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Effects of Seed Extracts of Pisum sativum on Tobacco Mosaic Virus Infectivity

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EFFECTS OF SEED EXTRACTS OF *PISUM SATIVUM* ON TOBACCO MOSAIC VIRUS INFECTIVITY

by

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VITA

The author, Douglas Gerard Mack, is the son of Stephen Mack and Irene (Majkszak) Mack. He was born May 22, 1956, in Chicago, Illinois.

His elementary education was obtained in the parochial school system at Mary Seat of Wisdom, Park Ridge, Illinois. He graduated from Maine South High School, Park Ridge, Illinois, in 1974, where he was elected to the National Honor Society.

He entered Northwestern University, Evanston, Illinois, 1974 and received the degree of Bachelor of Arts with a major in biology and psychology in June, 1978. In 1978, he was admitted to the Graduate School of Loyola University, Chicago, Illinois, leading to a Masters of Science degree in biology under the direction of Dr. William Cordes Ph.D.

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INTRODUCTION

The property of transmissibility is a fundamental characteristic of viruses as it is of other biological agents that cause disease. Since many viruses are highly infectious in nature and systemic in distribution it would seem likely that many viruses should be transmitted through the seed. This not the case. Crowley (1957) reported that out of some 300 plant viruses only 45 had been found to be seed transmitted. In view of the fact extracts from healthy plants sometime contain inhibitory substances to virus infection and produce interferon-like substances induced by viral agents, it seems possible that seeds may also contain virus inhibitors.

Finding virus inhibiting substances may be of great economic importance, since virus diseases often take a toll on the quality and quantity of many food crops. There is little in the literature to suggest any practical application for chemical virus inhibitors and inactivators discovered so far. Many of these inhibitors are phytotoxic or highly toxic to man. Naturally occurring inhibitors may be of more practical value, perhaps being more compatible with other biological systems. Seeds may contain such a suitable inhibitor. Furthermore, a general scheme may develop on how plants prevent the spread of virus diseases.

The purpose of this study is to determine if seeds from 4 varieties of Pisum sativum contain inhibitory substances to tobacco mosaic virus. Inhibitory effects were assessed on primary half leaves from 10 day old plants (Phaseolus vulgaris cv. pinto). Location of the inhibitor in the seed and characterization of the inhibitor were also studied.

REVIEW OF LITERATURE

Related topics of the literature reviewed are grouped to facilitate reading and understanding.

Seed Transmission of Viruses

Dugger (1930) suggested that tobacco mosaic virus in the tobacco seed was inactivated by some specific protein. Kausche (1940) found the presence of a heat stable substance extracted from ripened and germinating tobacco seeds that inactivated tobacco mosaic virus (TMV). This extract reduced infectivity by as much as 50%. He suggested that the inactivator had a surface effect on the TMV molecule which made it non-infective. Matthews (1970) suggested that natural cytokinins may play a part in controlling the distribution of some viruses in seeds and fruits. Nakagaki (1971) reported a reduction in TMV infection by kinetin.

Bennett (1936) explained that the lack of seed transmission of beet curly top virus by showing that this virus is restricted to the vascular tissue of the plant and thus unable to invade the embryo. Crowley (1955) commented that Bennett's explanation cannot account for the far greater number of plant virus diseases that are not restricted to the vascular tissue of their hosts.

A number of researchers have found that some

viruses enter the developing seed but are later excluded in the mature seed. von Stelzner (1942) indicated that potato X and Y viruses are inactivated in the seed during ripening, storage, and germination. Zaumeyer and Narter (1943) showed that the virus of southern bean mosaic was present in the seeds in fairly high concentrations of systemically infected bean plants in the early stages of development, but apparently are inactivated in the later stages of ripening and storage. Cheo (1955) with respect to southern bean mosaic virus found a decrease in seed transmission from about 80% in the immature seed to about 2% in the mature seed. The virus was present in all flower and fruit parts of the systemically infected bean plants. He postulated that the decrease was due to an inhibitor formed as the seed matured. Crowley (1957) reported that during the early stages of host development tobacco ringspot virus has been shown to infect the embryo and gametes, but is unable to infect the embryo during the later stages of development. Ford (1966) found that pea streak virus was transmitted in the immature seed and not in the mature seed. Cowpea chlorotic mottle virus is not seed transmitted in the cowpea because of deactivation during maturation. Active virus was recovered from the sepals, petal, stamens, seed coats, and the immature seeds. None was recovered from the endosperm or

the embryo in the mature seed (Gay, 1969).

Virus morphology is not the limiting factor in seed transmission of plant viruses (Ford, 1966). Small, isometric (tobacco ringspot virus = 28 mu dia.) and helical (bean common mosaic virus = 750 mu long) viruses are seed transmitted in large seed legumes (Desjardin et al., 1954; Reddick and Steward, 1919). The capacity of seed transmission is specific to both the host and virus. There is considerable variation in the rate of seed transmission between strains of individual viruses in certain hosts as well as between different hosts, including different cultivars of the same species, in relation to a certain virus. Neergaard (1977) reviewing previous research indicated that broad bean true mosaic virus and broad bean strain virus were seed transmitted, whereas broad bean mottle virus and broad bean vascular wilt virus were not. McLean (1941) described the mosaic disease of cowpea and indicated that highly suseptable varieties produced more virus infected seeds than slightly suseptable varieties. Anderson (1957) found 2 strains of cowpea mosaic virus and 1 strain of cucumber mosaic virus were more readily seed transmitted in cowpea cv. White Acre than in 3 other cultivars. Grogan, et al. (1959) showed that seed transmission of squash mosaic virus ranged from 0-20% in different types of cucurbits. Reyes (1961) reported that coffee ringspot virus is seed transmitted in Coffea excelsa, but is not through the seeds of Coffea arabica. Soybean mosaic virus was found seed transmitted; 20% in cv. Harosoy, 11% in cv. Lee, 1% in cv. Hill,

and not at all in cv. Merit (Kennedy and Cooper, 1967; Ross, 1969).

Virus Inhibitors from Plant Extracts

Extracts from healthy plants sometimes contain inhibitory substances to virus infection. Matthews (1970) reviewing the literature on inhibitory substances from higher plants found that inhibitors are widespread as is indicated by the fact that of 75 species (30 families) tested in one survey, 30 species yielded extracts which gave 95-100% inhibition of infection by TMV. Bawden (1954) showed that TMV was inhibited by polysaccarides, proteins, and tannins isolated from higher plants. He divided inhibitors in two categories: inhibitors of infection, and inhibitors of virus multiplication.

Spinacia oleracea (spinach) leaf extracts were found to inhibit cabbage mosaic on tobacco plants but would not inhibit TMV on the same host (Kuntz and Walker, 1947). Kassanis and Kleczkowski (1948) isolated and identified from Phytolacca esculenta (pokeweed) an inhibitor of mosaic disease of pokeweed. The inhibitor was identified as a glycoprotein. Owens et al. (1973) demonstrated that an inhibitor from Phytolacca americana inhibited synthesis of polypeptide in the host. They suggested that this inhibitor blocks in vivo the messenger function of viral RNA on the ribosomes of the host. Raychaudhuri (1952) showed that extracts of germinating Lycopersicon esculentum (tomato) seeds exerted an inhibitory

effect on TMV in Lycopersicon esculentum. Sills and Walker (1952) reported that extracts from healthy Cucumis sativus (cucumber) contained a substance inhibitive of infection by cucumber mosaic virus. The inhibitor was found in all parts of the plant, with the exception of the corolla. Francki (1964) stated that the loss of infectivity of cucumber mosaic virus on the exposure to Cucumis sativus leaf extracts could be due to the aggregation of virus particles, or the formation of a complex between some host material and virus particles, thus preventing infection. Cadman (1959) found that tannins in the leaves of Rubus idaeus (raspberry) inhibited virus infection. Ragetli and Weintraub (1962) postulated that an inhibitor from Dianthus caryophyllus (carnation) competes for essential sites through its e-amino group against possibly similar groups in TMV. Fantes and O'Neill (1964) found the Dianthus caryophyllus inhibitor to have a number of physical and chemical characteristics in common with chick interferon. Loebenstein and Ross (1963) reported that juices from apical leaves of Datura stramonium (jimson weed) to be inhibitory to TMV and TNV (tobacco necrosis virus). Zaitlin and Siegel (1963) found a heat stable protein in Nicotiana tabacum (tobacco) that inhibits virus infection. Dhaliwal and Dhaliwal (1970) reported that extracts from Allium cepa (onion) and Allium sativum (garlic) were inhibitory to whole TMV and TMV nucleic acid multiplication in Phaseolus vulgaris (bean).

Loebenstein (1974) pointed out that inhibitors of infection are not virus specific and the same substance may

inhibit very different viruses. Van Kammen et al. (1961) indicated that most inhibitors affect the host, and not the virus, by competing with virus for infection sites. Fischer and Nienhaus (1973) hypothesized that most inhibitors from plant extracts do not irreversibly inactivate viruses, because the original virus regains its infectivity when the mixture is diluted. Caldwell (1935), Bawden and Freeman (1952), and Slagle et al. (1952) established certain criteria for distinguishing whether an inhibitor affects the virus or the host; (1) instantaneous effect; (2) host dependent; (3) effect decreases with dilution; (4) effect when applied prior to virus inoculation.

Yarwood (1960) indicated that the presence of TMV induced local necrotic lesions on the leaf prevents the formation, on subsequent inoculation, of new lesions in the vicinity of the established lesion. Ross (1961) showed that localized inhibition is not virus specific. Lesions of a number of viruses inhibited subsequent production of lesions by several other viruses. Loebenstein and Ross (1963) observed that resistance was induced in other parts of the plant. When a leaf became infected other parts of the plant were less suseptable. They likened the the material to interferon, a protein or polypeptide substance that is produced by cells of many invertebrates and vertebrates in response to viral infection. Sela and his co-workers (1964) isolated an antiviral factor from virus infected plants which had interferon-like properties. The factor contained protein and nucleic acid.

Purified material had 5000 X the antiviral activity as did the crude extract. Cheo et al. (1964) reported that RNA isolated from plants other than the test species, but not RNA from the test species, was found to be effective in inducing host resistance to viral infection.

Chemical Inhibitors of Viral Infection

Lindner, et al. (1959) conducted an extensive study involving the screening of 233 chemicals for the ability to inhibit TMV and fruit ringspot disease. Of these chemicals only 15 were found to be effective inhibitors at a concentration of 1 mM or less. Leben and Fulton (1952) reported that 1 ug/ml concentration of potassium cyanide, sodium ozide, streptothricin hydrochloride, and terramycin hydrochloride prevented lesion production by 2 plant viruses. Lockhart and Semancik (1968) reported that the application of actinimycin-D caused inhibition of cowpea yellow mosaic virus. Hirai, et al. (1968) found that blasticidin-S was an effective inhibitor of plant viruses.

Inactivation of viruses by suitable base analogs have been reported. Commoner and Mercer (1952) found that TMV was inhibited by thiouracil. Matthews (1954) reported that 8-azoguanine delayed the development of turnip yellow mosaic virus in small chinese cabbage plants. This guanine analog was found to be incorporated into viral nucleic acid. Kurtzman, et al. (1957) found that virus multiplication was inhibited by 6-methylpurine and 6-chloropurine. Gordon and Stachelin

(1958) reported that the yield of TMV was reduced by 50% with 5-flourouracil in tobacco leaves. Verma (1968) showed that guanine carbonate inhibited the formation of tobacco necrosis virus in Phaseolus vulgaris cv. saxa. Cheo and Linder (1964) and Cheo (1969) reported that tannic acid and 2,4,-dichlorophenoxyacetic acid were inhibitory to TMV. Resistance to infection against TMV and tobacco necrosis virus was induced by the injection of polyacrylic into the tissues 2-3 days before inoculation.

Matthews (1970) suggested some possible mechanisms for viral inactivation: (1) interference with correct base pairing during the synthesis of viral RNA in a replicative structure; (2) production of faulty viral message leading to the production of nonfunctional or partly functional proteins required by the virus; (3) incorporation of the analog into the anticodons or enzyme recognition sites of transfer RNA's, giving misreadings to the viral message and again leading to faulty protein production; (4) reduction in the supply of precursors available for RNA synthesis through interference with enzymes involved in nucleotide synthesis,

MATERIAL AND METHODS

Cultivation of Tobacco Mosaic Virus

Seeds of Nicotiana tabaccum cv, Turkish were germinated in 6 x 20 x 30 cm. trays containing a mixture of Canadian sphagnum, top soil, and sand in a ratio of 2:2:1(v/v/v). The trays containing the seeds were covered with a glass plate to retain moisture and kept in a Hotpack incubator at a constant temperature of $28^{o}C \pm 2$, a continuous light intensity of 3000 LUX, and a relative humidity of approximately 70%. After 7 days the glass plate was removed and the trays containing the germinating seedlings were placed in the lab at $20^{o}C \pm 2$ under continuous illumination. When the primary leaves expanded to approxiamately 3 cm., the plants were transplanted into individual plastic pots and kept in a greenhouse at $24^{o}C \pm 4$ with a relative humidity of 40-60% and supplied with continuous artificial light (2000 LUX).

When the tobacco plants were 6 in. high, they were inoculated with a common strain of TMV, PV-135 obtained from American Type Culture, Rockville, Maryland. Older leaves were dusted with 320 mesh carborundum and a drop of virus suspended in neutral phosphated buffer was placed on the center of the leaf. The leaf was rubbed lightly with a finger, spreading the virus over the surface of the leaf, damaging the epidermal hairs allowing cells to become infected. The plants were harvested when the younger leaves showed mosaic symptoms.(Fig.1).

Fig. 1 Typical systemic infection due to tobacco mosaic virus infection Nicotiana tabaccum cv. Turkish. Leaves show mosaic pattern and chlorosis.

Purification of Tobacco Mosaic Virus

Leaf tissue from TMV infected plants was frozen in a deep freeze. The tissue was thawed and macerated in a Waring blender. The macerated tissue was passed through cheesecloth to remove the fibrous material. The extracted plant sap was centrifuged using an IEC clinical centrifuge for 15 min. @ 1000 rpm. to remove all cell fragments. The supernatent was mixed with polyethylene glycol and sodium chloride to form a 10-4 solution (10 = 10% polyethylene glycol and 4 = 4% sodium chloride). This mixture was passed through a 24 in., 2 in. diameter microcrystalline cellulose column (Fig. 2). A 5-2 solution was passed through the column eluting the chloroplasts and various other cellular components, thereby separating them from the virus. The addition of a 5-0 solution eluted the virus in its pure form.

The virus was precipitated by the addition of sodium chloride to the 5-0 solution containing the virus to make the equivalent of a 5-2 solution. This mixture was centrifuged at 4°C. for 15 min. @ 10,000 rpm. in a Sorvall RC2-B centrifuge. The supernatent was discarded and the pellet was resuspended in neutral phosphate buffer. The suspended pellet was centrifuged at 4°C. for 15 min. @ 10,000 rpm. This time the virus containing supernatent was saved. The titer of the virus was determined by serially diluting the virus solution and assaying each dilution on half leaves of Phaseolus vulgaris cv. Pinto. A concentration of virus forming 50-100 lesions per half was used throughout the entire study.

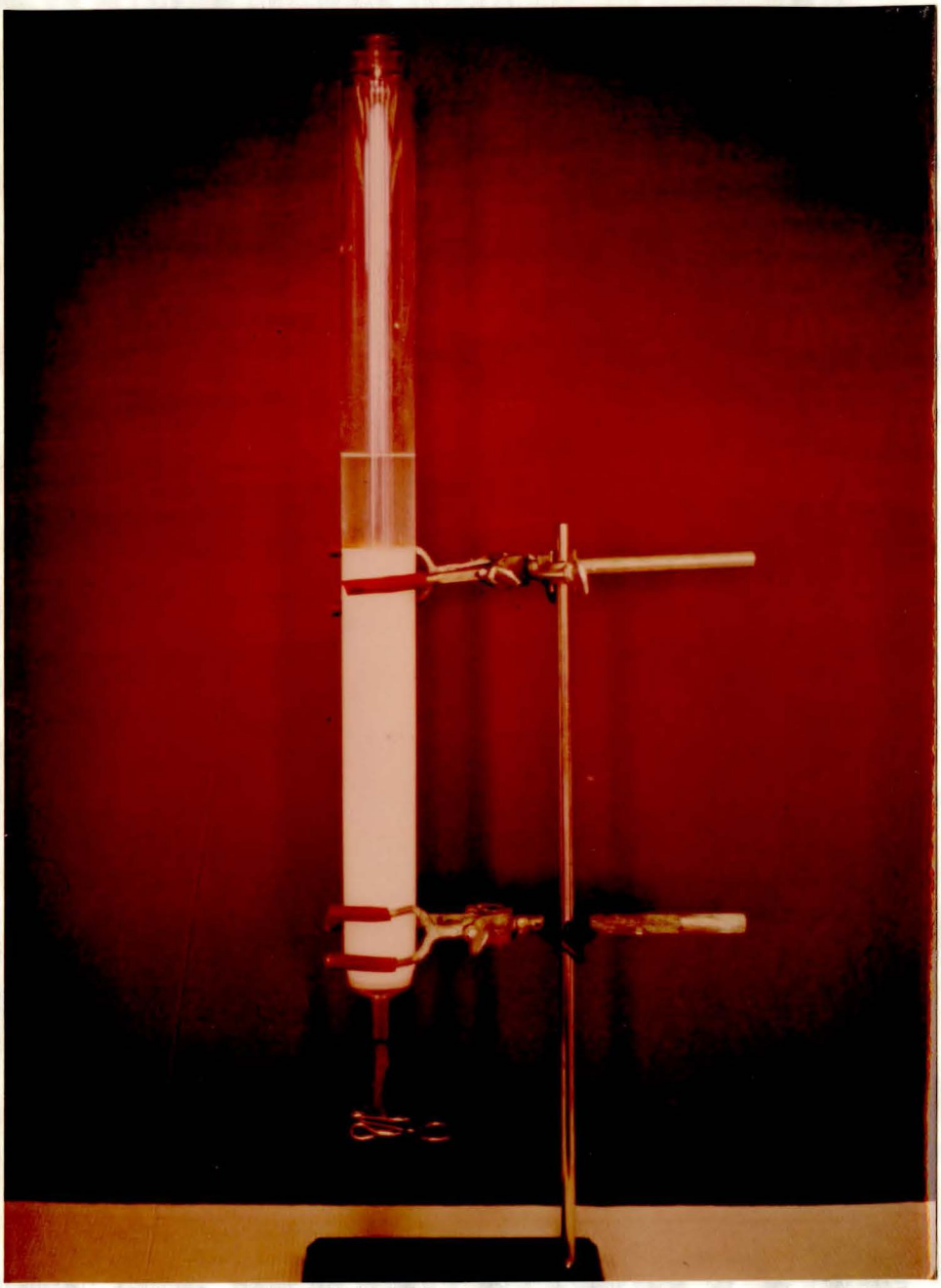

Fig. 2 Microcrystalline cellulose column used to purify TMV by chromatography.

Electron Microscopy of Tobacco Mosaic Virus

Grid Preparation:

The grid coating setup (Fig.3) was washed in Alconox detergent, alcohol, and distilled water (Bils, 1961). Several electrodeposited copper 300 mesh grids were cleaned with absolute alcohol. The cleaned grid holder was placed on a small beaker which in turn was placed in the center of a larger crystallizing dish. Distilled water was added to the crystallizing dish such that the water level was approximately 1 in. above the grid holder. The grids were placed shiny side up on the grid holder.

One drop of 1% Collodion in amyl acetate was deposited on the center of the water surface. The dry film was removed with forceps to clean the water surface of any dust. Another drop was cast. The water was siphoned at the edge of the dish to lower the film on the grids. The grids were allowed to dry on bibulous paper in half-open petri dish in an oven at (40-50°C.)

Negative Staining:

The purified, concentrated TMV suspended in neutral phosphate buffer was used for electron microscopy. Two drops of the virus suspension were placed on a Collodion coated 300 mesh grid. Excess virus was removed by touching the edge of the grid with bibulous paper. Two drops of 1% potassium phosphotungstate, pH 6.5 were placed on the grid. Any excess stain was removed. The grids were allowed to dry at room

Fig. 3 Grid coating setup for electronmicroscopy.

temperature in a petri dish. The virus was viewed under the electron microscope (Fig.4).

Ultrasonic Tobacco Mosaic Virus Bio-Assay (Lamborn, et al,1971)

Seeds of Phaseolus vulgaris cv. Pinto were germinated in 6 x 20 x 30 cm. trays containing vermiculite. The germinating seedlings were kept in a greenhouse at $24^{o}C \pm 4$ with a relative humidity of 40-60% and supplied with continuous artificial light (2000 LUX).

Primary leaves from 10 day old bean plants were harvested by cutting the plants at soil level. The leaves were treated by dipping the entire plant in $45^{o}C$ water for 1 min. Leaves were removed from the stems, cut into two halves; removing the midribs, and dusted with 320 mesh carborundum in a closed chamber using a gas pressure atomizer. One drop of inoculum from a 22 gauge needle was placed near the apical end of the half leaf. Detached half leaf was placed on a polyfoam pad; supporting and protecting the leaf from damage. Inoculum was spread over the surface of the leaf by passing the leaf slowly under the metal probe of a Branson W-140-C sonifier cell disruptor at 50 watts power level (Fig.5). Half leaves were placed on an absorbant paper strip and put into a 10 x 20 x 30 cm. airtight polyethylene container. The container with the leaves was placed in the dark at $38^{o}C \pm 2$ for 18 hrs. Each absorbent paper strip carried 6 half leaves and was placed on aluminum trays (4 x 20 x 30 cm.) containing 1% agar covered by a glass plate. Trays were incubated for 3 to 5 days at $22^{o}C \pm 2$ under continuous flourescent illu-

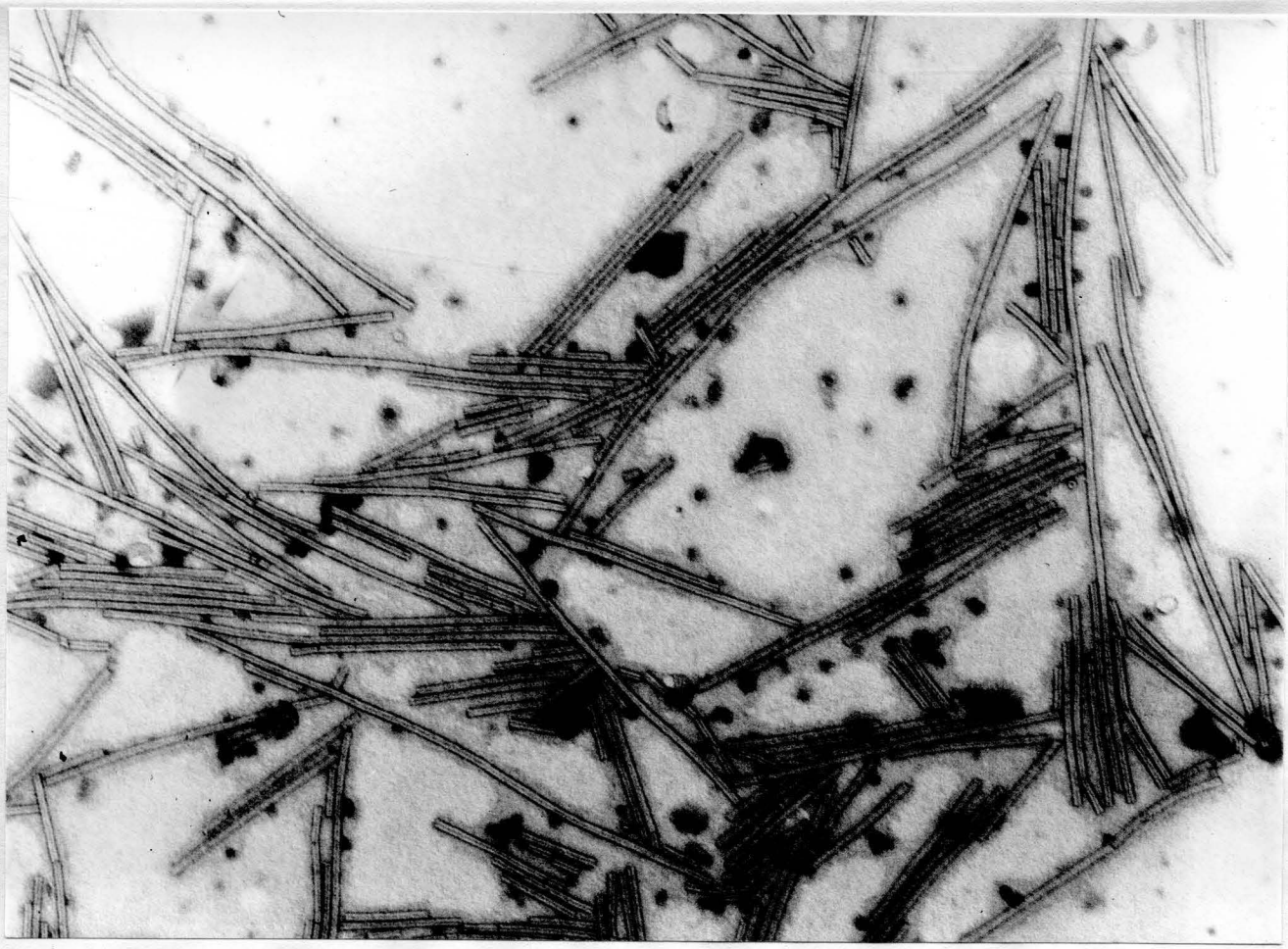

Fig. 4 Electron micrograph of tobacco mosaic rods stained with 1% phosphotungstate. Note penetration of stain in axial hole of the rods (X 32,000)

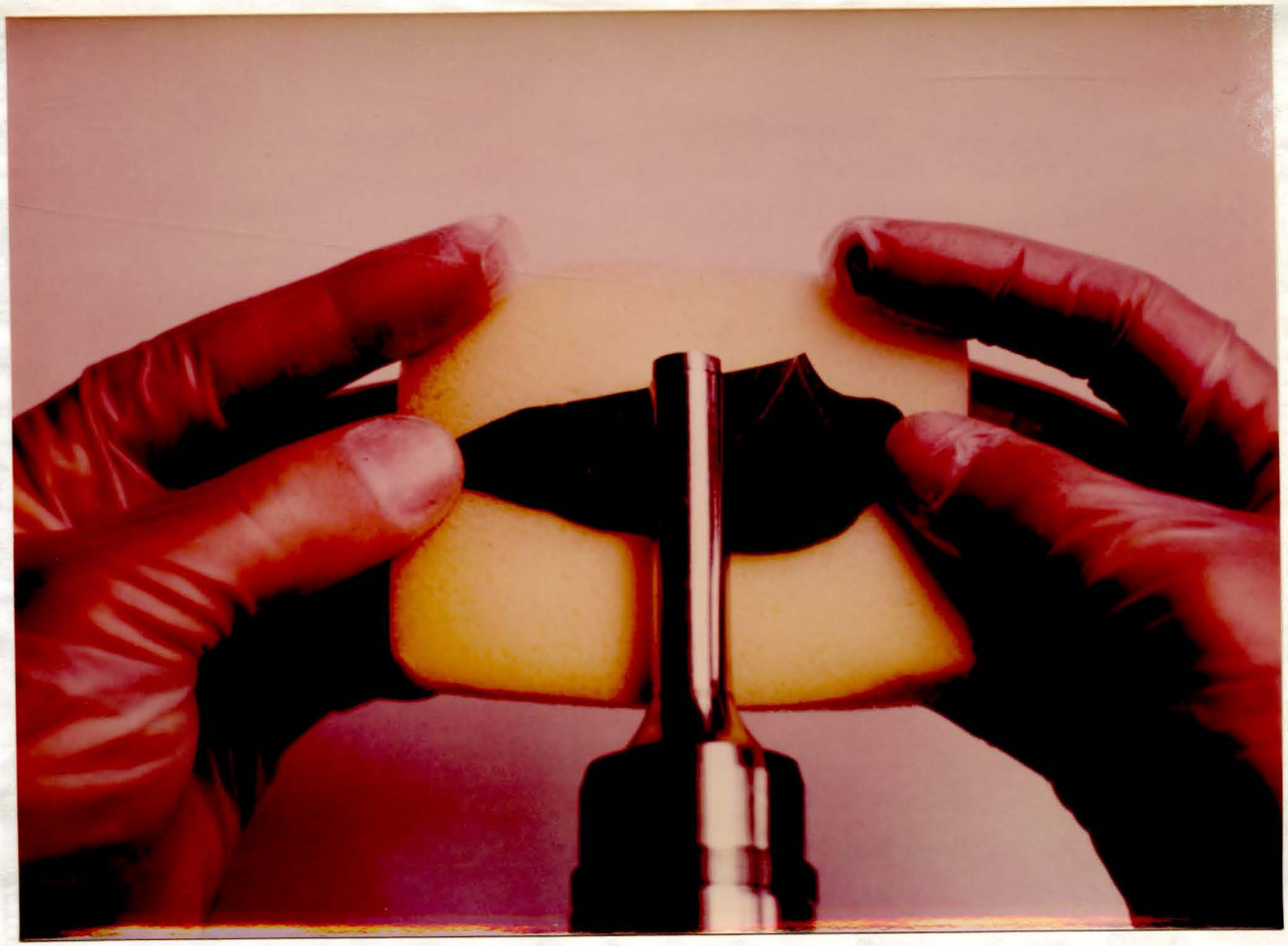

Fig. 5 Ultrasonic inoculation with tobacco mosaic virus of a half leaf. Phaseolus vulgaris cv..Pinto leaves were pushed against and drawn under the ultrasonic probe.

mination at 3,000 LUX. Lesions were counted using a Wild binocular microscope (Fig.6).

Preparation of Seed Extracts

Crude Extracts:

Seeds from 4 varieties of Pisum sativum; cv. Alaska, cv. Freezonian, cv. Perfection, and cv. Wyoming Wonder were used in the study. The seeds were soaked in distilled water for approximately 4 hrs., softening the seeds so that they could be separated into seed coats, cotyledon, and hypocotyl-radicle (Fig.7). The tissues were each macerated and mixed with distilled water in a ratio of 1:2 (w/v). The mixtures were allowed to stand at room temperature for about 3 hrs., then frozen in a deep freeze for 24 hrs. The thawed mixtures were strained through cheesecloth and filtrates centrifuged to remove cellular fragments using an IEC clinical centrifuge for 10 min. @ 5,000 rpm.

Prepared Extracts (Cheo 1955):

The crude extracts for each seed part were adjusted to pH 5 and a 40% solution of basic lead acetate was added until no further precipitation occurred. The mixture was centrifuged at 4°C. for 10 min. @ 10,000 rpm. in a Sorvall RC2-B centrifuge, the precipitated was discarded. The excess lead was removed by the addition of a 20% solution of dibasic sodium phosphate. The final solution was concentrated in a 50°C. vacuum oven to about half its original volume. This was again centrifuged and the precipitate discarded. The

Fig. 6 Typical necrotic lesions due to tobacco mosaic virus infection on a half leaf of Phaseolus vulgaris cv. Pinto.

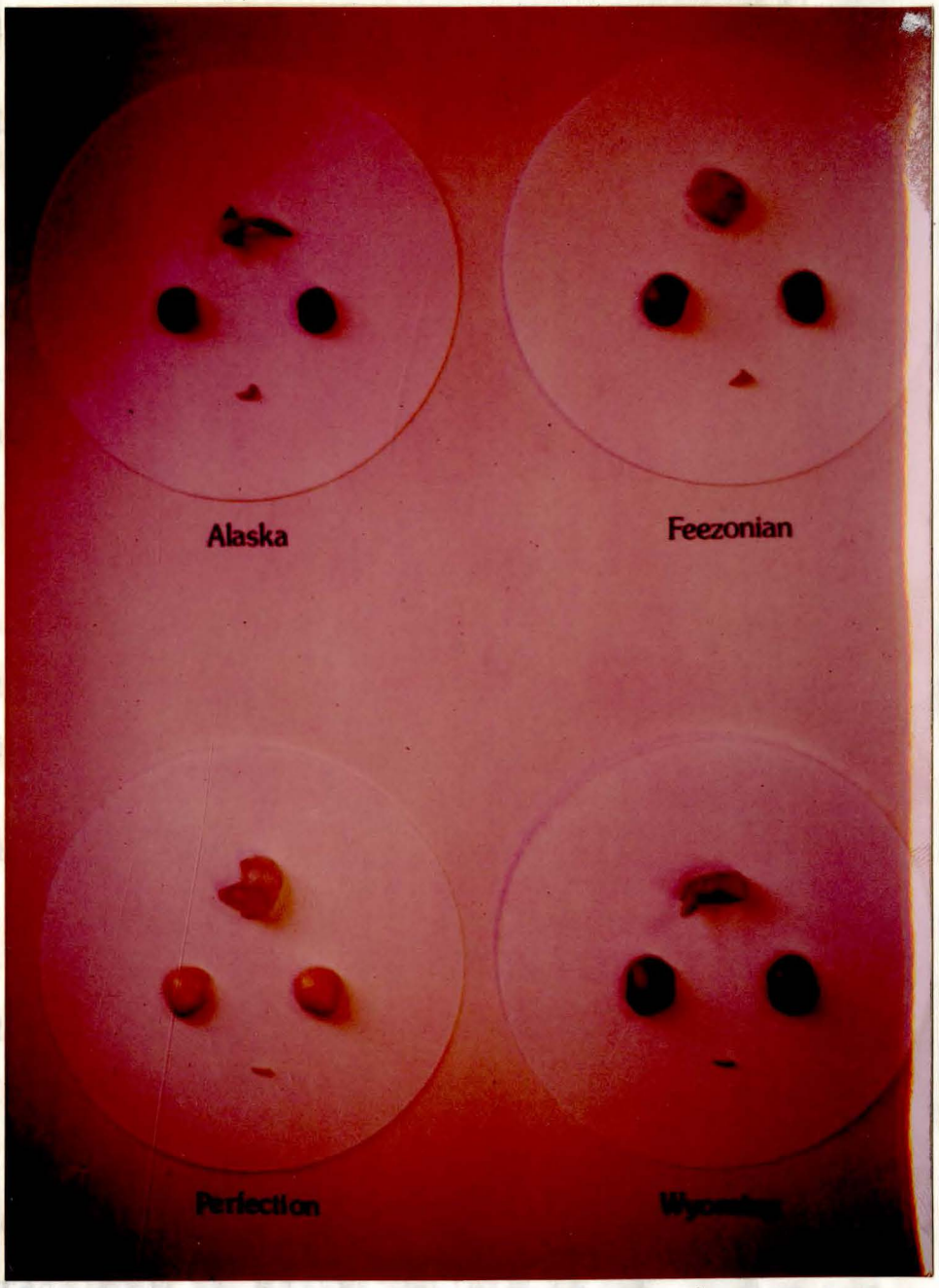

Fig. 7 Dissected seed parts; seed coats, cotyledon, hypocotyl-radicle from 4 varieties of Pisum sativum.

final extract had a pH of 6.2.

Testing Seed Extracts

Existence and Localization of the Virus Inhibitor:

The experimental inoculum consisted of equal volumes of suspended TMV and seed extracts. All control inoculum consisted of equal volumes of suspended TMV and neutral phosphate buffer. Inoculations were made using the ultrasonic bio-assay procedure.

Whole seed crude seed extracts for all 4 varieties of pea were tested for any inhibitory effect. Inoculation was either immediate or delayed 1, 2, 3, or 4 hrs. Crude extracts from individual seed parts; seed coat, cotyledon, and hypocotyl-radicle were tested for all varieties.

Characterization of the Virus Inhibitor:

Prepared extracts from each seed part were tested for all varieties. The crude seed extract from cv. Alaska and the control inoculum were each tested at dilutions of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}. The crude seed extract from cv. Alaska was tested after heating the extract in a 100^{0}C. water bath for 15, 30, 45, 60, 75 min. The crude seed extract from cv. Alaska was mixed with 6 molar hydrochloric acid, then neutralized with sodium hydroxide, then tested. The extract was also mixed with 6 molar sodium hydroxide, neutralized with hydrochloric acid, then tested.

RESULTS

One-way analysis of variance (ANOVA) with Kleczkowski transformations (1955) were used in the analysis of the data. For each bean plant, each of the four half leaves was put randomly in one of treatment groups. This violates an assumption of randomness in the statistical design but because variability between bean plants has been established (Holmes, 1929) and this variance is added to the error term the statistical tests should be conservative.

Effect of Whole Seed Extracts

This experiment consisted of five treatment groups; a control and extracts from four varieties of Pisum sativum Thirty replicates were in each treatment group. All four varieties significantly inhibited TMV (Table 1), reducing infectivity 16.45-27.89%. Varieties cv. Alaska and cv. Freezonian differed significantly from cv. Perfection and cv. Wyoming wonder.

Effect of Delaying Inoculation

This experiment consisted of five treatment groups; a control and extracts tested at time intervals of one, two, three, and four hours. Twenty-four replicates were in each treatment group. No significant difference was found due to the amount of time inoculation was delayed (Table 2).

TABLE 1 ANALYSIS OF VARIANCE: Effect of whole seed extract from 4 varieties of Pisum sativum on the infectivity of TMV. (Fig. 8).

	SS	DF	MS	F
Between	.2866	4	.0717	10.40***
Within	.9990	145	.0069	
Total	1.2856	149		

***p .001

Neuman Keuls Multiple Range Test

Three subsets:

(CONTROL)
(ALASKA, FREEZONIAN)
(PERFECTION, WYOMING WONDER)

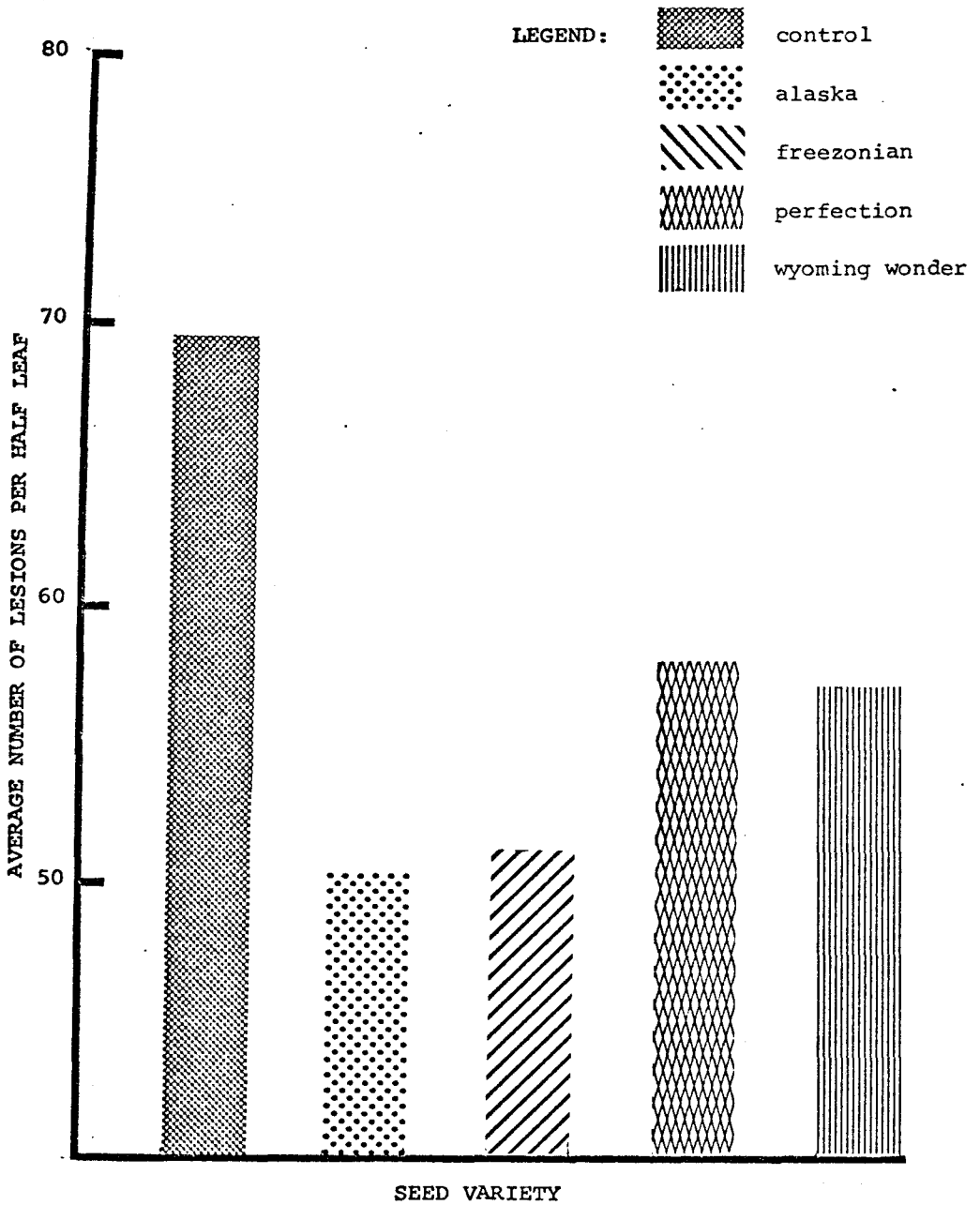

Fig: 8 Effect of whole seed extracts from 4 varieties of Pisum sativum on the infectivity of tobacco mosaic virus, using 10 day old half leaves of Phaseolus vulgaris cv. pinto as the assay plant

TABLE 2 ANALYSIS OF VARIANCE: Effect of delaying inoculation of virus mixed with whole seed extracts from 4 varieties of Pisum sativum on the infectivity of TMV. (Fig.9).

	SS	DF	MS	F
Between	.0719	4	.0180	2.36 N.S.
Within	.8763	115	.0076	
Total	.9481	119		

N.S.= Not significant

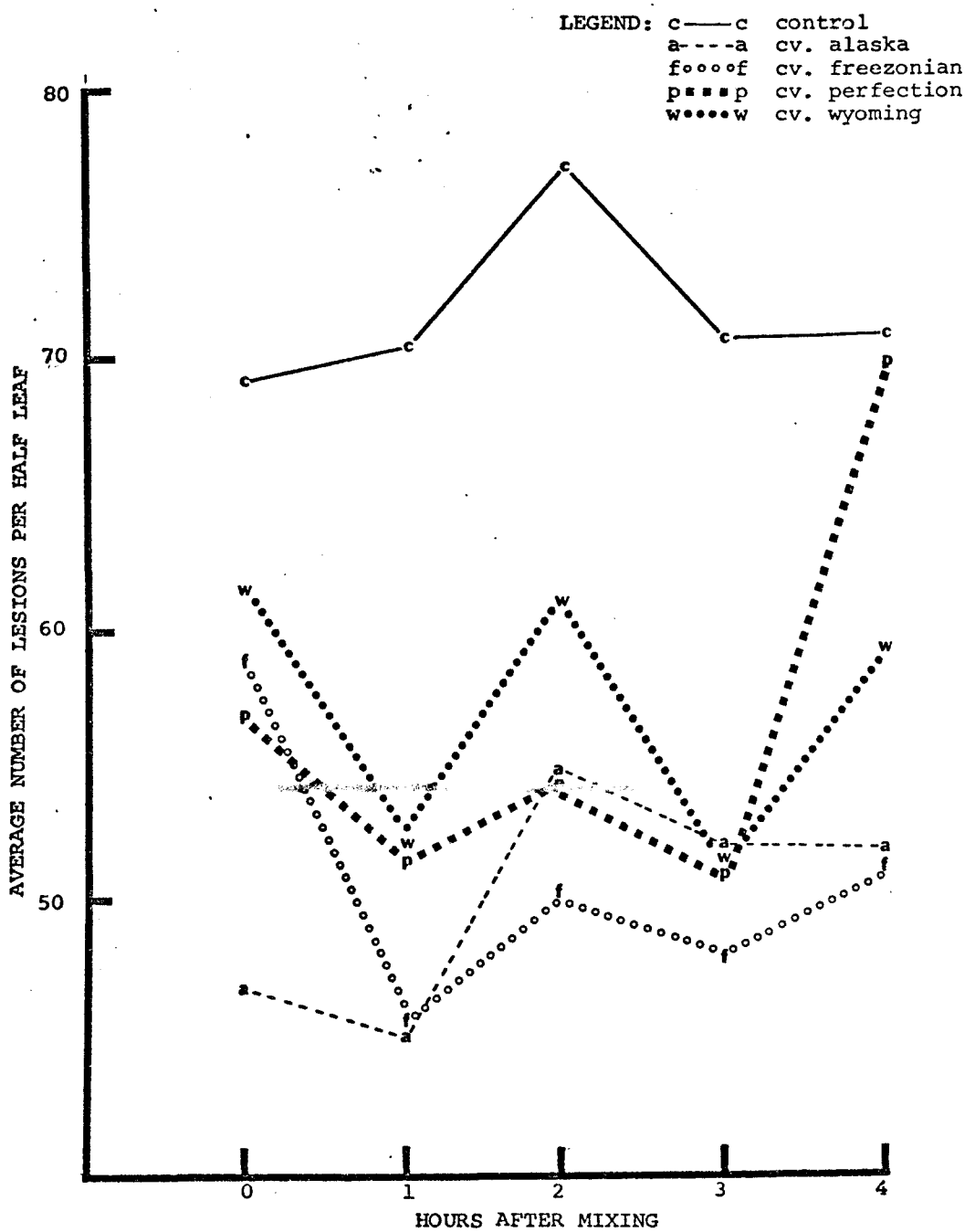

Fig: 9 Effect of delaying inoculation of virus mixed with whole seed extracts from 4 varieties of Pisum sativum on the infectivity of tobacco mosaic virus, using 10 day old half leaves of Phaseolus vulgaris cv. pinto as the assay plant

Effect of Seed Part Extracts

This experiment consisted of thirteen treatment groups; a control and twelve groups each comprised extracts of the seed coat, cotyledon, or hypocotyl-radicle of one of four varieties of Pisum sativum. Twelve replicates were in each treatment group. All seed parts except the seed coat in all the varieties significantly inhibited TMV (Table 3). Crude extracts from cv. Alaska of the cotyledon and hypocotyl-radicle reduced infectivity 30.44% and 20.80%, respectively. Extracts from cv. Freezonian of the cotyledon and hypocotyl-radicle reduced infectivity 37.59% and 19.83%. Extracts from cv. Perfection of the cotyledon and hypocotyl-radicle reduced infectivity 28.42% and 26.09%. Extracts from cv. Wyoming wonder of the cotyledon and hypocotyl-radicle reduced infectivity 15.09% and 27.70%.

Effect of Prepared Extracts

This experiment consisted of thirteen treatment groups; a control and twlve groups each comprised prepared extracts of the seed coat, cotyledon, or hypocotyl-radicle of one of four varieties of Pisum sativum. Twelve replicates were in each treatment group. No significant reduction in infectivity was found in seed parts from any of the varieties (Table 4).

Effect of Dilution of the Extract-Virus Mixture

Using a logarithmic transformation the slope of the

TABLE 3 ANALYSIS OF VARIANCE: Effect of crude seed part extracts from 4 varieties of Pisum sativum on the infectivity of TMV (Fig.10).

	SS	DF	MS	F
Between	.5100	12	.0425	5.82***
Within	1.0433	143	.0073	
Total	1.5532	155		

***p .001

Neuman Keuls Multiple Range Test

Four subsets:

(AC WHR PC PHR AHR FHR WC)
(AHR FHR WC FSR ASC)
(PHR AHR FHR WC FSC)
(FSC ASC WSC PSC CONTROL)

A= cv. Alaska
F= cv. Freezonian
P= cv. Perfection
W= cv. Wyoming wonder

C= cotyledon
HR= hypocotyl-radicle
SC= seed coat

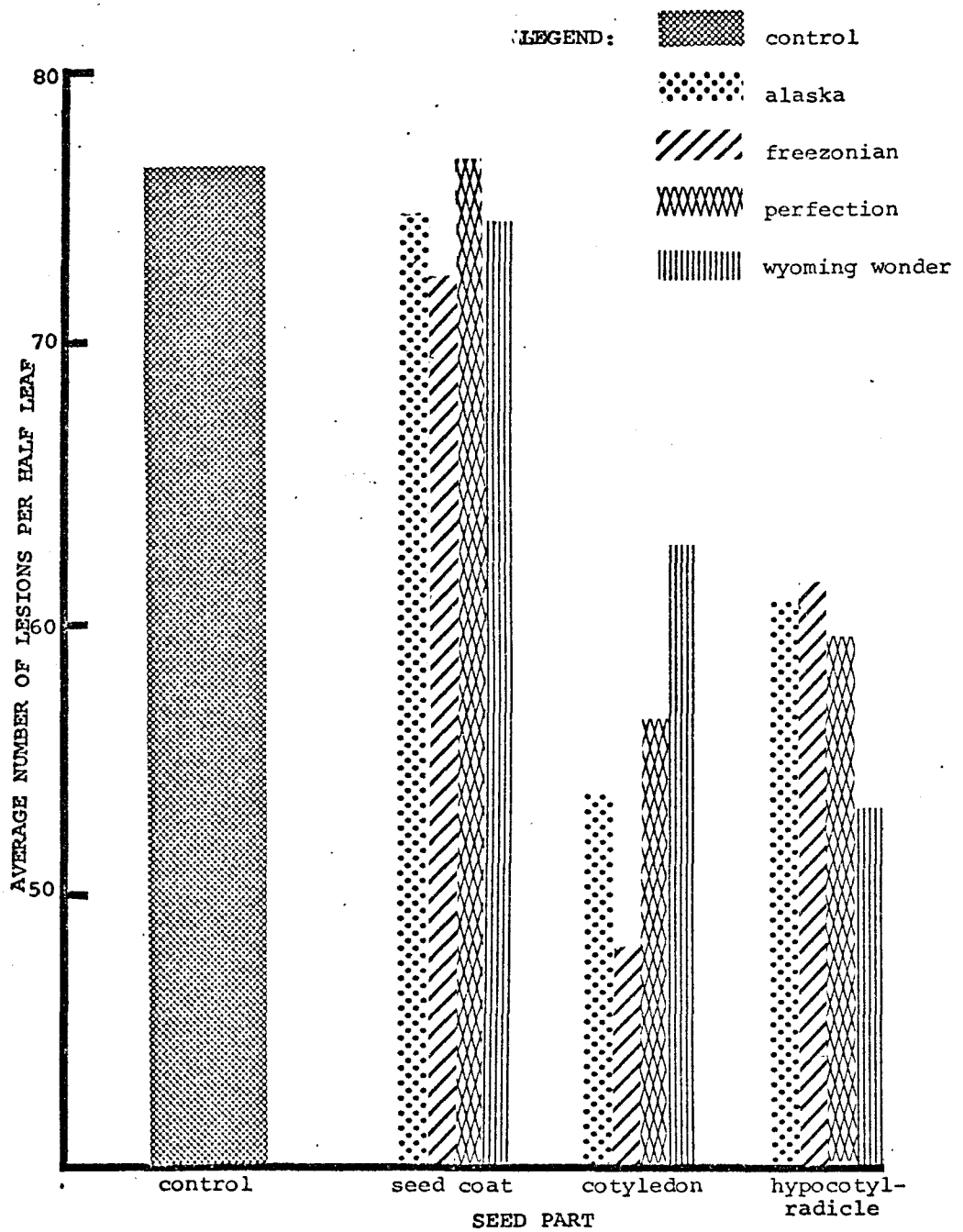

Fig: 10 Effect of seed part extracts from 4 varieties of Pisum sativum on the infectivity of tobacco mosaic virus, using 10 day old half leaves of Phaseolus vulgaris cv. pinto as the assay plant.

TABLE 4 ANALYSIS OF VARIANCE: Effect of prepared seed part extracts from 4 varieties of Pisum sativum on the infectivity of TMV (Fig.11).

	SS	DF	MS	F
Between	.0914	12	.0076	.6550 N.S.
Within	1.6630	143	.0116	
Total	1.7544	155		

N.S.= Not significant

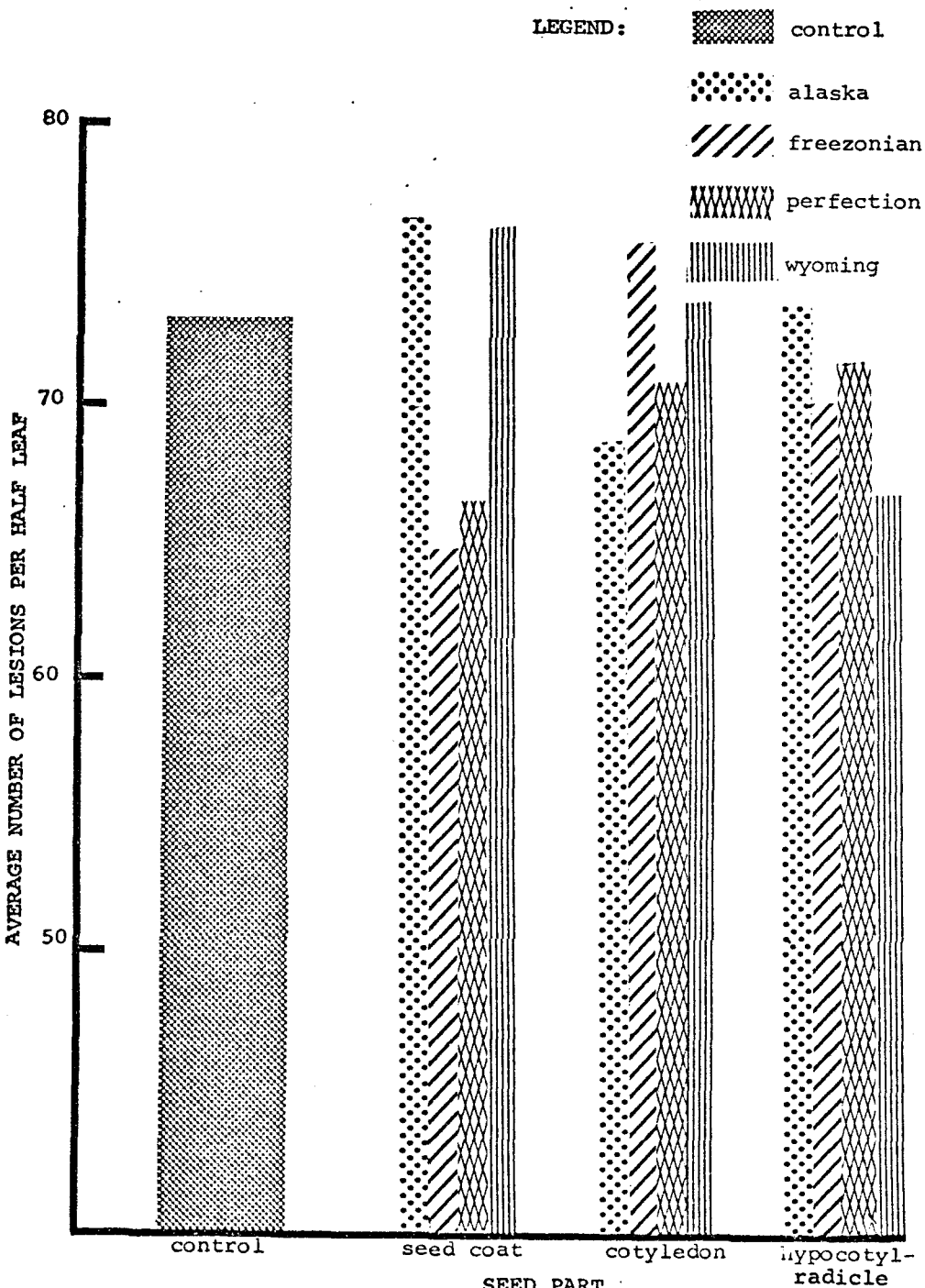

Fig:11 Effect of prepared seed part extracts from 4 varieties of Pisum sativum on the infectivity of tobacco mosaic virus, using 10 day old half leaves of Phaseolus vulgaris cv. pinto as the assay plant.

virus-extract mixture differed significantly from the slope of the virus-buffer mixture, $t_{0.05(2)115}$ 2.66. Dilution of the virus-extract mixture decreased infectivity less rapidly than did the dilution of the virus-buffer mixture (Figure 12).

Effect of Temperature on the Seed Extract

This experiment consisted of five treatment groups; a control and four groups comprised of heating the extract from cv. Alaska in 100°C water bath for 15,30,45,60,75 min. Twelve replicates were in each treatment group. The length of time in a 100°C water bath did not significantly alter the inhibiting effect of the seed extract (Table 5).

Effect of Acid and Alkali on the Seed Extract

This was two experiments: one tested the effect of 6 molar hydrochloric acid on the extract from cv. Alaska, the other tested the effect of 6 molar sodium hydroxide on the extract from cv. Alaska. Each had two treatment groups; a control and a group testing acid or alkali. Twelve replicates were in each treatment group. Acid significantly reduced the inhibiting effect of the extract, $t_{0.01(2)22}$ 3.64. Base had no significant effect on the inhibiting on the inhibiting action of the extract, $t_{0.05(2)22}$ 0.793.

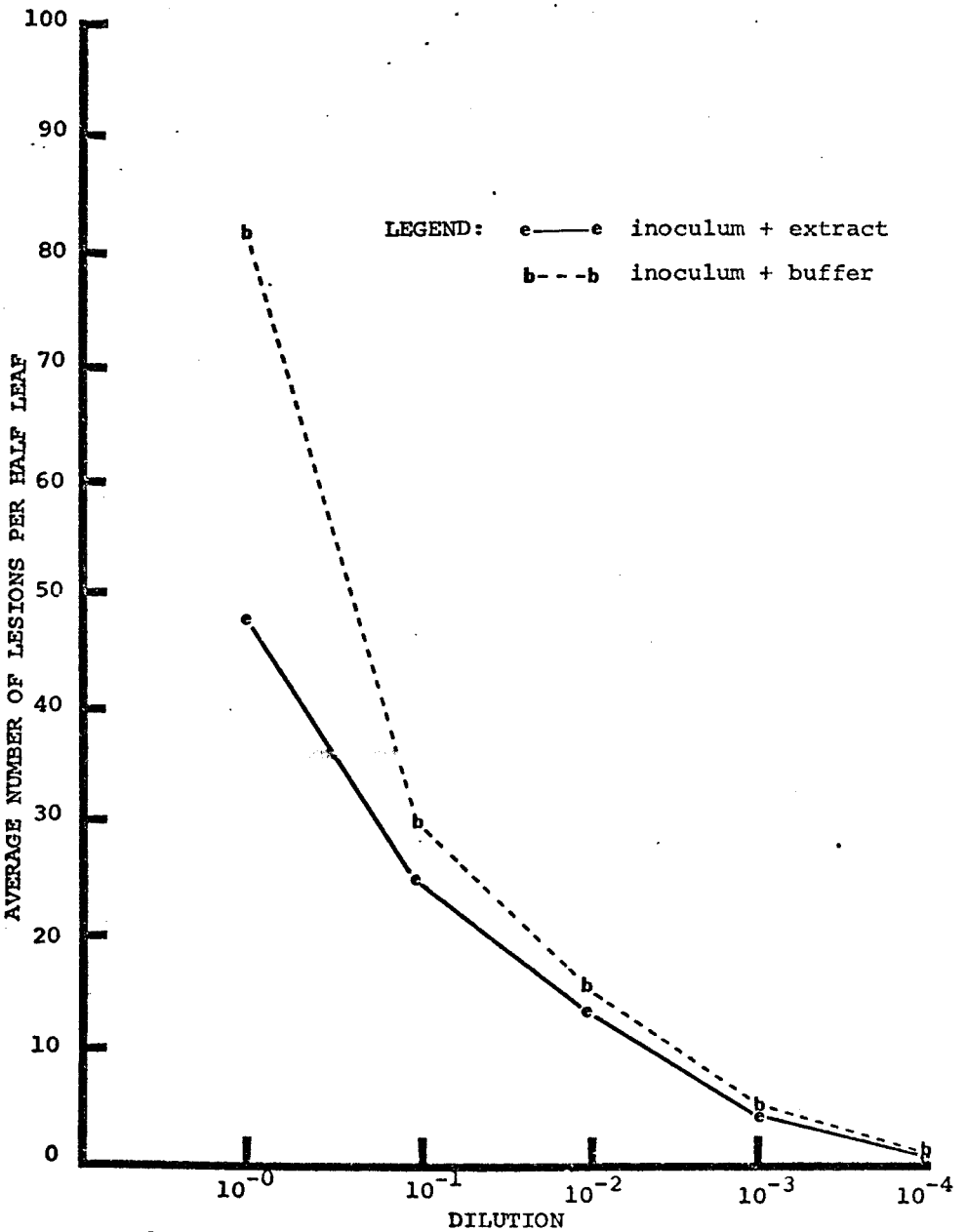

Fig: 12 Effect of diluting a mixture of tobacco mosaic virus inoculum and Pisum sativum seed extract with buffer, on infectivity of the mixture using half leaves of Phaseolus vulgaris cv. pinto as the assay plant.

TABLE 5 ANALYSIS OF VARIANCE: Effect of temperature of 100°C on the inhibiting action of crude seed extracts from Pisum sativum on the infectivity of TMV (Fig.13).

	SS	DF	MS	F
Between	.0167	4	.0042	.3767 N.S.
Within	.6093	55	.0111	
Total	.6260	59		

N.S.= Not significant

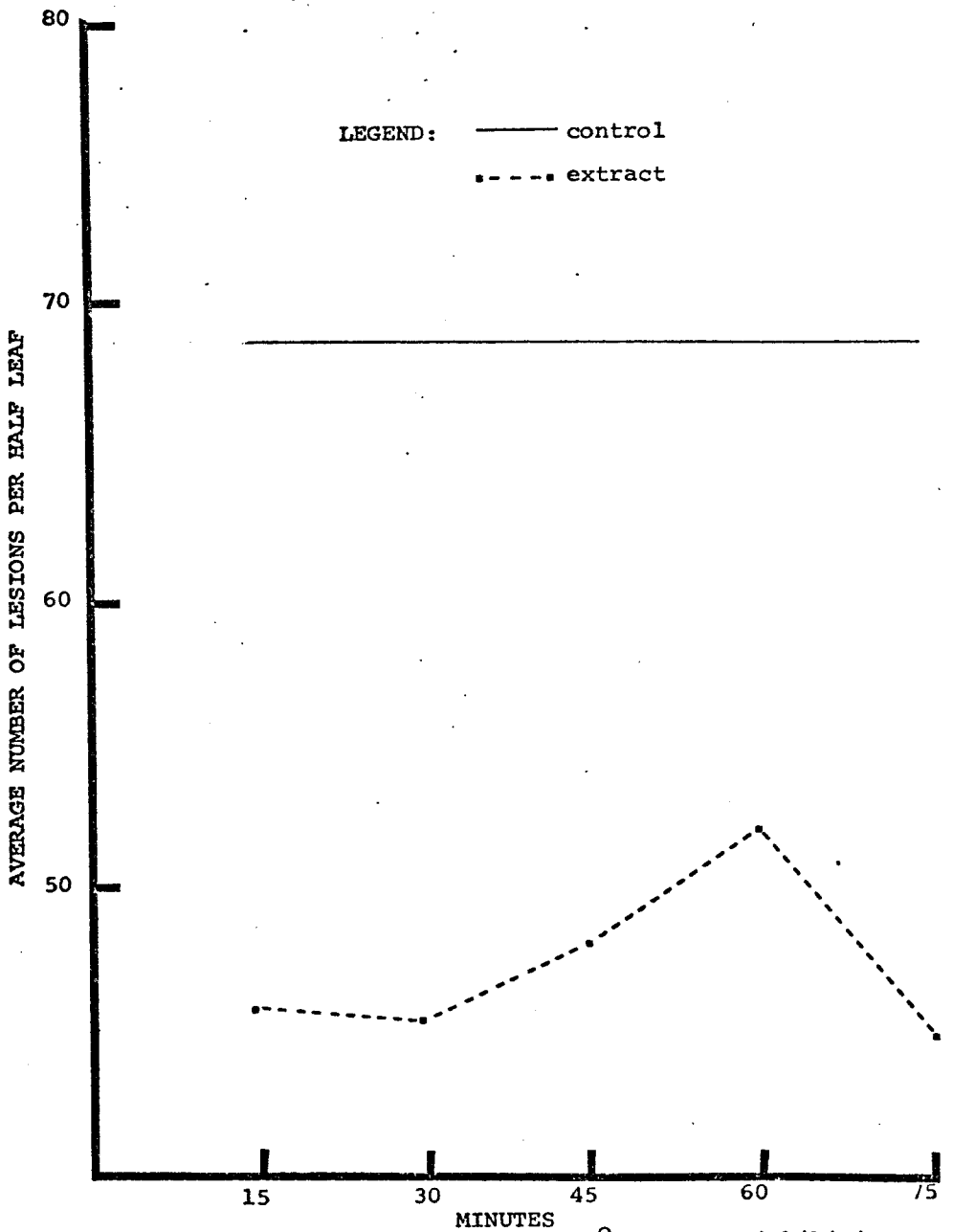

Fig. 13 Effect of temperature of 100°C. on the inhibiting effect of seed extracts from Pisum sativum on the infectivity of tobacco mosaic virus, using 10 day old leaves of Phaseolus vulgaris cv. pinto as the assay plant.

DISCUSSION

The presence of an inhibitor of tobacco mosaic virus in the seeds of Pisum sativum has been demonstrated. Furthermore the degree of inhibition varied between different varieties. The seed varieties of cv. Alaska and cv. Freezonian which produce mature plants in 60 and 63 days, respectively had a greater inhibitory effect than did cv. Perfection and cv. Wyoming Wonder which produce mature plants in 70 and 74 days. Perhaps growth substances are responsible for the inhibitory effect, since gibberellins, cytokinins and lectins are all known to occur in seeds. Nakagaki (1971) reported a reduction in TMV infection by kinetin. Sharon (1977) suggested lectins may act like plant antibodies. Uyemoto and Grogan (1977) in reviewing literature on lectins and their properties indicated that lectins commonly occur in high concentrations in legume seeds. The established interaction of lectins and carbohydrates (antigen-antibody-like activity) coupled with the fact that certain plant viruses possess glycoproteins on their viral caspid suggests a possible mechanism for virus inhibition in seeds. They also indicated that lectins increase as the seed matures. This would agree with other research showing that some viruses are present in the mature seed but are later eliminated as the seed matures. Entlicher et al. (1970) isolated two lectins from Pisum sativum seeds having

molecular weights of 53,000 and 54,000. Sharon and Lis (1974) reported that some lectins preferentially agglutinate mammalian tissue culture cells that have been transformed by oncogenic virus, which prompted investigations of the use of lectins as an inhibitor of malignant cells. They may have a similar effect in plants.

The failure of the seed extract to completely inhibit TMV is not really surprising, since the seeds for the extracts were obtained from apparently healthy plants. The amount or quality of the inhibiting substance may change when the plant becomes infected with virus, demonstrating interferon-like activity. Sela et al. (1964) found that juices from uninfected Nicotiana glutinosa leaves contained some antiviral activity, but that an additional substance was produced when the plants became infected with TMV.

The seed extract have an instantaneous effect. Caldwell (1935), Bawden and Freeman (1952) and Slagle et al. (1952) suggested that inhibitors that have an instantaneous effect characterize a class of inhibitors whose effect is primarily to affect the susceptability of the host.

The inhibitors were located primarily in the cotyledon and the hypocotyl-radicle. These two areas are responsible for protein synthesis and storage. The prepared extracts from which the proteins were removed produced little inhibition, suggesting the some protein is

responsible for the inhibitory effects.

Dilution of the inoculum and extract decreased infectivity less rapidly than the inoculum and buffer mixture. The ratio of the inoculum concentration and extract concentration did not alter throughout the series of dilutions, yet the reduction in the lesion production induced by the extract became less as the mixture was diluted. Crowley (1954) suggested that the converging nature of such dilution curves indicates that either the inhibiting constituents of the extracts are combined with virus in such a way that is readily dissociated by dilution, or the effect is on the host.

The seed extract inhibitor is heat stable and resistant to alkali, but not resistant to acid. The acid probably denatures the inhibiting substance, thereby making it inactive.

This study presents evidence for a heat-stable, alkali resistant, proteinous inhibitor or inhibitors in the seed from Pisum sativum effecting TMV infectivity. Future research may elucidate as to the exact chemical nature of the inhibitor in this study. Only then will it be possible to show any role inhibitors have in protecting plants against virus infection.

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The thesis is therefore accepted in partial fulfillment of the requirements for the degree of Masters of Science degree in Biology.

Aug 20, 1980
Date

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