers (1981) stated that they observed a diffuse and striking increase and dilation of the rough ER. They also observed a detachment of ribosomes and proliferation of the smooth ER which formed numerous rounded or ovoid vesicles in addition to curved membranes which developed by close apposition of two cisternae of ER. These circular structures had a diameter in the range of 120-300 nm and some of the rounded formations had electron-opaque material in between. The convoluted ER observed by Gravelle *et al.* (1982) was identical in morphology to the convoluted ER found in the cytoplasm of plant mesophyll and root-parenchyma cells infected with pea seedborne mosaic virus (Hampton *et al.* 1973). More recently, a report on the ultrastructural changes within patients infected with the human immunodeficiency virus (HIV) found that proliferation of the smooth ER in enterocytes was a striking feature (Mathan *et al.* 1990). Within dilated tubules of ER at the periphery of the area of smooth ER proliferation, clusters of electron-dense inclusions were present with occasional tubular structures. The nature of these inclusions is not known and HIV particles could not be detected although nucleotide probing demonstrated HIV infection. It is interesting to note that although these cytological changes were observed, no virus particles could be seen in any of the

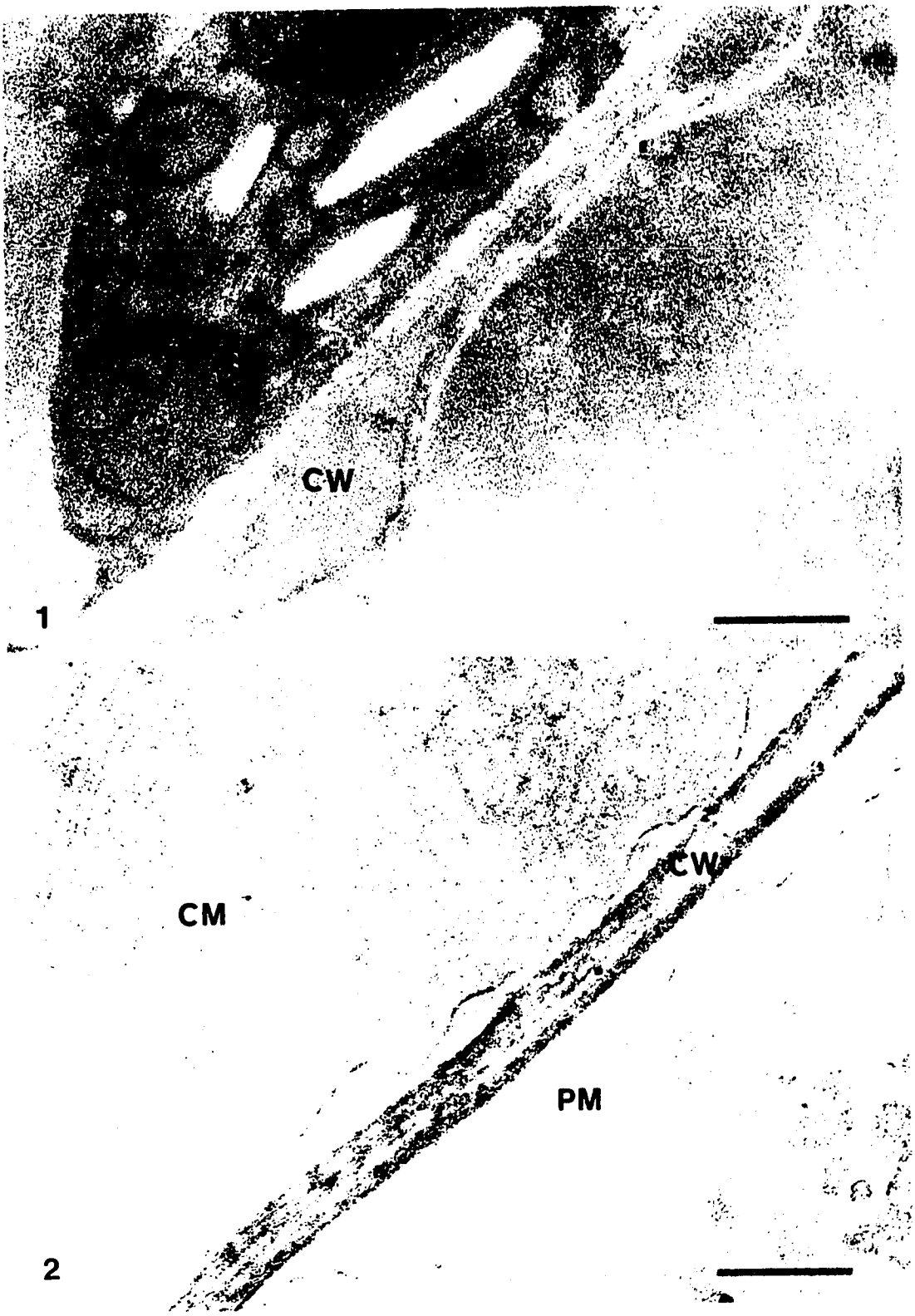
specimens. This has been the case thus far with WSpM since no virus particles have been observed and only the DMBs have been noted.

Therefore, the cytological changes induced in WSpM-affected plants in terms of proliferation of ER and the production of electron-dense bodies which appear to be physically connected to the ER, are features which have been observed in some plant and animal virus infections. Although it is not known if WSpM is caused by a virus infection, we have demonstrated that the WSpMBs can be isolated from affected tissue and that they are not present in healthy plant material. The identity of these structures, however, and their pathogenic significance, remains to be elucidated.

Figures II-1-2. Transmission electron micrographs of a thin section of WSpM-affected wheat fixed in glutaraldehyde and osmium tetroxide and embedded in Araldite resin.

Figure II-1. Specimen treated in 1% aq. periodic acid for 30 minutes. Note overall low contrast and poor resolution of fine features of cell wall (CW) and chloroplast (CH).
Bar = 0.5 μm

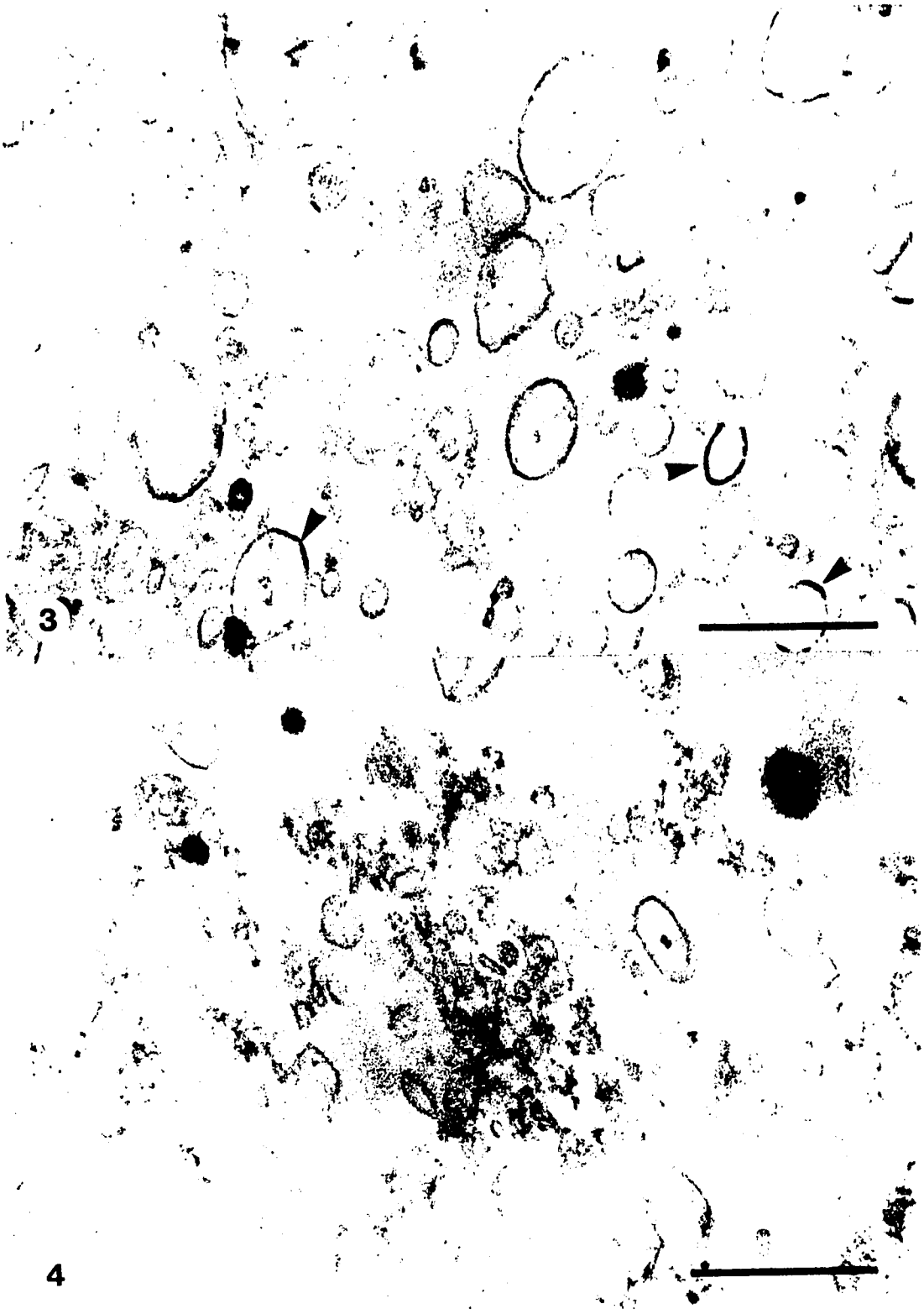
Figure II-2. Specimen treated in 1% aq. periodic acid and differential stain of 1% phosphotungstic acid in 10% chromic acid. Note the heavily stained cell wall and the stained plasma membrane (PM) along both sides of the cell wall. No stain deposition is observed on the chloroplasts or the chloroplast membranes (CM).
Bar = 0.5 μm


CW
1
CM
CW
PM
2

Figures II-3-4. Transmission electron micrographs of endoplasmic reticulum fractions fixed in glutaraldehyde and osmium tetroxide, embedded in Araldite resin, and stained with the plasma membrane-specific PACA stain.

Figure II-3. Fraction isolated from healthy wheat. Note the densely stained plasma membrane (arrowheads).
Bar = 0.5 μm

Figure II-4. Fraction isolated from WSpM-affected wheat.
Bar = 0.5 μm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Figures II-5-6. Transmission electron micrographs of endoplasmic reticulum fractions fixed in glutaraldehyde and osmium tetroxide, embedded in Araldite resin, and double stained with uranyl acetate and lead citrate.

Figure II-5. Fraction isolated from healthy wheat.
Bar = 0.5 μm

Figure II-6. Fraction isolated from WSpM-affected wheat. Note the numerous electron dense bodies which are not observed in the fraction from healthy wheat.
Bar = 0.5 μm

5

6

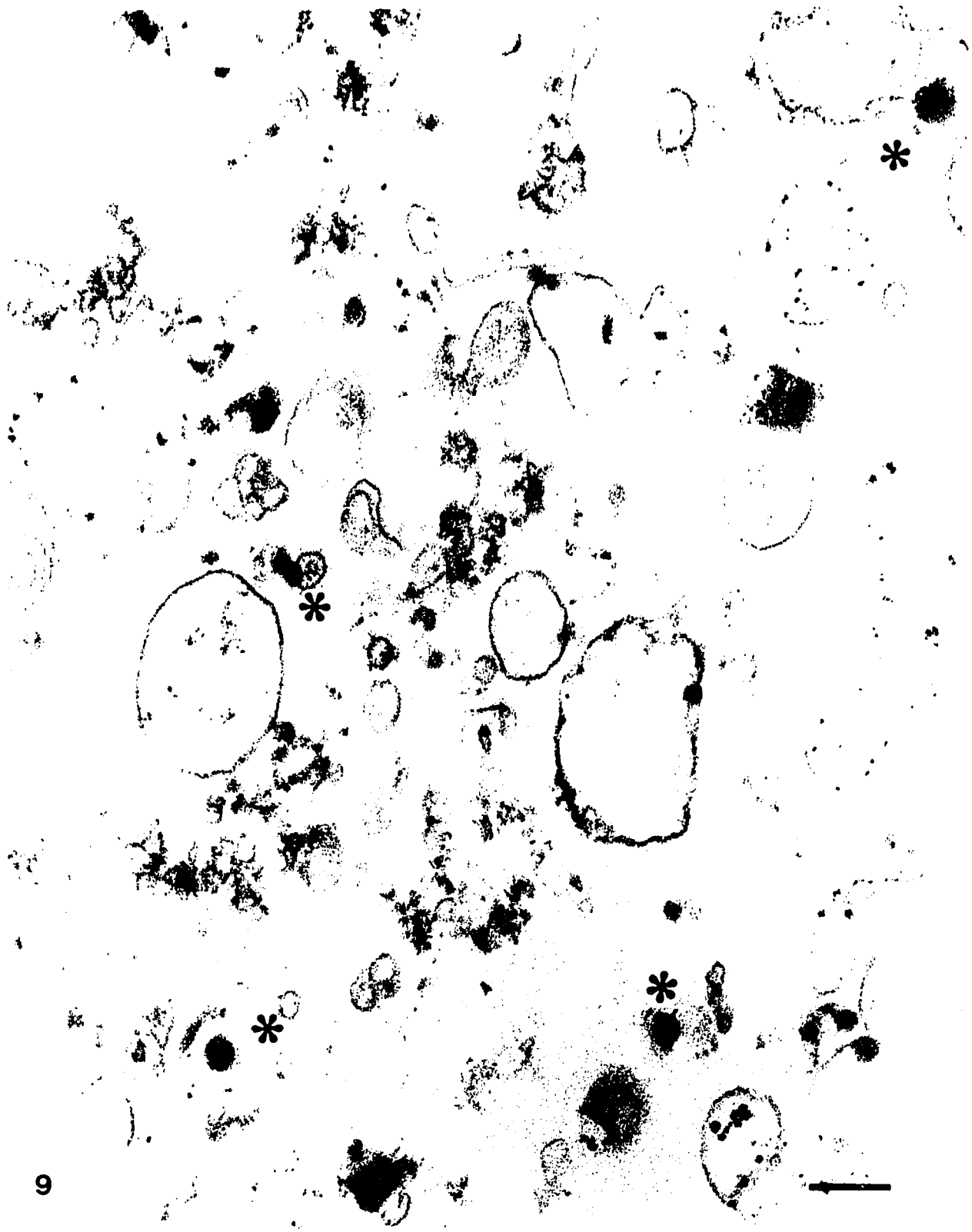
Figure II-7. Enlarged micrograph of figure 6 showing detail of isolated WSpM agent-like bodies (asterisks). Note the variation in electron density of the bodies and their physical attachment to the endoplasmic reticulum (arrows).
Bar = 0.2 μm

7

Figure II-8. Area of membrane fraction showing WSpM agent-like bodies. Bar = 0.2 μm

8

Figure II-9. Area of membrane fraction showing WSpM agent-like bodies. Bar = 0.2 μm



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CHAPTER III

DOUBLE-STRANDED RNA AND TOTAL PROTEIN ANALYSIS OF WHEAT SPOT MOSAIC-AFFECTED WHEAT

INTRODUCTION

The characteristics of wheat spot mosaic (WSpM) are such that it was first termed as a viral disease by Slykhuis (1956). A similar disease was isolated from wheat in Ohio, however, because mechanical inoculation was unsuccessful and the morphology and chemical composition of the pathogen were unknown, it was believed that the relationship and the viral nature of the cause should remain open (Nault *et al.* 1970). Ultrastructural examination of WSpM-affected tissue has thus far not revealed typical virus particles although Bradfute *et al.* (1970) observed ovoid, double membrane bodies (DMBs) (100-200 nm) in the cytoplasm of parenchyma, phloem, and epidermal cells. The DMBs have also been found in other eriophyid mite transmitted disorders such as fig mosaic (Bradfute *et al.* 1970; Plavsic and Milicic 1980; Appiano 1982; Appiano *et al.* 1986; Lovisolo *et al.* 1986), rose rosette in Arkansas (Gergerich *et al.* 1983) and pigeon pea sterility (Hiruki, C., personal communication).

Previously, a positive diagnosis of a viral pathogen could only be made if the virus particles were observed with the use of an electron microscope. However, recent advances in viral diagnosis, such as serology and nucleic acid hybridization, are proving to be more rapid and sensitive techniques. In addition, the analysis for double stranded (ds) RNA is rapidly becoming a very useful plant virus diagnostic technique since its development by Morris and Dodds (1979). Since dsRNA is not

normally found in healthy plants and over 90% of all known plant viruses are ssRNA viruses, the presence of dsRNA in plant tissue indicates the presence of the replicative form of a single-stranded (ss) RNA virus (Valverde 1990; Jordan and Dodds 1985; Dodds *et al.* 1984; Bar-Joseph *et al.* 1983).

Because of the viral-like nature of the WSpM disease, we attempted to extract dsRNA from WSpM-affected wheat and barley. Since virus infected plants often contain a number of virus specific proteins in addition to the coat protein, total protein analysis of WSpM-affected leaves was carried out to determine if any WSpM-specific proteins could be detected.

MATERIALS AND METHODS

1. dsRNA Analysis

a) Extraction

Leaf and root tissue from wheat spot mosaic affected wheat or barley was analyzed for the presence of dsRNA using a modified method of Morris and Dodds (1979). The cultivar, infection age, and amount of tissue analyzed is given in Tables 1 and 2. In addition to these samples, tissue of similar age and cultivar was also processed from healthy mite-free plants and mite-infested plants which were not infected with the WSpM agent. Barley infected with brome mosaic virus (BMV) was used as as positive controls. The BMV-infected plants were harvested 2 days after inoculation (9 days after seeding).

The following procedure was used for 10 g of tissue which was ground to a fine powder in liquid nitrogen using a mortar and pestle. An extraction medium was added to each sample which consisted of 10 ml 2xSTE buffer (STE: 50 mM tris 0.1

mM Na_2 EDTA, 100 mM NaCl, pH 8.0), 10 ml Tris saturated phenol (containing 0.1% 8-hydroxyquinoline), 2 ml 10% sodium dodecyl sulfate, and 5 ml chloroform:isoamylalcohol (24:1). When more or less tissue was analyzed, the volume of the reagents were adjusted by maintaining the same weight/volume ratio. The sample was stirred at room temperature for 1 hour and then centrifuged at 10,000*g* for 10 minutes in an SS 34 rotor using a Sorvall RC5B centrifuge. The supernatant was removed and adjusted with 95% ethanol to a final concentration of 15%. Cellex N1 (Bio Rad Laboratories, Richmond, CA) was added to the sample at a rate of 0.25 g/25 ml and the solution stirred slowly at 4°C overnight. After centrifugation at 10,000*g* for 10 minutes, the supernatant was decanted and 10 ml of 1xSTE buffer : 15% ethanol was added to the pellet. The pellet was resuspended and washed by centrifugation three more times. After washing, the pelleted material was again resuspended in 5 ml 1xSTE buffer : 15% ethanol and packed into a column prepared from a 5 ml pipet tip and a plug of glass wool. The column was washed with 50 ml of 1xSTE buffer : 15% ethanol and then eluted with 4 ml of 1x STE buffer. The elutant was collected and any contaminating DNA was digested by adding 5 µl of DNase and 40 µl of 3M $MgCl_2$ to each sample. This was then divided into eight 1.5 ml micro-centrifuge tubes (0.5 ml each) and incubated at 37°C for 30 minutes. After adding 50 µl of 3M NaOAc to each tube and then filling them with cold ethanol, the tubes were placed in a -20°C freezer overnight. The dsRNA was pelleted by centrifugiation for 10 minutes at 10,000*g* and the supernatant decanted before adding 400 µl of cold 70% ethanol to each tube. The content of the tubes were centrifuged once again at 10,000*g* for 5 minutes, the ethanol decanted, and the pellets dried in a Savant SVC 100 H SpeedVac Concentrator under vacuum for approximately 20 minutes. After drying, the pellets were carefully resuspended

in a total volume of 20 µl of distilled water. The samples were either electrophoresed immediately or stored in a -70°C freezer until use.

b) Gel Electrophoresis

The majority of the samples were analyzed under non-denaturing conditions on a 1% agarose gel using 0.3 g agarose (BRL, Gaithersburg, MD) in 30 µl 1xTAE buffer (50x stock solution = 242 g Tris base, 57.1 ml glacial acetic acid, 37.2 g $Na_2EDTA{\cdot}2H_2O$, pH 8.5) to which 3 µl of ethidium bromide was added. The gels were prepared in a mini gel apparatus (Tyler Research Instruments, Edmonton, Canada) and electrophoresed for 2 or 4 hours at 25 V in 1xTAE buffer. In the case of the WSpM-affected samples the entire volume from the resuspension step (20 µl) was loaded onto the gel along with 3 µl of 0.5% Bromophenol blue (BPB). The samples extracted from the BMV-infected material were divided into four 5 µl aliquots, and one aliquot, along with 3 µl of BPB, was electrophoresed per run. After electrophoresis the bands were visualized using a Spectroline Model TR-302 transilluminator and photographed with a polaroid through Kodak Nos. 23A and 2E Wratten gelatin filters.

For increased sensitivity, a number of samples were electrophoresed on 6% polyacrylamide slab gels (120 mm x 120 mm x 1 mm) run at 50 V for 12 hours at room temperature. The dsRNA was visualized with silver stain.

2. Protein Analysis

a) Extraction

Protein analysis of leaf tissue was carried out using 0.1 g of fresh or frozen leaves. The variety and infection age of the plants used in this experiment are explained in the legend of Figure 3. Portions of mature, fully expanded leaves with typical symptom expression were ground to a fine powder with liquid nitrogen in a mortar and pestle. To this, 500 µl of sample buffer (0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 2.5% 2-mercaptoethanol and 0.002% bromophenol blue) was added and the mixture transferred to a 1.5 ml micro-centrifuge tube. The proteins were dissociated by placing the tubes in boiling water for 3 minutes. The tubes were then centrifuged for 5 minutes at 8000*g* in a Beckman Microfuge 12 to remove insoluble materials.

b) Gel Electrophoresis

Polyacrylamide gel electrophoresis of protein samples was performed as described by Laemmli (1970) using 7.5 - 20% slab gels (120 mm x 120 mm x 1 mm) with 3% stacking gels. Thirty µl of each sample was loaded onto the gel being careful not to disturb the sediment in the bottom of the tubes. Electrophoresis was carried out at 50 V in a Bio-Rad Model 220 electrophoresis unit. The gels were stained for 30 minutes with Coomassie Brilliant Blue (0.25% w/v) in a solution containing 50% (v/v) methanol and 10% (v/v) acetic acid and destained in a 10% (v/v) methanol, 10% (v/v) acetic acid solution.

RESULTS AND DISCUSSION

1. dsRNA Analysis

Virus-specific dsRNAs were isolated from BMV-infected barley (Figure 1, Lane B). The molecular weights of the four dsRNAs respectively are; dsRNA 1 2.2 x 10^6, dsRNA 2 2.0 x 10^6, dsRNA 3 1.4 x 10^6 and dsRNA 4 0.6 x 10^6. This is in agreement with the reported molecular weights of single stranded (ss) RNA of BMV since they are approximately half the weight of dsRNA (Lane 1977). A fifth band with a molecular weight of 0.2 x 10^6 was also consistently isolated from the BMV-infected plants. These unexpected smaller dsRNA molecules have occured in extractions from other viruses (Dodds 1985) and may be an incomplete replicative form of the virus known as the replicative intermediate (Valverde 1990). No dsRNA bands could be detected in the leaves or roots of healthy barley (Figure 1, Lane A). The two large bands near the bottom of the gel (Figure 1, Lanes A and C) are ssRNA (probably tRNA) which was eluted with the dsRNA. These bands are commonly observed in RNA extractions for the detection of viroids (Morris and Wright 1975; Mosch *et al.* 1978).

In the case of the WSpM-affected plant material, only one extraction yielded specific bands after electrophoresis (data not shown). This was from 10 g of WSpM-affected TAi leaves, harvested 17 days after mite infestation. The molecular weight of the two bands was approximately 0.2 and 0.6 x 10^6 respectively. These results could not be repeated however, even by increasing the amount of starting material to 50 g or by using PAGE in combination with the sensitive silver staining technique. A typical agarose gel from a WSpM extraction is shown in Figure 2. The dsRNA from BMV is evident in Lane B whereas no bands can be observed from healthy (Lane A) or WSpM-affected wheat (Lane C). This is consistent with

initial studies of low molecular weight dsRNA analysis of WSpM-affected wheat (Ohki *et al.* 1985) and of a mite-transmitted, double membrane body (DMB) associated wheat disease in North Dakota (Edwards and McMullen 1988). Therefore, from the results of the dsRNA analysis, it appears that the WSpM-affected plants do not contain dsRNA or it may be present at a level which cannot be detected with the current techniques. Since some viral groups such as the luteoviruses and most potyviruses yield very low quantities of dsRNA (Valverde 1990), thus making detection difficult, this may be the case with WSpM. The amount of dsRNA obtained from plant viruses with elongated particles including the poty-, carla-, potex-, and tobamovirus groups can be affected by the incubation temperature of the host as well as the infection age of the harvested tissue (Valverde *et al.* 1986). This was the case with the tobamoviruses where highest dsRNA yields were obtained at 10 days after inoculation as opposed to 20 and 30 days. Also yields were highest from plants incubated at 27°C than those at 20 or 24°C. Since the temperature effect was not examined with the WSpM-affected material this area may require further attention.

Of the plant disorders associated with the eriophyid mites and the characteristic (DMBs), dsRNA has been isolated only from roses affected with rose rosette (Di *et al.* 1990). They consistently isolated four dsRNA bands of molecular weights 2.9, 1.2, 1.0, and 0.93 x 10^6 which were not obtained from healthy rose. However, their specific infectivities were not tested and it is not known what role the dsRNAs play in the disease syndrome.

Although dsRNA could be detected from rose rosette-affected material, the results of dsRNA analysis of WSpM-affected wheat and barley over a wide range of infection ages indicates that dsRNA is not associated with the WSpM disease. However, further studies of the other mite-transmitted, DMB-associated disorders

would be required to determine if dsRNA associated with rose rosette is an isolated case. The affected host may be a factor in this case.

2. Protein Analysis

Analysis by SDS-PAGE of total proteins from the leaves of brome mosaic virus (BMV) infected barley revealed the presence of a polypeptide with a molecular weight (M_r) of 20,000 (Figure 3, Lane H, I). This polypeptide is the coat protein of BMV and the M_r is in agreement with previously published data (Lane 1974; Lane 1977). The protein could not be detected in the healthy barley (Fig. 3, Lane F) or in barley which was analyzed 4 days after inoculation (Lane G). A quantitative difference can be observed in the protein 7 and 14 days post-inoculation (Fig. 3, Lane H and I respectively) indicating an accumulation of virus particles in the leaves. It is interesting to note that although coat protein could not be detected at 4 days post-inoculation, dsRNA from BMV could be detected as early as 2 days after inoculation. Alfalfa mosaic virus was detected in a similar manner using partially purified extracts and analyzing for the presence of coat proteins (Parent and Asselin 1984).

In the case of the WSpM-affected plants (Figure 3, Lanes C, J and L), no differences in the polypeptides obtained from the healthy (Lanes A and N) or healthy mite infested plants (Lanes B and M) could be observed. This was also the case with wheat with a mixed infection of WSMV and WSpM (Fig. 3 Lane K). Analysis of the WSMV infected leaf 14 days after inoculation revealed a minor band of a 66,000 dalton protein (Fig. 3 Lane E). This protein was also observed by White and Brakke (1983) who found that the quantity of this protein was affected by the age of the leaf, symptom severity, and whether the virus infection occured by systemic invasion or as a result of direct inoculation. This 66 kDa protein is the

cytoplasmic inclusion protein which forms the characteristic 'pinwheel' structure in the cytoplasm of cells infected with potyviruses (Shukla *et al.* 1991). The coat protein of WSMV could not be detected as in the case of BMV since it is a 45 kDa protein (Brakke *et al.* 1990) and migrates with the large subunit of ribulose diphosphate carboxylase (White and Brakke 1983).

Although no differences in polypeptides could be detected from the total protein extracts of WSpM-affected and healthy wheat, WSpM-specific proteins may have been masked by the migrating host proteins which has been demonstrated with WSMV. Further protein analysis of WSpM-affected plants could be carried out using two-dimensional gel electrophoresis in conjunction with partially purified extracts.

Table III-1

Double stranded RNA analyses of WSpM-affected wheat

Extraction number	Cultivar	weight of leaves (g)	Days after mite infestation	Gel*
1.	TAi	10	7	A
2.	TAi	10	10	A
3.	TAi	10 (roots)	12	A
4.	TAi	10	17	A
5.	TAi	10	17	A
6.	TAi	50	17	P
7.	Thatcher	10	14	A
8.	Thatcher	10	14	P
9.	Thatcher	10	21	A
10.	TAi	10	28	A
11.	HY320	10	35	A
12.	HY320	10	35	A
13.	HY320	10	35	A
14.	HY320	10	35	A
15.	HY320	10	35	A

Table III-2

Double stranded RNA analyses of WSpM-affected barley

Extraction number	Cultivar	weight of leaves (g)	Days after mite infestation	Gel*
1.	Leduc	10	3	A
2.	Leduc	10	10	A
3.	Gateway	10	12	P
4.	Leduc	10	14	P

*A-Agarose
P-Polyacrylamide

Figure III-1. Ethidium bromide stained 1% agarose gel electrophoresed for 2 hrs with samples from double stranded RNA analysis of:

Lane A - Healthy barley and Lane C - BMV-infected barley 4 days after inoculation.

Gel was loaded with one-fourth of sample obtained from 10 g of tissue.
Lane B - Hind III digested lambda DNA.

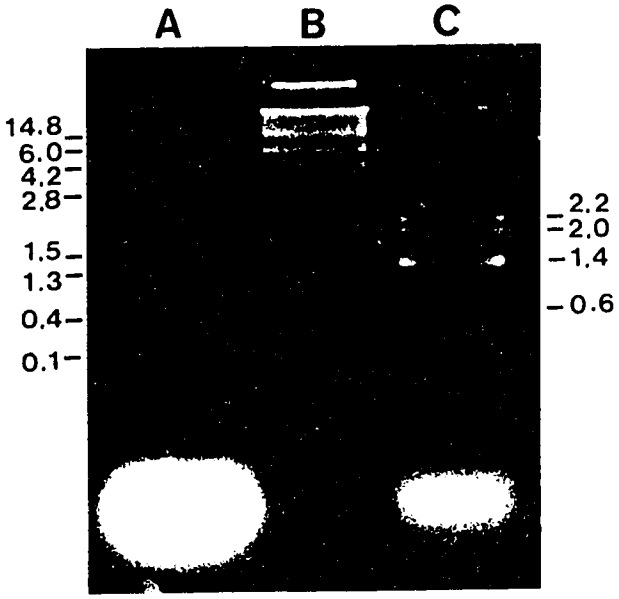

A
B
C
14.8
6.0
4.2
2.8
1.5
1.3
0.4
0.1
2.2
2.0
1.4
0.6

Figure III-2. Ethidium bromide stained 1% agarose gel electrophoresed for 4 hrs with samples from double stranded RNA analysis of:

Lane A - Healthy wheat.
Lane B - BMV-infected barley.
Lane C - Wheat spot mosaic-affected wheat.

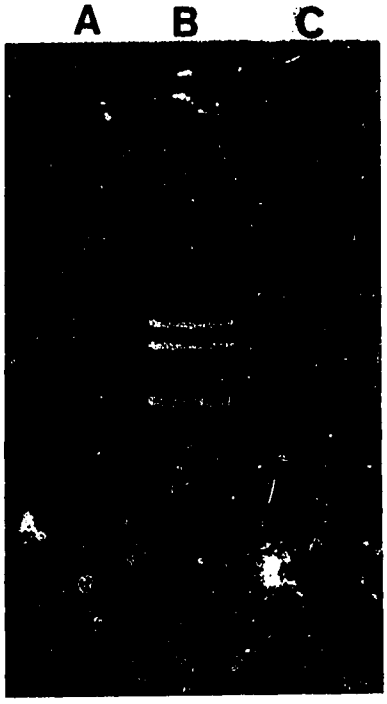
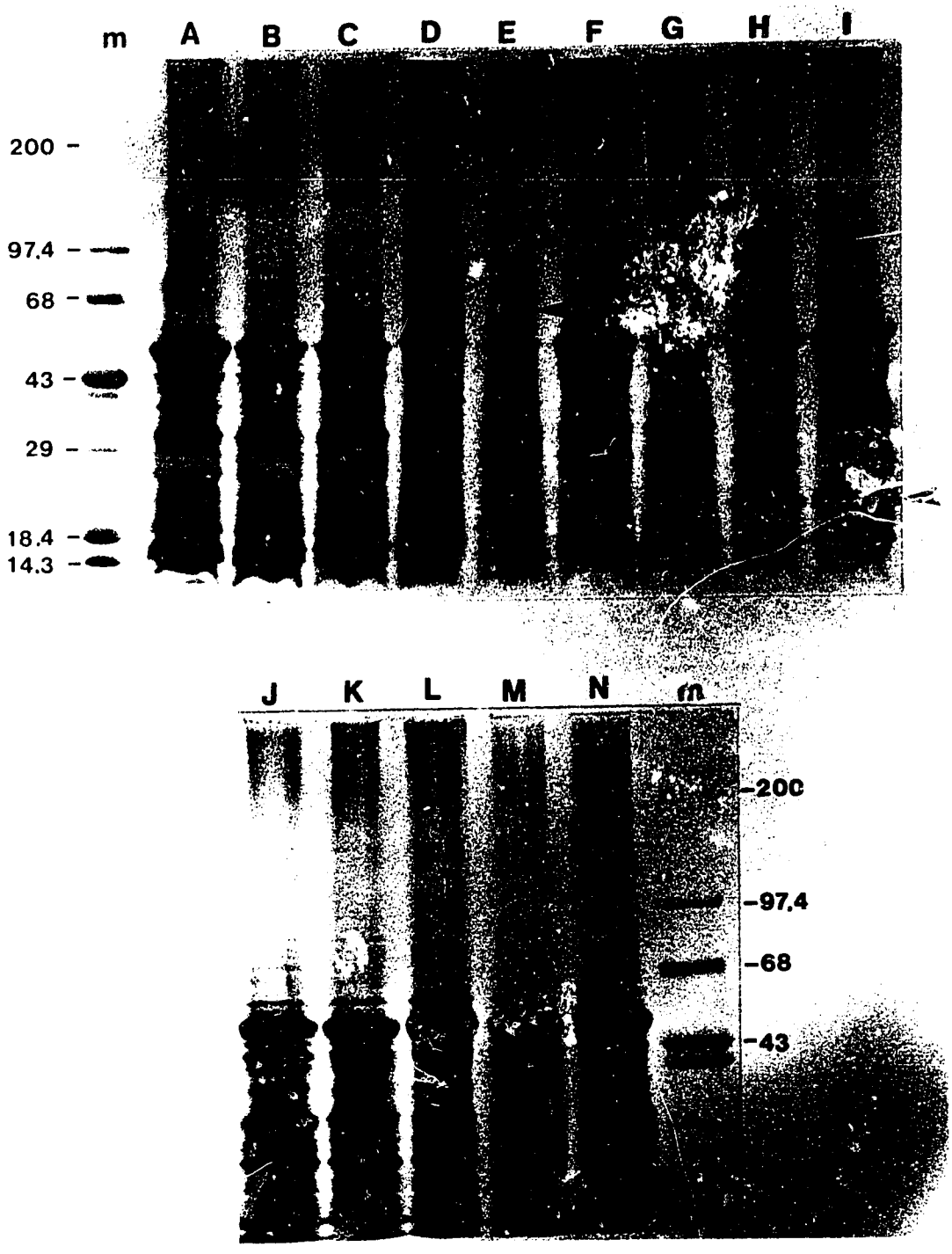

A
B
C

Figure III-3. SDS-PAGE of total leaf proteins on 7.5-20% gradient slab gels stained with Coomassie blue. Arrowhead in lane E denotes 66 kDa cytoplasmic inclusion protein of WSMV and arrowhead in lane H and I denotes 20 kDa coat protein of BMV. m - protein molecular weight standards

Lane	Cultivar	Age	Condition*	Days after inoculation/infestation
A	Conway wheat	21	H	---
B	Conway wheat	21	HM	14
C	Conway wheat	14	WSp	7
D	freeze-dried leaf	---	WS	---
E	Park wheat	21	WS	14
F	Galt barley	16	H	---
G	Galt barley	16	B	4
H	Galt barley	16	B	7
I	Galt barley	16	B	14
J	Conway wheat	22	WSp	14
K	Park wheat	28	WS/WSp	21
L	Park wheat	28	WSp	21
M	Conway wheat	21	HM	14
N	Conway wheat	21	H	---

*H - healthy
HM - infested with disease free mites
WSp - infested with mites and expressing wheat spot mosaic symptoms
WS - mechanically inoculated with wheat streak mosaic virus
B - mechanically inoculated with brome mosaic virus


m
A
B
C
D
E
F
G
H
I
200
97.4
68
43
29
18.4
14.3
J
K
L
M
N
m
200
97.4
68
43

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CHAPTER IV

DAPI STAINING AND PROTEIN-GOLD LABELLING OF WSPM-AFFECTED WHEAT

INTRODUCTION

Examination of leaf ultrastructure of wheat spot mosaic-affected wheat by electron microscopy revealed the presence of ovoid, double membrane bodies (DMBs) (0.1-0.2 μm) in the cytoplasm of phloem, parenchyma, and epidermal cells (Bradfute et al 1970). The membranes were more rigid and had less space between them than the double membranes bounding mitochondria, plastids, nuclei, and some bacteria. Leaves of *Ficus carica* systemically infected with a disease known as fig mosaic also contained the DMBs. Transmission of this disease agent is by the eriophyid mite, *Eriophves ficus*. The DMBs have also been reported associated with fig mosaic in Italy (Plavsic and Millicic 1930; Appiano 1982; Appiano et al 1986; and Lovisolo et al 1986), rose rosette in Arkansas (Gergerich et al 1983), pigeon pea sterility (Hiruki, C., personal communication) and with an unknown wheat disease in Eastern North Dakota (Edwards and McMullen 1988). In wheat spot mosaic affected wheat, the DMBs have been found in young roots as well as the leaves. The area surrounded by the double membrane is relatively electron dense and consists of dispersed fibrils. Although these double membrane structures are quite different from bacteria, mycoplasma-like organisms or typical virus particles, it has been suggested that they may contain nucleic acid (Bradfute et al 1970). In the case of rose rosette which is also trasmitted by an eriophyid mite (*Phyllocoptes fructiphilus* Keifer), the DMBs (120-150 μm) referred to as viral-like particles, were

found in the cytoplasm of parenchyma, phloem, and epidermal cells and were reported to be the causal agent (Gergerich and Kim 1983).

Since the role of these double membrane structures as a disease causing agent is not well understood, they were probed for the presence of nucleic acid. This was carried out in two separate studies. The first was a light microscopy study using the DNA specific stain 4',6-diamidino-2-phenylindole·2HCl (DAPI). This fluorescent stain, since its introduction in 1975 (Williamson and Fennell) has had numerous applications including its use to detect mycoplasmalike organisms in phloem elements of plants (da Rocha 1985). The second study was an electron microscopical examination of the double membrane structures with DNA and RNA specific colloidal gold probes.

MATERIALS AND METHODS

1. DAPI Staining

Specimens were collected from healthy wheat, mite infested wheat, and mite infested wheat expressing WSpM symptoms at 14 days after mite infestation and 21 days after seeding. All wheat used in this experiment was the cultivar TAi which is resistant to WSMV. Segments 3 mm in length were excised from the young curled leaves of the mite-infested plants and from the areas of severe chlorotic spotting in the case of the WSpM-affected plants. Portions of the older roots near the base of the plant as well as stem sections from approximately 1 cm above the crown were also collected and fixed for 2 hours in 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB) (pH 7.4).

Sections approximately 20 µm thick were cut using a Minotome microtome

(Damon/IEC Division, Needham MA). The sections were collected on a glass slide and stained with a 2 mg/ml solution of DAPI in an equal volume of glycerol. The specimens were then examined according to da Rocha (1985) with a Zeiss Universal fluorescence microscope (HBO 200) with barrier filter 50 and exciter filters BG 12 and BG 3.

As a control, sections of young petioles from healthy *Catharanthus roseus* (L.) G. Don and *C. roseus* infected with the potato witches broom isolate of a mycoplasma-like organism were processed and examined as above.

2. Protein-gold Labelling

Tissue Processing

Samples from young leaves and roots of WSpM-affected wheat and leaves from MLO-infected *C. roseus* were collected as outlined above and fixed for 1 hour in 1% glutaraldehyde in 0.1 M PB, pH 7.4. The samples were rinsed with buffer then postfixed in 1% OsO_4 in 0.1 M PB, pH 7.4, for 3 hours. Following two rinses with distilled water, the samples were either embedded in low-viscosity Spurr's resin or the hydrophilic LR White resin.

Spurr's

The samples were dehydrated in a 70-100% ethanol series and then infiltrated with a 1:1 ethanol and Spurr's solution for 30 minutes. This was followed by a 1:2 ethanol and Spurr's solution and two changes of 100% Spurr's for 1 hour each. The specimens were placed in a JBS No. 306 flat embedding mold (J.B. EM, Inc., Montreal) and the resin was polymerized at 70°C for 12 hours.

LR White

Tissue samples were infiltrated with LR White in a solution of 2:1 LR White and 70% ethanol and were gently shaken on a Lab-Line orbit shaker for 24 hours at room temperature. The specimens were rinsed with 100% LR White and were left on the shaker for another 24 hours in 100% LR White. The specimens were then embedded using the flat mold technique described by Simpson-Gomes and Simon (1989). The tissue was placed into either a No. 52090 Effa disposable transparent flat mold or a Beem No. 52180 flat mold (Ernest F. Fullam, Inc., Latham, NY). After filling the molds with resin, a small piece of Saran Wrap which was precut to the size of the mold, was carefully laid overtop to exclude any air bubbles. Catalyst was not added to the resin, therefore it was heat-cured at 45°C for 48 hours.

Gold Probe Preparation

Lactoferrin-gold complex

The lactoferrin-gold (LG) complex was prepared as outlined by Benhamou (1989). Unconjugated SPI-Mark colloidal gold with a mean particle diameter of 10 nm was purchased from SPI Supplies (Toronto, Ontario). Purified lactoferrin from bovine colostrum was obtained from Sigma Chemical Co. (St. Louis, MO).

To prepare the LG complex 250 µg of lactoferrin dissolved in 100 µl of distilled water was mixed with 10 ml of colloidal gold adjusted to pH 9.2 with 0.2 M K_2CO_3. Five drops of 1% aqueous polyethylene glycol 20,000 (PEG) was added to stabilize the gold. The solution was centrifuged at 10,000 *g* for 60 minutes at 4°C in a Sorval RC5B centrifuge with an SS34 rotor. The supernatant was carefully removed without disturbing the loosely

packed dark-red pellet. The pellet was resuspended in 0.5 ml of 0.01 M phosphate-buffered saline (PBS) and stored at 4°C until use.

RNase-gold complex

The preparation of this complex was similar to the LG complex with the exception that the appropriate pH of the colloidal gold and the quantity of RNase A (Sigma Chemical Co., St. Louis, MO) was determined by Bendayan (1981a,b) to be 1.0 and 0.5 mg respectively. After centrifugation, the pellet was resuspended in 3 ml of PBS and stored at 4°C until use.

Labelling

Ultrathin sections were obtained using a Reichert OMU2 ultramicrotome and were collected on 300 mesh nickel grids. The grids were previously dipped in 0.03% Formvar in chloroform to increase section adhesion.

The following procedure was carried out using a Falcon 30340 microtest plate (Falcon Plastics, Oxnard, CA) with 25 µl drops of the appropriate solution in each well and 1-2 ml of water around the perimeter of the plate to maintain humidity (Figure 1).

The grids were first floated section side down for 5 minutes on a drop of PBS-PEG. They were then transferred either to undiluted RNase-gold complex or to the LG complex diluted 1:8 in PBS-PEG, pH 7.4. The grids were incubated for 30 minutes at 37°C, transferred to a drop of PBS and then jet washed with PBS for 2 minutes/grid. After rinsing with distilled water the grids were dried and double stained with uranyl acetate and lead citrate in the staining device of Chen (1973).

The grids with the WSpM-affected specimens embedded in Spurr's resin,

were treated with a saturated aqueous solution of sodium metaperiodate for 2 hours prior to labelling as described by Bendayan and Puvion (1984) for osmicated tissue. The specimens embedded in LR White were not pretreated.

Controls

In order to assess the specificity of the probe, a number of controls were used which included obtaining and treating specimens in the manner described above but incubating them in unconjugated gold. As an additional test for the specificity of the LG probe, pure calf thymus DNA (2 mg dissolved in 200 µl of distilled water) was spotted onto Formvar coated grids and incubated as above in either conjugated or unconjugated gold. To further test the specificity of the conjugated probes, sections were cut from blocks of *Spiroplasma citri* which had been embedded in Spurr's since 1977.

RESULTS

1. DAPI Staining

Examination of the sections of healthy *C. roseus* which were stained with the fluorescent dye DAPI, revealed no significant areas of fluorescence (Figure 2). Autofluorescence of the xylem was observed as well as faint fluorescence from the other cell walls. The small fluorescent spots within many of the cells is a result of the dye binding to the DNA within the nuclei, chloroplasts and mitochondria. However, the phloem elements of the sections of MLO infected *C. roseus* fluoresced brightly indicating the presence of MLO's (Figure 3).

Fluorescence was occasionally observed in the vascular bundles of both healthy and WSpM-affected wheat which was stained in DAPI (Figures 4,5). Brightly fluorescing phloem cells could be detected whereas abnormal fluorescence was not observed in other regions of the tissue examined.

2. Protein-gold Probes

RNase

The RNase gold probe was found not to be specific enough to be a reliable RNA probe in this case. Binding of the colloidal gold to the specimens was often very sparse with a significant amount of non-specificity. Attempts at varying the treatment time in sodium metaperiodate, increasing incubation time, or decreasing the amount of jet washing all failed to increase the probe binding specificity.

Lactoferrin

Examination of the grids spotted with calf thymus DNA and incubated in unconjugated colloidal gold revealed an almost random distribution of non-

specifically bound gold particles (Figure 6). The LG complex, however, bound specifically to the DNA as seen in Figure 7. The electron dense areas on the grid, indicating the presence of the purified DNA, were heavily labelled whereas the electron luscent areas had very few gold particles and thus a low level of non-specific binding.

A similar result was observed when thin sections of MLO-infected tissue were incubated in the colloidal gold. Figure 8 illustrates the random distribution of gold particles over the MLOs, cytoplasm, and cell wall. Incubation of the MLOs in LG complex however, resulted in specific labelling over fibrillar areas within them while the cytoplasm and cell wall were free of gold particles (Figure 9). Intense labelling is also noted over the spiroplasmas with virtually no gold particles bound to the electron luscent resin surrounding the specimens (Figure 10).

The nuclei and chloroplasts of the plant tissue embedded in Spurr's resin were also labelled with numerous gold particles (Figures 11, 12). The nucleolus and dense chromatin were heavily labelled whereas the nucleoplasm, nuclear membrane, and cytoplasm were free of gold particles. Limited non-specific binding occured on the cell wall.

The use of LR White resin increased the intensity of labelling on the cell organelles as indicated by the heavy labelling on the chloroplasts (Figure 13). Very few gold particles were found on the cell walls or the cytoplasm immediately surrounding the labelled organelles.

Sections of roots and leaves from WSpM-affected plants showed a similar labelling pattern on the DNA-containing organelles, however, of all the sections examined, none of the double membrane bodies were labelled with gold particles. The specimens embedded in Spurr's resin and treated with sodium metaperiodate were labelled on the dense chromatin within the nucleus but the double membrane

bodies were free of gold (Figure 14). A similar labelling pattern was observed with the LR White embedded tissue, however, the labelling intensity was greatly improved as noted by the number of particles over the chloroplast and nucleus (Figure 15).

DISCUSSION

DAPI Staining

The positive fluorescence in the phloem of the MLO-infected *C. roseus* is in agreement with previous reports on the ability of DAPI to label the MLO DNA and thus allow for identification (Seemuller 1976; da Rocha 1985)

The observation of fluorescence in the phloem elements of the wheat indicates the presence of DNA. Similiar fluorescence has been observed in MLO-infected monocots in the case of lethal diseased coconut palms (Schuiling , 1981; Deutsch and Nienhaus 1983). Positive fluorescence of this nature has also been observed in healthy young actively developing phloem cells (Hiruki, C. personal communication). Examination of these areas by transmission electron microscopy revealed numerous mitochondria. Since mitochondria are adenine-thymine rich, they preferentially bind DAPI, thus forming a highly specific fluorescent complex (Williamson and Fennell 1975). Also, the fact that fluorescence was not observed in other regions of the tissue where DMBs are commonly observed by TEM, indicates that the fluorescence is not due to the presence of the DMBs.

Protein-gold Labelling

The results of the protein-gold labelling indicate that the LG complex has a high affinity for DNA. This is in agreement with previously published results (Berhamou 1989).

Therefore, to provide the optimum conditions for ultrastructural localization of DNA on the WSpM-affected tissues, two different treatments were utilized. The use of the oxidizing agent sodium metaperiodate to remove osmium from osmicated tissues has been demonstrated to yield immunolabellings of high intensity and specificity over well preserved organelles (Bendayan and Zollinger 1983; Bendayan and Puvion 1984). LR White resin has recently become established in cyto- and immunocytochemistry as an important embedding medium for colloidal gold labelling since it has a number of advantages over other resins (Herman 1989; Newman 1989).

Although the gold particles could be observed specifically bound to organelles which contained DNA, the DMBs were consistently free of labelling. This was the case even though the specificity and binding ability of the colloidal gold was improved by pretreating the sections in sodium metaperiodate or by embedding the specimens in LR White resin.

Therefore, the results using the LG probe indicate that the DMBs associated with WSpM do not contain DNA. Colloidal gold conjugated with antibodies specific to various cellular components such as ER may be valuable as a probe in further studies to determine the characteristics of the membrane and the internal contents of the DMBs.

Figure IV-1. Microtest plate used for labelling sections on electron microscope grids (g) with protein-gold complexes (pg).

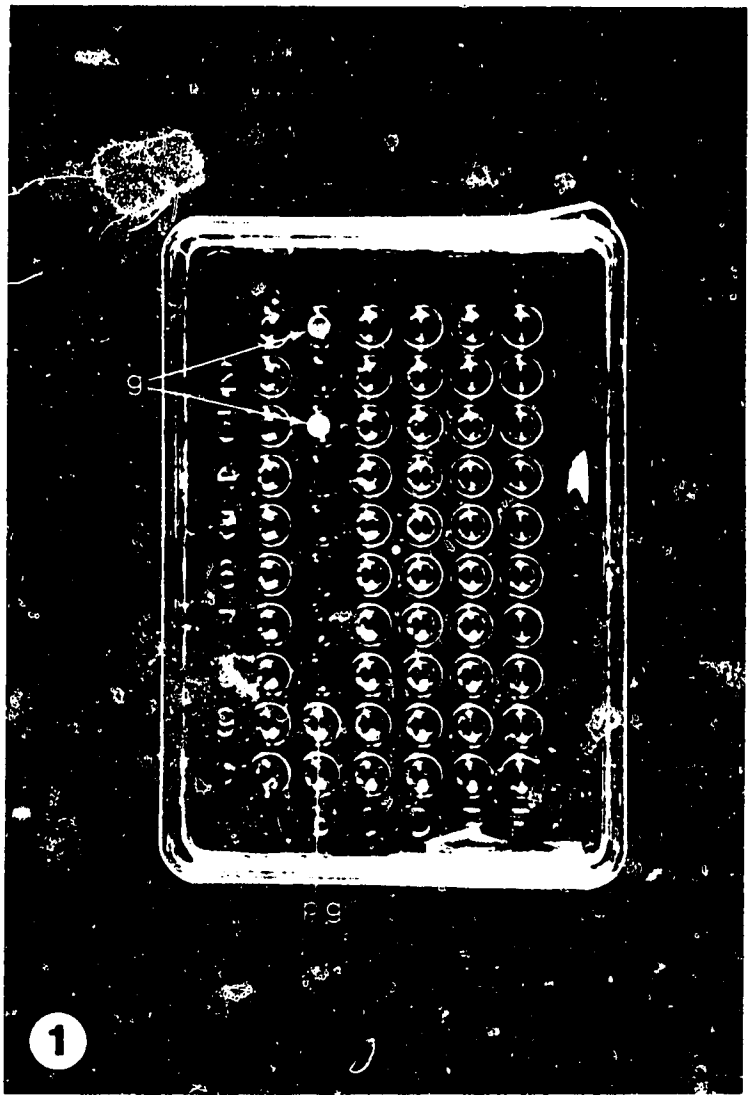

g
g
1

Figure IV-2. Cross section of glutaraldehyde fixed healthy *Catharanthus roseus* petioles stained with DAPI.
Bar = 0.2 mm.

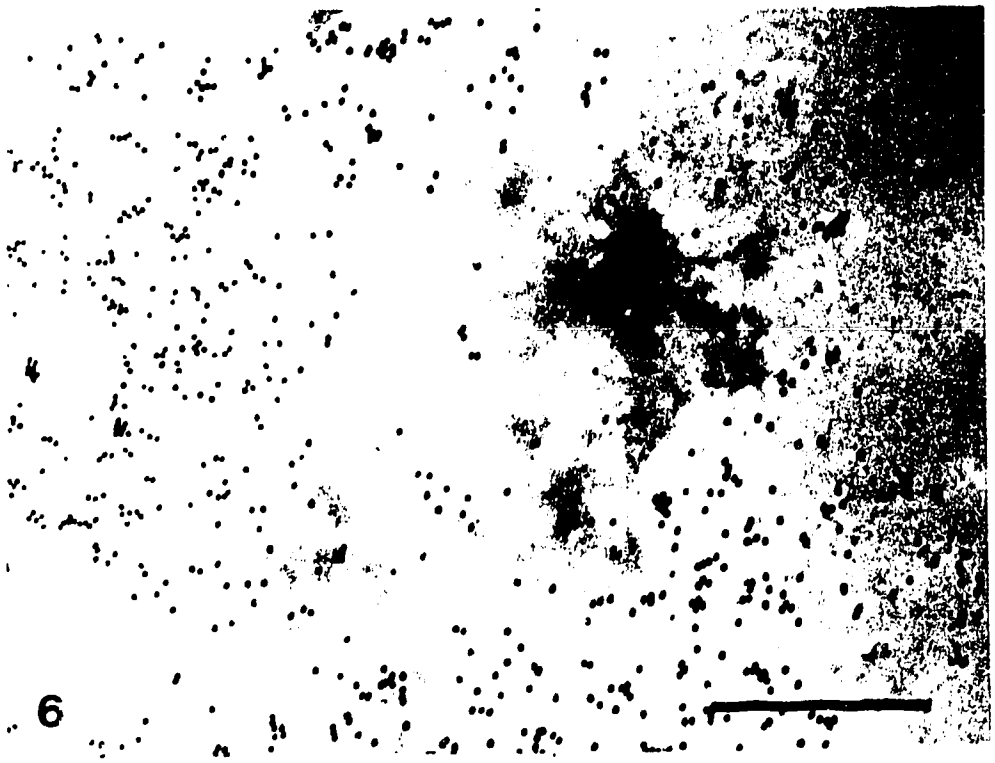
Figure IV-3. DAPI fluorescence in phloem of MLO-infected *C. roseus* (arrowheads).
Bar = 0.2 mm.

Figure IV-4. DAPI fluorescence (arrowheads) in cross section of leaf from healthy wheat.
Bar = 0.1 mm.

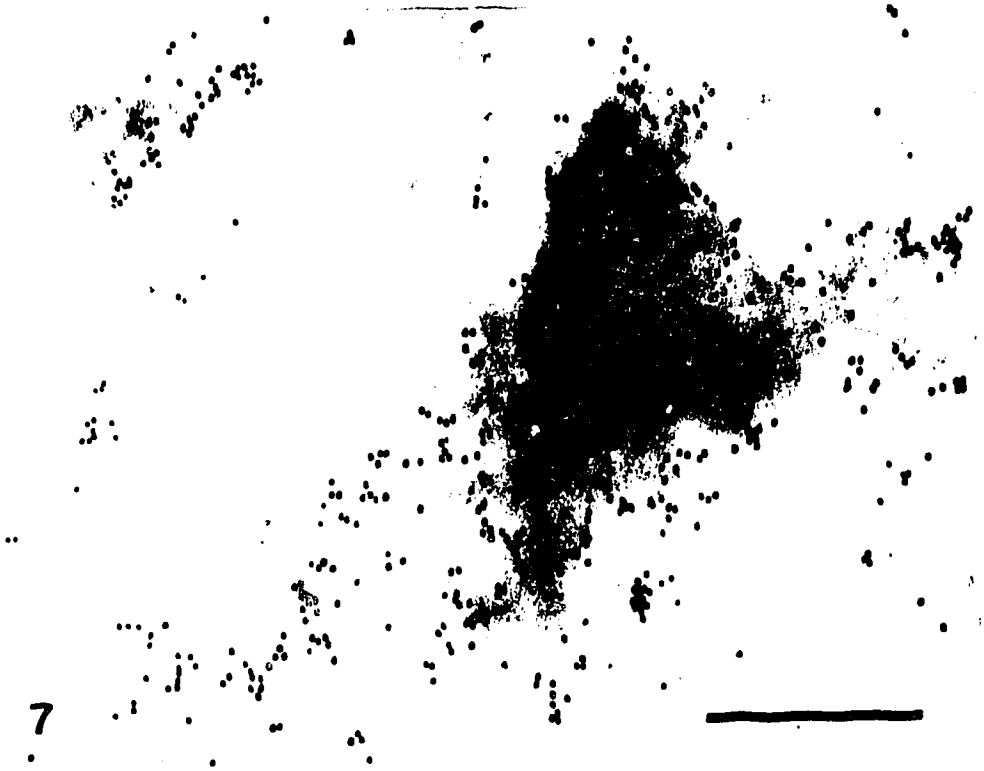
Figure IV-5. Cross section of vascular bundle from WSpM-affected wheat. Note DAPI fluorescence in phloem (arrowheads).
Bar = 0.1 mm.

Figure IV-6. Transmission electron micrograph (TEM) of calf thymus DNA incubated in unconjugated colloidal gold.
Bar = 0.5 μm.

Figure IV-7. TEM of calf thymus DNA incubated in colloidal gold conjugated with the DNA-specific protein, lactoferrin.
Bar = 0.5 μm.



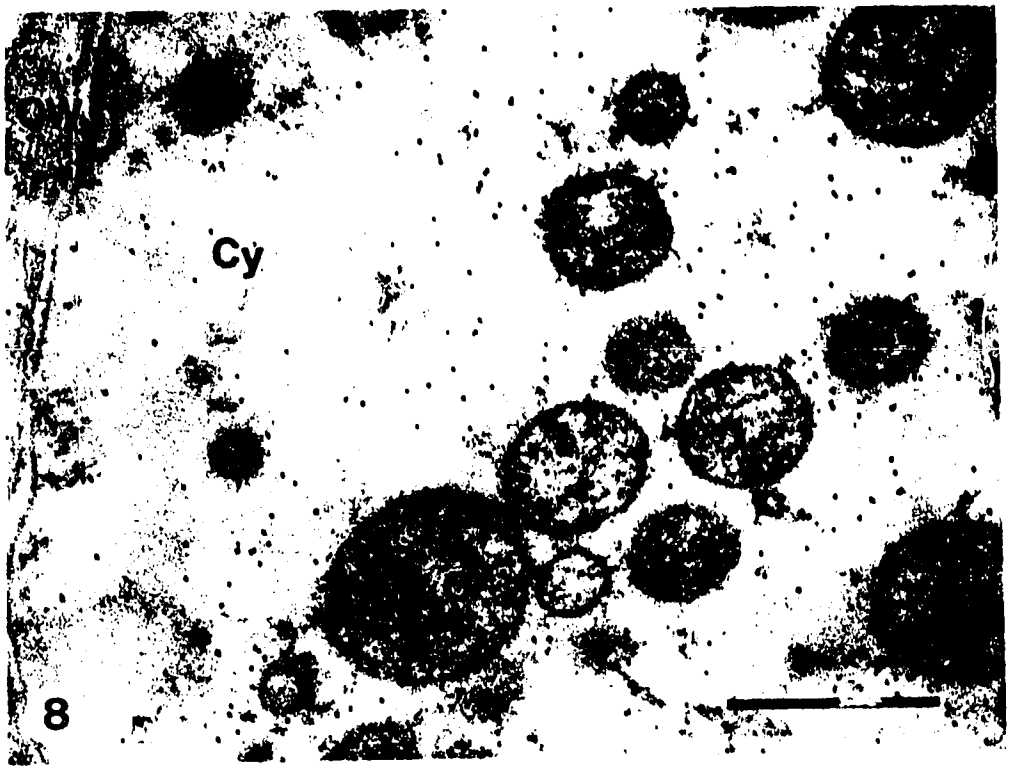
6



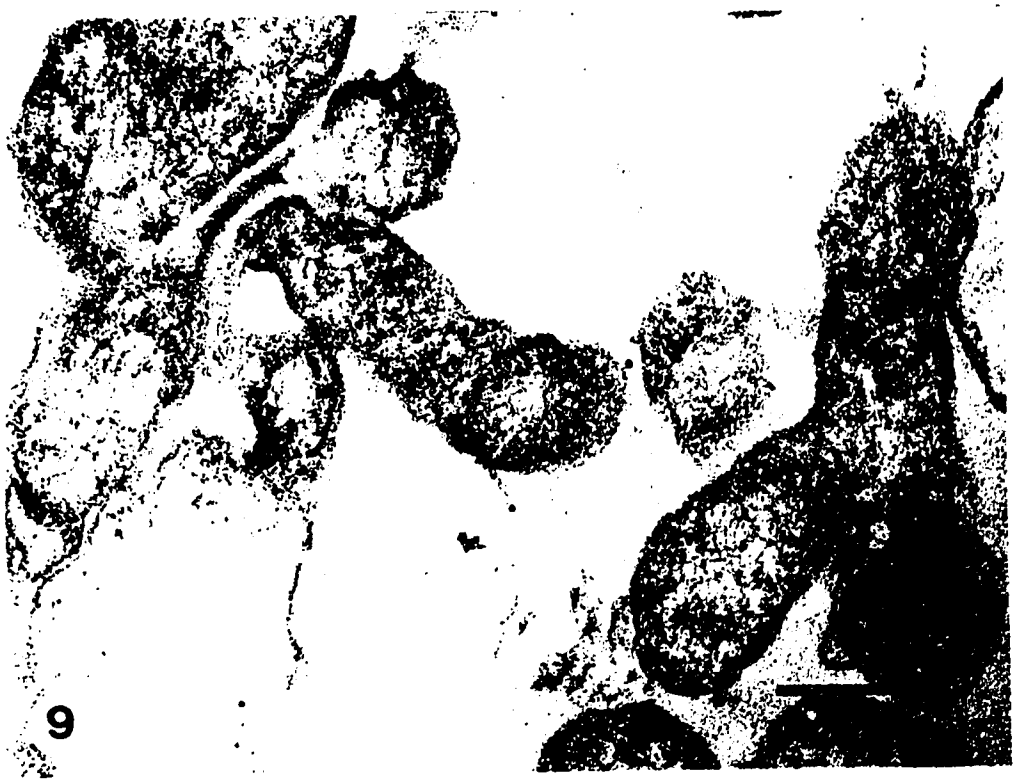
7

Figure IV-8. Transmission electron micrograph (TEM) of MLO infected *C. roseus* incubated in unconjugated colloidal gold. Note random distribution of gold particles over cell wall (CW), cytoplasm (Cy), and MLOs.
Bar = 0.5 μm.

Figure IV-9. TEM of MLO-infected *C. roseus* incubated in the DNA-specific lactoferrin-gold complex.
Bar = 0.2 μm.

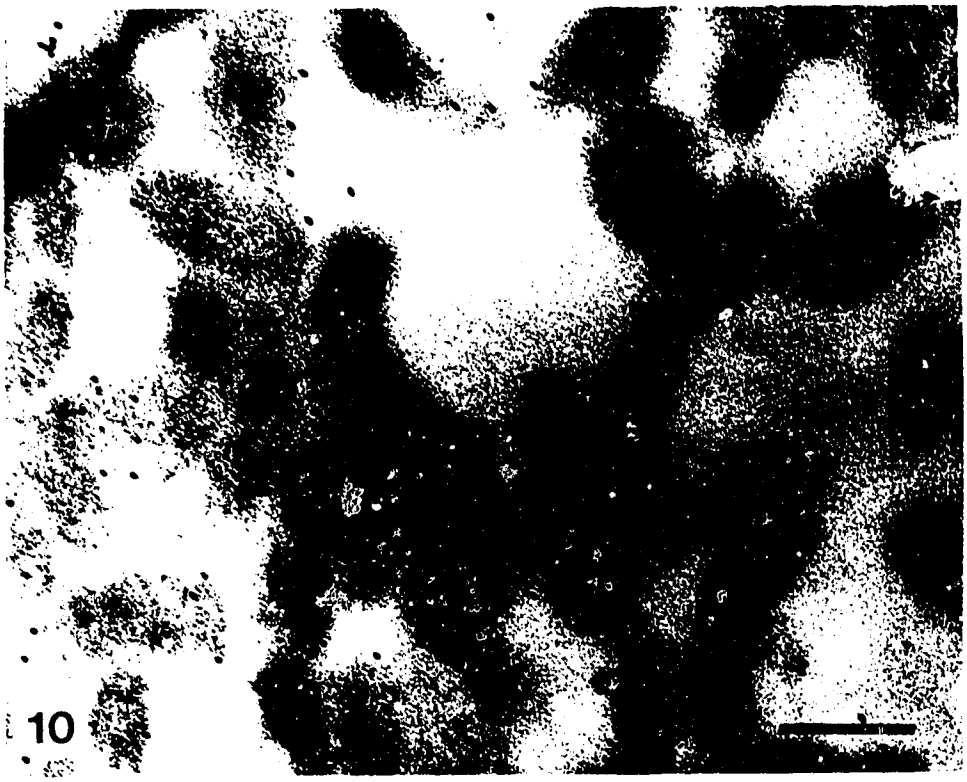


8



9

Figure IV-10. Transmission electron micrograph of an ultrathin section through a pure culture of spiroplasmas labelled with lactoferrin-gold complex.
Bar = 0.2 μm.

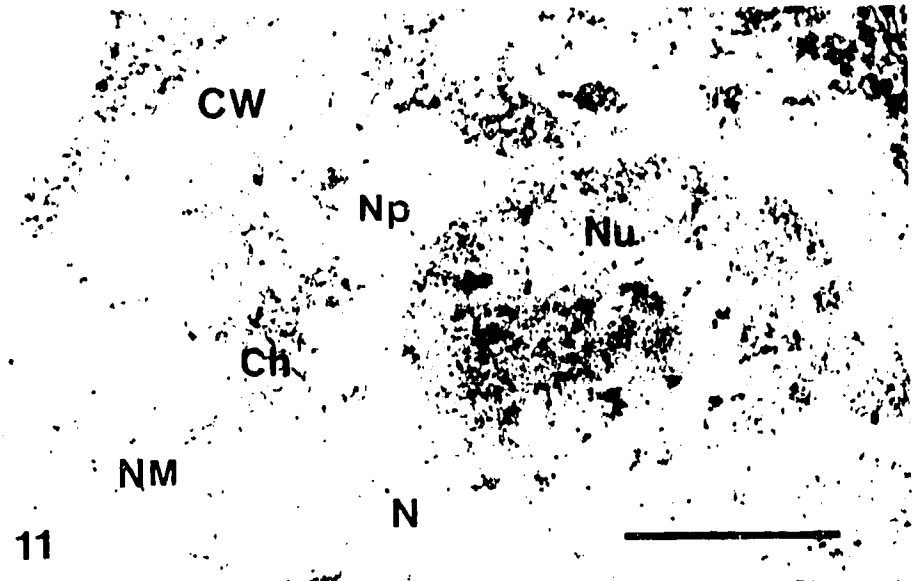

10

Figures IV-11-13. Transmission electron micrographs of DNA-containing organelles labelled with lactoferrin-gold complex.

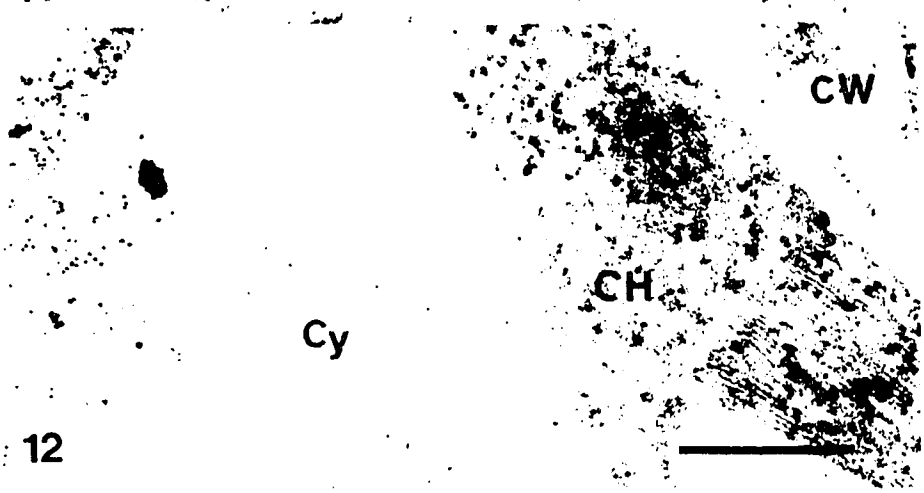
Figure IV-11. Nucleus (N) of *C. roseus* embedded in Spurr's resin with intense labelling over the nucleolus (Nu) and the electron dense areas of the chromatin (Ch) whereas the nuclear membrane (NM), nucleoplasm (Np) and cell wall (CW) are nearly free of gold particles.
Bar = 0.5 μm.

Figure IV-12. Gold labelled chloroplasts (CH) of *C. roseus* embedded in Spurr's resin with very few particles bound to the cell wall (CW) and cytoplasm (Cy).
Bar = 0.5 μm.

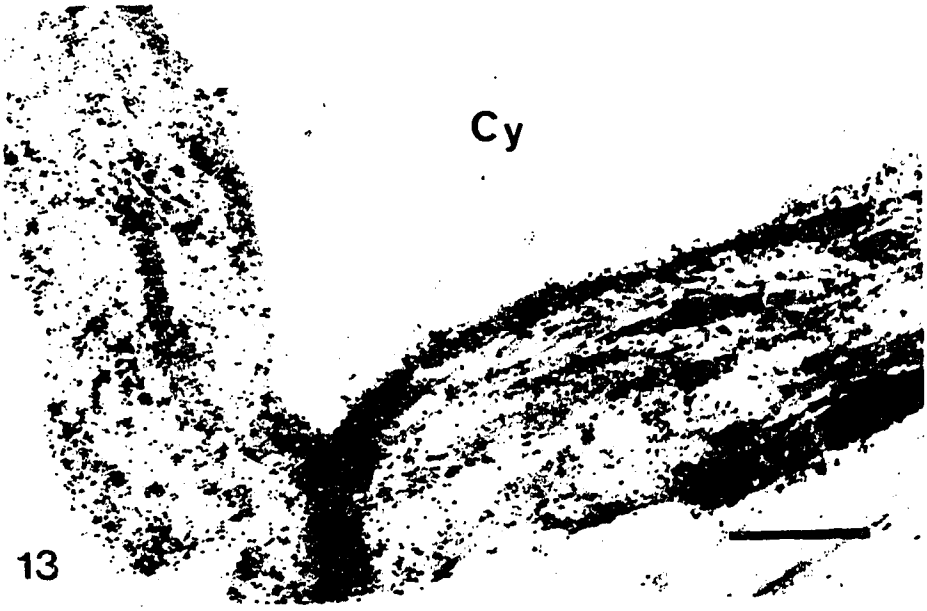
Figure IV-13. Heavy labelling on chloroplasts embedded in LR White resin. Note the absence of gold particles in the cytoplasm.
Bar = 0.5 μm.



11



12



13

Figure IV-14. Transmission electron micrograph of ultrathin section of WSpM-affected wheat root embedded in Spurr's medium and incubated in L-G complex. Note the gold particles (arrows) on the dense chromatin (Ch) within the nucleus (N). No labelling is noted on cell wall (CW), nucleoplasm (Np), or on the double membrane bodies (asterisks).
Bar = 0.5 μm.

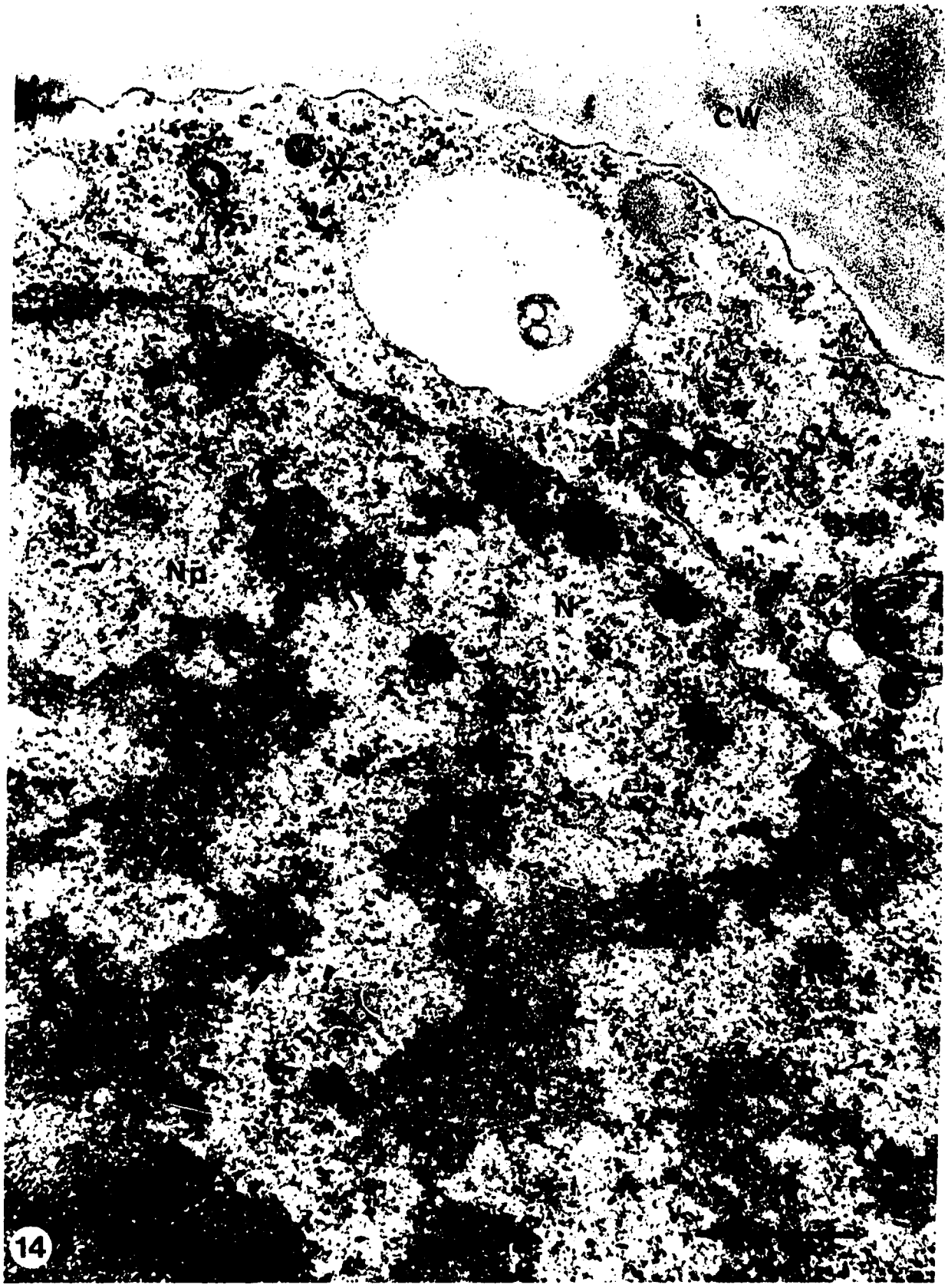

CW
Np
N
14

Figure IV-15. Transmission electron micrograph of ultrathin section of WSpM-affected wheat root embedded in LR White medium and incubated in L-G complex. Gold particles are found on the chromatin (Ch) within the nucleus and on the chloroplast (CH). The nucleoplasm (Np) and the double membrane (asterisk) are devoid of labelling.
Bar = 0.5 μm.


Np
15

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CHAPTER V

GENERAL DISCUSSION AND CONCLUSIONS

Electron dense structures approximately 100-200 nm in size which were physically attached to the endoplasmic reticulum (ER) were isolated from wheat spot mosaic (WSpM) -affected wheat. Similar structures were not observed in membrane fractions from healthy wheat. The relationship of the DMBs with ER is in agreement with the data presented by Chen and Hiruki (1990). The isolated bodies resembled the double membrane bodies (DMBs) which have been observed by transmission electron microscope examination of thin sections of WSpM-affected wheat and other DMB-associated disorders (Bradfute and Nault 1969; Bradfute *et al.* 1970; Hiruki *et al.* 1988; Hiruki 1989). Therefore, this isolation technique is useful for obtaining a partially purified fraction of the DMBs which may be used in further studies to characterize the structures and to determine the role they play in the etiology of the disease.

The viral etiology of WSpM on the basis of the dsRNA analysis is doubtful as no dsRNA could be isolated from the affected plants. This is in agreement with dsRNA analysis of a similar mite and DMB-associated wheat disease in North Dakota (Edwards and McMullen 1988). Although dsRNA was consistently isolated from rose rosette (Di *et al.* 1990), its relationship to the causal agent remains to be determined. The other DMB-associated disorders should be analyzed to determine if this is an isolated case. The possibility also exists that a DNA virus may be the cause however no virus particles typical of DNA or RNA viruses have ever been observed in the affected plants.

Total protein analysis of WSpM-affected leaves revealed no WSpM-specific bands. Further analysis of either the partially purified extracts from the plants or of the disease-associated mites would be beneficial in determining if a protein or proteins are associated with the disease.

It has been demonstrated through the use of DNA-specific DAPI stain and lactoferin conjugated colloidal gold that the DMBs do not contain any detectable amount of DNA. This data together with the relationship of the DMBs to the ER indicates that the DMBs are probably a host response to some mite-related factor. Although they are probably not the causal agent of WSpM, their relationship to and the exact nature of the causal agent remains unclear.

Further studies with the mite vector and of the factors affecting transmission may be useful to provide more information on this complex relationship.

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APPENDIX A

Maintenance of Wheat Spot Mosaic-affected Plant Material

Wheat spot mosaic (WSpM) was maintained on a Triticum x Agropyron hybrid (TAi) (seed provided by Dr. R.L. Conner, Agriculture Canada Research Station, Lethbridge) which is resistant to wheat streak mosaic virus. Seeds were sown in 13 cm pots in Terra-lite Metro-Mix (Grace Horticultural Products, Edmonton) and were kept in a Conviron Model E7 growth cabinet (Controlled Environments, Winnipeg) programmed for 20°C (day)/16°C (night) and 16 h light per day. The pots were placed in trays of water to maintain high humidity in the cabinet. Plants infested with mites which did not induce WSpM symptoms as well as mite-free plants were grown in separate growth cabinets in different rooms.

At one week after seeding, healthy plants were introduced into growth cabinets which contained either mite-infested WSpM-affected plants or mite-infested healthy plants. A fan placed at one end of the cabinet was used to facilitate the infestation of the healthy plants (Figure 1). A row of mite infested plants was placed in between the fan and the newly introduced seedlings. The fan was left on for a continuous 48 h period.

Figure A-1. Fan used in growth cabinet to facilitate movement of mites from infested to healthy plants.


1