Techniques

Murine Monoclonal Antibodies Produced Against Two Illinois Strains of Barley Yellow Dwarf Virus: Production and Use for Virus Detection

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ABSTRACT


A hybridoma cell line that secretes antibodies produced against an Illinois strain of barley yellow dwarf virus (BYDV) transmitted nonspecifically by Rhopalosipnum padi and Sitobion avenae (BYDVP-BAV) and two lines that secrete antibodies produced against an Illinois strain transmitted specifically by R. padi (BYDVP-RPV-IL) were produced by somatic cell fusion between mouse myeloma cell line SP2/0-Ag14 and spleenocytes from BALB/c mice immunized with either virus strain. Ascitic fluid produced by clones PAV-IL-1, RPV-IL-1, and RPV-IL-5 had titers of $10^6$, $10^8$, and $10^9$, respectively, in triple antibody sandwich enzyme-linked immunosorbent assay (TAS-ELISA) with sap extracted from virus-infected plants. All three ascitic fluids had titers of $10^9$ in ELISA with microtiter plates coated at pH 9.6 with purified virus for 2 hr at 37°C. Immunoglobulin subclasses were IgG1 for PAV-IL-1 and RPV-IL-1 and IgG2a for RPV-IL-5, all with kappa light chains. Each of the antibodies was used successfully to trap homologous virus in double-antibody sandwich ELISA. In TAS-ELISA, clone PAV-IL-1 detected 17 of 17 PAV-like isolates in dried tissue from four U.S. states and 10 other countries. Clones RPV-IL-1 and RPV-IL-5 both detected seven of seven RPV-like isolates in dried tissue from three states and three other countries. None of the clones detected any of 13 other BYDV isolates tested.

Barley yellow dwarf (BYD) is produced by several virus strains which were differentiated originally by vector specificity (10,22). On the bases of cytopathology (11), serology (1,23), dsRNA profile (9), and nucleic acid hybridization (2,26), the strains have been divided into groups I and II by the International Committee on Taxonomy of Viruses (18) and into two viruses in the Description of Plant Viruses by the Association of Applied Biologists (27). Two strains of barley yellow dwarf virus (BYDV) have been studied in Illinois: a vector-nonspecific strain transmitted by both Rhopalosipnum padi L. and Sitobion (Macrosiphum) avenae Fabr., designated BYDVP-BAV, and a strain transmitted specifically by R. padi, designated BYDVP-RPV-IL. BYDVP-BAV is a member of BYD group I, and BYDVP-RPV-IL is a member of group II.

BYD viruses belong to the luteovirus group of plant viruses (27). Luteoviruses occur in low concentrations in host plant tissue and are difficult to purify, probably because they are phloem limited. For these reasons, production of antiserum to BYDV strains has been difficult, and the worldwide supply is limited. Monoclonal antibodies provide a potentially unlimited supply of uniform antibody of defined specificity. Monoclonal antibodies
to some strains of BYDV have been produced and used for virus detection (8,14,21,24). However, none of the monoclonal antibodies produced to BYDV strains from North America is currently widely available. The objective of this research was to produce hybridomas secreting monoclonal antibodies useful for detection of BYDV strains and for examination of relationships among luteoviruses. The latter aspect is reported elsewhere (7).

MATERIALS AND METHODS

Viruses and purification. The virus strains used for immunization were BYDV-PAV-IL (6,12) and BYDV-RPV-IL (19,20). Purification procedures were as previously described (6,20). The origins and designations of BYDV strains and isolates used to test the specificity of cloned monoclonal antibodies are listed in Tables 1-3.

Cell lines and media. SP2/0-Ag14, a non-immunoglobulin-secreting mouse myeloma, was cultured in Dulbecco's Modified Eagle's Medium containing l-glutamine (Sigma Chemical Co., St. Louis, MO) with 4,500 mg/L of glucose and 10% fetal bovine serum (Cibeco, Grand Island, NY) as a base medium, containing 20 mg/ml of 8-azaguanine (Sigma Chemical Co.), for 10 days before lymphocyte cell fusion. Cells were cultured in a Steri-Cult Incubator (Forma Scientific, Marietta, OH) which maintains 7.2% CO_2 in an air, 98% humid, 37 C atmosphere.

Production of hybridomas. Female BALB/c mice 8-9 wk old were immunized by intraperitoneal injection with 45-75 μg of purified virus at each injection. Injection volumes were 100 μl of a 1:1 emulsification of virus in complete Freund's adjuvant (Difco Laboratories, Detroit, MI) for the primary injection and incomplete Freund's adjuvant for the following two booster injections. Immunizations were at intervals of 3-4 wk. Blood samples were collected 5 wk after the first injection by a retro-orbital venous plexus bleed using a 100-μl disposable micropet. The blood was allowed to clot at 4 C and centrifuged, and the sera were screened for specific circulating antibody using a triple antibody sandwich enzyme-linked immunosorbent assay (TASS-ELISA). Mice that were producing virus-specific antibodies were sacrificed and aseptically splenectomized, and splenocytes were isolated for use in polyethylene glycol-mediated lymphocyte cell fusion (15). Fused cells were plated in HAT medium, which contains hypoxanthine, aminopterin, and thymidine, as per Kennett et al (15) in 24-well sterile culture plates (Corning Glass Works, Corning, NY) and grown at 7.2% CO_2 in air atmosphere. After 2-3 wk, HAT-resistant colonies were selected and scored for antivirus activity. Positive cultures were expanded to be frozen; cultures of particular interest were cloned by limiting dilution (1 cell/well) to assure monoclonality. Clones were screened, and positive clones were expanded for freezing and production of ascitic fluid.

Production of ascitic fluid. Female BALB/c mice 10-12 wk old were injected intraperitoneally with 0.5 ml of 2,6,10,14-tetramethylpentadecane (Pristane, Sigma Chemical Co.) 7 days before receiving an intraperitoneal injection of 2 x 10^7 virus antibody secreting hybridomas suspended in 1.0 ml of phosphate-buffered saline (PBS), pH 7.2 (13). Ascitic fluid developed 2-3 wk after cell injection, and fluid was collected every other day for a period of 1 wk by intraperitoneal paracentesis using a sterile, 16-gauge hypodermic needle. Ascitic fluid was centrifuged at 8,000 rpm for 10 min. The cellular pellet was discarded, and the supernate was treated with 5% sodium dextran sulfate and 11.1% calcium chloride (2,25) to remove lipoproteins. After centrifugation at 10,000 rpm for 10 min, supernate was saved, and immunoglobulin was precipitated by 50% ammonium sulfate. Centrifugation at 10,000 rpm generated pellets which were resuspended in 1 x PBS, pH 7.2.

Assays. Culture fluids were tested for antibodies against BYDV-PAV-IL 27 and 33 days after fusion and for antibodies against BYDV-RPV-IL 15 and 17 days after fusion. Fluids were assayed by two types of ELISA on microtiter plates (Immuno 1, Dynatech Laboratories, Inc., Chantilly, VA). All reagents were 100 μl/well, except blocking steps which were 200 μl/well. In the first assay, plates were coated directly with purified virus (400 ng/ml in carbonate buffer, pH 9.6) for 2 hr at 37 C and blocked with

<table>
<thead>
<tr>
<th>BYDV isolate</th>
<th>Monoclonal antibody</th>
<th>Monoclonal antibody</th>
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</thead>
<tbody>
<tr>
<td>California</td>
<td>0.95†</td>
<td>0.01</td>
</tr>
<tr>
<td>Illinois</td>
<td>1.48</td>
<td>0.02</td>
</tr>
<tr>
<td>New York</td>
<td>1.21</td>
<td>0.01</td>
</tr>
<tr>
<td>North Dakota</td>
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<td>0.02</td>
</tr>
<tr>
<td>Australia-Victoria</td>
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<td>0.03</td>
</tr>
<tr>
<td>Canada-Y801</td>
<td>1.74</td>
<td>0.03</td>
</tr>
<tr>
<td>Canada-Y802</td>
<td>1.23</td>
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</tr>
<tr>
<td>England</td>
<td>0.98</td>
<td>0.04</td>
</tr>
<tr>
<td>Ethiopia-W9a-88</td>
<td>0.52</td>
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</tr>
<tr>
<td>Italy</td>
<td>0.97</td>
<td>0.01</td>
</tr>
<tr>
<td>Jordan-JW13-87</td>
<td>0.45</td>
<td>0.01</td>
</tr>
<tr>
<td>Jordan-JW43-87</td>
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<td>0.03</td>
</tr>
<tr>
<td>Morocco-M90-87</td>
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<td>0.01</td>
</tr>
<tr>
<td>South Africa-RSE</td>
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</tr>
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<td>0.02</td>
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<tr>
<td>Kenya</td>
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<td>0.03</td>
</tr>
<tr>
<td>Tunisia-TB3-87</td>
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</tr>
<tr>
<td>Uninoculated oats</td>
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<td>0.03</td>
</tr>
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<td>Buffer</td>
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<td>0</td>
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</table>

† Values are means of absorbances at A 405 of triplicate wells after overnight incubation at 4 C. Samples with mean values greater than twice the mean of the negative control (uninoculated leaves of Avena byzantina 'Coast Black') are considered positive.

<table>
<thead>
<tr>
<th>BYDV isolate</th>
<th>Monoclonal antibody</th>
<th>Monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>California</td>
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<td>1.03</td>
</tr>
<tr>
<td>Illinois</td>
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<td>1.08</td>
</tr>
<tr>
<td>New York</td>
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<td>1.27</td>
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<td>Australia-Victoria</td>
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<td>Canada-Classic</td>
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<td>Canada-Y803</td>
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<td>1.08</td>
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<td>Canada-Y804</td>
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<td>1.39</td>
</tr>
<tr>
<td>England</td>
<td>0.13†</td>
<td>1.69</td>
</tr>
<tr>
<td>Uninoculated oats</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Buffer</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

† Values are means of absorbances at A 405 of triplicate wells after overnight incubation at 4 C. Samples with mean values greater than twice the mean of the negative control (uninoculated leaves of Avena byzantina 'Coast Black') are considered positive.

Table 3. Isolates of barley yellow dwarf virus (BYDV) not detected in triple antibody sandwich enzyme-linked immunosorbent assay by monoclonal antibodies produced against two Illinois BYDV strains

<table>
<thead>
<tr>
<th>Group I: MAV-like</th>
<th>Group I: SGG-like</th>
<th>Group II: RMV-like</th>
</tr>
</thead>
<tbody>
<tr>
<td>California</td>
<td>New York</td>
<td>New York</td>
</tr>
<tr>
<td>New York</td>
<td>Canada-Y804</td>
<td>Australia-Queensland</td>
</tr>
<tr>
<td>Australia-Victoria</td>
<td>Canada-Y8516</td>
<td>Italy</td>
</tr>
<tr>
<td>Canada</td>
<td>Morocco-M90-87</td>
<td>England</td>
</tr>
<tr>
<td>Ethiopia-B9-88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* No samples had mean values greater than twice the mean of the negative control (uninoculated leaves of Avena byzantina 'Coast Black'); therefore, none are considered positive.
overnight at 4 C. Monoclonal antibodies purified from ascitic fluids were diluted 1:500 (RPV-IL-1 and RPV-IL-5) or 1:1,000 (PAYS-1) in PBS-T-M. Rabbit-anti-mouse (RAM) conjugate (Sigma Chemical Co.) was diluted 1:1,000 in PBS-T-M. Monoclonal antibodies and conjugate were each incubated for 2 hr at 37 C. Substrate (0.5 mg/ml) was incubated 1 hr at room temperature, then overnight at 4 C. Samples were considered positive when the mean absorbance of triplicate wells was greater than two times the mean absorbance of control wells (unincubated tissue).

RESULTS

Production of monoclonal antibodies. When hybridoma culture lines were assayed for production of monoclonal antibodies against each virus strain, 66 of 192 (34.4%) were positive for BVDV-BPV-1 and 10 of 48 (20.8%) were positive for BVDV-BPV-10. Two RPV lines and one PAV line that gave strong reactions with the homologous virus in infected rabbit sera were cloned, increased in ascitic fluid, and used in studies described herein. These monoclonal antibodies were designated PAYS-1, RPV-IL-1, and RPV-IL-5.

Characterization of monoclonal antibodies. Monoclonal antibody subclasses and titers determined with the homologous virus in ascitic fluids were determined in two assays. All reagents were used at 100 µl/well, except blocking steps which were 200 µl/well. In TAS-ELPlA, microtiter plates were coated with polyclonal IgG to trap virus from the ascitic fluid. In all assays, plates were blocked after coating with PBS-T plus 0.1% nonfat dry milk (PBS-T-M) for 1 hr at room temperature (approximately 25 C).

Electron microscopy. Purified BVDV-BPV-1 was pelleted, and equal amounts were resuspended in either phosphate buffer, pH 7.0, or carbonate buffer, pH 9.6, and incubated at 37 C for 2 hr. Drops of each virus preparation were placed on Parafilm, and allowed to dry. Glass beads were floated on the drops for 1 min. Grids were then moved sequentially to 2 drops of water to rinse them and were stained with 2% uranyl acetate by holding them with forceps and running 5 drops of stain over each grid. After drying on filter paper, grids were viewed in a Hitachi 600 electron microscope (Hitachi Instruments, San Jose, CA) operating at 75 kV.

Use of monoclonal antibodies as coating antibody. Purified IgG (1 µg/ml) from each of the three ascitic fluids was coated on microtiter plates (Linbro, Flow Laboratories, Inc., McLean, VA) either for 1 hr at 37 C or overnight at 4 C. Plates were blocked with PBS-T+M as above. Substrate was extracted from unincubated oat leaves and PAYS-1- or RPV-IL-infected oat leaves (1 g/3 ml PBS-T+M) was used for serial twofold dilutions which were added in duplicate wells and incubated at 4 C. Polyclonal conjugates were diluted 1:1,000 in PBS-T+M and incubated 2 hr at 37 C. Substrate (0.5 mg/ml) was incubated 1 hr at room temperature, then overnight at 4 C.

Detection of BVDV isolates. BVDV-infected and uninfected tissues, dried for shipment according to Lister et al (16), were requested from BVD researchers around the world. Only isolates of previously determined serotype were included in this study. Thirty-seven isolates were included (Tables 1-3): 17 PAYS-like, seven RPV-like, six MAV-like (transmitted specifically by S. avenae), four SVN-like (transmitted specifically by Schizophaeum graminum Rond.) and three RMV-like (transmitted specifically by R. maidis Fitch) (10,22). Detection of isolates was tested in TAS-ELPlA. Plates (Linbro) were coated with polyclonal IgG (1 µg/ml) homologous to the monoclonal antibody to be used for detection (1 hr, 37 C) and blocked with PBS-T+M as above. Samples were ground with mortars and pestles, first in liquid nitrogen, then in PBS-T+M (0.15 g/3 ml) and incubated in wells
mean absorbance in the PAV-IL-1 system. However, when its
mean absorbance (0.13) was compared with the mean absorbance
(0.09) of uninoculated oats of the same cultivar, A. sativa L.
'Vulcanise,' the sample was rated negative for PAV. None of
the three Illinois monoclonal antibodies was able to detect any
of the heterologous BYDV isolates tested, which included MAV-
like, RMV-like, and SVG-like isolates (Table 3).

**DISCUSSION**

Although monoclonal antibodies against North American
strains of BYDV have been produced (8,14), none is currently
widely available for use in large-scale projects such as breeding
or epidemiological studies. We report production of monoclonal
antibodies to strains of the two most commonly detected serotypes
of BYDV in North America: PAV and RPV. The monoclonal
antibodies are capable of detecting a wide range of isolates of
these serotypes both from North America and from many other
parts of the world.

When used at dilutions of at least 1,000-fold, all three
monoclonal antibodies had excellent signal-to-noise ratios for
detection of homologous virus in sap extracts. At dilutions of
100-fold, noise levels, that is, background in sap extracts from
uninoculated plants, were unacceptably high for all three
monoclonal antibodies. This same phenomenon has been noted
in other luteovirus TAS-ELISA systems (17) and is probably
due to nonspecific binding of IgG to the microtiter plates at high
IgG concentrations. Compared with DAS-ELISA systems used
in our laboratory for detection of BYDV strains, the TAS-ELISA
systems reported here have threefold to fivefold higher signal-
to-noise ratios. This improvement also has been reported for other
BYD monoclonal antibody systems (21). Because the monoclonal
antibodies were able to detect their homologous viruses at
antibody dilutions several orders of magnitude greater than 10¹,

they may be useful for detection of very low concentrations of
virus in infected tissue.

None of the three BYDV monoclonal antibodies reacted well
with virus that had been incubated at 37 C in the carbonate
buffer routinely used for coating microtiter plates. There is strong
evidence to indicate that BYDV strains, and probably other
luteovirus species, dissociate under these conditions (8). For example,
we have obtained similar results with BYDV-RPV-IL and with
potato leafroll virus (D'Arcy and Martin, unpublished data). It is
likely therefore that the BYDV monoclonal antibodies discussed
herein react with epitopes that are conformational and are lost
upon virion dissociation. The low level of reactivity remaining
after the virions were incubated 2 hr at 37 C in carbonate buffer
is probably due to reaction with particles that were incompletely
dissociated. The dissociation of BYDV strains in carbonate buffer
occurs quite rapidly during incubation at high temperatures and
more slowly at low temperatures. For example, when RPV-IL-1
and RPV-IL-5 were titrated against purified BYDV-RPV-IL
(50 ng/ml) coated in carbonate buffer overnight at 4 C, titers
were 10⁴ for both clones (Murphy and D'Arcy, unpublished data).
This suggests that, if plate-trapped virus is to be used in an ELISA
to screen for monoclonal antibodies, the temperature at which
coating is done will determine which monoclonal antibodies are
detected. Screening with virus coated at 37 C was used in an
attempt to detect monoclonal antibodies representing internal
virus epitopes (cryptoeptopes). No such monoclonal antibodies were
found.

All of the three monoclonal antibodies described herein were
used successfully to trap homologous virus in DAS-ELISA, most
efficiently when plates were coated overnight at 4 C. The apparent
inhibition of antigen binding by PAV-IL-1-coated antibody at high
sap concentrations is similar to that found for a U.K.
monoclonal antibody (MAC92) to an RPV-like isolate of BYDV (L.
Torrance, personal communication). MAC92 is more efficient as
a detecting antibody when sap is diluted 10⁴-10⁵-fold. Optimal
sap concentrations must be determined for each monoclonal
antibody used as a coating reagent.

Each of the monoclonal antibodies discussed herein has strong
cross-reactions with other luteoviruses (6; Murphy and D'Arcy,
unpublished data). PAV-IL-1 cross-reacts with potato leafroll
virus; RPV-IL-1 cross-reacts with bean leafroll virus; and both
RPV monoclonal antibodies cross-react with beet western yellos
virus. None of these cross-reactions will limit the usefulness
of the monoclonal antibodies for BYDV detection and diagnosis
in grass species because all the other luteoviruses detected infect
dicotyledonous species.

The use of monoclonal antibodies for detection and diagnosis
of luteoviruses has several advantages over polyclonal antisera.
Hybridoma cell lines provide a continuous supply of a defined
reagent. The clones described herein, which had been stored at
−80 C for almost 2 yr, recently have been regrown, and the
specificity and sensitivity of virus detection remain unimpaired.
A second advantage is the specificity of the reagents. Monoclonal
antibodies clearly differentiate among strains of BYDV within
groups I and II, where polyclonal antisera may not (21,23). Of
course, a panel of appropriate monoclonal antibodies will be
required for detection and diagnosis of all strains of BYDV present
in a location. There also is the possibility that monoclonal
antibodies may be so specific that they fail to detect some isolates
of the virus that lack the epitope to which they were made.
However, if a monoclonal antibody has been demonstrated to
recognize many known strains of the virus, as have the BYDV
monoclonal antibodies discussed herein, that possibility becomes
more remote.

**LITERATURE CITED**


3. Barbara, D. J., Kawata, E. E., Ueng, P. P., Lister, R. M., and Larkins,

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**TABLE 5. Reactions in triple antibody sandwich enzyme-linked
immunosorbent assay of monoclonal antibodies produced against
two Illinois barley yellow dwarf virus strains with extracts from leaves of
oats infected with the homologous virus strain or from uninoculated oats**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>PAV-IL-1</th>
<th>Control</th>
<th>PAV-IL-1</th>
<th>Control</th>
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<tbody>
<tr>
<td>Dilution</td>
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<td></td>
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<tr>
<td>10⁻²</td>
<td>&gt;2.00</td>
<td>1.51</td>
<td>0.64</td>
<td>0.30</td>
<td>0.97</td>
<td>0.62</td>
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<td>10⁻³</td>
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<td>10⁻⁴</td>
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<td>0.00</td>
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</tbody>
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*Extracts were 1 g tissue/3 ml of phosphate-buffered saline-Tween
plus 0.1% nonfat dried milk.
Values are mean of absorbances at A₄⁵⁰ of duplicate wells for each
sample after overnight incubation at 4 C.

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**Fig. 1.** Electron micrographs of purified barley yellow dwarf virus. PAV-
IL strain incubated in A, phosphate buffer, pH 7.0, or B, carbonate
buffer, pH 9.6, for 2 hr at 37 C and stained with 2% uranyl acetate.
×150,000.

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380 PHYTOPATHOLOGY


