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THE UNIVERSITY OF ALBERTA

Seed and pollen transmission of alfalfa mosaic virus in alfalfa

by

Zvezdana Pesic

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled Seed and pollen transmission of alfalfa mosaic virus submitted by Zvezdana Pesic in partial fulfilment of the requirements for the degree of Doctor in Philosophy in Plant Pathology.

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To my parents

Abstract

Double sandwich enzyme-linked immunosorbent assay (ELISA) and dot hybridization technique using a ^{32}P-labeled cDNA probe were compared for detecting alfalfa mosaic virus (AMV) in alfalfa pollen, purified virus preparations and purified virus added to extract from virus-free pollen. Dot hybridization was about ten times more sensitive than ELISA in detecting purified AMV, but the sensitivity was similar to that of ELISA in detecting AMV in crude extract from infected pollen and AMV added to the extract from virus-free pollen.

AMV was detected by ELISA in seeds, seedlings, seed coats and embryos of alfalfa cv. Beaver. Rate of AMV incidence in seed (20.6%) was significantly higher than in seedlings (7.3%). AMV was more frequently detected in seed coats than in embryos. The difference in detection levels in seeds and seedlings was related to the higher incidence of AMV in the seed coats and the lower incidence in embryos.

AMV was detected in alfalfa gametes by immunogold cytochemistry. AMV antigen was found in the cytoplasm and vacuoles of ovule integuments, and in the cytoplasm of microspores, mature pollen grains and anther tapetum cells. Raft-like aggregates of virus particles and large crystalline bodies were observed in the cytoplasm of pollen grains and anther tapetum cells, while non-aggregated virions were detected in the vacuoles and the cytoplasm of ovule integument cells.

The significant reduction in germination was observed in pollen from infected plants compared to pollen from virus-free plants of clones B-19, B-24 and B-39, after 30, 60, 90, and 120 min. Within each clone pollen from infected plants produced significantly shorter tubes than pollen from virus-free plants.

A rapid and sensitive method was developed for screening alfalfa for resistance to AMV. It was used to identify genotypes with the hypersensitive response to AMV in cv. Beaver. Out of 28 clones tested, 12 responded with the hypersensitive reaction and 16 with the systemic infection.

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1. INTRODUCTION

1.1 Alfalfa

Alfalfa (*Medicago sativa* L.) originated in southwest Asia, with Iran as the geographic center of origin (Bolton et al. 1972). It has become widely adapted in North and South America, South Africa, Australia, New Zealand, and Europe, and represents the most important forage legume crop on a world-wide basis (Bolton et al. 1972, Klinkowski 1933). In Canada alfalfa is grown in pure and mixed stands on an estimated area of 4 to 5 million ha (Goplen et al. 1982).

The alfalfa seed industry is centered in western Canada, with Alberta, Saskatchewan and Manitoba as the main producers of certified seed. Cross-pollination by bees is essential for attaining high seed yields. The introduction of the alfalfa leafcutting bee, *Megachile rotundata* of Euroasian origin, to replace the declining native species, has revived alfalfa seed industry in Canada. Thus, the seed production has doubled in the past twenty years to 3.5 million kg, with Alberta alone, producing 2 million kg of certified alfalfa seed (Anon. 1987).

1.2 Floral morphology

Alfalfa flower has a unique morphology and tripping mechanism. Flowers grow from leaf axis in clusters of 10 to 20 florets. They are usually blue or purple (*M. sativa*) but may be white or yellow (*M. falcata*). The pistil consists of a single carpel that develops a superior ovary. Campylotropous ovules are formed in alternate positions along the ventral suture of the ovary. The number of ovules per ovary may range from 6-18 (Barnes et al. 1972). The 10 stamens form a diadelphous tube in which 9 are fused and 1 is free. Alfalfa pollen is binucleate with three germination pores. The pollen

germinates in a stigmatic fluid and fertilization of alfalfa ovules occurs in 24-36 hours (Barnes et al. 1972).

1.3 Alfalfa mosaic virus

Alfalfa mosaic virus in the only member of its own group. Virus particles are built from a single polypeptide, MW 24.3×10^3, and positive-sense single stranded RNA (Hull 1969, Jaspars 1974).

Electron microscopic examination of purified virus preparations revealed the presence of a variety of particles, ranging from polyhedral, 18nm in diameter to bacilliform 56, 43, 35 and 30 nm long (Hull 1969, Jaspars & Bos 1980). Four components of AMV were separated by analytical ultracentrifugation: bottom (B), middle (M) and two top components (Ta and Tb), with the MWs of 6.9×10^6 (B), 5.2×10^6 (M), 4.3×10^6 (Tb) and 3.8×10^6 (Ta)(Jaspars & Bos 1980).

The tripartite genome of AMV consists of RNAs 1,2, and 3, with MWs of 1.04×10^6, 0.73×10^6, and 0.62×10^6, encapsidated in B, M and Tb particles, respectively. The subgenomic mRNA (MW 0.25×10^6) for coat protein found in Ta is encapsidated two molecules per particle (Heijtink & Jaspars 1974, Gerlinger et al. 1977). In plant cells, inoculated with four RNAs, RNA 4 is translated into coat protein that activates the genome (Bol et al. 1971). However, the activation of genomic RNA by the coat protein is not AMV specific. The coat protein of tobacco streak virus (Ilarvirus) which is serologically and chemically different from AMV protein activated AMV genomic RNAs (van Vloten-Doting 1975).

Virus particles are mainly stabilized by protein-RNA interactions, and dissociate at low concentrations of sodium dodecyl sulphate (Kaper 1973), or high concentrations of salt (Bol & Kruseman 1969). In the presence of

ribonuclease the particles lose RNA fragments and degrade into smaller structures (Bol & Veldstra 1969)). AMV-RNA has the capability of removing coat protein subunits from intact nucleoprotein particles (Verhagen et al. 1976).

AMV is moderately immunogenic with antibody titers of up to 1/1024 (Bancroft et al. 1960), and is not serologically related to any other virus (Jaspars & Bos 1980).

In crude sap, AMV has a dilution end-point of 10^{-3}-10^{-4}, thermal inactivation point between 50 and 70 C, and longevity *in vitro* from 1 to 4 days (Jaspars & Bos 1980).

1.4 Effect of AMV on its hosts

AMV was first reported by Weimer (1931, 1934) in the US and has been found since in most parts of the world. It occurs naturally in 150 species of herbaceous and woody plants belonging to 22 families (Schmelzer et al. 1973). Under experimental conditions the virus has been transmitted to over 430 plant species (Hull 1969, Schmelzer et al. 1973). AMV causes diseases of economic importance in alfalfa, clover, pea, potato, tobacco, pepper, tomato and celery (Jaspars & Bos 1980). Numerous strains of AMV that vary in host range, symptomatology and pathogenicity have been characterized: alfalfa virus 2 (Pierce 1937), alfalfa mosaic virus 1 (Zaumeyer 1938), potato calico strain (Black & Price 1940), celery mosaic strain (Snyder & Rich 1942), pepper strain (Berkeley 1947), potato necrosis strain (Oswald 1950), alfalfa yellow mosaic (Zaumeyer 1953), severe red clover strain (Hagedorn & Hanson 1963), and tobacco strain (Silber & Hegestad 1965).

The adverse effect of AMV on yield and growth of alfalfa was reported by several investigators (Baillis & Ollennu 1986, Crill et al. 1970a, Frosheiser 1969, Gates & Bronskill 1974, Gibbs 1962, Ohki et al. 1986, Tu & Holmes 1980). Working with different cultivars and AMV strains they found a variability in responses of alfalfa genotypes to AMV infection, ranging from masked and mild symptoms to severe stunting. Frosheiser (1969) and Crill et al. (1970b) reported that selected clones of alfalfa were resistant or tolerant to some isolates of AMV.

In infected leaves, AMV particles were found randomly distributed or in aggregates throughout the cytoplasm (de Zoeten & Gaard 1969, Hatta & Francki 1981, Hull et al. 1970, Wilcoxson et al. 1974). and in vacuoles (de Zoeten & Gaard 1969, Hull et al. 1970). There is no conclusive evidence regarding the presence of virus particles in cell organelles. However, the inhibitory effect of actinomycin D on the synthesis of host-directed RNA, and the incorporation of ^{3}H-labeled uridine into the nuclei and cytoplasm suggest organelle involvement in AMV-RNA synthesis (Bassi et al. 1970). Recently, the coat protein of AMV strain 425 was detected by immunocytochemistry in the cytoplasm and nuclei of infected tobacco leaves (van Pelt-Heerschap et al. 1987). The presence of a 32 K non-structural protein of AMV, P3, in the cell wall of infected tobacco cells suggested its role in facilitating cell-to-cell transport of AMV (Godefroy-Colburn et al. 1986).

1.5 Epidemiology of AMV

AMV is transmitted by at least 14 species of aphids in the nonpersistant manner (Crill et al. 1970c), mechanically by cutting equipment (Hiruki & Miczynski 1987), through seed (Belli 1961, Frosheiser 1964, 1970, 1974,

Hemmati & McLean 1977, Hiruki & Miczynski 1987, Tosic & Pesic 1975, Zschau & Janke 1962), via pollen and ovules (Frosheiser 1974, Hemmati & McLean 1977). Depending on the alfalfa cultivar and virus strain, transmission rates of AMV varied up to 55 % (Hiruki & Miczynski 1987, Zschau & Janke 1962) in commercial seed.

The high incidence and lasting longevity of AMV in seeds allows for primary infection from infected seeds (Frosheiser 1974, Hemmati & McLean 1977). Subsequent transmission of AMV within the field is by aphids with *Acyrthosiphon pisum* and *Myzus persicae* as the most efficient vectors (Crill et al 1970c; Frosheiser 1969). Thus, in 3- and 4-year-old stands, up to 80% of alfalfa plants were infected with AMV (Crill et al. 1970, Frosheiser 1964, Gates and Bronskill 1974, Gibbs 1962, Mueller 1965).

1.6 Objectives

The objectives of this thesis were to study: 1) incidence and localization of AMV in alfalfa seed, pollen and ovules, 2) mechanism(s) of AMV transmission to seed through pollen and ovules, 3) Effect of AMV on the germination and growth of alfalfa pollen, and 4) response of selected alfalfa genotypes to infection with AMV.

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2. COMPARISON OF ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) AND DOT HYBRIDIZATION FOR DETECTION OF ALFALFA MOSAIC VIRUS IN ALFALFA POLLEN [1]

2.1 Introduction

Enzyme-linked immunosorbent assay (ELISA) (Clark & Adams 1977) and dot hybridization technique which utilizes a ^{32}P-labeled complementary DNA (cDNA) probe (Owens & Diener 1981, Maule et al. 1983) have wide applications as diagnostic procedures in plant virology.

The most commonly used serological assay, ELISA, has been particularly useful for testing large numbers of individual seeds and/or seedlings for the presence of seed-borne viruses: tobacco ringspot and soybean mosaic (Lister 1978), cherry leaf roll (Cooper et al. 1984), prune dwarf (PDV) and prunus necrotic ringspot (PNRSV) (Kelley & Cameron 1986), blueberry leaf mottle (BBLMV) (Childress & Ramsdel 1986) and alfalfa mosaic (AMV) (Pesic & Hiruki 1986). Rapid and sensitive in quantifying viral antigen (Roggero & Pennazio 1984, Burrows et al. 1984), this method has also been used for localizing viruses associated with pollen, such as PNRSV (Cole et al. 1982, Hamilton et al. 1984, Kelley & Cameron 1986), PDV (Kelley & Cameron 1986), and BBLMV (Childress & Ramsdel 1986). Although nucleic acid hybridization has been applied for detecting both RNA and DNA viruses in plant tissue (Garger et al. 1983,Maule et al. 1983, Sela et al. 1984), it has a major application in diagnosis of diseases caused by viroids which are

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undetectable by immunological tests (Owens & Diener 1981, Flores et al. 1985, Lakshman et al. 1986, Schwinghamer & Scott 1986).

Previous comparison of dot hybridization and ELISA for detecting tobacco mosaic virus (TMV) in plant tissue and protoplasts indicated that the sensitivity of the former method was about twice as high as that of ELISA (Sela et al. 1984). The present investigation was undertaken to compare ELISA and dot hybridization for quantifying AMV in purified preparations and in plant tissue, and the applicability of these two methods for detecting AMV in small amounts of alfalfa pollen. Assays of alfalfa pollen are considered to be important due to the fact that AMV is transmitted to embryos of alfalfa seed through pollen (Pesio et al. 1988).

2.2 Materials and Methods

2.2.1 Virus

AMV isolate A-515 was propagated in *Nicotiana tabacum* L. cv. White Burley in the greenhouse at 23 C, and purified as described by van Vloten-Doting & Jaspars (1972).

2.2.2 Source of pollen

Pollen samples were collected from mature, untripped flowers of clone B-24 of alfalfa cv. Beaver. AMV-infected and virus-free clonal plants that served as a control were maintained in the greenhouse at 20 C, and a 16 h photoperiod. Plants were assayed by ELISA for the presence of AMV at 4-wk intervals. Pollen samples for ELISA and dot hybridization were prepared from the same pollen lot.

2.2.3 ELISA procedure

Double sandwich ELISA (Clark & Adams 1977) was used for AMV detection. ELISA was performed in flat bottom polystyrene microtest plates (Flow Laboratories Inc. McLean, Virginia). Plates coated with the γ-globulin fraction of anti A-515 antiserum (Pesic et al. 1988), cross-absorbed with virus-free plant antigen (da Rocha et al. 1986), in coating buffer (50 mM sodium carbonate, pH 9.6, containing 0.02% NaN_3) were incubated for 4 h at 37 C. After rinsing in 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20 and 0.02% NaN_3, 200 µl of sample preparations were added to each well. All samples were prepared in 0.01 M PBS-Tween, pH 7.4, containing 1% polyvinyl pyrrolidone 40 (PVP 40) and 0.1% ovalbumin (PBS-Tween-PVP-ovalbumin) (Sigma). The plates with samples were incubated for 16 h at 4 C and then washed in PBS-Tween. Alkaline phosphatase-conjugated γ-globulin diluted in PBS-Tween-PVP-ovalbumin was added 200 µl/well. Unless stated otherwise, the coating γ-globulin and enzyme-conjugated γ-globulin were used at a concentration of 2 µg/ml and 1/1000 dilution, respectively. Following incubation for 4 h at 37 C, the plates were washed in PBS-Tween and 200 µl of freshly prepared substrate (0.8 mg of p-nitrophenyl phosphate/ml of 10% diethanolamine-HCl, pH 9.8) was added to each well and incubated for 1 h at 37 C. The reaction was stopped by the addition of 50 µl/well of 3 N-NaOH and the absorbance of each reaction mixture at 405 nm was determined in a Titertek Multiscan photometer. All tests were replicated four times. The ELISA values were considered positive if the mean minus standard deviation (x-SD) was greater than the mean plus standard deviation (x+SD) of virus-free control values.

2.2.4 Isolation of AMV-RNA

Total AMV-RNA was isolated according to Gould (1981) and Maniatis et al. (1982). To 1 ml purified AMV (5.5 mg), 1 ml of 20 mM Tris buffer containing 1 mM ethylenediaminetetraacetate (EDTA), and 100 mM NaCl, pH 8.5, 1 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline and 200 µl of 10% sodium dodecyl sulfate (SDS) were added. The mixture was emulsified at room temperature and following centrifugation, the aqueous phase was re-extracted twice with an equal volume of phenol and once with chloroform and isoamyl alcohol (24:1, v/v) to remove protein from the preparation of nucleic acids. Traces of phenol and chloroform were removed with water-saturated ether. After adjusting the salt concentration to 200 mM with 3 M sodium acetate, pH 6.0, RNA was precipitated with 2.5 volumes of cold ethanol for 30 min at -70 C. The final pellet was resuspended in 10 mM Tris-Cl and 1 mM EDTA (TE), pH 8.0, and stored at -70 C.

2.2.5 Synthesis of cDNA

The cDNA probe to AMV-RNA was synthesized by the method of Taylor et al. (1975) and Maniatis et al. (1982). The reaction mixture of a final volume of 50 µl contained 5 µg Actinomycin D, 0.5 mM each of dATP, dGTP and dTTP, 125 µg of calf thymus deoxynucleotide primers, 20 mM dithiothreitol, 50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 100 mM KCl, 5 mM magnesium acetate, 2 µg of total AMV-RNA, 50 units of avian myeloblastosis virus reverse transcriptase (Pharmacia, Lot MM 92109) and 80 µCi (^{32}P) dCTP (sp. act. 3000 Ci/mol) (New England Nuclear). The mixture was incubated at 37 C for 2 h and the reaction was stopped by the addition of 5 µl of 5% SDS, 20 µl of 3 N NaOH and 125 µl of sterile water. The cDNA

was precipitated with ethanol using yeast tRNA as a carrier and the salt concentration was adjusted to 200 mM with 4 M ammonium acetate. Prior to hybridization, the radioactivity incorporated into ^{32}P-labeled cDNA was measured as described by Maniatis et al. (1982).

2.2.6 Preparation of pollen samples for dot hybridization

Crude extract was obtained by grinding 30 mg of pollen with a pestle and mortar in 0.01 M phosphate buffer, pH 7.5, containing 0.1% SDS to remove AMV capsid protein (Sela et al. 1984). After centrifugation at 12000 g for 10 min at 4 C the total volume of the supernatant was adjusted to 50 µl.

Nucleic acids were extracted from 30 mg of pollen in 0.05 M glycine buffer, pH 9.3, containing 0.1 M NaCl, 0.01 M EDTA, 0.1% SDS, 25% phenol and 25% chloroform (Sela et al. 1984). Following centrifugation and repeated extraction with water-saturated phenol until the interphase appeared to be clear, nucleic acids were precipitated with ethanol and resuspended in 50 µl of TE, pH 8.0. Serially diluted samples (10^{-1}- 10^{-5}) of nucleic acids and crude extracts were assayed 5 µl/dot.

2.2.7 Dot hybridization

The dot hybridization method applied in this study was adapted from Maniatis et al. (1982), Garger et al. (1983) and Maule et al (1983). Samples were manually spotted 5 µl/dot on a biodyne transfer membrane (Pall Ultrafine Filtration Co., Glen Cove, N.Y.). Membranes were baked at 80 C for 2 h and prehybridized at 42 C for 18 h in plastic bags containing 50% formamide, 5xSSPE (1xSSPE=0.12 M NaCl, 0.015 M sodium citrate, 0.013 M NaH_2PO_4, pH 6.5, 2mM EDTA), 4x Denhardt's solution (1xDenhardt's= 0.02% each of bovine serum albumin, Ficoll 400 and PVP-40), 100 µg/ml of

denatured calf thymus DNA and 0.1% SDS. Hybridization was carried out at 55 C for 48 h in a solution containing 50% formamide, 5xSSPE, 1xDenhardt's solution, 10% dextran sulfate, 100µg/ml of denatured calf thymus DNA, 0.1% SDS and a ^{32}P-labeled cDNA probe. Unless stated otherwise a cDNA probe of 10^6cpm/ml was used for hybridization. Hybridized membranes were washed twice for 15 min at room temperature and twice at 55 C in 2xSSC (1xSSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), and 0.1% SDS, and four times at 55 C in 0.1xSSC and 0.1% SDS. Dried membranes were autoradiographed at -70 C for 48 h using XAR-5 Kodak X-ray film and two Dupont Cronex Hi Plus XA intensifying screens.

Radioactivity counts in the biodyne membrane dots were taken seven days after autoradiographic exposure. Individual dots were cut and immersed in 10 ml of scintillation fluid (Econofluor, New Englans Nuclear) in plastic vials. Counting was done in the scintillation counter (Isocap/300, Searle Analytic Inc.) over a period of 10 min, and radioctivity was expressed in cpm.

2.3. Results

2.3.1 Sensitivity of ELISA

To determine relative sensitivity of ELISA in quantifying AMV antigen, coating γ-globulin (1 and 2 µg/ml) and enzyme-conjugated γ-globulin (1/1000, 1/2000 and 1/4000 dilutions) were assayed in various combinations against purified AMV in concentrations ranging from 0.25-1000 ng/ml. Crude leaf homogenate from virus-free tobacco in the extraction solution (1:3,w/v) was used as a control.

The quantitative response over the concentration range of AMV tested

indicated that ELISA was responsive to both variation in γ-globulin concentration and enzyme-conjugated γ-globulin dilution (Fig. 2.1). The greatest sensitivity of the ELISA for AMV detection 1 ng/ml, was obtained with the coating γglobulin at concentration of 2 µg/ml and enzyme-conjugated γ-globulin at 1/1000 or 1/2000 dilutions (Fig. 2.1A), or at 1 µg/ml and 1/1000 dilution, respectively (Fig. 2.1B). An increased dilution of enzyme-conjugated γ-globulin, 1/4000, reduced the levels of detection of AMV antigen to 16 ng/ml with the coating γ-globulin in concentration of 2 µg/ml (Fig. 2.1A), and to 250 ng/ml with γ-globulin at 1 µg/ml (Fig. 2.1B).

2.3.2 Detection of AMV in alfalfa pollen by ELISA

The effect of sap constituents on sensitivity of detection of viral antigen in pollen was investigated using a ten-fold dilution series (10-1000 ng/ml) of purified AMV and the equivalent dilutions of purified AMV added to the crude extract from virus-free pollen. ELISA was ten times more sensitive in detecting AMV antigen in purified preparations (1 ng/ml) than in crude extracts (10 ng/ml) (Fig. 2.2).

Detection levels of AMV in pollen were determined by testing serial dilutions up to 10^{-4} of extract from 30 mg of infected pollen. Pollen from virus-free plants was used as a control. The results indicated that 10^{-3} was the highest dilution in which AMV was detectable in this study (Fig. 2.3). According to the standard curve for AMV (Fig. 2.3), a 10^{-3} dilution of infected pollen had about 10 ng of AMV (Fig. 2.3). We also established, by testing samples containing various amounts of pollen (1-5 mg), that 3 mg was the optimum amount required for the efficient isolation of viral antigen.

In order to test the applicability of ELISA for screening alfalfa pollen for the presence of AMV under the conditions of this investigation, samples of

3 mg of pollen (30 - 50 flowers) were collected at random from each of fifty alfalfa plants. AMV was detected in pollen from thirty-three plants with absorbance values for individual samples ranging from 0.3-0.7 (Fig.2.4). The absorbance readings for virus-free pollen were 0.015-0.045. The additional testing of leaf extracts showed that only plants from which infected pollen was collected were positive for AMV.

2.3.3 Sensitivity of dot hybridization assay.

To study the sensitivity of RNA template detection by ^{32}P-labeled cDNA hybridization, cDNA probes with final specific radioactivities of $0.5x10^6$cpm/ml and 10^6cpm/ml were compared. Ten-fold dilutions of purified AMV and purified AMV added to crude extract from virus-free pollen were assayed in concentrations of 1.5 µg, 150 ng, 15 ng, 1.5 ng 150 pg, 15 pg and 1.5 pg/spot, and purified AMV-RNA, 250 ng, 25 ng, 2.5 ng, 250 pg, 25 pg, 2.5 pg and 0.25 pg/dot. Crude extract from virus-free pollen was spotted as a control.

Although AMV-RNA was detectable in quantities of 2.5 pg in purified AMV and RNA preparations by both cDNA probes, differences between the two probes were observed in the intensity of hybridization (Fig. 2.5). The radioactivity (cpm) in the biodyne membrane dots was greater for all samples hybridized with the cDNA probe of 10^6cpm/ml than with the probe of $0.5x10^6$cpm/ml (Table 2.1). The assay was less sensitive in detecting viral RNA in preparations of crude pollen extracts and purified AMV (1.5 ng AMV/dot). Considerable reduction in hybridization or radioactivity was observed for the lowest dilution of crude homogenate containing 1.5 µg AMV/dot, however, at the higher dilutions the response was linear (Table 2.1).

2.3.4. Detection of AMV-RNA in alfalfa pollen by dot-hybridization

To test whether host components interfere with the sensitivity of detection of viral RNA in alfalfa pollen, series of dilutions (10^{-1}-10^{-5}) of crude and nucleic acid extracts from infected pollen were compared. The highest dilutions of pollen in which AMV-RNA was detected were 10^{-3} for crude and 10^{-4} for nucleic acid extract (Fig. 2.6). Differences in quantities of detectable AMV-RNA were also observed between the two pollen extracts for the equivalent dilutions. The radioactivity in the membrane dots indicated that nucleic acids and crude extracts at 10^{-3} dilution contained about 250 pg and 25 pg of AMV-RNA, respectively, corresponding to approximately 1.5 ng and 150 pg of AMV (Table 2.2).

Although dot-hybridization assay was ten times more sensitive in detecting AMV-RNA in nucleic acid than in crude extracts, the applicability of crude extracts for screening of alfalfa pollen for the presence of AMV was further tested because of the considerably faster and simpler procedure for sample preparation. Crude extracts from 3 mg of pollen collected from eight infected and four virus-free alfalfa plants were assayed with the cDNA probe of 10^6cpm/ml. AMV-RNA was detected in all samples of pollen from infected plants, but no reaction was observed in pollen from virus-free plants (Fig. 2.7).

2.4 Discussion

In this investigation we have evaluated and compared direct double sandwich ELISA and dot hybridization method using AMV-specific ^{32}P-labeled cDNA probe for detection of AMV isolate A-515 in purified preparations and in alfalfa pollen.

In ELISA, the sensitivity of detection of AMV antigen was enhanced by a few hundred times using the coating γ-globulin at 2 µg/ml and enzyme-cojugated γ-globulin 1/1000 dilution, compared to 1 µg/ml and 1/4000 dilution, respectively. Increased sensitivity of ELISA was also observed for other plant viruses with the coating antibody 1-2 µg/ml (Converse 1978, Romaine et al. 1981), and low dilutions of enzyme-conjugated IgG (Clark & Adams 1977, Lister & Rochow 1979, Hewings & D'Arcy 1984). The correlation between the sensitivity of ELISA and variation in coating and conjugated immunoglobulins established in this study, and previously reported for other virus-ELISA systems (Clark & Adams 1977, McLaughlin et al. 1981, Hewings & D'Arcy 1984) suggested that optimization of ELISA conditions should be done for each virus-host combination. Detection level of 1 ng/ml for purified AMV was consistent with those reported for TRSV (Lister 1978), SMV (Hill et al. 1981), carnation mottle and carnation ringspot viruses (Lommel et al. 1982), beet western yellows virus (Hewings & D'Arcy), and CLRV (Massalski & Cooper 1984). Lower levels of detectable antigen (10 ng/ml) in extract from virus-free pollen containing purified AMV, and in extract from infected pollen, provided evidence that the presence of sap constituents reduced the sensitivity of ELISA for detecting AMV in alfalfa pollen. In a similar study SMV was detectable at 2.5 ng/ml in purified preparations or added to virus-free leaf and seed extracts, however, detection levels for viral anigen in infected tissue were not reported (Hill et al. 1981).

In this investigation viral RNA was detected by dot-hybridization in quantities of 2.5 pg in purified RNA and AMV preparations. These results coincide with those previously reported for TMV-RNA (Sela et al. 1984). The removal of coat protein from intact virions with the SDS treatment

applied in this study enhanced binding of nucleic acid to the biodyne membrane. According to Hull (1984) baking of membranes also released viral RNA. Greater sensitivity of detection of viral RNA was found in nucleic acid extracts than in crude extracts from infected pollen and purified AMV added to extract from virus-free pollen, and this result indicated that the removal of sap constituents from plant tissue increased binding of viral RNA to the solid phase and its hybridization with the cDNA probe (Owens & Diener 1981, Maule et al. 1983).

The sensitivity of dot hybridization assay was about ten times greater than that of ELISA in detecting AMV in purified preparations, 15 pg and 200 pg, respectively. Both methods were less sensitive in detecting AMV added to the extract from virus-free pollen, with detection levels of 1.5 ng by dot hybridization and 2 ng by ELISA. Although in crude extracts from infected pollen a dilution of 10^{-3} was the highest in which AMV was detectable by either method, samples containing a few milligrams of pollen were required for virus isolation in testing of alfalfa pollen. Similar quantitaitve responses for AMV added to pollen extract and AMV in crude extracts from infected pollen suggested that both ELISA and dot hybridization are reliable and sensitive methods for screening of small amounts of alfalfa pollen for the presence of AMV. Pollen testing with highly sensitive techniques such as ELISA and dot hybridization ensures the use of AMV-free sources of pollen for alfalfa breeding programs.

Table 2.1. Radioactivity counts in individual dots containing purified AMV-RNA, AMV, AMV added to crude extract, or crude extract from virus-free pollen, hybridized with the AMV-specific cDNA probe

Sample	cDNA probe (cpm)	Radioactivity (cpm)						
		A	B	C	D	E	F	G
AMV-RNA*	0.5×10^6	1950	1512	889	540	124	56	25
	10^6	2524	1870	1309	935	260	89	21
AMV**	0.5×10^6	2080	1632	1016	217	123	107	14
	10^6	2791	2170	1478	643	189	151	29
AMV+sap**	0.5×10^6	73	1147	393	45	26	18	29
	10^6	159	1410	1176	136	34	17	22
Virus-free***	0.5×10^6	11	15	10	11	17	12	19
	10^6	20	23	16	19	25	18	27

* AMV-RNA, A-G: 250 ng, 25 ng, 2.5 ng, 250 pg, 25 pg, 2.5 pg, and 0.25 pg, respectively.

** Purified AMV and AMV added to crude extract from virus-free pollen, A-G: 1.5 µg, 150 ng, 15 ng, 1.5 ng, 150 pg, 15 pg, and 1.5 pg, respectively.

*** Ten-fold dilutions of crude extract from virus-free pollen.

Table 2.2. Radioactivity counts in individual dots containing crude or nucleic acid extracts from infected or virus-free pollen, or purified AMV-RNA, hybridized with the cDNA probe of 10^6cpm/ml

Sample	Radioactivity (cpm)					
	A	B	C	D	E	F
Crude extract*	825	470	172	21	26	-
Nucleic acid extract*	2217	1784	535	98	16	-
Virus-free**	16	23	20	18	25	21
AMV-RNA***	1928	1561	680	216	135	-

* Ten-fold dilutions of crude and nucleic acid extracts from 30 mg of infected pollen, A-E: 10^{-1} to 10^{-5}, respectively.

** Ten-fold dilutions of crude (A-C), and nucleic acid (D-F) extracts from 30 mg of virus-free pollen: 10^{-1} to 10^{-3}, respectively.

*** AMV-RNA, A-E: 25 ng, 2.5 ng, 250 pg, 25 pg and 2.5 pg, respectively.

Figure 2.1. Detection of purified AMV by ELISA. Coating γ-globulin 2 µg/ml (A), and 1 µg/ml (B), and enzyme-conjugated γ-globulin 1/1000 (⊡—⊡), 1/2000 (■—■), and 1/4000 (□—□). Antigen concentrations: 0.25 ng/ml (a), 1 ng/ml (b), 4 ng/ml (c), 16 ng/ml (d), 63 ng/ml (e), 250 ng/ml (f), and 1000 ng/ml (g). Extract from virus-free tobacco leaves (◇—◇). Each point represents the mean of five wells. The range of virus-free control absorbance values at 405 nm was 0.015-0.045 with the SD=0.008-0.01. Standard deviation for absorbance values of antigen samples ranged from 0.01-0.02.

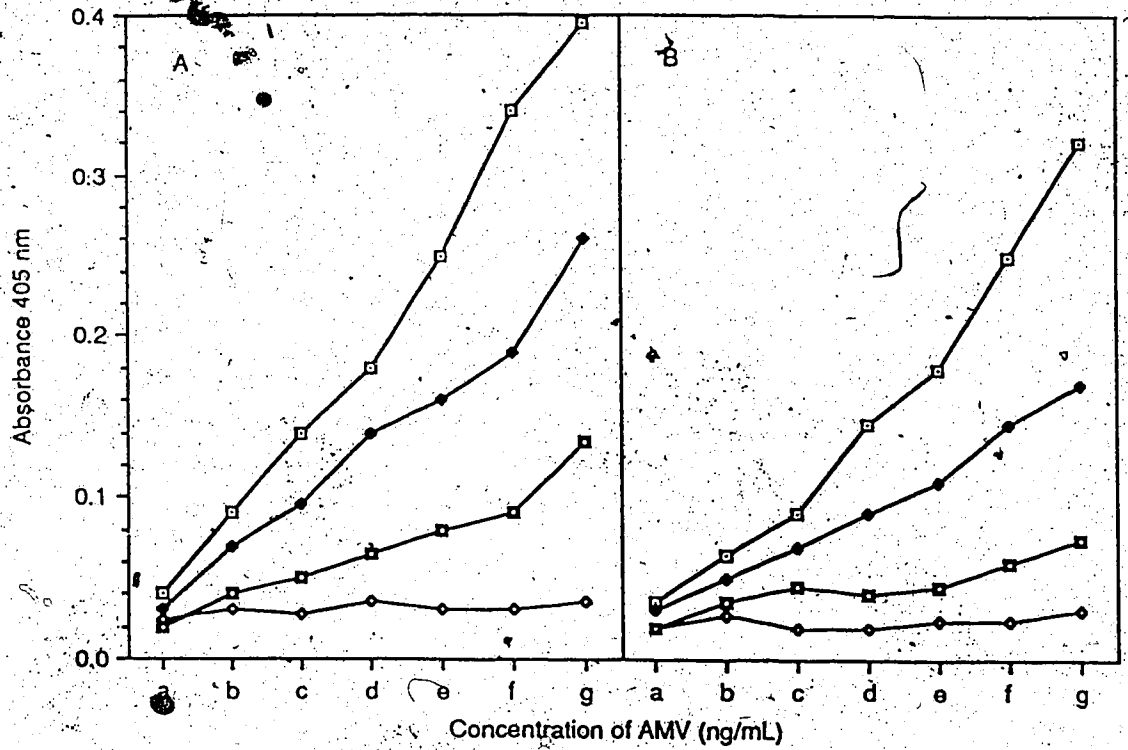
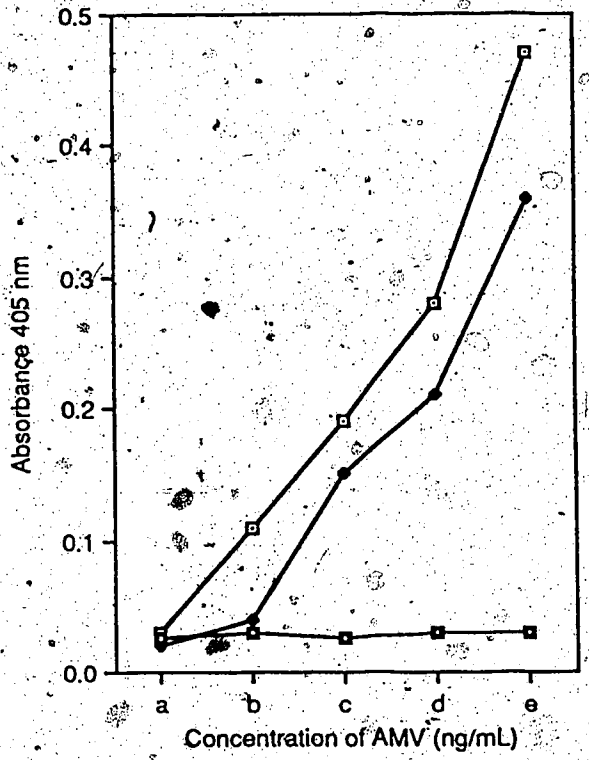

A
B
0.4
0.3
0.2
0.1
0.0
Absorbance 405 nm
a
b
c
d
e
f
g
Concentration of AMV (ng/mL)

Figure 2.2. Effect of plant sap on the sensitivity of detection of AMV in alfalfa pollen by ELISA. The concentrations of purified AMV (⊡—⊡), and purified AMV added to crude extract from virus-free pollen (■—■) : 0.1 ng/ml (a), 1 ng/ml (b), 10 ng/ml (c), 100 ng/ml (d), and 1000 ng/ml (e); crude extract from virus-free pollen (□—□). Crude extract was prepared in the extraction solution (1:3,w/v). Coating γ-globulin was applied at 2 µg/ml and enzyme-conjugated γ-globulin at 1/1000 dilution. Each point represents the mean of five wells. Absorbance values for control samples ranged from 0.02-0.045 with SD=0,01. Standard deviation for test samples was 0.01-0.02.


0.5
0.4
0.3
0.2
0.1
0.0
Absorbance 405 nm
a
b
c
d
e
Concentration of AMV (ng/mL)

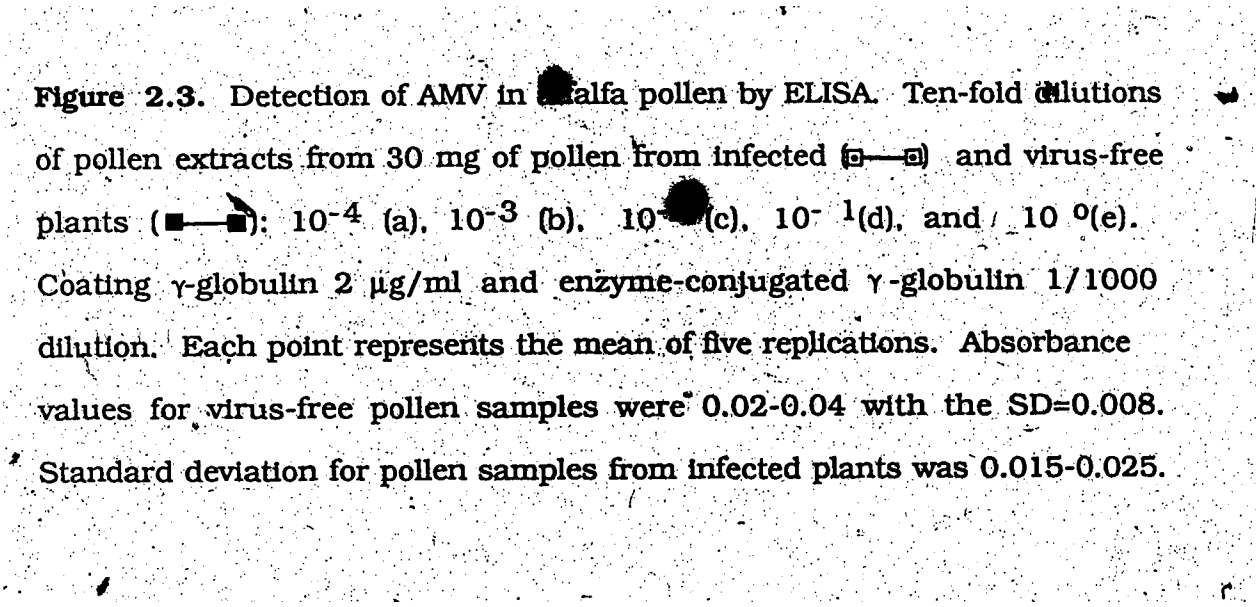


Figure 2.3. Detection of AMV in alfa pollen by ELISA. Ten-fold dilutions of pollen extracts from 30 mg of pollen from infected (▫—▫) and virus-free plants (■—■): 10^{-4} (a), 10^{-3} (b), 10^{-2} (c), 10^{-1} (d), and 10 ^{0} (e). Coating γ-globulin 2 μg/ml and enzyme-conjugated γ-globulin 1/1000 dilution. Each point represents the mean of five replications. Absorbance values for virus-free pollen samples were 0.02-0.04 with the SD=0.008. Standard deviation for pollen samples from infected plants was 0.015-0.025.

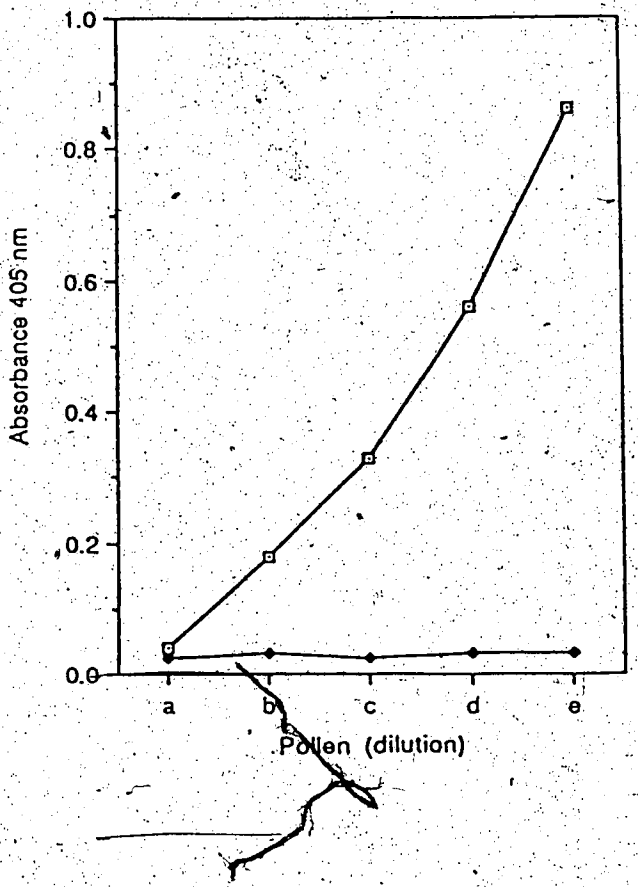

1.0
0.8
0.6
0.4
0.2
0.0
Absorbance 405 nm
a
b
c
d
e
Pollen (dilution)

Figure 2.4. Frequency distribution for ELISA absorbance values of extracts of alfalfa pollen. Pollen samples (3 mg/well) from infected (■), and virus-free plants (⧈). Coating γ-globulin 2 µg/ml and enzyme-conjugated γ-globulin 1/1000. The absorbance values for virus free-pollen ranged from 0.015-0.045.

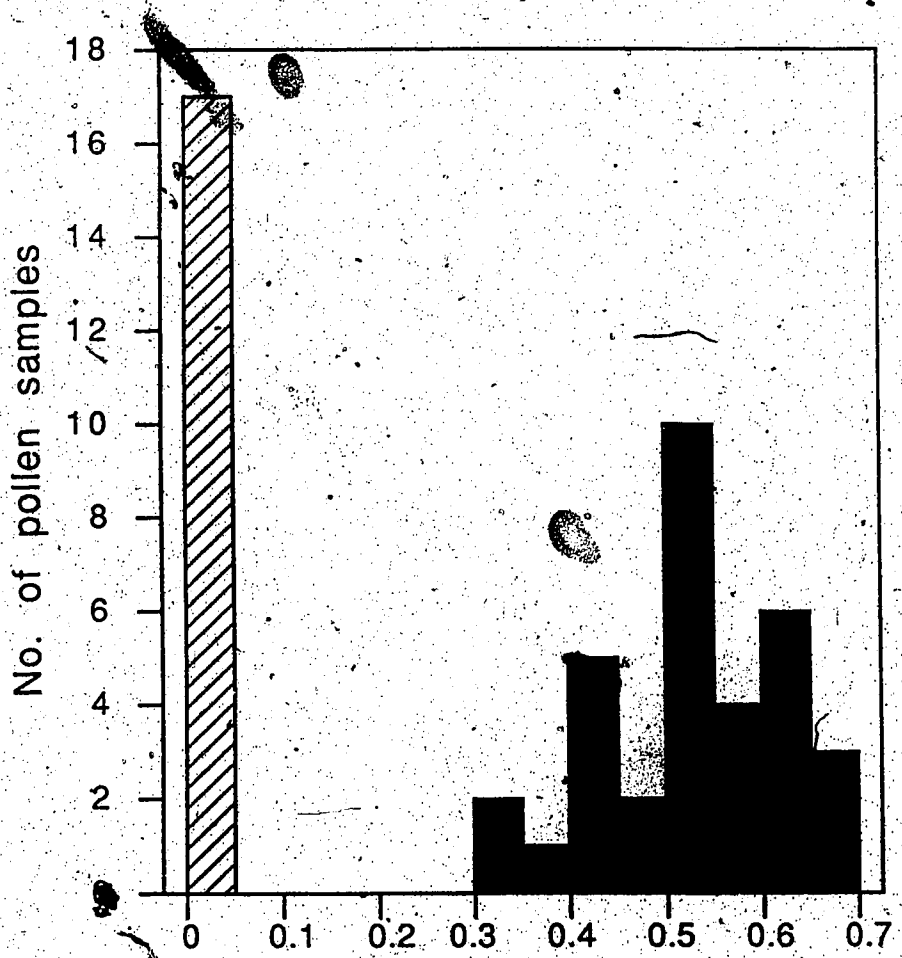

No. of pollen samples
18
16
14
12
10
8
6
4
2
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Absorbance at 405 nm

Figure 2.5. Effect of ^{32}P-labeled cDNA probe on the sensitivity of detection of AMV-RNA by dot hybridization assay. **A**, cDNA probe with 0.5×10^6 cpm/ml, and **B**, cDNA probe with 10^6cpm/ml hybridization solution. Row **a** (1-6), ten-fold dilutions of purified AMV added to crude extract from virus-free pollen, and row **b** (1-6), purified AMV: 1.5 µg, 150 ng, 15 ng, 1.5 ng, 150 pg and 15 pg/dot, respectively. Row **c** (1-6), purified AMV-RNA in TE, pH 8.0: 250 ng, 25 ng, 2.5 ng, 250 pg, 25 pg, 2.5 pg/dot, respectively. Row **d** (1-6), ten-fold dilutions of crude extract from virus-free pollen. Dilutions of purified AMV, AMV in crude extract, and crude extract(1:3,w/v) were prepared in 0.01 M phosphate buffer, pH 7.5, containing 0.1% SDS.

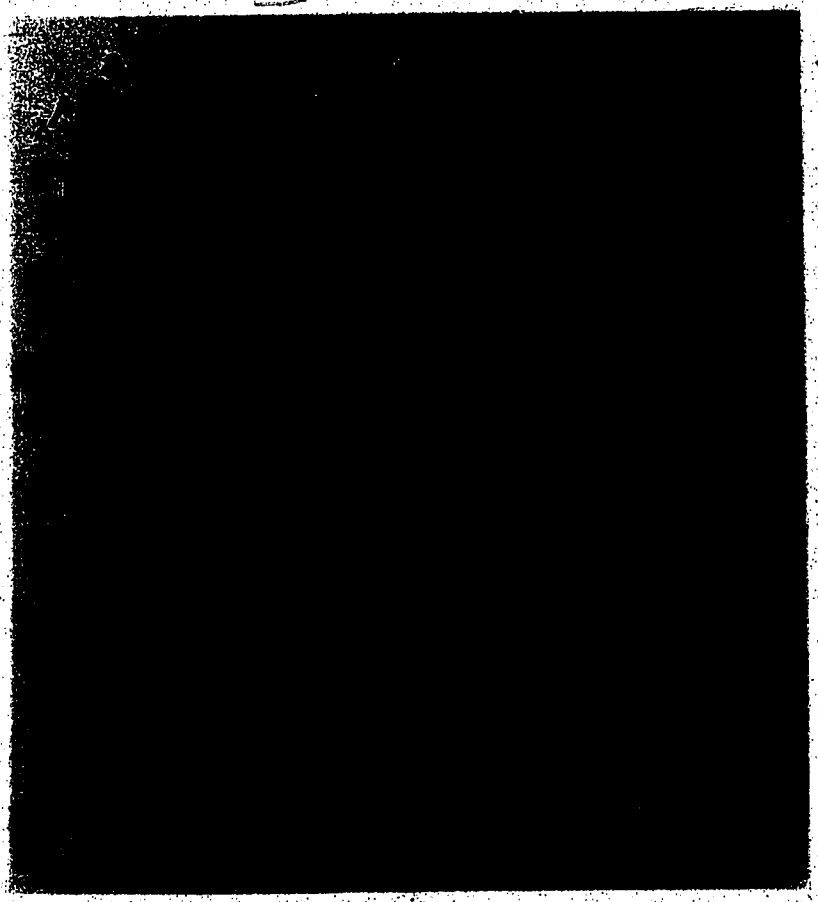
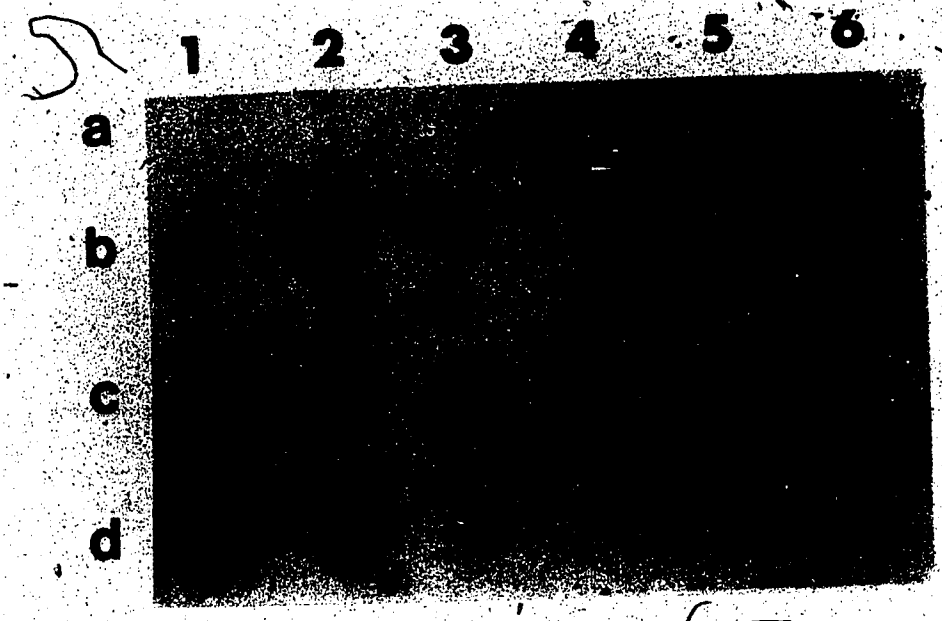


Figure 2.6. Effect of crude and nucleic acid extracts on the sensitivity of detection of AMV-RNA by dot-hybridization in alfalfa pollen. Row **a**, ten-fold dilutions of crude (1-3), and nucleic acid (4-6) extracts from 30 mg of virus-free pollen: 10^{-1} - 10^{-3}, respectively. Row **b** (1-5) ten-fold dilutions of crude, and row **c** (1-5) nucleic acid extracts from 30 mg of infected pollen: 10^{-1} - 10^{-5}, respectively. Row **d** (1-5), purified AMV-RNA: 25 ng, 2.5 ng, 250 pg, 25 pg and 2.5 pg/dot, respectively.


1
2
3
4
5
6
a
b
c
d

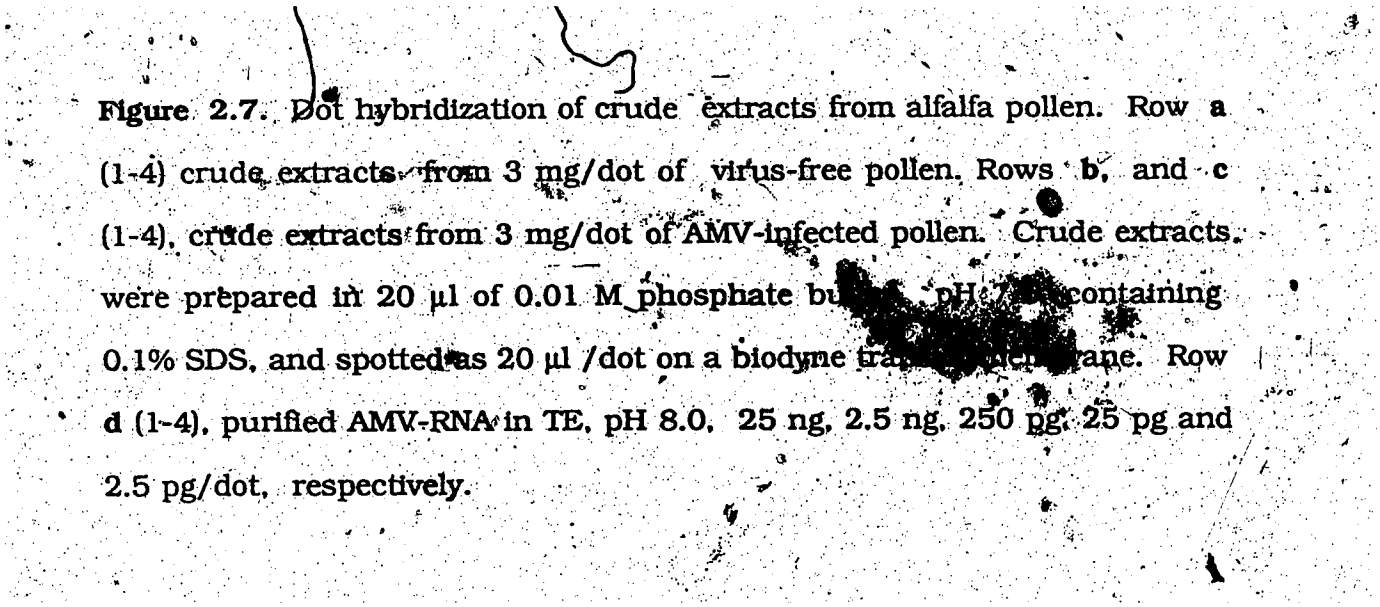
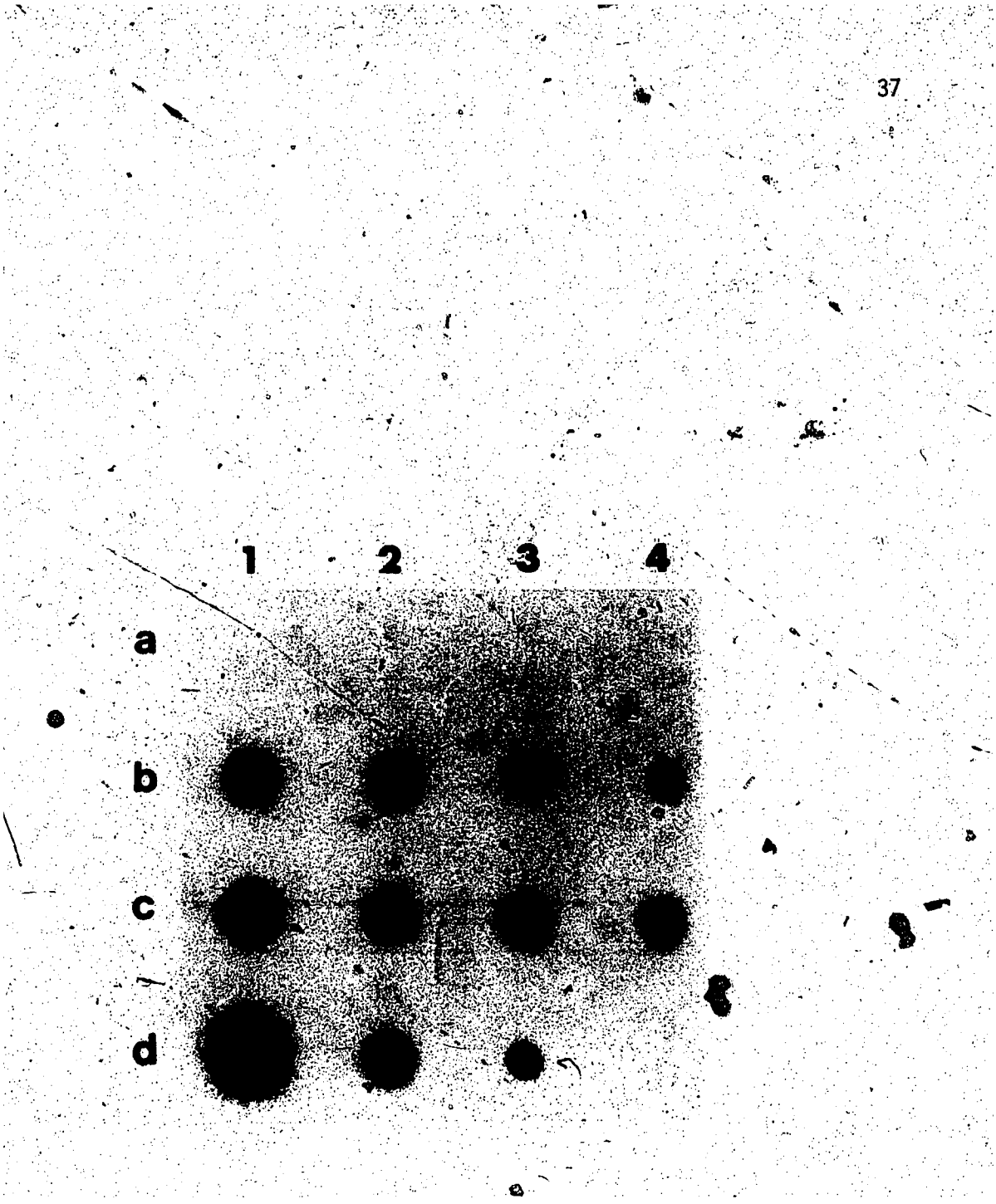


Figure 2.7. Dot hybridization of crude extracts from alfalfa pollen. Row **a** (1-4) crude extracts from 3 mg/dot of virus-free pollen. Rows **b**, and **c** (1-4), crude extracts from 3 mg/dot of AMV-infected pollen. Crude extracts were prepared in 20 µl of 0.01 M phosphate bu[illegible] pH 7.[illegible] containing 0.1% SDS, and spotted as 20 µl /dot on a biodyne tra[illegible]ane. Row **d** (1-4), purified AMV-RNA in TE, pH 8.0, 25 ng, 2.5 ng, 250 pg, 25 pg and 2.5 pg/dot, respectively.


1
2
3
4
a
b
c
d

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3. DIFFERENT RATES OF ALFALFA MOSAIC VIRUS INCIDENCE IN SEED COAT AND EMBRYO OF ALFALFA SEED [1]

3.1. Introduction

The important role of infected seed in the epidemiology of alfalfa mosaic virus (AMV) has been recognized since high incidence and longevity of AMV in alfalfa seed were established (Zschau & Janke 1962, Frosheiser 1970, 1974). As reported by Frosheiser (1974) the extent of virus transmission depends on virus strain, alfalfa cultivar or clone and environmental conditions. Frosheiser (1974) and Hemmati & McLean (1977) observed transmission of AMV through pollen and ovules of alfalfa. AMV was also found in the embryonic cotyledons of alfalfa (Wilcoxson et al. 1975) and *Nicandra physaloides* L. (Gallo & Ciampor 1977).

Although various aspects of AMV transmission through seed have been studied, little is known as to whether infection of alfalfa seed and seedlings by AMV differ significantly. In a comparative study of direct seed and seedling assay with two seed lots containing about 20% of infected seeds, Frosheiser (1974) reported that the percentage of infected seeds detected was essentially the same in both assay methods. However, a recent preliminary investigation indicated that the rates of AMV incidence in seeds (24%) and seedlings (16%) were significantly different (Pesic et al. 1984). Because infected seedlings represent the main source of AMV in the field (Mueller 1965, Gates & Bronskill 1974), the relationship between

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incidence rates of AMV in alfalfa seed and seedlings was further investigated. This paper reports a comparative study on rates of detection of AMV in seed and seedlings, of alfalfa cv. Beaver as well as on the high incidence of AMV in the seed coats and its relatively low incidence in embryos of the same cultivar.

3.2. Materials and Methods

3.2.1 Virus and virus purification

An Alberta isolate of AMV (A-515) was used for seed inoculation. AMV was propagated in *Nicotiana tabacum* L. cv. White Burley in the greenhouse at 23 C and harvested ten days later. Virus was purified as described by van Vloten-Doting & Jaspars (1072).

3.2.2 Source of seed

Two naturally infected seed lots of alfalfa cvs. Beaver and Vista, from commercial sources in Alberta were selected for this study. Preliminary seed testing showed a seed transmission rate for AMV of 24% in cv. Beaver and 0.5% in cv. Vista.

3.2.3 Virus assay

An infectivity assay on the primary leaves of 10-day old *Phaseolus vulgaris* L. cv. Red Kidney in the greenhouse at 23 C, and enzyme-linked immunosorbent assay (ELISA) (Clark & Adams 1977) were used for AMV detection. Samples for ELISA were prepared in 0.01 M phosphate-buffered saline (PBS), pH 7.4, containing 0.05% Tween 20, 1% polyvinyl-pyrrolidone and 0.1% egg albumin. Individual seeds were homogenized in 0.8 ml,

seedlings in 1.5 ml and seed coats and embryos in 0.4 ml of PBS, and the supernatant was tested in 200 µl aliquots. Coating γ-globulin was prepared at 2 µg/ml and the alkaline phosphatase conjugate (1/1000 dilution) was used with p-nitrophenyl phosphate as a substrate (0.8 mg/ml substrate buffer). The incubation period was 40 min at 37 C and the quantitation of the reaction product was determined at 405 nm on a Titertek Multiskan photometer. Readings above 0.1 were considered to be positive for the presence of AMV. This value was obtained by multiplying three times the highest absorbance value for virus-free material used as a negative control and the background absorbance for wells from which test samples were excluded.

3.2.4 Seed and seedling assay

To estimate the rate of AMV incidence in intact alfalfa seed, a total number of 1022 seeds of cv. Beaver were individually assayed by ELISA in nine replicates. In order to compare the incidence of AMV in seed and seedlings, 682 seven-day-old seedlings grown in a soil mixture (soil:peat:sand, 1:1:1) were assayed in four replicates

3.2.5 Seed coat and embryo assay

For this study 594 randomly selected seeds of cv. Beaver were placed on wet filter paper in Petri plates and incubated for 48 h at room temperature. Each seed was dissected with a sterile razor blade under a stereomicroscope and seed coat and embryo were washed in PBS and assayed by ELISA.

To determine if AMV remaining on the seed coat served as a source of infection for seedlings during germination, 300 seeds of cv. Vista were

soaked for 30 min in a suspension of AMV, 1 mg/ml 0.01 M NaH_2PO_4, pH 7.0, and air-dried at room temperature for 30 min. Following this treatment seed coats were removed from 100 seeds and homogenized in groups of 20 in 1.5 ml PBS. The remaining 200 seeds were placed in two cheesecloth bags in pots with a soil mixture. Three and seven days later the bags containing free seed coats, seedlings and ungerminated seed were recovered from the soil. Seed coats were removed from ungerminated seeds, and a total of 100 seed coats was collected each time and homogenized in lots of 20 in 1.5 ml PBS. The equivalent number of seedlings and/or embryos was homogenized in the same way. Alfalfa seed of cv. Vista soaked in 0.01 M NaH_2PO_4, pH 7.0, was used as a control in this experiment. Seed coats, seedlings and/or embryo samples were prepared according to the above procedure. ELISA and infectivity assay on Red Kidney beans were used for AMV detection in seed coats, seedlings and/or embryos. Each sample was inoculated on 12 half-leaves previously dusted with 22 µm Carborundum. The opposite half-leaves were inoculated with purified AMV, 5.5 mg/ml PBS as the internal control.

3.3. Results and Discussion

3.3.1 Seed and seedlings

According to the results obtained by ELISA, the average incidence of AMV in alfalfa seed cv. Beaver was 20.6% but varied among replicates, ranging from 18.1 to 24.1%. The average incidence for seedlings was 7.3% (Table 3.1) or three times lower than the incidence in seeds (20.6%). However Frosheiser (1874) reported that detection levels for seed and seedling

assays were essentially the same, and that small differences were probably due to the uneven distribution of infected seeds in a given seed lot.

3.3.2 Seed coat and embryos

Since AMV was found in tissues of embryonic cotyledons of alfalfa (Wilcoxson et al. 1975), its transmissibility was expected to be related to embryo infection. If the virus was present only in alfalfa embryos, detection rates for seed and seedlings would have been comparable. However a higher detection rate for seed and a lower detection rate for seedlings suggested that not only the embryo but also the seed coat was involved in determining the incidence of AMV in seed. To test this hypothesis, seed coats and embryos of individual seeds were assayed separately by ELISA, and the possibility of AMV remaining on the seed coat surface as a source of infection for seedlings during germination was examined.

AMV remaining on the seed coat was not detected by the infectivity assay. However AMV was detected by ELISA in the seed coat samples prepared immediately after soaking of seed in the AMV suspension. AMV was not detected in seed coats, seedlings, and/or embryos three and seven days following the treatment with AMV or in untreated samples by either method.

Thus, the results obtained by ELISA indicate that although AMV can be detected on the seed coat surface, the possibility that active virus remaining on the seed coat serves as an infection source for seedlings during germination appears to be remote.

Based upon the results of seed coat and embryo assays, infected seeds could be grouped in three categories according to virus incidence in seed coats and embryos (Table 3.2), viz., (1) seeds with infected seed coats, (2)

seeds with infected embryos, and (3) seeds with infected seed coats and embryos. Since the seed coat separates from the embryo during germination and AMV on the seed coat does not serve as a source of infection for seedlings according to the evidence obtained in this study, seeds from the first category will produce virus-free plants. On the other hand, seeds from the remaining two categories that contain infected embryos will generate virus-infected plants.

It is confirmed with these results that the seed coat infected during its development, plays an important role in influencing the incidence of AMV in alfalfa seed and that the difference in detection rates of AMV in seed and seedlings was due to the high incidence of AMV in seed coats.

In order to differentiate between seed and seedling infection, terminology such as 'seed transmission rate' and 'embryo transmission rate' was adopted (Table 3.3). Seed transmission rate was used to express both seed coat- and embryo infection and corresponds to detection rates for whole seeds (20.6%) and/or seed coats and embryos combined (18.1%). Embryo transmission rate refers only to embryo infection and is equivalent to detection rates for seedlings (7.3%) and embryos (9.0%).

Taking into consideration the results obtained for this particular AMV-host combination, it is concluded that the embryo transmission rate is more appropriate than the seed transmission rate in expressing the extent of AMV transmission through seed; also, seedling and embryo assays were more accurate than the whole seed assay in determining the actual rate of AMV transmission through seed. The observed difference between Frosheiser's data (1974) with two different AMV strains and the present data indicate that additional work needs to be done.

Table 3.1. Detection of AMV in alfalfa cv. Beaver seedlings by ELISA

Replicate	No. of seeds sown*	Germination rate (%)+	No. of seedlings		Detection rate (%)
			Tested	AMV-detected	
1	250	64.8	162	12	7.4
2	250	70.8	177	13	7.3
3	250	69.6	174	15	8.6
4	250	67.6	169	10	5.9
Total	1000		682	50	
Average		68.2			7.3

* Alfalfa seed sown 250/tray or 50/row in a soil mixture.

\+ Germination rate (68.2%) was similar to the one obtained for alfalfa seed germinated on wet paper towels (70%) in a separate test using the same seed lot.

Table 3.2. Detection of AMV in seed coats and embryos of alfalfa cv. Beaver by ELISA

Replicate	No. of seeds tested	AMV-detected				
		seed coat*	embryo*	seed coat and embryo**	seed coats and embryos combined (%)+	embryos (%)++
1	55	3	2	3	8 (14.5)	5 (9.1)
2	60	5	1	5	11 (18.3)	6 (10.0)
3	73	8	2	5	15 (20.5)	7 (9.6)
4	58	1	3	4	8 (13.7)	7 (12.0)
5	58	4	1	4	9 (15.5)	5 (8.6)
6	58	6	1	6	13 (22.4)	7 (12.0)
7	58	6	3	4	13 (22.4)	7 (12.0)
8	58	9	0	3	12 (20.6)	3 (5.1)
9	58	5	0	3	8 (13.7)	3 (5.1)
10	58	7	0	4	11 (18.9)	4 (6.8)
Total (average)	594	54	13	41	108 (18.1)	54 (9.0)

* AMV was detected in either the seed coat or the embryo of alfalfa seed.
** AMV was detected in both the seed coat and the embryo of alfalfa seed.
\+ Total number of alfalfa seeds in which AMV was detected.
++ Total number of alfalfa embryos in which AMV was detected.

Table 3.3. Detection of AMV in seed and seedlings of alfalfa cv. Beaver by ELISA

Source	Samples tested (No.)	Detection rate (%)*	Seed transmission rate (%)†	Embryo transmission rate (%)**
Seed	1022	20.6	20.6	----
Seed coats and embryos combined	594	18.1	18.1	----
Seedlings	682	7.3	----	7.3
Embryos	594	9.0	----	9.0

* Represents experimental values for seed infection rates.
† Designates seed infection and corresponds to detection rate for whole seeds and seed coats and embryos combined.
** Designates embryo infection and corresponds to detection rates for seedlings and embryos.

3.4. References

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4. DETECTION OF VIRAL ANTIGEN BY IMMUNOGOLD CYTOCHEMISTRY IN OVULES, POLLEN AND ANTHERS OF ALFALFA INFECTED WITH ALFALFA MOSAIC VIRUS [1]

4.1 Introduction

Although alfalfa mosaic virus (AMV) is transmitted to alfalfa seed through both male and female gametes, the mechanism of virus transmission is still unknown. A previous electron microscope study on the localization and aggregation forms of two strains of AMV in alfalfa gametes revealed the presence of rafts, and star-like aggregates, of virus particles in the cytoplasm of anther parenchyma cells and pollen grains (Wilcoxson et al. 1975). However, the virus was not detected in thin sections of alfalfa ovules using a heavy-metal staining method. Recently, AMV was detected by enzyme-linked immunosorbent assay (ELISA) in the seed coat and embryo of alfalfa seed (Pesic & Hiruki 1986). In individual seeds, AMV was detected in either the seed coat or the embryo, or in both of them simultaneosly. These results can be interpreted as a random distribution of AMV in alfalfa seed and suggest that virus distribution within infected pollen and ovules and the mode of virus transmission may influence the type of seed infection.

Immunogold cytochemistry has been introduced as an alternative to the heavy metal staining for virus identification in leaf-dip preparations and *in situ* localization of intact virus particles or viral antigen by electron and light microscopy (Giband et al. 1984, Lin & Langenberg 1983, Lin 1984, Louro &

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1 Accepted for publication in Phytopathology (1988).

Lesemann 1984, Tomenius et al. 1983). It is particularly advantageous for detection of small polyhedral viruses in thin sections of plant tissue where virus particles can not be readily distinguished from cytoplasmic ribosomes (Hatta 1976, Lawson & Hearon 1970, Wilcoxson et al. 1975). Digestion of ribosomes with ribonuclease is another method employed for a similar purpose (Hatta & Francki 1979, 1981).

The present immunocytochemical investigation was undertaken to detect the distribution of AMV in pollen, ovule integuments and embryo sacs of alfalfa and to ascertain their role in AMV transmission to alfalfa seed.

4.2 Materials and Methods

4.2.1 Virus and virus assay

AMV isolate A-515 (Hiruki & Miczynski 1987) was propagated in *Nicotiana tabacum* L. cv. White Burley in the greenhouse at 23 C and a 13 h photoperiod. AMV was purified from leaves with symptoms harvested ten days after inoculation (van Vloten-Doting & Jaspars 1972). Direct double antibody sandwich ELISA (Clark & Adams 1977) was used for virus detection in alfalfa plants.

4.2.2 Plants

Three clones from Beaver alfalfa, B-19, B-24 and B-39 (Pesic & Hiruki 1985), were inoculated at the 6-8 leaf stage with purified AMV preparation, 1 mg/ml of 0.025 M phosphate buffer, pH 7.0. Inoculated and virus-free clonal plants were maintained on separate benches in the same greenhouse at 18 C with a 16 h photoperiod. At four week intervals plants were assayed by ELISA for the presence of AMV.

4.2.3 Antiserum

Antiserum against AMV isolate A-515 was produced in rabbits using purified virus preparation in a combination of intravenous and intramuscular injections. After the first, intravenous injection (1 mg/ml in 0.01 M NaH_2PO_4, pH 7.0), a series of intramuscular booster injections was given at weekly intervals with a mixture (1:1, v/v) of AMV and Freund's incomplete adjuvant. Rabbits were exsanguinated when the titer of antiserum determined by double diffusion tests reached 1/256. To remove the antibodies to host proteins, the antiserum was absorbed with acetone-extracted powder of virus-free tobacco plants (da Rocha et sl. 1986). The powder obtained from 10 g of frozen tobacco leaves was washed with 0.01 M phosphate-buffered saline (PBS), pH 7.4 and incubated with 10 ml of antiserum for 2.5 h at 40 C in a water bath. The antiserum was separated from the precipitate by low speed centrifugation. The immunoglobulin fraction of the antiserum was precipitated by mixing equal volumes of 4 M $(NH_4)_2SO_4$ and antiserum for 2 h at room temperature. After centrifugation for 10 min at 1000 g, the precipitate was resuspended in half strength PBS, pH 7.4, and dialyzed overnight at 4 C against three changes of PBS. The concentration of purified immunoglobulin was determined by measuring the absorbance at 280 nm using the extinction coefficient of 1.35 $(mg/ml)^{-1}\ cm^{-1}$ (Johnstone & Thorpe 1982). The immunoglobulin was stored at -20 C.

4.2.4 Tissue preparation

Anthers and ovaries were collected from selected flowers of infected and virus-free plants by tripping the flower and dissecting it under a stereomicroscope. Tissues were vacuum infiltrated with 0.01 M PBS, pH

7.4, to remove air and to assist in fixative penetration. They were fixed with 1% glutaraldehyde in 0.01 M PBS, pH 7.4, at 4 C overnight, rinsed in three changes of PBS, 1 h each, and post-fixed in 2% OsO_4 for 2 h at room temperature. After rinsing in distilled water, anthers and ovaries were dehydrated in a graded series of acetone (70-100%), with three changes, 20 min each, in absolute acetone. Anthers and ovaries were then passed through a mixture of equal parts of acetone and Spurr's resin for 2 h, then Spurr's resin overnight and cured at 65 C for 10 h. Thin sections were stained with 2 % uranyl acetate for 60 min and lead citrate for 2 min. For immunogold cytochemistry, low-acid water-soluble GMA embedding mixture consisting of glycol methacrylate (2-hydroxyethyl methacrylate), benzoil peroxide and butyl methacrylate was used for dehydration, infiltration and embedding of plant tissue. After primary fixation in 1% glutaraldehyde in 0.01 M PBS, pH 7.4, and rinsing in PBS the samples were dehydrated and infiltrated simultaneously in aqueous solutions of GMA (85 and 97% GMA) and kept in the final non-prepolymerized mixture at 4 C overnight. For final embedding the samples were placed in prepolymerized GMA in size 00 gelatin capsules (J.B.EM. Services Inc., St Lawrence, Quebec, Canada), and polymerized at 4 C for 18 to 24 h in an ultraviolet light apparatus equipped with the long wavelength (3150 A) UV lamps (G.E. #F6T5, BL) at a distance of 1.5 cm from the light source.

4.2.5 Chemicals and immunogold reagents

SPI-CHEM low acid GMA water-soluble embedding kit for transmission electron microscopy (SPI Supplies Division of Structural Probe Inc., Box 342, West Chester, PA), Spurr's low-viscosity embedding resin (J.B.EM. Services Inc., St Lawrence, Quebec, Canada), and protein A (Pharmacia,

Dorval, Quebec, Canada) were used in this study. Gold-labelled-goat anti-rabbit IgG with colloidal gold particles of the nominal size of 15 mm (Jansen Life Sciences Products, A division of Jansen Pharmaceutica, B-2340, Beerse, Belgium) and protein A-gold were used for immuno-cytochemical staining staining of plant tissue. Protein A was coupled to colloidal gold particles of the nominal size 10-15 nm (Roth 1982, Slot & Geuze 1981). The final solution of gold-IgG and protein A-gold was prepared in 0.5% bovine serum albumin (BSA) in 0.01 M PBS, pH 7.4, containing 0.05% Tween 20.

4.2.6 Immunocytochemical staining

Grids with thin sections attached were floated face down on 0.5% BSA-PBS-Tween at room temperature for 5 min to block the non-specific binding sites. The grids were transferred without rinsing to a Falcon 3034 microtest plate (Falcon Plastics, 1950 Williams Dr., Oxnard, CA 93030, USA) containing antiviral immunoglobulin and incubated at 37 C for 2 h. The concentration of immunoglobulin was adjusted to 5, 10 and 20 µg/ml 0.5% BSA-PBS-Tween. The sections were rinsed with 1% BSA in 0.01 M PBS, pH 7.4, for 20 sec using a plastic spray bottle, then placed in PBS for 5 min, rinsed with PBS and blotted on filter paper. The grids were placed on drops of immunoglobulin-gold complex diluted in 0.5% BSA-PBS to an absorbance of 0.1 at 520 nm or of protein A-gold complex (20-fold dilution of the stock solution) for 1 h at room temperature. The grids were rinsed with PBS for 30 sec, immersed in PBS for 10 min, rinsed a second time with PBS and finally rinsed with distilled water. The sections were subsequently stained with 2% uranyl acetate for 20 min and lead citrate for 5 min.

Quebec, Canada) were used in this study. Gold-labelled-goat anti-G with colloidal gold particles of the nominal size of 15 mm (Jansen ences Products, A division of Jansen Pharmaceutica, B-2340, Belgium) and protein A-gold were used for immuno-cytochemical staining of plant tissue. Protein A was coupled to colloidal gold of the nominal size 10-15 nm (Roth 1982, Slot & Geuze 1981). l solution of gold-IgG and protein A-gold was prepared in 0.5% erum albumin (BSA) in 0.01 M PBS, pH 7.4, containing 0.05% 0.

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4.2.6 Immunocytochemical staining

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tapetum cells (Fig. 4.4A). In anther tapetum cells stained with the protein A-gold complex, the intensity of antigen labelling was lower than with the gold-IgG complex (Fig. 4.4B). In the majority of cells examined, aggregates of virus particles were densely distributed throughout the cytoplasm. A high intensity of labelling was associated with virus particles in raft-like aggregates and in crystalline bodies (Fig. 4.4C).

4.3.5 AMV detection in alfalfa pollen

AMV was detected in microspores stained with the gold-IgG complex (Fig. 4.5A) and mature pollen grains stained with heavy metals (Fig. 4.5B). In microspores, the differentiation of the wall of the pollen grain was noticeable, as well as the large cytoplasmic bridges connecting microspores to one another in the early stages of development. Long rafts and crystalline aggregates of virus particles similar to those in the anther tapetum cells were observed in the cytoplasm (Fig. 4.5A and 4.5B). AMV was also present on the surface of pollen exine and in the cytoplasmic bridges (Fig. 4.5A). The aggregation and distribution of virus particles in mature pollen grains were similar to those observed in microspores (Fig. 4.5B).

4.3.6 Comparison of alfalfa clones

There was no difference in detection of AMV in ovules, anthers and pollen of three alfalfa clones (B-19, B-24 and B-39) of cv. Beaver (data not shown).

4.3.7 Comparison of antigen-antibody reaction

In purified AMV preparations stained with gold-goat anti-rabbit IgG (Fig. 4.6B) or protein A-gold complex (Fig. 4.6C), labelling of virus particles with

gold-IgG was higher than with protein A-gold complex (Table 4.1). Only a few particles exposed to normal rabbit serum prior to immunogold staining were labelled with colloidal gold (Fig. 4.6A).

4.4 Discussion

For the first time in any study of seed transmission of AMV, virus particles were localized in alfalfa ovules. Thus far, AMV transmission through female gametes has been demonstrated only in cross-pollination experiments between infected and virus-free alfalfa plants (Frosheiser 1974, Hemmati & McLean 1977). The mechanism of virus transmission to seed, however, has been unknown mainly because of the scarcity of any direct evidence of virus distribution in infected ovules. Localization of AMV in the integuments of alfalfa ovules in this study and previous detection of AMV in the seed coat of alfalfa by ELISA strongly indicate that seed coat infection results from ovule transmission of AMV (Pesic & Hiruki 1986). The fact that AMV was not detected in the embryo sac of alfalfa ovules by either the immunocytochemistry or by the heavy-metal staining method in this investigation but was previously found in the embryo of alfalfa seed (Pesic & Hiruki 1986) suggests that embryo infection occurs solely through infected pollen during fertilization. A similar mechanism of virus transmission to seed through gametes has been reported recently for cherry leaf roll in birch (Cooper et al. 1984). Cross-pollination experiments with various combinations of infected and virus-free gametes are needed for a more comprehensive understanding of the mechanism of seed transmission of AMV.

Viral antigen and AMV virions were detected in the cytoplasm and vacuoles of infected gametes and anther tapetum cells. Two strains of AMV,

F1 and U21, were found previously in the cytoplasm of anther parenchyma cells and pollen grains, but were not associated with any of the cell organelles (Wilcoxson et al. 1975). However, an isolate of AMV from pepper was detected in aggregated form in vacuoles of pepper mesophyll cells (De Zoeten & Gaard 1969). The results obtained in this study and those previously reported suggest that virus distribution within the cell is both strain- and host-specific. Localization of AMV in the cytoplasmic bridges indicated that movement of virions might occur between microspores in the early stages of development of pollen grains (Pacinf & Cresti 1977).

Consistent differences in aggregation forms of virus particles were observed between pollen and ovules in each of three alfalfa clones. Non-aggregated virus particles were detected in the cytoplasm and vacuoles of ovule integument cells. Large crystalline bodies and rafts of virus particles randomly distributed in the cytoplasm of anther tapetum cells, microspores and mature pollen grains morphologically resemble those of strains F1 and U21 in the cytoplasm of pollen grains and anther parenchyma cells (Wilcoxson et al. 1975). Differences in aggregation in various alfalfa organs including pollen and anthers were also reported for AMV strains F1 and U21 (Wilcoxson et al. 1975).

Although AMV was localized in the ovules, pollen and anthers by both immunogold cytochemistry and heavy-metal staining, the advantage of using the immunogold technique was in detecting viral antigen and complete virions. Fixation of plant tissue in 1% glutaraldehyde, dehydration in glycol methacrylate, and low temperature embedding preserved cellular organization and the antigenic properties of virus particles as shown by the high intensity of gold-IgG labelling and the formation of antigen-antibody

complex. These results are comparable with those previously reported for AMV (Stussi-Garaud et al. 1987) and barley stripe mosaic virus (Lin & Langenberg 1983). The specificity of the immunogold method for detection of viral antigen was also demonstrated by the absence of gold labelling in ultrathin sections of virus-free tissue subjected to anti-A-515 immunoglobulin and in infected tissue treated with normal rabbit serum. The results obtained in this study and those previously reported for *in situ* localization of cauliflower mosaic virus in turnip (Giband et al. 1984) and red clover mottle virus in pea (Tomenius et al. 1983) clearly indicate that the antigen specific and sensitive immunogold technique can be successfully applied for identification and localization of plant viruses in infected tissue.

The binding of colloidal gold to AMV particles in purified preparations was much higher with gold-IgG than with protein A-gold complex (Table 4.1). Two factors might be involved in the resulting differences in the specificity of antigen-antibody complex in this investigation, namely 1) the indirect method of immunogold labelling and 2) use of colloidal gold-labelled-goat anti-rabbit IgG that will react specifically with rabbit IgG to AMV antigen ((Lin & Langenberg 1983).

In summary, the results of this investigation indicated that AMV can be effectively localized in alfalfa ovules, pollen and anthers by immunogold cytochemistry using gold-IgG and protein A-gold complexes as markers of viral antigen, and provided additional evidence toward a better understanding of seed transmission of AMV.

Table 4.1. Detection of AMV by immunogold staining in purified preparations

First antibody	Second antibody	Number of Virions Total	Labelled	% labelled
Anti-A-515 immunoglobulin	Gold-IgG	504	395	78.4±5.6
	Protein A-gold	437	218	49.8±6.0
Normal rabbit serum	Gold-IgG	356	10	2.8±2.9
	Protein A-gold	425	10	2.3±2.3

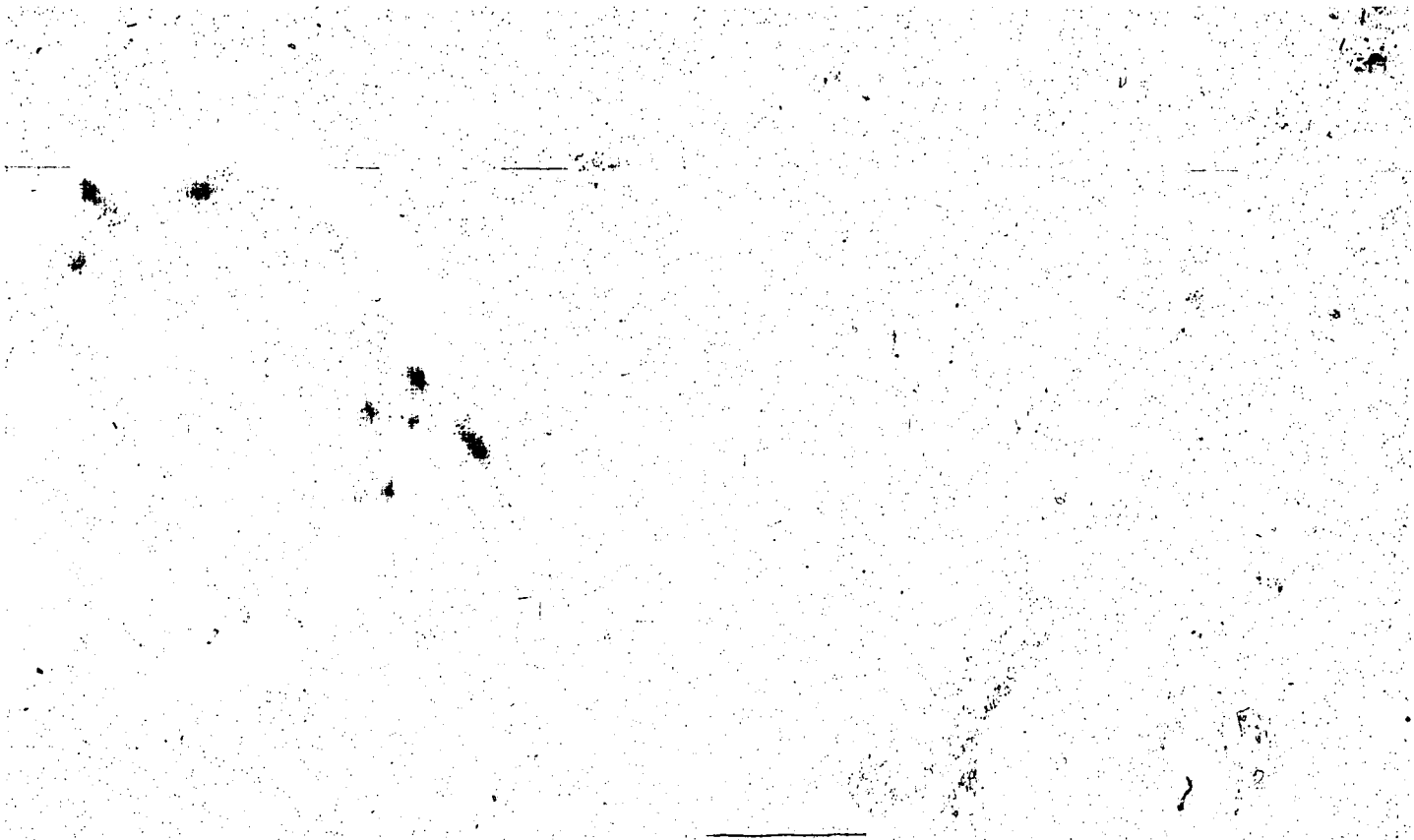


Fig. 4.1. Thick section of alfalfa anther of clone B-24 infected with AMV. Anther wall (an arrow head), and mature pollen grains (arrows) (x610). Bar represents 50 μm.

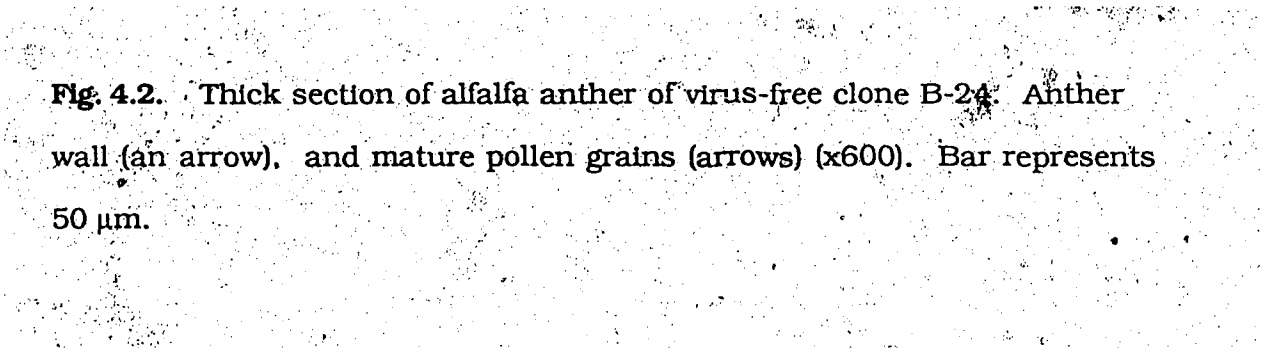


Fig. 4.2. Thick section of alfalfa anther of virus-free clone B-24. Anther wall (an arrow), and mature pollen grains (arrows) (x600). Bar represents 50 μm.

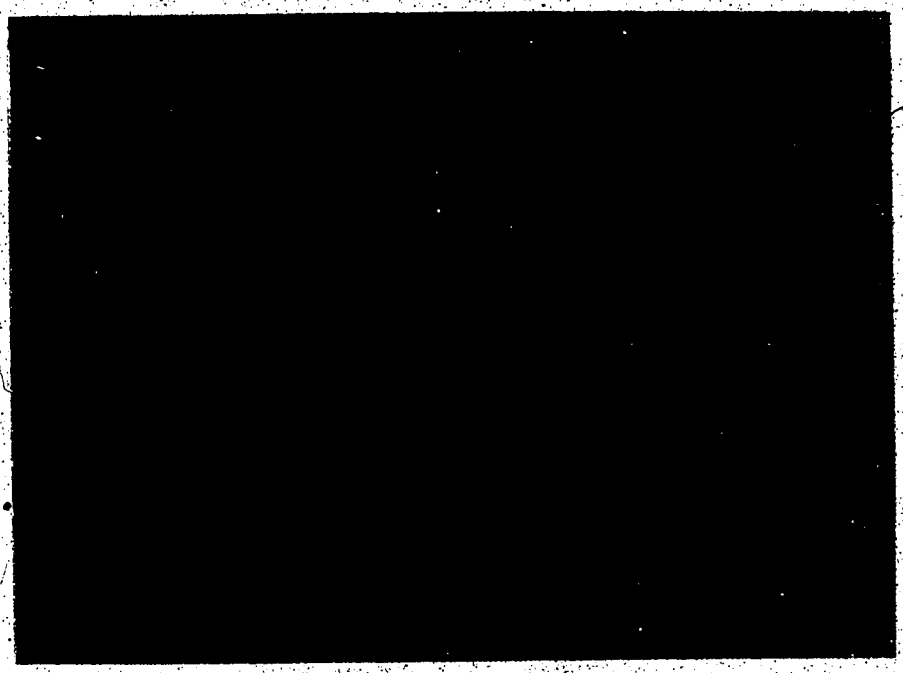


Fig. 4.3. AMV-infected ovules of alfalfa clone B-24. **A.** Thick section of alfalfa ovule. IN, integuments surrounding embryo sac (arrow) (x120). Bar represents 100 µm. **B.** Localization of AMV in ovule integument cell stained with gold-goat anti-rabbit IgG. Gold-labelled viral antigen is found in the cytoplasm and vacuoles (V). N, nucleus, CH, chloroplast, (x29,800). **C.** AMV-infected integument cell exposed to normal rabbit serum and stained with gold-IgG complex. Non-specific staining is present in low concentrations over the cytoplasm and nucleus (N), (x17,500). **D.** Localization of AMV in thin sections of the integument cell stained with uranyl acetate and lead citrate. Non-aggregated virions are located in the cytoplasm (arrows), M, mitochondrion, N, nucleus, CW, cell wall, (x45,000). Bar represents 0.5 µm unless otherwise indicated.

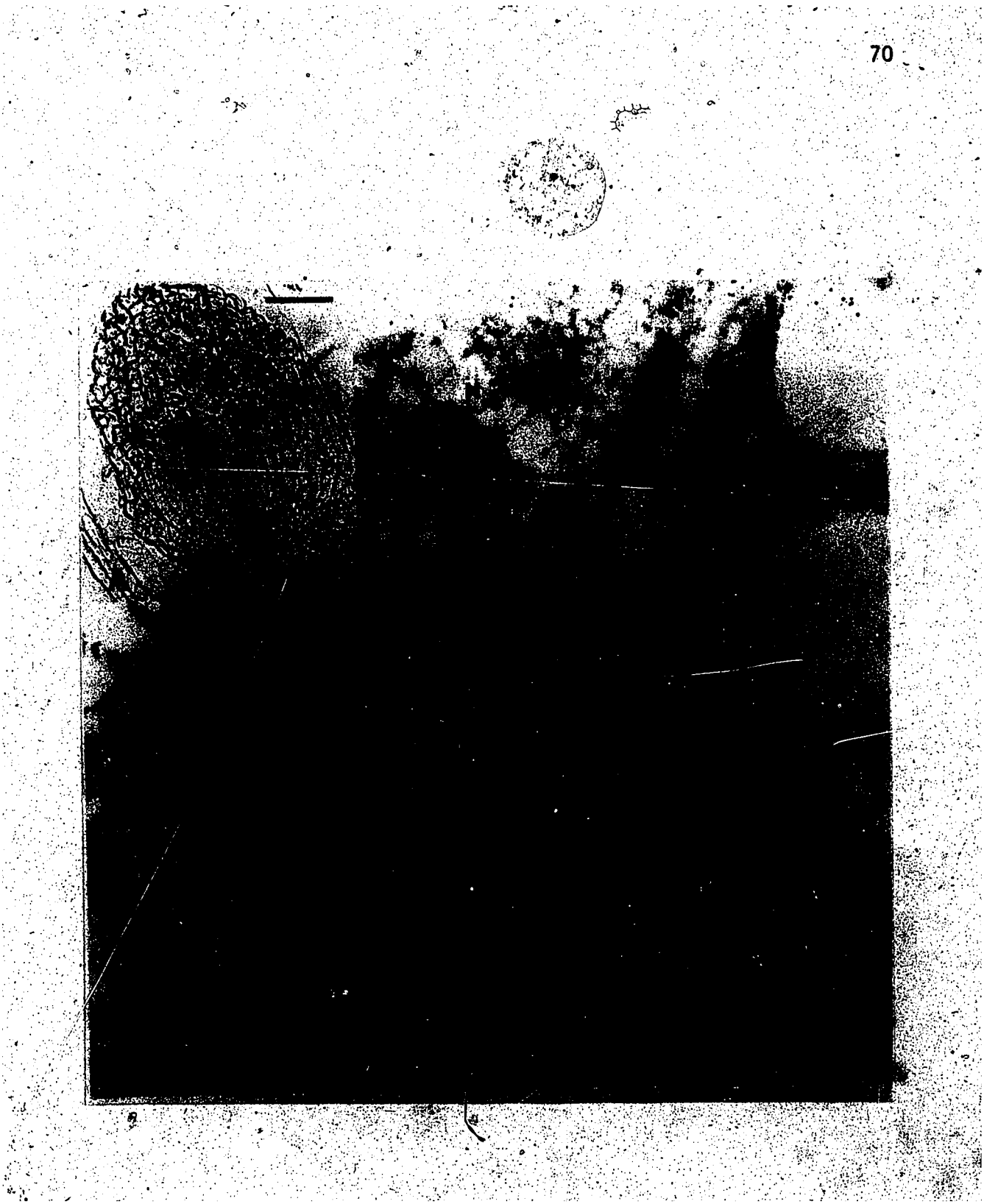


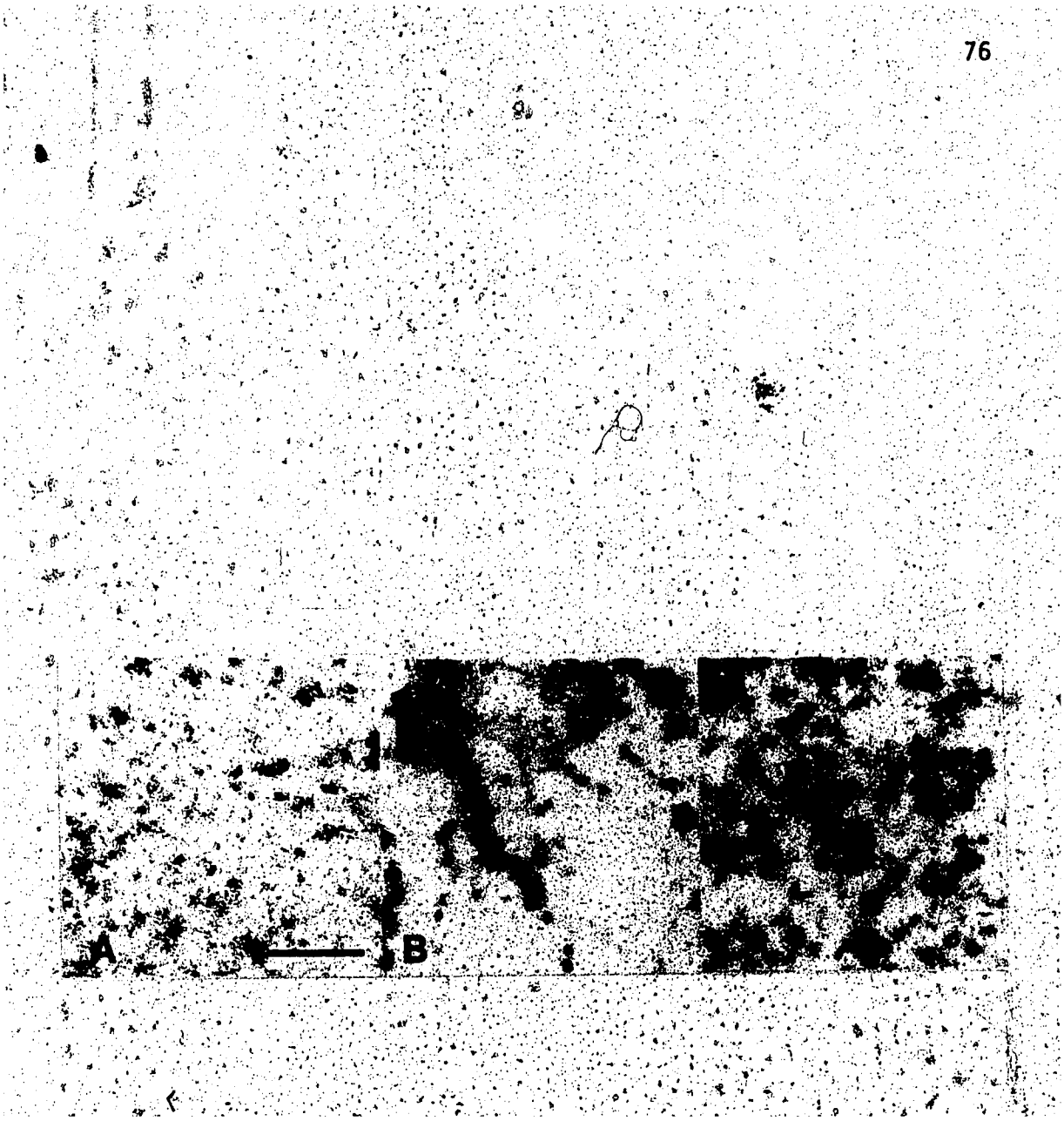
Fig. 4.4. Localization of AMV in anthers of alfalfa clone B-24 infected with isolate A-515. **A.** Anther tapetum cells stained with gold-IgG complex, (x65,400). **B.** Anther tapetum cells stained with protein A-gold complex, (44,800). **C.** A raft aggregate of AMV particles (small arrow head) and large crystalline bodies (large arrow head) in the cytoplasm of anther tapetum cells stained with gold-IgG complex, (x51,400). Bar represents 0.5 μm.


A

Fig. 4.5. AMV-infected pollen of alfalfa clone B-24. **A.** Localization of AMV in the microspore by immunogold staining with gold-IgG complex. An arrow head indicates the area magnified in an inserted micrograph (x64,500). Aggregates of virus particles are located in the cytoplasm. Viral antigen is also present in the cytoplasmic bridges (arrow) and on the surface of developing pollen exine, (x19,700). Bar represents 0.3 µm.

B. Aggregates of virions in the cytoplasm of mature pollen grain stained with uranyl acetate and lead citrate, (x137,000). Bar represents 0.25 µm.

Fig. 4.6. Detection of purified AMV by immunogold staining. **A.** AMV particles exposed to normal rabbit serum prior to staining with protein A-gold complex. **B.** Labelling of viral antigen with gold-IgG. **C.** Protein A-gold complex. (x57,400). Bar represents 0.25 µm.


A
B

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5. EFFECT OF ALFALFA MOSAIC VIRUS ON GERMINATION AND TUBE GROWTH OF ALFALFA POLLEN [1]

5.1 Introduction

Alfalfa mosaic virus (AMV) is transmitted to alfalfa seed through both male and female gametes (Frosheiser 1974, Hemmati & McLean 1977). In experiments with different combinations of AMV strains and alfalfa clones, Frosheiser (1974) found that the frequency of virus transmission through pollen (up to 26.5%) was considerably higher than through ovules (up to 9.5%). Similar results were obtained by Hemmati & McLean (1977), who also demonstrated that AMV was pollen-transmitted only to seed, and not to virus-free plants..

Taking into consideration the evidence that infection of alfalfa seed occurs primarily through infected pollen, the effect of AMV on pollen viability and tube growth was further investigated.

5.2 Materials and Methods

5.2.1 Virus and virus purification

The AMV isolate A-515 was used for inoculation of alfalfa clones. The virus was propagated in *Nicotiana tabacum* L. cv. White Burley in the greenhouse at 23 C and purified according to van Vloten-Doting & Jaspars (1972).

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[1] Accepted for publication in Can. J. Plant Pathol. (1987).

5.2.2 Virus assay

Direct double sandwich enzyme-linked immunosorbent assay (ELISA) (Clark & Adams 1977) was used for AMV detection in alfalfa clones. Samples were prepared and assayed as previously described by Pesic & Hiruki (1986). AMV antigen was cross-absorbed with virus-free plant antigen (da Rocha et al. 1986). Coating -globulin was used at 2 µg/ml with the alkaline phosphatase conjugate at a 1/1000 dilution.

5.2.3 Source of alfalfa clones

The three clones, B-19, B-24 and B-39, included in this investigation were established from alfalfa (*Medicago sativa* L. cv. Beaver) (Pesic & Hiruki 1985) and vegetatively propagated by stem cuttings. The cuttings were treated with Stim Root No. 2 rooting powder containing 0.4% indole-3-butyric acid (Plant Products Co., Bramalea, Ontario) and rooted in Perlite on a mist propagation bench at about 20 C and a 13 h photoperiod. Clonal plants with well-developed roots were inoculated with *Rhizobium meliloti*, strains NRG-43-4 and NRG-85-1 (The Nitrogen Co., Milwaukee, Wis., USA), and were transplanted into 15 cm pots containing a regular soil mix (soil:peat:sand, 1:1:1,v/v). Alfalfa plants were maintained in the greenhouse at 18 C and a 16 h photoperiod. Two weeks after transplanting, one half of the clonal plants was inoculated with an inoculum containing 1 mg/ml AMV in 0.025 M phosphate buffer, pH 7.0; the other half was treated with the same buffer solution only. One month later, all the clonal plants were assayed by ELISA and three AMV-infected and three virus-free plants of each clone were selected. Infected and virus-free clonal plants were kept on separate benches in the same greenhouse compartment.

5.2.4 Germination medium

Pollen growth characteristics were studied *in vitro* on an agar-sucrose medium consisting of 100 ml of distilled water, 1.5 g of Bacto-agar and 20 g of sucrose. The pH was adjusted to 7.0 with 1 N NaOH. Sterilized medium was kept in Petri plates at 4 C.

5.2.5 Pollen sampling

A total of 250 untripped flowers was collected from each plant and pollen samples from ten randomly selected flowers were used immediately for plating. Pollen was dispersed over the medium by tripping one flower at a time with a pair of forceps under a stereo microscope. Petri plates were incubated in a moist chamber at 29 C in darkness. Pollen germination was stopped by adding a small amount of lactophenol cotton blue solution. The plates were then kept at 4 C in a refrigerator until observation. Pollen grains still remaining in the flowers were assayed by ELISA for AMV.

5.2.6 Pollen growth characteristics

For pollen germination tests, counts were made following incubation periods of 80, 60, 90 and 120 min. Germ-tube elongation was measured after 20 h. To determine germination rate, only singly separated pollen grains visibly filled with cytoplasm were counted. Pollen was considered germinated if the length of the pollen tube exceeded the diameter of the pollen grain. Pollen tube length was measured on individual pollen grains and it included the size of the pollen grain (about 34 µm on agar medium plus the length of the pollen tube in all cases). Pollen germination and pollen tube measurements were made at 100x magnification under a Leitz Ortholux microscope with an ocular scale.

5.2.7 Experimental design

A completely randomized design was used for tests on pollen germination and pollen tube growth. All tests were done in three replicates per clone including two treatments; AMV-infected and virus-free. Pollen germination counts were made on six Petri plates/replicate (500 grains/plate), or a total of [illegible]00 pollen grains per treatment for each incubation time. The germination rate (%) was calculated per plate. Six plates for each replicate were averaged, with the mean being used for further analysis. For pollen tube growth, the length of 450 germ tubes per treatment was measured (25 germ tubes/plate). The analysis of variance was used for the comparison of two treatments and Duncan's multiple range test for the comparisons among three clones.

5.3 Results

5.3.1 Pollen germination

Pollen from infected plants of alfalfa clones B-19, B-24 and B-39 had lower germination rates compared to pollen from virus-free plants, following incubation for 30, 60, 90 and 120 min at 29 C (Table 5.1). Between 40 and 50% of all pollen grains from virus-free and 30-40% from infected plants germinated during the first 30 min. The analysis of variance has shown that differences in germination rates of pollen from infected and virus-free plants were significant for each clone at all incubation times, except for B-39 at 30 min. The comparison of pollen viability indicated that the highest germination rate after 120 min was for pollen samples from virus-free clone B-24 (82.1%), followed by B-39 (78.3%), and B-19 (67.2%) (Table 5.1).

The corresponding values for pollen from infected plants were 64.3, 59.7 and 54.7%, respectively.

No visible changes in the morphology of germinating pollen grains were observed after 120 min of incubation (Fig. 5.1). Sterile pollen grains, oval shaped and smaller, were observed in pollen samples from both infected and virus-free plants of all clones; however, they were excluded from counting in germination tests. AMV was detected by ELISA in pollen from infected plants. Results were negative for pollen from virus-free plants, with the absorbance readings up to 0.03.

5.3.2 Pollen tube growth

Pollen from infected plants of clones B-19, B-24 and B-39 produced shorter tubes than pollen from virus-free plants after 20 h of germination at 29 C (Table 5.1). Pollen tube growth for virus-free and infected clones was also studied by compsring means of frequency distributions for pollen tube lengths (Table 5.2). The distribution of pollen tube lengths for both treatments (AMV-infected and virus-free) was unimodal. For infected plants of clones B-19 and B-39, the majority of germ tubes (44.0 and 38.0%, respectively) were between 0.6 and 0.9 mm long. For clone B-24, only 17.5% of germ tubes belonged to this category, while about 25.0% of all tubes were 1.2-1.5 mm long. Pollen from virus-free plants of clone B-19 produced about 39.1% of germ tubes 0.9-1.2 mm long. The length of about 69.0% of germ tubes in clone B-24 were between 0.9 and 1.8 mm, and in clone B-39, 56.0% of all grains had tubes between 0.9 and 1.5 mm, long.

The longest tubes were produced by pollen from virus-free plants of clone B-24 (2.4-2.7 mm), followed by B-39 (2.1-2.4 mm) and B-19 (1.5-1.8 mm). In AMV-infected plants the longest tubes were found in pollen samples from

alfalfa clone B-24 (1.8-2.1 mm), followed by B-39 (1.5-1.8 mm) and B-19 (1.2-1.5 mm).

The comparison of pollen tube growth in virus-free and infected plants of three alfalfa clones revealed that the average lengths of tubes produced by pollen from virus-free plants of clones B-24 and B-39 were 1.5 and 1.2 mm, respectively (Table 5.1). The average lengths of tubes of pollen from virus-free plants of clone B-19 and infected plants of clone B-24 were 1.1 mm. The shortest tubes were produced by pollen from AMV-infected plants of clones B-19 and B-39 (0.8 mm).

5.4 Discussion

The investigation on the effect of AMV on germination and tube growth of alfalfa pollen established that clones of cv. Beaver differed in their response to infection with AMV isolate A-515. Germination of pollen from infected plants was significantly reduced within and among clones compared to pollen from virus-free plants. The results also indicated that within each clone, pollen from infected plants produced germ tubes shorter than those of pollen from virus-free plants (Table 5.1). However, in a mixture of pollen from virus-free plants of clone B-19 and B-39 and infected plants of clone B-24, pollen from infected plants will be able to compete efficiently in fertilizing alfalfa ovules, and transmitting AMV to seed, because a certain number of pollen grains from infected plants of clone B-24 produced longer tubes than pollen from virus-free plants of clone B-19 (Table 5.2).

Significant differences were found among clones in germination and tube length of pollen from virus-free plants. Sexsmith & Fryer (1943) also reported differences in pollen germination among clones, while Barnes & Cleveland (1963a, 1963b) and Straley & Melton (1970) showed significant

differences in average pollen tube length among alfalfa clones in *in vitro* studies. A possible explanation for observed differences is that pollen tube growth is controlled by inherent genetic factors. However it is important to point out also, that these authors did not consider the possible effect of virus infection on pollen germination and pollen tube growth and no testing of alfalfa clones for the presence of AMV has been reported.

The effect of AMV on pollen viability *in vitro* was studied on an agar-sucrose medium that provided a combination of good germination and tube growth for hand-collected pollen (Lehman & Puri 1964). Although the tube lengths of pollen germinated *in vitro* were expected to be shorter than those of pollen germinated on stigma, according to Barnes & Cleveland (1963b) *in vitro* measurements were good indicators of pollen viability and could be used for rapid estimation of the potential pollen tube growth *in situ*.

The implications of the results obtained in this investigation on the effect of AMV on pollen viability should be explained in relation to some genetic and reproductive characteristics of alfalfa. Alfalfa is a cross-pollinated perennial with a low incidence of selfing (Busbice et al. 1972). It is a heterozygous autotetraploid with a great variability of germplasm. In nature, alfalfa is pollinated by bees, and a mixture of pollen from both virus-free and infected plants from different clones is randomly deposited on stigma. The effect of virus infection on pollen viability and tube growth and variability among clones may be important factors involved in determining the extent of fertilization and pollen transmission of AMV to alfalfa seed. Frosheiser (1974) has reported that the extent of AMV transmission to seed depended on the alfalfa clone, cultivar, AMV strain and environmental conditions.

Another important aspect that should be pointed out in regard to the epidemiological role of pollen in disease spread under field conditions, is the age of alfalfa stands, particularly those used for seed production. Taking into consideration the differences among clones in pollen tube length of pollen from virus-free and infected plants in one-year-old alfalfa stands with a low incidence of infected plants, pollen from virus-free plants of clones B-19, B-24 and B-39 will fertilize ovules more frequently than pollen from infected plants of the same clones, particularly those at the base of the ovary. As a result, a larger number of virus-free seeds per pod will be produced. However, in the third and fourth years of cultivation, the number of infected plants within the field was shown to increase considerably (Gates & Bronskill 1974, Mueller 1965). Consequently, the frequency of pollination and fertilization by pollen from infected plants of clones B-19, B-24 and B-39 may increase, resulting in a higher incidence of contaminated seeds. Since pollen from infected plants has shorter tubes, the probability that it will fertilize basal ovules is remote, however. Poor seed set per pod will result in the overall reduction in seed yield.

While we have shown that under certain conditions pollen from infected plants can compete successfully with pollen from virus-free plants in fertilizing alfalfa ovules, the opposite results were obtained for some other virus-host combinations. Yang & Hamilton (1974) reported for tobacco ringspot virus (TRSV) in soybeans that the role of infected pollen in seed transmission was negligible due to the poor germination and slow elongation of tubes. Results of cross pollination experiments indicated that TRSV was transmitted to soybean seed mainly through infected megagametophytes. Recently, Childress & Ramsdell (1986) have demonstrated that pollen from highbush blueberry infected with blueberry leaf mottle virus had reduced

germination and slowed tube elongation, and hence could not compete successfully with virus-free pollen.

Although the impact of virus infection on pollen viability was previously investigated, this is the first report on the effect of AMV on viability and germ tube growth of alfalfa pollen *in vitro*. This study has established that the effect of AMV isolate A-515 was manifested by the overall reduction in germination and in germ tube growth of pollen from clones B-19, B-24 and B-39 of alfalfa cv. Beaver, as well as clonal differences in response to virus infection. Cross pollination experiments are in progress to determine the *in situ* role of alfalfa pollen in transmission of AMV to alfalfa seed.

Table 5.1. Frequency distribution for pollen tube length of AMV-infected and virus-free clones of alfalfa cv. Beaver

Alfalfa clone	Pollen Grains (%) Tube length (mm) 0-0.3	0.31-0.6	0.61-0.9	0.91-1.2	1.21-1.5	1.51-1.8	1.81-2.1	2.11-2.4	2.41-2.7
B-19	3.1/0*	21.5/4.0	44.4/25.8	25.7/39.1	5.1/22.9	0/8.0	0/0.2	0/0	0/0
B-24	1.3/0	12.4/0.4	17.5/5.3	29.3/21.1	25.3/24.4	10.6/24.2	3.3/13.1	0/7.7	0/3.5
B-39	3.3/0	18.2/5.1	38.0/14.9	27.5/26.9	12.2/29.3	0.6/16.9	0/6.2	0/0.6	0/0

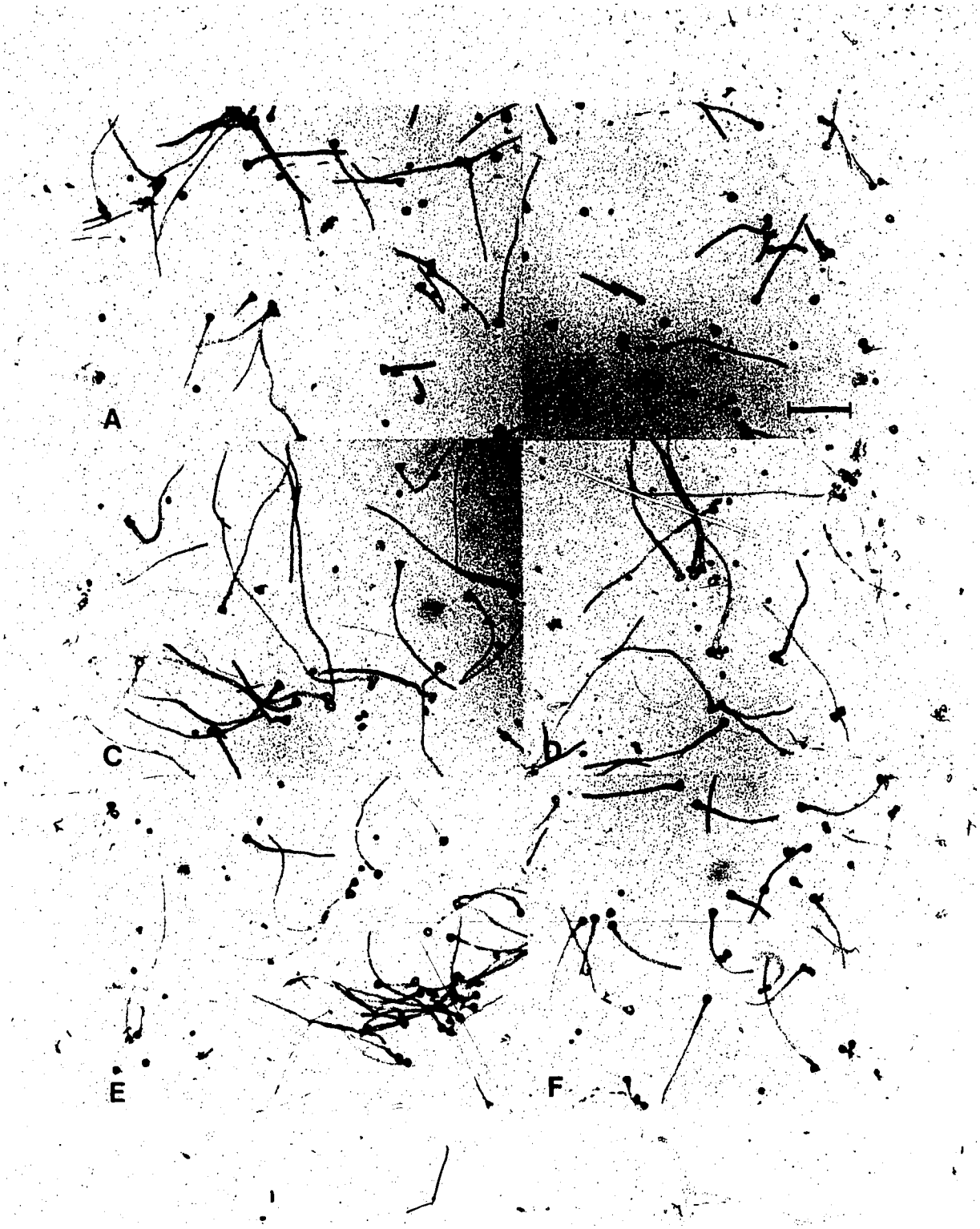
* Numerator represents % of pollen grains from infected clones.
Denominator represents % of pollen grains from virus-free clones.

Table 5.2. Comparison of pollen viability and tube length of AMV-infected and virus-free clones B-19, B-24 and B-39 of alfalfa cv. Beaver

Clone/ Treatment	Germination Rate (%)				Tube Length (mm)
	30 min.	60 min.	90 min.	120 min.	20 h
B-19/virus-free	42.6_{CD}	54.2_{C}	61.3_{B}	67.2_{B}	1.1_{B}
B-19/AMV	31.5_{A}	42.0_{A}	50.9_{A}	54.7_{A}	0.8_{A}
B-24/virus-free	47.9_{D}	60.1_{A}	72.7_{C}	82.1_{C}	1.5_{D}
B-24/AMV	34.5_{AB}	43.9_{A}	55.9_{AB}	64.3_{B}	1.1_{B}
B-39/virus-free	46.7_{D}	61.5_{D}	69.8_{C}	78.3_{C}	1.2_{C}
B-39/AMV	38.9_{BC}	49.1_{B}	57.6_{B}	59.7_{AB}	0.8_{A}

Means with the same letter within a column are not significantly different at the 5% level.

Fig. 5.1. Germinated alfalfa pollen grains. Pollen grains were germinated for 2 h on a medium consisting of 1.5 g Bacto-agar and 20 g of sucrose per 100 ml H_2O, pH 7.0. Pollen from virus-free plants of clones B-19 (A), B-24 (C) and B-39 (E) and pollen from infected plants of the same clones, B, D, and F, respectively. The bar represents 250 μm.


A
C
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6. HYPERSENSITIVE REACTION IN ALFALFA (*Medicago sativa* L.) INDUCED BY ALFALFA MOSAIC VIRUS[1]

6.1 Introduction

The inheritance of resistance to some isolates of AMV has been studied previously for several alfalfa clones (Crill et al. 1971, Kehr et al. 1972). However, no alfalfa cultivars resistant to AMV have been developed so far. Probable reasons for this are: 1) the diversity of AMV strains in pathogenicity (Bancroft et al. 1960, Crill et al 1971, Gibbs 1962, Frosheiser 1969, Paliwal 1982), and 2) the absence of accurate and consistent screening procedures for identifying resistant plants.

Frosheiser (1969) reported that several clones of alfalfa were immune to all AMV isolates tested, and that one clone, resistant to 27 isolates, remained virus-free under field conditions in a mixture with naturally infected plants. It has also been shown that plants resistant to any one AMV strain can be found in many alfalfa cultivars (Crill et al. 1971, Frosheiser 1969).

Development of reliable and sensitive screening procedures for selecting genotypes of alfalfa immune and resistant to AMV is essential for the production of new cultivars through breeding programs. Because of the variability in symptom expression and the frequent absence of visible symptoms in infected alfalfa plants under both greenhouse and field conditions (Burke 1963, Diachun & Hanson 1957, Gibbs 1962, Frosheiser 1964), indicator hosts and serological methods have been routinely used for indexing alfalfa for the presence of AMV (Kehr et al. 1972). A screening

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program was developed using AMV in crude sap as inoculum and a bioassay on indicator hosts to identify AMV-infected plants (Crill et al. 1970). A major disadvantage of this method is that detection of AMV is significantly influenced by the age of indicator hosts and the environmental conditions, and as such optimum conditions should be determined for each particular virus-host combination. The assay procedure is also time-consuming and requires controlled growth facilities.

During preliminary screening of Beaver alfalfa for resistance to an isolate of AMV, Pesic & Hiruki (1985) observed a hypersensitive reaction on inoculated leaves of about 40% of all clones tested. Hypersensitivity represents a form of resistance in which the virus is confined to the necrotic cells at the site of infection and consequently is unable to invade a plant systemically. This paper reports on the conditions for the expression of the hypersensitive reaction of selected alfalfa clones and their use in identifying alfalfa genotypes resistant to AMV.

6.2 Materials and Methods

6.2.1 Source of alfalfa clones

Twenty-eight clones were established from virus-free alfalfa seedlings of cv. Beaver (Pesic & Hiruki 1985), and vegetatively propagated by stem cuttings (Pesic & Hiruki 1987). Clonal plants, treated with *Rhizobium meliloti* , strains NRG-43-4 and NRG-85-1 (The Nitrogen Co., Milwaukee, Wis.), were transplanted into plastic rootrainers, 4 x 5 x 20 cm (Spencer-Lemaire Ind., Edmonton, Alta.), containing regular soil mix (soil:peat:sand, 1:1:1, v/v/v), and maintained in the greenhouse at 20 C and a 16 h photoperiod.

6.2.2 Virus and virus purification

An Alberta isolate of AMV, A-515 (Hiruki & Miczynski 1987) was propagated in *Nicotiana tabacum* L. cv. White Burley in the greenhouse at 23 C and a 16 h photoperiod. Leaves with severe mosaic symptoms were harvested 2 weeks after inoculation and were kept frozen at -70 C until used. The virus was purified according to van Vloten-Doting & Jaspars (1972).

Frozen tobacco leaves (100 g) were homogenized in a blender with 100 ml of 0.1 M K_2HPO_4, 0.1 M ascorbic acid and 0.02 M ethylenediaminetetraacetate adjusted to pH 7.1 with KOH. Following low-speed centrifugation at 14,000 g for 20 min in a Sorval refrigerated RC2-B centrifuge, the virus was precipitated from the clarified supernatant by adding an aqueous solution of 30% polyethylene glycol (Fisher Sc. MW 15,000-20,000) to a final concentration of 5%, and a 30 min incubation at 4 C with constant stirring. The precipitated virus was collected by low-speed centrifugation at 20,000 g for 20 min, and the pellets were resuspended in 0.01 M NaH_2PO_4 adjusted to pH 7.0 with NaOH. The suspension was subjected to two cycles of differential centrifugation and virus pellets were resuspended in 0.5 ml of 0.01 M NaH_2PO_4, pH 7.0. High-speed centrifugation was carried out at 78,000 g in a Beckman L5-75 preparative ultracentrifuge.

Partially purified AMV preparations were subjected to sucrose gradient centrifugation to separate AMV and host material. Resulting AMV preparations (0.5 ml) were layered on a linear-sucrose gradient of 10-40% prepared in 0.01 M NaH_2PO_4, pH 7.0, and centrifugation was carried out in a Beckman SW 28 rotor for 2 h at 95,000 g. Viral fractions, collected from the sucrose gradient, were subjected to high-speed centrifugation, and the final pellet was resuspended in 0.1 M NaH_2PO_4, pH 7.0. The concentration

of AMV (mg/ml) in purified preparations was determined from the ultraviolet absorbance at 260 nm using an extinction coefficient of 5.2 $(mg/ml)^{-1}cm^{-1}$.

6.2.3 Inocula

Purified AMV and AMV in crude sap were used for inoculation of alfalfa clones. Crude sap was obtained by grinding 10 g of frozen tobacco leaves with a pestle and mortar on ice and straining the homogenate through a double layer of cheesecloth. Dilutions of purified virus and crude sap were prepared in 0.025-M phosphate buffer (Na_2HPO_4-NaH_2PO_4), pH 7.0.

6.2.4 Inoculation protocol

Clonal plants were manually inoculated at the five- to seven-leaf stage by rubbing three compound leaves located in the middle portion of the stem with virus inoculum to which 600 mesh (22 µm) Carborundum was added. After rinsing with distilled water plants were maintained in the greenhouse at 20 C and a 16 h photoperiod.

6.2.5 Enzyme-linked immunosorbent assay (ELISA)

Clonal plants were tested for the presence of AMV four and eight weeks after inoculation by direct double sandwich ELISA (Clark & Adams 1977). Samples were prepared by grinding individual petioles (about 1.5 cm long) in 0.4 ml, and three leaflets per plant in 1.5 ml of phosphate-buffered saline, pH 7.4, containing 0.05% Tween 20, 1% polyvinyl-pyrrolidone, and 0.1% egg albumin. Crude sap from infected and virus-free tobacco plants served as a control. The γ-globulin fraction of anti A-515 antiserum (Pesic et al. 1988)

was used at 2 µg/ml and alkaline phosphatase conjugated γ-globulin at 1/1000 dilution.

6.2.6 Infectivity assay

Relative infectivity of AMV in virus suspensions used fro inoculation of alfalfa clones was measured by a half-leaf local lesion assay on the primary leaves of ten-day-old beans, *Phaseolus vulgaris* L. cv. Red Kidney. Prior to inoculation plants were given a dark treatment for 24 h. Leaves rubbed with phosphate buffer alone served as a control. Each dilution of purified AMV and AMV in crude sap was paired with another dilution on the opposite half leaf six times for each possible combination of dilutions. The inoculated leaves were rinsed with distilled water immediately after inoculation and were covered with wet paper towels. The plants were maintained in the greenhouse at 20 C and a 16 h photoperiod. Local lesion counts were made 2-3 days later using a stereomicroscope and a manual counter.

6.3 Results

6.3.1 Clonal response to AMV

Twenty-eight alfalfa clones with 30 plants per clone, were assayed for resistance to AMV isolate A-515 using purified virus, 1 mg/ml in 0.025 M phosphate buffer, pH 7.0.

The hypersensitive reaction was observed on the inoculated leaves of 12 clones, whereas the uninoculated leaves were symptomless (Fig. 6.1A). On hypersensitive leaves light brown, necrotic local lesions developed in 3 to 4 days, reaching up to 1 mm in diameter (Fig. 6.1B). All the plants of hypersensitive clones inoculated with AMV consistently produced local

lesions. Testing of clonal plants by ELISA showed that AMV was confined to the area of local lesions only, and was not present in the leaf petioles or the rest of the plant.

The remaining 16 alfalfa clones were systemically infected, with the mosaic symptoms developing in 7 to 10 days after inoculation (Fig. 6.1C). The symptoms varied among clones, from masked to mild and severe mosaic. The percentage of infected plants per clone was between 73% and 96% in eight clones, and 100% in another eight clones.

6.3.2 Comparison of purified virus and crude sap inocula

Virus-free plants of resistant clones B-8 and B-48, and susceptible clones B-19 and B-20, were inoculated with serial dilutions of purified AMV (1 µg/ml-1000 µg/ml), and crude sap (10^{-3}-10^{0}). The total number of plants per treatment is shown in Table 6.1.

The hypersensitive reaction was observed on the inoculated leaves of clones B-8 and B-48 for all dilutions of purified AMV. However, inoculations with the higher dilutions of AMV resulted in lower numbers of local lesions. Thus, only a few local lesions developed on leaves inoculated with AMV at 1 µg/ml. While 100% of plants inoculated with AMV at 1000 µg/ml responded with the hypersensitive reaction, only about 10% of plants developed local lesions following inoculation with AMV at 1 µg/ml. Visual observations were confirmed by ELISA.

In plants inoculated with crude sap, visible local lesions were absent on inoculated leaves. However, AMV was detected by ELISA in about 40% of plants in clone B-8, and 35% in clone B-48, inoculated with the undiluted sap, and 48% and 43% of plants, respectively, inoculated with the sap dilution of 10^{-1}. Testing by ELISA also indicated that in plants inoculated

with purified virus or crude sap. AMV was localized in inoculated leaves, and did not spread systemically throughout the plants (Table 6.1).

Clones B-19 and B-20 responded to inoculation with purified virus and crude sap with systemic infection and mild mosaic symptoms on newly developing leaves. The highest percentage of infected plants, 90.4% for B-20 and 79.5% for B-19, was obtained with purified virus at 1000 µg/ml. Only 2.7% of plants were infected in clone B-19 and none in B-20 with AMV at 1 µg/ml (Table 6.1). In the case of crude sap, infection rates were slightly higher with a sap dilution of 10^{-1} (Table 6.1). Only about 2.0% of the plants in either clone were infected with the crude sap at 10^{-2} dilution.

The bean-infectivity assay of purified AMV and crude sap used for inoculation of clonal plants showed that purified AMV was infective at all dilutions, with the average number of local lesions per half leaf ranging from 216 for 1000 µg/ml to 5 for 1 µg/ml (Fig. 6.2). The infectivity of the crude sap was slightly lower for the undiluted sap than for the dilution of 10^{-1} (Fig. 6.2).

6.4 Discussion

In this investigation two distinct reactions of AMV isolate A-515 of alfalfa cv. Beaver were observed: 1) localized infection resulting from hypersensitive reaction, and 2) systemic infection in susceptible clones. Previously the hypersensitive response to AMV was found in assay species such as *Phaseolus vulgaris* , *Vigna sinensis*, *Chenopodium amaranticolor* and *C. quinoa*. The first two species were used in screening programs as indicator hosts when testing alfalfa for the presence of AMV (Crill et al. 1970, Rosheiser 1969). In this study, however, the hypersensitive reaction was used for direct identification of clones of alfalfa cv. Beaver with resistance to AMV isolate A-

515. Furthermore, the hypersensitive reaction was also observed in several other alfalfa cultivars tested against four isolates of AMV, including A-515 (Pesic & Hiruki, unpublished data).

Since hypersensitive reaction in alfalfa was induced by the purified AMV only, and the percentage of plants with local lesions was directly proportional to virus concentration in inoculum, the amount of AMV 1 mg/ml of 0.025 M phosphate buffer, pH 7.0, was found to be the most suitable for screening alfalfa for resistance to AMV.

According to the results obtained in this investigation, the disadvantages of using crude sap inoculum were: the absence of visible symptoms on inoculated leaves of resistant clones (Crill et al. 1970, Frosheiser 1969), and low rates of AMV transmission to alfalfa for both undiluted and diluted sap (Frosheiser 1969). This means that repeated inoculations of clonal plants (Tu & Holmes 1980) and testing by ELISA for the presence of AMV are required for detecting resistant plants.

In summary, the method developed for assaying Beaver alfalfa for resistance to AMV isolate A-515 using purified virus as inoculum and hypersensitive response for identification of resistant genotypes is rapid, specific and sensitive, and as such is recommended for routine screening of alfalfa in breeding programs. Further investigation must be conducted on the response of resistant clones of cv. Beaver to aphid and pollen transmission of AMV.

Table-6.1. Response of alfalfa clones cv. Beaver to purified and crude sap inocula

Alfalfa clone	Inoculum: Purified AMV (µg/ml) 1000	100	10	1	Crude sap (dilution) 10^{0}	10^{-1}	10^{-2}	10^{-3}
B-8	0/44	0/40	0/38	0/47	0/49	0/44	0/44	0/53
B-48	0/44	0/40	0/47	0/38	0/42	0/47	0/42	0/47
B-19	31/39 (79.5)	29/41 (70.7)	13/40 (32.5)	1/37 (2.7)	21/45 (46.7)	21/40 (52.5)	1/38 (2.6)	0/44 (0.0)
B-20	47/52 (90.4)	36/53 (67.9)	14/52 (26.9)	0/37 (0.0)	28/55 (50.9)	30/56 (53.6)	1/48 (2.0)	0/49 (0.0)

Numerator represents number of clonal plants systemically infected.
Denominator represents number of clonal plants inoculated.
Figure in brackets indicates percentage of infection.

Fig. 6.1. Responses of alfalfa cv. Beaver to AMV isolate A-515. Clones B-48 (1A and 1B), and B-24 (1C) were inoculated with purified AMV 1 mg/ml of 0.25 M phosphate buffer, pH 7.0. 1A hypersensitive reaction on inoculated leaf (left) and the absence of symptoms on a new leaf (right); virus-free control (centre). 1B a close-up of necrotic local lesions distributed over the entire inoculated surface. 1C, masked symptoms on inoculated leaf (left), and mosaic on a new leaf (right); virus-free control (centre).

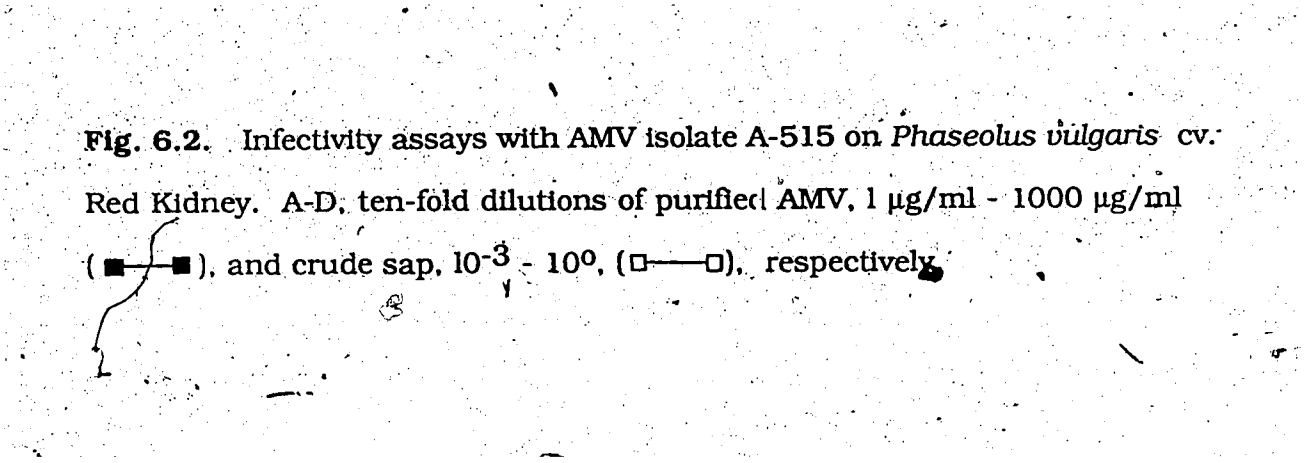
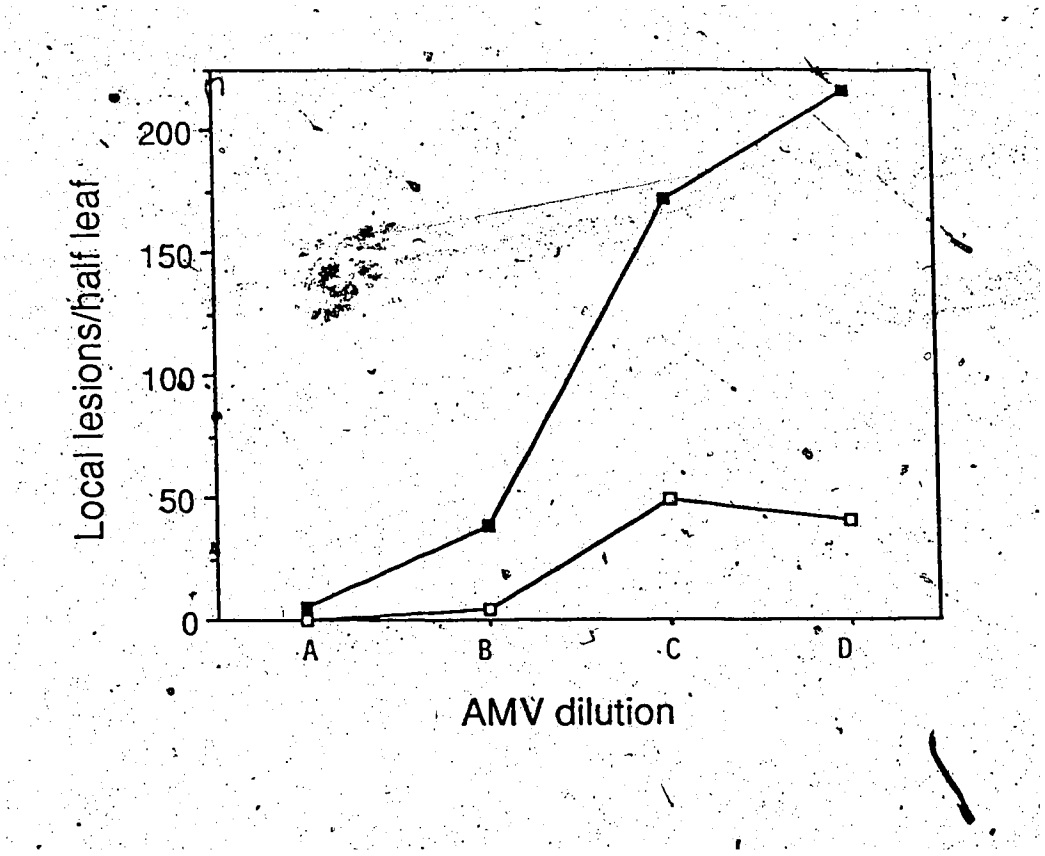


Fig. 6.2. Infectivity assays with AMV isolate A-515 on *Phaseolus vulgaris* cv. Red Kidney. A-D, ten-fold dilutions of purified AMV, 1 µg/ml - 1000 µg/ml (■——■), and crude sap, 10^{-3} - 10^{0}, (□——□), respectively.


Local lesions/half leaf
200
150
100
50
0
A
B
C
D
AMV dilution

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7. DISCUSSION AND CONCLUSIONS

Transmission of AMV through seed and gametes was previously established (Frosheiser 1974, Hemmati & McLean 1977) but little was known about the mechanism(s) of virus transmission through pollen and ovules and the incidence of AMV in alfalfa seed. Therefore, a more comprehensive study of these and related aspects was carried out for a better understanding of AMV transmission through seed.

In this investigation the emphasis was on (1) a development of rapid and sensitive techniques for AMV detection in infected tissues, (2) elucidation of the mechanism(s) of AMV transmission through pollen and ovules, (3) the role of infected pollen and seed in the epidemiology of AMV, and (4) selection of clonal materials of alfalfa resistant to AMV.

ELISA and dot hybridization using a ^{32}P-labeled AMV- specific cDNA probe were rapid, sensitive and reliable methods for detecting AMV in minute quantities of alfalfa pollen. As such they are recommended for routine screening of pollen to ensure the use of virus-free sources of pollen in breeding programs. ELISA was also more sensitive and faster in detecting AMV in seed and seedlings than the infectivity assay (Frosheiser 1974, Tosic & Pesic 1975).

Immunogold cytochemistry using an immunoglobulin fraction of anti AMV antiserum and gold-labeled IgG or protein-A gold complexes enabled localization of AMV virions and viral antigen in alfalfa pollen, and for the first time in alfalfa ovules.

Localization of AMV in the integuments of alfalfa ovules and in the seed coat indicated that seed coat infection was the result of ovule transmission of AMV. The absence of AMV in the embryo sac of alfalfa ovules and its

localization in embryos of alfalfa seed indicated that embryo infection occurred through infected pollen during fertilization. Cherry leaf roll virus is transmitted to birch seed in a similar manner (Cooper et al. 1984).

The overall reduction in germination and tube growth of pollen from AMV-infected clones compared to virus-free pollen is important in accomplishing the production of virus-free seed, particularly in newly established stands with a low incidence of AMV infection. However, a certain number of pollen grains originating from infected plants that produce tubes longer than virus-free pollen, will be able to compete efficiently with virus-free pollen in fertilizing alfalfa ovules and generating infected seeds. The role of virus-infected pollen in the epidemiology of AMV is particularly significant in aging alfalfa stands with a high incidence of infected plants (Crill et al. 1970, Mueller 1965) The prevalence of infected pollen in such fields will result in a higher frequency of AMV transmission to seed.

The method developed in this study for assaying alfalfa for resistance to AMV is rapid, specific and sensitive, and enabled identification of genotypes of cv. Beaver with the hypersensitive response to AMV. This method should be incorporated in breeding programs for routine screening of alfalfa. Clones with the hypersensitive response to infection with AMV isolate A-515 represent genetic material suitable for the development of new alfalfa cultivars.

Although a clean seed program can effectively eradicate virus diseases as in the case of pea-seed borne mosaic virus (Hampton et al. 1976), the use of virus-free seed in establishing alfalfa stands is limited by the following factors: 1) alfalfa is a perennial cross-pollinated species and infected plants represent a long lasting source of infected pollen and seed in the field.

and 2) AMV has a wide host range that includes weeds and other forage legumes, frequently grown in mixed stands with alfalfa, and is efficiently transmitted by aphids in a non-persistent manner. Therefore, the use of alfalfa cultivars with resistance to AMV is practical and is considered as a long term solution in achieving effective control of AMV, and the production of virus-free certified seed.

Continuing study on AMV-host interactions, with alfalfa cultivars and AMV strains prevalent in western Canada and particularly in Alberta, will contribute to the rapid development of alfalfa cultivars resistant to AMV.

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