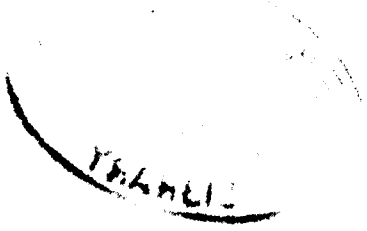
1979

A Comparative Study of Tmv Infected Mesophyll Protoplasts of Nicotiana tabaccum CV. White Burley and Nicotiana glutinosa

Allan Louis Campione
Loyola University Chicago

A COMPARATIVE STUDY OF TMV INFECTED MESOPHYLL PROTOPLASTS OF NICOTIANA TABACCUM CV. WHITE BURLEY AND NICOTIANA GLUTINOSA



by

ALLAN LOUIS CAMPIONE, B.S.

A Thesis Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Master of Science

January

1979

ACKNOWLEDGEMENTS

I am thankful to Dr. A. S. Dhaliwal, Chairman of my thesis committee and my advisor, for his interest, help and continued support throughout this project. I am also thankful to the other members of my committee, Dr. W. Cordes and Dr. M. Goldie, for their suggestions and guidance concerning the experimental procedure and the preparation of this manuscript. Also, I am deeply grateful to my fiancee, Pat, and Dave Hong for their assistance in analyzing the data and preparing the thesis manuscript. I am also thankful to my mother, Josephine, and my father, Louis, for their patience and financial support and for the funds allocated for this project by the Biology Department of Loyola University without which this project could not have been completed. Finally, my thanks to Dr. G. K. Dhaliwal for her help and editorial comments.

VITA

The author, Allan Louis Campione, is the son of Louis Frank Campione and Josephine (Moskalski) Campione. He was born May 4, 1951, in Chicago, Illinois.

His elementary education was obtained in the parochial school system of the archdioceses of Chicago, and graduated from De La Salle High School, Chicago Illinois, in 1969.

He entered Loyola University of Chicago in September, 1969 and received the degree of Bachelor of Science with a Major in Biology in June, 1974. While attending Loyola University, he was presented the University honors research award in 1973. In 1974, he became a graduate member of Beta Beta Beta Biological Society, Lambda Omega Chapter. In 1978, he became an affiliated member of the American Biological Photographic Association, a student member of the American Association for the Advancement of Science, and the American Institute of Biological Science.

In September, 1974, he was admitted to the Graduate School of Loyola University leading to a Masters of Science degree in Biology under the direction of Dr. Amrik Singh Dhaliwal Ph.D. He presented a seminar at the Annual American Institute of Biological Society Meetings at Oregon State University in 1975, and at the Illinois Academy of

Science Meetings at the University of Illinois, Normal, Illinois in 1978. He is co-author of "The Action of Chloramphenicol and Dimethylsulfoxide on the Morphology of Blue-green Bacteria and Plaque-forming ability of Blue-green Bacteria Virus," Journal of Phycology, 1975.

The author taught several microbiology and virology laboratory courses from 1972 to 1977. He was also, appointed a faculty position as instructor of biology, teaching microbiology and seminars in virology at Loyola University of Chicago in 1978.

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INTRODUCTION

Early studies on isolated eukaryotic protoplasts were limited because adequate quantities of protoplasts could not be prepared by available mechanical isolation techniques (Klercker 1892; Seifriz 1928; Chambers 1931). To increase the yield of protoplasts, another technique utilized enzymes that degraded cell walls in the isolation procedure to obtain viable protoplasts. Giaja (1919) successfully isolated yeast protoplasts, and Cocking (1960) reported the first successful isolation of higher plant protoplasts.

Since 1960, the development and utilization of improved enzymatic techniques, as well as improved methods for cultivation of higher plant cells in vitro have advanced plant virology. Living mesophyll protoplasts were isolated from Nicotiana tabaccum cv. Bright Yellow (Takebe, Otsuki and Aoki 1968). These protoplasts were isolated through enzymatic means using non-purified Macerozyme and Cellulase P1500, supplied by Kinki Yakult Manufacturing Company. Suspensions of plant protoplasts cultivated in vitro have been infected with Tobacco Mosaic Virus (TMV), (Takebe and Otsoki 1969), TMV RNA (Aoki and Takebe 1969), and other plant viruses (Motoyoshi 1973). Infectivity has been measured in several ways including: immunofluorescence, cytopathic

effects upon susceptible hosts using local lesion counts, and electron microscopic counts of particles.

With the isolation and culture of various defined animal tissue cell lines, extensive studies of virus replication, cytopathic effects, and the physiological changes within animal cells have been conducted. Not until recently with the acceptance of the enzymatic isolation process of higher plant protoplasts, have investigators extensively studied virus replication, physiological and morphological changes within a single cell. In the past, cell changes within an infected plant could not be efficiently studied because of problems associated with infection and the slow rate of replication of the virus.

The purpose of this project is to isolate tobacco protoplasts through enzymatic digestion using Macerozyme R-10 and Cellulase R-10 and to determine the rate of replication of TMV in two species of tobacco, Nicotiana tabaccum cv. White Burley, a systemic host (Fig. 1), and Nicotiana glutinosa, a local lesion host (Fig. 2). In order to assess the rate of replicaton of TMV in both species of isolated protoplasts, tobacco mesophyll protoplasts were infected with a common strain of Tobacco Mosaic Virus. Samples were then tested on primary half leaves from 10 day old plants (Phaseolus vulgaris cv. Pinto).

Fig. 1 Typical systemic infection due to tobacco mosaic virus infection of tobacco. Leaves show mosaic pattern and chlorosis.

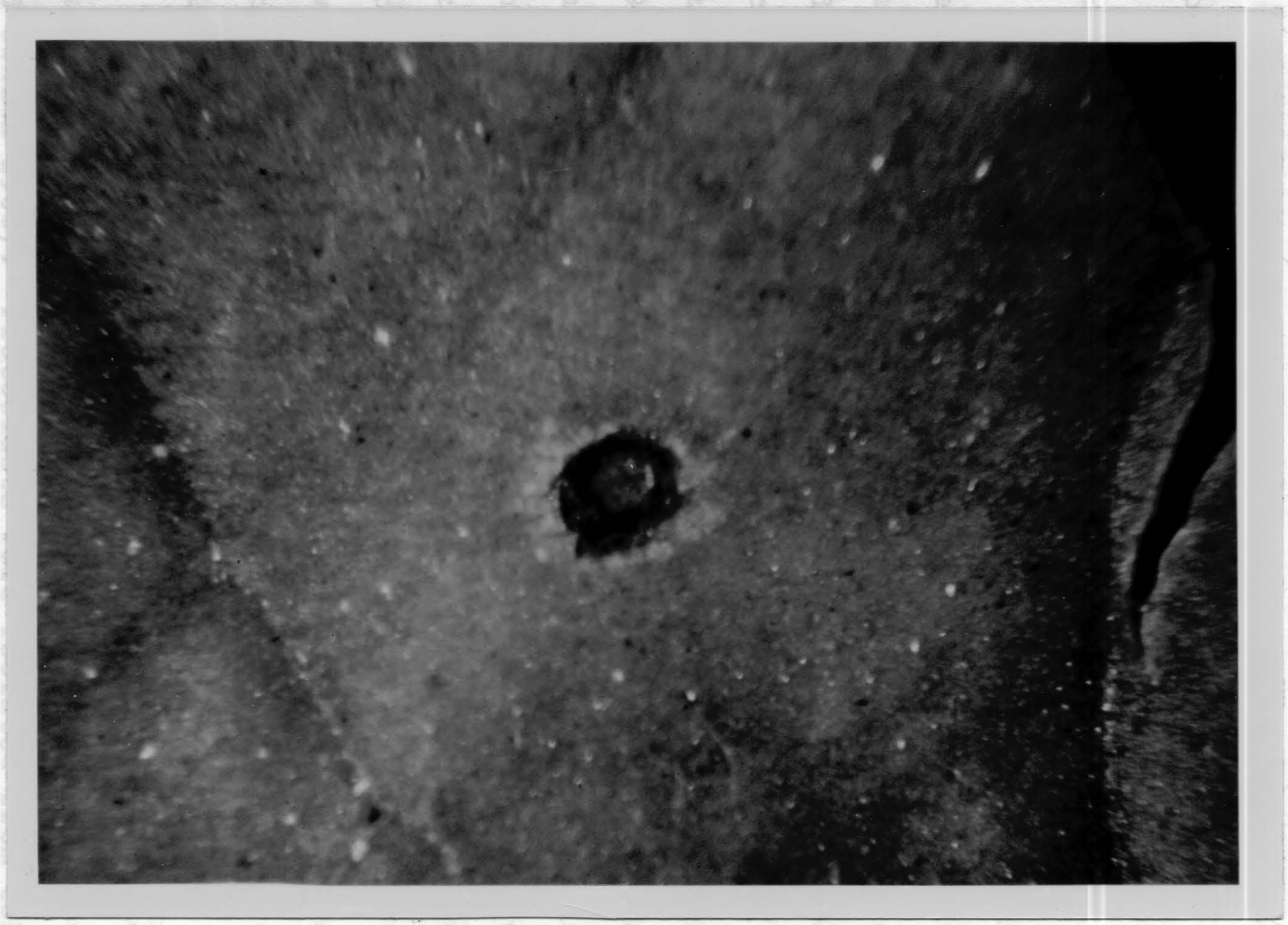

Fig. 2 Typical necrotic lesion due to tobacco mosaic virus infection of tobacco. Leaf show reddish-brown lesion.

REVIEW OF LITERATURE

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

Protoplast Isolation:

Mesophyll tissue of the leaves of higher plants was used to prepare protoplasts. Matthews (1970) suggested that mesophyll cells constituted two-thirds of the leaf area and the greatest number of these were palisade tobacco cells. Recently, large amounts of mesophyll cells have been released enzymatically from tobacco leaves. Takebe, Otsuki and Aoki (1968) reported 50 to 90% of cells released from tobacco leaf tissue were intact mesophyll cells, and were easily converted to protoplasts. This high yield of cells was obtained by allowing enzymes to come directly in contact with mesophyll cells. Takebe *et al*. (1968) removed the lower epidermis of the leaf by placing fine-tipped forceps into the midrib of a wilted leaf and gently pulling the epidermis away so that mesophyll tissue was exposed.

Several methods have been employed to isolate intact mesophyll protoplasts (Bonnett and Eriksson 1974; Motoyoshi and Oshima 1968). Protoplasts were isolated from tobacco leaves by treating leaf pieces with a solution of 1% Macerozyme and then with a solution of Meicelase P, both in osmotic stabilizer (Watts and King 1973). Takebe *et al*. (1968)

isolated cells of Nicotiana tabaccum cv. Bright Yellow in large quantities using 0.5% Macerozyme, potassium dextran sulfate and then a solution of Cellulase P1500, both in osmotic stabilizer. A simplified method of obtaining protoplasts devised by Kassanis and White (1974) consisted of soaking tobacco leaves lacking lower epidermis for an extended period in a mixture of 0.4% Macerozyme, 1.2% Cellulase and 0.7 M D-mannitol. Large yields of protoplasts were obtained by simplifying the laborious method of the two-step isolation technique and eliminating potassium dextran sulfate from the maceration medium. The one-step isolation technique of Kassanis and White (1974) facilitated protoplast release from cells of both the spongy and palisade mesophyll layers. In comparison to the two-step enzymatic isolation technique, during mesophyll release spongy and palisade cells were released separately. Palisade cells are more suitable for viral infection (Takebe et al. 1968).

Factors Influencing Protoplast Isolation

Plant choice:

Protoplasts have been isolated enzymatically from many herbaceous plants besides tobacco (Bonnett and Eriksson 1974; Cocking and Pojnar 1969; Watts and King, 1973). After macerozyme treatment and incubation for various times with cellulase to convert mesophyll cells to protoplasts (Otsuki and Takebe 1969), a large yield of protoplasts have been

obtained from Petunia hybrida (petunia), Spinacia oleracea (spinach), Hyacinthus orientalis (hyacinth) and Vigna sinensis (cowpea).

Motoyoshi, Watts and Bancroft (1974) and Watts, Motoyoshi and King (1973) reported specific problems encountered in the preparation of protoplasts. They outlined the conditions of growth necessary to obtain tobacco protoplasts. The age of the plants and environmental conditions were the critical factors in obtaining protoplasts. Nicotiana tabaccum cv. White Burley is an excellent plant for use in the enzymatic isolation of protoplasts. In comparison to other tobacco plants, White Burley leaves turn yellow to white upon senescence, whereas other tobacco cultivars remain a brilliant green throughout the vegetative life of the plant masking the age and onset of senescence of the plant. It is suggested that etiolated plants are not suitable for protoplast isolation. Kassanis and White (1974) and Takebe et al. reported that leaves of 60 to 90 day old plants about 30 to 35 cm. in length and weighing 12 to 16 grams were satisfactory for protoplast isolation. Young or old protoplasts isolated by enzymatic procedures did not survive in liquid culture for an extended period of time (Takebe 1975). Takebe et al. (1968) used increased shaking in their two-step isolation procedure so that liberated protoplasts were healthy. Protoplasts isolated in this manner were preferred over those from the one-step isolation procedure (Kassanis

and White 1974; Power and Cocking 1970).

Requirements necessary for the isolation of viable protoplasts:

In 1974 Ushimiya and Murashige evaluated the parameters necessary to obtain the maximum yield of protoplasts. Variables studied included: (1) concentration of enzymes; (2) pH of enzyme solution; (3) length of incubation; (4) relationship of cell number to volume of enzyme; (5) temperature; (6) agitation of enzyme and tissue mixture; (7) concentration of osmotic stabilizers; (8) potassium dextran sulfate requirements and (9) nutrient requirements. The study was conducted using cells of Nicotiana tabaccum cv. Bright Yellow in broth. Unlike previous investigators who varied only one or a few of the parameters, Uchimiya and Murashige's investigation combined many variables to define more clearly the conditions most important for successful isolation of protoplasts. A solution of 1% Cellulase and 0.2% Macerozyme was found to be the optimum concentration for protoplast release. An increase in these concentrations did not affect protoplast release. A pH of enzyme solution ranging from 4.7 to 5.7 allowed satisfactory yield of protoplasts. These investigators and Otsuki and Takebe (1969) showed that periods of enzyme incubation varied with plants. Both groups of researchers found that a period of 2 hours in macerozyme and 2 to 7 hours in cellulase were necessary for isolation of mesophyll cells and protoplasts respectively. Uchimaya indicated that not more than 500 mg. tissue in 5 ml. of an

enzyme solution gave maximum yield. Deviations from this proportion showed a reduction in the number of cells released. Temperature, a critical factor in catalyzed reactions, had little effect on protoplast yield if maintained between 22 and 37°C. Maximum protoplast release occurred between 50 and 100 revolutions per minute. For 150 to 200 revolutions per minute, the yield of released protoplasts was very poor due to mechanical damage of the plasma membrane. Concentrations of osmotic stabilizer ranging from 0.01M to 0.09M was analyzed along with a variety of different sugars. Use of 0.7M to 0.8M concentrations of D-mannitol yielded maximum quantities of protoplasts. Since no significant increases of yield were found for any of the other sugars tested, and a drastic decrease was found when sucrose was used, mannitol was judged the best stabilizer for protoplast release. With the introduction of potassium dextran sulfate in the initial isolation of mesophyll cells, Takebe et al. (1968) found no significant improvement in the isolation of protoplasts from tobacco cells. Although the samples used in this investigation were tobacco cells in culture, the experimenters suggested that these same parameters would prove an excellent guide for higher plant protoplast isolations.

Protoplast culture:

Isolated protoplasts can be maintained in a tissue culture in several ways, either as protoplasts (Aoki and Takebe 1969; Takebe et al. 1968) with or without minor

modifications, or as solid masses of callus (Nagata and Takebe 1971; Powers and Cocking 1969; Usui and Takebe 1969; Takebe, Libib and Melchers 1971; Uchimaya and Murashige 1974). In most experiments utilizing leaf isolated mesophyll protoplasts, cells were suspended in liquid media of Aoki and Takebe (1969), a modification of the elements used by Murashige and Skoog (1962).

Virus replication in protoplasts:

Since isolated protoplasts support TMV multiplication, the presence of a cell wall is not required for cells to become infected and establish virus infection (Nagata and Takebe 1970; Meyer and Abel 1974). These workers reported little increase in RNA and protein synthesis after TMV infection of the isolated protoplasts. Takebe (1975) stated that the nutrients found in Aoki and Takebe's (1969) media did not contribute to virus synthesis and that more work is needed in this area. The effect of plant growth substances on the viability of protoplasts has also been studied. Watts, Motoyoshi and King (1973) suggest that increased levels of naphthalene acetic acid (NAA) were favorable for the maintenance of viable Nicotiana tabaccum cv. White Burley protoplasts. They found that younger cells were able to compensate for low auxin levels but older cells relied heavily on supplied auxin.

Inoculation of Protoplasts

Virus infection:

The process of virus infection (without the necessity of mechanical injury to the host) and replication of virus in tobacco protoplasts were investigated by Takebe, Otsuki and Aoki (1968). Large amounts of tobacco mesophyll cells were released in an intact state and subsequently infected with TMV. Synthesis of TMV within the protoplasts was shown by using specific RNA and protein synthesis inhibitors. A reduction in viral particles was noted when either of the inhibitors was used. In 1969 Takebe and Otsuki showed by fluorescence antibody staining method, that specific staining for TMV antigen with an antibody labeled with fluorescein isothiocyanate can determine the presence of TMV in a protoplast. An approximate percentage of infected protoplasts can also be determined (Clark and Shepard 1963). The experiment of Takebe et al. (1969) indicated that a poly-cation (poly-L-ornithine) was necessary for the infection of tobacco protoplasts. They usually infected the protoplasts with TMV in potassium citrate buffer and poly-L-ornithine. Poly-L-ornithine stimulation of virus attachment appears to be significant as reported by Takebe and Otsuki (1969) and Takebe and Nagata (1973).

Zhuravler, Pistskaya,Shumilova, Musoroh and Peifman (1975) stated that many TMV particles can be absorbed on the isolated mesophyll protoplasts even in the absence of poly-L-ornithine, but in order for infection to occur the

presence of poly-L-ornithine was necessary. Virus uptake by pinocytosis was stimulated by poly-L-ornithine possibly by the formation of TMV poly-L-ornithine complex (Cocking 1966; Mayo and Cocking 1969; Takebe and Otsuki 1976). Burgess, Motoyoshi and Fleming (1973) found no evidence in their electron microscopic studies to support the theory of pinocytosis. Burgess et al. (1973) suggested that poly-L-ornithine causes stress on the cell membrane causing "lesions" which were favored sites for binding of TMV particles allowing entry of the virus particles with no actual participation of the host.

Protoplasts incubated with TMV for 10 to 60 minutes showed a marked increase in virus content (Burgess et al. 1973; Takebe et al. 1968; Cocking et al. 1968). Protoplasts were also washed several times with sterile 0.8M D-mannitol containing $CaCl_2$, after incubation with virus to remove all unabsorbed virus particles. However, the percentage of protoplasts infected varied. Takebe and Otsuki (1969) showed 26% infection whereas Honda, Natsui, Otsuki and Takebe (1973) showed 70 to 93% of the protoplasts isolated were infected as determined by FITC-antibody staining technique.

Range of virus infected tobacco protoplasts:

The efficiency of virus infection of protoplasts tends to diminish any means of determining viral specificity. Tobacco protoplasts have not only been infected by tobacco

mosaic virus, but by cucumber mosaic virus (Otsuki and Takebe 1973), potato X virus (Shalla and Peterson 1973), cowpea chlorotic mottle virus (Motoyoshi, Hull and Flack 1975). Virus infection of tobacco protoplasts by nonspecific viruses, such as those mentioned above, were less than that by TMV and TMV-RNA, except in the case of cowpea chlorotic mottle virus where progeny yield per protoplast was sometimes ten times higher than that of TMV (Motoyoshi et al. 1973). The efficiency of the protoplast system with regard to absorption and infection was found to be far superior to that of the whole plant.

Takebe (1975) stated that about 80,000 virus particles are required to infect one protoplast at a concentration of 1 ug./ml. of TMV and 2,500 virus particles are required to infect one protoplast at a concentration of 0.01 ug./ml. of TMV. In infected leaf tissue, 1,000,000 or more virus particles are necessary for the infection of one cell. The large amount of particles required for infection may be explained by a study by Furmoto and Wildman (1963). They reported that at least one of ten TMV particles is infected in purified preparations. These numbers could be reduced if uniform tobacco mosaic virus particles were obtained by the procedure outlined by Boedther and Simmons (1975). The amount of virus particles adsorbed to a protoplast varies. Takebe (1969) estimated that between 100 and 100 virus particles were adsorbed per protoplast. The calculations

of Zhuravlev et al. (1975) showed 600 particles attached per protoplast by carbon 14 labeling of TMV and assay of radioactive emission.

Viral Replication

Virus penetration:

The process of virus entry in isolated protoplasts has been examined using protoplasts fixed and embedded for electron microscopy immediately after infection with virus. Possible theories have also been derived by Motoyoshi, Watts and Bancroft (1974), but the events in the establishment of infection of protoplasts have not yet been conclusive. Motoyoshi et al. (1974) found it difficult to determine the actual means of penetration but suggest that after Cellulase treatment, protoplasts must be concentrated by centrifugation and resuspended before infection. This procedure was essential for a high efficiency of infection, especially if virus entry was caused by active transport. If, however, adsorption had occurred then damage may have been caused to the plasmalemma by centrifugation, allowing entrance of the virus.

Electron microscopy:

Presence of virus particles found attached to the plasmalemma, in the cytoplasmic vesicles, as well as numerous bays and infoldings in the plasmalemma, have led investigators to conclude that the process of virus entry into

tobacco protplasts is by pinocytosis (Cocking and Pojnar 1969; Takebe et al. 1969; Cocking 1970; Hibi and Yora 1972; Otsuki, Takebe, Honda and Matsui 1972). Cocking examined the direct evidence for pinocytosis in isolated protoplasts after incubation in which isolated fruit protoplasts were incubated with ferritin and showed accumulation of ferritin particles in cytoplasmic vesicles. Other investigators agree with Cocking's conclusions that inoculum particles adsorbed to the cell membrane were taken up by pinocytotic processes in a matter of minutes. The virus particles disappear from the pinocytotic vesicles and are not found in the protoplasts for several hours (eclipse phase). Takebe (1975) postulated that a TMV-poly-L-ornithine complex induced adsorption to the plasmalemma and pinocytosis occurred.

A completely different means of virus entry was postulated by Burgess, Motoyoshi and Fleming (1973). No evidence was found to suggest that protoplasts incubated with virus take up the virus through pinocytosis. Burgess et al. (1973) suggested that poly-L-ornithine, a compound necessary for infection of protoplasts, causes stress to the cell membrane. Electron micrographs showed local damage to the membrane and it was at these points of damage that the virus particle entered.

Replication of virus in protoplasts:

Most investigators reported that virus apparent at zero time was due to the virus adsorbed on the protoplasts,

and that this effect decreased as virus was broken down into its components (removal of protein coat). Coutts, Cocking and Kassanis (1972) reported little increase in virus at 15 hours after incubation, but a drastic increase was reported after 48 and 72 hours incubation. Takebe et al. (1969) suggested an increase at 6 hours post infection and a logarithmic reduction in the rate preceding 72 hours. Sensitivity of assay methods plays an essential role in determining replication rate in viral infected protoplasts (Matthews 1970).

Viral assay:

Plant virus appears unable to naturally penetrate the intact plant leaf cuticle (Holmes 1929). This problem could be resolved either by avoiding the need to penetrate (as in infection of plant protoplasts where the cell wall is removed) or by some method involving penetration through a wound in the cuticle (as in mechanical inoculation). Our knowledge about virus transmission is far from complete, but attempts have been made to examine several possibilities. Holmes (1929) mechanically inoculated Nicotiana glutinosa leaves with TMV. Mechanical inoculation involved the introduction of infectious virus or its RNA into a wound made in the plant surface. When virus established itself successfully in the cell, infection occurred. This method as well as improvements and modifications of existing methods (Yarwood 1968; Lamborn, Cochran and Chidester 1971) are of

great importance for the study of interactions between a virus and susceptible cells.

Simultaneous applications of two or more assay methods that depend upon different properties of the virus are useful and essential in obtaining a valid picture of virus replication (Coutts, Cocking and Kassanis 1972). Of the many assay procedures devised to determine virus replication in protoplasts, Coutts et al. (1972) and Kavravlev et al. (1975) utilized the three most sensitive procedures. Local lesion counts on half leaves of tobacco, the most accurate assay, distinguishes between infectious and non-infectious virus. Other procedures include serological determination in which measurements can be made on very small amounts of virus, and electron microscopy in which actual counts of physical particles are made giving a very crude but rapid indication of relative numbers of virus particles present in a sample. In general, local lesion assays are much more sensitive than physical and chemical methods (Matthew 1970). Some serological tests approach the sensitivity level of infectivity assays (Sampson and Taylor 1968). The use of radioisotopes to label plant or virus is more sensitive than infectivity assay for detecting very small amounts of virus in early stages following infection (Khuravler, Pisetskaya, Schumilova, Musorok and Reifman 1975).

MATERIALS AND METHODS

Tobacco Plant Cultivation

Seeds of Nicotiana tabaccum cv. White Burley and Nicotiana glutinosa were germinated in 6 x 20 x 30 cm. trays containing Redi-earth potting soil comprised of Canadian sphagnum and horticultural vermiculite. To retain moisture, trays containing seeds were covered with 20 x 30 cm. single weight glass plates for two days, afterwhich the glass plates were removed. Trays with germinating seedlings were kept in a Hotpack incubator at a constant temperature of 28°C ± 1, a continuous light intensity of 3,000 LUX, and a relative humidity of approximately 70%. Approximately 14 days after germination, primary leaves expanded to 0.5 to 1.0 cm. in length depending upon the species of plant. When this leaf size was reached, seedlings were transplanted to individual pots 15 to 16 cm. containing sphagnum, loam soil and horticultural vermiculite. Plants were kept in a shaded greenhouse at 20 to 28°C where relative humidity was approximately 50 to 60% and were supplied with continuous artificial light (2,000 LUX) during fall and winter growing periods.

Protoplast Preparation

Leaf preparation for protoplast release:

Tobacco leaves 25 to 30 cm. in length for Nicotiana tabaccum cv. White Burley (Fig. 3) and 15 cm. in length for Nicotiana glutinosa (Fig. 4) of 60 to 90 day old plants were used as a source for tobacco mesophyll protoplasts. Leaves were selected randomly and were washed for 5 minutes in a 1% Alkonox detergent and then were surface sterilized by dipping them in 70% ethyl alcohol for 30 seconds and subsequently in 0.5% sodium hypochlorite for 2 minutes. The leaves were rinsed 4 times in sterile, de-ionized, double-glass distilled water. All the following procedures were carried out under strict aseptic conditions. To facilitate the mechanical removal of the lower leaf epidermis, the leaves were allowed to wilt in a sterile pan.

Stripping of epidermis:

The lower epidermis was removed by placing fine-tipped forceps into the midrib of a wilted leaf and gently pulling the epidermis away from the midrib so that the mesophyll tissue was exposed (Fig. 5). Leaves with stripped epidermis were placed in a petri dish containing 0.8 M D-mannitol so that the exposed tissue was in contact with mannitol.

Release of mesophyll cells:

Mesophyll cells were released by the use of Macerozyme R10, a polygalacturonase which degrades pectin. The maceration medium consisted of 0.5% Macerozyme R10, 0.8M

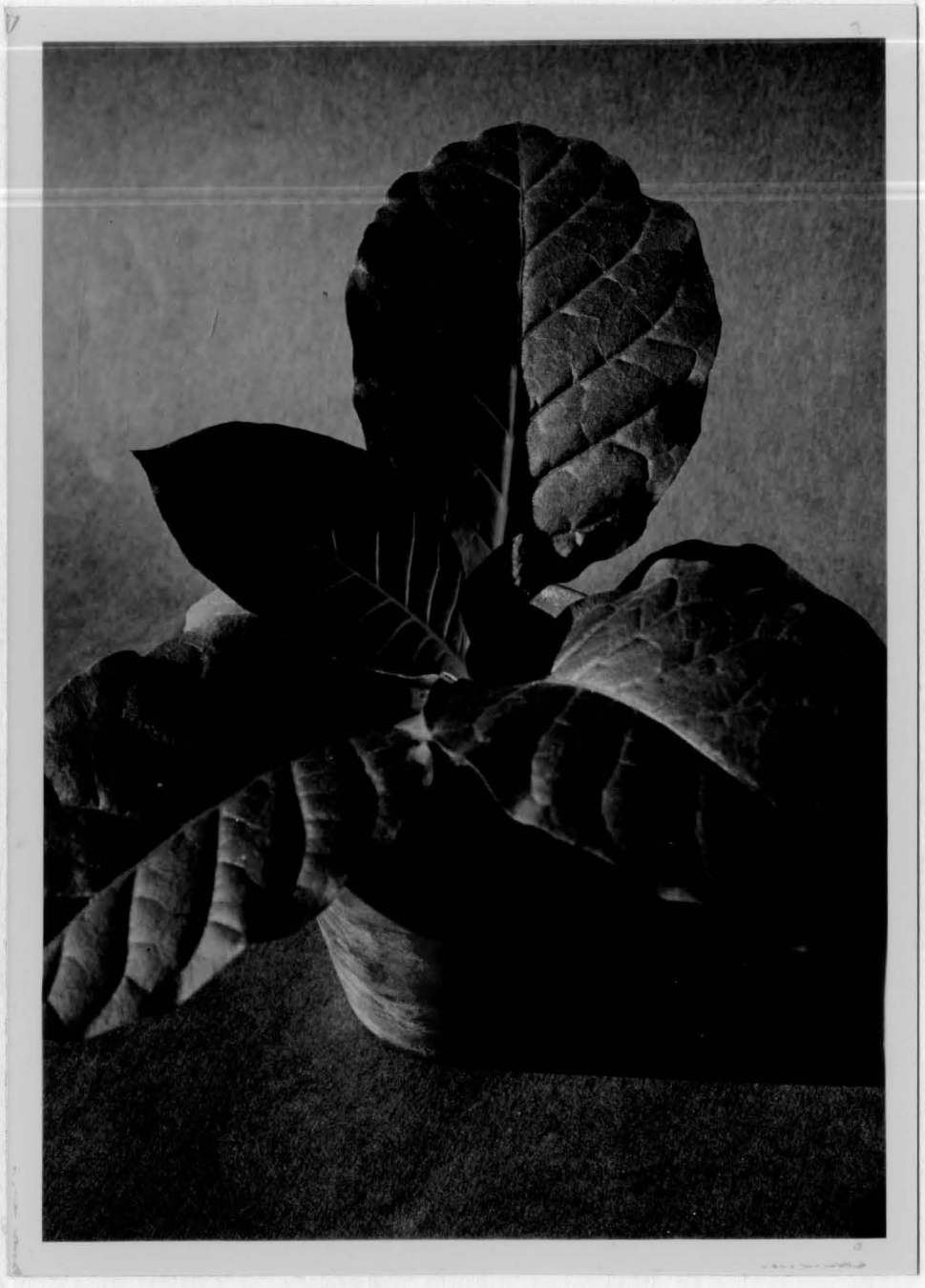

Fig. 3 60-90 day old plant of Nicotiana tabaccum cv. White Burley. Tobacco leaves 25-30 cm. in length.

Fig. 4 60-90 day old plant of Nicotiana glutinosa. Tobacco leaves 15 cm. in length.

Fig. 5 Removal of the lower leaf epidermis by placing a fine-tipped forcep into the midrib of a wilted tobacco leaf and gently pulling the lower epidermis away. Leaf tissue was exposed for enzymatic release of cells.

D-mannitol as osmotic stabilizer, and a 0.3% solution of potassium dextran sulfate (sulfur content 17.8%, obtained from Meito Sangyo Co., Nagoya, Japan). The pH of the maceration medium was adjusted to 5.7 with 2N HCl (Appendix B). Two grams of leaf tissue were placed in a 100 ml. Erlenmeyer flask containing 20 ml. of maceration medium (a ratio of 500 mg. tissue to 5 ml. maceration medium) and evacuated for 3 min. at 381 mm. Hg by electric millipore vacuum pump, 115 v. 60 Hz. The tissue was then shaken at a frequency of 120 revolution/min. on an Eberbach rotator exposed to continuous light intensity of 3,000 LUX. After a brief 15 min. incubation (Fig. 6), the enzyme medium was decanted and replaced with 20 ml. of fresh maceration media in order to remove any broken or fragmented cells produced during epidermal stripping. Afterwards, the maceration medium was replaced every 30 min. for a period of 2 hr. until maceration was completed. For the first 30 min., the reaction medium contained cells of the spongy parenchyma (Fig. 7), whereas the third and fourth 30 min. incubations contained cells mainly of the palisade parenchyma (Fig. 8).

Release of protoplasts:

Palisade parenchyma cells obtained were washed with 0.8M D-mannitol three times to remove macerozyme using low speed centrifugation at 100 g. for 3 min. each time. This procedure was carried out at 23^{o}C. Washed mesophyll cells were then suspended in a 0.3% solution of cellulase contain-

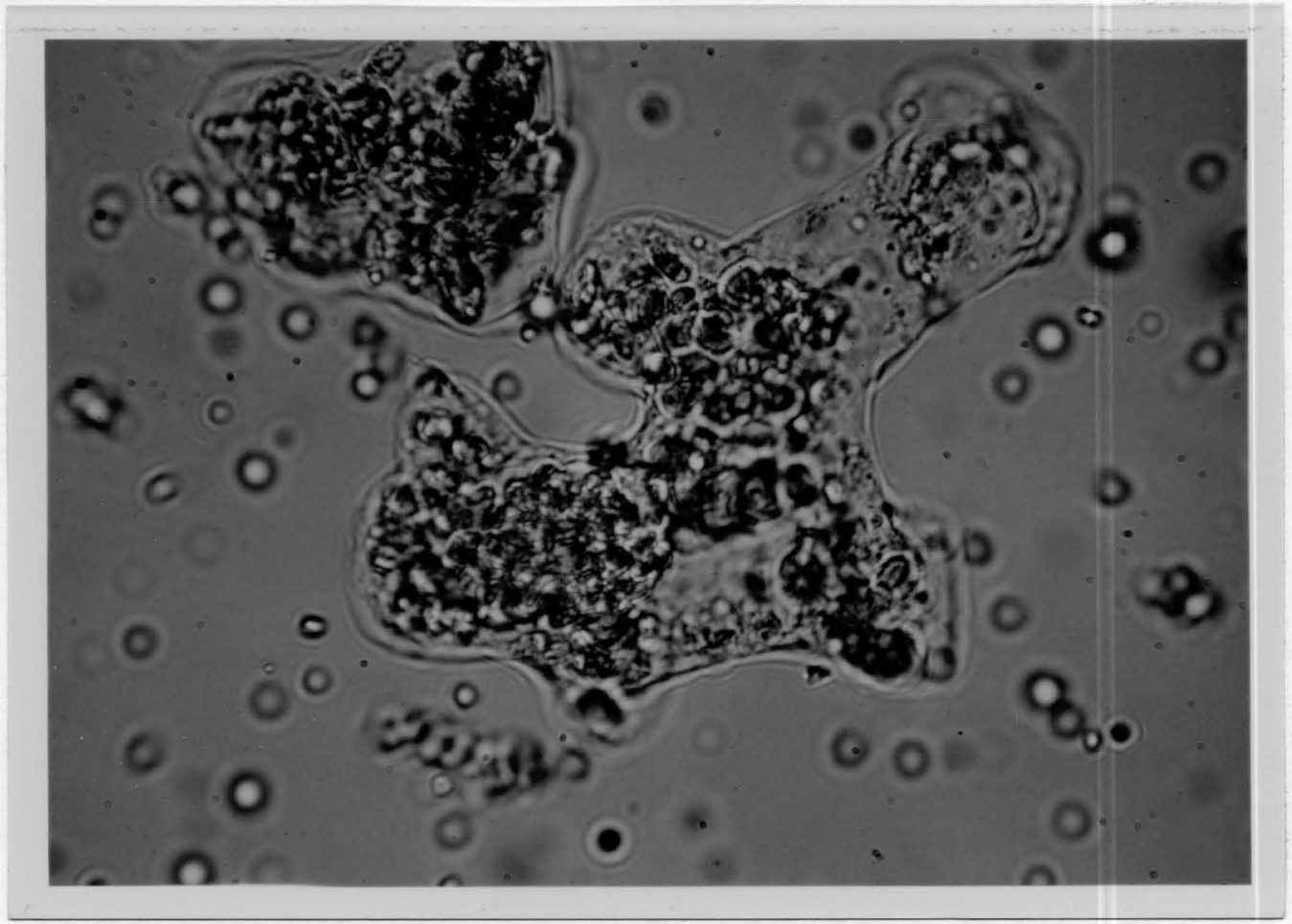

Fig. 6 Cell fragments released during macerozyme treatment of tobacco leaf tissue. After a brief 15 minute incubation in macerozyme medium, broken cells and fragments produced during epidermal stripping were removed. Magnification 400X.

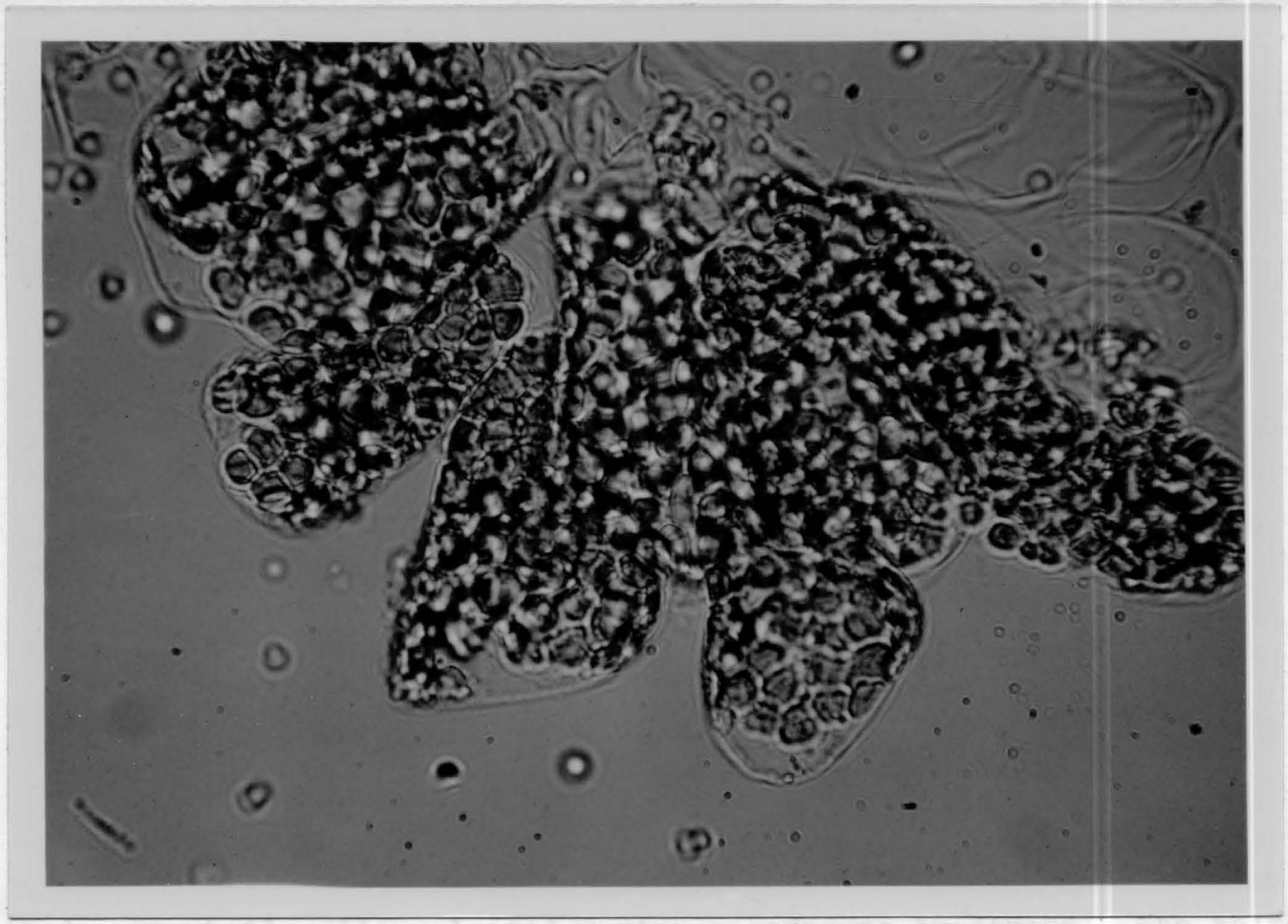

Fig. 7 Spongy mesophyll cells released during macerozyme treatment. The first 30 min. of incubation with macerozyme released cells of the spongy parenchyma type. Magnification 400X.

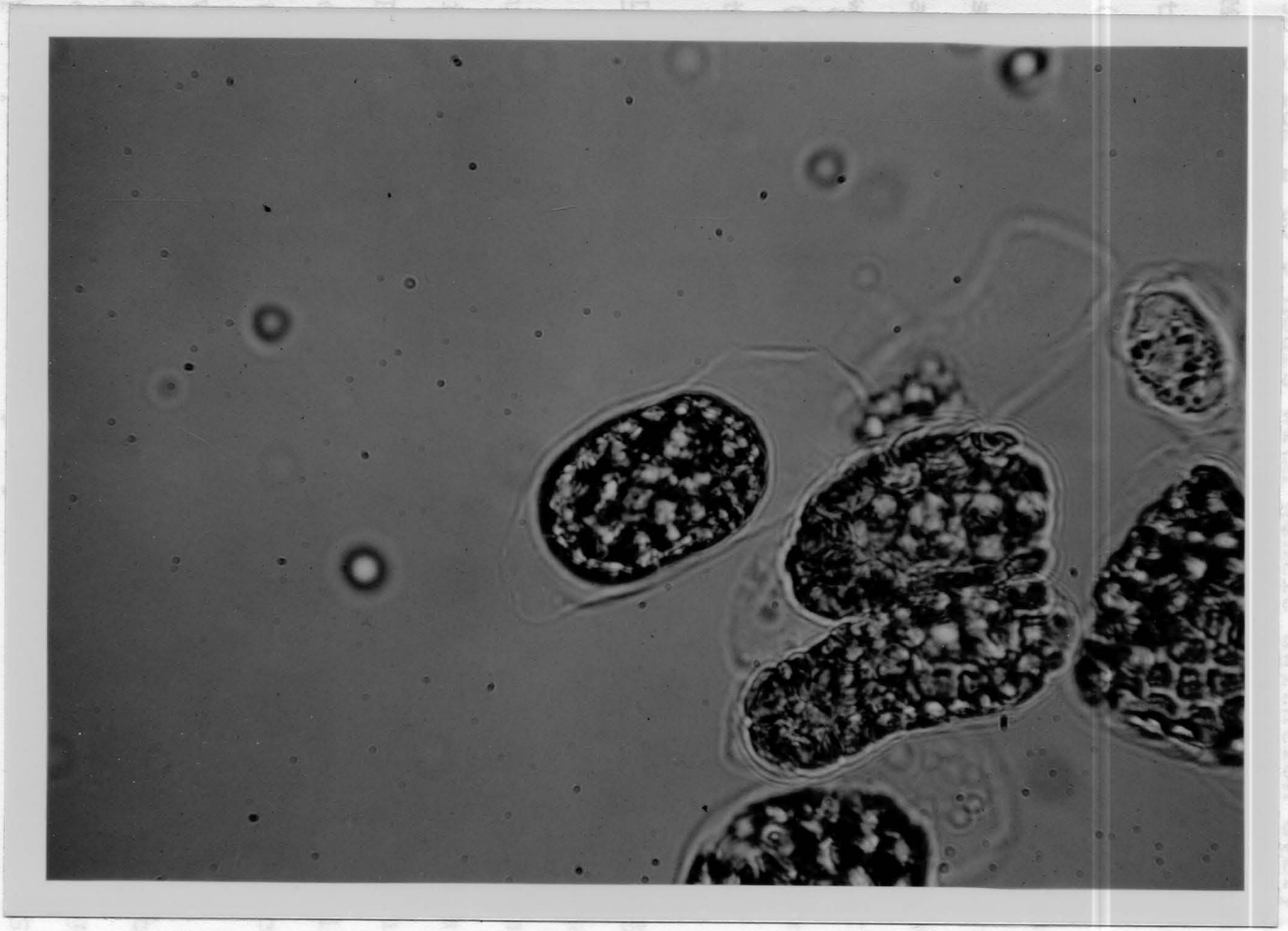

Fig. 8 Palisade mesophyll cells released during macerozyme treatment. The third and fourth 30 min. incubations of leaf tissue with macerozyme medium released cells of the palisade parenchyma. Cell walls can be seen still surrounding the plasmalyzed cytoplasm. Magnification 400X.

ing 0.8M D-mannitol and the pH was adjusted to 5.4 with 2N KOH (Appendix B). The suspended cells were incubated at 38°C for 2 to 3 hours with gentle agitation every 15 min. Microscopic examination of samples were taken to determine the degree of cell wall removal and protoplast release (Figs. 9 and 10).

Isolated protoplasts were filtered through a nylon sieve cloth 150 μ mesh size. This filter removed any cell aggregates and large debris. Protoplasts were washed 4 times with 0.8M D-mannitol at 23°C using low speed centrifugation (100 g.) for 3 min. each time. Following these washes, protoplasts were free of cell debris and cellulase.

Determination of the number of protoplasts released:

Volume of intact, viable protoplasts was measured using 1 ul. micropipetts manufactured by Clay Adams. Counting of protoplasts was facilitated by the use of a specially prepared cover slip .15 x 25 x 50 mm., supported at the edges by a glass strip .15 mm. in thickness and placed on the glass slide. A Carl Zeiss tri-occular microscope equipped with a 40X iris apochromatic objective, 1.0 N.A., 1.25 optibar, Ukatron 60 flash unit, and Nikkon F_2 photomic camera back, was used to examine as well as photograph the protoplasts. Kodak high-speed Ektachrome film, ASA 160, was used for recording results obtained.

Protoplast medium:

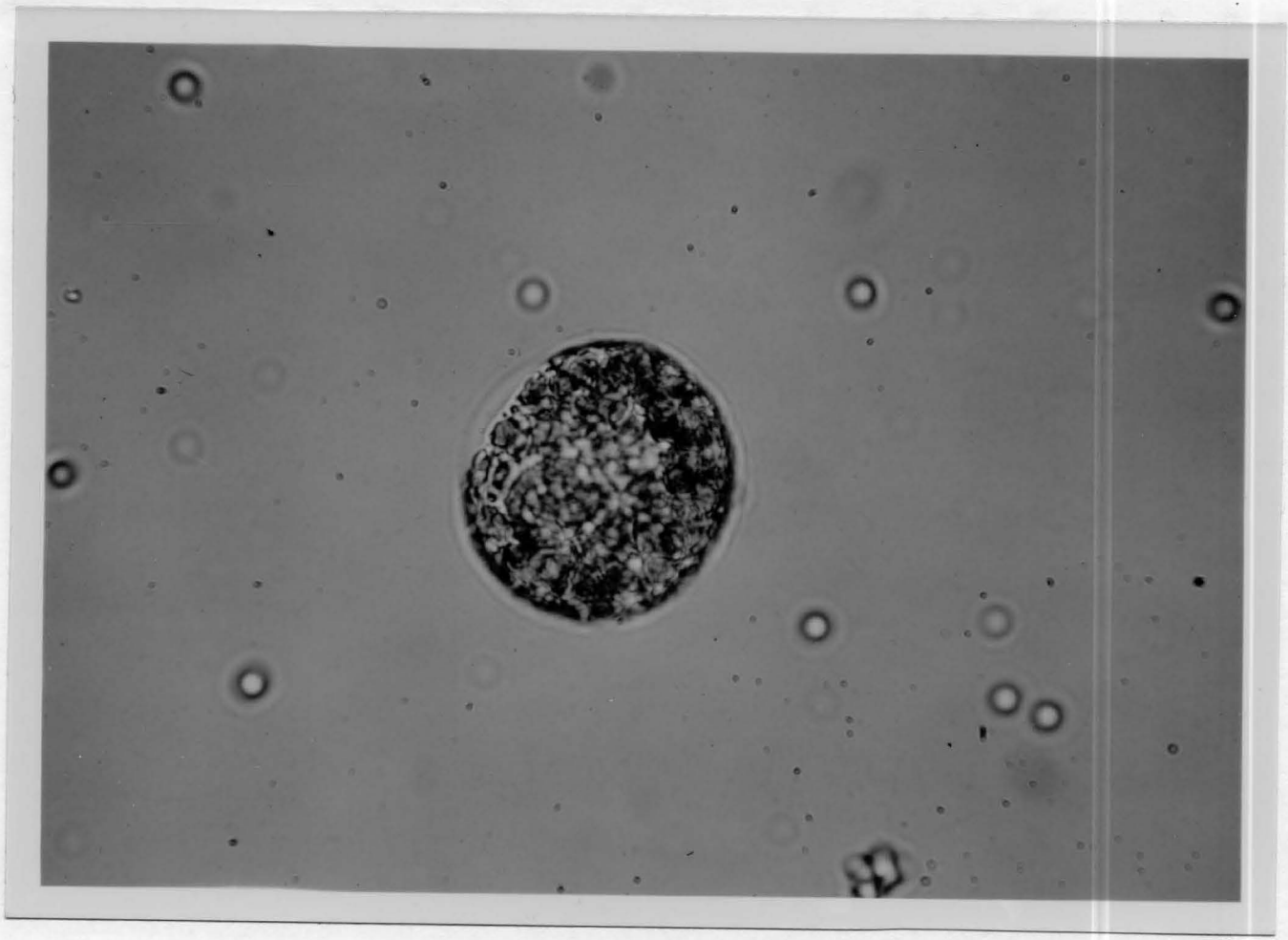

Fig. 9 Protoplast of N. tabaccum cv. White Burley produced after 2 hr. incubation in cellulase medium at 38°C. Cell walls have been removed. The cytoplasm is very condensed due to the hypertonic environment in which protoplasts are maintained. Magnification 400X.

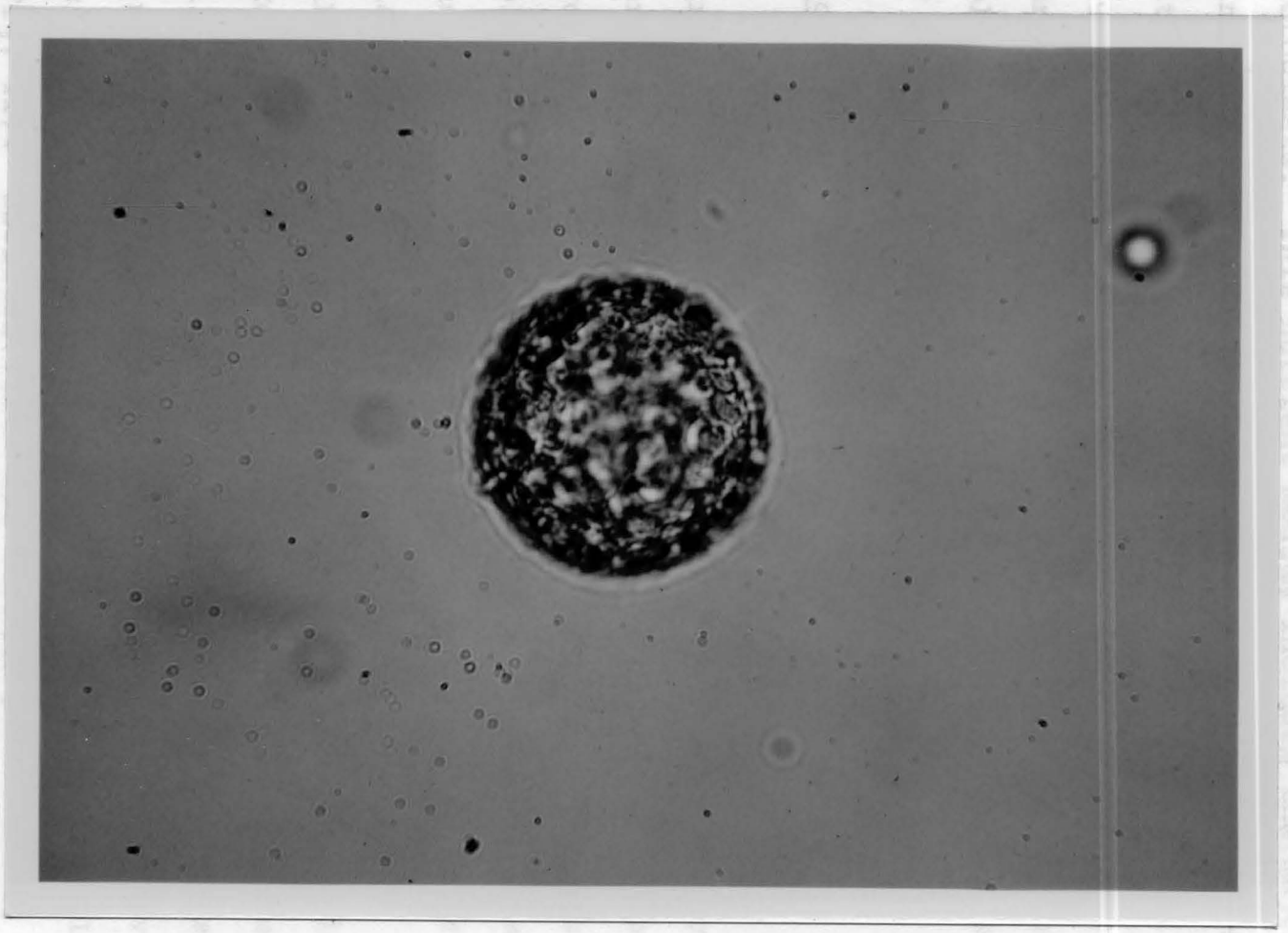

Fig. 10 Protoplast of N. glutinosa obtained after 2 hrs. of incubation in cellulase medium at 38°C. Cell walls have been removed and the cytoplasm is very condensed. Magnification 400X.

The medium given in Appendix A was used for maintaining the viability of freshly isolated palisade mesophyll protoplasts. This nutrient medium includes the major and minor elements used by Nagata and Takebe (1971), a modification of the elements used by Murashige and Skoog (1962). Organic substances were those formulated by Uchimiya and Murashige (1974). All nutrient media, as well as enzyme solutions, were filter sterilized using Millipore GS-0.22 um., 47 mm. membrane filters and glass filter apparatus.

Isolation of tobacco mosaic virus:

Thirty day old plants of Nicotiana tabaccum cv. Turkish were mechanically damaged by rubbing and subsequently were infected with a common strain of TMV, PV-135 obtained from American Type Culture Collection, Rockville, Maryland. Following a 30 day incubation period, 1000 gm. of leaf tissue was frozen and macerated in a Waring blender. Macerated plant material was passed through a cheese cloth to remove fibrous material. Plant extract was centrifuged using an IEC Clinical centrifuge at 1000 g. for 15 min. to remove all large cell fragments. Supernatant was then passed through a microcrystalline cellulose column to isolate the virus using polyethylene glycol and sodium chloride as solvents (Fig. 11). Isolated virus was further purified and concentrated using ultra-centrifugation (Sorvall oil Turbine Drive #2) for 2 hr. at 171,000 RCF. Pellets of virus were resuspended in phosphate buffer pH 7.0. The titer of the virus was

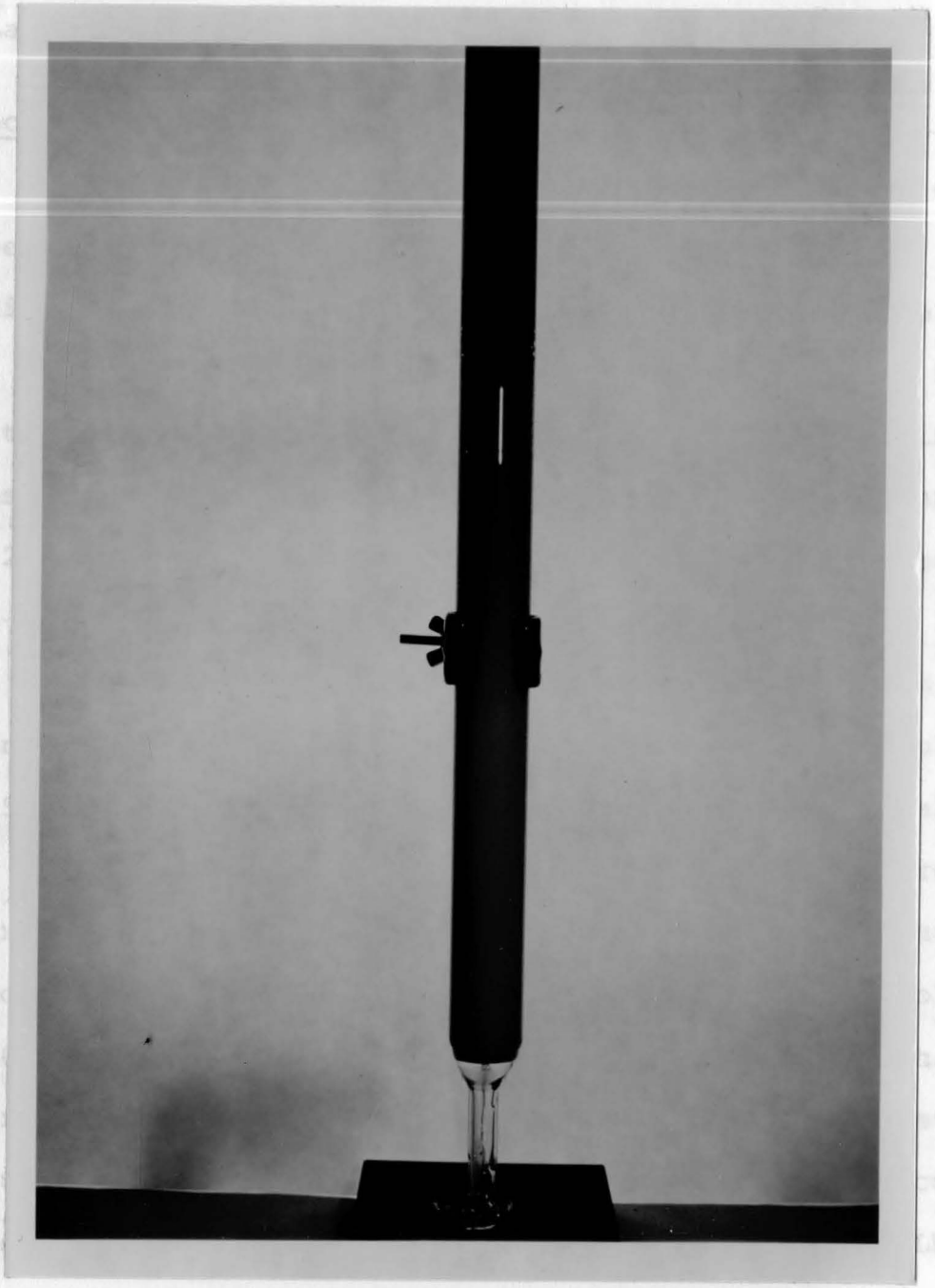

Fig. 11 Microcrystalline cellulose column used to purify TMV by chromatography.

determined by serial dilution and assayed on half leaves of Phaseolus vulgaris cv. Pinto, following the technique of Lamborn, Cochran, and Chidester(1971).

Infection of protoplasts with tobacco mosaic virus:

Protoplasts obtained from enzymatic processes described earlier were inoculated with TMV under aseptic conditions. Approximately 5 to 9 x 10^5 protoplasts per ml. in a nutrient media were used as experimental units. Protoplasts were infected with a solution of TMV 1:25 ml. in 0.02M potassium citrate buffer (pH 5.2) containing 0.8M D-mannitol and 2 µg./ml. poly-L-ornithine (2 x 10^9 virus particles/10 ml.). This mixture was incubated for a period of 10 min. at 27°C. Five ml. of the infectious medium was added to a 100 ml. Erlenmeyer flask containing concentrated washed protoplasts and 5 ml. of 0.8M D-mannitol solution. Following a half hour incubation at 26°C ± 1, protoplast washings and removal of excess inoculum was accomplished by 6 low speed centrifugations at 100 g. for 3 min. each using 0.8M D-mannitol and 0.1 mM $CaCl_2$ pH 5.4 as washing medium. After washing and removing the unadsorbed virus, protoplasts were returned to 20 ml. of nutrient medium (Appendix A) and were incubated at 26°C ± 1 with continuous light intensity of 3,000 LUX. Samples of tobacco protoplasts infected with TMV were removed from the incubation medium at various times and concentrated by low speed centrifugation using 100 g. for 3 min. Pelleted protoplasts were re-suspended in phosphate buffer pH 7.0 and

stored at 4°C until needed for bio-assay.

Ultrasonic Tobacco Mosaic Virus Bio-Assay

Frozen protoplast samples were allowed to thaw at 24°C and were sonicated for 7.5 sec. at 70 watts power level, using a Branson W 300 sonifier cell disruptor. One micro-liter of sonified protoplast sample was placed on a half leaf of Phaseolus vulgaris cv. Pinto previously dusted with 320 mesh Carborundum. Detached half leaves containing .001 or .1 ml.samples were passed slowly under the metal probe of the sonifier, supported by a polyfoam pad (Fig. 12). Half leaves were placed on an absorbent paper strip and put into a 10 x 20 x 30 cm. air tight polyethylene container. This container with leaves was placed in the dark at 26°C ± 1 for 18 hrs. Each absorbent paper strip carried 6 half leaves and was placed on trays (4 x 20 x 30 cm.) containing 1% agar and covered by a 20 x 30 cm. single weight glass plate. Trays were incubated for 3 to 5 days at 20 to 25°C under continuous flourescent light intensity of 3,000 LUX. Lesions were counted using a Wild binocular microscope (Fig. 13). The data was analyzed statistically using log transformations, analysis of variance and t-tests for independent samples.

Fig. 12 Ultrasonic inoculation with tobacco mosaic virus of a half-leaf. Phaseolus vulgaris cv. Pinto leaves were pushed upon and drawn under the metal probe.

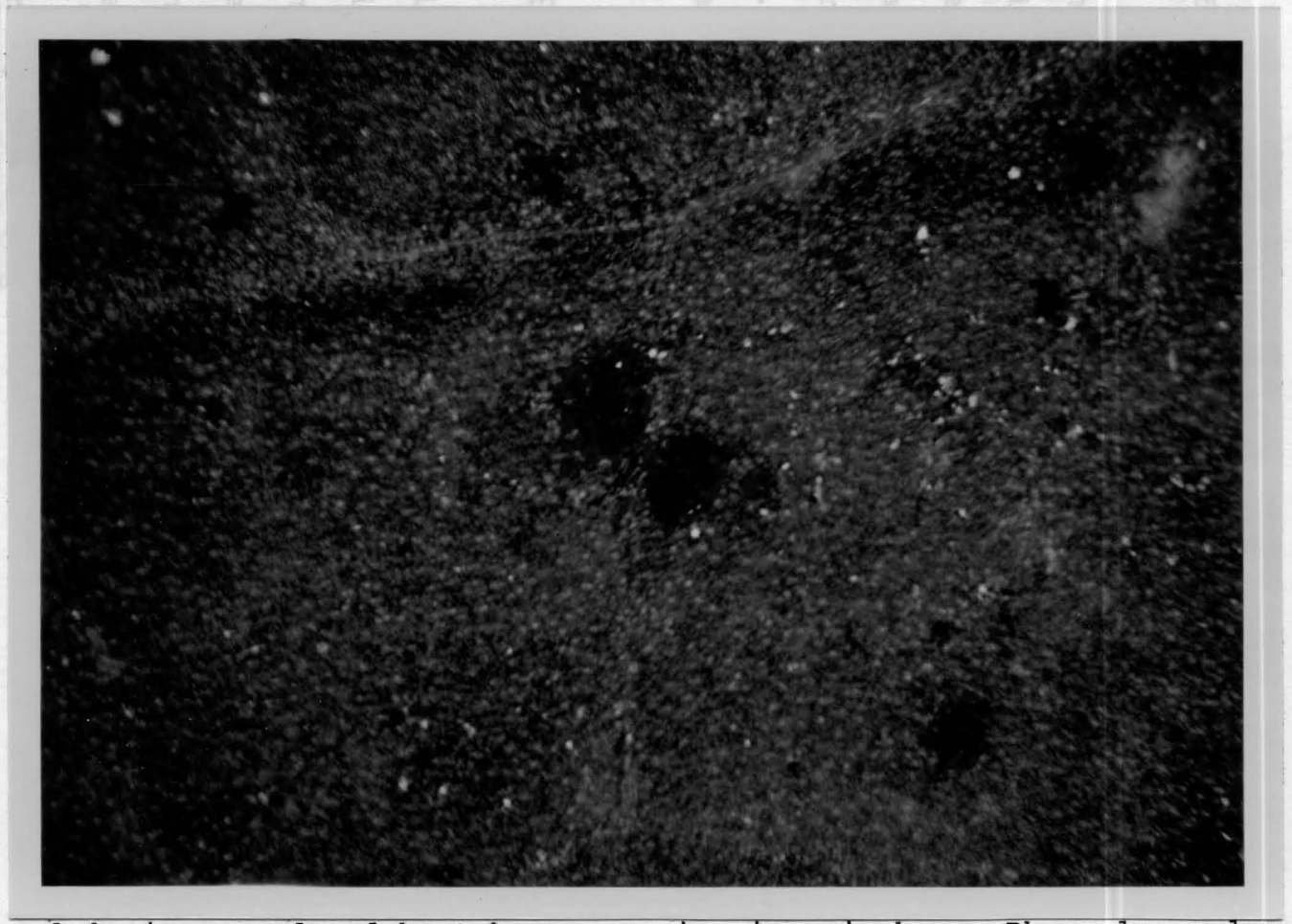

Fig. 13 Local lesions produced by tobacco mosaic virus in bean, Phaseolus vulgaris cv. Pinto, incubated 3 to 5 days at 20 to 25°C. One lambda of TMV infected protoplasts were placed on a half leaf previously dusted with 320 mesh carborundum and passed slowly under the probe of a sonifier.

RESULTS AND DISCUSSION

In order to facilitate the reader's understanding of the experimental data, discussion accompanies the results.

Mesophyll protoplasts of N. tabaccum cv. White Burley and N. glutinosa were isolated using Takebe's procedure (1968). Microscopic examination of samples taken throughout cell release in maceration medium and cell wall removal in cellulase medium showed protoplasts were intact. Both types of protoplasts appeared similar in color and shape, except that N. glutinosa protoplasts were deeper green and had larger vacuoles than N. tabaccum cv. White Burley protoplasts (Figs. 9 and 10).

Preliminary Investigation

Four preliminary experiments were conducted to determine the optimum age and length of tobacco leaves for isolating protoplasts and to aid the experimenter in mastering the isolation technique for subsequent research. Data indicated that 60 to 90 day old leaves, 25 to 30 cm. in length for White Burley and 15 cm. in length for glutinosa, gave the best results.

Protoplast Counts

Numbers of protoplasts released:

Four grams of leaf tissue released 5×10^5 to 4×10^6 protoplasts from N. tabaccum cv. White Burley and 3×10^5 to 2×10^6 from N. glutinosa. The number of protoplasts obtained from White Burley does not differ considerably from numbers reported by other investigators. Kassanis and White (1974) obtained 5 to 10 $\times 10^6$ protoplasts from a petri dish well-covered with leaf pieces of N. tabaccum cv. White Burley. Small variations in the number of protoplasts can be attributed to differences in isolation techniques and environmental and geological growing conditions. No results have been reported on the isolation of N. glutinosa protoplasts from leaf tissue. Therefore comparisons cannot be made.

Effects of plant type and time on isolated protoplasts:

Tobacco plants were grown for five consecutive months from September to January and protoplasts were isolated after a 60 to 90 day growing period from November to March. In order to compare the number of isolated protoplasts two factors were studied: the type of plant (N. tabaccum cv. White Burley and N. glutinosa); and the time at which plants were grown. Using the analysis of variance technique (ANOVA)* for a 2 (White Burley and glutinosa) x 5 (November, December, January, February and March) factorial design, it was found that both main effects (due to plant type and time)as well as the interaction effect (a particular plant at a particular time) were significant p's $< .01$ (Table 1). The direction of the effect due to plant type can be readily determined by referring to

TABLE: 1 ANALYSIS OF VARIANCE: Protoplasts isolation of N. tabaccum cv. White Burley and N. glutinosa.

SOURCE (FIG:14)	SS	d.f.	MS	F
Plant	5,925.20	1	5,925.20	23.25**
Time	50,857.17	4	12,714.29	49.89**
Interaction	141,465.89	4	35,366.47	138.77**
Error	15,036.22	59	254.85	-

* p .05
** p .01

ANALYSIS OF VARIANCE: Bio-assay of the washings of TMV infected protoplasts of N. tabaccum cv. White Burley and N. glutinosa, tested after a half hour incubation, on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto. Numbers of local lesions produced were transformed for statistical analysis.

SOURCE (FIG:15)	SS	d.f.	MS	F
Plant	.17	1	.17	3.09
Time	7.14	5	1.43	26.00**
Interaction	.95	5	.19	3.46*
Error	3.13	57	.05	-

* p .05
** p .01

ANALYSIS OF VARIANCE: Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto. Numbers of local lesions produced were transformed for statistical analysis.

TABLE: 1 Continued

SOURCE (FIG:16)	SS	d.f.	MS	F
Plant	.11	1	.11	.27
Time	2.27	7	.32	4.63**
Interaction	.23	7	.03	.48
Error	2.49	35	.07	-

* p .05
** p .01

SOURCE (FIG: 17)	SS	d.f.	MS	F
Plant	3.43	1	3.43	28.58**
Time	8.55	6	1.43	11.88**
Interaction	1.08	6	.18	1.5
Error	9.86	80	.12	-

* p .05
** p .01

SOURCE (FIG:18)	SS	d.f.	MS	F
Plant	.12	1	.13	1.08
Time	19.76	11	1.80	14.96**
Interaction	3.81	11	.35	2.88**
Error	2.67	115	.12	-

* p .05
** p .01

TABLE: I Continued

SOURCE (FIG: 19)	SS	d.f.	MS	F
Plant	.25	1	.25	4.88*
Time	1.53	19	.08	1.55
Interaction	.73	19	.04	.74
Error	10.16	196	.05	-

* p .05
** p .01

SOURCE (FIG: 20)	SS	d.f.	MS	F
Plant	1.66	1	1.66	33.78**
Time	7.77	24	.32	6.60**
Interaction	2.30	24	.10	1.95**
Error	11.78	243	.05	-

* p .05
** p .01

Figure 14. White Burley produces a significantly larger proportion of protoplasts than N. glutinosa, $F(1,59) = 23.25$ $p < .01$.

In order to determine the locus of the significant effects for time, $F(4,59) = 49.89$, $p < .01$ and the interaction effect, $F(4,59) = 138.77$, $p < .01$, post hoc t tests for independent samples were calculated. A significantly larger proportion of protoplasts were isolated from plants in January, February and March than in November and December, df (1,67) $t = 9.05$, $p < .01$. Furthermore, the proportion of protoplasts isolated in February, during the fourth isolation period, was significantly larger than those isolated in January, df (1,27) $t = 4.17$ $p < .01$. However, there was no significant difference between the proportion of protoplasts isolated in February and March, isolations 4 and 5 respectively.

Since the plants grown in a greenhouse over five months were supplied with continuous artificial illumination and maintained at a constant temperature, the only variable changing over time was the experimental day length and light intensity. As the environmental day length and light intensity increased so did the efficiency of the protoplast isolation technique, with maximum yield produced during February and March. From these results it can be concluded that the environmental conditions under which tobacco plants are grown play an essential role in the production of viable

*For an example of ANOVA and t formulas see Appendix C and D.

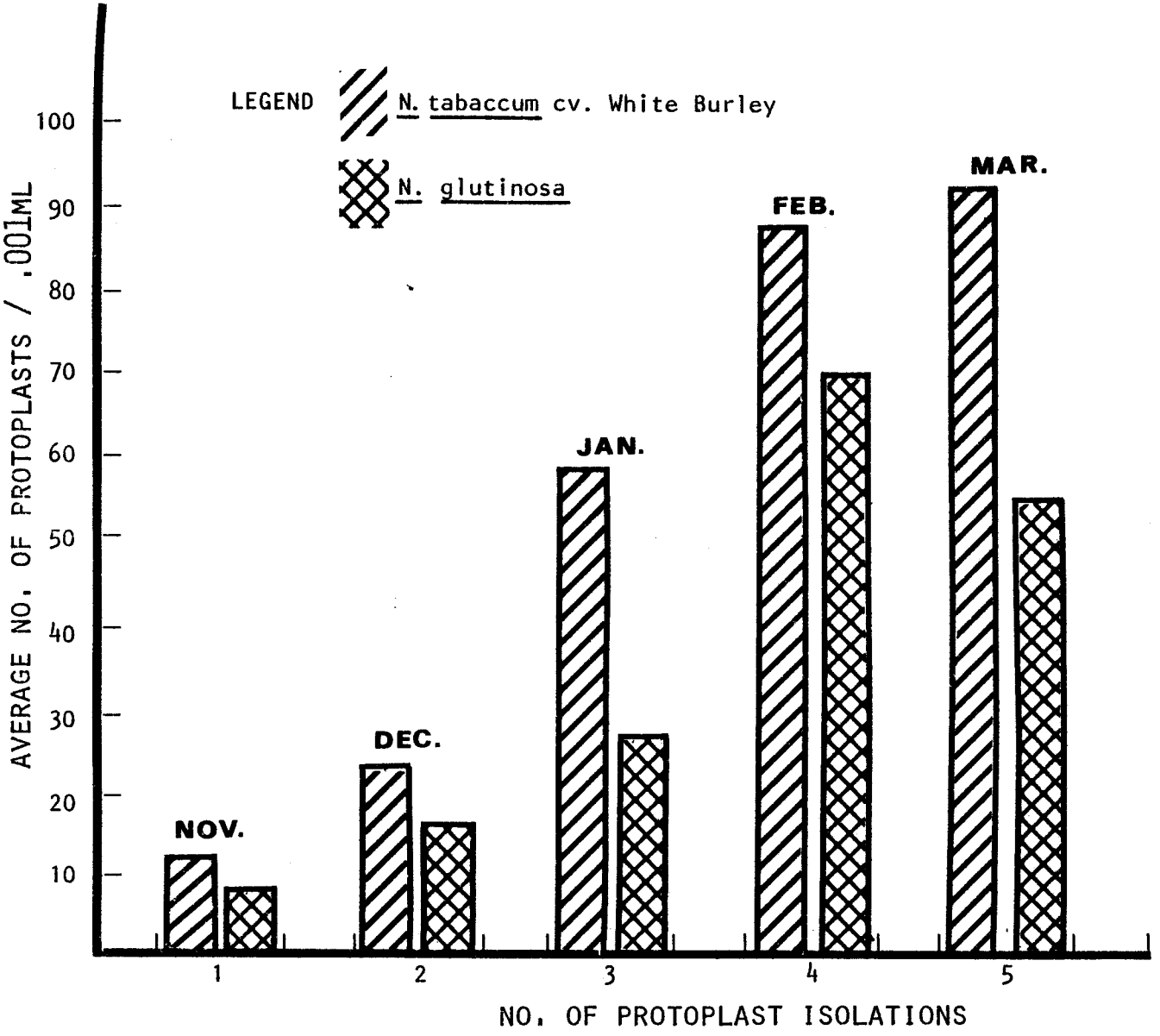

Fig: 14 Protoplast isolation of N. tabaccum cv. White burley and N. glutinosa grown in the green house at 20°C ± 5 under varying light intensities

protoplasts. Elevated or reduced temperatures and light conditions reduce the number of viable protoplasts produced during isolation.

Using t-tests to assess the interaction effect, it was found that N. tabaccum cv. White Burley protoplasts were significantly more abundant than N. glutinosa protoplasts in January, df (1,10) $t = 4.06$ $p < .01$ and in March, df (1,14) $t = 3.59$ $p < .01$. No signficant differences were found between the plants for the three remaining isolation periods.

One must be cautious in interpreting the results obtained for the main effect due to plants and the interaction effect. From the results presented, it appears that White Burley yields a greater number of protoplasts than N. glutinosa. Since the number of cells per gram weight was not calculated however, this conclusion cannot be made. It would be difficult to get an accurate weight of leaf material before isolation because leaves are wilted. Therefore, these results remain tentative.

Virus Replication in Protoplasts

Protoplast culture:

Protoplasts obtained from the enzymatic processes described earlier were inoculated with TMV media containing approximately 2×10^9 virus particles per 10 ml (Appendix B). From this estimation of virus particles and data presented in Figure 14, it appears that the ratio of virus particles

to protoplasts ranged from 1:5.4 x 10^2 to 1:6.7 x 10^3. Takebe and Otsuki (1969) and Hiba and Yore (1972) estimated that between 1 x 10^1 and 1 x 10^2 virus particles were adsorbed per protoplast. These figures indicate that large numbers of virus particles were present permitting massive adsorption of TMV by viable protoplasts. It is necessary for inoculum to contain large numbers of virus particles because virus infection of protoplasts is not a one to one hit ratio as seen in bacteriophage. TMV lacks the mechanism to inject its RNA into the host cell. Therefore, TMV must enter by other means. In leaf cells it has to be introduced by mechanically damaging the cell. In the case of protoplasts, entry of TMV occurs by adsorption by the plasma membrane or through damaged areas in the membrane or possibly pinocytosis.

After exposure to the infectious media, protoplasts were washed six times to remove excess inoculum and suspended in protoplast medium (Appendix A). After being incubated for 86 hours in protoplast medium, both species of tobacco were examined under light microscope, and protoplasts were found to be intact.

Removal of excess inoculum:

Samples of washing medium were bio-assayed to determine how effective washes were in removing unadsorbed virus (Fig. 15). Since the number of virus particles do not vary equally from the mean for small numbers, a log transformation was performed on the data as suggested by Kleczkowski 1955

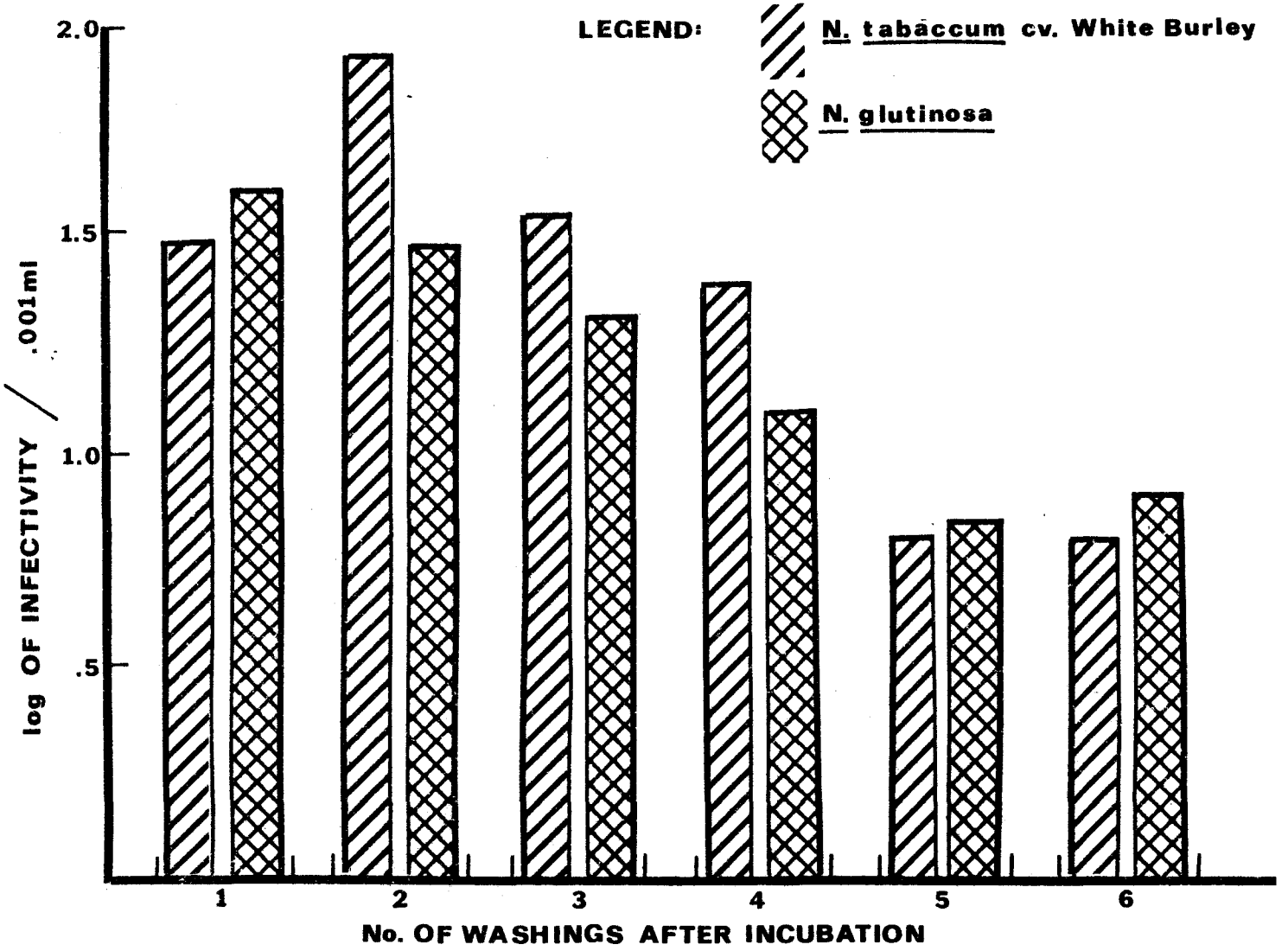

Fig: 15 Bio-assay of the washings of TMV infected protoplasts of N. tabaccum cv. White Burley and N. glutinosa, tested after half hour incubation, on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto. Numbers of local lesions produced were transformed for statistical analysis.

(Appendix E).* All subsequent statistical analysis were performed on transformed data. Analysis of variance for a 2 (White Burley and glutinosa) x 6 (first through sixth washings) factorial design showed no overall significant effect due to plant type, showing plants were not differentially affected by washings (Table 1). However, a significant effect was found due to time $F(5,57) = 26.00$ $p < .01$ and interaction between plant and time $F(1,5) = 3.46$ $p < .05$. Furthermore, t-tests revealed that washing 1 was found to be significantly different from washing 5, df (1,22) $t = 6.84$ $p < .01$ and washing 6, df (1,22) $t = 6.42$ $p < .01$ in the number of virus particles removed. Washing 5 and 6 did not differ significantly in the amount of virus particles removed, indicating that almost all virus particles had been removed by the fifth washing and that six washings were effective in removing excess inoculum. Although viable virus paticles were not completely eliminated, it may be that the later washings contained fragments from ruptured protoplasts and wirus particles which had adhered to the incubation container. Washings helped provide a one-step growth curve by eliminating the chance of protoplasts becoming reinfected.

For both White Burley df (1,10) $t = 3.71$ $p < .01$ and glutinosa df (1,10) $t = 6.18$ $p < .01$, a significantly greater amount of virus particles were removed in the first

*Actual data for all bio-assays are listed in Tables 2 to 11, and Figures 21 to 26 found at the end of the results and discussion section.

washing as compared with the fifth and the sixth washing. At first it appears that the second washing for White Burley removed a greater number of virus particles than the first washing. However, this difference was not found to be statistically significant. Furthermore, it may be that because of the polar nature of TMV, the two ends of the particles are different, resulting in a bundling of particles at high concentrations tend to reduce the number of local lesions on an assay host. This explanation may account for the difference in number of virus particles removed for White Burley after first and second washings. Also, it may be that this effect was not found for glutinosa because glutinosa protoplasts, though smaller in number, could have adsorbed a greater number of virus particles.

Early events following infection:

Viral infection of a cell means the introduction of new genetic information in a host cell. Infection results in the viral directed cell synthesis of viral nucleic acid, coat protein, and finally recombination of coat protein and nucleic acid into a viral particle. TMV replication did occur in both species of protoplasts, N. tabaccum cv. White Burley and N. glutinosa. Replication was evident in samples assayed for TMV after infection. Figure 16 illustrates the typical growth curve of early virus replication in an infected protoplast. Using ANOVA for a 2 (White Burley and glutinosa) x 8 (1 to 12 hours) factorial design (Table 1), it

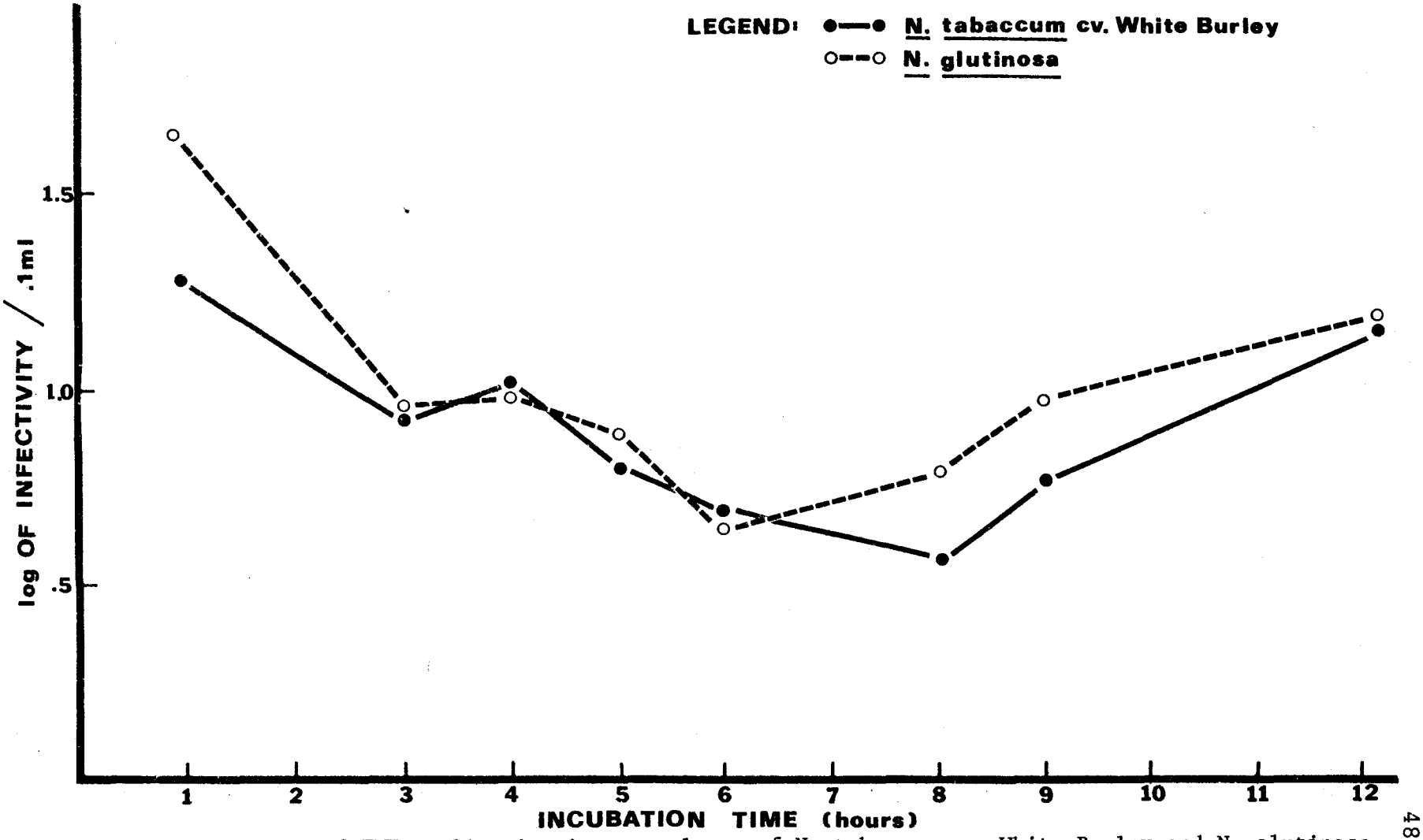

Fig: 16 Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto. Numbers of local lesions produced were transformed for statistical analysis.

was found that the number of lesions produced over time differed significantly $F(1,7) = 4.63$ $p < .01$. In order to determine the locus of the effect, a post hoc t-test was calculated and revealed that the large amount of virus present in the sample at one hour diminished by the third hour and remained low until the sixth hour, df (1,10) $t = 4.26$ $p < .01$. At the first time period, infectivity was due to the fact that inoculum virus adsorbed to protoplasts. A large inoculum at time one could be interpreted as follows. First, the entry of various particles into the protoplasts requires at least an hour and a half. Second, the long held notion that only one virus particle enters the cell might be incorrect because virus particles initially in a high ratio to protoplasts at one hour are not present 3 hours after infection.

The decrease after 6 hours reflects the eclipse phase in which protein coats of virus particles are removed. The eclipse phase detected 3 to 6 hours after infection represents the in vivo dissociation of TMV and release of viral RNA. Other investigators provide evidence to support the findings that disassociation of TMV could not occur at the cell membrane because release of viral RNA at this point would allow cytoplasmic ribonucleases access to naked viral RNA. However, since TMV-RNA has been found in the nucleus, viral dissociation is said to possibly occur on the nuclear membrane or endoplasmic reticulum associated with the nucleus (Reddi 1972).

A significant increase of TMV in inoculum was observed from 6 to 12 hours, df (1,13) $t = 3.31$ $p < .01$. This increase could mean two things; that progeny viral RNA has been synthesized from parent RNA, and that replication of virus is evident 6 hours post-infection and continues until 12 hours. Previous investigations (Takebe 1975) examined protoplasts 6 hours after infection using an electron microscope and observed progeny virus particles which, when counted, produced an essentially similar curve to that of a bio-assay. Additional research is needed to further clarify this event. No other significant differences were found for number of lesions between plants or interaction of plants across time. It is clear from these findings that assays of both species had an equal number of virus particles present in their samples at any given time.

Late events following infection:

Four experiments were conducted to determine the growth curve of TMV in infected tobacco mesophyll protoplasts of N. tabaccum cv. White Burley and N. glutinosa (Figs. 17 to 20). The events following adsorbtion and uncoating of the virus particles were of particular interest. Complete virus particles accumulate in the cytoplasm after assembly. The rate (determined by bio-assay) at which virus particles accumulate is an indication of the efficiency of virus replication in the two species.

General trends show a significant effect due to

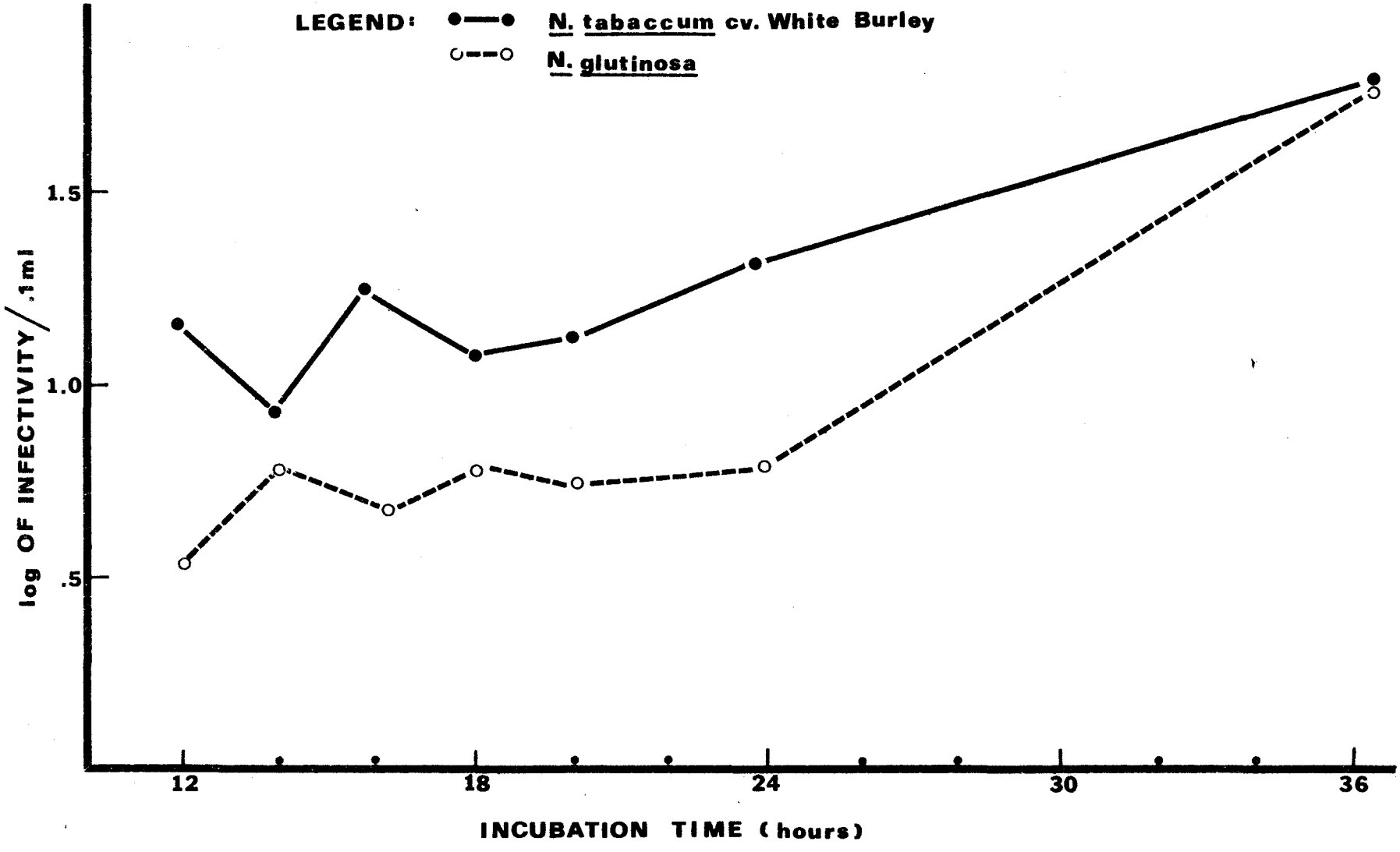

Fig: 17 Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto. Numbers of local lesions produced were transformed for statistical analysis.

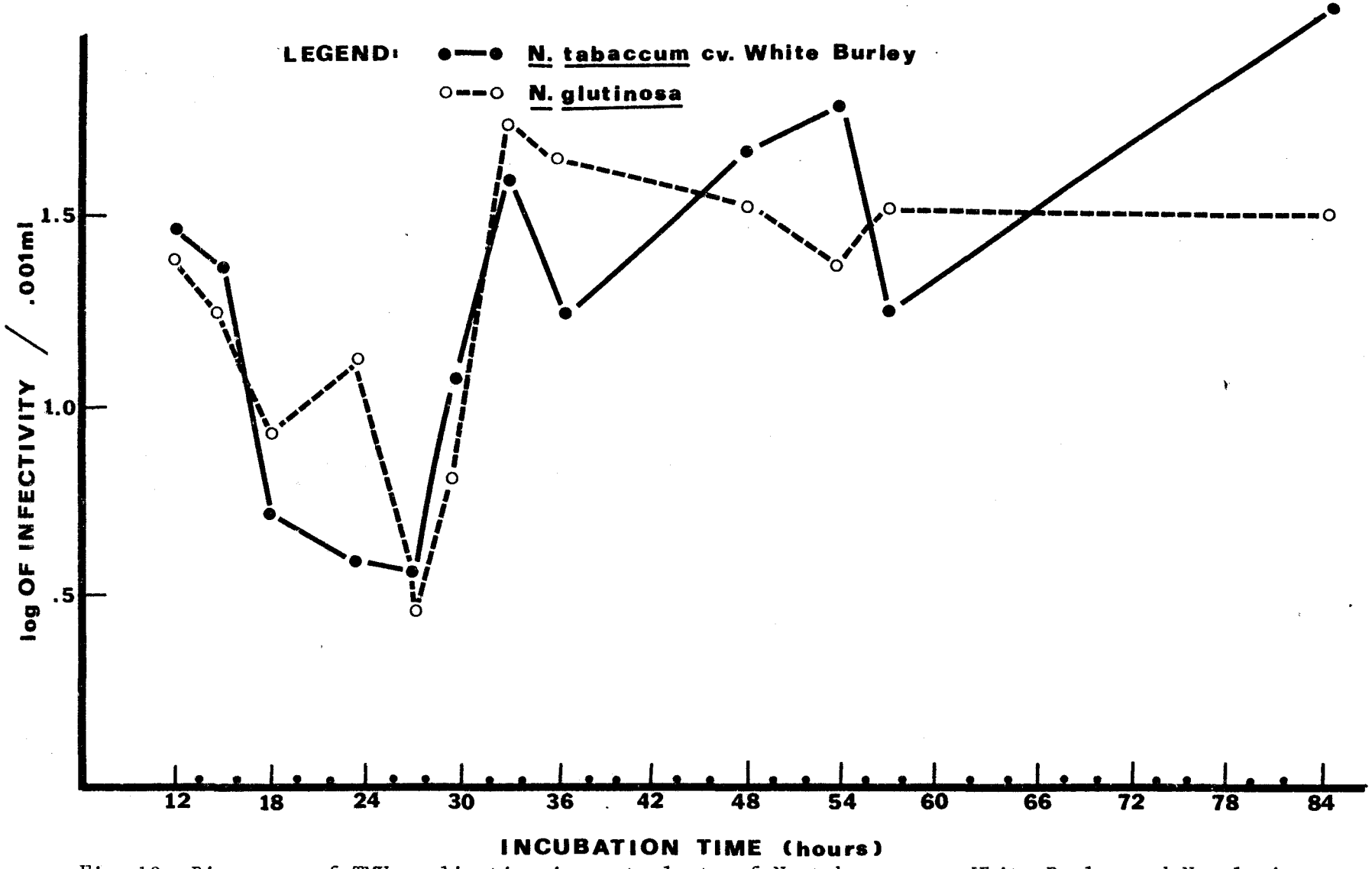

Fig: 18 Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto. Numbers of local lesions produced were transformed for statistical analysis.

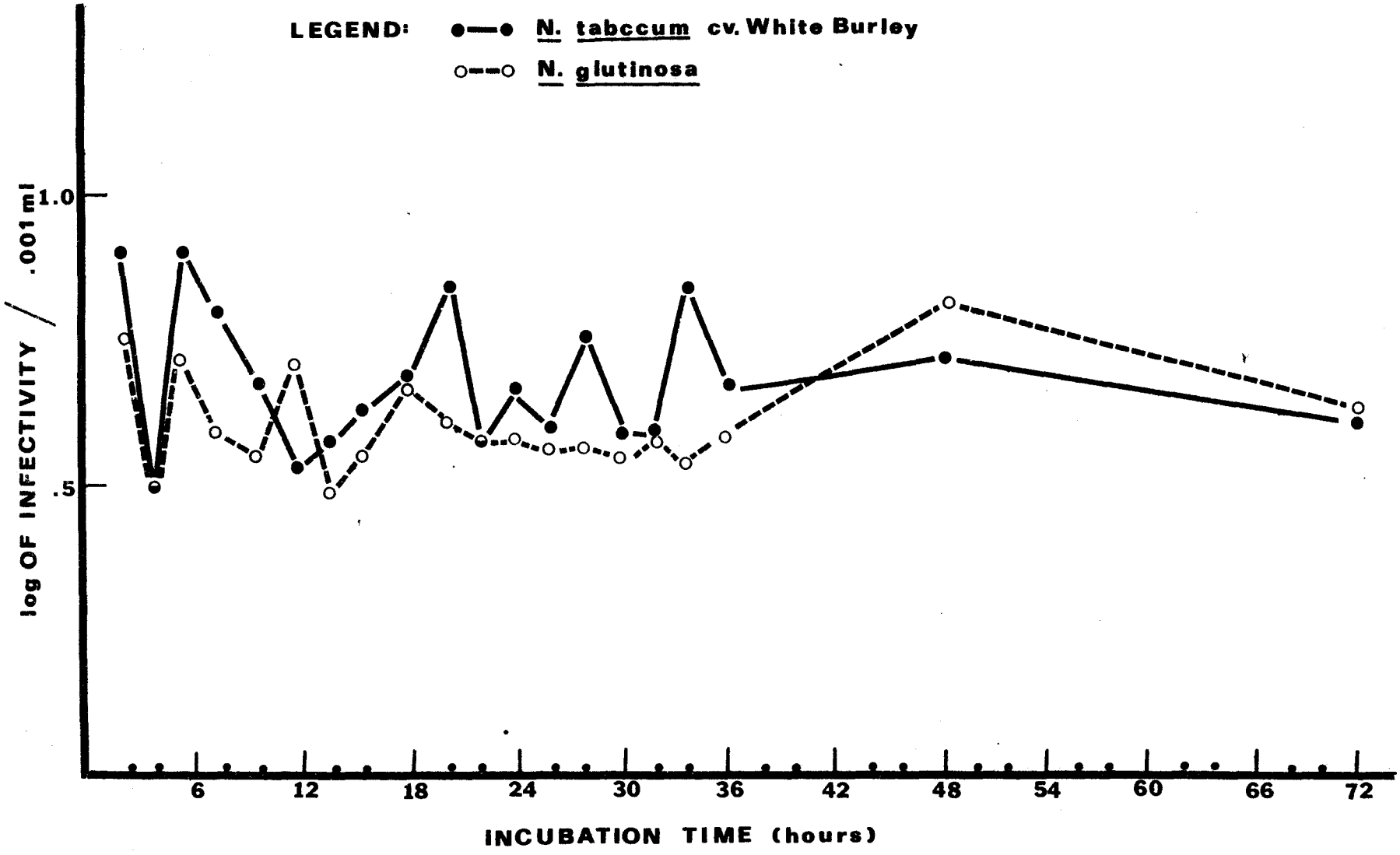

Fig: 19 Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto. Numbers of local lesions produced were transformed for statistical analysis.

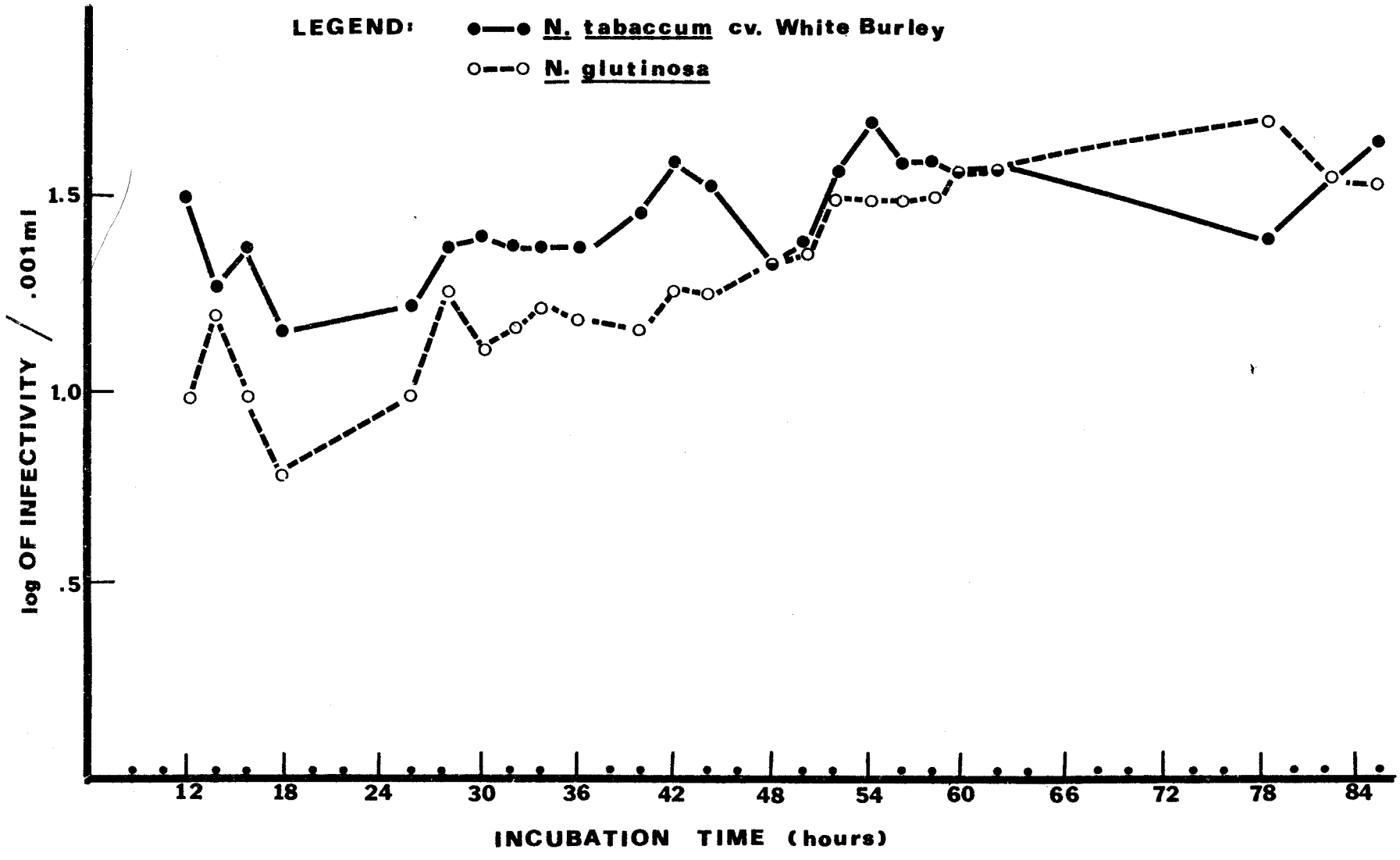

Fig: 20 Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto. Numbers of local lesions produced were transformed for statistical analysis.

plant type indicating that White Burley consistently produces a significantly greater number of virus particles than glutinosa ($p < .01$). Results supporting this conclusion can be drawn from Table 1 in which it can be seen that the analysis of variance calculated yielded significant differences for plants. Furthermore, it can be seen that overall there is a significant effect due to virus particles produced at certain hours after inoculation ($p < .01$) as shown in Table 1. Since readings in each of three experiments were taken for different time intervals across a variety of time spans, the effects of time will be discussed for each individual experiment.

First, for the first experiment results depicted in Figure 17, it can be seen that there is a significant increase in the number of virus particles produced in the inoculum from 12 to 36 hours, df (1,23) $t = 5.02$ $p < .01$ for both plants. For the second experiment (Fig. 18), a large number of virus particles are detected 12 hours after infection for both plants. From 12 to 27 hours a significant decrease is detected in number of virus particles, df (1,22) $t = 9.03$ $p < .01$. These results are similar to those found by Takebe and Otsuki (1969) who detected a reduction in the growth curve after 24 hours. Their explanation for this loss of infectivity was attributed to shaking. However cultures were not shaken in the present investigation. It would appear that some other factor is involved, rendering virus particles non-infectious.

In the fourth experiment, refer to Figure 20, a significant rise in infectivity can be seen from 18 to 86 hours, df (1,20) $t = 7.33$ $p < .01$. These results taken together with the results over time for experiment one, lend support to Takebe's later findings which showed no decline in the growth curve after 24 hours. Since protoplasts for experiment one were infected during December, protoplasts for experiment two were infected during January, and protoplasts for experiment four were infected during March, it is difficult to make any clear, direct comparisons for time across experimental sessions.

Lastly, the following interaction effects were statistically significant for the second and fourth experiment ($p < .01$). In Figure 18, it is evident that a significant increase occurs from 27 to 33 hours after inoculation for White Burley, df (1,10) $t = 4.70$ $p < .01$ and glutinosa, df (1.8) $t = 10.78$ $p < .01$. TMV replication slows for both species after 33 hours. A similar rise in infectivity occurs in experiment four in White Burley from 12 to 54 hours and in glutinosa for 18 to 84 hours (Figure 20). The rate of accumulation of virus particles in the cytoplasm is reduced after 2 days, indicating maximum accumulation of virus particles had been reached. Given the short time period for virus replication, it appears that TMV is easily produced in protoplasts. The infected cells in both species are not damaged and there seems to be a limit to the replication of

virus, possibly controlled by an interferon-like substance. A decrease in infectivity is present for White Burley between 54 and 78 hours, df (1,9) $t = 3.24$ $p < .01$, and for glutinosa between 14 and 18 hours, df (1,10) $t = 3.50$ $p < .01$. A possible explanation for the decrease observed during late virus replication could be the synthesis of an unknown substance which acts as a defense mechanism associated with cell resistance.

Local lesion response:

N. glutinosa responds to TMV infection by producing necrotic local lesions at the primary infection site. In past research (Hibi et al. 1972; Otsuki et al. 1972), the necrotic response was not demonstrated for TMV infected protoplasts in N. tabaccum cv. Xanthi nc. In the present experiments, N. glutinosa protoplasts showed no necrosis 84 hours after infection with TMV. Reasons for the loss of the necrotic response are not known. However reddish-brown local lesions have been produced indicating necrosis in callus tissue of N. glutinosa, N. tabaccum cv. (NN Samsun), (NN burley) and (Xathi nc) when infected with TMV (Beachy and Murakisha 1971). Callus cells were connected by plasmadesmata and protoplasts are separate from each other. There appears to be a need for cell to cell contact. Perhaps materials passing between cells are responsible for the local lesion response. Protoplasts are not connected, therefore, cell to cell contact is absent. Further research must be

undertaken to determine other factors responsible for necrosis.

TABLE: 2 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of Nicotiana tabaccum cv. White Burley on half leaves of Phaseolus vulgaris cv. Pinto

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER .1ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
1	1	10	14.50
	2	26	
	3	15	
	4	7	
3	1	4	3.50
	2	3	
4	1	4	6.60
	2	9	
	3	7	
5	1	7	3.66
	2	1	
	3	4	
6	1	5	2.25
	2	3	
	3	0	
	4	1	
8	1	1	.66
	2	0	
	3	1	
9	1	6	4.00
	2	3	
	3	0	
	4	3	
12	1	17	7.50
	2	2	
	3	6	
	4	5	

TABLE: 3 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of Nicotiana glutinosa on half leaves of Phaseolus vulgaris cv. Pinto

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .1ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
1	1	38	38.00
3	1	4	4.00
	2	4	
	3	3	
	4	5	
4	1	5	5.00
5	1	0	6.00
	2	6	
	3	12	
6	1	0	2.32
	2	0	
	3	7	
8	1	0	9.00
	2	1	
	3	0	
	4	35	
9	1	5	6.50
	2	11	
	3	4	
	4	6	
12	1	7	8.25
	2	9	
	3	7	
	4	10	

TABLE: 4 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of Nicotiana tabaccum cv. White Burley on half leaves of Phaseolus vulgaris cv. Pinto

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .1ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
12	1	8	15.83
	2	1	
	3	16	
	4	1	
	5	54	
	6	15	
14	1	33	5.86
	2	0	
	3	3	
	4	3	
	5	0	
	6	1	
	7	1	
16	1	62	19.14
	2	0	
	3	10	
	4	5	
	5	17	
	6	30	
	7	10	
18	1	10	14.57
	2	4	
	3	3	
	4	1	
	5	7	
	6	2	
	7	75	
20	1	16	11.86
	2	28	
	3	13	
	4	12	
	5	2	
	6	0	
	7	12	
24	1	21	20.00
	2	9	
	3	15	
	4	30	
	5	15	
	6	50	
	7	0	

TABLE: 4 Continued.

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .1ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
36	1	3	84.00
	2	35	
	3	155	
	4	138	
	5	39	
	6	134	

TABLE: 5 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of *Nicotiana glutinosa* on half leaves of *Phaseolus vulgaris* cv. Pinto

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .1ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
12	1	2	.86
	2	3	
	3	0	
	4	0	
	5	1	
	6	0	
	7	0	
14	1	4	4.00
	2	10	
	3	1	
	4	9	
	5	0	
	6	0	
16	1	0	1.86
	2	5	
	3	0	
	4	1	
	5	0	
	6	3	
	7	4	
18	1	0	4.43
	2	6	
	3	8	
	4	15	
	5	0	
	6	2	
	7	0	
20	1	9	3.43
	2	1	
	3	0	
	4	12	
	5	0	
	6	0	
	7	2	
24	1	0	2.57
	2	5	
	3	2	
	4	2	
	5	1	
	6	3	
	7	5	

TABLE: 5 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .1ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
36	1	65	81.16
	2	128	
	3	75	
	4	0	
	5	75	
	6	144	

TABLE: 6 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of Nicotiana tabaccum cv. White Burley on half leaves of Phaseolus vulgaris cv. Pinto

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
12	1	99	34.40
	2	20	
	3	28	
	4	2	
	5	23	
	6	34	
15	1	13	22.80
	2	13	
	3	57	
	4	24	
	5	7	
18	1	2	2.00
	2	2	
	3	4	
	4	4	
	5	0	
	6	0	
24	1	1	1.20
	2	1	
	3	3	
	4	0	
	5	0	
	6	2	
27	1	1	1.50
	2	7	
	3	0	
	4	0	
	5	0	
	6	1	
30	1	4	14.80
	2	1	
	3	0	
	4	51	
	5	9	
	6	24	

TABLE: 6 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	64	
	2	3	
	3	111	
33	4	125	60.50
	5	10	
	6	50	
	1	4	
	2	2	
	3	5	
36	4	52	20.00
	5	17	
	6	40	
	1	25	
	2	81	
	3	137	
48	4	7	62.80
	5	72	
	6	54	
	1	26	
	2	95	
	3	156	
54	4	65	74.30
	5	95	
	6	9	
	1	58	
	2	0	
	3	1	
57	4	56	29.80
	5	34	
	1	18	
	2	70	
	3	21	
60	4	50	42.00
	5	91	
	6	2	
	1	106	
	2	153	
	3	60	
84	4	44	96.60
	5	133	
	6	84	

TABLE: 7 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of Nicotiana glutinosa on half leaves of Phaseolus vulgaris cv. Pinto

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
12	1	16	24.80
	2	10	
	3	55	
	4	17	
	5	28	
	6	23	
15	1	18	19.70
	2	5	
	3	6	
	4	3	
	5	14	
	6	72	
18	1	12	6.50
	2	2	
	3	3	
	4	0	
	5	18	
	6	4	
24	1	47	12.00
	2	7	
	3	1	
	4	6	
	5	7	
	6	4	
27	1	2	.33
	2	0	
	3	0	
	4	0	
	5	0	
	6	0	
30	1	4	1.00
	2	0	
	3	0	
	4	1	
	5	0	
	6	1	
33	1	73	57.00
	2	22	
	3	80	
	4	53	

TABLE: 7 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	42	
	2	68	
	3	51	
36	4	20	45.20
	5	41	
	6	49	
	1	69	
	2	57	
	3	41	
48	4	4	34.50
	5	0	
	6	36	
	1	58	
	2	0	
54	3	1	29.80
	4	56	
	5	34	
	1	4	
	2	7	
	3	32	
57	4	28	32.00
	5	91	
	6	30	
	1	28	
	2	6	
	3	8	
84	4	49	28.30
	5	47	
	6	32	

TABLE: 8 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of Nicotiana tabaccum cv. White Burley on half leaves of Phaseolus vulgaris cv. Pinto

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
2	1	4	3.67
	2	4	
	3	6	
	4	4	
	5	2	
	6	2	
4	1	0	.50
	2	0	
	3	2	
	4	0	
	5	0	
	6	1	
6	1	3	3.83
	2	5	
	3	5	
	4	3	
	5	5	
	6	2	
8	1	1	2.83
	2	3	
	3	1	
	4	1	
	5	9	
	6	2	
10	1	4	1.83
	2	1	
	3	0	
	4	1	
	5	5	
	6	0	
12	1	0	.66
	2	0	
	3	0	
	4	2	
	5	2	
	6	0	

TABLE: 8 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	1	
	2	0	
	3	4	
14	4	0	1.00
	5	1	
	6	0	
	1	1	
	2	0	
	3	0	
16	4	4	1.16
	5	1	
	6	1	
	1	0	
	2	1	
18	3	0	3.75
	4	14	
	1	2	
	2	4	
	3	2	
20	4	1	1.60
	5	6	
	6		
	1	0	
	2	0	
	3	3	
22	4	1	.83
	5	1	
	6	0	
	1	0	
	2	2	
	3	11	
24	4	2	2.50
	5	0	
	6	0	
	1	2	
	2	0	
	3	1	
26	4	0	.83
	5	1	
	6	1	

TABLE: 8 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
28	1	2	2.50
	2	4	
	3	1	
	4	0	
	5	7	
	6	1	
30	1	2	.83
	2	1	
	3	1	
	4	0	
	5	1	
	6	0	
32	1	0	1.00
	2	0	
	3	3	
	4	2	
	5	0	
	6	1	
34	1	6	3.33
	2	6	
	3	1	
	4	1	
	5	1	
	6	5	
36	1	0	1.16
	2	3	
	3	1	
	4	1	
	5	1	
	6	1	
48	1	3	2.33
	2	3	
	3	1	
	4	0	
	5	0	
	6	7	
72	1	1	.83
	2	2	
	3	1	
	4	0	
	5	0	
	6	1	

TABLE: 9 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of Nicotiana glutinosa on half leaves of Phaseolus vulgaris cv. Pinto

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
2	1	0	5.33
	2	1	
	3	13	
	4	0	
	5	18	
	6	0	
4	1	0	2.20
	2	0	
	3	11	
	4	0	
	5	0	
	6	0	
6	1	6	2.50
	2	0	
	3	5	
	4	2	
	5	2	
	6	0	
8	1	2	.83
	2	0	
	3	1	
	4	0	
	5	1	
	6	1	
10	1	0	.36
	2	0	
	3	0	
	4	1	
	5	2	
	6	1	
12	1	0	1.66
	2	1	
	3	2	
	4	2	
	5	2	
	6	3	

TABLE: 9 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	1	
	2	0	
	3	1	
14	4	0	.33
	5	0	
	6	0	
	1	0	
	2	1	
	3	1	
16	4	3	1.00
	5	0	
	6	1	
	1	0	
	2	1	
	3	0	
18	4	1	1.83
	5	3	
	6	6	
	1	0	
	2	0	
	3	1	
20	4	4	1.50
	5	0	
	6	4	
	1	1	
	2	1	
	3	1	
22	4	0	.66
	5	1	
	6	0	
	1	0	
	2	0	
	3	1	
24	4	3	1.00
	5	2	
	6	0	
	1	0	
	2	18	
	3	0	
26	4	0	3.00
	5	0	
	6	0	

TABLE: 9 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	0	
	2	0	
	3	2	
28	4	1	.83
	5	2	
	6	0	
	1	0	
	2	0	
	3	1	
30	4	1	.44
	5	2	
	6	0	
	1	0	
	2	0	
	3	5	
32	4	2	1.40
	5	0	
	1	0	
	2	1	
	3	4	
34	4	0	.83
	5	0	
	6	0	
	1	0	
	2	1	
	3	3	
36	4	0	1.00
	5	2	
	6	0	
	1	1	
	2	6	
	3	4	
48	4	1	3.50
	5	0	
	6	9	
	1	0	
	2	5	
	3	0	
72	4	2	1.50
	5	0	
	6	2	

TABLE: 10 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of Nicotiana tabaccum cv. White Burley on half leaves of Phaseolus vulgaris cv. Pinto.

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	15	
	2	74	
12	3	24	36.67
	4	15	
	5	84	
	6	8	
	1	13	
	2	15	
14	3	15	16.50
	4	11	
	5	32	
	6	13	
	1	11	
	2	25	
16	3	31	21.17
	4	25	
	5	26	
	6	9	
	1	4	
	2	14	
18	3	11	10.33
	4	13	
	5	8	
	6	12	
	1	15	
	2	6	
26	3	7	17.33
	4	5	
	5	13	
	6	58	
	1	12	
	2	20	
28	3	70	24.67
	4	17	
	5	7	
	6	22	

TABLE: 10 Continued

INCUBATION TIME (HRS)	NO. OF HALF LEAVES INNOCULATED	PER. .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO.OF LESIONS
	1	28	
	2	15	
30	3	16	27.83
	4	9	
	5	21	
	6	78	
	1	4	
	2	4	
32	3	65	30.00
	4	14	
	5	20	
	6	73	
	1	13	
	2	48	
34	3	20	22.17
	4	15	
	5	25	
	6	12	
	1	24	
	2	25	
36	3	15	16.33
	4	13	
	5	17	
	6	27	
	1	35	
	2	18	
40	3	99	35.33
	4	23	
	5	18	
	6	19	
	1	6	
	2	38	
42	3	48	46.00
	4	78	
	5	63	
	6	43	
	1	28	
	2	16	
44	3	34	27.33
	4	24	
	5	40	
	6	22	

TABLE: 10 Continued

INCUBATION TIME (HRS)	NO. OF HALF LEAVES INNOCULATED	PER .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	23	
	2	12	
48	3	46	20.67
	4	24	
	5	9	
	6	10	
	1	28	
	2	10	
50	3	15	20.83
	4	25	
	5	26	
	6	21	
	1	59	
	2	10	
52	3	33	40.17
	4	60	
	5	39	
	6	40	
	1	59	
54	2	72	53.20
	3	32	
	4	62	
	5	41	
	1	14	
	2	35	
56	3	26	48.17
	4	100	
	5	94	
	6	20	
	1	54	
	2	41	
58	3	24	40.33
	4	41	
	5	32	
	6	50	
	1	28	
	2	44	
60	3	45	34.17
	4	32	
	5	21	
	6	35	

TABLE: 10 Continued

INCUBATION TIME (HRS)	NO. OF HALF LEAVES INNOCULATED	PER .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	47	
	2	12	
62	3	23	35.33
	4	84	
	5	19	
	6	27	
	1	18	
	2	16	
78	3	55	24.83
	4	23	
	5	14	
	6	23	
	1	30	
	2	15	
82	3	54	33.00
	4	33	
	5	19	
	6	47	
	1	12	
	2	57	
84	3	31	31.67
	4	15	
	5	50	
	6	25	
	1	70	
	2	33	
86	3	35	48.00
	4	36	
	5	37	
	6	77	

TABLE: 11 Assay of Tobacco Mosaic Virus infected mesophyll protoplasts of *Nicotiana glutinosa* on half leaves of *Phaseolus vulgaris* cv. Pinto

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	3	
	2	10	
	3	8	
12	4	7	6.33
	5	5	
	6	5	
	1	12	
	2	4	
14	3	8	16.60
	4	11	
	5	28	
	6	20	
	1	9	
	2	8	
16	3	2	7.33
	4	10	
	5	8	
	6	7	
	1	3	
	2	5	
18	3	3	3.33
	4	5	
	5	3	
	6	1	
	1	10	
	2	4	
26	3	7	6.50
	4	4	
	5	7	
	6	7	
	1	23	
	2	16	
28	3	26	25.30
	4	28	
	5	5	
	6	8	

TABLE: 11 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	12	
	2	0	
30	3	16	11.00
	4	15	
	5	7	
	6	16	
	1	11	
	2	7	
32	3	10	10.50
	4	13	
	5	12	
	6	10	
	1	22	
	2	8	
34	3	12	13.83
	4	16	
	5	6	
	6	19	
	1	9	
	2	10	
36	3	10	12.17
	4	24	
	5	17	
	6	3	
	1	37	
	2	19	
40	3	2	14.00
	4	2	
	5	15	
	6	9	
	1	18	
	2	26	
42	3	24	24.00
	4	19	
	5	6	
	6	7	
	1	17	
	2	19	
44	3	9	15.00
	4	19	
	5	20	
	6	6	

TABLE: 11 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	41	
	2	16	
48	3	13	19.00
	4	19	
	5	14	
	6	11	
	1	13	
	2	14	
50	3	20	18.33
	4	37	
	5	14	
	6	12	
	1	91	
	2	19	
52	3	42	36.17
	4	9	
	5	21	
	6	35	
	1	30	
54	2	160	53.25
	3	15	
	4	8	
	1	27	
	2	23	
56	3	6	30.67
	4	60	
	5	17	
	6	51	
	1	37	
	2	43	
58	3	32	27.33
	4	9	
	5	21	
	6	22	
	1	24	
	2	19	
60	3	27	29.83
	4	24	
	5	26	
	6	59	

TABLE: 11 Continued

INCUBATION TIME (HRS.)	NO. OF HALF LEAVES INNOCULATED	PER .001ML SAMPLE NO. OF LESIONS / HALF LEAF	AVERAGE NO. OF LESIONS
	1	29	
	2	44	
62	3	13	32.67
	4	41	
	5	39	
	6	30	
	1	58	
	2	60	
78	3	100	54.67
	4	25	
	5	40	
	6	45	
	1	35	
82	2	23	30.75
	3	23	
	4	42	
	1	18	
	2	42	
84	3	32	35.83
	4	35	
	5	35	
	6	53	
	1	35	
86	2	29	32.50
	3	24	
	4	42	

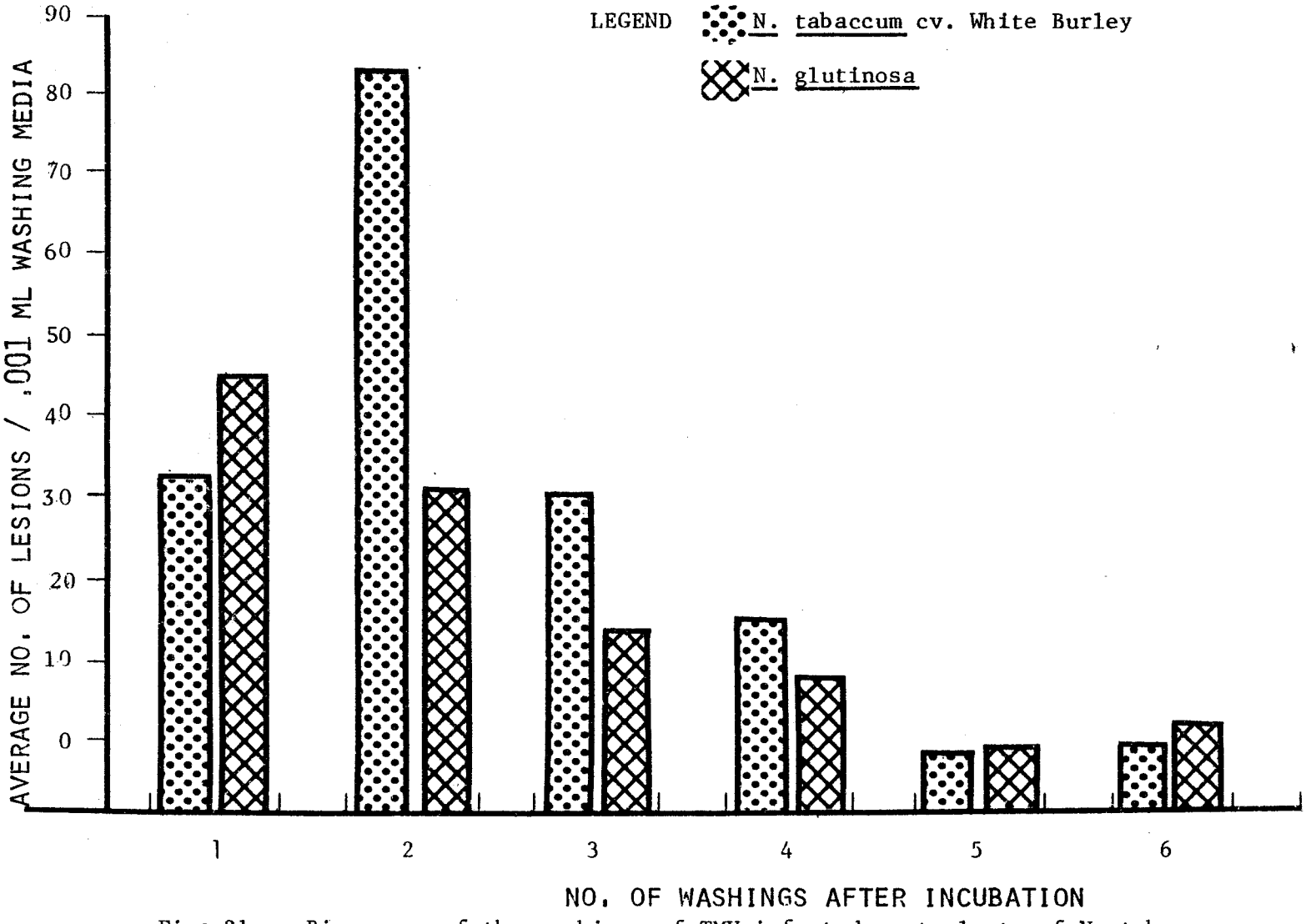

Fig: 21 Bio-assay of the washings of TMV infected protoplasts of N. tabaccum cv. White Burley and N. glutinosa, tested after half hour incubation, on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto

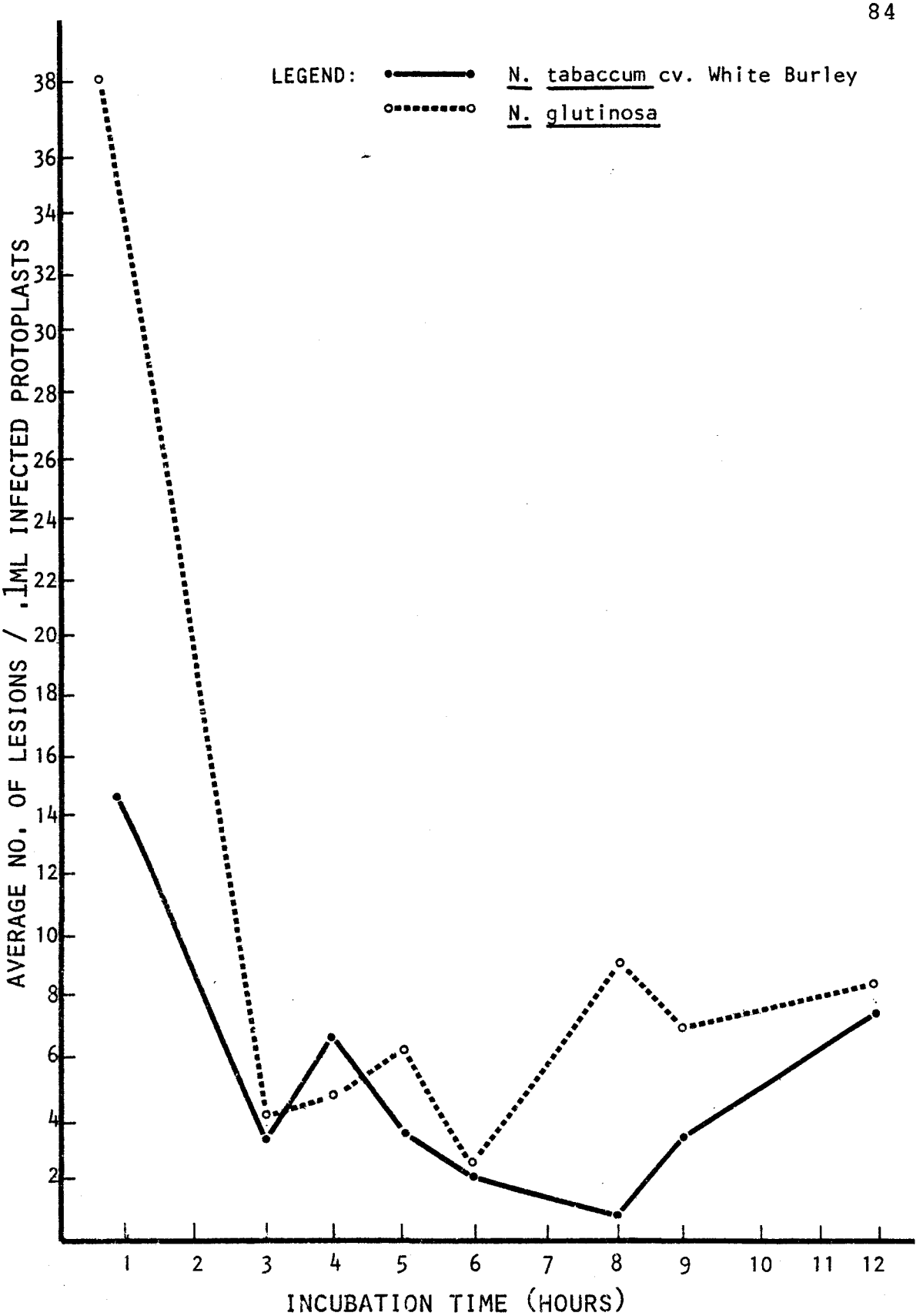

Fig:.22 Bio-assay of TMV replicatiom in protoplasts of N. tabaccum cv. White Burley and N. glutinosa, tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto

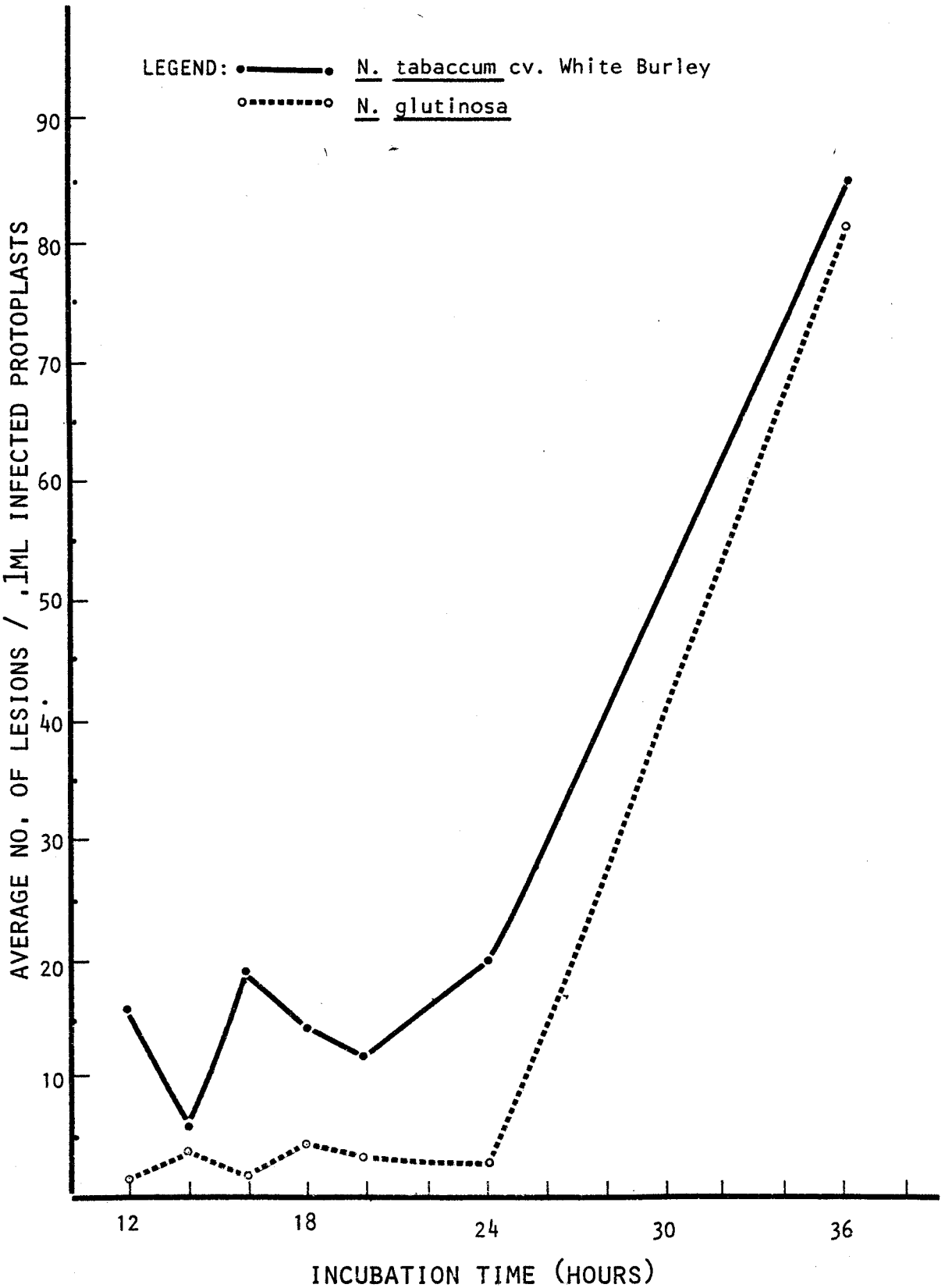

Fig:.23 Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa, tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto

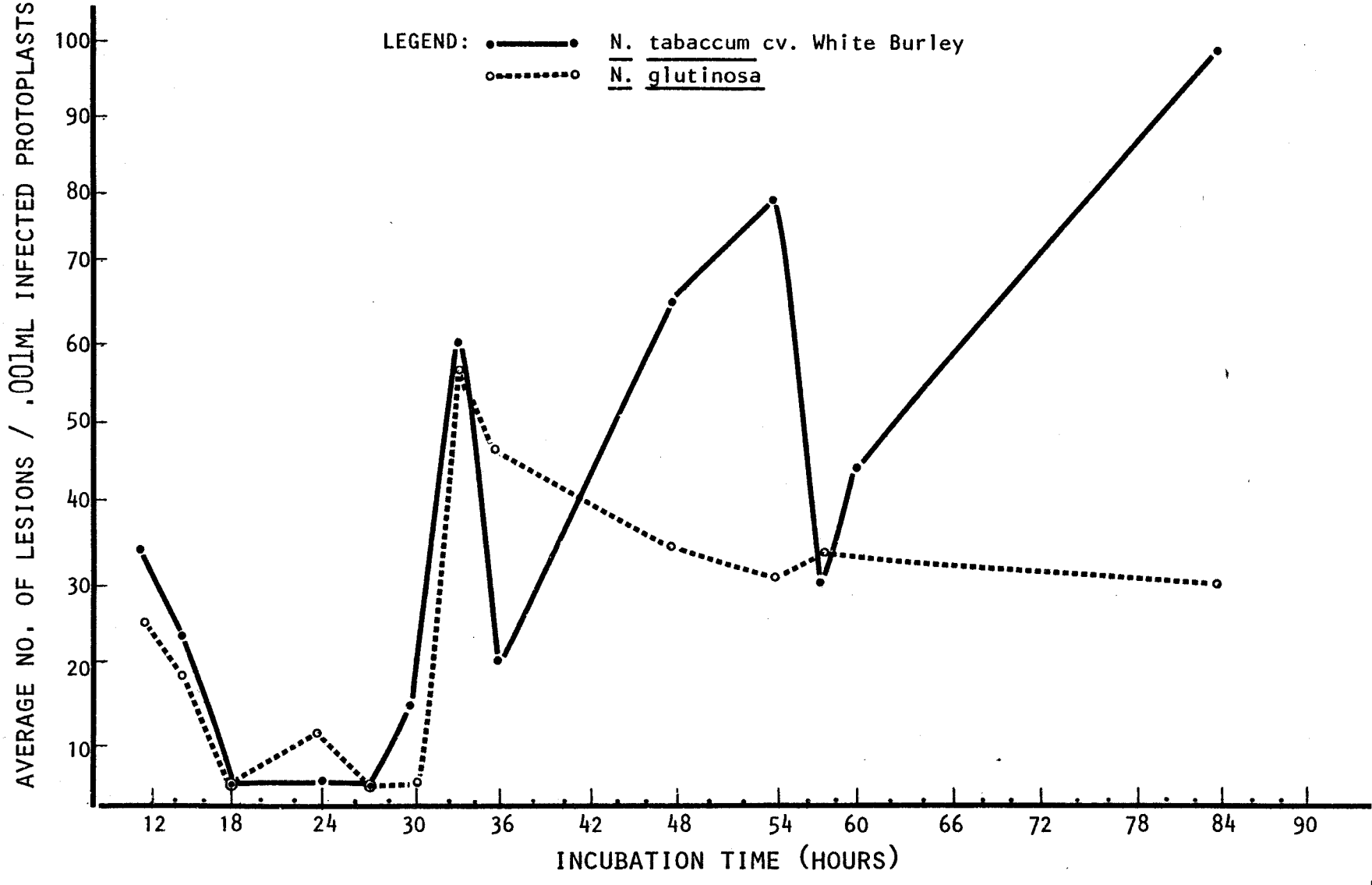

Fig: 24 Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa, tested on 10 day old half leaves of Phaseolus vulgaris cv. Pinto

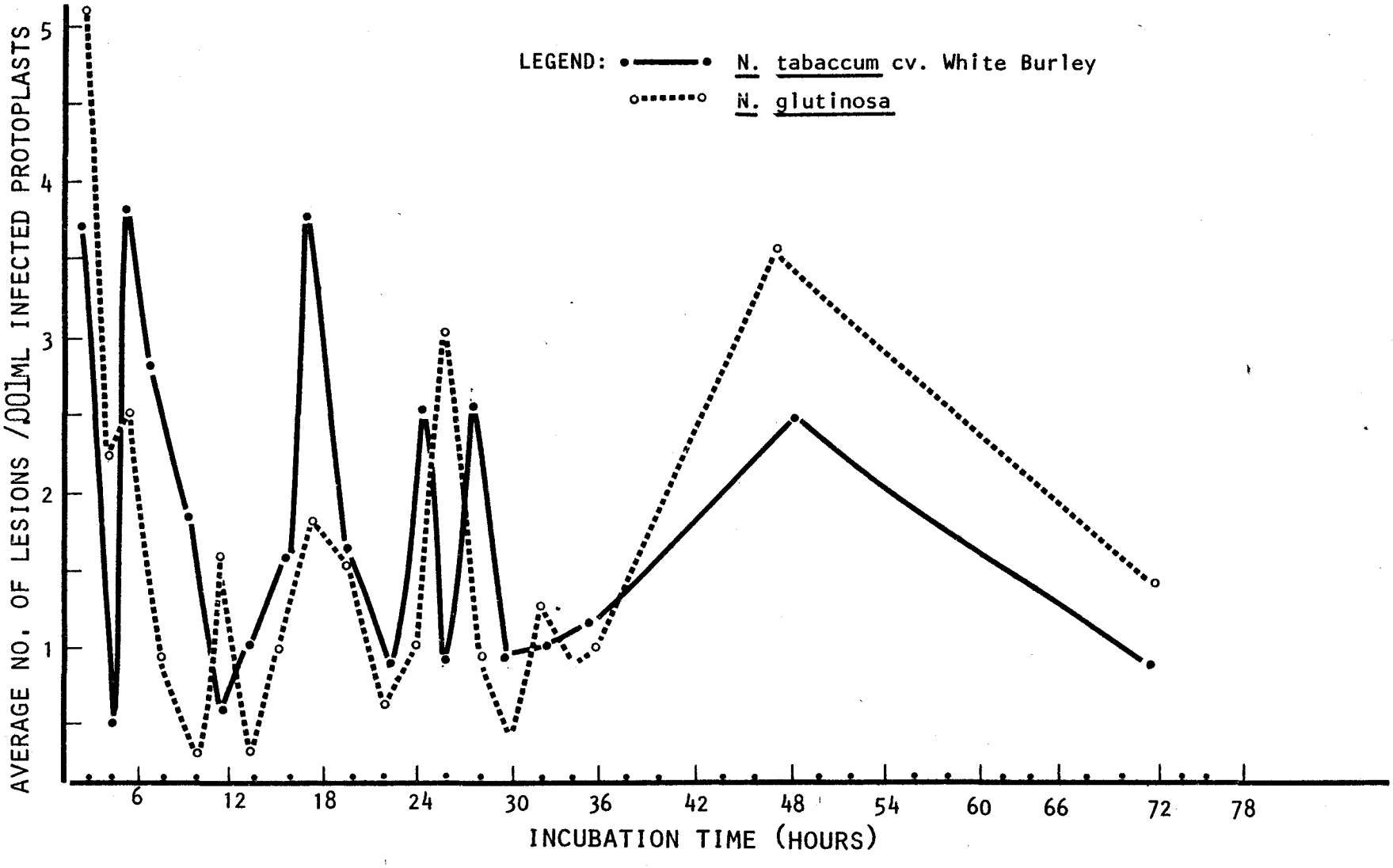

Fig: 25 Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa, tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto

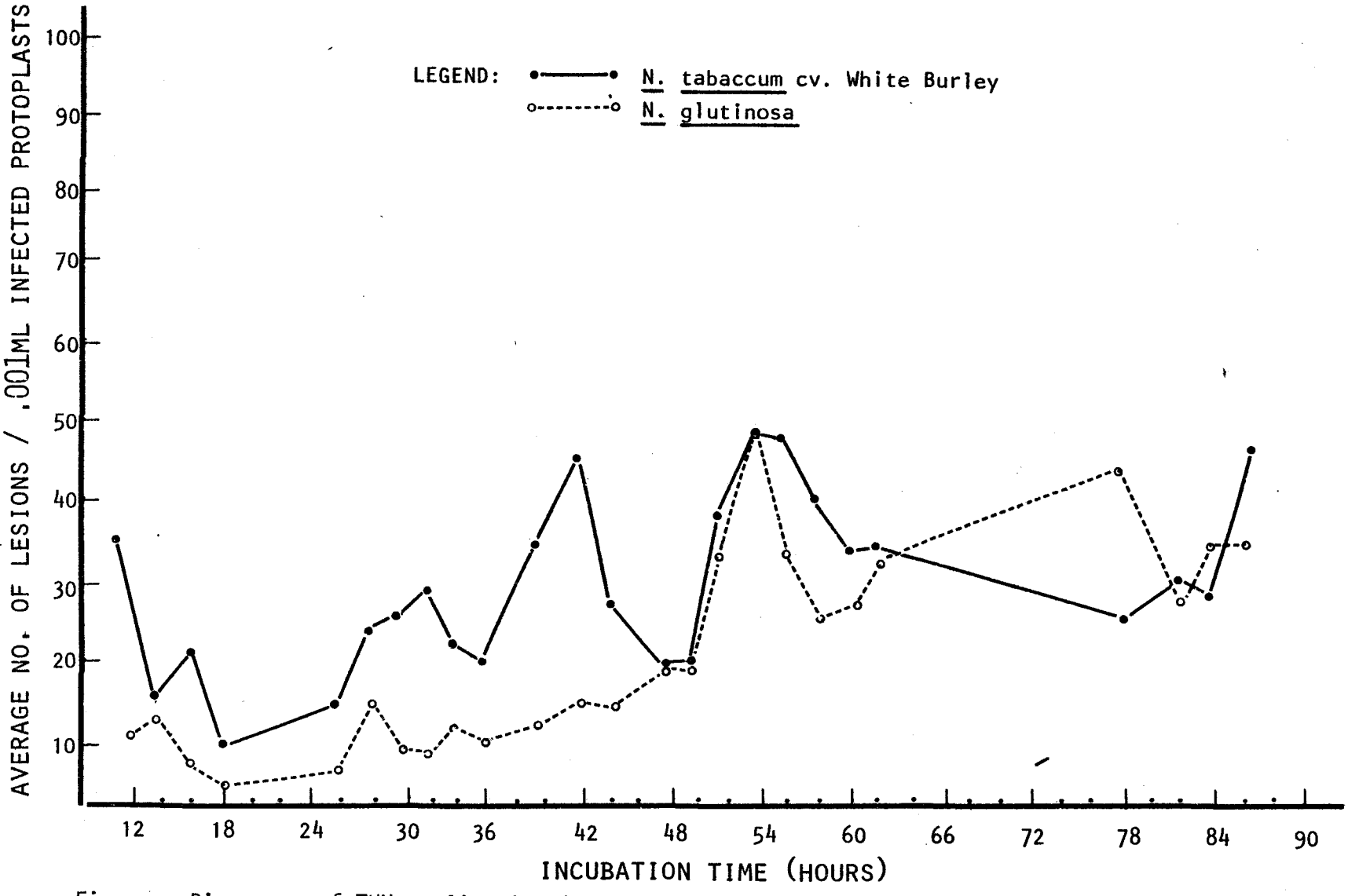

Fig: 26 Bio-assay of TMV replication in protoplasts of N. tabaccum cv. White Burley and N. glutinosa, tested on 10 day old primary half leaves of Phaseolus vulgaris cv. Pinto

SUMMARY AND CONCLUSIONS

This research is divided into two parts, isolation of protoplasts from two Nicotiana species and virus replication in isolated protoplasts.

Objectives

The main objective of this phase of research was to isolate mesophyll protoplasts from leaf tissue of N. tabaccum cv. White Burley and N. glutinosa in large quantities.

Requirements

The optimum age and length of tobacco leaves was determined by four preliminary experiments. 60 to 90 day old leaves of 25 to 30 cm. in length for White Burley and 15 cm. in length for glutinosa were found to be satisfactory.

Morphology

Mesophyll protoplasts of White Burley and glutinosa upon isolation were similar in color and shape, except that glutinosa protoplasts were deeper green and had larger vacuoles than White Burley. Numbers of protoplasts reported by other investigators did not differ considerably from

numbers that were obtained, indicating successful isolations of viable protoplasts from both species.

Yield

Tobacco plants were continually grown from September to January, and protoplasts were isolated after a 60 to 90 day growing period from November to March. Protoplasts isolated in November and December were less in number than those isolated later in the year January, Feburary and March. It was found that White Burley protoplasts were significantly more abundant than glutinosa protoplasts in January and March. Environmental conditions under which tobacco plants are grown play an essential role in the production of viable protoplasts.

Caution must be taken in interpreting the results obtained for protoplast yield, because a accurate measurement of the number of leaf cells initially present for isolation is difficult. Until as accurate means of measuring the numbers of cells is devised, their numbers can only be estimated.

Virus Replication in Protoplasts

Objective:

The main objective of this phase of experimentation was to determine the efficiency of virus infection and viral replication in isolated protoplasts of N. tabaccum

cv. White Burley and N. glutinosa. The morphology of both species was also examined

Removal of inoculum:

Six washings appear adequate for the removal of excess inoculum, in protoplasts infected with TMV. White Burley adsords less virus particles than glutinosa, as measured from particles present in early washings.

Early events:

TMV replication occured in both species of tobacco. A large inoculum obtained from 1 hour sampling indicated adsorbtion of virus particles in both species. An eclips phase was indicated by a low inoculum for 6 hours after infection. This finding confirms results obtained by other investigators using bio-assays. The process of viral replication in protoplasts during early synthesis needs further clarification to determine how quickly virus is adsorbed. Additional research should also examine RNA synthesis as well as recombination with protein coat.

Late events:

N. tabaccum cv. White Burley consistently produced a significantly greater number of virus particles than N. glutinosa. A loss of infectivity is detected in samples from 12 to 27 hours in both species of tobacco. N. glutinosa protoplasts showed no necrosis 84 hours after infection

with TMV. Infected protoplasts in both species showed no sign of deterioration but there was a limit to the amount of virus synthesized. Lastly, TMV replication diminishes for both species 33 hours after infection, indicating termination of virus synthesis and the ease with which TMV is produced in protoplasts.

Concluding remarks:

Protoplasts are useful in studying the mechanism of plant virus replication, hybredization of plants through protoplast fusion and the cultivation of virus resistant mutants. Protoplasts may be useful in the investigation of other aspects of plant virology, including gene activation of specific functions and the factors responsible for the local lesion response. Additional experiments can be conducted to improve existing techniques, to examine the effects of specific virus on various species of plants and to assess the factors limiting virus replication in cells.

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APPENDIX: A

CHEMICAL COMPOUNDS USED FOR MAINTAINANCE AND GROWTH OF ISOLATED PALISADE MESOPHYLL PROTOPLASTS.

MAJOR COMPOUNDS	MG/L	MINOR COMPOUNDS	MG/L
NH_4NO_3	825.000	H_3BO_3	6.200
KNO_3	950.000	$MnSO_4 \cdot 4H_2O$	22.300
$CaCl_2 \cdot 2H_2O$	220.000	$ZnSO_4 \cdot 4H_2O$	8.600
$MgSO_4.7H_2O$	1233.000	KI	.830
KH_2PO_4	680.000	$Na_2MO_4 \cdot 2H_2O$	.250
Na_2-EDTA	37.300	$CoSO_4 \cdot 7H_2O$	.030
$FeSO_4 \cdot 7H_2O$	27.800	$CuSO_4 \cdot 5H_2O$	.025
pH is adjusted with KOH to 5.8 before autoclaving			

ORGANIC COMPOUNDS	MG/L		
NAA	.06	Myo-inositol	100.00
Kinetin	.10	Glycine	2.00
Thiamine-HCl	10.00	Sucrose	15,000.00
Nicotinic acid	5.00	D-Mannitol	97,500.00
Pyridoxine-HCl	10.00		

APPENDIX: B

MACERATION MEDIUM
.5% MACEROZYME R-10
.8M D-MANNITOL
.35 POTASSIUM DEXTRAN SULFATE
pH adjusted to 5.7 w/ 2N HCl

CELLULASE MEDIUM
.3% CELLULASE
.8M D-MANNITOL
pH adjusted to 5.4 w/ 2N KOH

INFECTION MEDIUM
TOBACCO MOSAIC VIRUS
.02M POTASSIUM CITRATE BUFFER pH 5.2
.8M D-MANNITOL
2 ug/ml POLY L-ORNITHINE

APPENDIX: C

Computational procedures for an unweighted means analysis for unequal numbers of observations will be described. Suppose that the levels of a factor A represent two species of plants, White Burley and glutinosa, and levels of factor B represent five periods during which protoplasts were isolated, December to March. The number of protoplasts counted for each sample for each time period are arranged in the table below.

	November	December	January	February
W.B.	8,6,4,10,	23,14,5,10,	30,5,26,29,	17,87,91,80,63,
	10,7,	31,15,	48,27,	54,75,58,85,

	March
W.B.	47,35,47,51,
	77,88,67,44,

	November	December	January	February
glu.	6,8,18,18,	35,21,15,17,	57,77,55,60,	90,72,75,96,
	12,10,	31,18,	49,44,	87,80,126,60,

	March
glu.	68,114,76,65,
	87,114,114,102,

Next, observations for each cell in the table were counted (n), and scores within each cell are summed (ΣX), squared and summed (ΣX^2) and the Sum of the Squares is calculated ($SS = \Sigma X^2 - \frac{(\Sigma X)^2}{n}$) as shown below.

n					
	W.B.	6	6	6	9	8
	glu.	6	6	6	8	8

Appendix: C (Continued)

ΣX	W.B.	45	98	165	610	456
	glu.	72	137	342	686	740
ΣX^2	W.B.	365	2,036	5,475	45,638	28,342
	glu.	992	3,465	20,140	61,570	71,586

SS W.B. $365-\frac{(45)^2}{6}$ $2,036-\frac{(98)^2}{6}$ $5,475-\frac{(165)^2}{6}$

SS W.B. $45,638-\frac{(610)^2}{9}$ $28,342-\frac{(456)^2}{8}$

SS glu. $992-\frac{(72)^2}{6}$ $3,465-\frac{(137)^2}{6}$ $20,140-\frac{(342)^2}{6}$

SS glu. $61,570-\frac{(686)^2}{8}$ $71,586-\frac{(740)^2}{8}$

Thirdly, the harmonic means of the cell frequencies is computed ($\bar{n}_h$).

$$\bar{n}_h = \frac{\text{levels of factor A} \cdot \text{levels of factor B}}{1/n_1 + 1/n_2 \ldots\ldots\ldots\ldots\ldots\ldots 1/n_{10}}$$

Fourthly, the variance within samples which constitutes error variance, is calculated by summing all the sums of squares in this case totaling 15,036.22.

Next, the mean of the respective cells are calculated as summarized below. All the following calculations are carried out on the row and column totals of these means.

	Nov.	Dec.	Jan.	Feb.	Mar.	Totals
W.B.	7.50	16.33	27.50	67.78	57.00	176.11
glu.	12.00	22.83	57.00	85.75	92.50	270.08
Totals	19.50	39.16	84.50	153.53	149.50	446.19

Appendix: C (Continued)

Compute:

1. $\frac{(\text{Grand Total})^2}{\text{levels of A x levels of B}} = \frac{(446.19)^2}{(2)(5)} = 19{,}908.55$

2. $\frac{\text{Sum of the means for W.B. squared + Sum of the mean for g squared}}{\text{levels of B}}$

$= \frac{(176.11)^2 + (270.08)^2}{5} = 20791.59$

3. $\frac{\text{Sum of means for Nov. squared + Sum of means for Dec. squared +.....+ Sum of means for Mar.sq.}}{\text{levels of A}}$

$= \frac{(19.50)^2+(39.16)^2+(84.50)^2+(153.53)^2+(149.50)^2}{2} = 27{,}487.86$

4. Each cell mean squared and summed $= 7.50^2+16.33^2+\ldots\ldots\ldots\ldots +92.50^2 = 28{,}745.82$

Than calculate the sum of squares using these four quantities.

SS for plants $= \bar{n}_h(\#2 - \#1) = 6.71\ (20{,}791.59-19{,}908.55)$

$= 5925.20$

SS for time $= \bar{n}_h(\#3 -\#1) = 6.71\ (27{,}487.86-19{,}908.55)$

$= 50{,}857.17$

SS for interaction $= \bar{n}_h\ (\#4-\#2-\#3+\#1) = 6.71\ (28{,}745.82-$

$20{,}791.59 - 27{,}487.86 + 19{,}908.55) = 141{,}465.89$

Next, determine the degrees of freedom, df, associated with each effect. So that, df for plants = number of species of plants - 1 = 2-1=1

df for time = number of time periods - 1 = 5-1=4

Appendix: C (Continued)

df for interaction of plants times time = (df for plants) X (df for time) = (1) X (4) = 4

df for within group variation (error)= total number of individual observations - (levels of factor A)X(levels of factor B)= 69-10=59

In order to determine the mean square value for each effect, divide each sum of squares value by its own df. Lastly, divide mean square values (MS) by the MS value for within group variation to determine F ratio values. Calculations are summarized in the table below.

SOURCE	SS	df	MS	F	
A Plants	5,925.20	1	5,925.20	23.25	
B Time	50,557.17	4	12,714.29	49.89	
A X B	141,465.89	4	35,366.47	138.77	(interaction)
Within	15.036.22	59	254.85		(error)

Using an F distribution statistics table, look up the degrees of freedom associated with the effect being considered and the degrees of freedom for within variation, and determine the critical F value. In this case, for the effect due to plants, the df are 1, 59 and the critical F value is 7.08. Since the observed F value for plants, 23.25 exceeds the critical F value, the experimenter may conclude that there is a significant difference between plants for number of protoplasts produced. This effect is significant at p .01, determined from the tabled value, which means

Appendix: C (Continued)

that the experimenter would expect to get such a result only 1 out of 100 times by chance alone. The experimenter concludes that White Burley produces a significantly greater number of protoplasts than glutinosa. The same procedure is carried out on the F value for time and the F value for interaction. Readers who are interested in additional details of the procedure should refer to Winer (1971).

APPENDIX: D

T-Test for Independent Sample Means

The t statistic is used to test differences between two sample means to determine if one is significantly larger or smaller (two-tailed test) than the other. In the present investigation, these tests were conducted after the overall analysis of variance values were found to be significant in order to determine the locus of the effect. An example follows below.

	White Burley January	glutinosa January
# of protoplasts		
	30	57
	5	77
	26	55
	29	60
	48	49
	27	44
N=	6	6
Mean	27.50	57.00
Variance	187.42	129.28

$$t = \frac{\text{Mean of group 1(W.B.)} - \text{Mean of group 2(glu)}}{\sqrt{(1/N_1 + 1/N_2) \times \left[\frac{(N \text{ group } 1-1) \times (\text{var. group1}) + (N\text{group } 2-1) \times (\text{var. group } 2)}{N \text{ for group } 1 + N \text{ for group } 2-2}\right]}}$$

$$= \frac{27.50 - 57.00}{\sqrt{(2/6 \; \frac{5(187.42) + 5(129.28)}{10}}} = -4.06$$

Appendix: D (Continued)

Next, determine the df, which are equal to $N_1 + N_2 - 2 = 10$. Check the table for the critical t value = 3.17. Since the calculated value exceeds the tabled value, you conclude there is a significant difference between the means of the plants during January.

APPENDIX: E

KLECZKOWSKI TRANSFORMATION

"When the mean values of X are greater than 10, the transformation $Z = \log_{10} (x + c)$ (where c is constant. 5 to 15), is satisfactory but inapplicable with smaller numbers. In some work the use of poorly infective inocula is unavoidable, and to allow statistical analysis of results in such work a transformation $Z = \log_{10} \frac{1}{2} (x + c + \sqrt{x^2 + 2cx}$ is used, when mean values are less than 10."

Z = individual scores transformed by either of the two formula dependent upon the mean score.

Example:

x = 1,1,3,5 $\Sigma x = 2.5$ mean 10

Z = .67, .67, .85, .97 $\Sigma Z = 2.4$

x = 10, 15, 25, 40 $\Sigma x = 90$ mean 10

Z = 1.17, 1.30, 1.48, 1.65 $\Sigma Z = 5.6$

APPROVAL SHEET

The thesis submitted by Allan Louis Campione has been read and approved by the following committee:

Dr. Amrik S. Dhaliwal, Director
Professor, Biology, Loyola University

Dr. William C. Cordes
Assistant Professor, Biology Loyola University

Dr. Mark Goldie
Assistant Professor, Biology Loyola University

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the committee with reference to content and form.

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

12/12/1978
Date

A. S. Dhaliwal
Director's Signature