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Chloroplast involvement in the replication of maize dwarf mosaic virus

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Chloroplast involvement in the replication of maize dwarf mosaic virus

by

Dennis E. Mayhew

A Thesis Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
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Major: Plant Pathology

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Iowa State University
Ames, Iowa

1973

TABLE OF CONTENTS

INTRODUCTION

Maize dwarf mosaic virus (MDMV) is a long flexuous rod-shaped virus which causes severe mosaic symptoms in corn, sorghum, and other grasses. It is transmitted in the field either mechanically or by aphids. The two strains of MDMV (A and B) used in this study differ primarily in their host range. The A strain will infect Johnson grass, while the B strain will not. MDMV does not seem to replicate in high concentrations in any host; the known local lesion host has not been shown to be sensitive enough to MDMV infection to perform quantitative assays.

Little is known about how MDMV replicates in host tissue, or with what cellular organelle or organelles replication and assembly are associated. Most of the research on sites of virus synthesis has been done with viruses that replicate in large numbers in their respective hosts, or viruses which are readily purified and assayed on quantitative local lesion hosts, which is not true of MDMV. Of the work that has been done, chloroplasts and nuclei are the organelles most often implicated in virus synthesis.

The purpose of this study is to provide new evidence that chloroplasts of corn leaves are involved in the synthesis and perhaps assembly of maize dwarf mosaic virus. A preliminary report of part of this study has already been published (Mayhew et al., 1972).

LITERATURE REVIEW

For many years there has been great interest in and controversy over the determination of the sites of synthesis and assembly of plant viruses in the cells that they infect. There are numerous reports presenting data obtained by a variety of techniques, which implicate various cellular organelles, or which contradict previous evidence for the involvement of a particular organelle. Those organelles most often implicated are the chloroplast and the nucleus, but there are many reports providing evidence that viral synthesis and assembly occurs only in the cytoplasm. There are three good review articles which cover the topic of sites of viral synthesis and discuss the evidence presented prior to 1966 (Bald, 1966; Esau, 1967; Schlegel et al. 1967).

Most of the evidence presented so far suggests that chloroplasts are involved in virus replication. Much of the work was done with the aid of electron microscopy. Earliest reports show virus particles in, or associated with chloroplasts, with or without chloroplast degeneration or abnormalities (Carroll, 1970; Chalcroft and Matthews, 1966; Esau and Cronshaw, 1967; Granett and Shalla, 1970). Some workers have found tobacco mosaic virus (TMV) particles in plastids of etiolated tobacco, implying independence of virus synthesis on photosynthesis (Honda and Matsui, 1971; Pratt, 1969). Ushiyama and Matthews (1970) found that chloroplasts in plants infected with turnip yellow mosaic virus (TYMV) had abnormal vesicles in which was found a fine stranded material, but upon wilting, virus inclusions formed only in the cytoplasm. This suggested to them that RNA synthesis occurs in the chloroplasts and virus assembly occurs in

the cytoplasm. Using ferritin-labeled antibodies, Shalla (1968) localized TMV in chloroplasts and further demonstrated that infectivity could be associated with isolated chloroplast fractions prepared from infected tissue. Nagaraj (1965) localized TMV protein near chloroplasts, using fluorescein-labeled antibodies, but the relative permeability of chloroplasts to antibodies is still unknown.

Studies involving maize dwarf mosaic virus (MDMV) have been unsuccessful in showing virus particles in chloroplasts (Krass and Ford, 1969; Langenberg and Schroeder, 1970); however, Tu and Ford (1968) report a reduction in number of chloroplasts in mesophyll parenchyma cells of infected corn leaves, as well as increased plastid size.

Many workers have obtained chloroplast fractions from diseased tissue and have associated infectivity with these isolated organelles (Matsushita, 1965; Shalla, 1968; Singer, 1972; Zaitlin and Boardman, 1958). Cech (1967) reported, however, that virus activity in chloroplasts is delayed 40 hours after inoculation and never attains one-tenth of total infectivity. Others isolating chloroplast fractions and using various labeling techniques find that labeled virus protein or RNA is associated with these fractions (Boardman and Zaitlin, 1958; Ralph and Clark, 1966; Ralph and Wojcik, 1969; Ralph et al., 1971b; Singer, 1972). Bove et al. (1965) and Bove (1966) found a double stranded RNA with the characteristics of the replicative form of TYMV associated with a chloroplasts and nuclei fraction of TYMV-infected Chinese cabbage leaves. Working with intact chloroplasts from healthy tobacco and tobacco infected with tobacco etch virus (TEV), Hampton et al. (1966) demonstrated identical rates of uptake of labeled glycine, but the preparation from infected leaves had only two-thirds as

many chloroplasts.

There are several reports of RNA changes in chloroplasts of infected tissue. Cooper and Loring (1957) show an increase in total RNA in TMV-infected chloroplasts, while Fraser (1972) also working with TMV-infected plants demonstrates an increase in cytoplasmic and chloroplast rRNA and tRNA, and a rapid degeneration of normal chloroplast RNA soon after inoculation. Other results show, however, that synthesis of chloroplast rRNA is inhibited by TMV infection, while cytoplasmic rRNA is unaffected (Oxelfelt, 1971). Also the amount of degraded rRNA greatly exceeds virus RNA accumulation. Another worker, studying six different viruses, showed that 18S RNA in chloroplasts was reduced after inoculation (Pratt, 1967).

A report submitted by Sela and Kaesberg (1969) indicates that from a ribosomal, cell-free system isolated from tobacco chloroplasts, to which is added TMV RNA, one can obtain TMV coat protein which, and when the newly formed protein is mixed with TMV RNA, they combine to form complete virus particles. Kataoka et al. (1969) found that chloramphenicol, which inhibits chloroplast ribosomes, does not inhibit TMV synthesis, so these ribosomes may not play a major role in virus protein synthesis.

There are reports of reduced amounts of photosynthetic pigments in leaves of virus infected plants (Pratt, 1967; Zwolinska and Grela, 1969); however, there are conflicting reports of the effect of viruses on photosynthesis. Goffeau and Bove (1965) working with TYMV, demonstrated an increase in the Hill reaction and cyclic and noncyclic photophosphorylation in chloroplasts from leaves in active virus replication stages, while Tu and Ford (1968) report a decrease in photosynthesis in leaves infected with MDMV. Nakagaki and Hirai (1971) suggest that there is no direct

relationship between chlorophyll degradation and TMV replication.

Several workers have tested the effect of the amount of light supplied to whole plants or tissue cultures on the amount of virus produced. There are as many reports showing an increase in virus titer in reduced light (Bawden and Roberts, 1947; Matthews, 1953), as in full illumination (Cheo, 1971; Murakishi et al., 1971). Virus replication has been reported in plants that are mutant for photosynthetic pigments or some photosynthetic pathways (Holmes, 1934; Wenzel, 1970; Yamaguchi, 1968), or in etiolated plants (Doke, 1972; Holmes, 1934).

Most, if not all, of the evidence implicating the nucleus as the site of viral synthesis has been based upon light and electron microscopy, with numerous reports of virus particles in or around the nuclei (De Zoeten and Gaard, 1969; Honda and Matsui, 1971; Martelli and Castellano, 1969; Reddi, 1964b; Rubio-Huertos and Hidalgo, 1964; Shikata and Maramorosch, 1966). The use of labeled antibodies has localized virus protein in the nucleus (Hirai and Hirai, 1964; Langenberg and Schlegel, 1969; Shalla, 1964). Incorporation of uridine-^{3}H into nuclei and nucleoli of cells treated with actinomycin D suggests that viral RNA is also replicating in these areas (Smith and Schlegel, 1965; Yasuda and Hirai, 1964), although Babos and Shearer (1969) suggest that labeled RNA in nucleoli is host RNA. The first report of the use of acridine orange staining to determine RNA changes in infected tissue revealed an increase of RNA in the nucleus (Hirai and Wildman, 1963). Reports of cytological changes in nuclei and nucleoli of infected plants have appeared (Bald, 1964; Martelli and Castellano, 1969; Shikata and Maramorosch, 1966), but Milne (1966) reports no changes in nuclei of tobacco palisade cells infected with TMV.

Much of the evidence for cytoplasm involvement in virus replication is also based upon light and electron microscopy. Reports of virus particles in the cytoplasm of infected cells are numerous (De Zoeten and Gaard, 1969; Ie, 1971; Langenberg and Schlegel, 1969; Langenberg and Schroeder, 1970; Milne, 1966; Shalla, 1964). Labeled antibodies have also been used to localize virus protein in the cytoplasm (Nagaraj, 1962, 1965; Schlegel and Delisle, 1971). Biochemical evidence for cytoplasm involvement is varied. The use of cycloheximide to inhibit cytoplasmic ribosomes has been shown to inhibit TMV replication, but not accumulation (Harrison and Crockatt, 1971). Babos (1969) demonstrated the association of labeled RNA with cytoplasmic ribosomes in tissue treated with actinomycin D. Hirai and Wildman (1967, 1969) found that most of the newly synthesized ^{32}P-labeled TMV nucleoprotein was in the cytoplasmic fraction of infected cells, and found no label associated with chloroplast ribosomes or rRNA.

Electron microscopic evidence for the involvement of other organelles includes the association of virus particles and cytological changes in mitochondria (Harrison and Roberts, 1968; Hatta et al., 1971; Weintraub et al., 1966), and association of the double stranded replicative form of TMV RNA with membrane bound ribosomes (Ralph et al., 1971a). Reddi (1964a) provided evidence that microsomes are not involved in TMV synthesis.

MATERIALS AND METHODS

The virus under investigation was the B strain of maize dwarf mosaic virus (MDMV-I 188) isolated from a sweet corn field in southeast Iowa and supplied by Dr. Richard Ford, Iowa State University, Ames, Iowa (Ford et al., 1967).[1] This virus is a flexuous rod with properties similar to those of other viruses in the potato virus Y group (Figure 1). The isolate was maintained in, and all infectivity assays were made on Golden Bantam sweet corn grown under greenhouse conditions in a loam:sand:peat mixture (2:1:1). Inoculations were performed by applying solutions with cheesecloth to Carborundum-dusted (600 mesh) seedlings which were at the 2-leaf stage of development. Mosaic symptoms usually appeared within 7-10 days in newly expanded leaves (Figure 2).

In order to determine the effect of chloroplast and pigment development on virus replication, the susceptibility of several pigment-deficient mutants of corn (supplied by Dr. Don Robertson, Iowa State University, Ames, Iowa) to infection with MDMV was investigated. The plants were grown in the same soil mixture as described above but maintained in growth chambers at 25°C with 12 hrs of light (2000 foot candles). Inoculations were performed as described above with either the B strain of MDMV or the A strain (I 74). Since infected plants were still symptomless 7 days after inoculation (the maximum survival time of mutants under the above growing conditions), individual plants were ground in 0.01M KPO_4 buffer, pH 7.2,

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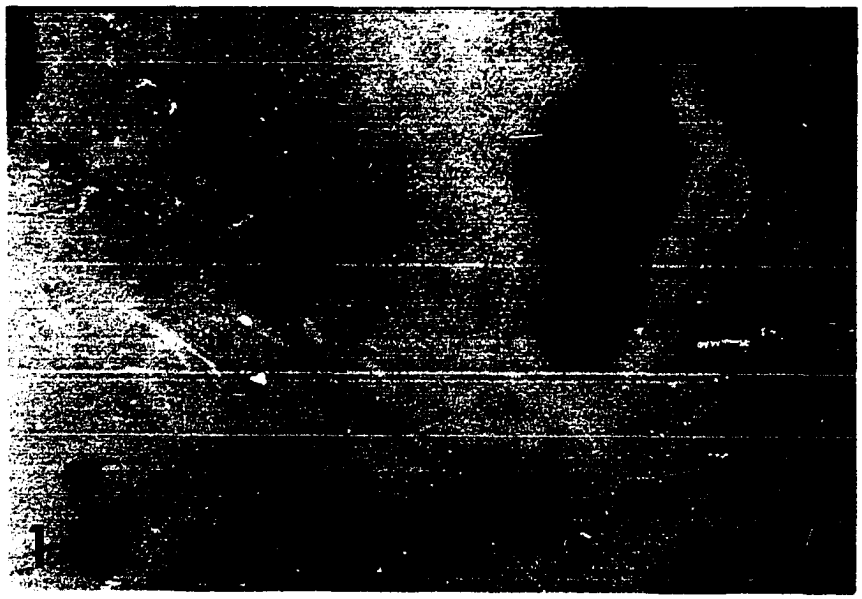
Figure 1. B strain of maize dwarf mosaic virus prepared by mixing sap expressed from systemically infected corn leaves with equal volumes of 4% phosphotungstic acid and placing a small droplet on a Formvar coated grid and allowing it to air dry. Bar represents 100 nm

Figure 2. Systemic mosaic symptoms in corn leaves infected with the B strain of maize dwarf mosaic virus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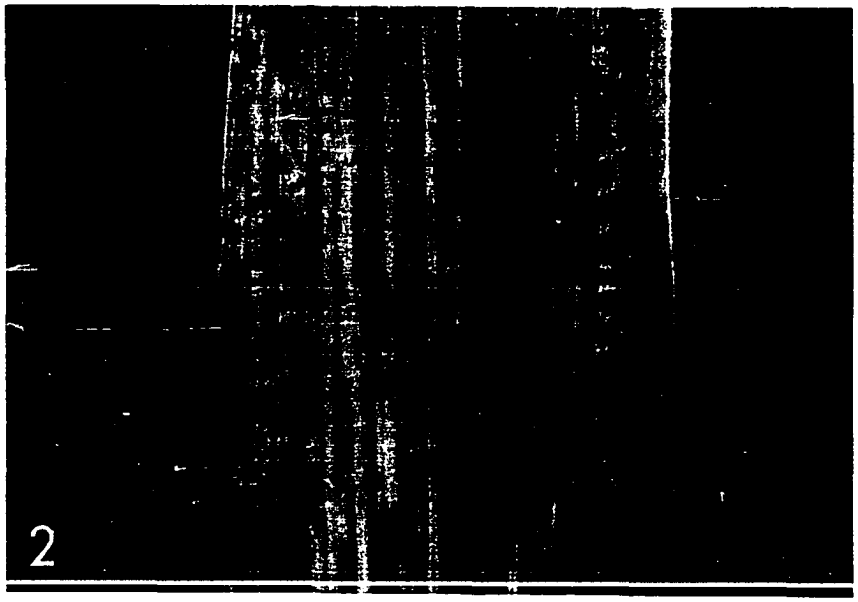
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with a mortar and pestle, and assayed on sweet corn. The susceptibility of several genotypes was investigated, but for further experimentation four representative genotypes were selected. These were $\underline{lw}_1$ (albino), $\underline{w}_1$ (albino), $\underline{w}_{8896}$ (yellow), and $\underline{pas}_{8686}$ (pale green) (Demerec, 1923, 1925; Everett, 1949; Robertson, 1961).

Since it has been reported that some etiolated mutants produce plastids and quantities of pigments similar to those found in normal etiolated corn (Bachmann et al., 1969), the effect of etiolation on the ability of MDMV to replicate in mutants was investigated. Mutants were grown under greenhouse conditions in either full light or in a dark chamber. The plants were inoculated and assayed as previously described.

Cytological Techniques

Sweet corn seedlings were inoculated with either a solution made by grinding 3 week old virus-infected tissue in 0.01M KPO_4 buffer, pH 7.2, in a 1:1 ratio or virus-free buffer which served as a control. Inoculated leaves were harvested at intervals of 1 and 3 days after inoculation, and systemically infected leaves were collected 18 days after inoculation. Entire leaves were sliced into 1 cm sections, fixed in formalin-acetic acid (FAA), dehydrated in a tertiary butanol series, and embedded in Tissuemat (52.5°C) according to the procedure described by Jensen (1962). The paraffin-embedded tissue was cut into 10μ sections with an AO rotary microtome, and the sections were mounted on glass slides coated with Haupt's adhesive. Leaf tissue from $\underline{w}_1$ albino plants was prepared in the same manner.

Similar tissue was embedded in plastic in preparation for thin

section light microscopy. Leaf material taken from an area 4 cm long in the middle portion of each leaf, and from which was removed the midrib, was cut into pieces 1 mm^2 and fixed in 3% glutaraldehyde in 0.02M KPO_4 buffer (pH 7.3) for 48 hrs. The tissue was then washed in buffer, post-fixed in 1% OsO_4 for 1 hr, dehydrated in an ethanol series, and embedded in Spurr low-viscosity embedding media.[2] Sections 1μ thick were cut with an LKB Ultratome III using glass knives. The sections were stained with 1% toluidine blue and viewed and photographed with a Leitz microscope and Wetzlaer automatic camera.

Fluorescence Microscopy

In order to determine qualitatively the distribution and changes of RNA in healthy and infected leaf tissue, a histochemical technique utilizing acridine orange fluorescence was performed as described by Von Bertalanffy (1963). Paraffin-embedded sections were deparaffinized in a xylene-ethanol series and rehydrated to distilled water. The sections were then treated with either a 0.1% solution of pancreatic ribonuclease (Nutritional Biochemical Co., 5X) in distilled water and adjusted to pH 6.8 with NaOH, or a distilled water control for 4 hrs at 37°C. The sections were subsequently stained with 0.1% acridine orange in 0.15M KPO_4 buffer, pH 6.0, and viewed and photographed with a Leitz fluorescence microscope and Wetzlar automatic camera. A barrier filter (490-470μ) and lamp housing filters BG38 and UG1 were used. Single-stranded RNA fluoresces a brilliant

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[2]Materials and procedures available from Polysciences, Inc., Paul Valley Industrial Park, Warrington, Pennsylvania 18976. (Data sheet #127).

orange-red when stained with acridine orange, while DNA and reportedly double-stranded RNA (Spendlove, 1967) fluoresces yellow-green. Positive transparencies were made with Kodak High Speed Ectachrome film balanced for daylight, with camera settings at 400 ASAd and D50%. Negatives for prints were made by photographing positives with either Kodak Kodacolor-X or Panatomic-X films.

The acridine orange staining technique was also performed on smears of chloroplasts isolated from healthy and inoculated leaf tissue collected 3 days after inoculation with MDMV or buffer control. The chloroplasts were prepared by the following procedure:

1. 10 gms of leaf tissue ground in 50 ml FAA with a Waring blendor for 30 sec
2. brei filtered through 4 layers of gauze
3. centrifuged at 1000Xg for 5 min (supernatant discarded)
4. pellet resuspended in 25 ml FAA
5. process repeated twice more
6. centrifuged 500Xg for 90 sec (pellet discarded)
7. centrifuged 1000Xg for 10 min (supernatant discarded)
8. resuspend pellet in 4.5 ml FAA
9. layer 1.5 ml on three discontinuous sucrose density gradients made with 60, 50, 40, and 30% sucrose in distilled water (all 1 ml except 1.5 ml 40%) in Spinco SW50 tubes
10. gradients centrifuged in Beckman SW50 rotor for 50 min at 45,000 rpm
11. chloroplast fractions removed, mixed with 2 volumes FAA
12. centrifuged 1000Xg for 5 min (supernatant discarded)

13. chloroplasts resuspended in just enough FAA to cover pellet. The isolated chloroplasts were smeared on glass slides, allowed to air dry, and stained with acridine orange as described previously.

In order to determine what role protein plays in the resistance of RNA to enzymatic attack, paraffin sections of healthy and MDMV-infected corn (harvested 3 days after inoculation) were pretreated with protease (Nutritional Biochemical Co.) before treating with ribonuclease and staining with acridine orange as described previously. The sections were first coated with 0.5% parloidian and then placed in a protease solution [1 mg/ml 0.1M BPES buffer, pH 7.0, consisting of 1.608 gm/l Na_2HPO_4, 0.274 gm/l NaH_2PO_4, and 0.372 gm/l EDTA in 0.1M NaCl (Zyskind, 1968)] for 12 hrs at 37°C. The sections were rinsed for 1 min each in 95% EtOH and distilled water before subsequent treatment with ribonuclease.

In an attempt to localize viral protein in infected tissue, an indirect fluorescent antibody staining procedure was used. Sections of leaf tissue harvested 18 days after inoculation were deparaffinized and rehydrated to distilled water. The tissue was then covered with either rabbit or goat antiserum prepared against MDMV-B (188) and incubated for 1 hr at 37°C. After rinsing in borate-buffered saline (Campbell et al., 1964) for 30 min, the tissue was stained with either Difco FA goat globulin antiglobulin or rabbit globulin antiglobulin and incubated for 1 hr at 37°C. Following incubation, the slides were again rinsed in borate-buffered saline for 30 min, covered with Difco FA mounting medium, and viewed in UV light with a Leitz fluorescence microscope.

Infectivity Assays

To obtain additional evidence that chloroplasts are involved in virus replication, infectivity assays of fractionated cells were made. Preliminary studies were performed by grinding 20 gms of systemically infected leaf tissue (from seedlings inoculated with MDMV-B 3 weeks earlier) in one of two grinding media (2 ml/gm tissue) in a Waring blendor for 30 sec. One medium consisted of 0.5M Na citrate buffer (pH 8.0) containing 0.5% mercaptoethanol; and the other was made up of 0.02M Hepes, 0.3M mannitol, 0.1% bovine serum albumin, 0.05% cysteine, and 1mM EDTA (buffer adjusted to pH 7.1). After grinding, the brei was treated in the following manner:

1. filtered through 2 layers of gauze
2. centrifuged 500Xg for 20 min (pellet saved for assay)
3. centrifuged supernatant 11,000Xg for 20 min (supernatant saved for assay)
4. pellet resuspended in 6 ml grinding medium (save portion for assay)
5. layered on discontinuous sucrose density gradients consisting of 30, 40, 50, and 60% sucrose in distilled water (7 ml each) in Beckman SW25.1 tubes
6. centrifuged in Beckman SW25.1 rotor at 25,000 rpm for 4 hrs
7. fractions collected with ISCO Density Gradient Fractionator
8. all UV absorbing peaks as well as pellets and supernatants assayed directly by rubbing them on Carborundum-dusted sweet corn leaves.

The fractionation and assay procedures were performed on the pigment-deficient mutants as well.

In order to assay the infectivity of chloroplasts isolated from infected tissue, a procedure described by Shalla (1968) was used. The procedure was as follows:

1. 25 gms of systemically infected corn leaf tissue (9-21 days after inoculation, all with symptoms) were ground in 100 ml Honda medium consisting of 2.5% ficoll, 4.0% dextran, 0.25M sucrose, 0.025M Tris buffer, 1 mM Mg acetate, and 4 mM mercaptoethanol

2. filtered through 2 layers of gauze

3. centrifuged 500Xg for 1 min (pellet discarded)

4. supernatant centrifuged 10,000Xg for 20 min (supernatant discarded)

5. sides of centrifuge tubes rinsed with distilled water

6. pellet resuspended in 10 ml of Honda medium

7. repeated twice more

8a. 1/2 chloroplasts resuspended in 7 ml Honda medium

8b. 1/2 chloroplasts resuspended in borate buffer made with 0.68 gms H_3BO_3/l and 1.124 gms $NaB_4O_7.10H_2O$/l pH 8.7

9. after 10 min, centrifuged 10,000Xg for 20 min and supernatant assayed for infectivity

10. chloroplasts from 8a resuspended in 7 ml borate buffer with trace of sodium dodecyl sulfate and assayed immediately.

Since the washed chloroplasts prepared in this manner still contained some contamination, the procedure was modified by layering 2 ml of washed chloroplasts which had been resuspended in 6 ml of Honda medium of three discontinuous sucrose density gradients prepared as suggested by Tewari and Wildman (1966), that is, 10 ml each of 60% and 45% sucrose in Honda

medium and 5 ml of 20% sucrose in Honda medium in Beckman SW25.1 tubes. The gradients were centrifuged in a Beckman SW25.1 rotor for 2 hrs at 25,000 rpm. The chloroplast bands were removed, 2.5 volumes of Honda medium added, and the solutions centrifuged for 20 min at 10,000Xg. The chloroplast pellet was then treated as described in steps 8a-9 above.

The above procedures (steps 1-9) were also performed with leaves harvested 3 days after inoculation with MDMV, with two modifications. Fifty grams of tissue were used instead of 25, and the isolated chloroplasts were allowed to sit at room temperature at step 8 for one hour before continuing.

All statistical analyses were performed using chi-square.

RESULTS

Replication of strain A and B of maize dwarf mosaic virus could be demonstrated in several pigment-deficient mutants of corn (Table 1). Of the nine mutants tested, six supported replication of MDMV-A and eight supported replication of MDMV-B. Of the four mutants selected for further study, the $\underline{w}_1$ albino was the most efficient in supporting MDMV replication, $\underline{pas}_{8686}$ and $\underline{w}_{8896}$ were intermediate, and the $\underline{lw}_1$ albino relatively resistant to infection.

Table 1. Ability of pigment-deficient mutants of corn to support replication of maize dwarf mosaic virus

Genotype	Phenotype	No. plants infected/inoculated		
		MDMV-A	MDMV-B	
$\underline{lw}_1$	albino	0/6	1/96[a]	X[b]
$\underline{w}_1$	albino	1/2	8/22[a]	Y
$\underline{w}_{8657}$	albino	0/6	0/6	
$\underline{l}_{10}$	yellow	3/5	3/5	
$\underline{w}_{8896}$	yellow	1/6	18/13[a]	Z
$\underline{l}_{Brawn\ \#1}$	yellow-green	3/6	3/4	
$\underline{pas}_{8686}/\underline{w}_3$	pale-green	2/4	2/4	
$\underline{pas}_{8686}$	pale-green	2/4	21/104[a]	Z
$\underline{l}_7$	pale-green	0/0	1/1	

[a]Selected for further study.

[b]Results followed by the same letter are not significantly different at 1% level.

In the etiolation experiments (Table 2), the data suggest that etiolated mutants are better able to support viral infection than those grown in full illumination, even though the differences are not significant at the 5% level. Unfortunately, mutant corn seed supplies were limited, and many genotypes had very poor germination, which limited the possibilities for replication of experiments.

Table 2. Effect of etiolation on the ability of pigment-deficient mutants of corn to support replication of maize dwarf mosaic virus

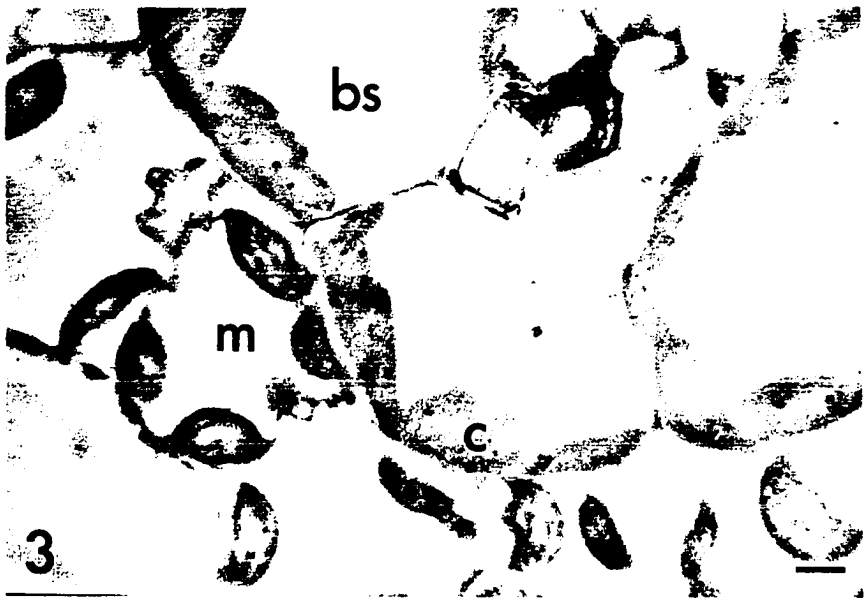
Genotype	Phenotype	No. plants infected/inoculated	
		Etiolated	Full light
$\underline{lw_1}$	albino	2/30	0/30
$\underline{w_{8896}}$	yellow	4/21	2/29
$\underline{pas_{8686}}$	pale-green	2/30	0/30

Cytology

Cross sections of normal corn leaf tissue embedded in paraffin and plastic, reveal the size, type, and distribution of cell nuclei and chloroplasts (Figure 3). Other cellular organelles are not discernible in this tissue. The chloroplasts are relatively large, and, in many cases, equal in size to the nucleus. The plastids are of two types, the very large and numerous, agranal plastids of the bundle sheath parenchyma cells, and the less numerous, granal plastids of the mesophyll cells. With both types, the plastids are distributed around the periphery of the cell, closely appressed to the cell wall. In albino tissue (Figure 4) the

Figure 3. Healthy corn leaf tissue (X.S.) embedded in Spurr low viscosity embedding medium, cut to 1 μ thickness, and stained with toluidine blue. Note the orientation and lack of grana in the chloroplasts (C) of bundle sheath parenchyma cells (BS), and the more swollen, granal chloroplasts of the mesophyll cells (M). Bar represents 10 μ

Figure 4. $\underline{w}_1$ albino leaf tissue (X.S.) embedded in Spurr low viscosity embedding medium, cut to 1 μ thickness, and stained with toluidine blue. The chloroplasts are small and degenerate and closely appressed to the cell wall (arrow). Bar represents 10 μ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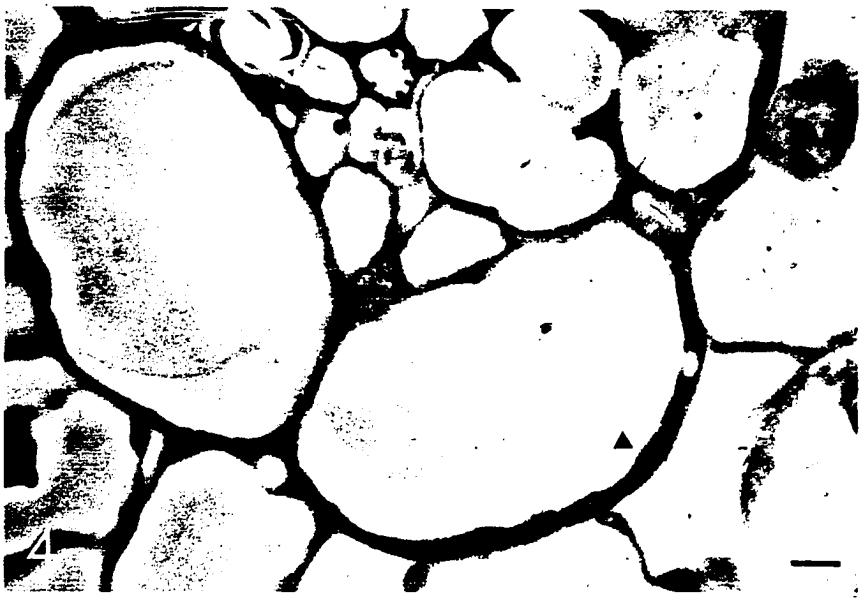
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plastids are small and degenerate and indistinguishable in terms of granal structure. As in the normal tissue, the plastids are closely appressed to the cell wall.

Fluorescence Microscopy

In cross sections of paraffin-embedded corn leaf tissue, stained with acridine orange, and viewed in UV light, most of the fluorescence was in the orange-red spectrum and located primarily in chloroplasts and nuclei. Enzymatic analyses indicated that the orange-red fluorescence was due primarily to ribonucleic acid. In tissue harvested 24 hrs after inoculation (Figure 5) there was a sharp contrast in the amount of RNA present in tissue infected with MDMV which had been treated with ribonuclease, as compared to healthy tissue treated in a similar manner. In the healthy tissue, ribonuclease treatment removed almost all of the RNA present, while a relatively large amount of ribonuclease-resistant RNA (rr-RNA) remained in the chloroplasts of infected tissue. The greatest amount of rr-RNA appeared to be in the chloroplasts of the bundle sheath parenchyma cells. The same phenomenon is seen in tissue harvested 3 and 18 days after inoculation (Figures 6 and 7). In the nuclease treated infected tissue harvested at three days (Figure 7D), it was observed that adjacent areas in the tissue had varying amounts of rr-RNA. This was also observed in one day old tissue. The most dramatic contrast in the effect of ribonuclease treatment on healthy and infected tissue can be seen in the systemically infected tissue harvested 18 days after inoculation. Virtually all of the RNA present in healthy tissue was removed by ribonuclease, while most, if not all, of the RNA present in untreated

Figure 5. Paraffin-embedded corn leaf tissue (X.S.) harvested 1 day after inoculation (inoculated leaves), stained with 0.1% acridine orange, observed and photographed with a Leitz fluorescence microscope. Ribonucleic acid fluoresces orange-red in UV light. A) Healthy tissue, untreated. B) Healthy tissue treated with 0.1% pancreatic ribonuclease for 4 hrs at 37°C prior to staining. C) Tissue inoculated with maize dwarf mosaic virus strain B, untreated. D) Infected tissue pretreated with ribonuclease. Note the distribution of ribonuclease-resistant RNA in the chloroplasts of treated infected tissue (arrows). Bar represents 10 μ

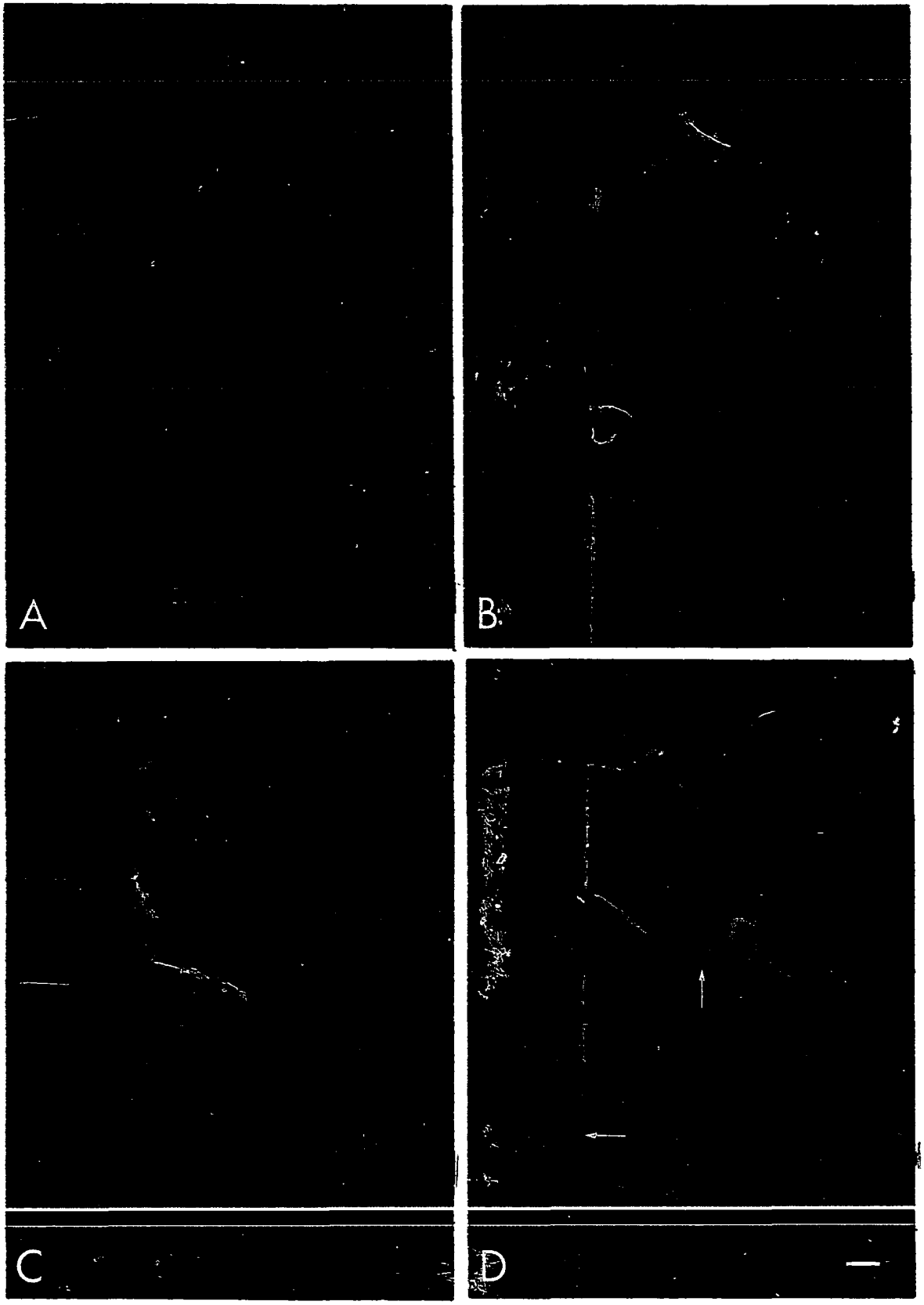
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Figure 6. Paraffin-embedded corn leaf tissue (X.S.) harvested 3 days after inoculation (inoculated leaves), stained with 0.1% acridine orange, observed and photographed with a Leitz fluorescence microscope. Ribonucleic acid in chloroplasts and nuclei appears white in these micrographs. A) Healthy tissue, untreated. B) Healthy tissue treated with 0.1% pancreatic ribonuclease for 4 hrs at 37°C prior to staining. C) Tissue inoculated with maize dwarf mosaic virus strain B, untreated. D) Infected tissue, pretreated with ribonuclease. Note that chloroplasts in cells of adjacent bundles of treated, infected tissue react differently (D), suggesting the presence of "hot spots" where viral RNA may be replicating. Bar represents 10 μ

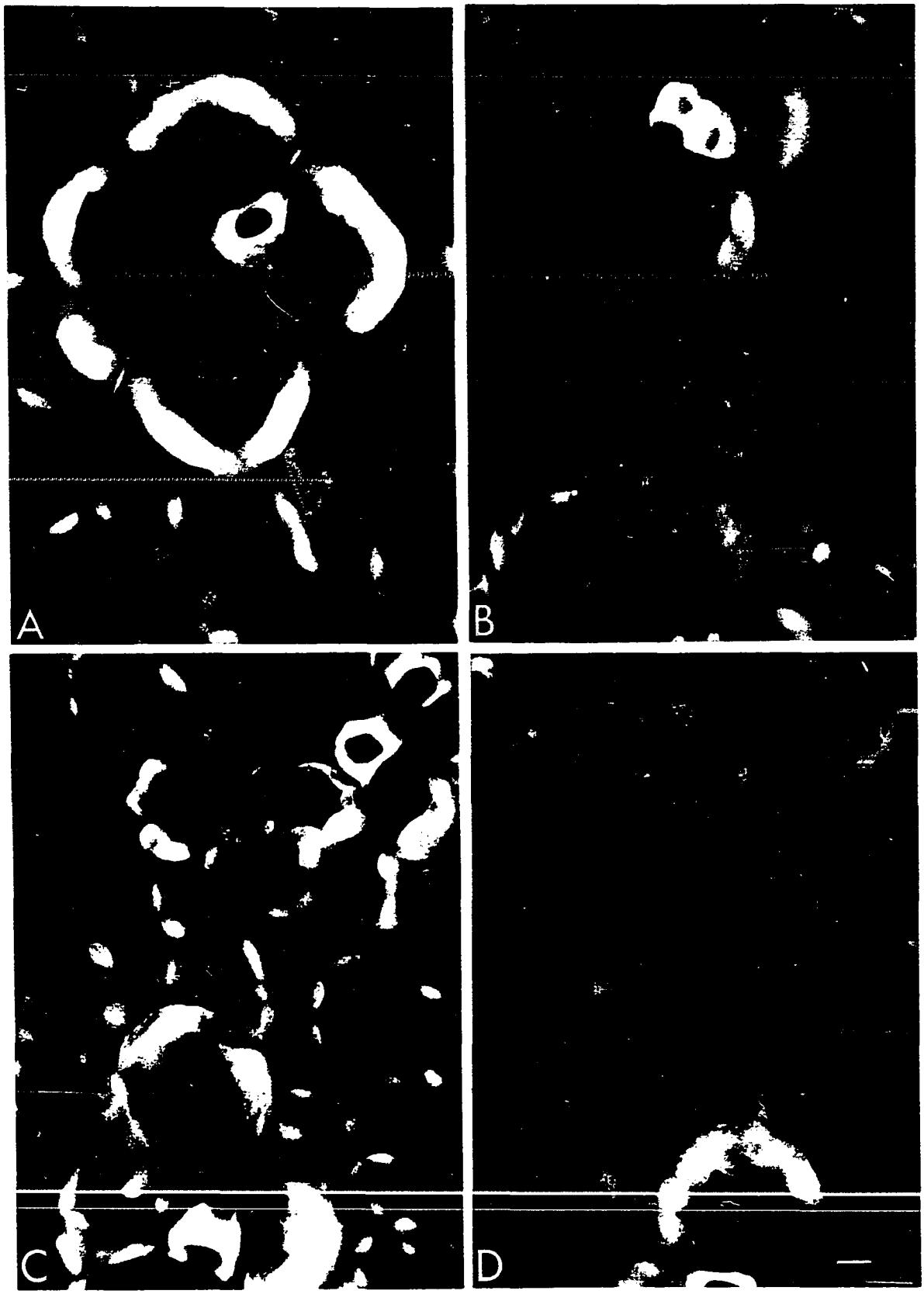
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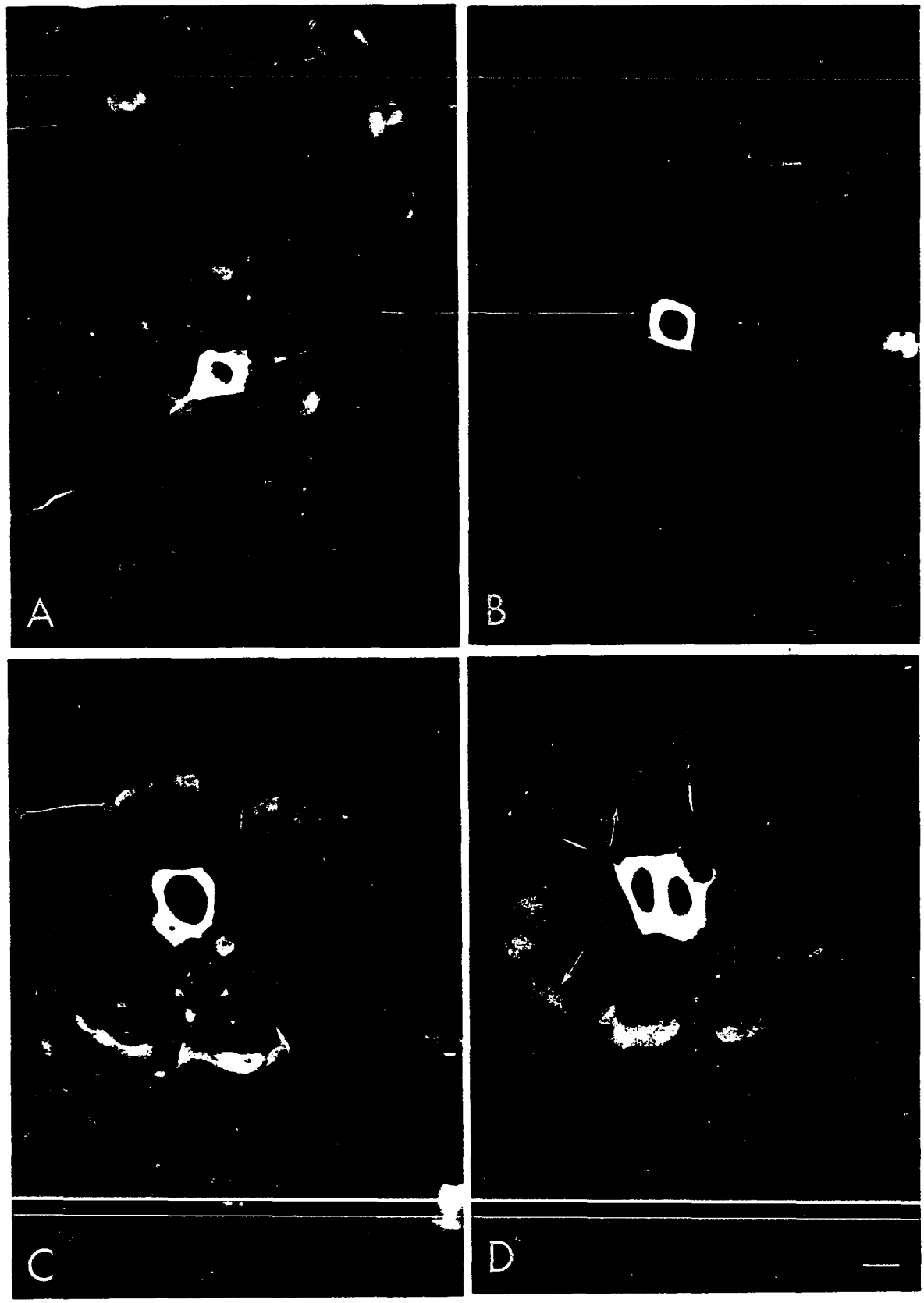
Figure 7. Paraffin-embedded corn leaf tissue (X.S.) harvested 18 days after inoculation (systemically infected), stained with 0.1% acridine orange, and observed and photographed with a Leitz fluorescence microscope. Ribonucleic acid in chloroplasts and nuclei appears white in these micrographs. A) Healthy tissue, untreated. B) Healthy tissue treated with 0.1% pancreatic ribonuclease for 4 hrs at 37°C prior to staining. C) Tissue inoculated with maize dwarf mosaic virus strain B, untreated. D) Infected tissue pretreated with ribonuclease. Note the presence of ribonuclease-resistant RNA primarily in chloroplasts of bundle sheath parenchyma cells of treated, infected tissue (arrows). Bar represents 10 μ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infected tissue appears to be of the rr-RNA species. The presence of rr-RNA was also shown in the albino tissue (Figure 8) which was treated in a similar manner as the normal tissue. Again the rr-RNA appeared to be localized in the small, degenerate plastids, and primarily those of the bundle sheath parenchyma cells.

The indication that the chloroplasts of infected tissue contained rr-RNA, suggested by the results obtained with paraffin sections, was confirmed by acridine orange staining and ribonuclease treatment of chloroplasts isolated from healthy and infected tissue (Figure 9).

Pretreatment of paraffin sections of corn with protease prior to ribonuclease treatment and acridine orange staining, revealed that one or more proteins may be involved in imparting nuclease resistance to the RNA found in nuclease treated, infected tissue (Figure 10). There is a significant reduction in rr-RNA in the protease treated infected tissue, but in no case did it equal the reduction of RNA observed in ribonuclease treated, healthy tissue.

Infectivity Assays

Assays performed on cellular fractions isolated from density gradients indicate that infectivity is associated with the chloroplast fraction (Table 3). Infectivity could not be demonstrated in fractions containing mitochondria, microsomes, or nuclei. Results of assays using washed chloroplasts treated with either an isotonic or hypotonic buffer were quite dramatic. Infectivity of the hypotonic buffer (borate) which caused the chloroplasts to swell, was 43 times greater than the isotonic buffer (Honda) which has little or no effect on the plastids. Borate buffer

Figure 8. Paraffin-embedded w_1 albino corn leaf tissue (X.S.) harvested 7 days after inoculation, stained with 0.1% acridine orange, photographed in UV light with a Leitz fluorescence microscope. Ribonucleic acid in the nuclei and abnormal plastids appears white in these micrographs. A) Healthy tissue, untreated. B) Healthy tissue treated with 0.1% pancreatic ribonuclease for 4 hrs at 37°C prior to staining. C) Tissue inoculated with maize dwarf mosaic virus strain B, untreated. D) Infected tissue pretreated with ribonuclease. Note the presence of ribonuclease-resistant RNA in plastids of treated, infected tissue (arrows). Bar represents 10 μ

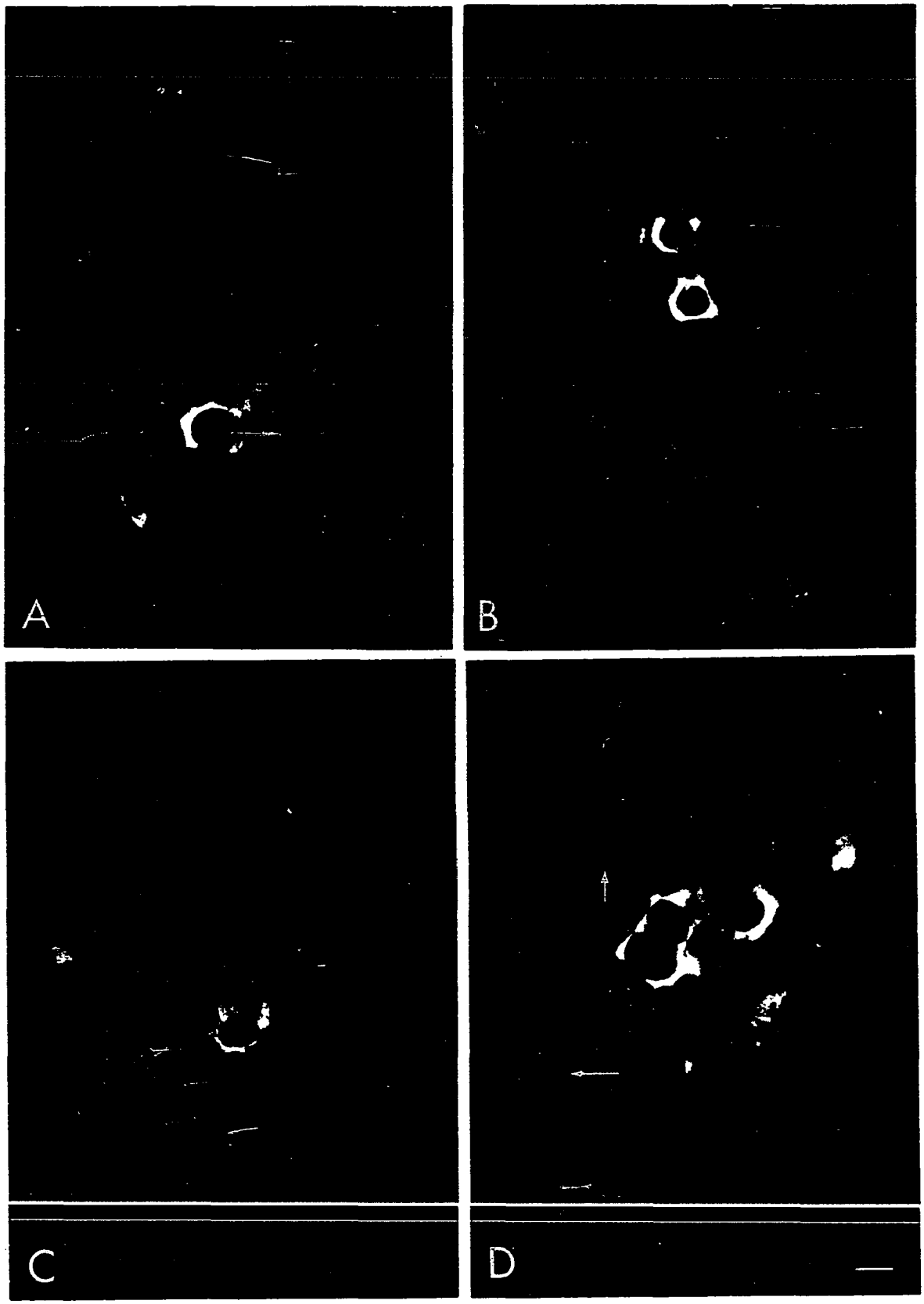
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Figure 9. Chloroplasts isolated from corn leaf tissue harvested 3 days after inoculation (inoculated leaves), smeared on glass slides, stained with 0.1% acridine orange, and photographed in UV light with a Leitz fluorescence microscope. Ribonucleic acid appears white in these micrographs. A) Chloroplasts from healthy tissue, untreated. B) Chloroplasts from healthy tissue, treated with 0.1% pancreatic ribonuclease for 4 hrs at 37°C prior to staining. C) Chloroplasts from tissue inoculated with maize dwarf mosaic virus strain B, untreated. D) Chloroplasts from infected tissue, pretreated with ribonuclease. Note the presence of ribonuclease-resistant RNA in chloroplasts of treated, infected tissue (D). Bar represents 10 μ

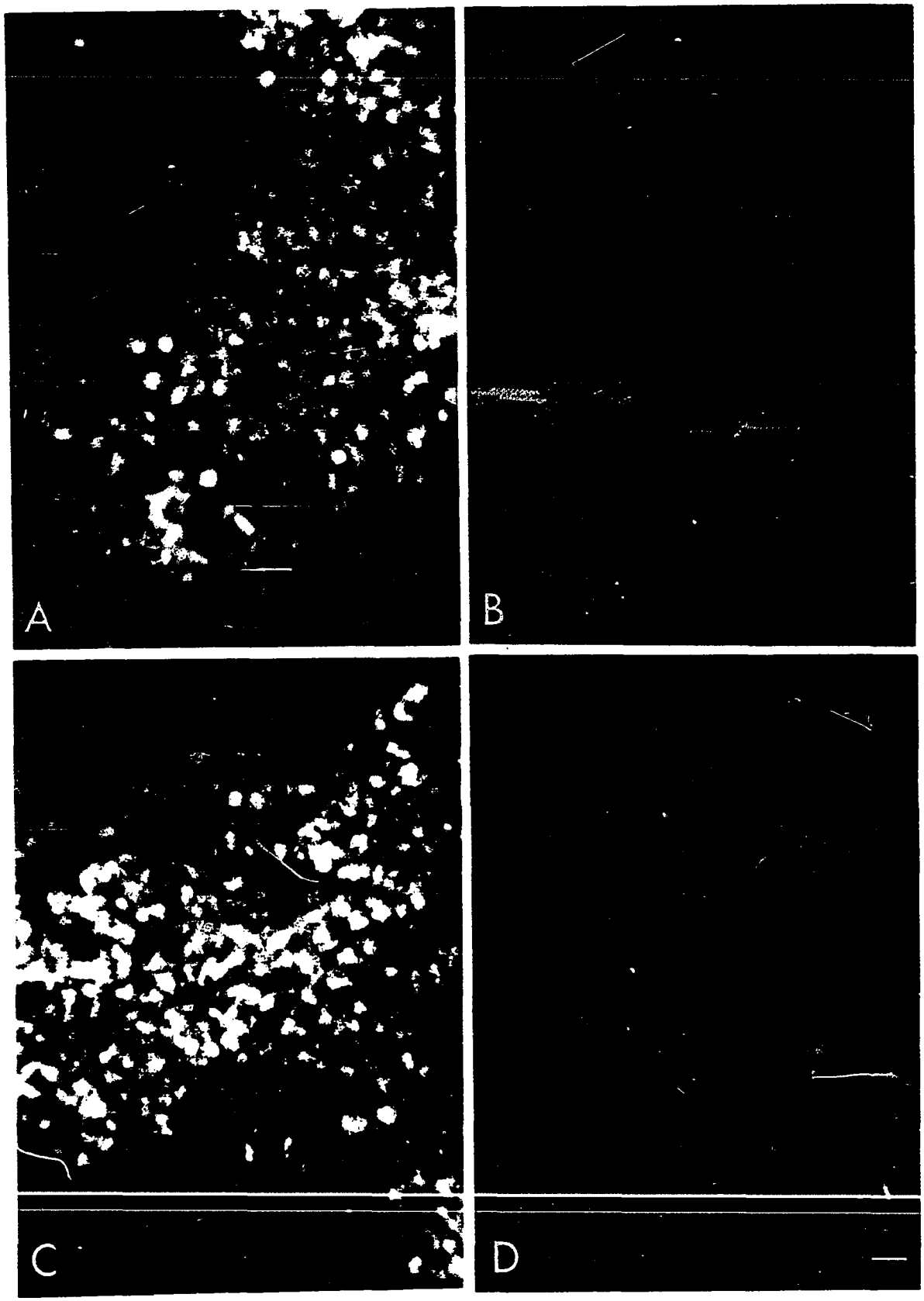
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Figure 10. Paraffin-embedded corn leaf tissue (X.S.) harvested 3 days after inoculation, stained with 0.1% acridine orange, and photographed in UV light with a Leitz fluorescence microscope. Ribonucleic acid in the nuclei and chloroplasts fluoresces orange-red. A) Healthy tissue treated with protease (1mg/ml of 0.1M BPES buffer, pH 7.0) for 12 hrs at 37°C prior to staining. B) Healthy tissue pretreated with protease and further treated with 0.1% pancreatic ribonuclease for 4 hrs at 37°C prior to staining. C) Maize dwarf mosaic virus (strain B) infected tissue pretreated with ribonuclease. D) Infected tissue pretreated with both protease and ribonuclease. Note the reduction of ribonuclease resistant RNA in chloroplasts of infected tissue pretreated with protease (D). Bar represents 10 μ

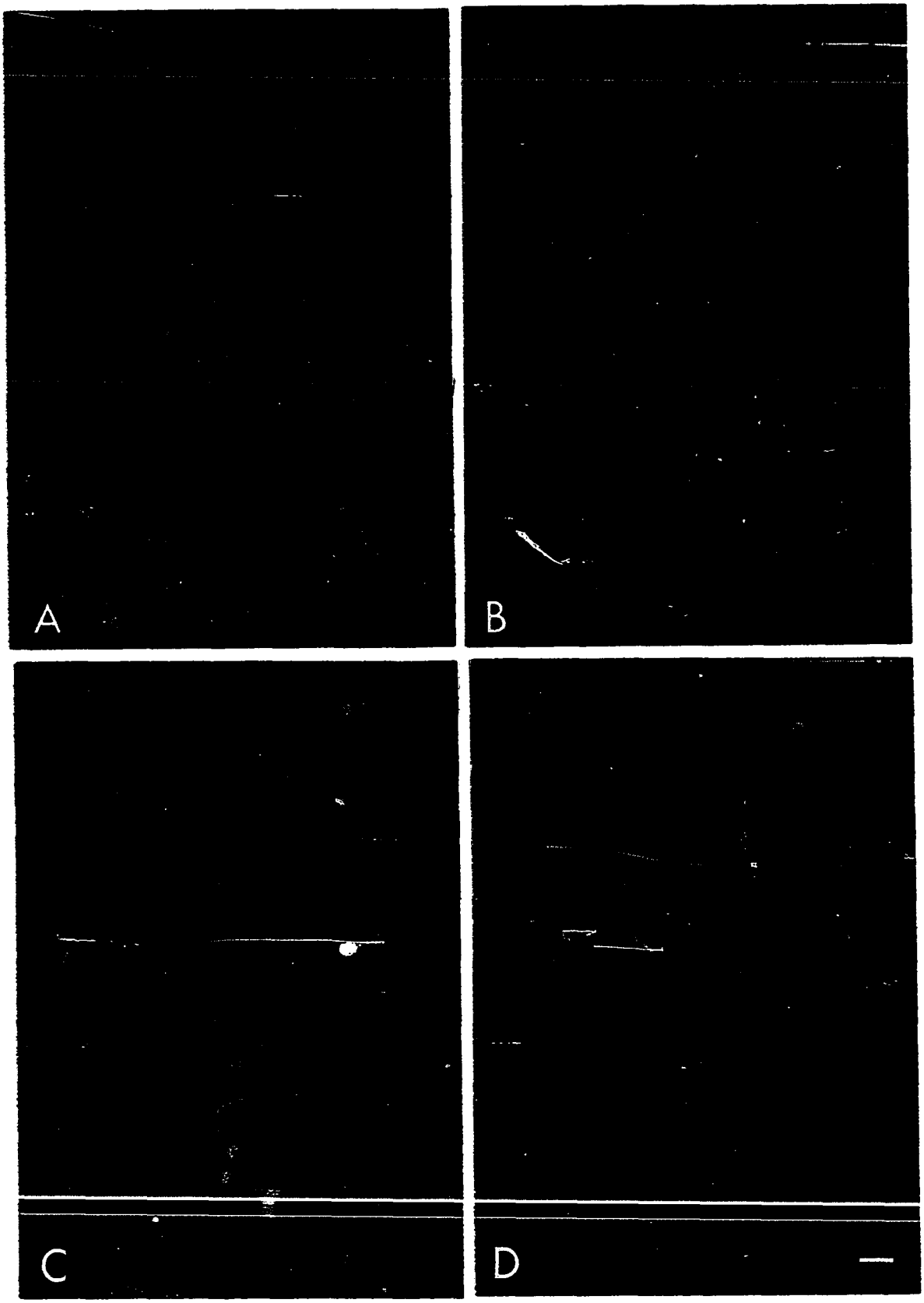
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Table 3. Infectivity of cellular fractions prepared from sweet corn leaves inoculated with maize dwarf mosaic virus strain B

Treatment	No. plants infected/ no. inoculated	% infected (relative infectivity)
Total cell assay:		
pellet	14/60	23.3
supernatant	18/80	22.5
mitochondria	0/80	0
chloroplasts	10/89	11.2
microsomes	0/80	0
nuclei	0/80	0
Washed chloroplasts:		
Honda	1/237	0.4
borate	41/234	17.5*
borate + SDS	14/210	6.7*
Density gradient treated:		
Honda	0/240	0
borate	0/240	0
Modified DSDG tmt:		
Honda	1/259	0.3
borate	14/348	4.0*
3 day inoculated leaves:		
Honda	0/80	0
borate	0/80	0

*Difference significant at 1% level.

(plus SDS) treatment of chloroplasts collected from the Honda treatment displayed a 15-fold increase in infectivity over the isotonic buffer. In a separate experiment, Honda buffer had no effect on infectivity of a crude virus preparation.

Similar results could not be obtained with chloroplasts isolated from discontinuous sucrose density gradients. Because infectivity could not be demonstrated with these chloroplasts, two modifications of the technique were made. Since many plastids are lost by centrifugation, the total amount of chloroplasts per ml of buffer to be layered on gradients was increased two-fold. The other modification was increasing the time that the chloroplasts remained in isotonic and hypotonic buffers from 10 min to 1 hour. With these modifications, infectivity was shown to be associated with chloroplasts, and, in addition, there was a 13-fold increase in infectivity with the hypotonic buffer treatment as compared to the isotonic treatment.

Infectivity could not be associated with the chloroplasts isolated from tissue harvested 3 days after inoculation, even with the modified technique.

DISCUSSION

This study has provided several new lines of direct evidence which implicate the chloroplasts as sites of viral synthesis. The most important new evidence is the discovery of a ribonuclease-resistant species of RNA (rr-RNA), presumed to be viral RNA, in the chloroplasts of infected tissue, which cannot be demonstrated in chloroplasts of healthy tissue. The rr-RNA was detected in both inoculated and systemically infected leaves, and was demonstrated in inoculated leaves as early as 24 hrs after inoculation even though no symptoms could be detected, implying that viral RNA synthesis is not the primary cause of mosaic symptoms in host tissue. In addition, the presence of rr-RNA in tissue harvested 1 and 3 days after inoculation (inoculated leaves) appeared to be localized in small areas, which would be expected assuming a random distribution of infectious virions by mechanical inoculation. The fact that isolated chloroplasts prepared from infected tissue contained rr-RNA, eliminates the possibility that localization of the rr-RNA in the cytoplasm or in close proximity to the surface of chloroplasts would explain all of the fluorescence seen in paraffin sections.

It appears that at least part of the resistance of rr-RNA to attack by pancreatic ribonuclease is due to the presence of one or more proteins in close association with the rr-RNA. Pretreating paraffin sections with protease resulted in a partial decrease in rr-RNA, which may be expected if RNA transcription is still occurring and only some of the nascent RNA is being coated by newly formed viral protein. The results with protease do, however, raise some interesting questions as to virus assembly and

mechanisms by which uncoated or nascent RNA is protected from nuclease attack in the host cell. The protein or proteins involved in imparting nuclease resistance to viral RNA may be viral or host. If it is viral, it may or may not be the coat protein, and in any case, may provide evidence for complete virus assembly in the chloroplasts. If the protein(s) is host in its origin, its presence or absence may be a factor in host specificity of the virus, and resistance to infectivity in the host.

The results obtained with the various infectivity assays substantiate the hypothesis that chloroplasts are the site of viral replication. In crude assays of organelle fractions, infectivity was associated only with the chloroplast fraction and the supernatant, but no other organelles. With washed chloroplasts, the results are dramatic. Treating the washed chloroplasts with a hypotonic buffer, which causes the plastids to swell markedly, greatly increases infectivity. Even though there is contamination of these preparations with other organelles and cell debris, the results indicate that the virus or infectious RNA is located in an organelle. To avoid such contamination, the chloroplasts were further purified on discontinuous sucrose density gradients. No infectivity was detected with chloroplasts treated this way. The problem, however, seemed to be in the technique, since modification of the procedure restored some measure of detectable infectivity in a pure chloroplast fraction treated with a hypotonic buffer.

It is interesting that infectivity assays performed with chloroplasts isolated from leaf tissue harvested 3 days after inoculation were negative. Even using the modified isolation procedure, no infectivity was ever detected. This may indicate that the production of coat protein and

subsequent assembly of virus particles is very slow and inefficient, while the opposite is true of RNA transcription. Since, at the time of inoculation, the inoculated leaves are fully expanded and mature, the chloroplasts are also physiologically mature, and may be less efficient in protein synthesis, or resistant to alteration by the presence of viral RNA. RNA transcription is not necessarily dependent upon chloroplast machinery and could continue as long as raw materials were supplied from the cytoplasm. This would explain the presence of rr-RNA in 3 day chloroplasts, as well as the inability to obtain infectious entities and complete lack of symptoms in inoculated leaves.

Since symptoms do not occur in inoculated leaves, but only in young, newly formed leaves, there is another bit of indirect evidence that may implicate chloroplast involvement in viral synthesis. In general, the titer of MDMV in infected corn tissue is quite low, and the most efficient purification techniques employ low molarity buffers for grinding, and chloroform for clarification (Hill et al., 1972). Both of these treatments degrade chloroplast membranes, which would release virus particles if present.

The results obtained from studies involving pigment-deficient mutants are quite significant. The presence of rr-RNA in the degenerate plastids of the albino mutant infected with MDMV further substantiates plastid involvement. The indication that etiolation may increase infectivity is further evidence, considering that Bachmann et al. (1969) report that the plastids of etiolated mutants have similar structure and pigments as etiolated normal plants, and, presumably, the more normal the plastids, the better able they are to support viral infection. The results of

etiolation experiments also indicate that viral replication, if it does occur in chloroplasts, may be independent of photosynthesis, which is not biologically unreasonable, and agrees with results of other workers (Bawden and Roberts, 1947; Matthews, 1953).

Based upon evidence obtained in this study and that presented and discussed by other workers in earlier papers (Boardman and Zaitlin, 1958; Doke, 1972; Esau and Cronshaw, 1967; Matsushita, 1965; Pratt, 1967; Ralph and Wojcik, 1969; Sela and Kaesberg, 1969; Singer, 1972; Ushiyama and Matthews, 1970), a generalized hypothetical replication cycle of maize dwarf mosaic virus can be presented. After the virus particle enter the host cell, either by insect feeding or mechanically, it is partially or entirely uncoated in the cytoplasm. The RNA, protected in some manner from nuclease attack, probably by some remaining coat protein, migrates to and enters a chloroplast. RNA transcription begins and the nascent RNA becomes coated, or at least associated in some way with a protein which protects it from nuclease attack. This protein is probably coat protein which is being synthesized in the chloroplast. If the chloroplast is mature, very little coat protein is produced and the RNA translocates to the shoot apex, where it enters the developing chloroplasts of newly formed leaves. These chloroplasts are very efficient in synthesizing viral coat protein and the translocated RNA plus any nascent RNA is coated to form intact viral particles. These young chloroplasts are so altered by viral protein synthesis that their abnormalities result in measurable disease symptoms. The altered chloroplasts swell, begin to break down with senescence and release intact virions to the cytoplasm and eventually to the phloem, where they are picked up by a feeding insect, which completes the

replication cycle.

A study of this nature will naturally raise more questions as to the actual events of virus replication. Some of the more obvious, concern the quantities of rr-RNA produced in chloroplasts in relation to the amount of virus obtained from infected tissue, and the nature of the protein or proteins which impart nuclease resistance to the rr-RNA.

Besides the questions that are raised by this study, there are some potential benefits. The pigment-deficient mutants used in this study, which were shown to support virus infection, may provide model systems for the further study of virus-host interactions. Another benefit which may result from the proof that chloroplasts are the actual site of viral replication and assembly, is the development of cell-free systems for the replication or assay of plant viruses.

SUMMARY

Maize dwarf mosaic virus (MDMV) was shown to replicate in a series of pigment-deficient mutants of corn. Of nine mutants tested, six supported replication of the A strain of MDMV, and eight supported replication of the B strain. Etiolated mutants were shown to be more susceptible to MDMV than those grown in light. Because of these findings, an attempt was made to determine what role chloroplasts play in MDMV replication.

Healthy and MDMV infected normal corn leaf tissues were fixed in Formalin-acetic acid, embedded in Tissuemat, sectioned, and treated with either 0.1% pancreatic ribonuclease (in H_2O, pH 6.8) or distilled H_2O (pH 6.8) at 37°C for 4 hr. The sections were stained with 0.1% acridine orange and viewed in ultraviolet light with a fluorescence microscope. All RNA fluoresced orange-red. In healthy tissue, treatment with ribonuclease removed all fluorescent RNA from chloroplasts and cytoplasm. Infected tissue, however, even 1 day after inoculation, showed the presence of ribonuclease-resistant RNA (rr-RNA) in the chloroplasts, primarily in the bundle sheath parenchyma cells. This rr-RNA is presumed to be viral RNA. Ribonuclease-resistant RNA was also shown to be present in the degenerate plastids of infected albino mutant tissue prepared in a manner similar to the normal corn. Chloroplasts isolated from healthy and infected normal tissue, when treated and stained in a similar manner, reacted in the same way as paraffin sections. Pretreating paraffin sections with protease (12 hrs at 37°C) prior to treatment with RNAase and staining, revealed that one or more proteins may impart nuclease

resistance to the rr-RNA.

Infectivity assays performed on organele fractions of infected corn tissue resulted in the association of infectivity with the chloroplast fraction only. Chloroplasts were subsequently isolated from infected leaves, washed several times, and treated with either a hypotonic or isotonic buffer. The infectivity of the hypotonic buffer in which infected chloroplasts were treated, was 30 times greater than the isotonic buffer. Hypotonic buffer treatment of washed chloroplasts which were further purified by discontinuous sucrose density gradient centrifugation, also showed higher infectivity than the isotonic treatment.

The results of this study implicate the chloroplast as the site of viral RNA synthesis, and possibly protein synthesis and virus assembly. This evidence, as well as that reported by other workers, form the basis for a hypothetical life cycle for maize dwarf mosaic virus, which is discussed in detail.

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