SYMPTOM EXPRESSION AND VIRUS MULTIPLICATION IN RHABDOVIRUS-INFECTED PLANTS

BY

FATHI ALI ASKEER

A thesis submitted for the degree of PhD

University of Glasgow
Department of Botany

September 1993

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 – 1346

DEDICATION

I would like to record my sincere gratitude to my parents, my wife, Naima and to my children, Fouad, Fadel and Assiah, to all my family, for their continued patience, encouragement and good spirit as always and specially during the period of my research. To them this work is dedicated.

TABLE OF CONTENTS

CHAPTER THREE

Symptom expression and virus multiplication in *Nicotiana* species

CHAPTER FOUR

The effect of SYNV on photosynthesis rates.

ACKNOWLEDGEMENTS

I gratefully like to convey my sincere thanks and appreciation to my supervisor Dr. J. J. Milner for his excellent guidance, unending and invaluable support and encouragement throughout the period of study and especially at the time when I was experiencing some technical and English language difficulties. I am deeply grateful, in particular to Dr. P. J. Dominy for his fruitful discussion and helpful advice during the use of oxygen-electrode. I am also very grateful, firstly to E. Robertson for helpful advice about the preparation of samples for, and the use of the electron microscope and to Dr. I. D. Hamilton (Department of Biochemistry, University of Glasgow) for the preparation of anti-EMDV antiserum.

Further sincere thanks are extended to Mr. Tait for all his help in the photography during the research and for help in the preparation of the final copies of the photographs, Dr. G. Adam (D. S. M. Braunschweig, Germany) for providing EMDV (DSM 0031 isolate) and to staff members at Garscube for their help in maintaining plants in the greenhouse. My warm thanks again are due to Drs. J. J. Milner and P. J. Dominy for critical reading of various parts of this thesis. Many people, at various stages of my research, gave assistance, helpful advice, to them I am very grateful.

I wish also to acknowledge the Libyan People Government, Ministry of Education for their scholarship which made this study possible.

Final thanks go to my parents and my family to whom I am eternally indebted for all their support and encouragement throughout the years.

DECLARATION

I hereby declare that the whole of work now submitted in this thesis is the result of my own investigation except where reference is made to published literature and where assistance is acknowledged.

No part of this thesis has been previously presented for any degree.

SUMMARY

Two plant rhabdoviruses Sonchus yellow net virus (SYNV) and eggplant mottled dwarf virus (EMDV) have been investigated in three hosts *N. glutinosa, N. clevelandii* and *N. edwardsonii*, a hybrid of the previous two. Particles of both viruses accumulated in the perinuclear space of infected cells. The symptoms of the two viruses were characterised by vein-clearing and yellowing in the case of SYNV, appearing after 2-3 weeks post-inoculation and in the case of EMDV, vein-clearing, yellowing and mottling of the leaves appearing after 3-4 weeks post-inoculation. Vein-clearing and yellowing symptoms of SYNV, eventually disappeared after 6 weeks post-inoculation and yellow mottled spots appeared in new leaves of all three hosts. These were accompanied by reduction in the numbers of virus particles observed in infected cells. In contrast, EMDV-infected plants continued to show severe symptoms. The severity of SYNV and EMDV symptoms depended to a great extent on the age of the plants at the time of inoculation. EMDV symptoms were much more severe than the symptoms of SYNV in three hosts studied. In this study, the appearance of the symptoms depended not only on the type of the virus but differed between two closely related hosts.

Using slight modification of technique originally developed for purifying SYNV, pure infection preparations of EMDV, essentially free of host contaminants were made. Yields of EMDV on a μg/gram fresh weight of leaves basis was two to three times that of SYNV. Purified EMDV was used for the preparation of antisera.

Structural polypeptides of SYNV and EMDV were separated by polyacrylamide-gel electrophoresis. Both viruses showed four bands corresponding to the four major structural proteins. Both SYNV and EMDV virions contain what were originally believed to be two membrane matrix (M) proteins (M1 and M2) in addition to the glycosylated (G) protein and the nucleocapsid (N) protein.

Levels of virus antigen in various hosts were estimated using ELISA. The levels of SYNV and EMDV in infected tissues initially increased, reached a maximum, and then declined. The levels of SYNV virus antigen in *N. glutinosa* and *N. edwardsonii*, rose between 5-15 days after inoculation. During this period, the virus appeared to multiply and spread rapidly, as indicated by the concentration of virus antigen. This time period coincided with the increase in symptom severity and the appearance of vein-clearing in all unexpanded leaves in both hosts. During this phase of rapid synthesis and spread, large areas of viroplasm, containing scattered patches of granular matrix were observed within infected cells. These areas of viroplasm appear to be involved in the synthesis, replication or assembly of nucleocapsids and labelled strongly in immunogold labelling of thin sections. Following the maximum of virus concentration 15 days after inoculation, the levels of virus antigen declined in unexpanded leaves in both hosts, accompanied by a reduction in numbers of virus particles observed in infected cells by electron microscopy.

After 30 days post-inoculation, the virus levels in infected *N. edwardsonii* and *N. glutinosa* reached a plateau and declined only very slowly thereafter. During this stage, the decline in virus concentration was accompanied by the appearance of yellow mottled spots in unexpanded leaves in *N. glutinosa* and a few *N. edwardsonii* plants whereas most *N. edwardsonii* plants started to show signs of recovery. *N. glutinosa* plants appeared to be the more susceptible host to SYNV infection. Not only did they replicate and accumulate to greater levels but the symptoms, specially stunting, were more severe than in *N. edwardsonii*. Although virus antigen levels rose in a comparable manner in both hosts over the period 5-15 days, antigen levels were consistently two to three fold greater in *N. glutinosa*.

The concentration of EMDV antigen in *N. edwardsonii* was much higher than SYNV and symptoms were much more severe. The concentration of virus antigen rose during the first two weeks and reached a maximum after 3 weeks at which time the infected plants showed severe symptoms. The maximum levels of virus concentration correlated with the appearance of large numbers of virions in almost all cells of infected leaves. After 4 weeks, the virus antigen levels dropped sharply reaching a plateau and continuing up to 24 weeks. However, the levels of EMDV antigen were always greater than levels of SYNV in the same host.

The effect of SYNV and EMDV in the cytology of plant-infected cells have been studied by electron microscopy, following the infection for a period of time after inoculation. The most dramatic changes induced by the two viruses were observed in the nuclei. SYNV and EMDV particles accumulated in the perinuclear space causing swelling and large areas of viroplasm developed. These changes could be observed in the nuclei after 2 weeks post-inoculation in SYNV-infected cells and persisted for the rest plant life. The effect on the nuclei became less in the late stage of infection (after 20 weeks) in infected *N. glutinosa* and far less in infected *N. edwardsonii*. After 20 weeks post-inoculation, nuclei from SYNV-infected cells showed vesicles in the perinuclear space and in the nuclei containing virus-like particles but with an abnormal morphology. These reacted very strongly to the immunogold labelling, using antibodies to total SYNV protein and to the G protein. In contrast, in infected *N. glutinosa*, the vesicles were often filled with a mixture of mature virus particles and abnormal virus particles. The nuclei of EMDV-infected cells showed large numbers of virus particles in the perinuclear space which persisted for the period of infection.

Other abnormalities in the nuclei of SYNV-infected cells included alterations of chromatin distribution and cytoplasmic invaginations into the nuclei. Similar abnormalities were observed in nuclei of EMDV-infected cells. Chloroplasts from infected cells showed reduction in thylakoids and the development of large areas of starch.

Immunogold labelling of EMDV using antiserum to total EMDV 5 and 23 weeks post-inoculation showed similar reaction to the antigen in spite the differences in times, and the gold particles reacted only to the nuclei.

The levels of individual SYNV proteins in infected *N. glutinosa* at different times during the infection period were estimated using western blots. The levels of the major structural proteins reach a maximum at 15 days post-inoculation and then decline gradually with increasing time after inoculation. In contrast, to the situation reported for SYNV in *N. edwardsonii*, G, N and M1 proteins continued to be detectable up to 60 days post-inoculation. Similar experiments were carried out with EMDV. The levels of EMDV proteins detectable on western blots rose and fell as anticipated from the ELISA results. The individual virus proteins reacted to varying degrees. G and N gave strong bands whereas M2 reacted poorly and M1 was almost undetectable, except in lanes containing purified virus. The levels of G, N and M2 relative to each other did not obviously differ in samples taken early and late in infection.

In SYNV-infected *N. edwardsonii* and *N. clevelandii* plants short particles which have the characteristics of defective interfering (DI) particles are observed. DI particles were produced in the late stages of infection (over 6 weeks after inoculation) and their numbers increased with age of infected plants. However, in infected *N. glutinosa*, the appearance of short particles were very rare compared to the other two hosts. Short bacilliform particles were also observed in plants infected with EMDV showing chlorotic lesions after 7 weeks post-inoculation.

The effect of SYNV on photosynthesis rates in *N. edwardsonii* and *N. glutinosa* plants was determined at different times after inoculation using an oxygen-electrode. Two models were used to estimate the photosynthetic parameters. SYNV infection had a significant effect in the chlorophyll levels in both hosts. The results showed that the virus had no effect on the net photosynthesis rates of infected *N. edwardsonii* but showed signiificant effect on the dark respiration rates which increased. In contrast, in *N. glutinosa* infection significantly reduced net photosynthesis rates but not dark respiration rates. Other parameters (quantum efficiency of photosynthesis at low light intensity, and the ratio of physical to total resistance of CO_2 diffusion) were unaffected by infection in both hosts.

Chapter One

General Introduction

INTRODUCTION

1.1- Definition:

The meaning of the word virus has changed considerably during the last century. In old time the word virus meant poison. Since the beginning of this century the modern concept of the word virus and its study virology, has taken on a more specific meaning to denote a group of extremely small, obligately parasitic, pathogenic agents.

In 1950, a virus was described by Bawden as "an obligately parasitic pathogen with dimensions of less than 200 nm" but this and other early definitions (Lwoff, 1957; Pirie, 1962) were based on the small size of particles, pathogenicity, possession of nucleic acid and an inability to multiply outside a living cell. As knowledge of viruses and associated disease agents increased, it became clear that those definitions were not entirely satisfactory. They failed to distinguish between viruses and other disease agents, such as mycoplasma and rickettsia, and excluded large animal viruses such as the pox virus.

Recently, Matthews (1991) has more specifically defined a virus as " A set of one or more nucleic acid template molecules, normally encased in a protective coat, or coats of protein or lipoprotein, which is able to organise its own replication only within suitable host cells. Within such cells virus production is (a) dependent on the host's protein synthesising machinery. (b) organised from pools of the required materials rather than binary fission and (c) located at sites which are not separated from the host cell contents by a lipoprotein, bilayer membrane.". Such a definition clearly distinguishes viruses from other plant disease agents such as viroids, mycoplasma and rickettsia.

1.2- The development of plant virology as a science:

During the seventeenth century in Holland, colour variegation or striping of tulip petals in plants infected with tulip mosaic virus was much prized by Dutch tulip growers. Although the cause of the tulip petal symptoms was unknown at the time, some growers knew the condition could be grafted to a normal flowered bulb. It was not until 1926 that tulip " breaking" was associated with a virus and shown to be transmitted by infected sap or by aphids (McKay and Warner, 1933).

In 1886 Mayer, an agricultural chemist working in Holland, found that the tobacco mosaic disease could be transmitted to healthy tobacco plants in juice extracted taken from infected plants. A few years later, Ivanowski (1892) worked on two diseases in the same plant and described one as pox disease, and the other as a mosaic disease similar to the one reported by Mayer. He confirmed Mayer's report that the mosaic disease could be sap transmitted and showed that the sap was still infectious after it has been passed through a Chamberland filter, which was known to retain bacteria. Mayer suggested that the mosaic disease might be caused by a toxin produced by a bacterium, or a new small bacterium which led Beijerinck (1898) to demonstrate that the causal agent could diffuse through agar and concluded that the disease was not caused by a microbe, but by a pathogenic agent "contagium vivum fluidium". He thought that the contagium could reproduce itself in the living plant and used the word virus to describe it. For further details on the development of plant virology see (Walkey, 1985).

1.3- Plant virus classification:

The requirement for a sound classification system for plant viruses, based on well defined characteristics, became essential during the 1950's and 60's as more and more information on individual plant viruses was accumulated. This requirement was met by the appointment at the International Congress of Microbiology held in Moscow in 1966, of the committee to investigate virus

taxonomy. The committee later became known as the International Committee on Taxonomy of Viruses (ICTV). As the result of the work of this committee and its plant virus sub-committee, a system for plant virus classification was introduced, based on such characteristics as virus particle morphology, type and quantity of nucleic acid, genome structure and type of vector. This classification system has now become widely accepted by most plant virologists. The characteristics of plant virus groups which have received international approval by the ICTV were documented and have been described in more detail by Matthews (1979 and 1982).

At the present time, plant viruses are classified into twenty-seven groups of which three are referred to as families. Unlike the animal virus groups, the plant virus groups are not normally referred to as families, but three families, the rhabdoviridae, the reoviridae and the bunyaviridae, include viruses that can infected plants, arthropods and vertebrates. Of the remaining twenty-five groups, nineteen have been given ICTV approved names and the other six groups are at present known by the name of the type member of the group. In this classification scheme, the viruses have been grouped according to their known physical, chemical and biological characters. Frequently only a few essential characters need to be known in order to identify a virus as belonging to a particular group. In many cases the most characteristic feature of a virus is the nature of its genome. The characters of the virus genome, if RNA or DNA, and if it is double stranded (ds) or single stranded (ss), together with the distinct morphologies of the virus particles, immediately identifies members of these groups. For more details in this subject see Matthews, (1979,1982), Kurstak, (1981).

1.4- The family rhabdoviridae:

The occurrence of rhabdoviruses in vertebrates, invertebrates and in plant hosts makes them one of the largest and most important families in virology.

The term "rhabdovirus" was first suggested by Melnick and Coombs in 1966 and the International Committee on nomenclature of viruses recommended its adoption in 1970 (Wildy, 1971). The name rhabdovirus is derived from the Greek "rhabdos" meaning rod, but in fact the virus has a bullet shape or bacilliform morphology.

The first member of this family studied morphologically was vesicular stomatitis virus (VSV) in 1950s. The distinctive bullet-shaped morphology was also found for rabies virus (Almeida *et. al.*, 1962), and since that time, more than 100 viruses have been classified as members or possible members of the family on the basis of their morphology (Brown *et. al.*, 1979). The consequence of reliance on a distinctive morphology for classification, is that many potential members have not been studied and characterised in a satisfactory way. Wagner (1975) has defined six important characteristics of rhabdoviruses;

1- Rhabdoviruses have rod-shaped particles which vary considerably in length (60-400 nm) but are of fairly uniform width (60-85 nm).

2- Animal rhabdoviruses tend to be bullet-shaped in appearance, flat at one end and a tapered sphere at the other. Plant rhabdoviruses are usually bacilliform in shape, quite elongated and with two round ends.

3- All rhabdoviruses appear to be surrounded by a membrane envelope with protruding spikes. All rhabdoviruses contain lipids and are, therefore susceptible to disruption by ether and detergent.

4- The nucleocapsid inside the envelope of rhabdoviruses is a ribonucleoprotein (RNP) core which gives the appearance of striation when viewed by electron microscopy. All rhabdoviruses examined contain one molecule of single stranded RNA which is not by itself infectious and does not serve as a messenger. Therefore, rhabdoviruses are generally classified along with the myxoviruses, paramyxoviruses and bunyaviruses as negative-strand viruses.

5- Many, if not all, rhabdoviruses contain an RNA-dependent-RNA

polymerase (transcriptase) as part of the nucleocapsid, which renders it infectious in the absence of the envelope.

6- A common characteristic of animal rhabdoviruses, conceivably also of plant rhabdoviruses, is the frequent occurrence of defective interfering particles (DI) which are non infectious because a considerable segment (one third to two-thirds) of the RNA genome is deleted.

The most detailed work has been done with vertebrate viruses and two major genera in the family have been defined, the Vesiculovirus genus and the Lyssavirus genus. The Vesiculovirus genus contains three groups; (1) The vesicular stomatitis viruses, related to the Indiana serotype.(2) The vesicular stomatitis viruses, related to the New Jersey serotype. (3) The vesicular stomatitis viruses, related to the Piry Chandipura, and Isfahan serotypes. The viruses in group (1) were demonstrated very clearly related to each other (Cartwright and Brown, 1972). The Lyssavirus genus also contains three serogroups of which the most important is that containing rabies, Mokola, Lagos bat, and Duvenhage viruses (Frazier and Shope, 1979). Although all the members of the group are clearly closely related, they are sufficiently different in cross-neutralisation and cross-protection tests to be considered as separate species (Brown, 1987).

Rhabdoviruses can also be divided into two groups on the basis of their host range. These that infect animals and those that infect plants. The two groups share many morphological, physical and biochemical properties (Hummeter, 1971; Francki, 1973; Knudson, 1973; Wagner, 1975; Francki and Randles, 1980; Francki, *et. al.*, 1981).

Although most animal rhabdoviruses replicate and assemble in the cytoplasm (Wagner, 1975), plant rhabdoviruses differ markedly in their morphogenesis and site of accumulation. The characteristic rhabdovirus structure is easily distinguished from normal cellular components by electron microscopy.

This technique has permitted several detailed ultrastructural studies of the cytopathology of infected animal and plant cells. The infectious units of all animal rhabdoviruses appear to be by electron microscopy structurally similar to that of the VSV (Howaston, 1970; Wagner, 1975). The infectious standard virion of VSV is bullet-shaped, rounded at one end and flat at the other and they measure about 180 nm in length and 65 nm in width. The bullet-shaped particles are similar to those seen in preparations of most vertebrate rhabdoviruses. The structure of the VSV virion, when disrupted by detergent, shows two major components. The envelope, when disrupted by detergents results in release of the nucleocapsid core, which contains single-stranded RNA tightly encased by the major nucleocapsid protein designated N (Gallione, *et. al.*, 1981). The infectious nucleocapsid also contains two other minor proteins. The larger of the two polymerase proteins is designated L (M.W. 241,012); the other polypeptide required for polymerase activity has been designated NS. The envelope itself or limiting membrane is associated with two proteins, the integral, externally oriented glycoprotein G (spikes protein) and the peripheral matrix (M) protein, which lines the inner surface of the membrane in close association with the nucleocapsid core (Patzer, *et. al.*, 1979; Zakowski and Wagner, 1980). The G protein is the major antigenic determinant responsible for type specificity and gives rise to neutralising antibody (Kelley, *et. al.*, 1972; Volk, *et. al.*, 1982). The approximate composition of VSV, which is probably like that of all other rhabdoviruses, is 74% protein, 20% lipids, 3% carbohydrate, and 3% RNA (McSharry and Wagner, 1971; Wagner, 1975).

The lyssavirus genus which includes rabies virus as a type member, shares the same structure with VSV except that no NS is present. Rabies was originally believed to contain two perpherial matrix proteins M1 and M2. However, M1 is now believed to correspond to NS in VSV while M2 corresponds to M in VSV (Cox. *et. al.*, 1981).

The various conditions to which animal or plant rhabdoviruses are

exposed during purification and preparation for electron microscopy could have some effect on their morphology under electron microscope. Orenstein *et. al.*, (1976) found that the bullet-shape of VSV is an artefact of fixation and staining and the infectious particles are bacilliform with two rounded end. However, the internal nucleocapsid is itself bullet-shaped. Preparative artefacts almost certainly occur also with all plant rhabdoviruses. However, some plant viruses may fail to retain the original structure (e.g. potato yellow dwarf virus (PYDV); Macleod, 1968) more than others (e.g. lettuce necrotic yellow virus; LNYV) (Wolanski, *et al.* 1976; Wolanski and Francki, 1969). Nevertheless, the plant rhabdovirus structure is that of a bacilliform particle comprising a viral envelope enclosing a long strand of nucleoprotein wound into a helix of low pitch (Francki and Randles, 1980). The most significant differences between plant and animal rhabdoviruses are in their size and sedimentation properties (Table 1.1).

Table 1.1: physical properties of plant and vertebrate rhabdoviruses.

Property	LNYV	SYNV	VSV	Rabies
Virions				
Dimension (nm)			ns	ns
In section	360x52	220x70	175x68	180x75
Sedimentation coefficient(s)	945	1045	625	600
Nucleocapsids				
sedimentation	260	250	140	200
Infectivity	Yes	Yes	Yes	No
Ribonucleic acid				
Sedimentation	43	44	36-45	45
Strandedness	Single	Single	Single	Single
Infectivity	No	No	No	No
Molecular weight ($x10^6$ daltons)	4.2	4.4	3.6-4.5	4.6

ns= Negative staining

Basic on this information and on studies on the animal and plant rhabdoviruses, plant rhabdoviruses can probably be divided into at least three groups depending on the site of nucleocapsid formation and assembly of the virions (Francki and Randles, 1980; Francki, *et. al.*, 1981). One group includes viruses such as sonchus yellow net virus (SYNV), potato yellow dwarf virus (PYDV), and eggplant mottled dwarf virus (EMDV) that mature in association with the inner nuclear membrane and accumulate in the perinuclear spaces. A second group of viruses, includes lettuce necrotic yellows virus (LNYV), broccoli necrotic yellows virus, and maize sterile streak virus, appear to mature in association with the endoplasmic reticulum and accumulate in the vesicles of the endoplasmic reticulum. A third group of viruses, represented by barley yellow striate mosaic virus (BYSMV) and northern cereal mosaic virus (NCMV), mature in association with membrane-bound granular structures called "viroplasm". After virus particles bud from membranes associated with the viroplasm, they accumulate in vacuole-like spaces. The latter two groups of viruses may be difficult to distinguish during routine electron microscopy.

1.5- The plant rhabdoviruses:

1.5.1- Members of the group, geographical distribution and host range:

There are thirty eight confirmed members of the plant rhabdoviruses and another 30 possible members listed by Peters (1981) in addition 43 members are listed by Jackson *et. al.*, (1987), (Table 1.2). None of the rhabdoviruses infecting plant has been assigned to either the Lyssavirus or to the Vesiculovirus genus (Matthews, 1979).

Plant rhabdoviruses are easily distinguished from members of other groups of plant viruses. Very little work has been done to trace possible relationships among them because of the scanty data available. The same virus might have been entered under different names and it is conceivable that some of the viruses might be sufficiently closely related to be considered as strains of the same virus. In many descriptions of rhabdoviruses, differences in reported particle dimensions are given as evidence that two viruses are distinct. However, because an individual measurement of rhabdovirus particles depends on fixation and staining procedures, Francki and Randles (1980) suggested that the morphology of two rhabdoviruses in negatively stained preparations can be taken as distinct only when significant differences are evident in a mixture of the two viruses.

A further division of the plant rhabdoviruses by Peters (1981) was based on the previous division into three groups, according to the cellular location of particle assembly, kinetics of transcriptase activity and protein composition of virus particles. According to the Peters division, the particles of viruses in subgroup I occur in the cytoplasm, contain matrix protein (M) and possess transcriptase activity that is readily detectable in *vitro*. In these properties, they resemble the vesiculovirus subgroup of animal rhabdoviruses and includes lettuce necrotic yellows virus from plant rhabdoviruses. Subgroup II viruses, accumulate in the perinuclear spaces, possess matrix proteins (M1 and M2) and

have low in *vitro* transcriptase activity. They share these properties with the lyssavirus and include potato yellow dwarf virus, SYNV and EMDV.

Most plant rhabdoviruses are transmitted by aphids or leafhoppers, but some are reported to be transmitted by other agents such as lace bugs and mites (Table 1.2). The virus and its vector relationship is highly specific, and in all cases that have been examined, rhabdoviruses have been shown to replicate in their vector (Francki, 1973; Harris, 1979; Jackson *et. al.*, 1981; Jackson *et. al.*, 1987).

Table 1.3: Members of the plant rhabdoviruses.

Name of virus member [$]	Size and shape	Site of accumulation*	Vector	Sap Trans.
Barley yellow striate mosaic (BYSMV)	45x330	Cyt	Au	No
Beet leaf curl (BLCV)	80x250	Nuc	Gy	No
Broccoli necrotic yellows (BNYV)	64x297	Cyt	Ap	Yes
Carrot latent (CLV)	70x220	Nuc	Ap	No
Cereal chlorotic mottle (CCMV)	65x230	Nuc	Au	No
Coffee ringspot (CRV)	65x183	Nuc	Au	Yes
Colocasia bobone disease (CBDV)	50x300/335	Nuc	Au	No
Cow-parsnip mosaic	90x265	Nuc	-	Yes
Cynara	75x260	Cyt	-	Yes
Digitaria striate (DSV)	55x280	Cyt	Au	No
Egg plant mottle dwarf (EMDV)	66x220	Nuc	-	Yes
Euonymus fasciation	-	Cyt	-	No
Festuca leaf streak	61x330	Cyt	-	No
Finger millet mosaic	80x285	Nuc	Au	No
Gomphrena (GV)	75x230	Nuc	-	Yes
Laburnum yellow vein	89x245	Nuc	-	-
Lettuce necrotic yellows (LNYV)	52x360	Cyt	Ap	Yes
Lucerne enation (LEV)	85x250	Nuc	Ap	No
Maize mosaic (MMV)	75x300	Nuc	Au	No
Melilorus latent (MLV)	80x300	Nuc	-	-
Northern cereal mosaic (NCMV)	60x300	Cyt	Au	No
Oat striate mosaic (OSMV)	75x210	Nuc	Au	No
Parsley latent (PLV)	87x214	Cyt	Ap	Yes

Pelargonium vein clearing	70x250	Nuc	-	Yes
Pisum	45x240	Cyt	-	Yes
Pittosporum vein yellowing (PVYV)	80x245	Nuc	-	Yes
Plantago lanceolata	63x330	-	-	-
Potato yellow dwarf (PYDV)	75x380	Nuc	Au	Yes
Raspberry vein chlorosis (RVCV)	65x430	Cyt	Ap	No
Rice transitory yellowing (RTYV)	93x325	Nuc	Au	No
Sonchus yellow net (SYNV)	94x248	Nuc	Ap	Yes
Sonchus (SV)	50x250	Cyt	-	Yes
Sowthistle yellow vein (SYVV)	95x200	Nuc	Ap	No
Strawberry crinkle (SCV)	69x190/380	Cyt	Ap	No
Wheat chlorotic streak mosaic (WCSMV)	55x355	Cyt	Au	No
Wheat rosette stunt (WRSV)	50x320	Cyt	Au	No
Wheat (American) Striate mosaic (WSMV)	75x250	Nuc/Cyt	Au	No
Winter wheat (Russian) mosaic (WWMV)	60x260	-	Au	No
Atropa belladonna	55x310	-	-	-
Cajanus cajan	-	-	-	-
Callistephus chinensis	-	-	-	-
Chanderilla juncea	58x135	Nuc	-	-
Chrysanthemum sp.	-	-	-	-
Clover enation	80x200	Nuc	-	-
Dendrobium sp.	-	-	-	-
Gebera sp.	70x300	-	-	-
Holcus lanatus	-	-	-	-
Iris grmanica	52x320	Cyt	-	-
Ivy vein-clearing	55x325	Cyt	-	-

Gebera sp.	70x300	-	-	-
Holcus lanatus	-	-	-	-
Iris grmanica	52x320	Cyt	-	-
Ivy vein-clearing	55x325	Cyt	-	-
Laelia red leafspot	80x280	-	-	-
Lemon scanted thyme	72x219	Nuc	-	-
Lorus streak	90x300/340	Cyt	-	-
Lupin yellow vein	89x250	-	-	-
Manihot esculenta	-	-	-	-
Melon variegation	60x320	Cyt	-	-
Pineapple chlorotic leaf streak	60x200	Nuc	-	-
Phalaenopsis sp.	-	-	-	-
Pogostemon patchouli	-	-	-	-
Ranunculus repens	-	-	-	-
Raphanus sp.	-	Cyt	-	-
Red clover mosaic (RCMV)	65x300	Nuc	-	-
Ryegrass bacilliform	68x287	Nuc	-	-
Saintpaulia leaf necrosis	60x200	-	-	-
Sambucus vein clearing	80x275	-	-	-
Sarracenia purpurea	-	-	-	-
Triticum aestivum	-	-	-	-
Vigna sinensis	-	-	-	-
Zea mays	50x325	Cyt	-	-

* = Virus accumulates predominantly in the prenuclear space (Nuc) or cytoplasm (Cyt). $= Peters (1981).

! = See also " Affinities with other groups" Ap = Aphids, Ac = Mite and tick, Au = Leafhopper, Gy = Mirid, Piesmid, or Tingid bug, Ve = None of the above.

1.5.2- The economic importance of plant rhabdoviruses:

Plant rhabdoviruses are capable of infecting many major crop plants and many instances of serious disease have been reported from most parts of the world including tropical, subtropical and temperate regions. Among 68 members of the group, known and suspected listed by Peters (1981), several are known to cause diseases of economic importance although these are usually distributed in limited geographical areas (Francki *et. al.*, 1981). Both monocotyledonous and dicotyledonous plants can act as hosts of rhabdoviruses but as far as its known, none of the viruses can infect members of both plant groups (Francki, 1973).

Lettuce necrotic yellows virus (LNYV), a plant rhabdovirus causes serious losses in lettuce crops in Australia (Stubbs and Grogan, 1963). Lettuce plants infected with LNYV show acute symptoms, becoming chlorotic, and assume a flattened appearance. Degrees of necrosis can occur, survivors usually enter a chronic condition, remaining stunted and fail to produce a marketable head (Stubbs and Grogan, 1963). LNYV is dependent on infection of sowthistle for its survival in nature and is transmitted in a persistent manner by two aphid species, (Boakye and Randles, 1974). PYDV was first found in 1917 in the north-eastern United states. The virus which has a wide host range can be transmitted by several leafhopper species, which colonises mostly on red clover which is a symptomless host of PYDV (Black, 1970). Perennial weeds provide the source for regular annual outbreaks of the disease. Serious crop losses occurred in the 1930s in dry years. Since that time, however, the incidence of the disease has decreased, and major losses have not occurred since the 1940s (Black, 1979). PYDV has recently been isolated from periwinkle in California (Falk *et. al.*, 1981). However, its presence does not cause a serious disease. Infected potato plants develop a leaf chlorosis that causes the plant to have a yellowish cast, stem necrosis, stunting and reduced tuber production. Those tubers that are produced become necrotic and show difficulty sprouting (Wagner, 1987).

Maize mosaic virus (MMV) and rice transitory yellowing virus (RTYV) cause serious diseases in the field, infecting monocotyledonous hosts. MMV is widely distributed throughout the world, in central and south America to Africa, India, Australia and has recently been identified in the southern part of the United States (Bradfute and Tsai, 1983). The virus is spread by plant hoppers which migrate from several grass species known to be reservoirs of the virus. The symptoms of plants infected with MMV depend on the strain of the virus but in general, infected plants initially develop stripes between the leaf veins, and later turn yellow and become necrotic. If the infection occur early, the plants may be stunted and cobs deformed. The disease is most severe in areas where corn is grown continuously throughout the year and in some cases, 100% yield losses have been reported (Brewbaker, 1981). RTYV was first recorded in Taiwan in 1960 (Chiu and Jean, 1969) and causes a serious disease in the rice crops. Infected plant leaves, first turn yellow, then plants become stunted and tillering is reduced. However, the leaf symptoms gradually disappear, hence the name of the disease. RTYV is transmitted in a persistent manner by three leafhopper species of the genus *Nephotettix* and the damage to the rice crop depends to a great extent on the age of the plants at the time of inoculation (Chiu *et. al.*, 1968).

1.5.2.1- The economic importance of eggplant mottled dwarf virus (EMDV):

Eggplant mottled dwarf virus (EMDV) was first reported by Martelli (1969) in eggplant (*Solanum melongena* L.) from southern Italy. Symptoms were a severe stunting of the affected plants accompanied by pronounced mottling and crinkling of the leaves and generalised unfruitfulness.

The virus has been transmitted only by grafting and by sap inoculation. The vector is not known (Martelli and Russo, 1973). Recently EMDV has been reported in a variety of countries mainly from the Mediterranean region, stretching from Portugal, Morocco, Algeria, Tunisia, in the west to Jordan, Iran,

and Turkey in the east (El.Maataoui, *et. al.*, 1985; Martelli and Hamadi, 1986; Martelli, *et. al.*, 1984; Cherif and Martelli, 1985; Al.Musa and Lockhart, 1990). The virus was mainly reported infecting tomatoes. The virus which was found in Morocco infected tomato was originally reported and named as tomato vein-yellowing virus (TVYV), and was characterised by vein-yellowing, stunting and plant deformation symptoms (El.Maataoui, *et. al.*, 1985). The virus reported has major cytopathological effects on its host. Yellow areas of infected leaves contained various alterations of cell structures. Nuclei were enlarged by accumulations of virus particles bounded by the inner membrane of the nuclear envelope which protruded into the interior of the nuclei. The virus accumulation in the perinuclear space also extended into the cytoplasm and filled adjacent elements of the endoplasmic reticulum (El.Maataoui, *et. al.*, 1985). The virus particle morphology was similar to those described earlier for EMDV and other plant rhabdoviruses in subgroup II. The virus particles were bullet-shaped or bacilliform, depending on the fixation and staining procedures. The particles after staining in glutaraldehyde, measured 192x75 nm but were observed after fixation with OsO_4, to be up to 234 nm long (El-Maataoui, *et al.*, 1985). Later, Adam and his co-workers (1987) used two different serological techniques to compare three different isolates of EMDV including TVYV and concluded that TVYV is a strain of EMDV. EMDV has also been reported from Iran in potato (Danesh and Lockhart, 1989). The symptoms of primary infection by EMDV in potato consisted of severe stunting, folding of the young leaflets chlorosis, and wilting of the entire plant, starting at the stem apex and progressing downward. Secondary symptoms in plants grown from infected tubers consisted of slow growth, folding of young leaflets, chlorosis and lethal systemic necrosis (Danesh and Lockhart, 1989).

The severity of EMDV in its hosts and the widespread occurrence from the Mediterranean region to the Middle East, makes the virus more economically important than previously thought. The virus has been reported to be transmitted

through potato tubers in various cultivars of potato (Danesh and Lockhart, 1989), and has been found in weed hosts such as *Solanum sodomeum* (El.Maataoui, *et. al.*, 1985) and *S. nigrum* (Lockhart, 1987). In addition, with the possibility of a widespread vector, (Martelli and Russo, 1973), the economic importance of the virus is much more potentially significant, with two major crops potato and tomato in which EMDV is endemic.

1.5.2.2- The economic importance of sonchus yellow net virus (SYNV):

Sonchus yellow net virus (SYNV), the most extensively studied plant rhabdovirus was originally found in central and south Florida, USA infecting sowthistle (*Sonchus oleraceous*) and *Bidens pilosa* (Christie, *et. al.*, 1974). Later the virus was found in lettuce (*Lactuca sativa*) in Florida (Falk, *et. al.*, 1986). The symptoms in sowthistle were distortion and general yellowing of the leaves whereas in lettuce, they were bright interveinal clearing in old leaves (Falk, *et. al.*, 1986). The original sowthistle isolate and the lettuce isolate appear to represent different but antigenically related strains of SYNV (Ismail and Milner, 1988; Ismail, 1988; McElwee and Milner, unpublished). SYNV has not been reported elsewhere in the world.

1.5.3- Morphology and structure of plant rhabdoviruses:

On the basis of evidence from studies of particle morphology observed in thin sections of infected cells and negatively stained particles isolated by virus purification methods designed to preserve particle integrity, all plant rhabdoviruses have a bacilliform shape (Peters and Kitajima, 1970; Ahmed, *et. al.*, 1970; Russo and Martelli, 1973). Rhabdovirus particles appear smaller in sections of infected cells than in negatively stained preparations, presumably due to shrinkage during fixation and embedding and flattening during air drying (Francki, *et. al.*, 1981).

Considerable variation has been observed in both the length and width of

plant rhabdovirus particles. The width range from 32-35 nm for orchid fleck virus particles, which appear to lack a membrane (Doi, *et. al.*, 1977), to 70-80 nm for SYNV and EMDV (Ismail, 1988; Russo and Martelli, 1973). The length of the virion ranged from 216 nm for SYNV (Ismail, 1988) and 210 nm, for RTYV (Chen and Shikata, 1971). Although the accuracy of the measurements obtained in different laboratories is difficult to evaluate, the results indicate that different plant rhabdoviruses vary markedly in length and width. The chemical composition of the few plant rhabdoviruses studied have been estimated to be about 70% protein, 20-25% lipid, and 2-3% RNA. The virions also contain a small amount of carbohydrate.

Different models of the physical organisation of the rhabdovirus virion have been proposed based on electron micrographs (figure 1.1) (Francki, *et. al.*, 1981; Hull, 1976). Three distinct layers of varying electron density are observed in cross sections of the virion. These layers are thought to be composed of surface projections, a membrane, and a helical ribonucleoprotein surrounding a central canal. The spike projections protrude 6-10 nm from the lipid membrane that surrounds a nucleoprotein is about 15-20 nm thick. The lipoprotein envelope of the rhabdovirus particle, which is essential for maintaining the structure of the virion and for its maximum infectivity (Francki and Randles, 1975; Jackson, 1978) differ in composition and in the proportion of individual lipids from plant to animal rhabdoviruses. This probably reflects chemical differences between the lipids of plant and animal membranes from which the envelope has originated (Selstam and Jackson, 1983; McSharry and Wagner, 1971).

All the plant rhabdoviruses which have been investigated contain a number of polypeptides which can be separated by polyacrylamide-gel electrophoresis. Two of the major virion proteins are released with the envelope when disrupted with detergent, the glycosylated (G) protein which compresses the surface projections and the matrix protein (M). The glycosylated (G) protein in animal rhabdoviruses functions in attachment of the virion to the host receptor

sites on the plasma membrane during the early stages of infection and also thought functions in assembly of virions (Repik, 1979; Etchison and Summers, 1979; Wagner, *et. al.*, 1984). A G protein has been identified in all plant rhabdoviruses test with a molecular weight ranging from 70-90kD and staining positively for carbohydrate (Francki and Randles, 1975; Ziemiecki and Peters, 1976; Jackson, 1978; Falk and Weathers, 1983; Adam and Hsu, 1984). The G protein is thought to be associated with the viral envelope (Dale and Peters, 1981; Jackson, 1978; Ziemiecki and Peters, 1976) and exposed on the surface of the virions, since it becomes heavily labelled when intact virions are iodinated by various *in vitro* labelling techniques (Jackson, 1978; Ziemiecki and Peters, 1976). However, the G protein of three strains of PYDV have been shown to be different both in molecular weight and in the polypeptides produced after partial proteolysis. The G protein appears to has function in binding to host receptor sites on the leafhopper vector cell surface (Falk and Weathers, 1983; Adam and Hsu, 1984; Jackson, *et. al.*, 1987).

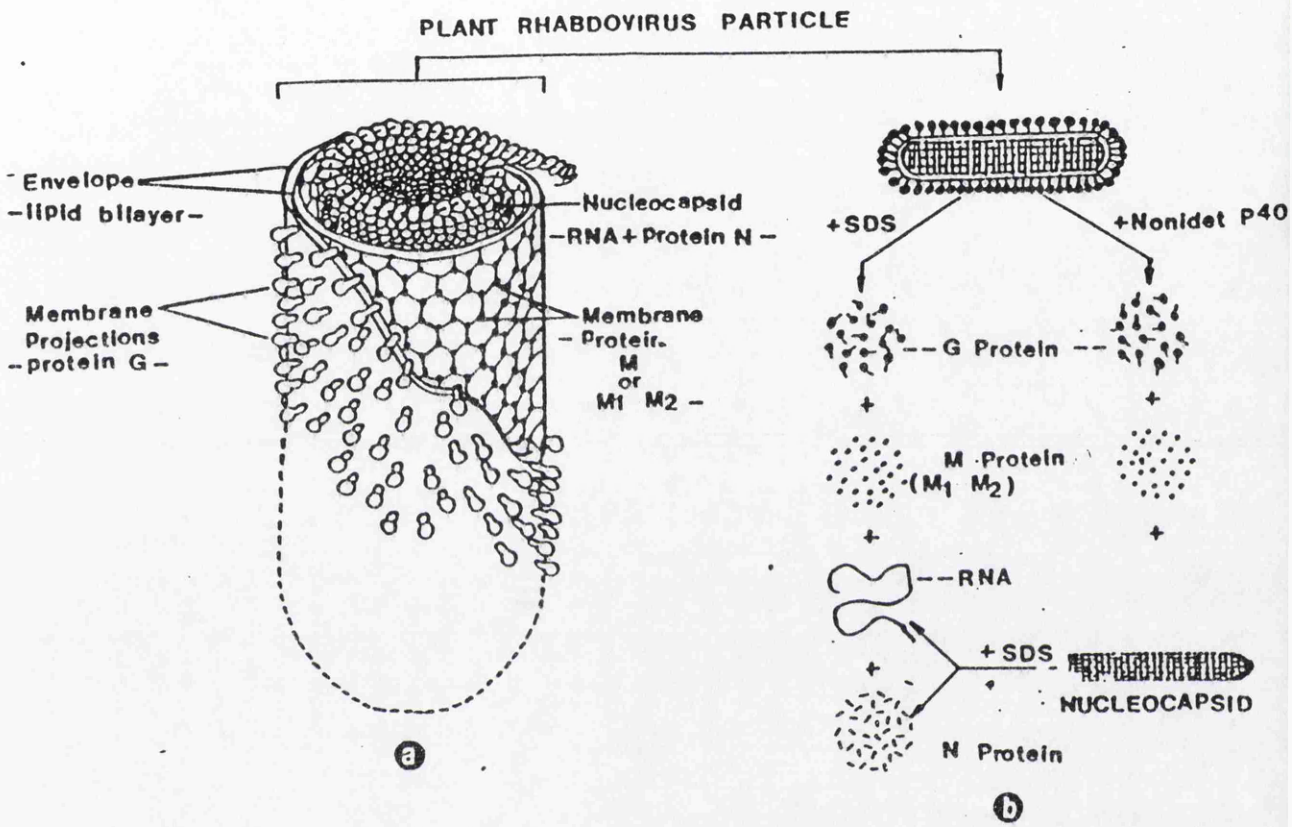


Figure 1.1:

(a) Model of plant rhabdovirus particle cut open to show the various internal structural components. (b) Disassembly of (a) with SDS and Nonidet P40 detergents (from Francki, *et. al.*, 1981).

Two membrane matrix (M) proteins classes were originally designated. One class includes the protein of VSV, the prototype of the vesiculovirus group .with one M protein (McSharry, 1979). Rabies virus, and other members of the lyssavirus group, was assigned two M proteins (Sokol, *et. al.*, 1971). The M protein of VSV is associated with the lipid of viral envelope and with G protein and has an important role in maintaining viral structure and possibly an involvement in the regulation of the genome replication (McSharry, 1979; Wagner, *et. al.*, 1984; Patton, *et. al.*, 1984). Studies concerning the actual location and function of the two M-proteins of rabies virus, indicate that the M2 protein of rabies virus and M protein of VSV have a similar location in the virion and on analogous function while the M1 protein of rabies virus appear to be similar to the VSV non-structural protein (NS). The rabies virus M1 protein appears to be closely associated with the nucleoprotein complex whereas, M2 protein appears to be closely associated with the viral envelope. The G and M2 proteins are both localised on the surface of the cell infected with rabies virus. In contrast, the NS and M1 proteins are located within the cells (Cox, *et. al.*, 1981).

One of the rhabdovirus characteristics which led to the division of plant rhabdoviruses in to two subgroups was the assignment of one or two matrix (M) protein similar to the old division in the animal rhabdoviruses. the M protein of plant rhabdoviruses has a molecular weight ranging from 19-45kD and is thought to be associated with membrane envelope. New studies on the location and function of M proteins of rabies virus led to a reconsideration of the location and function of M proteins of those plant rhabdoviruses which have M1 and M2. Protein studies with PYDV showed that M1 protein was the only membrane-associated protein while M2 protein found associated with nucleoprotein (Gaedigk, *et. al.*, 1986). Recent studies on the genome of SYNV sequences (see below section 1.5.4) that M2 protein may be a nucleoprotein correlating with NS and M1 proteins of VSV and rabies viruses respectively (Heaton, *et. al.*, 1987;

Cox, *et. al.*, 1981). It likely that all the rhabdoviruses with only one M protein are assembled and distributed in the cytoplasm whereas those which contain both M1 and M2 proteins are largely confined to the perinuclear space of infected cells (Table 1.3).

The nucleoprotein assigned as non-structural (NS) protein is a minor component of VSV virions but has an important role in RNA transcription and probably also functions in replication of the genome in infected cells (Bell and Prevēc, 1985; Clinton and Huang, 1981; Hsu, *et. al.*, 1982; De and Banerjee, 1985; Patton, *et. al.*, 1984). Several plant rhabdoviruses have protein patterns in polyacrylamide gels thought that resemble the NS protein of VSV (Dale and Peters, 1981). However, lack of information and structural data concerning plant rhabdovirus NS proteins make it premature to designate NS-protein assignments to the plant rhabdoviruses (Jackson, *et. al.*, 1987).

The nucleocapsid (N) protein is a structural protein, associated with the viral RNA and is an integral component of the transcriptase complex in VSV (Hunt, *et. al.*, 1979). Several studies have suggested that the N protein has a direct role in replication of genomic RNA and regulation of the switch from transcription to replication (Blumberg, *et. al.*, 1981; Mark, *et. al.*, 1985; Patton, *et. al.*, 1984; Perrault, *et. al.*, 1983; Amheiter, *et. al.*, 1985). All the plant rhabdoviruses that have been studied have an N protein that is tightly bound to the viral RNA (Ziemiecki and Peters, 1976; Jackson, 1978; Dale and Peters, 1981). The core of the virion can be visualised in the electron microscope as a loosely coiled structure that consists of RNA and N protein (Jackson, 1978). Studies with SYNV and LNYV have shown that the intact virus was more infectious than the nucleocapsid (Jackson, 1978; Randles and Francki, 1972). Moreover, recent studies with SYNV showed that the N protein has diverged considerably in amino acid sequence from rabies and two strains of VSV viruses.

A fifth protein of large molecular weight (241kD) designated L protein is

present in small amounts found in the VSV virion, rabies virus and other animal rhabdoviruses and associated with the nucleocapsid (Harmison, *et. al.*, 1984; Heyward, *et. al.*, 1979). L protein is the major component of the transcriptase and is believed that it is an integral requirement for all rhabdoviruses (Belle-Isle and Emerson, 1982; De and Banerjee, 1984; Morgan and Kingsbury, 1981). Tests with some plant rhabdoviruses failed to reveal the presence of an L protein in the nucleocapsid preparations. However, high molecular weight polypeptides are usually present in plant rhabdovirus preparations. In fact, an L protein associated with isolated nucleocapsid preparations has actually been detected only in LNYV (Dale and Peters, 1981). In some other viruses such as SYNV, there is indirect evidence favouring the presence of an L protein namely the presence of a mRNA coding for such a protein (Rezaian, *et. al.*, 1983; Choi, 1993).

1.5.3.1- Morphology and structure of EMDV:

EMDV is one of the plant rhabdoviruses of subgroup II characterised by accumulation of virus particles in the perinuclear space and the possession of two matrix proteins (M1 and M2) in its virion structure. EMDV particles have been observed in thin sections of infected cells, lodged in the nuclear membrane in the nuclei early in infection. Virus particles have not been seen in other sites of the infected cells (Russo and Martelli, 1973). EMDV virions are bacilliform particles measuring 220-230 nm in partially purified preparations fixed in glutaraldehyde before negative staining with phosphotungstate. The virus particles exhibit surface projections 6 nm long, a helical internal nucleocapsid with periodicity of 4.5 nm and an outer envelope of 10-12 nm thick (depending on the fixing and staining procedure) (Russo and Martelli, 1973). In tissue sections "immature" particles (those still connected to the nuclear envelope) were bullet-shaped whereas the "mature" particles (free from the inner nuclear membrane) were bacilliform (Martelli, 1969; Martelli and Costellano, 1970). Although the virus particles were mainly found in the perinuclear space, late in infection, some virus

particles could be seen scattered in the cytoplasm in clusters surrounded by a membrane.

The structural proteins of EMDV virus have been analysed by polyacrylamide gel electrophoresis. Like other type II rhabdoviruses, EMDV contains four major proteins, Glycosylated (G) protein which has a molecular weights of 83kD and is the spike protein that protrudes from the envelope of the virion. The nucleocapsid (N) (M.W. 61kD) a structural protein is associated with the viral RNA. The two additional major proteins, M1 and M2 have molecular weights of 27kD and 21kD respectively. In the light of reassessment of the roles of M1 and M2 in SYNV, PYDV and in the rabies virus, the function of M1 and M2 remains uncertain (Heaton, *et. al.*, 1987; Gaedigk, *et. al.*, 1986; Cox, *et. al.*, 1981). Tests with EMDV failed to reveal the presence of any large (L) protein in nucleocapsid preparations, but showed that high-molecular weight proteins are present in the soluble fraction after dissociation of the viral envelope with non-ionic detergent (Dale and Peters, 1981). Little is known about the structure of genomic RNA.

1.5.3.2- Morphology and structure of SYNV:

The SYNV virions are bacilliform particles measuring 94x248 nm after fixation in glutaraldehyde and negative staining. Internal cross-striations with a periodicity of 4.1 nm was observed as was an outer envelope through which 6 nm long surface projections protrude (Jackson and Christie, 1977). Bullet-shaped particles were observed when leaf dips of infected *N. edwardsonii* were stained with phosphotungstate (Ismail, 1988). SYNV consists of RNA, proteins, lipids and carbohydrate associated with G protein (Jackson, 1978).

Several lines of evidence have demonstrated the single strandedness and negative sense of SYNV RNA; RNA is susceptible to ribonuclease (RNase) under high ionic strength conditions and can act as a template for cDNA synthesis (Milner and Jackson, 1979). RNA hybridises to polyribosomal RNA

from SYNV infected tobacco. RNA is not infectious and comprises only about 0.47% of the mass of the virions assuming that a virion of about $9x10^8$ daltons contains a single copy of RNA (Jackson and Christie, 1977; Rezain *et. al.*, 1983; Milner and Jackson, 1979, 1983; Milner *et. al.*, 1979) which encodes six putative messenger (m) RNAs (Heaton *et. al.*, 1989).

Four major and some minor electrophoretically distinguishable polypeptides were found in SYNV purified virions (Jackson, 1978; Dale and Peters, 1981) as shown in (table 1.3). However, sequencing studies of the SYNV genome demonstrated the molecular weight of the N protein and M2 protein to be 53.641 Da and 38.332 Da respectively (Zuidema *et. al.*, 1987; Heaton *et. al.*, 1987).

In addition to the four major proteins, several high molecular weight proteins have been reported of which one is presumably the L protein (Jackson, 1987; Dale and Peters, 1981). The G protein is reported to be glycosylated. (Jackson, 1978).

Studies on the lipid composition of SYNV showed that the lipid fraction represents about 18% of the virion. SYNV-lipids consist of 62% phospholipids, 31% sterols and 7% triglycerides. The fatty acids were more unsaturated in SYNV than those of animal rhabdoviruses such as VSV. These differences probably reflect the differences between the plant and animal membranes from which the viral envelopes are derived (Selstam and Jackson, 1983; Harwood, 1980; Mazliak, 1977; Compans and Klenk, 1979).

1.5.4- The sequences of plant rhabdovirus genomes:

Considerable progress has been made in understanding the organisation of rhabdovirus genomes. The complete sequence of SYNV is now known (Heaton, *at. al.*, 1987 & 1989; Zuidema, *et. al.*, 1987; Hillman, *et. al.*, 1990; Goldberg, *et. al.*, 1991; Choi and Jackson, 1993) and a start has been made on the analysis

of the LNYV genome (Wetzel, *et. al.*, 1993). The organisation of the SYNV and LNYV genomes are similar to that of the VSV and rabies virus genomes (Rezaian, *et. at.*, 1983; Heaton, *et. al.*, 1989; Jackson, *et. al.*, 1987) except that contains an additional sixth open reading frame (ORF). The gene order from the 3' to 5' ends of the SYNV RNA is l-N-M2-sc4-M1-G-L. Infected tobacco leaves contain transcripts that are complementary to more than 95% of the viral RNA (Milner and Jackson, 1979). The complementary transcripts are associated with polyribosomes, are polyadenylated, and are mRNAs. The five smaller RNA species are correspond to the mRNAs for the four most abundant viral polypeptides and an additional non-structural polypeptide. Although tests with SYNV failed to reveal the presence of an L protein in the nucleocapsid preparations. A mRNA of approximately 6600 b coding for a putative L protein with some homology to the polymerase of VSV and rabies virus has been detected and sequenced (Jackson, *et. al.*, 1987; Choi, 1993).

The deduction of the complete sequence of SYNV, and the recent analysis of the gene arrangement of LNYV have underlined the similarities and differences between plant and animal rhabdoviruses. The sixth ORF, located between the M1 and M2 genes may be a common feature of plant rhabdoviruses. Its function is unknown. The sequence analysis of SYNV has permitted accurate determinations of the molecular weights of SYNV proteins and has elucidated their functions (Heaton, *at. al.*, 1987 & 1989; Zuidema, *et. al.*, 1987; Hillman, *et. al.*, 1990; Goldberg, *et. al.*, 1991; Choi and Jackson, 1993; Choi, 1993).

1.5.5- Plant rhabdoviruses purification:

Purification of viruses from plant tissues requires efficient methods of clarifying plant crude material extracts before the virus is concentrated. The effect of host and environmental factors can affect the amount of virus yield. The age of the plant, light and temperature, and the length of the infection are critical factors that can drastically alter the amount of virus recovered. For instance,

adequate amount of PYDV (Hsu and Black, 1973), LNYV (Wolanski and Chambers, 1971), and SYNV (Jackson and Christie, 1977) can be recovered only between 10 and 20 days after inoculation, since the amount of virus in tissue declines rapidly thereafter (Ismail, *et. al.*, 1987).

All rhabdoviruses that have been studied have thermal inactivation points below 60°C and pH optima near neutrality in *vitro*. Thus extraction should take place near 0°C and pH should be slightly above or at pH7 (Jackson *et. al.*, 1987). Reducing agents are also necessary to preserve infection of some rhabdoviruses (Jackson and Christie, 1977; Peters and Kitajima, 1970; MacLeod, 1967; Falk and Weathers, 1983). However, some agents may influence the stability of rhabdoviruses differently.

The most difficult problem in virus purification is to remove contaminants such as chloroplasts and other membrane fragments from the preparations; these may sometimes be removed by careful filtration through thin pads of Celite before the density-gradient step (Ahmed, *et. al.*, 1970; Greber and Gowanlock, 1970). The viruses are usually concentrated from plant sap by centrifugation but this can have some adverse effects, such as virus aggregation. These can be avoided by removing as much host material as possible before centrifugation. Alternatively, polyethylene glycol (PEG) precipitation has been used to reduce the damage caused by centrifugation. Most purification schemes designed for rhabdoviruses, have employed density-gradient centrifugation (Jackson, *et. al.*, 1987). Two alternative approaches to density-gradient centrifugation have been applied to plant rhabdoviruses; chromatography on calcium phosphate gels (Lin and Campbell, 1972; McLean and Francki, 1967) and electrophoresis in sucrose density gradients (Ahmed, *et. al.*, 1970; Peters and Kitajima, 1970; Sinha, *et. al.*, 1976), but neither of these methods have proved satisfactory for general use with rhabdoviruses (Jackson, *et. al.*, 1987).

1.5.5.1-Purification of SYNV and EMDV:

Jackson and Christie (1977) reported the purification of SYNV by using celite pad filtration and sucrose density gradient centrifugation. The final purified virus was 200-445 μg/100g leaf fresh weight although the yield was very dependent on plant age at inoculation, harvesting time after inoculation and the conditions under which the plants were grown (Selstam and Jackson, 1983).

The methods of EMDV purification were almost the same methods which used for most plant rhabdoviruses such as SYNV with slight differences. Celite pad filtration, sucrose density gradient centrifugation and high speed centrifugation were the main procedures in virus purification (Russo and Martelli, 1973; El-Maataoui, *et. al.*, 1985; Martelli, 1973). Although various procedures for purifying EMDV have been published (Russo and Martelli, 1973; El-Maataoui, *et. al.*, 1985; Martelli, 1973; Adam, *et. al.*, 1987), none of them is reported to yield pure preparations, uncontaminated by host proteins.

Table 1.3: Structural proteins of plant rhabdoviruses:

Protein[4]	PYDV[1]	EMDV[5]	SYNV[2]	LNYV	WSMV[3]
L	+[a]	-	+	171	145
G	78	83	70	71	92
N	56	61	56	56	59
NS	-[b]	-	-	38	-
M1	33	27	41	39	25
M2	22	21	38		

a = Detected but molecular weight not estimated accurately. b = Not detected.
1 = Knudson and MacLeod (1972). 2 = From sequences data; Zuidema, *et al.*, (1987), Heaton, *et al.*, (1987) and Goldberg, *et. al.*, (1990) 3= Trefzger and Lee (1977), 4 = Wagner *et al*, (1972). 5 = Dale and Peters (1981).

1.5.6- Serology of plant rhabdoviruses:

Antisera have been prepared to over 17 members of the plant rhabdoviruses (Jackson, *et. al.*, 1987). Several serological techniques have been applied to study the relationship between plant rhabdoviruses and their hosts, and the relationships between different the members of the family. The most sensitive and suitable technique for diagnostic application is ELISA. This assay has been used to distinguish many plant rhabdoviruses from other viruses (Chu and Francki, 1982). In addition, the ELISA technique has been used to distinguish between different strain of EMDV. Antisera raised against EMDV and tomato vein yellowing virus (TVYV) were tested in ELISA against virus proteins of EMDV and TVYV revealed no differences in antibodies binding. These results suggested that the TVYV is in fact a strain of EMDV (Adam, *et. al.*, 1987). The ELISA technique has been used to detect and locate the movement of SYNV in infected plants. The virus antigen was detectable 24h after inoculation and reached its maximum 10 days after inoculation (Ismail, *et. al.*, 1987). Immunogold labelling has been used to localise the virus protein(s) and establish their distribution within infected cells. Plants infected with SYNV, and labelled

with immunogold, revealed the distribution of the virus protein within the infected cell. In the early stages of infection, the immunogold particles bound heavily in the perinuclear space and in the viroplasm. Furthermore, viral proteins were detected within the cell wall and chloroplasts (Ismail, 1988). Information gained by serological tests have been extensively documented by Jackson, *et. al.*, (1987) and the serology of plant rhabdoviruses has been discussed in a number of reviews (Francki, 1973; Francki and Randles, 1980; Francki, *et. al.*, 1981; Peters, 1981)

SYNV-infected *N. edwardsonii* showed ultrastructural changes to the cell wall. Channels or tubular structures were observed interconnecting adjacent infected cells; these channels were not modified plasmodesmata. Immunogold labelling demonstrated that these unusual structures were able to bind anti-SYNV antibodies (Ismail, 1988). At various times after inoculation, immunogold labelled cells consistently showed bound clusters of gold particles associated with regions of the cell wall. Binding of gold particles to the inner surface of the cell wall was also occasionally observed, suggesting the presence of viral protein(s) in the plasma membrane (Ismail, 1988).

1.5.7- Plant rhabdovirus replication:

VSV replication has been the most extensively studied of all rhabdoviruses. The replication of VSV starts with uncoating and release of the nucleocapsid (NC) in the cytoplasm. The nucleocapsid contains a negative polarity RNA strand which serves initially as a template for transcription of five subgenomic mRNAs, each of which is translated to give a different protein and later, a full-length positive RNA strand is synthesised. Primary transcription of the incoming genome and translation of the resulting mRNAs generates the proteins necessary for replication (N, NS, L) (Dubois-Dalcq, *et. al.*, 1984; Hill, *et. al.*, 1981; Blumberg, *et. al.*, 1983). VSV has five functional proteins. N protein was first detected in the cytoplasm following infection and NS protein

was found to be abundant in the cytoplasm of infected cells. NS antigen was co-localised with N antigen. M protein was found distributed diffusely throughout the cytoplasm of infected cells and did not co-localise with any other protein. N, NS and M proteins were synthesised on free polysomes (Knips, *et. al.*, 1977). G protein was synthesised on membrane-bound ribosomes, glycosylated and transported to the plasma membrane (Lodish and Rothman, 1980; Morrison, 1980; Bergmann, *et. al.*, 1981; Rose and Bergmann, 1982). The assembly of virus components starts with the condensation of N protein and viral RNA. The aggregating property of the N protein is essential for the formation of a helical nucleocapsid (NC) (Hsu, *et. al.*, 1977; Blumberg, *et. al.*, 1983). The condensation can be influenced by two factors, salt concentration and M protein. Reducing the amount of M protein led to a reduced helix patch; increasing the ratio of M protein to purified NC, increased NC compaction at low ionic strength (Heggeness, *et. al.*, 1980; De, *et. al.*, 1982).

Depending on the cell surface, G protein may play a more or less important role in attracting NCs to the infected cell membrane. When G protein synthesis was blocked in VSV-infected cells by monensin, virus buds were not detected at the cell surface (Johnson and Schlesinger, 1980). Several possible models of interaction between NC and envelope protein have been proposed (Dubois-Dalcq, *et. al.*, 1984);

1- M protein may first bind to NC forming a M-NC complex, this probably takes place on the membrane and might involve recognition of the carboxyl-terminal end of the G protein, inducing clustering of G molecules and the formation of spikes. Therefore, two binding sites on the M protein are required, one to NC and one to G, but the later binding site would be less specific since certain host membrane proteins can enter the viral envelope when G is absent.

2- M protein first forms a patch in the membrane by self aggregation and

binds to G molecules and/or cellular membrane proteins. Subsequently, a NC recognises this patch by association of N and G and induces further clustering (Jacobs and Penhoel, 1982).

3- NC recognises and binds directly to the carboxyl-terminal end of G, and M only plays a role in coiling of NC (Odenwald, *et. al.*, 1984).

During these transmembrane assembly events, an increasing number of NC coils are incorporated into the bud, and the virus envelope grows tightly packed with virus protein molecules and excluding most host cell protein. Generally, the most frequent sites of rhabdovirus budding are the plasma membrane, viroplasm, inner nuclear envelope and endoplasmic reticulum (Brown and Riedel, 1977; Dubois-Dalcq, *et. al.*, 1979, 1984).

Once the rhabdovirus bud has reached its final shape and contains the entire coiled NC, it is released from the cell as a bacilliform or bullet-shaped particle. The NC length, number of turns, and the virus length and shape depend on the type of rhabdovirus.

The available information on the replication of plant rhabdoviruses comes from experimentation on both systemically infected plants and protoplasts. Whole plant experiments have some disadvantages because infection of individual cells occur synchronously and systemic spread of virus is difficult to control. These problems are exacerbated because many rhabdoviruses can be transmitted only by the insect vector. Virus replication has been described in some detail in the reviews of Francki (1973), Knudson (1973), Francki and Randles (1980), Francki, *et. al.*, (1981), Peters (1981) and Jackson, *et. al.*, (1987). On the basis of the site of replication and assembly of virus particles, plant rhabdoviruses have been divided into two groups; those viruses which replicate and assemble in the nuclei and those which replicate and assemble in the cytoplasm. Studies of *N. edwardsonii* infected with SYNV (member of first group), investigated the amount and location of virus proteins in infected cells

over the period of infection. These showed that virus proteins were detected 12hr post-inoculation in inoculated leaves and that the virus antigen reached its maximum 10 days after inoculation (Ismail, *et. al.*, 1987; Ismail, 1988). Immunogold labelling of SYNV-infected leaves from *N. edwardsonii*, showed gold particles bound to the nucleus 5 days after inoculation. Binding was much more intense at 20 days and additionally gold particles were found scattered in the viroplasm. In addition, gold particles were found associated with the cell wall 5, 10 and 15 days after inoculation. Fewer gold particles were found in the cell wall in infected plants than in the nucleus (Ismail, *et. al.*, 1987; Ismail, 1988). Wolanski and Chambers (1971) studied the events in the multiplication of LNYV (a member of the second group of plant rhabdoviruses) in *N. glutinosa* leaf cells by sampling the first systemically infected leaf at various time intervals after inoculation of the lower leaves. The experiments involved both autoradiography of cells treated with actinomycin D and labelled with ^{3}H-uridine, and direct electron microscopic observations. They concluded that the initial events associated with virus synthesis took place in the nucleus, and later the cytoplasm become involved. Autoradiography of ^{3}H-labelled cells of leaf-tissue fragments that had been treated with actinomycin D revealed accumulation of radioactivity in the nucleus during virus-induced blistering of the nuclear envelope. Subsequently, aggregates of virus particles were detected in the perinuclear spaces, but as infection progressed, virus particles began to appear in cytoplasmic vesicles near the endoplasmic reticulum. During this time, ^{3}H incorporation decreased in the nucleus and increased in the cytoplasm. These results led Francki (1973) to conclude, that initially, viral nucleic acid is synthesised in the perinuclear space and the failure to detect nucleocapsids in the nucleoplasm may be due to their rapid assembly into complete virus particles. The absence of virus particles in the perinuclear spaces at later stages of infection could be due to their transport in the enchylema of the endoplasmic reticulum.

The development of methods of infecting protoplasts with plant rhabdoviruses gives a more precise delineation of the specific events that occur during replication. Van Beek and his co-workers (1985a, b) were able to infect cowpea protoplasts with SYNV and festuca leaf streak virus (FLSV) with the help of polyethylene glycol (PEG). This provides a system that should be extremely useful for studies of the replication of plant rhabdoviruses. Infectivity of SYNV was detected in extracts of cowpea protoplasts 11-12hr post-inoculation and increased until about 30hr later then started to decline. Rhabdovirus particles were present in the perinuclear space and in the cytoplasm of infected protoplasts at 67hr post-inoculation. The investigation of the synthesis of SYNV proteins in SYNV-infected protoplasts of cowpea showed that N protein was detected at 8hr and M2 protein at 9hr post-inoculation while G and M1 proteins were not detected in the first 13hr post-inoculation (Van Beek, *et. al.*, 1986). However, the assembly of SYNV particles in the protoplasts began 10hr post-inoculation (Van Beek, *et. al.*, 1985b). Host proteins from the infected protoplasts obscured the detection of the four virus proteins before 13hr (Van Beek, *et. al.*, 1986).

At the early stage of infection, up to 7hr after inoculation, no cytopathological changes were observed. Nine to ten hours later, following increased the numbers of polysomes in the cytoplasm, nucleocapsids appeared at the edge of a granular matrix in the nucleus and particles in the process of budding from the inner nuclear membrane were evident. Particles began to accumulate in the perinuclear spaces and to enter the lumen of the endoplasmic reticulum 12hr after inoculation. From 12hr to 24hr post-inoculation, virions appeared in the cytoplasm in association with loose fitting membrane and both enveloped virions and nucleocapsids were present in the cytoplasm. The matrix, nucleocapsids, and budding virions were subsequently observed (Van Beek, *et. al.*, 1985b). Protoplasts from SYNV-infected *N. edwardsonii* and *N. benthamiana* have been found to be suitable for studies of replication (Jones and

Jackson, 1990). SYNV messenger RNAs were detected within 2hr post-inoculation, accumulated to a maximum within 24hr, and subsequently declined to undetectable levels by 60hr. The four major SYNV structural proteins were detected by western blot by 24hr after inoculation and were present in highest concentrations between 43 and 60hr post-inoculation (Jones and Jackson, 1990).

Throughout the course of infection, the number of particles appeared to increase in the cytoplasm, suggesting that virus replication continued throughout the period of experiment despite an observed drop in extractable virus during the last part of the experiment (Van Beek, *et. al.*, 1985b). Direct evidence for nuclear involvement in SYNV assembly was obtained in infected protoplasts in the presence of the glycosylation inhibitor Tunicamycin. The results showed that in Tunicamycin treated protoplasts, the granular matrix almost filled the nucleus and no enveloped virus particles were observed. In addition, no nucleocapsids were evident in the cytoplasm 24 and 47hr after inoculation. These results suggest that nucleocapsids are unable to emerge from the nucleus into the cytoplasm without first having become enveloped at the nuclear membrane (Jackson, *et. al.*, 1987).

The replication of plant rhabdoviruses in their insect vectors has been investigated to a limited extent. Such studies have enormous potential because of the unique opportunity for direct comparison of responses of plants and animals to infection by the same virus. Some rhabdoviruses have been shown to be propagative in their vectors. Direct evidence, based on serial transfer of inoculum by injection through a number of generations of insect, has been obtained for SYVV, SCV, PYDV, WSMV, RTYV and NCMV (Francki, 1973; Jackson, *et. al.*, 1981). In the case of viruses, for example, LNYV, BNYV, MMV and SYNV virus particles have been seen in thin sections of vector tissues. This is strong evidence that they are propagative (Jackson, *et. al.*, 1981). Virus particles have been detected in different parts of the insect vector. LNYV and SYVV particles have been detected in salivary glands, brain, muscle, fat body, mycetomes,

ovaries, and oesophagus but not in embryos (O'Loughlin and Chambers, 1967; Sylvester and Richardson, 1970). Particles of SYVV have been seen in the nucleoplasm and cytoplasm, and it was suggested that the nucleocapsids acquire their envelope while passing from the nucleoplasm into the perinuclear space (Sylvester and Richardson, 1970). Broccoli necrotic yellows virus (BNYV) particles accumulated in random arrangements within nuclei, and it was suggested that unlike the situation in the plant, where cytoplasmic vesicles were thought to be the site of virus maturation, the nuclei were the main sites of virus development in the aphid (Garrett and O'Loughlin, 1977; Francki and Randles, 1980). The effect of rhabdoviruses on their vectors indicate that some of them can be considered as insect as well as plant pathogens. Both LNYV and SYVV infection significantly shortens the life of their vectors and one isolate of SYVV also reduces the rate of larviposition and excretion (Boakye and Randles, 1974; Sylvester, 1973).

It has been suggested that rhabdoviruses may be considered as "Zoophytic" or "bridging" viruses between plant and vertebrate hosts. That the "bridge" may also be extended to vertebrate hosts has been suggested by the demonstration that VSV (generally considered to be a vertebrate virus) multiplies in *Peregrinus maidis*, the vector of MMV (Sylvester, 1973; Conti and Plumb, 1977; Lastra and Esprza, 1976).

Cultured insect cells are more attractive than intact insects for virus replication studies, because they can be synchronously infected and single-hit kinetics of infection can be obtained (Hsu and Black, 1973; Black, 1979). Established cell lines as well as primary cultures of insect vector cells can be infected *in vitro* with plant rhabdoviruses (Black, 1969; Peters and Black, 1970; Falk and Weathers, 1983; Adam and Hsu, 1984; Adam and Gaedigk, 1986).

1.6- Movements and transmission of plant virus:

1.6.1- Movements of plant virus:

Soon after inoculation of a plant with a virus, only a very small number of cells become infected. The virus replicates in these primarily infected cells and then moves to the neighbouring healthy cells. It has been widely accepted that the cell-to-cell movement and systemic spread of the virus is a passive process either in the form of free RNA in the case of positive stranded RNA viruses or as a nucleocapsid in the case of negative stranded RNA viruses and/or as mature virion particles (Atabekov and Dorokhov, 1984). During the primary infection of the plant, virus particles penetrate through microinjuries into epidermal cell and probably into occasional cells of mesophyll (Sulzinski and Zaitlin, 1982). Further systemic spread of infection takes place in two ways: (1) Slow cell-to-cell movement (short distance transport) in the parenchyma and (2) Rapid migration over long distances (long-distance transport). The long distance transport of plant viruses occurs usually in the phloem, and in a few cases in the xylem (Atabekov and Dorokhov, 1984).

It is believed that the plasmodesmata play the role of the transport channels through which the infective principle is transferred from cell to cell. The diameter of plasmodesmata and their numbers are varied in different tissues and depends on the plant species. However, it has been reported that the virions of plant viruses may occasionally be observed inside the plasmodesmata of infected tissues. Virus infection may lead to modification in the fine structure of the plasmodesmata which can permit to the virus genome to be transported through the gate to the healthy cell (Atabekov and Dorokhov, 1984). Much of the recent evidence indicates that cell to cell spread is mediated by a virus-coded product. The original evidence came from the studies of tobacco mosaic virus by Leonard and Zaitlin (1982) Atabekov and Morozov (1979) which suggested the 30k gene product was the most probable candidate for a movement protein,

involved in the cell to cell spread. The accumulating evidence suggests that more than one mechanism is involved in short-distance movement. One of the two proposed mechanisms of movement is examplified by TMV, in which the 30k protein interacts with plasmodesmata to facilitate the passage of a non-virion form of the virus, the other examplified by cowpea mosaic virus (CPMV) and cauliflower mosaic virus (CaMV), in which virus particles appear to pass along tubular structures through plasmodesmata (Linstead, *et. al.*, 1988; Tomenius, *et. al.*, 1987).

The rapid migration over long distances via the conducting tissues has not been gives as much attention as has cell to cell transport. It was believed, from the systemic spread behaviour of some viruses such as TMV and tobacco rattle virus (TRV), that long-distance transport of viruses involves mature virus particles (Sanger, 1968; Siegel *et. al.*, 1962; Dawson *et. al.*, 1988; Hull, 1989). Studies on the movement of SYNV in infected *N. edwardsonii*, (Ismail, *et. al.*, 1987) using ELISA, showed that the virus was detectable as little as 24hr after inoculation away from the site of inoculation in leaves and roots. The levels of virus antigen increased within roots and unexpanded leaves between 1 and 4 days after inoculation, suggest that virus multiplication occurred within these tissues. They reported that progeny from these initially infected cells were transported, presumably via the vascular system, throughout the plant and during the period between 5 and 10 days post-inoculation, the virus appeared to multiply and spread rapidly, as indicated by the levels of virus antigen within infected tissues (Ismail, *et. al.*, 1987).

1.7- Effects of virus infection on photosynthetic rates:

The replication of a virus can have a dramatic effect on its plant host. The symptoms are most probably induced by secondary events rather than virus replication, because some viruses replicate to a substantial degree but produce mild or even no symptoms (Zaitlin and Hull, 1987). Over the years, plant

physiologists and plant virologists have described the effects of viruses upon respiration, photosynthesis, and secondary metabolism (Zaitlin, 1979; Goodman, *et. al.*, 1986). Virus infections have been reported to reduce photosynthetic rates, in some cases where symptoms are mild, or apparently absent. The results appear to depend on the virus strain, the type of host studied and the protocol of the experiment. The loss of the chlorophyll that accompanies viral infection is most commonly reported to cause a reduction or loss of photosynthetic activity in the host cell. However, viral infections have also been reported to have no effect or even stimulate photosynthesis (Magyarosy, *et. al.*, 1973; Smith and Neales, 1977), whilst in some other cases viral infections have been reported to induce changes in the carbon metabolism of photosynthesis of cells, resulting in the accumulation of various amino acids and organic acids (Bedbrook and Matthews, 1972; Magyarosy, *et. al.*, 1973).

Various methods of measuring photosynthesis rates involve monitoring CO_2 assimilation in leaves or whole plants. This usually achieved by an infrared gas analyser (IRGA) or by an oxygen evolution measurement method which is relatively inexpensive and simple polarographic method for evaluating CO_2-dependent oxygen evolution in leaf discs are available (Delieu and Walker, 1981).

One of the models which describes the photosynthesis light response is the rectangular hyperbola model of Rabinowitch (1951). The model describes the relationship between photosynthesis based on the biochemical reactions within the chloroplasts and irradiance (light intensity) in terms of a rectangular hyperbola and this relationship has been used by many later workers. The model uses three related parameters to model photosynthesis rates; maximum gross photosynthesis rate (Pgmax), quantum efficiency of photosynthesis rate at low light intensities (α) and dark respiration rate (Rd). Recently, Marshall and Biscoe (1980) described a model which combined a simplified description of the biochemical reactions occurring within the chloroplasts with the physical

diffusion of CO_2 from the atmosphere. The response curve is a non-rectangular hyperbola, and uses four parameters; maximum gross photosynthesis rate (Pgmax), dark respiration rate (Rd), quantum efficiency of photosynthesis at low light (α) and ratio of physical to total resistance to diffusion of CO_2 (θ). Further description of the two models will be given in chapter two.

1.8- Symptomology and cytopathology of virus-infected plant:

In natural plant communities undisturbed by man it is probable that many plant viruses cause symptomless or almost symptomless infections. However, in agricultural and horticultural plant communities both cultivated and weed species often respond with some form of macroscopically observable disease. The response of plant to virus infection is extremely varied (Matthews, 1991). The symptoms characteristic of a particular disease will arise because of the effect of virus infection on particular cells or classes of cells. These cellular response are in turn dependent on the organelle changes which arise because of biochemical and molecular biological events that occur during the establishment and replication of the virus. The initially infected cell may give rise to a macroscopically visible local lesion which may consist of a patch of dead cells or of cells that have lost some chlorophyll. Subsequently, the virus may move systemically through the plant. The kinds of symptoms which develop following systemic infection are various. The major kinds of disease symptoms that occur in response to virus infection are summarised in Table 1.4.

Table 1.4: Important host plant responses to systemic virus infection:

Plant part	Symptoms	Comments*
Whole plant	1- Reduction in size	-The commonest response
	2- Wilting	-May lead to death of the plant
	3- Generalised necrosis	-Lead to rapid cell death
Leaves	1- Vein clearing	-Translucent tissue near veins
	2- Mosaic	-A very common response
	3- Blistering & distortion of lamina	-Associated with mosaic
	4- Vein banding	-Darker green tissue near veins
	5- Yellowing of veins	-
	6- Generalised yellowing	-
-	7- Etched ringspot & line patterns	-Due to death of superficial layer of cells.
	8- Reduction of lamina	-In extreme examples only midrib & veins may develop
	9- Enations	-Abnormal leaflike growths from veins & midrib
	10- Epinasty & leaf abscission	-
Flowers	1- Variegation or "breaking" of petal pigmentation	-Associated with mosaic on leaves
	2- Malformation	-
	3- Necrosis	-
Fruits	1- Mottling patterns	-
	2- Ring & line patterns	-
	3- Reduced size	-
	4- Malformation	-
	5- Necrosis	-
Stem, roots, leaf veins & petioles	Tumorlike growths	-Characteristic of plant members of the Reoviridae.

* = Matthews, (1980).

Because of the physical structure of plants, the cuticle and the cellulose walls must be penetrated before virus particles can gain entry into the cell. Thus many plant viruses have formed associations with insects, nematodes, or other organisms that can induce the necessary wounds and in most cases these associations are highly specific. Other mechanisms for mechanical damage include the breakage of leaf hairs, implicated in the spread of some viruses that accumulate to high concentration such as TMV and PVX, and the act of fertilisation.

1.8.1- Cytopathological effects of plant virus infection:

1.8.1.1- Nuclear changes:

Among the most important cytological effects of virus infection are changes in cell nuclei. Various viruses have been observed within the nucleus, including southern bean mosaic (SBMV) (Weintraub and Ragetli, 1970), tomato bushy stunt and many rhabdoviruses (Russo and Martelli, 1972). Pea enation mosaic virus (PEMV) has been observed to cause the breakdown of nuclei as the virus multiplies in infected pea leaves (Shikata and Maramorosch, 1966) and the nucleolus of the bean plants infected with bean golden mosaic virus (BGMV) has been reported to increase in size to fill three-quarters of the nuclear space (Kim, *et. al.*, 1978).

Plant rhabdoviruses which assemble in the perinuclear space have been reported to cause distortions to the nucleus accompanied by accumulation of the particles in the perinuclear spaces. Internal disorganisation of the nuclei may also occur involving reduction or disappearance of chromatic material, swelling of the nucleolus and appearance of viroplasm-like structures (Chen and Shikata, 1968; 1971; Lee, 1970; Christie, *et. al.*, 1974; Martelli and Russo, 1977; Ismail, *et. al.*, 1987; Ismail, 1988). Ismail and co-workers carried out an extensive examination of the ultrastructural effects of SYNV infection on the cells of *N. edwardsonii* (Ismail, *et. al.*, 1987; Ismail, 1988). They found that virions often accumulated in disordered inclusions around the nucleus and caused the swelling of the nucleus. Viroplasm-like regions have been observed in the nuclei of some infected cells in early stage of infection. The chromatin was less abundant, its distribution was altered, being commonly located adjacent to aggregates of nucleocapsids at the periphery of these nuclei. When these sections of infected leaves were immunogold labelled using anti-SYNV antibodies, particles reacted very extensively to the granular fibrallar viroplasm regions, but not to the chromatin, indicating the presence of large amounts of free viral proteins within

the viroplasm. These data, combined with the presence of virions in the perinuclear spaces suggests that the nucleus has a crucial role in SYNV assembly (Jackson, *et. al.*, 1987).

1.8.1.2- Effects on mitochondria:

Alteration in mitochondrial ultrastructure have been reported following the infection with many plant virus. The long rod particles of tobacco rattle virus have been reported closely appressed to mitochondrial membrane without penetrating it in infected cells (Harrison and Roberts, 1968; Kitajima and Costa, 1969). Mitochondria in cells of various hosts infected with cucumber green mottle mosaic virus developed small vesicles bounded by a membrane and which lay within the perimitochondrial space and in the cristae (Hatta, *et. al.*, 1971; Hatta and Ushiyama, 1973; Sugimura and Ushiyama, 1975). It is generally reported that normal mitochondria or degenerated ones may aggregate during virus infection of *Datura* cells by henbane mosaic potyvirus (Kitajima and Lovisolo, 1972). SYNV infection of *N. edwardsonii* caused dramatic effects on mitochondria. In later stages of infection, mitochondria were substantially altered. Enlarged, very irregularly shaped multiple vesicles of different sizes were observed (Ismail, 1988). In his study, mitochondria in infected cells, observed in ultrathin sections, had lost most of their cristae and some of the mitochondrial matrix early in infection. Clusters of electron dense finely granulated material were observed within the matrix, and in some infected cells, mitochondria were often difficult to recognise (Ismail, 1988). However, there is no direct evidence that mitochondria play any part in plant rhabdovirus replication (Peters, 1981; Ismail, 1988).

1.8.1.3- Effects on chloroplasts:

Two views have emerged to explain the primary effect of virus infection on leaves: (1) the view of Beijerinck and Konig (1899), that virus infection is primarily a disease of chloroplasts and manifests itself by chloroplast damage

and ultimate disintegration (Nelson, 1932); and (2) that the primary effect of virus infection does not involve chloroplasts, may lead to a reduction in the number of chloroplasts but it does not initially cause major changes in their photosynthetic activity (Woods, 1909; Ivanowski, 1903). Although chloroplast activity may be altered in the late stages of disease, such changes would be a result of the infection rather than a primary cause of the infection (Buchanan, *et. al.*, 1981).

Chloroplasts of Chinese cabbage plant infected with Turnip yellow mosaic virus (TYMV) were morphologically and biochemically altered by infection (Bedbrook and Matthews, 1972, 1973). Infection caused a shift in photosynthetic products from sugar to organic acids and amino acids. Such a shift was also reported by other investigators examining various virus diseases (Platt, *et. al.*, 1979). Bove and Bove (1985) demonstrated that TYMV replicates at the chloroplast envelope of Chinese cabbage leaf cells. Chloroplasts became rounded and clumped together in the cell, and eventually developed large vesicles or fragment. At such a stage, photosynthetic activity may be considerably reduced (Matthews, 1980, 1981). Not all plant viruses have a severe effect on the photosynthetic activity of chloroplasts. In TMV-infected tobacco, photosynthesis is not reduced. Chlorophyll content in the light areas decreased, but this loss was compensated for by higher chlorophyll content in the dark areas (Platt, *et. al.*, 1979). In contrast, in many other mosaic or yellows diseases, photosynthesis was severely reduced, even when calculated on a chlorophyll basis. Yellowing such as induced by beet yellows virus in sugar beet, decreased photosynthesis up to about 50% (Hall and Loomis, 1972). In peanut infected with peanut green mosaic virus, leaves developed a light green mosaic. Although chlorophyll loss was relatively small, photosynthesis was severely reduced due to an inhibition of electron transport at the reducing site of photosystem II. It has been suggested that this inhibition results from a decrease in the content of plastoquinone (Naidu *et. al.*, 1984a, b). Although TMV replicates and

accumulates primarily in the cytoplasm, virus-like particles have been observed in chloroplasts (Esan, 1968). More recently, both TMV coat protein (Reinero and Beachy, 1986; 1989) and TMV genomic RNA (Schoelz and Zaitlin, 1989) have been isolated from percoll-purified chloroplasts. Reinero and Beachy (1989) found a positive correlation between the amount of coat protein found in chloroplasts and the severity of visible symptoms. Work with a severe strain of TMV by Shalla and co-workers (Ganett and Shalla, 1970; Shalla, 1968) demonstrated that chloroplasts from tissue infected with the U5 strain of TMV often contained many virus-like particles whereas chloroplasts from tissue infected with U1 strain contained few. Nevertheless, the U1 strain induced more severe symptoms than those induced by the U5 strain. These results (Holt *et. al.*, 1990) demonstrate that while TMV is responsible for the development of mosaic symptoms in systemically infected leaves, the correlation between the presence of coat protein or virions in chloroplasts and symptom expression may be coincidental rather than causative. Recent work (Culver, *et. al.*, 1991) indicates that the 126/183k gene production may be somehow involved in the prevention of normal chloroplast development in systemically infected leaves.

The morphological changes in chloroplasts from plants infected with rhabdoviruses have been very well documented (Francki and Randles, 1980). *N. glutinosa* leaf cells infected with LNYV were examined and within one day of symptom appearance, chloroplast ribosomes declined in concentration and were completely undetectable 1 to 3 days later. The chloroplasts were also reduced in size (Randles and Coleman, 1970, 1972). Electron microscopy of infected chloroplasts showed displacement and disorganisation of the lamellae the appearance of vacuoles, osmiophilic granules, and other inclusions (Francki and Randles, 1980). Alterations in *N. edwardsonii* chloroplasts infected with SYNV have been reported. Loss of the thylakoid network, development of membrane-bound inclusion bodies and the accumulation of a large amount of starch within the chloroplasts were observed. In addition, immunogold labelling has indicated

the presence of free virus protein within chloroplasts (Ismail, 1988).

1.8.1.4- Effects on the cell wall:

Abnormalities have been observed in or near the cell wall of infected cells. Abnormal thickening has been observed, due to the deposition of callose within infected cells and cells at the edge of virus-induced lesions (Hiruki and Tu, 1972). Other changes observed between the cell wall and the plasma membrane were depositions of electron-dense material induced by oat necrotic mottle virus (Gill, 1974); these may be associated with plasmodesmata as observed with barley stripe mosaic virus (BSMV) (McMullen, *et. al.*, 1977).

1.9- Defective interfering particles (DI):

Repeated passage of many types of animal virus results in attenuation of virulence. This attenuation often follows the generation of defective interfering (DI) particles (Huang and Baltimore, 1977). DI particles contain the same protein components as the standard virus, require the presence of standard virus for replication and interfere with the replication of standard virus (Huang, 1973). Rhabdovirus DI particles are more extensively characterised than those of any other virus because of the relative ease of separating rhabdovirus DI particles and nucleocapsids from each other and from infectious virus. The generation of DI particles of vesicular stomatitis virus (VSV) has been well studied (Huang and Wagner, 1966; Bellett and Cooper, 1959; Holland, 1987). Following several passages of the virus, short particles accumulated near the cell surface. Their genomic RNAs differ from the standard virus as the result of deletion and rearrangements. These rearrangements involve the termini and sometimes internal regions of the genome (Faulkner and Lazzarini, 1980).

The plant rhabdoviruses closely resemble the animal rhabdoviruses with which they share many biochemical and morphological properties (Jackson, *et. al.*, 1987). PYDV has been reported to generate a defective strain following

repeated passages of sap from PYDV-infected leaves to *N. rustica* (Adam, *et. al.*, 1983). Plant virus defective strains may be generated by mutations, deletions or, in the case of viruses with multipartite genomes, loss of entire genome segments of the parental virus (see Roux, *et. al.*, 1991). Recently Ismail and Milner (1988) isolated defective interfering particles from *N. edwardsonii* chronically infected with SYNV. These DI particles were generated naturally during chronic infection of individual plants and characterised by a short bacilliform particles ranging from 130 nm to near full size 210-220 nm (Ismail, *et. al.*, 1987). Analysis of their genomes suggest that they arise by sample deletion encompassing most of the L gene and extending into adjacent gene (McElwee and Milner, unpublished results).

1.10- Aim of this work:

The aim is to investigate two plant rhabdoviruses from subgroup II, (SYNV and EMDV), in particular their spread and symptom expression in different hosts of *Nicotiana species*. The project involves an investigation into the multiplication and behaviour of SYNV and EMDV in three species of *Nicotiana* (*N. glutinosa, N. clevelandii* and *N. edwardsonii*) and their interaction with the plant hosts. Although the effect of SYNV infection in *N. edwardsonii* has been studied extensively, further investigation is needed to determine whether the virus has different effects on different hosts.

1- Where is the virus located within infected tissues and is this different with different hosts?

2- Does virus protein concentration vary with different stages of infection and with different hosts?

3- Does virus multiplication remain at the same level during the infection period and if not, what is or are the factor(s) which change during the process?

4- Given the previous reports of ultrastructural changes in chloroplasts of

infected plants, can any changes in photosynthetic function on the host be measured directly?

Chapter Two

Materials and Methods

Materials and methods

2.1- Source of materials:

2.1.1- Source of seeds:

Nicotiana edwardsonii (*N. clevelandii* x *N. glutinosa*; Christie, 1969), *N. clevelandii* and *N. glutinosa* were allowed to flower and used as sources of seeds.

2.1.2- Source of inoculum:

Sonchus yellow net virus, type isolate (ATCC PV-263) was originally obtained from Prof. A. O. Jackson. (Formerly of Purdue University, West Lafayette Indiana, USA) in 1981 and was maintained in *N. edwardsonii* by regular passage in the greenhouse.

Eggplant mottled dwarf virus, type isolate (DSM-0031) was originally obtained from Dr. G. Adam (D.S.M. Braunschweig, Germany). The virus was maintained in *N. edwardsonii* or *N. glutinosa* by regular passage in the greenhouse.

2.2- Growth conditions and inoculation:

2.2.1- Germination and growth of plants:

Seeds of *N. edwardsonii, N. clevelandii* and *N. glutinosa* were surface sterilised by soaking in 0.3-0.5% (v/v) bleach (Chlorox) for 16 hr or overnight at room temperature, recovered by filtration through Whatman N0·1 filter paper and air dried at room temperature.

Sterilised plant seeds were scattered on the surface of sterile damp potting compost in a tray, covered with a thin layer of the same compost and maintained at 23-25^{o}C in the greenhouse. Seedlings appeared above the surface

7-10 days after sowing and were transplanted individually into different size pots containing sterile potting compost to grow.

All plants were grown in the greenhouse with supplemental lighting (16 hours light provided by 400W lamp and 8 hours dark) at 23-25^{o}C.

2.2.2- Inoculation of plants with SYNV and EMDV:

Once the plants had the first four to six fully expanded leaves for SYNV or ten leaves when used for EMDV, they were inoculated with the virus.

The mortar and pestle, and 9-12 cm square pieces of muslin were autoclaved for 15 min at 15lb/in^2 and then chilled on ice-cold. Leaves from well-infected plants, usually 2 to 3 weeks after inoculation were ground up in 1% Na_2SO_3 in a ratio of approximately 1g leaf material to 1 ml solution, in the mortar and pestle (Christie, *et. al.*, 1974). At least three to four leaves from each plant were lightly sprinkled with carborundum (silicon carbide, super fine, about 600 grit. BDH) which act as an abrasive. The inoculum was soaked up into the muslin pad and rubbed on to the carborundum-dusted leaves. The inoculated plants were then left to grow as described above.

Some plants were inoculated with EMDV at a later stage (10 leaves or over). The plants inoculated at this stage live much more longer than those inoculated at an earlier stage, these usually died very soon after the symptoms appeared.

2.3- Purification of SYNV and EMDV:

Both viruses were purified by the method described by Jackson and Christie (1977) with some modifications (Milner and Jackson, 1979). The buffers used in the procedure were autoclaved before use and all the steps were carried out on ice.

Systemically infected leaves showing normal vein-clearing symptoms were harvested 3-4 weeks post-inoculation. Usually between 100-150 g of fresh infected leaves were used. Infected leaves were processed immediately or stored at 4°C for not longer than two days. They were hand cut into small pieces and placed in a large liquidizer with viral extraction buffer (VEB) in the ratio of 1g fresh leaf to 2-3 ml of cold VEB (0.1M tris-HCl pH 8.4; 0.01M magnesium acetate; 0.04M Na_2SO_3; 0.001 $MnCl_2$). VEB was prepared, autoclaved and stored in 4°C and used next day; the last two components were added just before use. Infected leaves were homogenised for 30-50 seconds and filtered through two layer of muslin. The resulting suspension was centrifuged at 3,000-4,000 rpm in a Beckman J2-21 centrifuge for 10-15 min. The supernatant was filtered through two layers of soft Kleenex tissue. The filtrate was layered onto tubes 25.4x89 mm polyallomer centrifuge over discontinuous sucrose gradients formed from 8 ml (bottom) and 5 ml (top) of 60% (w/v) and 30% (w/v) respectively, sucrose was made up in cold viral maintenance buffer (VMB) (0.1M tris-HCl pH 7.5; 0.01M magnesium acetate; 0.04M Na_2SO_3; 0.001M $MnCl_2$). VMB was prepared from stock as described above for VEB. These discontinuous gradients were centrifuged in an AH629 or AH627 rotor at 27,000 rpm for 45 min in a Sorvall OTD-65B ultracentrifuge. Three layers were visible in each tube. The green band between the pale yellow upper layer (30% sucrose layer) and the lower clear layer (60% sucrose) was collected, combined from each tube and diluted with an equal volume of VMB.

A celite pad was prepared by suspending celite (diatomaceous earth, Sigma), sufficient to fill three 50 ml beaker in the case of SYNV purification and two beakers in the case of EMDV, in 250 ml of VMB. This was carefully poured over two Whatman N0.1 filter papers in a Buchner funnel (15 cm in diameter) and the liquid pulled through under gentle vacuum. The celite pad was washed with about 100-150 ml VMB and stored in 4°C.

The crude preparation from the discontinuous gradients was mixed with sufficient celite to half fill a 50 ml beaker. The mixture was vacuum-filtered through the celite pad, followed by about 100 ml VMB. The light brown filtrate containing the virus was centrifuged in the Sorvall T865 rotor at 30.000 rpm for 25 min. The pellets were collected and suspended in 2-4 ml VMB. The concentrated virus suspension was layered onto 5-30% sucrose gradients (prepared in VMB) and centrifuged in the Sorvall AH629 or AH627 rotor at 25,000 rpm for 20 min at 4°C. Gradients were fractionated and scanned at 254 nm using an ISCO Model 185 density gradient fractionator attached to an ISCO Model UA5 absorbance monitor. The light milky light-scattering virus bands were collected in a centrifuge tube, diluted with VMB to the shoulder of the tube and centrifuged in the Sorvall T865 rotor at 30,000 rpm for 30 min at 4°C. The pellet was resuspended in 1-2 ml VMB and stored in liquid nitrogen (-196°C).

2.3.1- Partial purification of SYNV and EMDV:

Partial purification of SYNV and EMDV was carried out as described by Jones and Jackson (1990). The procedures were the same as described above except that the centrifugation through discontinuous sucrose gradients was omitted. The supernatant from the first low speed centrifugation was passed through a celite pad as described above. The resulting straw-colored filtrate was collected and centrifuged in a T865 rotor for 35 min at 40,000 rpm. The supernatant was decanted and the pellets from each tube were resuspended in a minimal volume (0.5-1 ml) of VEB. A 40% aqueous stock solution of PEG (polyethylene glycol, MW 1540, Polysciences) was added to the highly enriched virus samples to obtain a final concentration of 2% PEG and aliquots were stored at -70°C or in liquid nitrogen.

2.3.2- Determination of the virus protein concentration:

The coomassie blue binding assay (Bradford, 1976) was used to determine the concentration of virus protein in preparation of SYNV and EMDV. Bovine serum albumin (BSA) was used as a standard.

Two mg/ml BSA was prepared in sterile water and used as a stock. Concentrated dye-reagent (Bio-Rad) was diluted to 1:4 with sterile water and filtered through Whatman N0.1 filter paper. Duplicate aliquots of 5 µl, 10 µl, or 20 µl of purified SYNV or EMDV and the same of 2 µl, 5 µl, 10 µl or 20 µl of BSA stock solution were added to a series of tubes containing 1 ml of diluted dye reagent and the final volume made up to 1.1 ml with sterile water. The A_{595} of each sample was determined against a blank sample containing 1 ml diluted dye-reagent and 100 µl sterile water. The A_{595} for BSA samples were plotted against concentration and used to determined the concentration of virus protein.

2.4- Gel electrophoresis:

2.4.1- Electrophoresis of proteins on polyacrylamide gels:

Protein from purified SYNV and EMDV and total cellular plant proteins were fractionated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) using the method described by Laemmli (1970).

Gels were formed between two glass plates (19.5x18 cm and 16.5x18 cm, separated by 1.0 mm or 2.0 mm thick rubber spacer). The plates were thoroughly washed with detergent (Decon 90), rinsed with distilled water, wiped with methanol and air dried before use. The stock solutions (prepared as shown in table 2.1) were prepared and stored at 4°C.

Table 2.1: Solutions for gel electrophoresis:

Solution	Chemicals
Acrylamide	- 30% (w/v) Acrylamide (Koch-Light. - 0.66% (w/v) N'N'methylene-bis-acrylamide.
Stacking gel buffer	- 0.5M tris-HCl, pH 8.3. - 0.4% (w/v) SDS.
Separating gel buffer	- 1.5M tris-HCl, pH 8.8. - 0.4% (w/v) SDS.
Electrolyte buffer	- 25mM tris-HCl-base. - 0.192M glycine. - 0.2% (w/v) SDS. pH adjusted to 8.3-8.4 with HCl.
Boiling buffer	- 62.5mM tris-HCl, pH 8.0. - 2% (w/v) SDS. - 10% (v/v) glycerol. - 2% (v/v) 2-mercaptoethanol. - 0.001% (w/v) bromophenol blue.

Gels were prepared to a final acrylamide concentration of either 12.5% or 15% for the separating gel and 4.5% for the stacking gel using stock solution as indicated in table 2.2.

Table 2.2: The amount of forming electrophoresis gels:

Solution	Separating gel		Stacking gel
	12.5%	15%	4.5%
30% acrylamide	8.3 ml	10 ml	3 ml
Separating gel buffer	5 ml	5 ml	-
Stacking gel buffer	-	-	5 ml
Distilled water	6.7 ml	5 ml	12 ml
10% (w/v) APS*	0.2 ml	0.2 ml	0.2 ml
TEMED$	0.05 ml	0.05 ml	0.02 ml

*APS = ammonium persulphate, freshly prepared.
$TEMED = N'N'N'N'-tetramethylenediamene.

The separating gel solution was first applied to the pre-assembled plates, leaving about twice the length of the comb teeth to a stacking gel. Three to five ml of distilled water were carefully applied on the top of the separating gel to form a flat interface. After the separating gel had polymerised, the water at the interface was removed, the stacking gel was added and the comb was put in place. The gel was allowed to polymerise.

The protein samples were denatured by adding 1-2 volumes of boiling solution and boiled for 2 min. Standard marker proteins of known molecular weight (BDH, Limited) were run on each gel. Gels were stained with Coomassie Brilliant Blue or silver stained.

2.4.1.1- Coomassie Brilliant Blue staining for proteins in polyacrylamide gels:

The gels were stained overnight with approximately 500 ml of 0.2% Coomassie Brilliant Blue R250 in 10% acetic acid and 7% methanol and destained in 10% acetic acid for appropriate changes over a period of 24 hr.

2.4.1.2- Silver stain for proteins in polyacrylamide gels:

The gels were stained by the method described by Morrissey (1981). The gels were prepared as described in (section 2.4.1). They were prefixed in 50% methanol and 10% acetic acid for 30 min, followed by 5% methanol and 7% acetic acid for another 30 min. Then the gels were fixed for 30 min in 10% glutaraldehyde and rinsed in a large volume of distilled water overnight or with several changes for 2 hr each. The gels were then soaked for 30 min in dithiothreitol (DTT) (50 mg/ml). The DTT solution was poured off and without rinsing, 0.1% (w/v) silver nitrate was added to the gels and left for 30 min. The gels were then rinsed very rapidly with a small amount of distilled water and then twice rapidly, with a small amount of developer (50 µl of 37% formaldehyde in 100 ml of 30% (v/v) sodium carbonate). The gels were soaked in developer until the desired level of staining was attained. Developing was stopped by adding 5 ml of 2.3 M citric acid directly to the developer and leaving for 10 min. This solution was then discarded and the gels were washed several times in distilled water. The gels were handled carefully with rinsed plastic gloves throughout the procedures.

2.5- Electron microscopy:

2.5.1- Standard preparation:

Samples of healthy or infected tissues (approximately 1-2 mm square pieces) were fixed in 3% glutaraldehyde in 0.2 M sodium cacodylate buffer pH 7.2 at room temperature for 12-16 hr. Samples were initially in filtered under vacuum to remove air and allow penetration of the fixative and thoroughly washed in 0.2 M sodium cacodylate buffer for four changes over 24 hr, followed by fixation in 1% (v/v) OsO_4 in the same buffer for 3 hr. They were then washed in distilled water (3 changes over 30 min) then block-stained in 2% (w/v) aqueous uranyl acetate for 2 hr. Samples were dehydrated through graduated

ethanol series (25%, 50%, 75%, for 2 hr each and overnight in 100%), embedded in Spurr resin and polymerised at 60°C for 24 hr.

Section of approximately 60 nm thickness sections were cut on an LKBIII ultramicrotome using a diamond knife and mounted on 300 mesh copper grids. Sections were stained with saturated uranyl acetate in 50% methanol for 20 min, and lead citrate (Reynolds, 1963) for 5 min. The grids were examined using a Philips EM 301, JEOL 100C or ZEISS 902 electron microscope at 60 and 80KV.

2.5.2- Sample preparation for immunogold labelling:

Samples treatment was identical to the standard preparation method (section 2.5.1) except that post-fixation with OsO_4 and block-staining with uranyl acetate were omitted. After being dehydrated, embedded in LR-white resin and polymerised for 30 hr. Sections were mounted on 300 mesh nickel grids. After immunogold labelling, the sections were stained with 2% aqueous uranyl acetate and lead citrate (Reynolds, 1963).

2.6- Detection of virus protein:

2.6.1- Preparation of antisera:

Anti-SYNV and anti-EMDV antisera were raised under licence by Dr. I. D. Hamilton. (Department of Biochemistry, Glasgow University). The anti-SYNV antisera were raised against total virus proteins (type strain) and have been described previously (Ismail, *et. al.*, 1987). The antiserum against the SYNV G protein was a kind gift from Mr. J. Allen.

For the preparation of anti-EMDV serum, virus was purified from *N. edwardsonii*. Virus concentration was determined as described in sections 2.3 and 2.3.2. The purified EMDV (750 μg protein) was divided into three; one part was emulsified in 50% Freund's complete adjuvant and injected subcutaneously

at different sites in a New Zealand white rabbit. Six weeks later the rabbit was boosted with the second injection of the virus as before. Two weeks later, the rabbit was injected with the third injection (250 μg virus proteins).Two weeks later, blood was collected, allowed to coagulate, the clot removed and the whole sera stored in 1 ml aliquots at -20°C.

2.6.2.- Pre-absorption of anti-virus antiserum:

Although, purified virus samples still might have contained low levels of contaminating plant material protein which were potentially immunogenic. Any antibodies to the host proteins were removed from anti-EMDV serum by incubating the latter overnight at 4°C with crude sap from healthy *N. edwardsonii* plants in a ratio 1:4. Pre-absorbed antiserum was recovered following 5 min centrifugation at 13,000 rpm in microcentrifuge. This removed precipitates formed from the reaction between antibodies to host proteins present in the healthy sap. The supernatant was stored in 0.5 ml aliquots at -20°C.

2.6.3- Preparation of immunoglobulin G from anti-EMDV:

IgG was prepared from anti-EMDV antiserum by salt precipitation using the method described by Clark, *et. al.*, (1986) with some modification.

One to two ml of saturated $(NH_4)_2SO_4$ was added very slowly to 2 ml of anti-EMDV serum while steering. A flocculent precipitate developed, this was collected by centrifugation for 10-15 min at 3,000 rpm. The pellet was resuspended in PBS (170 mM NaCl; 3.4 mM KCl; 10 mM Na_2HPO_4; 1.8 mM KH_2PO_4. pH 7.2). The resuspended pellet was precipitated again as above a total of 3 times. The precipitate was dissolved in half strength PBS transferred to dialysis tubing and dialysed against 4 changes of 500 ml of half-strength PBS over a period of 32 hr. The final preparation of IgG was stored in 0.5 ml aliqouts at -20°C.

2.6.4- Immunogold labelling of cell and tissue for electron microscopy:

Sections of SYNV and EMDV infected and healthy plant tissues were fixed and cut as previously described (section 2.5.2). These sections were labelled at room temperature in petri dishes containing sheets of dental wax (Agar Aids). All buffers, solutions and distilled water were filtered through Whatman No.1 filter paper before use. Grids were handled throughout the procedures with sections face downwards.

Grids were placed for 30-45 min on droplets of 20-30 µl of immunogold labelling buffer (IGL) (20 mM Tris-HCl; 0.5 M NaCl; 0.05% (v/v) Tween 20; 0.1% (w/v) BSA; pH 7.4). Five percent (v/v) normal goat serum (Scottish Antibody Production Unit) was added to IGL. The grids then were washed in 20 µl droplets of distilled water, 4 changes of 5 min each. The grids transferred to the droplets of 20 µl of IgG (diluted to 1:15 with IGL) and then incubated for 90 min at 20°C. Sections were incubated with wet tissue in petri dishes to keep the moist. Unbound IgG was removed by washing the grids 5 times with 20 µl droplets of IGL buffer for 5 min each. The grids were transferred to 20 µl droplets of gold conjugated goat-anti-rabbit IgG diluted 1:20 with IGL buffer, and incubated as above for 90 min. Grids were washed thoroughly in a series of five 50 µl droplets of microfiltered water for 5 min each, drained onto filter paper and dried at room temperature. Sections were stained with 2% uranyl acetate and lead citrate and examined by electron microscopy.

Two controls were carried out for both healthy and infected tissues; anti-EMDV IgG was omitted and replaced with IGL buffer, and anti-EMDV IgG was replaced by non-immune rabbit serum.

2.6.5- Enzyme-linked-immunosorbent assay (ELISA) for virus protein:

Plant leaf tissue of healthy or infected with SYNV or EMDV were homogenised using a glass rod homogenizer with 1:5 volumes of PBS-TPO

(0.136 M NaCl; 9.2 mM Na_2HPO_4; 0.87 mM KH_2PO_4; 2.68 mM KCl; 0.05% (v/v) Tween-20; 2% (w/v) polyvinylpyrollidone (PVP) and 0.2% (w/v) BSA; pH 7.2, Clark, *et. al.*, 1986) and clarified by centrifugation for 2 min in a microcentrifuge. Homogenisation procedures were carried out at 4°C in an ice bucket and the samples were stored at -20°C. One hundred µl of each sample were made up to a final 2% SDS, heated to 65°C for 5 min and cooled at room temperature. Ten µl of each denatured sample were diluted (1:25 to 1:1600) with coating buffer (15 mM Na_2CO_3; 35 mM $NaHCO_3$; pH 9.6) containing 5% (v/v) normal donkey serum added just before use.

ELISA was carried out according to the method of Lommel *et. al.*, (1982). Duplicate 200 µl sample diluted with coating buffer were applied to the microtitre wells (U-well plate, Sterilin Ltd, Feltham, England) and incubated for 90 min at 35°C. The wells were emptied to remove unattached antigen, washed with buffer (0.154 mM NaCl; 0.05% (v/v) Tween-20; pH 7.4) and left for 1 min. Three further washes were carried out, any residual liquid was shaken out and the plates were dried by inverting them on the tissue at room temperature. Preabsorbed anti-EMDV or anti-SYNV antiserum was diluted 1:75 with PBS-TPO containing 5% normal donkey serum, 200 µl were added to each well and the plates then incubated, washed and dried as above. Donkey anti-rabbit IgG conjugated to horseradish peroxidase (Scottish antibody production unit) was diluted 1:1000 with PBS-TPO containing 5% normal donkey serum and 200 µl added to each well. The plates were then incubated, washed, and dried as above.

The developer contained O-phenylenediamine 0.5 mg/ml in 25 mM sodium acetate pH 5.5 and 0.03% (v/v) H_2O_2 (100 volume). One hundred and fifty µl were added to each well and left for 5-10 min in the dark. The reactions were stopped by adding 50 µl of 4 M H_2SO_4. The plates were shaken gently and absorbance at 492 nm was determined using a Titertek multiscan MC or Anthos reader 2001 plate reader.

Known concentration of purified EMDV or SYNV were run in parallel with the samples from infected plants, as well as samples from healthy plants. For an additional control, anti-EMDV or anti-SYNV serum was omitted and replaced with PBS-TPO buffer.

2.6.6.- Immunoblotting of EMDV and SYNV:

Protein samples from purified SYNV, EMDV, healthy and infected plant tissues were prepared and fractionated by polyacrylamide gel electrophoresis as described previously (section 2.4.1). Proteins were blotted onto a nitrocellulose membrane (Schleicher & Schuell, BA 85) by the contact diffusion method (Bowen *et. al.*, 1980) or by electrophoretic transfer (Burnette, 1981, with minor modification).

The contact-diffusion method was carried out by using a glass plate placed over a larger plastic tray containing 1.5L of transfer buffer (25 mM tris-HCl; 192 mM glycine; 20% (v/v) methanol) (Towbin *et. al.*, 1979; Burnette, 1981). A piece of 3MM Whatman paper (35x15 cm) was layered over the glass plate, forming a bridge.

After running the gel, the stacking gel was removed, and separating gel was carefully placed on the top of the pre-wetted 3 MM Whatman paper bridge. A piece of nitrocellulose membrane cut to size 1 cm longer than the separating gel on each side, was prewetted with transfer buffer and carefully layered on the top of the gel. Care was taken to avoid any air bubbles between the 3 MM Whatman paper and the separating gel, and between the gel and the nitrocellulose membrane. Ten dry pieces of 3 MM Whatman paper (the same size as the separating gel) were layered on the top of the membrane, followed by a glass plate, larger than the size of Whatman paper and a 2 to 2.5 Kg weight placed over the glass plate to ensure a good contact between the gel and the membrane. Transfer was left to continue for 24 to 36 hr at room temperature.

Electrophoretic transfer of samples proteins was carried out by the method described by Burnette (1981). A "sandwich" was prepared with the following successive layers; a black plastic plate cassette followed by a foam pad, a sheet of Whatman No.1 filter paper followed by the polyacrylamide gel (stacking gel removed). The nitrocellulose membrane was placed in contact with the gel, care being taken to avoid air bubbles between them, followed by filter paper, foam pad and finally the white plastic cassette plate. All components in contact with the gel were pre-wetted in the electrode buffer (20 mM tris-HCl; 150 mM glycine, and 20% methanol). The sandwich was secured with clips and inserted between the electrodes of a Canalco gel destainer with the nitrocellulose toward the anode. The chamber was filled with transfer solution and electrophoretic transfer carried out at 40 mA overnight. The membrane was removed, air dried, and the proteins were fixed to the membrane by 15 min incubation at 4^{o}C (Hawkes. *et. al.*, 1982).

Two buffers were prepared, TBS buffer (0.05 M tris-HCl; 0.2 M NaCl; pH 7.4) and blocking solution buffer (3% (w/v) BSA or 5% (w/v) non fat dried milk powder made up in TBS and containing 5% (v/v) normal donkey serum). The filters were individually placed into a convenient tray containing blocking solution (0.5 ml/cm^2), and incubated at room temperature with gently shaking for 10-20 min. Blocking solution was removed by aspiration and the filters reacted with anti-SYNV or anti-EMDV antiserum. Preabsorbed serum was diluted 1:500 with blocking solution; 0.5 ml per cm^2 was added to the filters and incubated as above but for 90 min. Unbound antiserum was removed followed by three washes of 5 min each with TBS buffer (100 ml each) with gentle shaking. Donkey anti-rabbit IgG conjugated with horseradish peroxidase was diluted 1:1000 with blocking solution; 0.5 ml of this per cm^2 was added to the filters and incubated for 90 min as above. Unreacted second antibody was removed by five washes with 100 ml each of TBS buffer. To detect the antibody-antigen complex, 4-chloro-1-naphthol (97%, Aldrich Chemical Co. Ltd; 3 mg/ml in methanol) was

diluted 1:4 with TBS containing 0.01-0.03% (v/v) H_2O_2 (100 volume) prepared freshly on each occasion. Filters were incubated with 0.5 ml/cm^2 of the above developer; positive signals appeared as blue bands against the white background of the membrane. Filters were washed several times with distilled water, dried between 3 MM Whatman paper at room temperature and stored in the dark until ready for photography.

2.7.- Measurement of photosynthetic oxygen evolution:

Photosynthetic oxygen evolution was carried out using a Hansatech gas leaf electrode (Hansatech Ltd., Paxman Road, Hardwick Industrial Estate, King's Lynn, Norfolk) as described by Delieu and Walker (1981).

2.7.1- The principle of oxygen measurement:

In photosynthesis, light energy is absorbed by chlorophyll and used to drive the reduction of carbon dioxide to carbohydrate. The major end-product of photosynthesis in higher plants is usually sucrose and temporally as starch. These substances can be represented by a purely nominal carbohydrate (CH_2O) and the overall process by the equation:

$$CO_2 + H_2O \text{-----}h\nu\rightarrow CH_2O + O_2$$

Accordingly, if a leaf is enclosed in a chamber and provided with carbon dioxide and then illuminated, oxygen will be evolved. In the Hansatech LD2, a leaf-disc is used and CO_2 is provided in the gas-phase or in form of sodium biocarbonate.

$$NaHCO_3 \text{----------}\rightarrow NaOH + CO_2$$

The oxygen which accumulates in the gas-phase during photosynthesis can then be detected, polarographically, by a "Clark-type" Pt/Ag/$AgCl_2$ electrode (Delieu and Walker, 1972). The O_2-electrode is contained in a hard plastic disc consists of an electrochemical cell containing a relatively large (2 mm) platinum

cathode and a silver anode immersed in a solution of KCl. The cathode at the centre of the plastic disc and the silver anode in a circular groove (electrolyte reservoir). The electrode is protected by a thin wick (cigarette paper) and a thin polythene membrane, which is permeable to oxygen, is secured smoothly over the surface of the platinum cathode by an O-ring. The diagram of a gas phase oxygen electrode shown in figure (2.1)

2.7.1.1- The leaf chamber:

The leaf chamber is made of anodised aluminium which affords good temperature control when circulated by a thermostatically-controlled water bath (23 -/+ 0.01°C). The chamber itself is cylindrical and accommodates a leaf disc of 10 cm^2 area on a perforated stainless steel plate which prevents light falling directly on the cathode. The electrode rests on the base which presses it against an O-ring so that an air-tight seal is effective. The cathode is exposed to the atmosphere in the leaf chamber through a small hole in its floor. The roof of the chamber is the Perspex window of the upper water jacket, through which the leaf is illuminated. When the chamber is closed, the upper section is fixed in position by two clips supported by O-ring to give an air-tight seal. Two taps, fitted to the outside of the leaf chamber, enable the chamber to be calibrated by flushing with air or nitrogen. To prevent the carbon dioxide content of the leaf chamber becoming exhausted, sodium bicarbonate ($NaHCO_3$) buffer was used as a source of CO_2. The buffer (0.4 ml of 1M solution) was applied to the capillary matting to facilitate rapid equilibration. The leaf tissue was protected from the alkaline buffer by a second stainless steel disc and a foam rubber disc which were also inserted into the chamber.

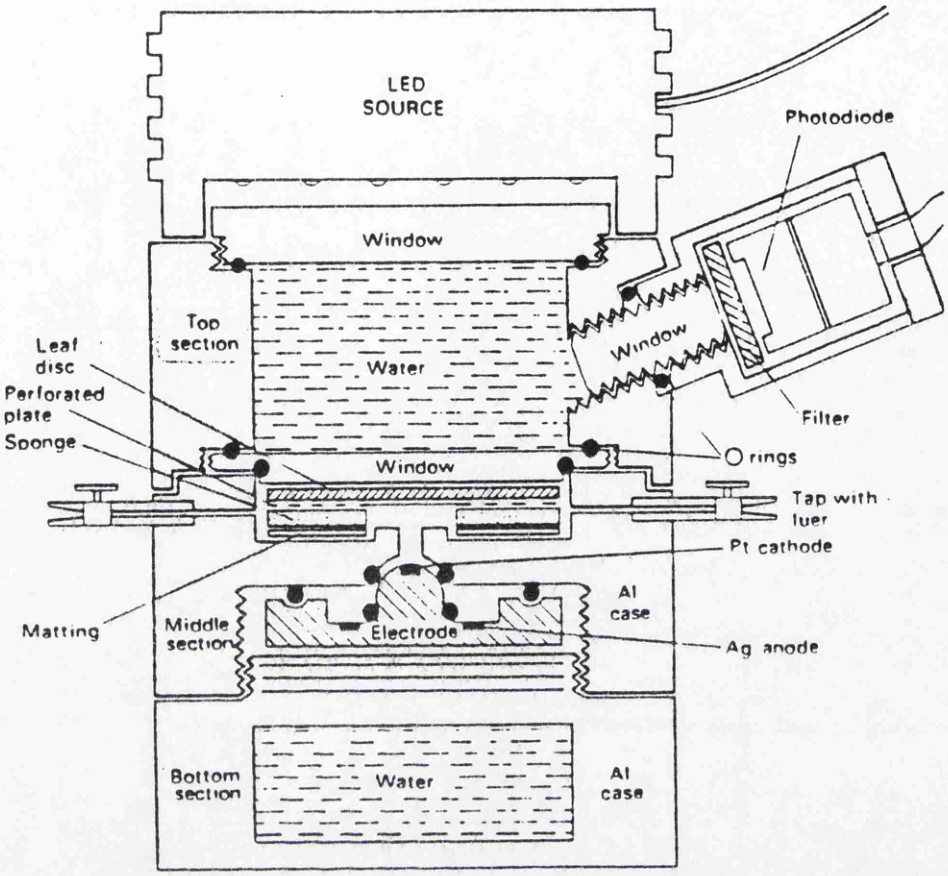


Figure (2.1):

Schematic diagram of gas-phase oxygen electrode. The leaf-disc, or leaf pieces, are supported on a stainless steel mesh in a chamber which is located in the middle section of the apparatus. The O_2 sensor (Clark type electrode) lies beneath the leaf chamber with its Pt cathode exposed to the atmosphere within it. The leaf tissue is pressed lightly against the temperature-controlled roof of the chamber by a foam disc which also separates it from carbonate/biocarbonate buffer carried on the capillary matting. The leaf is illuminated through this window which also allows fluorescence to reach a probe. The clips which draw the top section on to the middle section (so that the roof of the leaf chamber is sealed against an O-ring) and the tubes which carry temperature-controlled water to the top and bottom sections are not shown. The taps (with luers) are for calibration and adjustment of the gas phase. (Figure and description from David Walker, 1987).

The measurements of oxygen evolution from infected or control tobacco plants were taken at 23°C and illumination was provided by a 24°, 50W Dichroic quartz-hologen spot lamp (WOTAN). The incident light intensity was altered using Balzar neutral density filters (NDF) placed on the top of the window. Eleven NDFs, ranging from 2.7% to 78% were used and the percentage of transmission expressed in moles of photons. $S^{-1}.m^{-2}$.

2.7.2- Calibration and calculation:

Using a gas-tight syringe, a volume of air or nitrogen was flushed through one tap while the other one was open. As a result, electrical output of oxygen sensor indicated on chart recorder as voltage, rose to a steady state and withdrawn in a similar way when flushed with nitrogen. The difference between the electrical output of the electrode in air and nitrogen is a measure of the partial pressure of oxygen in the internal atmosphere. The deflection recorded in the chart caused by flushing nitrogen (zero oxygen) and air recorded in millivolts, corresponded to the number of moles of oxygen at a particular temperature (T°C).

The amount of oxygen in 1 ml of air at the standard temperature and pressure (s.t.p) is 9.37 μ moles oxygen (containing 21% O_2 by volume), therefore, at any temperature (T), the amount can be calculated by multiplying 9.37 by (273/273+T). At the temperature of 23°C the temperature maintained throughout the experiment, and volume of 5 ml (the volume of the chamber), the amount of oxygen in 5 ml air is given by:

$$5\ [\ 9.73\ (273/273+23)\ ]\ =\ 43.20\ \mu\ \text{moles}\ O_2$$

Details of the oxygen electrode and its calibration are found in Delieu and Walker (1981).

2.7.3- Photosynthesis rate calculation:

After calibration, the leaf chamber was opened and a leaf disc (10 cm^2) was placed in stainless steel mesh supported on a capillary mat containing 400 µl of 1M $NaHCO_3$. The chamber was then sealed and left to equilibrate for approximately 20 min in the dark until the sample had fully reached equilibrium. The measurements were taken from 0% light intensity up to 100%. At each intensity, the chart recorder was left to 5-7 min to come to a steady state before taking a measurement. The measurements were calculated on the basis of moles of $O_2.s^{-1}.m^{-2}$. Gross photosynthesis rate (Pg), net photosynthesis rate (Pn) and the dark respiration (Rd) were all calculated on the same basis. Rd was calculated after placing the leaf disc in the dark for about 15 min. The maximum gross photosynthesis (Pgmax), the maximum net photosynthesis (Pnmax), the quantum efficiency of photosynthesis at low light intensity (α), dark respiration (Rd) and the ratio of physical to total resistance to diffusion of CO_2 (θ) were estimated using model of Marshall and Biscoe (1980) and with Rabinowitch model (1951) only three parameters were estimated (Pgmax, Pnmax and α).

2.7.3.1- Analysis of data:

The models of Rabinowitch (1951) and Marshall and Biscoe (1980), which both describe the response of photosynthesis to changes in light intensity, were used to fit the measurements of photosynthesis.

One of the earliest models to describes a response of photosynthesis derived by Blackman (1905). He proposed that the response of photosynthesis increases linearly with the light intensities with the only limiting factor being light (light limited phase) until the available supply of CO_2 prevents a further increase in the rate of photosynthesis with light intensity (CO_2-limited). Rabinowitch (1951) found that Blackman's model did not reveal a sharp discontinuity between the light-limited and CO_2-limited phases and proposed a

describes the relationship between photosynthesis and light intensity in terms of a rectangular hyperbola as shown in the equations below.

$$Pg = \frac{Pgmax\ \alpha\ .\ I}{Pgmax\ +\alpha\ .\ I} \qquad (1)$$

$$Pg = Pn + Rd \qquad (2)$$

Where, Pg is the gross photosynthesis rate, Pgmax is the maximum gross photosynthesis rate, α is the quantum efficiency of photosynthesis at low light intensity, I is the irradiance, Pn is the net photosynthesis rate, and Rd is dark respiration rate.

The equation was modified by Thornley (1976a & b) for a simple enzyme-substrate reaction as;

$$Pg = \frac{\alpha\ .\ I\ (Cr/rx)}{\alpha\ .\ I\ +\ (Cr/rx)} \qquad (3)$$

Where Cr is CO_2 concentration at the site of fixation, and rx is the chemical or carboxylation resistance.

The rectangular hyperbola model, accounts only for the biochemical reactions within the chloroplast. Before fixation, CO_2 must diffuse through the leaf boundary layer, the stomata, and mesophyll layer which present some resistance. These resistance layers were not taken into account in the rectangular hyperbola model. A model of non-rectangular hyperbola was derived by Thornley, using quadratic equation to discribe gross photosynthesis rate and later modified by Marshall and Biscoe (1981) for discription of net photosynthesis rate. The model combines a simplified description of the biochemical reactions occurring within the chloroplast with the physical diffusion of CO_2. The model is a non-rectangular hyperbola and uses four parameters: Pn max, Rd, α, and θ. A brief derivation of the model, as described by Marshall and Biscoe (1981), is given below.

The net flux of CO_2 from the atmosphere to the site of fixation is.

$$Pn = (Ca - Cr)/rp \qquad (4)$$

Where Ca is CO_2 concentration in the air, and rp is physical diffusion resistance.

Assuming that equation (3) is an accurate description of the biochemical reactions and that equation (2) defines Pg, Cr can be eliminated from equations (3) and (4) and Pg substituted from equation (2) to give (Thornley, 1976b).

$$Pn + Rd = \frac{\alpha . I\ (Cr/rx - Pn\ rp/rx)}{\alpha . I + (Cr/rx - Pn\ rp/rx)} \qquad (5)$$

To remove uncertainties created by rx, the equation multiplied and denominator by rx/(rp + rx) to give.

$$Pn + Rd = \frac{\alpha . I\ (Pgmax - \theta\ Pn)}{(1 - \theta)\alpha . I + (Pgmax - \theta Pn)} \qquad (6)$$

Where Pgmax = Cr/(rp + rx), θ = rp/(rp + rx).

On expansion, equation (6) gives.

$$\theta Pn^2 - (Pgmax + \alpha I - \theta Rd)Pn + \alpha I(Pgmax - (1-\theta)Rd) - Rd\ Pgmax = 0 \quad (7)$$

Where α is still the initial slope at zero irradiance, and θ, the ratio of physical to total resistance, describes the degree of the curvature at the shoulder of the photosynthesis-light response (PLR). The equation (7) describes the PLR as a non-rectangular hyperbola and a quadratic non-liner equation. The maximum rate of net photosynthesis (Pn max) is calculated from the equation.

$$Pnmax = Pgmax - (1 - \theta)\ Rd \qquad (8)$$

The simple quadratic equation is.

$$Y = 0 = a + bX + cX^2$$

By substituting the variables C, b, and a from equation (7).

$$C = \theta$$

$$b = (Pgmax + \alpha I - \theta Rd)$$

$$a = (Pgmax - (1 - \theta)Rd)$$

The data were fed to the computer, and the coefficients Pmax, α, θ and Rd solved using a non-liner least squares minimisation algorithm (conjugate method of the solver routine in Microsoft Excel 4.0 program for windows).

2.7.4- Determination of chlorophyll concentration:

Chlorophyll concentration was determined by the method described by McKinney (1941) using methanol as an organic solvent to extract the chlorophyll from leaf tissue.

Leaf discs as used for oxygen electrode experiments were kept at 4°C and assayed the next day or processed immediately for chlorophyll extraction. A centrifuge tubes containing 19 ml of methanol was prepared and a leaf disc immersed in the tube and maintained in a heated water bath at 65°C for 30 min. The centrifuge tube was covered with aluminium foil to prevent photo-oxidative loss of the pigments from the light. The leaf disc was removed and the centrifuge tube was centrifuged for 2-3 min at 3,000-4,000 rpm to remove any fragments of leaf. The supernatant was transferred to a volumetric flask and adjusted to 25 ml with methanol. The absorbance was measured against methanol at 665 and 650 nm using methanol as a baseline. The amount of chlorophyll was calculated using the following formulae:

$$\mu g\ Chl\textit{a}.\ ml^{-1} = 16.5(A_{665}) - 8.3(A_{650})$$

$$\mu g\ Chl\textit{b}.\ ml^{-1} = 33.8(A_{650}) - 12.5(A_{665})$$

Chapter Three

Symptom expression and virus multiplication in *Nicotiana* species

Symptom expression and virus multiplication in *Nicotiana* species

3.1- Introduction:

The effect of plant rhabdoviruses on their hosts have been discussed in a number of reviews (Francki, 1973; Francki and Randles, 1980; Francki, *et al.*, 1981; Jackson, *et al.*, 1987). Symptoms, often include either chlorotic or necrotic lesions or systemic vein clearing and leaf cupping or mottling. Although, symptomology is of limited use for virus diagnosis and identification, host symptoms are still very important to the applied plant virologist. In the field, symptoms give the first clue to a virus's identity, and in the laboratory, the symptoms produced in a range of test plants may often be of considerable diagnostic value. For the grower, symptoms may be the most important aspect of virus infection. The nature and severity of the disease symptoms will determine the economic importance of a particular virus, in terms of yield loss and reduced quality. The interaction between a plant virus and its host may result in visible or other detectable abnormalities in plants, which are recognisable as symptoms. Symptoms may appear on infected plants several days to several weeks after inoculation.

Studies on virus movement and spread through the plant and the way virus levels vary within the host during the infection period, can add to our understanding of virus-host interaction. This information might be of importance in designing means to control virus replication and pathological effects. In several plant rhabdoviruses, virus levels have been determined by infectivity assays, and show an initial increase within various tissues, reach a maximum, and then decline (Jackson and Christie, 1977). Ismail, *et. al.*, (1987) determined the virus movement of SYNV in infected *N. edwardsonii* by using ELISA and immunoblotting assays. They reported that the virus proteins were detectable systemically after as little as 24 hr. Systemic spread occurred in plants from

which the inoculated leaves were removed 24 h. after inoculation but not in plants from which the infected leaves were removed 12 h. after inoculation.

The sites of multiplication of several plant rhabdoviruses have been investigated (Francki, *et al.*, 1981; Jackson, *et al.*, 1987, Ismail, 1988). Information about the effects of plant rhabdoviruses on the ultrastructure of their host cells is limited. The studies of LNYV in infected *N. glutinosa* (Wolanski, 1969), and SYNV in infected *N. edwardsonii* (Ismail, 1988) are the most comprehensive studies using electron microscopy and immunocytochemical techniques of the cytopathological effects of plant rhabdoviruses .

Cytopathological effects of plant rhabdoviruses were most severe at the site of virus particle assembly. In cells infected by type II plant rhabdovirus, the most affected organelle was the nucleus which showed dramatic changes in chromatin content and exhibited swollen perinuclear spaces where the virus particles accumulated (Ismail, *et al.*, 1987; Jones and Jackson, 1990; Martelli and Russo, 1977). Other organelles were reported to have also shown abnormal changes in their structure during the infection period (Martelli and Castellano, 1970; Russo and Martelli, 1972; Martelli and Russo, 1977; Ismail, 1988; Lin, *et al.*, 1987).

The main aim of the work described in this chapter was to study changes in symptom expression, levels of virus antigens and cytopathological effects induced by the two plant rhabdoviruses, SYNV and EMDV in three *Nicotiana* species (*N. glutinosa, N. clevelandii,* and *N. edwardsonii*) and to determine their intracellular location in infected cells of these plants.

3.2- Purification of SYNV and EMDV:

There has been no simple and generally applicable procedure yielding adequate amounts of pure virus. A number of different purification procedures have been discussed (Jackson, *et al.*, 1987; Jones and Jackson, 1990). The yields

of purified plant rhabdoviruses, are comparatively low compared to the many other plant viruses (Matthews, 1981; Jackson, *et al.*, 1987).

Although a number of methods have been previously reported for purification of EMDV, none has given satisfactory yields of purified virus. Russo and Martelli (1973) succeeded in purifying EMDV but the purified virus was not infectious. Adam, *et al.*, (1987) purified EMDV using a different method, but the partially purified virus was substantially contaminated with plant cell components.

The method which was used to purify EMDV in this research was based on that used for SYNV purification by Jackson and Christie (1977) and is described in detail in section (2.3). The amount of celite was critical in the purification of EMDV. With over 150g of celite, the filtered liquid was straw coloured and clear of chloroplast fragments but no virus was recovered. With much less than 150g of celite, large amounts of virus were recovered but they were still contaminated with chloroplast fragments. However, with 150g of celite a substantial amount of virus was recovered with little or no contaminating chloroplast fragments. The yield of both SYNV and EMDV was estimated by measuring the virus protein using the coomassie blue binding assay as described in section (2.3.2). The yield of SYNV was ranged between 12 μg and 16 μg/g leaf while EMDV ranging between 18 μg and 27 μg/g leaf.. Typical absorbance profiles of the final gradient rate-zonal sucrose are shown in figure (3.1 a & b). For partly purified viruses (omitting the rate zonal sucrose gradient centrifugation), the yield was much higher but the pelleted virus was still contaminated with host cell components which co-sedimented.

Purified SYNV and EMDV were both tested for their infectivity after storage in liquid nitrogen for over one year as described in section (2.3 & 2.3.1). The two viruses retained their infectivity towards *N. edwardsonii* and in the case

of EMDV, over 90% of the test plants became infected and showed severe symptoms after 3 weeks.

SYNV and EMDV were fractionated by SDS polyacrylamide gel electrophoresis as described in section (2.4.1). The gels were stained over night with approximately 5 volumes of 0.2% Coomassie Brilliant Blue R250 in 10% acetic acid and 7% methanol and destained in 10% acetic acid for three changes over a period of 24 h. Both viruses showed four clear bands corresponding to the four major structural proteins (Figure 3.2). By using SYNV proteins as molecular weight standards the sizes of EMDV proteins were calculated to be G protein, 86 kD, N protein, 56 kD, M1 protein, 35 kD, and M2, 23 kD. The size of each protein was based on the average of five separate gels.

3.3- Measurement of virus protein concentrations:

In order to assess the concentration of virus proteins in infected plants during the infection period, five infected leaves from five infected plants were taken at different time and one gram from each infected leaf was homogenised in 5 ml of PBS-TPO buffer (0.136 M NaCl; 2.68 mM KCl; 9.2 mM Na_2HPO_4; 0.87 mM KH_2PO_4; 0.05% (v/v) Tween-20; 2% (w/v) PVP; 2% BSA. pH, 7.2). These samples were clarified by 5 min centrifugation at 13000 r.p.m. The pellets were discarded and the supernatants were stored at -20°C. Ten microliter from each infected leaf sample and healthy control samples of the same age were used in ELISA as described in section (2.6.5).

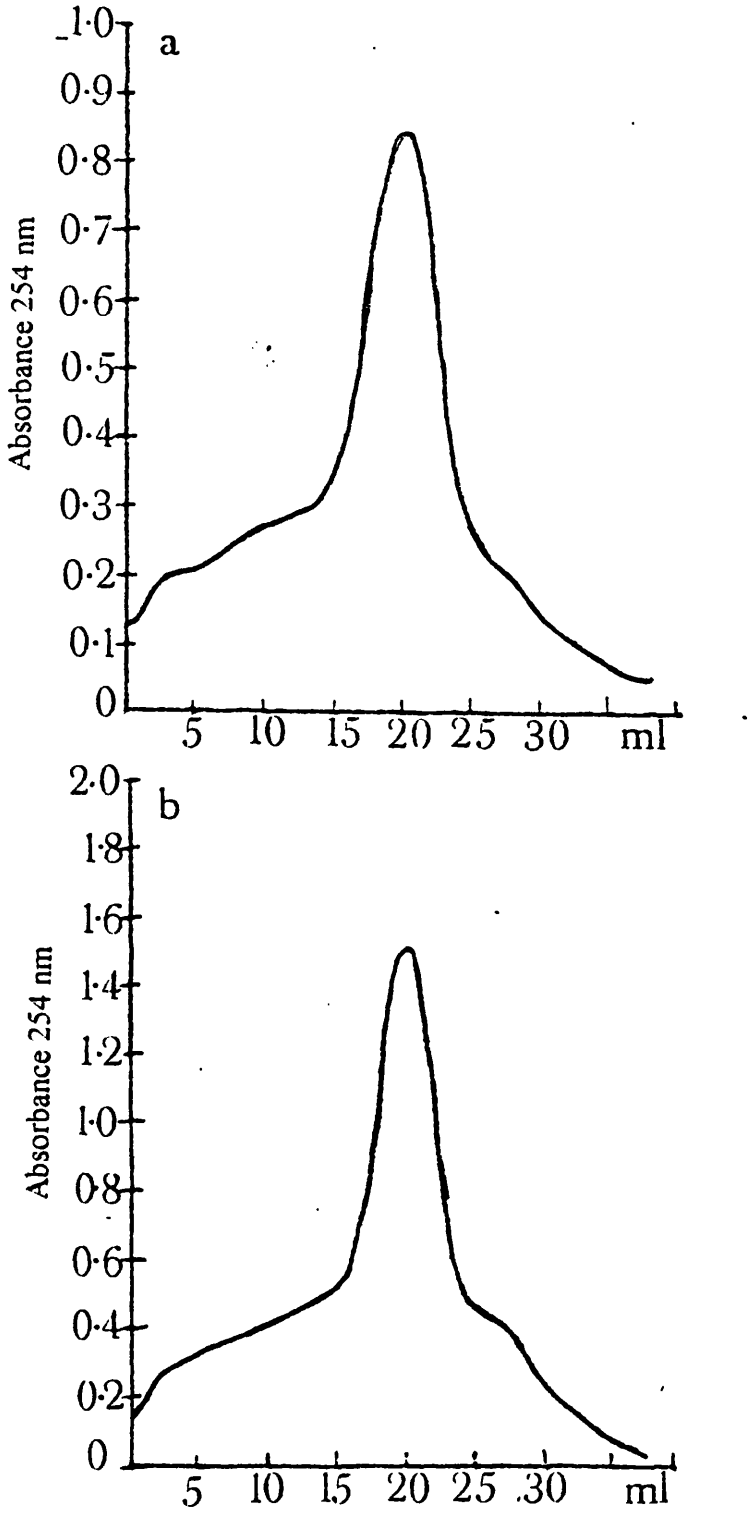


Figure 3.1:

Photometeric scan at 254 nm of 5-30% rate zonal sucrose gradient of (a) SYNV, and (b) EMDV. Leaves were harvested from infected plants 4 weeks post-inoculation.

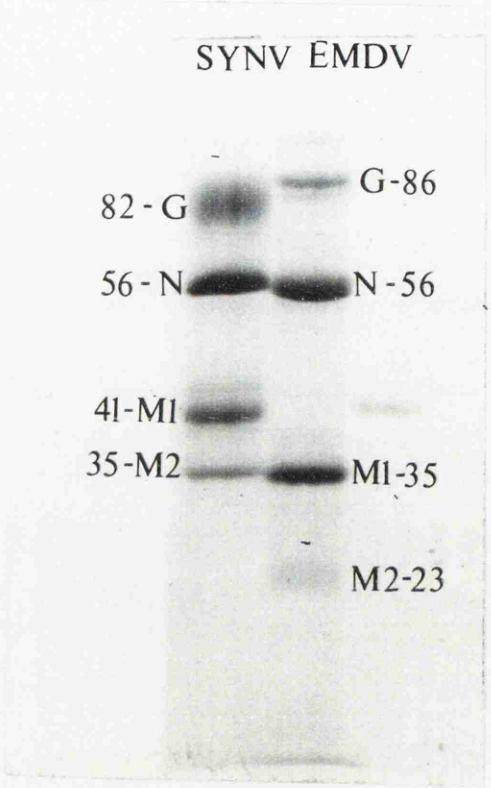


Figure 3.2:

Fractionation of SYNV and EMDV proteins by SDS polyacrylamide gel electrophoresis, showing a clear four bands, corresponding to the four major structural proteins. Sizes of the proteins are shown in kD.

Infected leaves from *N. edwardsonii* plants infected with EMDV were taken starting from 7 days up to 168 days post-inoculation and the concentration of virus proteins in infected plants determined by ELISA. The concentration of virus proteins reached at maximum in infected plants between three and four weeks (21-28 days) after inoculation, then started to decline gradually. After 23 weeks, the concentration of virus proteins reached a steady state (figure 3.3a).

Leaves from *N. glutinosa* and *N. edwardsonii* plants infected with SYNV were taken, starting from 5 days up to 65 days after inoculation and determined by ELISA. The concentration of SYNV proteins in *N. glutinosa* reached a maximum 15 days post-inoculation then gradually declined. This period of decline coincided with the appearance of chlorotic mottling symptoms. The concentration of virus proteins reached a minimum steady state by 30 days and (13.3 μg/g) (figure 3.3b) had hardly decreased even 230 days post-inoculation (12.1 μg/g).

The maximum concentration of SYNV proteins in infected *N. edwardsonii* was far lower than in *N. glutinosa.* The concentration of virus proteins reached a maximum after 15 days (13.4 μg/g) after inoculation and started to decrease gradually after 20 days. By 45 days post-inoculation, the virus protein concentration reached a steady state of (8.5 μg/g) and declined only slightly thereafter. Similar results were obtained in other experiments with other batches of *N. glutinosa* and *N. edwardsonii* infected plants. These results are from only one of the experiments.

3.4- Symptoms of SYNV-infected plants:

Three species of *Nicotiana* were used as hosts of SYNV (*N. glutinosa, N. clevelandii* and *N. edwardsonii,* which is a hybrid of *N. glutinosa* and *N. clevelandii*). The plants were inoculated and maintained as described in chapter 2 section (2.2). All three hosts responded in broadly similar manners to the virus

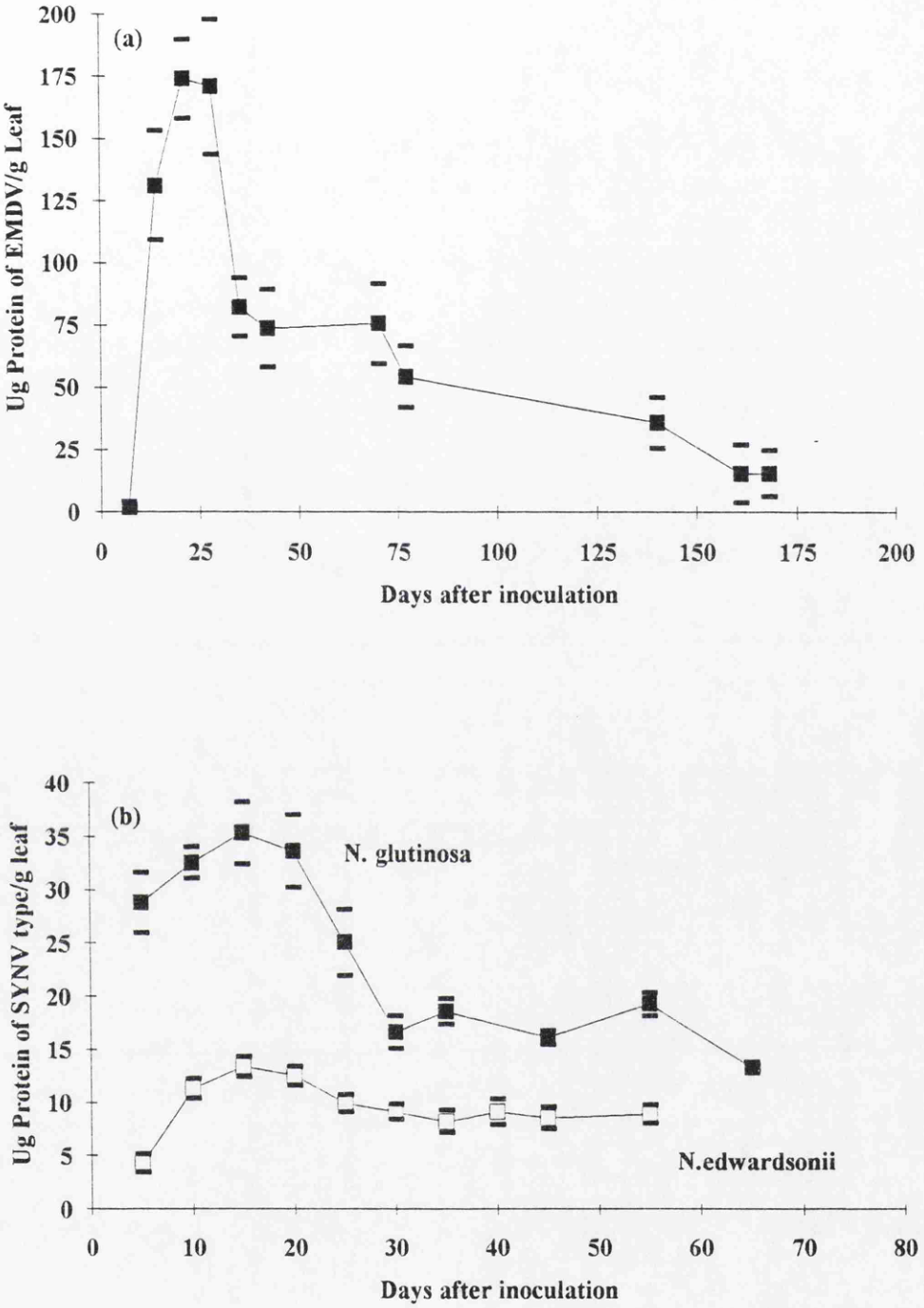


Figure 3.3:
(a)- Concentration of EMDV antigen in infected *N. edwardsonii.*
(b)- Concentration of SYNV antigen in infected *N. glutinosa* and *N. edwardsonii.* (Each point is the mean of 5 measuements +/- SE.)

infection. However, differences in the severity and time of appearance of symptoms were apparent.

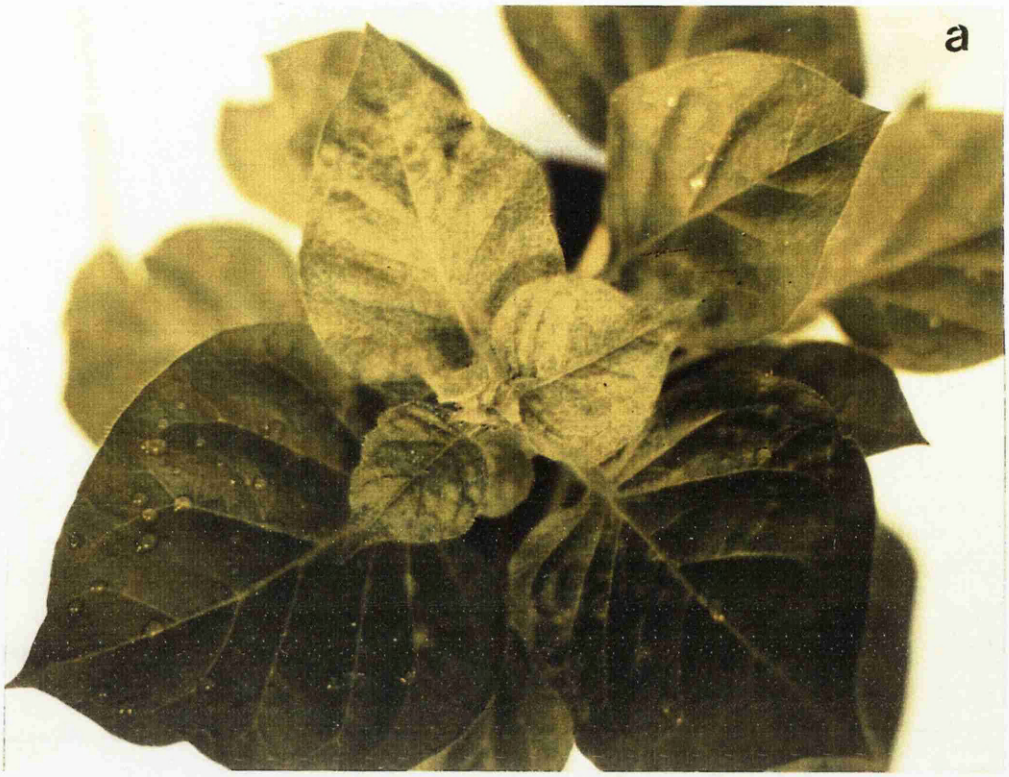
N. edwardsonii plants infected with SYNV showed typical systemic symptoms of vein-clearing and leaf cupping in unexpanded leaves 10-14 days after inoculation. Expanded leaves failed to develop further on occasions, local lesions appeared on inoculated leaves. Systemic vein clearing symptoms gradually disappeared after 4 weeks and mottled spots began to develop after 6 weeks in all unexpanded leaves (figure 3.4a & b). Although the yellow mottled spots persisted in most infected plants from 6 weeks and thereafter, some infected plants underwent a recovery where symptoms disappeared. These plants were hard to distinguish from control plants by the late stages of infection (over 12 weeks). The time course of development of symptoms, and recovery of infected plants were similar to earlier reports (Ismail, *et al.*, 1987; Ismail, 1988). The severity of symptoms was less than observed in *N. glutinosa* or in *N. clevelandii* and infected plants grew to a similar size and at the same rate as healthy controls.

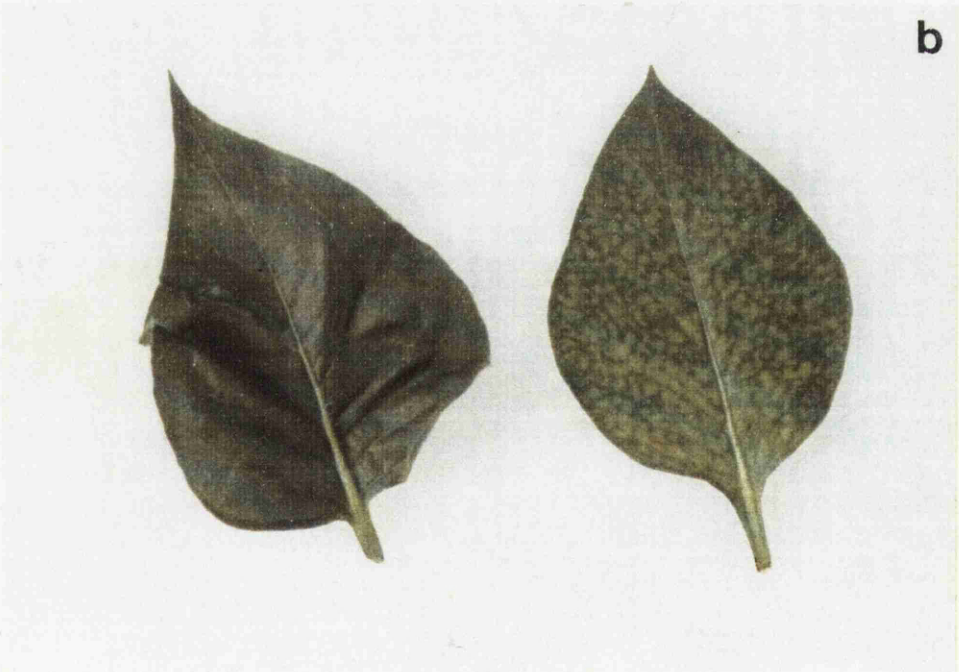
N. glutinosa, infected with SYNV, responded with typical vein-clearing and yellowing after 10-14 days under greenhouse conditions. Virus infection had a dramatic effect on plant height. In addition to vein-clearing and yellowing, plants were stunted and internodes shortened compared to the control plants. The infected plants very rarely showed local lesions in inoculated leaves but these leaves developed necrotic areas. After 6 weeks of infection, the plants started to show a change in symptoms with mottled spots throughout the leaf lamellae and large mottled spots starting to appear in place of the vein-clearing observed earlier (figure 3.5a & b).

Figure 3.4:

(a)- SYNV infected *N. edwardsonii* plant showing symptoms of vein-clearing and yellowing of leaves 3 weeks post-inoculation.

(b)- Symptoms of yellow mottled spots in SYNV-infected *N. edwardsonii* leaves 6 weeks post-inoculation. (right) infected plants. (left) Healthy control plant.


a


b

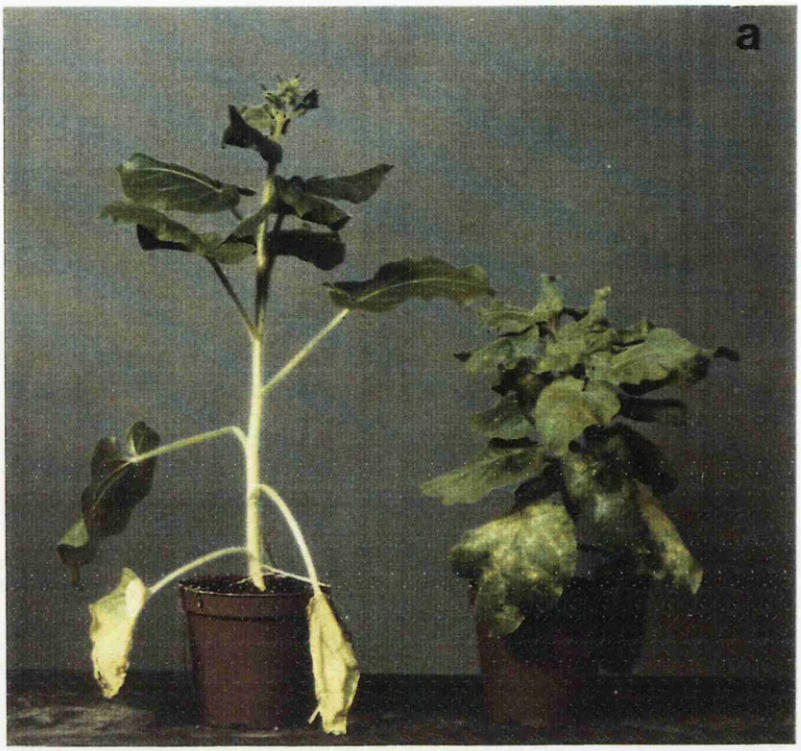
Infected plants usually started flowering approximately 2 weeks earlier than control plants and yellow mottling symptoms were observed in the petals of infected plants. At this late stage of infection (6 weeks), the symptoms had spread throughout the infected plants. Symptoms of this sort were observed on all leaves and persisted for much more than a year.

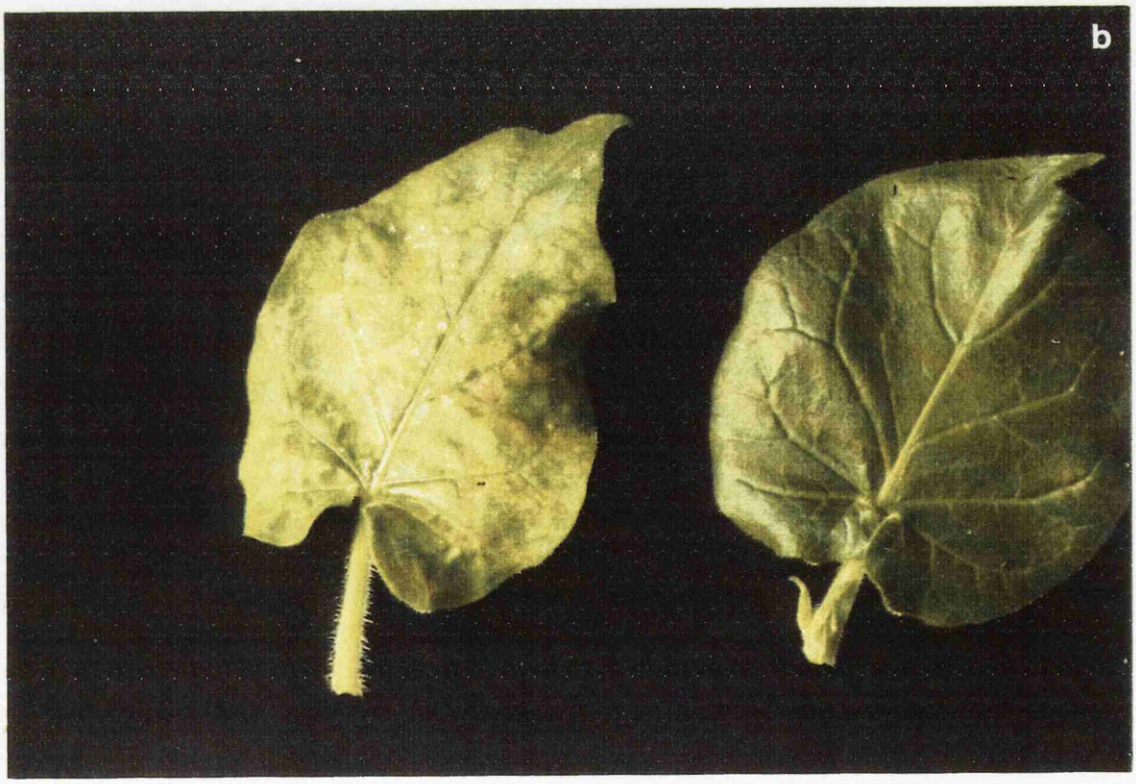
N. clevelandii infected with SYNV started to show symptoms of vein-clearing and yellowing earlier than *N. glutinosa* 8-10 days after inoculation. The symptoms (vein-clearing and leaf cupping) were severe in unexpanded leaves whereas expanded leaves showed less severity. The development of symptoms could be divided into two stages as for *N. glutinosa.* The first stage (up to 5 weeks) involved distinct vein-clearing especially in unexpanded leaves; the second stage involved mottled spots and leaf curling. These were clearly visible 6 weeks after inoculation. The symptoms in general were less severe than in *N. glutinosa* but the infected plants still showed stunting and in some cases rosette leaves. Infected plants survived up to 12 weeks from inoculation but were stunted compared to the controls (figure 3.6 a & b). Table 3.1 summarises the main feature of the symptoms exhibited by the three hosts used when infected by SYNV.

Figure 3.5:

(a)- *N. glutinosa* plants infected with SYNV showing severe stunting of (right) the infected plant, (left) healthy control plant.

(b)- *N. glutinosa* plant leaf showing SYNV symptoms of chlorotic yellow spots and vein-clearing. (left) Infected leaf. (right) Control plant leaf.


a


b

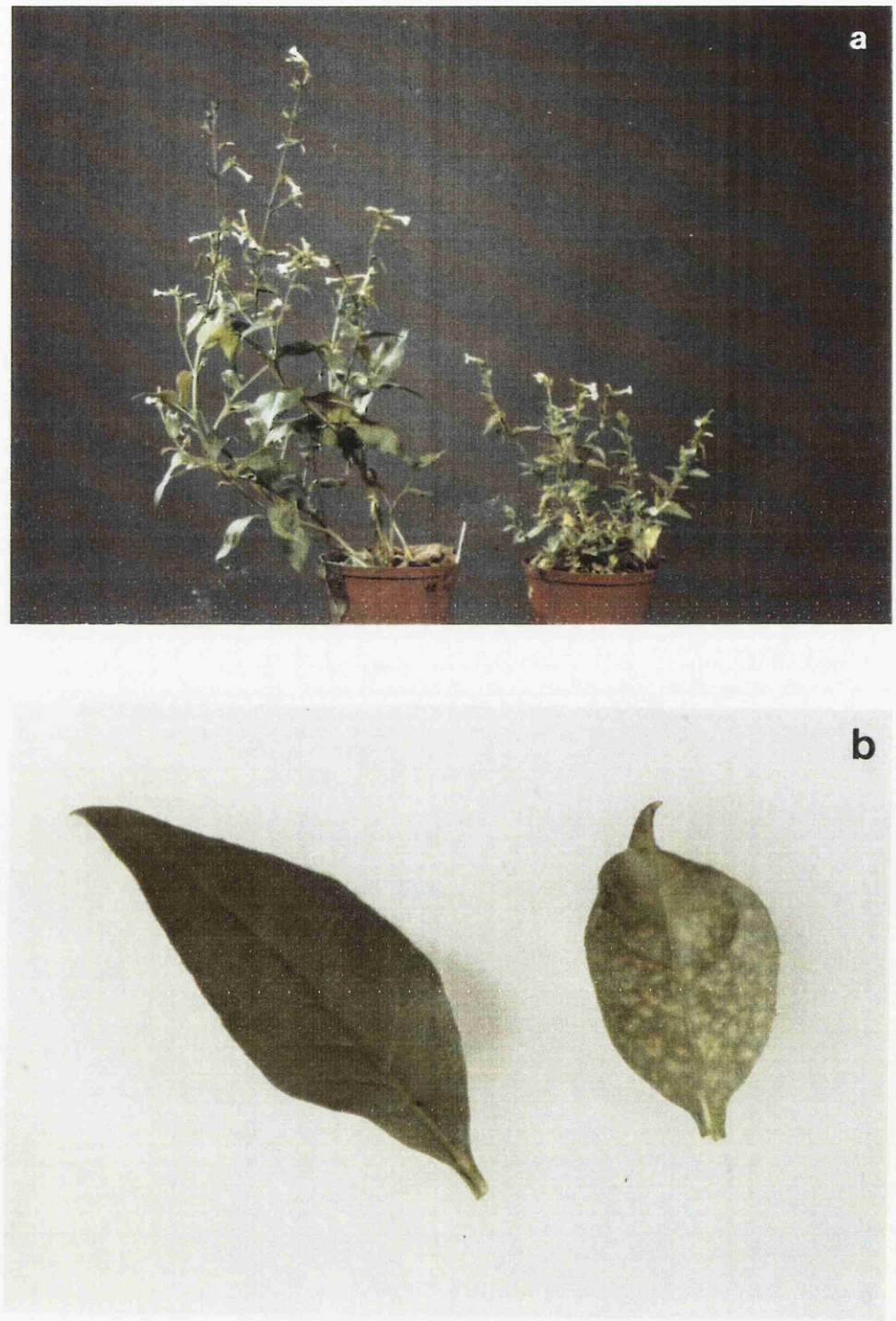


Figure 3.6:

(a)- *N. clevelandii* plants infected with SYNV, (right) showing severe stunting 6 weeks post-inoculation. (left) Control plant. **(b)-** *N. clevelandii* leaf plant showing (right) SYNV symptoms 6 weeks post-inoculation, of yellow mottled spots, (left) control plant leaf.

Table 3.1: Summary of the SYNV symptom expression in the three hosts.

Hosts	Symptoms
N. glutinosa	- Symptoms appeared after 10-14 days with vein-clearing, yellowing and stunting. Necrotic areas in inoculated leaves. - Chlorotic and mottling spots symptoms appeared after 6 weeks. - Symptoms were severe if plants inoculated early (4-6 leaves). - Some infected plants survived for more than 1 year.
N. clevelandii	- Symptoms appeared after 8-10 days with severe systemic and no stunting to the plants. - Chlorotic spots appeared after 5 weeks. - The symptoms were less severe than *N. glutinosa.* However, the infected plants did not live more than 15 weeks.
N. edwardsonii	- Symptoms observed after 10-14 days with vein-clearing and yellowing and some local lesions. -Mottling spots observed after 6 weeks. - Symptoms were less severe with time. Recovered after 12 weeks, symptoms were mild. Infected plants survived as long as the control healthy plants.

3.5- Morphology, structure and intracellular location of SYNV particles:

3.5.1- Morphology and structure of SYNV:

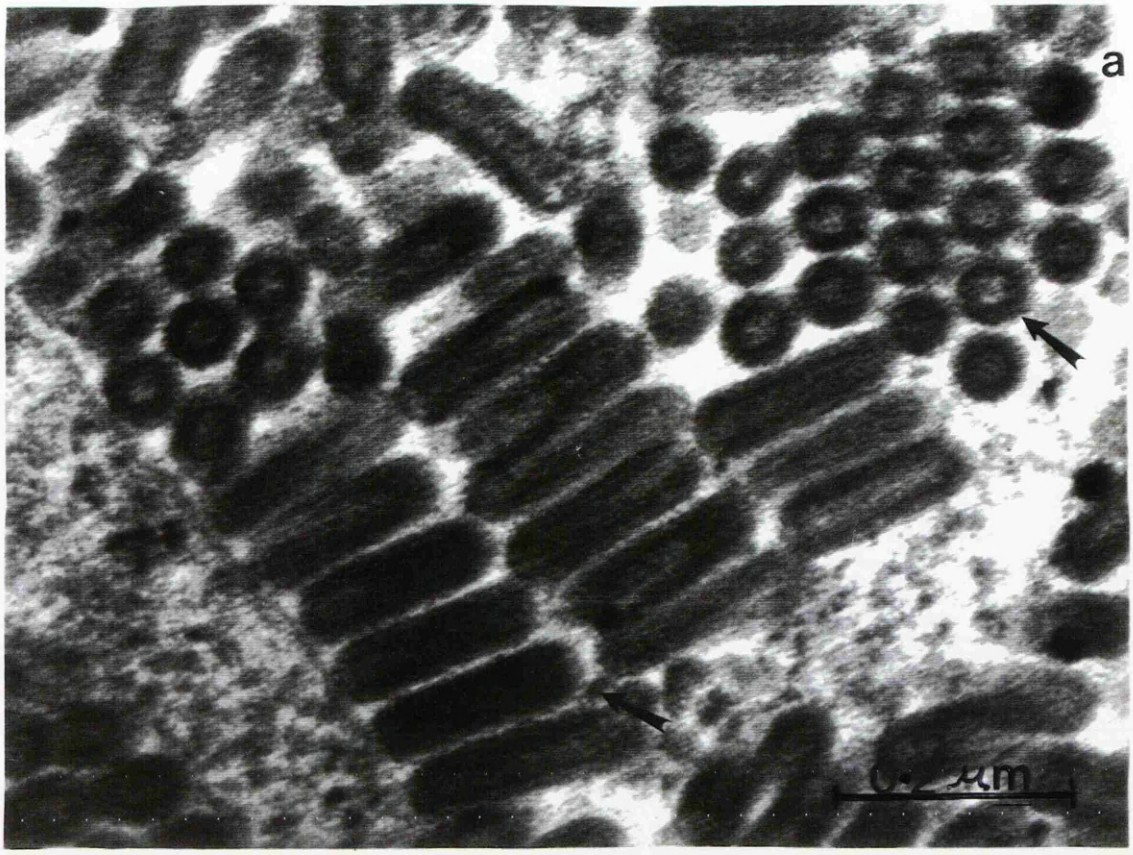
Electron microscopy of ultrathin sections of SYNV-infected *N. edwardsonii, N. glutinosa* and *N. clevelandii* leaves revealed the distinct morphological structures of bacilliform virus particles. These particles were lodged in the perinuclear space in great numbers and the dimensions of these particles taken 3 weeks post-inoculation were 200-220 nm in length and 60-70 nm in wide. Particles in cross section revealed a number of concentric rings corresponding to the nucleocapsid and envelope with its projections (Figure 3.7a). Immature particles with a bullet-shape were observed still connected to the inner nuclear membrane at their base (Figure 3.8a). Particles in cross section show concentric rings indicating the helical organisation of the nucleocapsid in the intact particle. With the progress of the infection, the number of virus particles of standard length were consedrably reduced but shorter particles were also observed in most infected cells. These short particles which have been reported as defective interfering (DI) particles (Ismail, 1988), ranged from 165-180 nm and showed the same bacilliform shape as standard virus particles (Figure 3.7b). However, short particles were observed in *N. clevelandii* and *N. edwardsonii* infected leaves by 6 weeks post-inoculation whereas in *N. glutinosa* they were not observed at this time.

3.5.2- Cytology of SYNV-infected cells:

The early stage of infection (2-5 weeks post-inoculation) were characterised by the appearance of typical vein-clearing symptoms; abnormal changes appeared in the nuclei of infected cells regardless of the host species. Four weeks after inoculation, infected *N. glutinosa* and *N. clevelandii* cells showed very distinctive abnormalities of the nuclei and chloroplasts. Nuclei contained large numbers of virus particles in perinuclear inclusions. The nuclei of *N. glutinosa*

Figure 3.7:

(a)- Electron micrograph of a thin section of SYNV-infected *N. edwardsonii* leaf after 3 weeks post-inoculation, showing virions of standard size in elongated and in cross-section (standard length in longitudinal and cross section are arrowed). **(b)**- Short particles in SYNV infected *N. clevelandii* after 8 weeks after inoculation (short particle is arrowed).


a

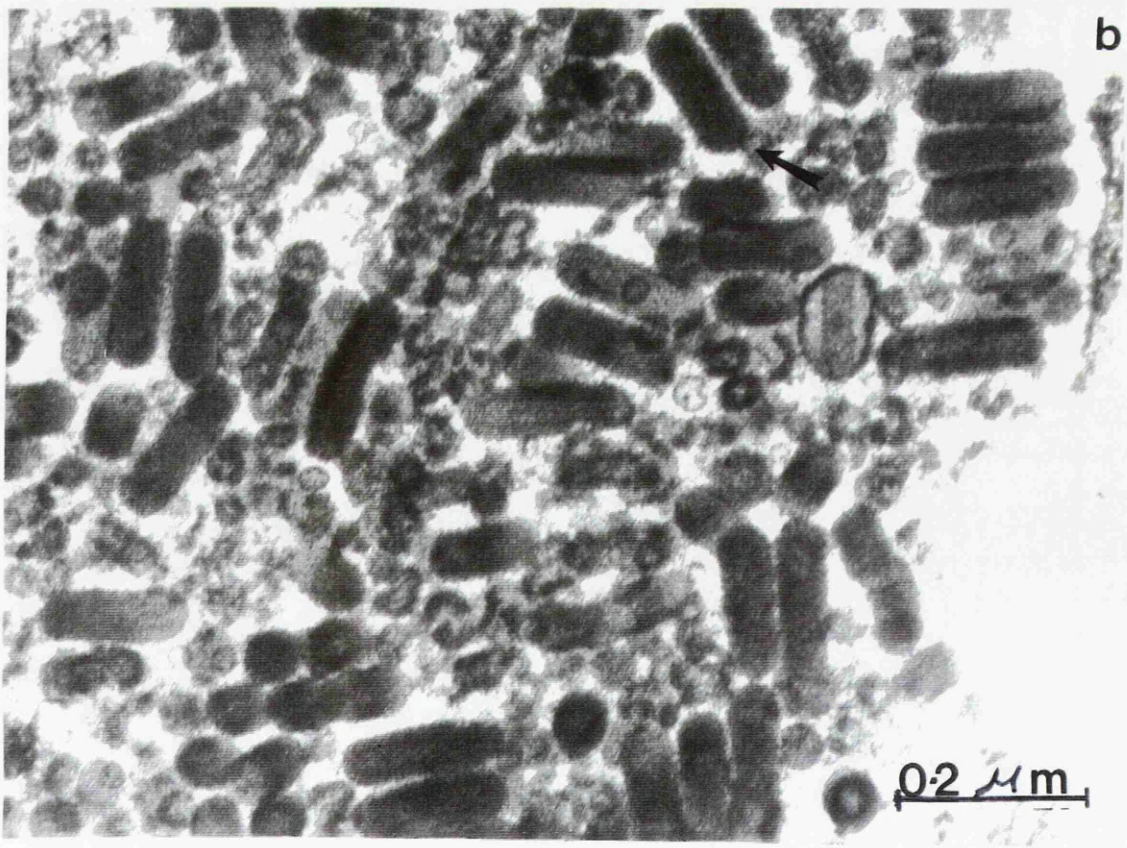

b
0·2 μm

Figure 3.8:

(a)- Electron micrograph of a thin section of SYNV-infected *N. glutinosa* leaf 8 weeks post-inoculation, showing immature virus particles in the perinuclear space, some still connected by their bases to the inner nuclear membrane.

(b)- Electron micrograph of a thin section of SYNV-infected *N. edwardsonii* leaf 3 weeks post-inoculation, showing massive numbers of virus particles in the nucleus.

Standard abbreviations:

N = Nucleus.

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

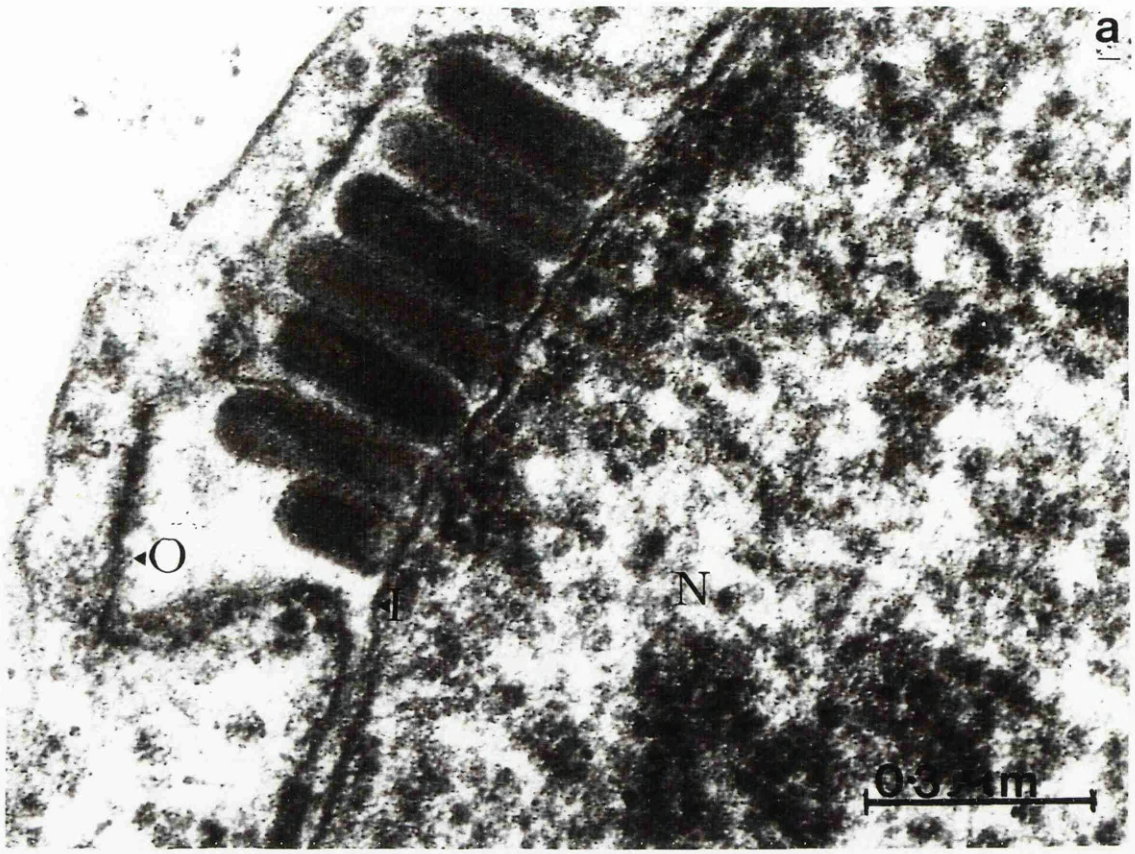
M = Mitochondrium.

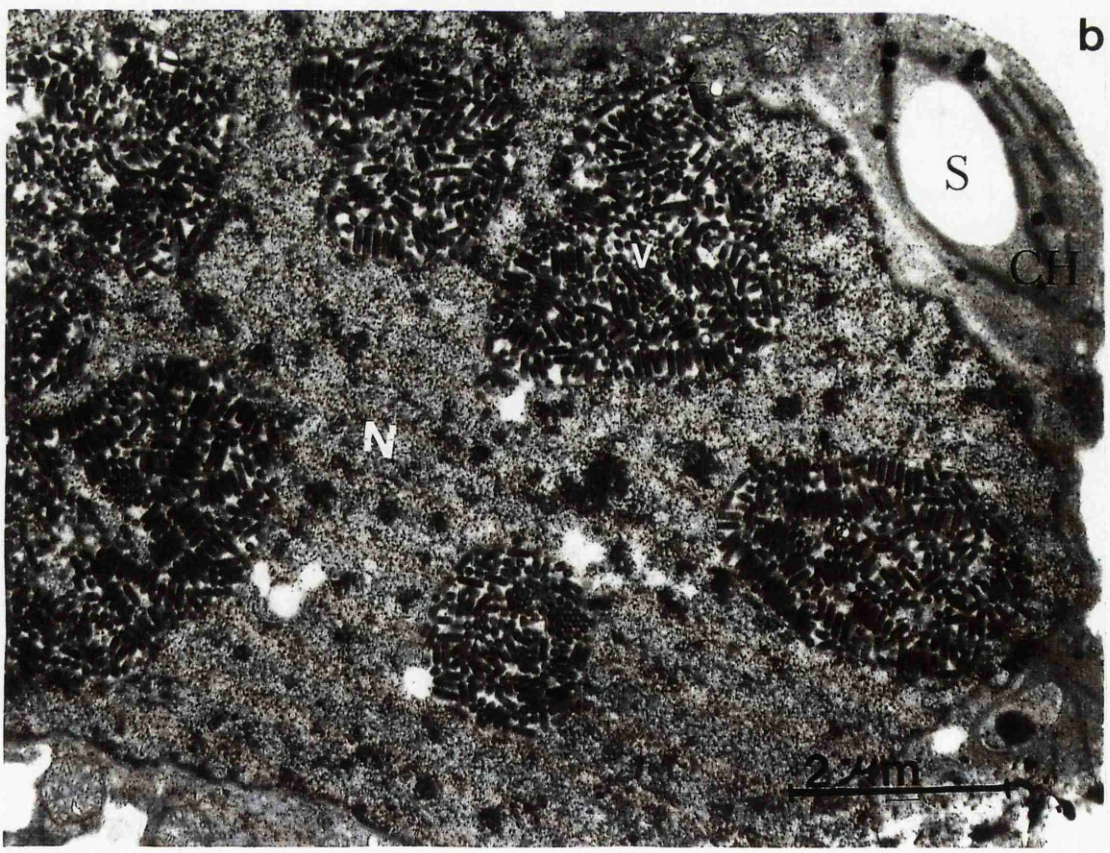
CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
O
I
N


b
S
CH
V
V
N
2·4 m

and *N. clevelandii* cells showed three distinct areas; electron-dense viroplasm in the centre taking up a large proportion of the nucleus were surrounded by a strip of chromatin and outwith that, the expanded perinuclear space was filled in some regions areas with virus particles (Figure 3.9a & b). These perinuclear inclusions were invaginated in the nucleus. *N. clevelandii* nuclei contained a mixture of mature and immature virus particles, some of them still connected by their base to the inner nuclear membrane (Fig. 3.8a). *N. edwardsonii* infected cells, showed fewer abnormalities compared to *N. glutinosa* and *N. clevelandii*. Large viroplasm regions did not developed in the infected cells at this stage (Fig. 3.8b), although, large number of virus particles were seen in the nucleus.

Chloroplasts also started to show abnormalities in their structure in the three hosts, a large area of starch developed in the centres. Disruption and reduction of the thylakoids were also evident (Figures 3.8b, & 3.9a &b).

N. clevelandii was the only host which, by three weeks developed distinct local lesions in inoculated leaves. Infected cells from the green areas of inoculated leaf showing local lesions, revealed no changes compared to the healthy controls whereas cells from the yellow areas showed abnormalities similar to those described above.

By 6 weeks after inoculation (second stage), analysis of the concentration of viral antigen had already shown that SYNV levels had declined in the infected host. The cytological changes in cells during this stage were characterised by a reduction in the numbers of virus particles in infected cells and also with a change in symptoms to yellow mottled spots.

Over the period 6-8 weeks post-inoculation, infected cells from the three hosts showed similar changes in the nuclei. Large areas of viroplasm were present in the centre but the numbers of virions lodged in the perinuclear space were reduced compared to earlier in infection. Large viroplasm regions similar to these which developed in *N. glutinosa* and *N. clevelandii* early in infection (Fig.

3.9a &b) developed in *N. edwardsonii* later (6 weeks) after inoculation. Chloroplasts developed large areas of starch in the centre (figure 3.9).

From 6 weeks post-inoculation, symptoms in infected plants started to change from vein-clearing to yellow mottled spots in all three infected hosts. Leaves of *N. glutinosa*, 8 weeks after inoculation and showing yellow mottled spots were sectioned and examined. A green area from an infected leaf showing yellow mottled spots revealed no virus particles in the cells examined (figure. 3.10b) and cells appeared ultrastructurally relatively normal although the nuclei often contained dense regions of condensed chromatin (figure 3.10b). Pale yellow or yellow mottled areas showed a different picture. Nuclei of all the cells examined contained large areas of viroplasm and contained virus particles in perinuclear inclusions (Figure 3.11a & b). Infected *N. glutinosa* cells showed little or no ultrastructural changes from 8 weeks up to 20 weeks post-inoculation. Although the virus particles fewer in number during this stage than earlier in infection they still appeared of normal length (200-220 nm) and bacilliform in shape. In contrast in the other two hosts short particles were often seen in the nucleus together with mixed with mature virus particles of standard length. These were apparently defective interfering particles (see later). However, the virus particles in infected cells of all three hosts were very clearly reduced in number compared to earlier in infection (Figure 3.10a).

Twelve weeks after inoculation, yellow mottled spots were well established in the leaves. The cells of these *N. clevelandii* leaves contained few virus particles compared to the first 2-3 weeks post-inoculation. Most of these were shorter than standard length. Further work has been carried out with these plants by McElwee and Milner (unpublished results). These workers purified virus from *N. clevelandii* 12 weeks after inoculation and the genomic RNAs were compared to the RNA from standard SYNV particles in Northern blots and by sequencing studies. The results showed the virus purified from infected *N.*

Figure 3.9:

Electron micrographs of thin sections of cells from SYNV-infected *N. glutinosa* leaf (a) 4 weeks, and (b) *N. clevelandii* 3 weeks, showing three distinguishable areas in the nucleus and disrupted chloroplasts.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

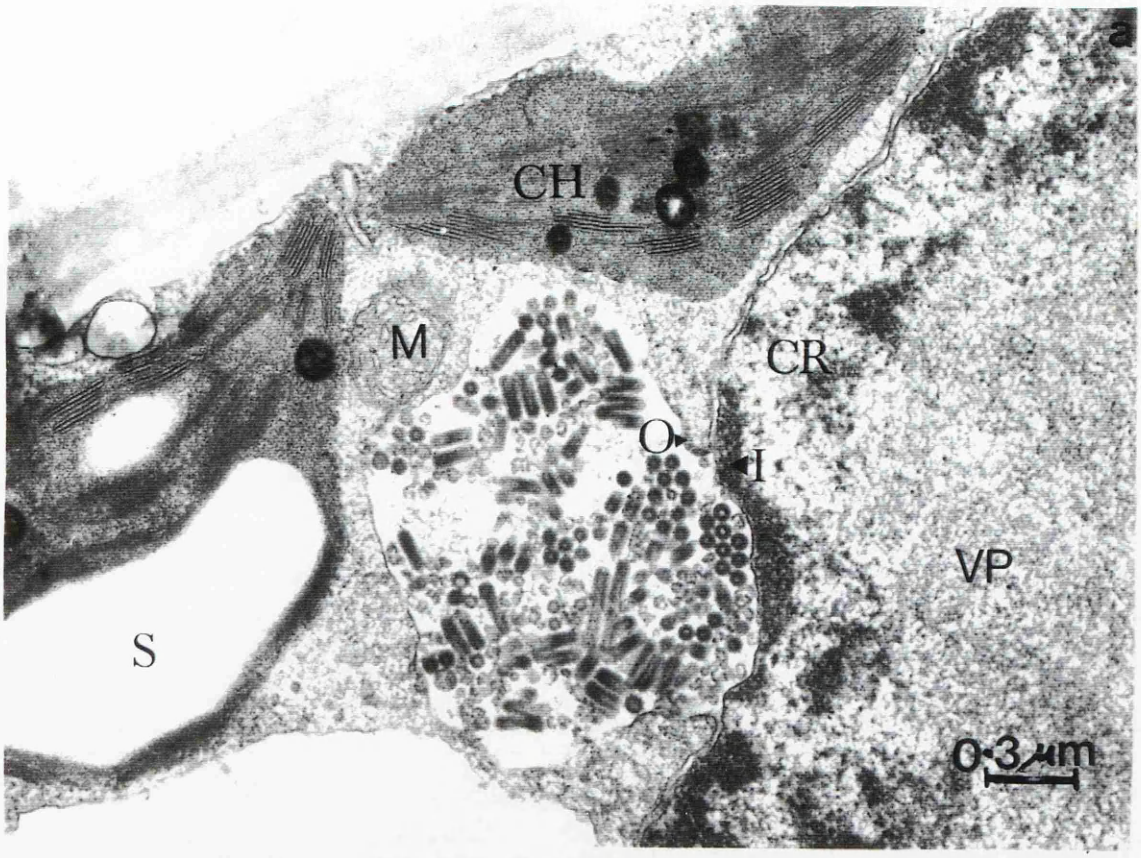
M = Mitochondrium.

CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
CH
M
CR
O
I
VP
S
0·3 µm

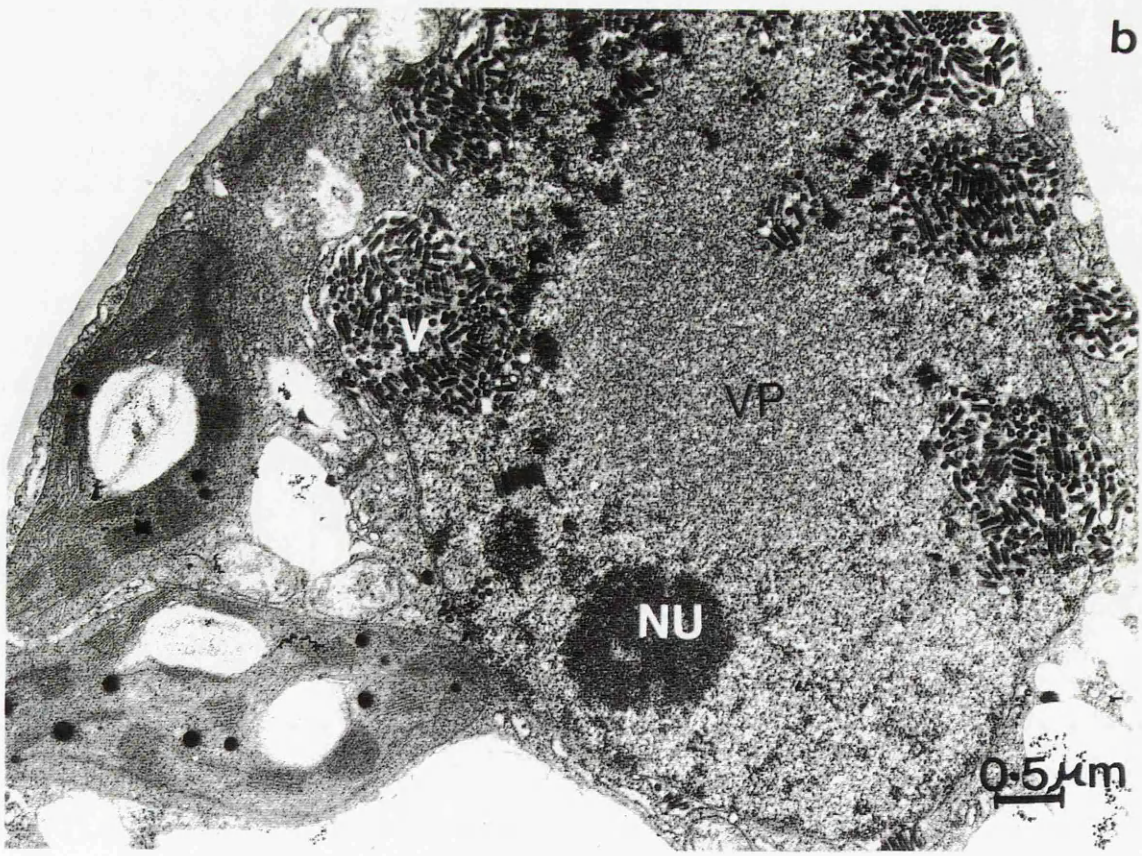

b
V
VP
NU
0·5 µm

Figure 3.10:

Electron micrographs of thin sections of SYNV-infected *N. glutinosa* leaf 8 weeks post-inoculation. (a) From a yellow area of leaf showing a nucleus with large numbers of virus particles. (b) Green area in infected leaf, showing intensely stained chromatin material in the nucleus but no virus particles.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

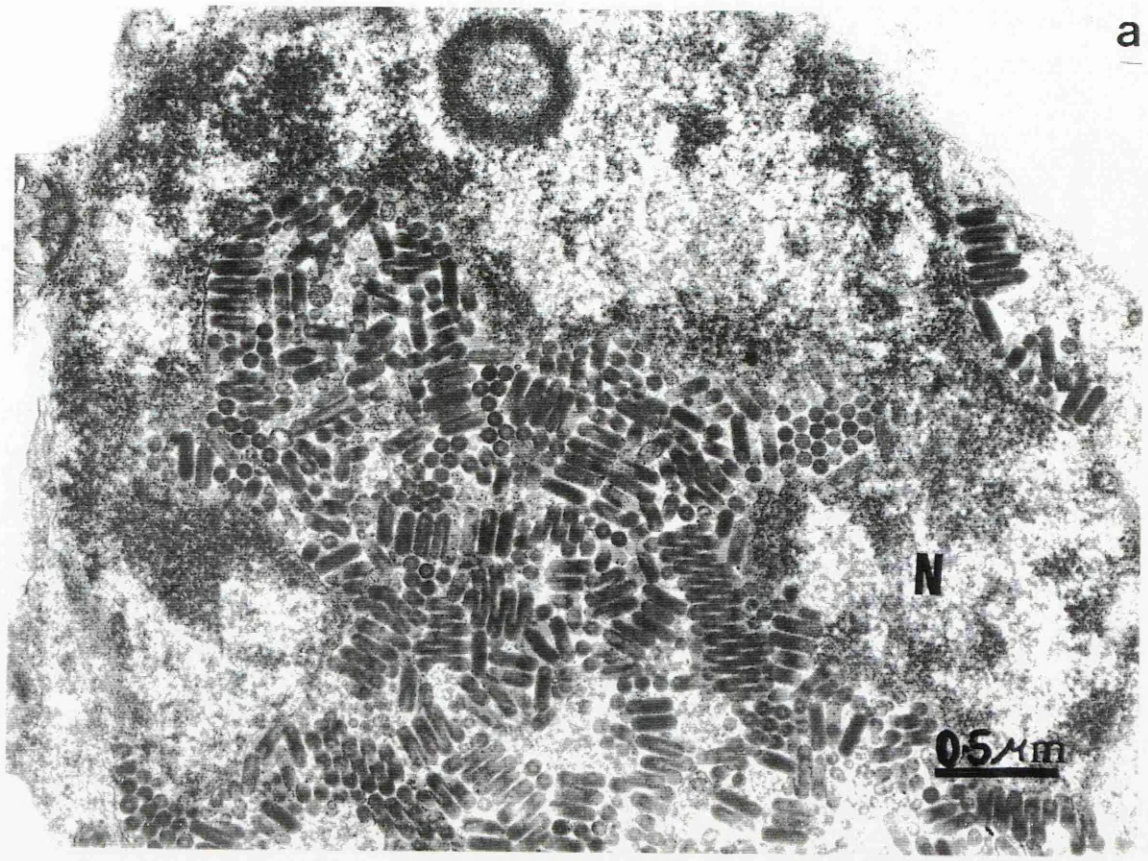
M = Mitochondrium.

CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
N
0·5 µm

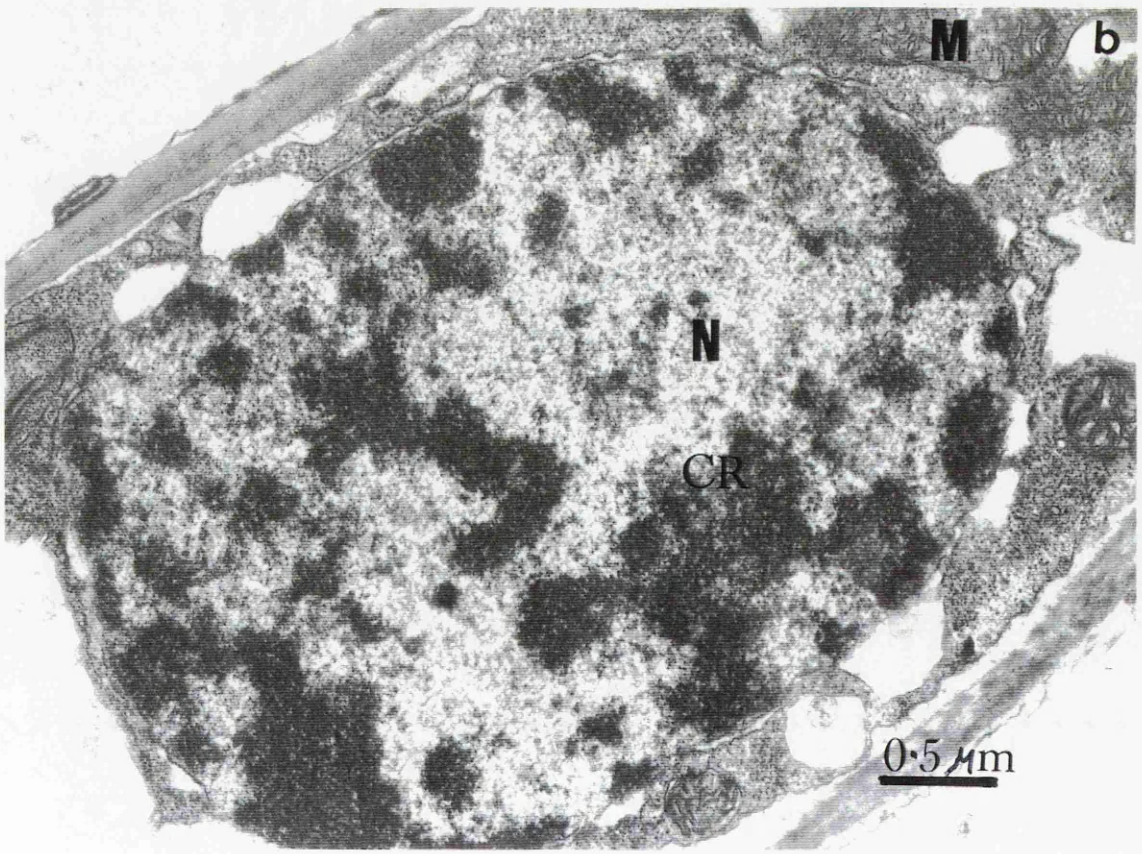

M
b
N
CR
0·5 µm

Figure 3.11:

Electron micrograph of a thin section of yellow area of SYNV-infected *N. glutinosa* leaf 8 weeks post-inoculation, showing abnormalities in the nucleus, and a few virus particles.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

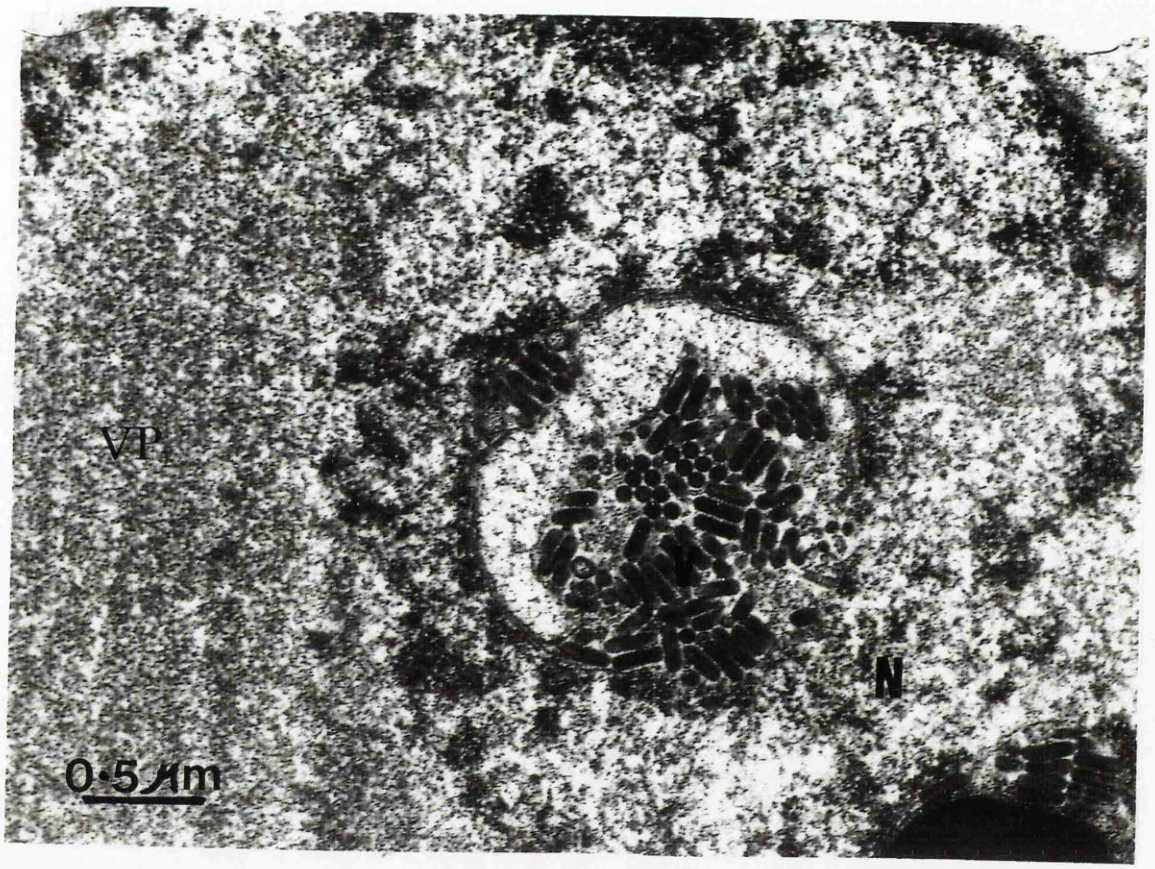
M = Mitochondrium.

CI = Cytoplasmic invagination.

I = Inner membrane.

O= Outer membrane.

UV = Atypical virus-like particle.


VP
N
0·5 μm

clevelandii 12 weeks post-inoculation contained as well as full length genomic RNAs at least three shorter species derived from the standard genome as a result of deletion of most of L, G and SC40 genes. These results and the previous studies on *N. edwardsonii* chronically infected with SYNV (Ismail, *et. al.*, 1987), Ismail, *et. al.*, (1987) demonstrate that these virus particles have all the characteristics of DI particles and resemble those reported previously in other members of the family rhabdoviridae. DI particles seem to be produced in large numbers compared to the standard virus particles and start to appear early in infected *N. clevelandii,* 8 weeks after inoculation (Figure 3.7b).

During the middle stages of infection (5-7 weeks) *N. edwardsonii* showed similar ultrastructural changes to those observed in *N. glutinosa* and *N. clevelandii.* However, changes in *N. edwardsonii* often occurred 5-7 days later after infection than in the other two hosts. Ismail, *et al.* (1987) and Ismail, (1988) reported that the ultrastructural changes in infected *N. edwardsonii* cells started as early as 8 days after inoculation and that abnormalities in chloroplasts and other cytoplasmic components were observed in the later stages of infection (over 20 days). They also reported that short virus particles were found within infected *N. edwardsonii* plants 150 days (22 weeks) after inoculation and that these had all the characteristics of DI-particles. Although the results obtained in the course of this research on SYNV-infected *N. edwardsonii* were similar to those of Ismail (1988), differences were found in the timing after inoculation at which the changes took place. These differences might be related to the conditions under which the infected plants were grown.

Although at the later stages of infection, 20 weeks post-inoculation, infected cells of *N. edwardsonii* appeared ultrastructurally abnormal, infected leaves appeared only mildly symptomatic. Many cells from infected leaves showed large areas of viroplasm in the centre of the nuclei and a few membrane-bound sacs at the edge of the nuclei (Figure 3.12a & b). These were filled with atypical virus-like particles and in some other cells were mixed with a few

Figure 3.12:

Electron micrographs of thin sections of SYNV-infected *N. edwardsonii* leaf 20 weeks, showing (a) three distinct regions in the nucleus. (b) higher magnification of the region containing atypical virus-like particles.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

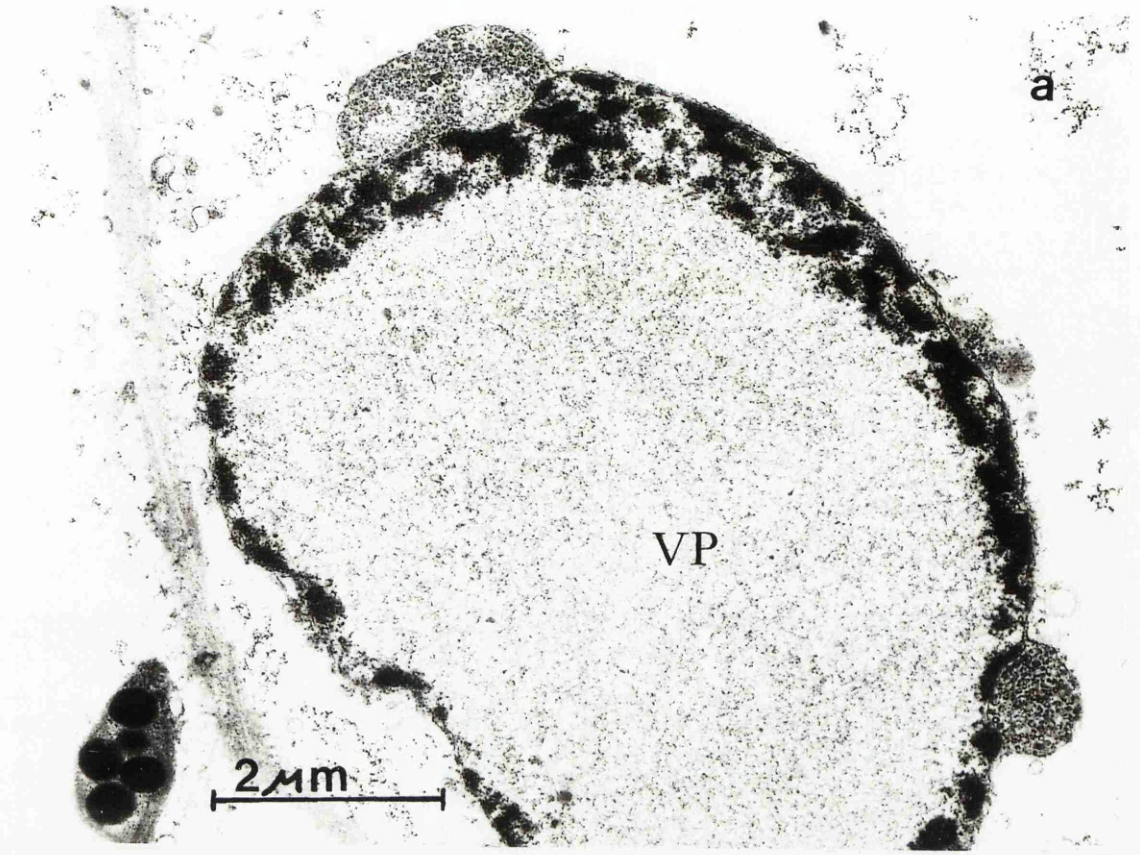
M = Mitochondrium.

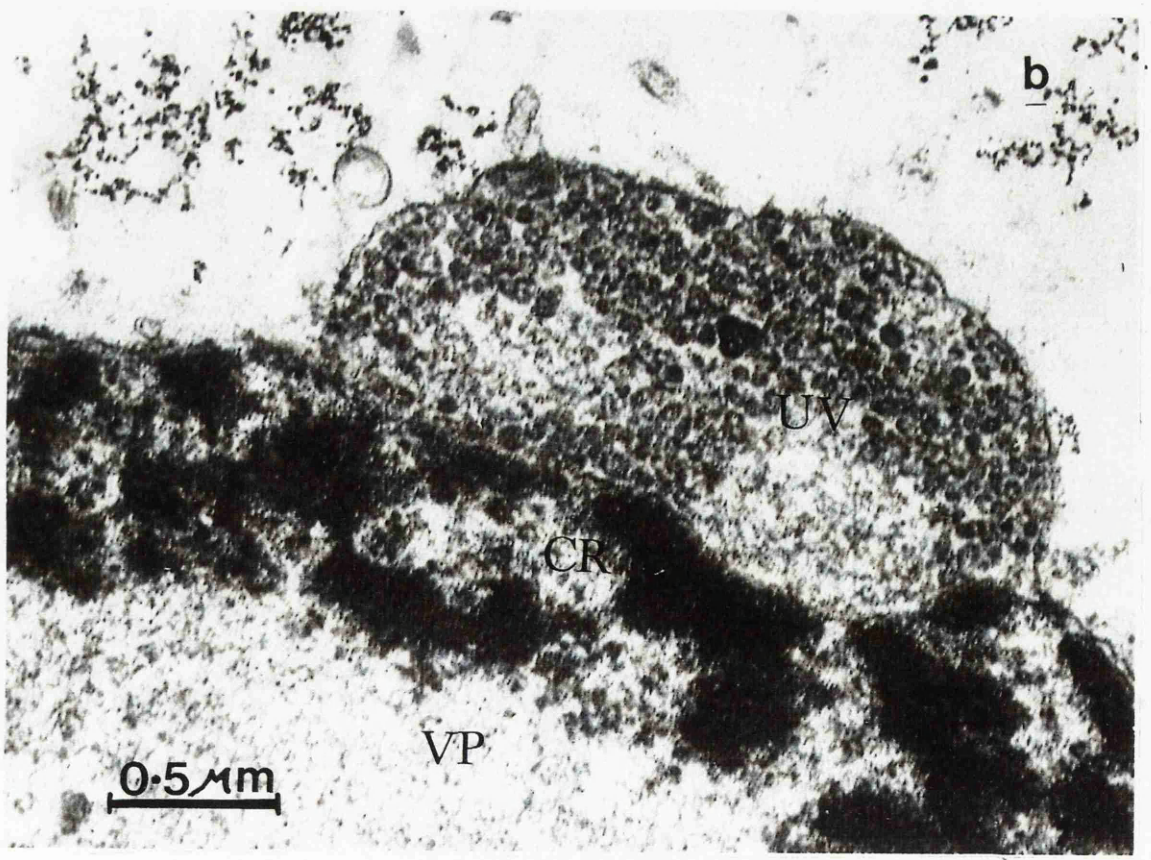
CI = Cytoplasmic invagination.

I = Inner membrane.

O= Outer membrane.

UV = Atypical virus-like particle.


a
VP
2µm


b
UV
CR
VP
0·5µm

bacilliform standard and short particles. These atypical or unformed virus-like particles can be seen in figure 3.12. Evidence will be presented later that they are viral in origin. Twenty weeks after inoculation and thereafter, infected plants started to produce new leaves which showed no outward virus symptoms although older expanded leaves continued to show yellow mottled spots. Ultrathin sections from expanded leaves, 24 weeks after inoculation, revealed typical features of virus infection in cells. The nuclei showed distinctive areas of viroplasm in the centre and the nuclear envelope was associated with perinuclear inclusions containing bacilliform and atypical virus-like particles. Chloroplasts were disrupted and disformed (Figure 3.13a & b). Unexpanded leaves showed only a few yellow mottled spots. Sections showed that the nuclei contained membrane bound sacs filled with atypical virus-like particles and invagination of the nucleus and disruption of chromatin (Figure 3.14a & b). Chloroplasts appeared normal in most of the cells examined. By the late stage of infection in *N. edwardsonii*, after 24 weeks, few if any bacilliform particles were observed in newer leaves. However, a proportion of the nuclear showed the presence of membrane-bound sacs filled with atypical "unformed" virus-like particles (Figure 3.15a & b).

When sections of infected leaves, 30 weeks after inoculation were immunogold labelled using anti-SYNV antibodies (Anti-SYNV antibodies were produced against a whole virus protein by I. Ismail in Botany department, Glasgow University), gold particles bound very extensively to the contents of membrane-bounded sacs but not to the chromatin, indicating the presence of large amounts of viral proteins within the sacs (Figure 3.16a). None of gold particles were seen in the viroplasm or in chromatin. Sections from the same plants were treated with anti-G protein antibodies of SYNV and immunogold labelled, the section revealed a large number of gold particles were binded to the content of membrane-bounded sacs and a few gold particles were observed in the viroplasm (Figure 3.16b).

Figure 3.13:

Electron micrographs of thin sections of SYNV-infected *N. edwardsonii* expanded leaf 24 weeks, showing (a) three distinct regions in the nucleus. (b) higher magnification of the region containing virus and atypical virus-like particles.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

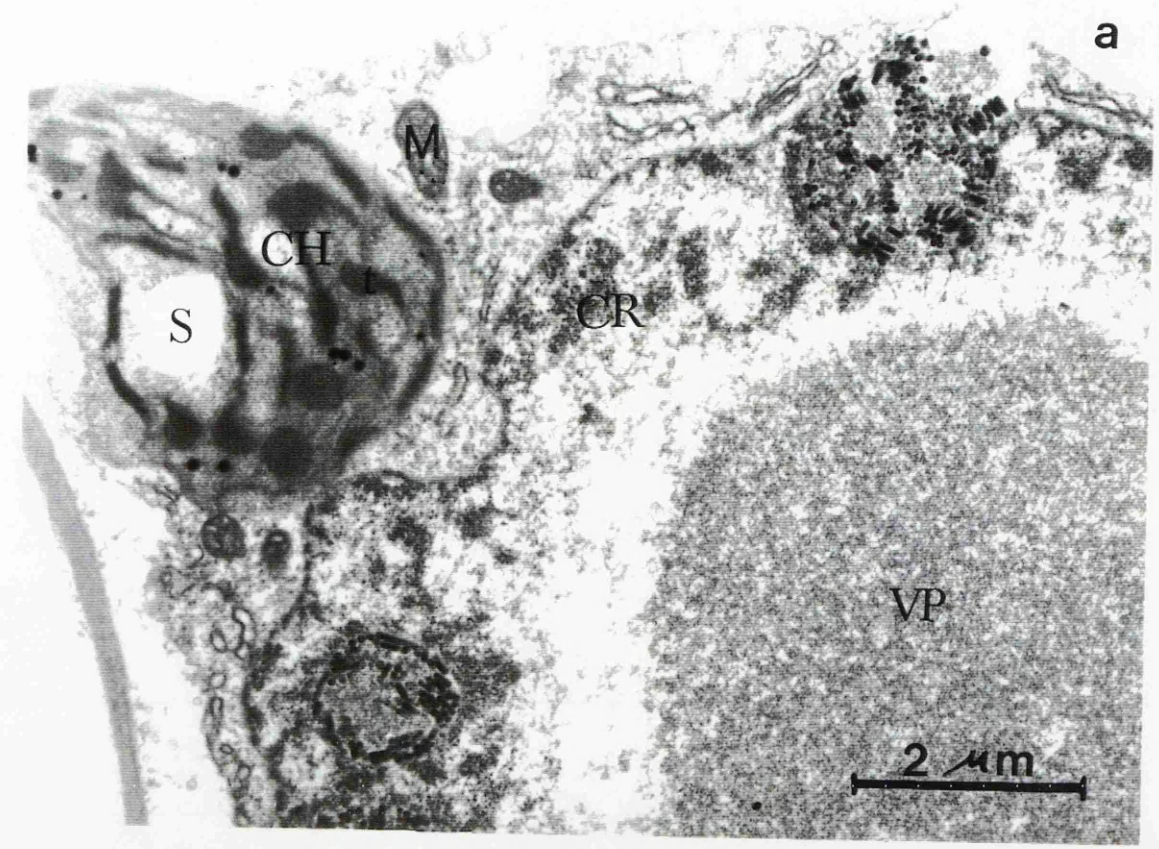
M = Mitochondrium.

CI = Cytoplasmic invagination.

I = Inner membrane.

O= Outer membrane.

UV = Atypical virus-like particle.


a
M
CH
S
CR
VP
2 µm

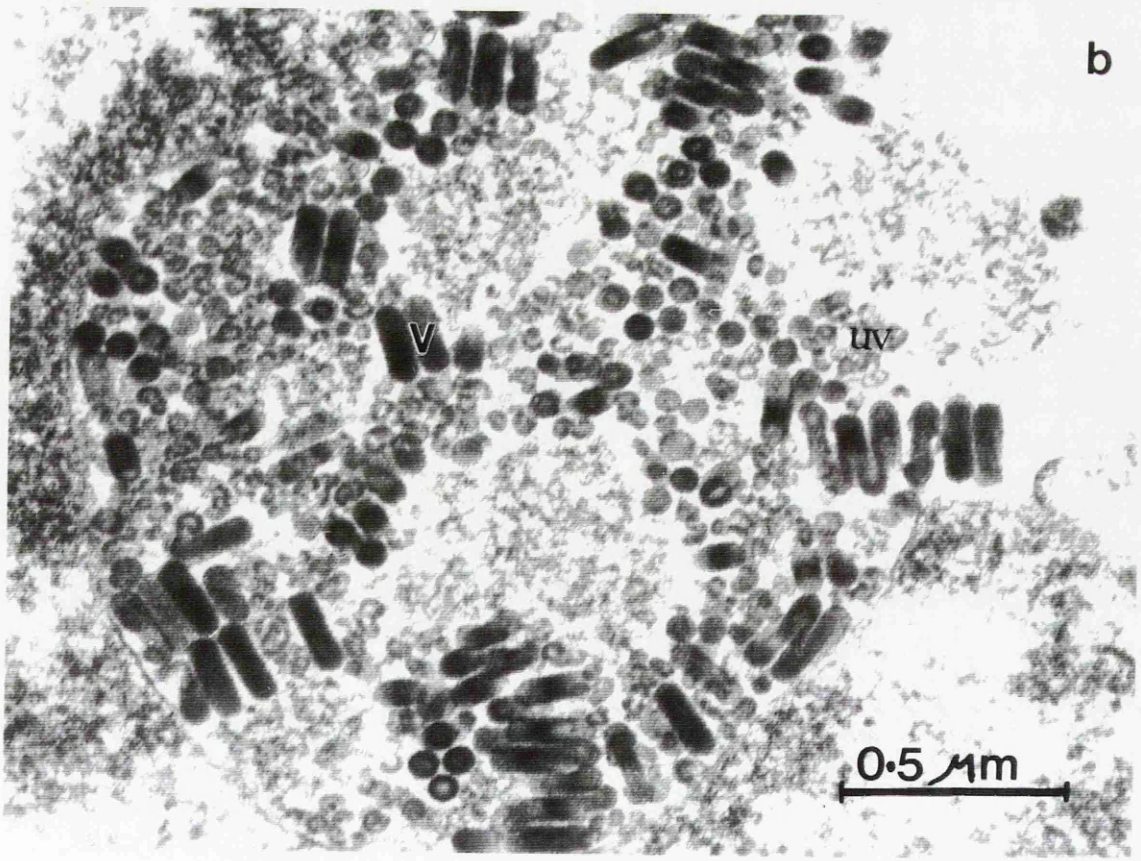

b
V
uv
0·5 µm

Figure 3.14:

Electron micrographs of thin sections of asymptomatic unexpanded leaves of SYNV-infected *N. edwardsonii* 24 weeks after inoculation, showing (a) few membrane-bound sacs filled with atypical virus-like particles and invagination in the nucleus. (b) higher magnification of the region containing atypical virus-like particles.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

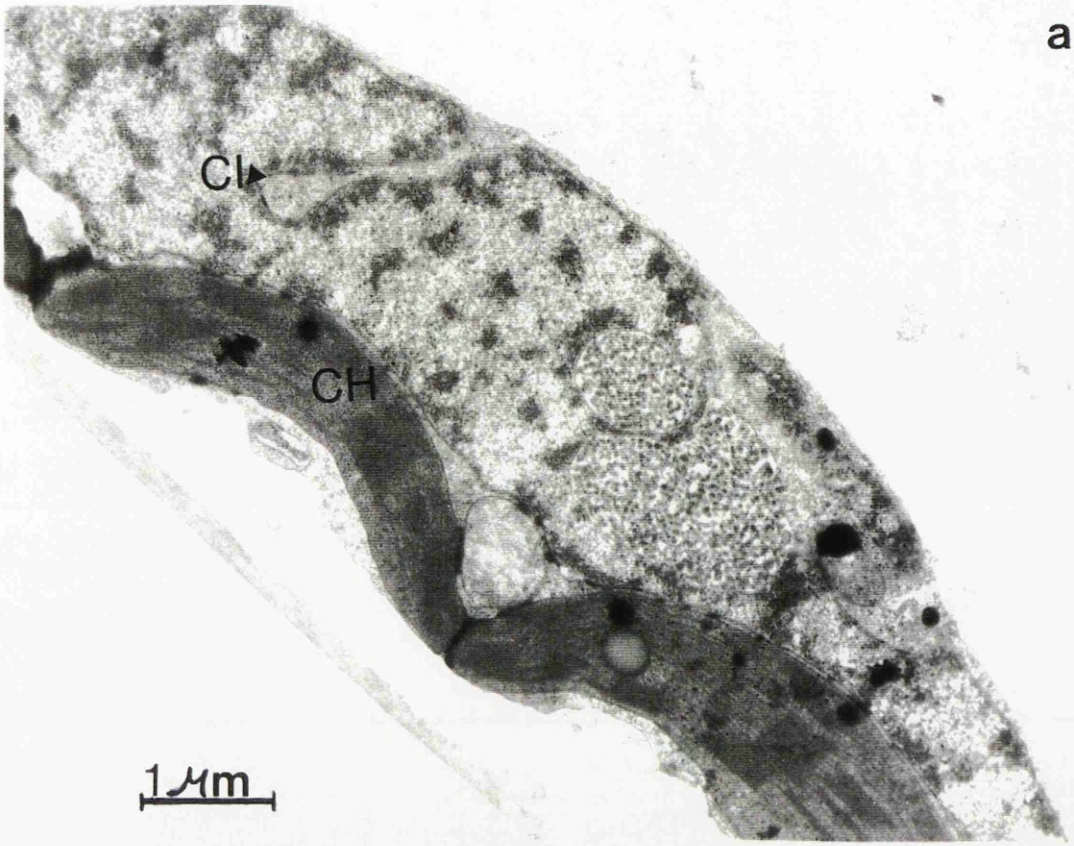
M = Mitochondrium.

CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
Cl
CH
1 µm

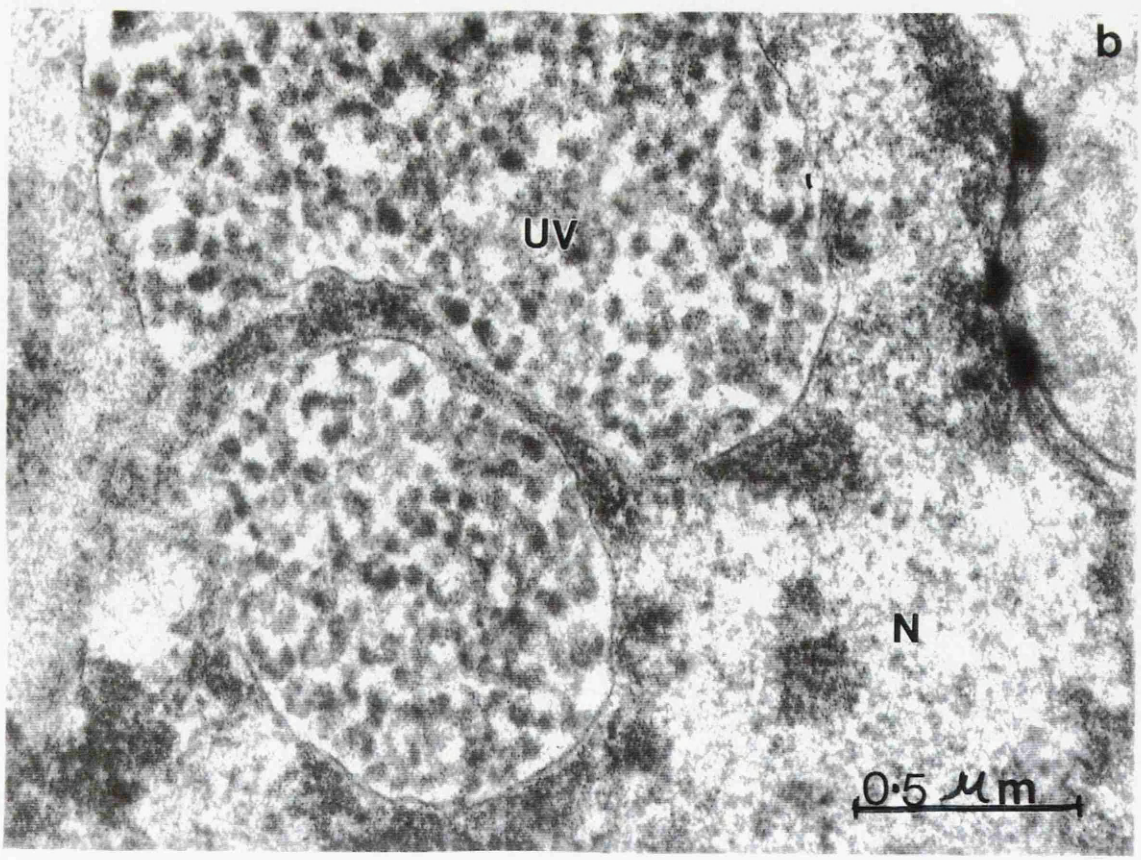

b
UV
N
0·5 µm

Figure 3.15:

Electron micrographs of thin sections of SYNV-infected *N. edwardsonii* leaf 31 weeks, (a) showing several membrane-bounded sacs filled with atypical "unformed" virus-like particles and nucleocapsid. (b) Higher magnification of membrane-bound sac.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

M = Mitochondrium.

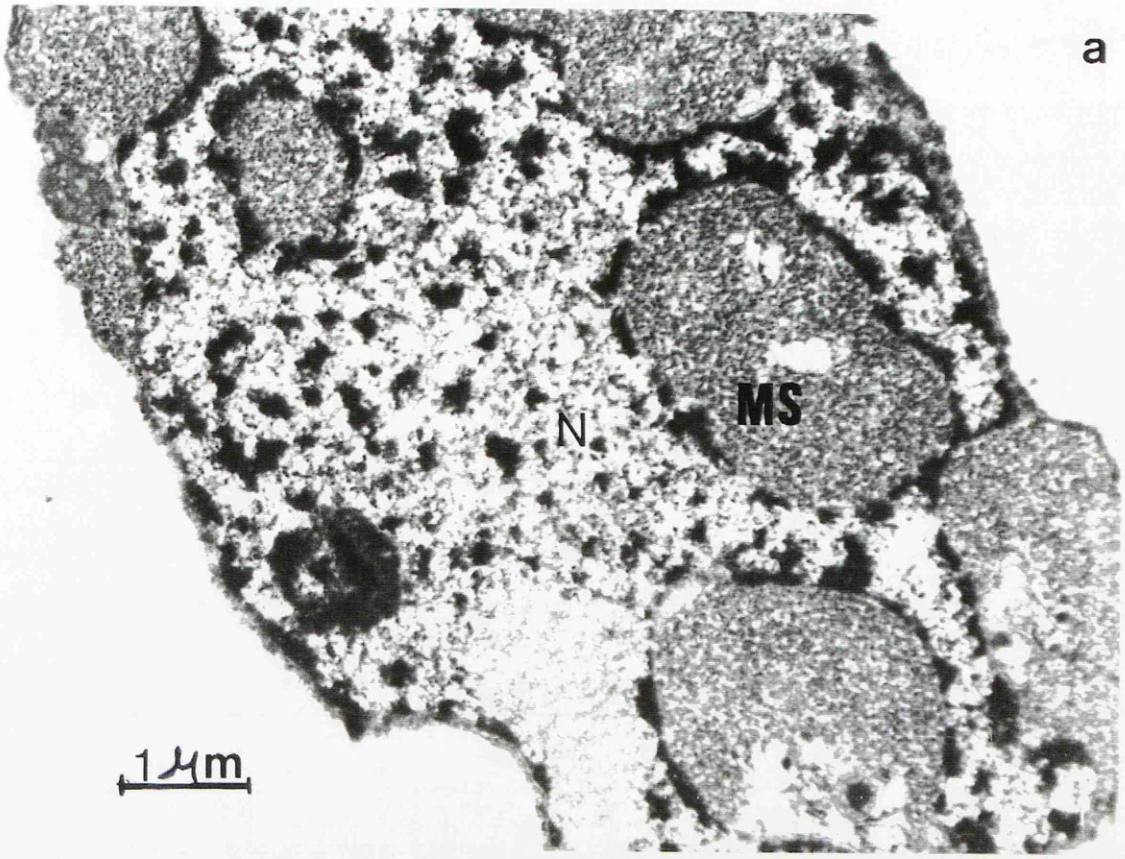
CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.

MS = Membrane-bound sacs.


a
N
MS
1 µm

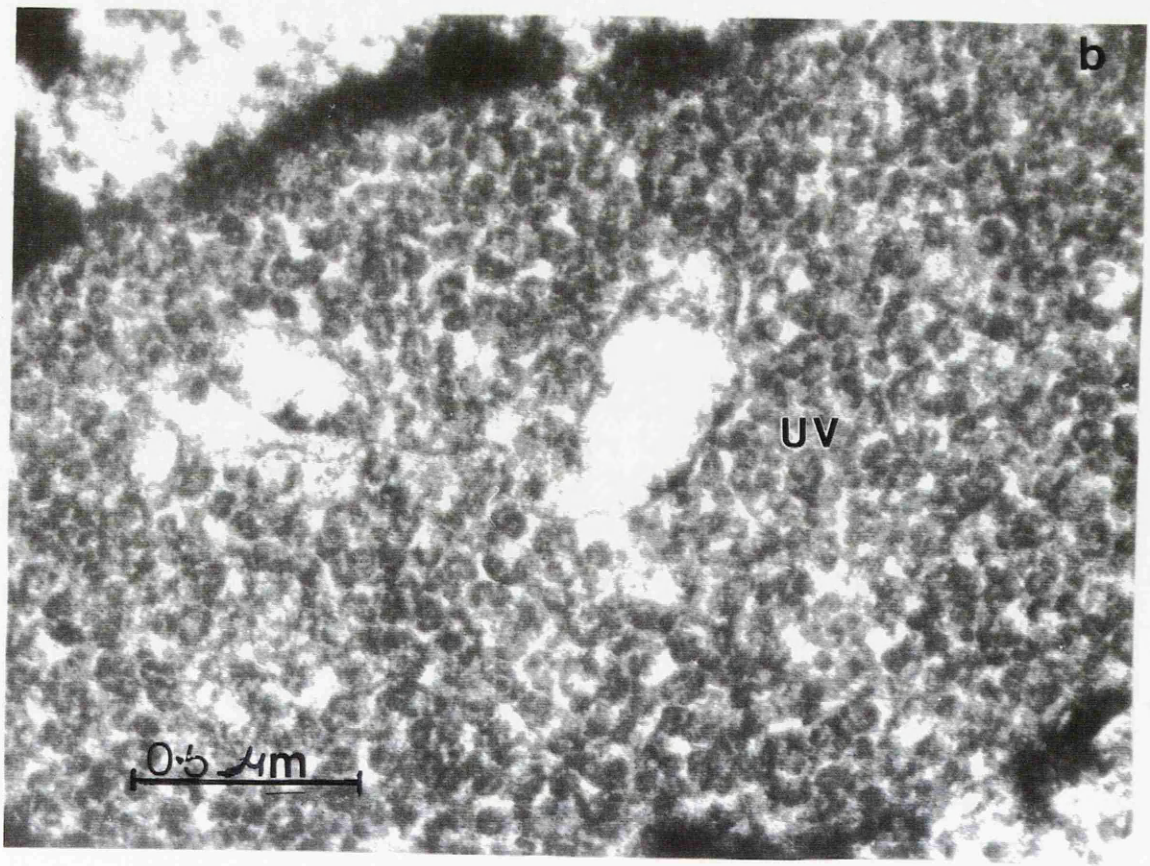

b
UV
0·5 µm

Figure 3.16:

Electron micrographs of thin sections immunogold labelled SYNV-infected *N. edwardsonii* leaf 31 weeks, (a) using anti-SYNV antiserum, (b) anti-SYNV G protein antiserum (b). A large number of gold particles are bound to the contents of membrane-sacs while very few gold particles can be seen in the viroplasm.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

M = Mitochondrium.

CI = Cytoplasmic invagination.

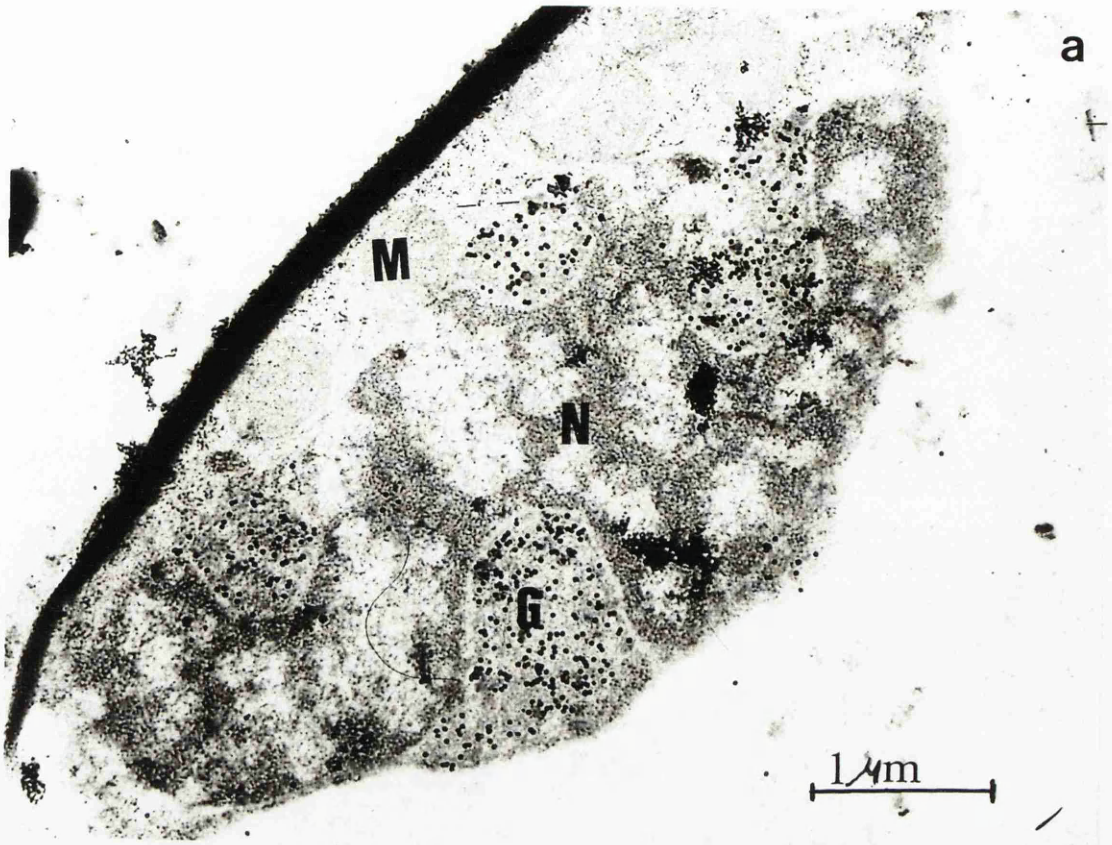
I = Inner membrane.

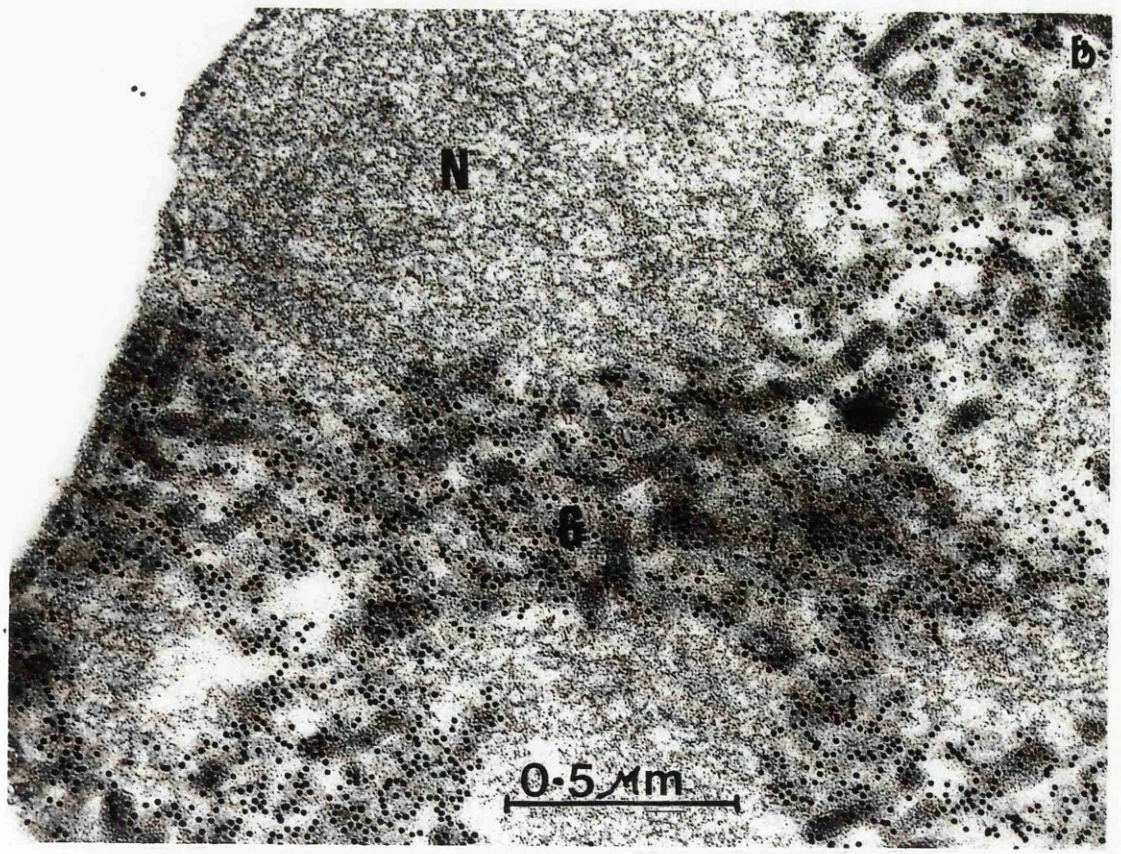
O = Outer membrane.

UV = Atypical virus-like particle.

MS = Membrane-bounded sacs.

G = Gold particles.


a
M
N
G
1 µm


b
N
G
0·5 µm

In later stages of infection, 28 weeks post-inoculation, *N. glutinosa* leaf cell components still showed ultrastructural changes induced by infection, especially in the nucleus. Chromatin was disrupted, and aggregated near the edge of the nucleus. Membrane-bound inclusions could be observed at the edge of the nucleus. In contrast to *N. edwardsonii,* most of the cells contained a mixture of normal and atypical virus-like particles (Figure 3.17a & b), although the number of virus particles was fewer compared to the previous two stages of infection.

Infected *N. clevelandii* plants responded similarly to *N. glutinosa* in the final stages, after 20 weeks. Electron micrographs of infected cells 23 weeks after inoculation showed large areas of viroplasm surrounded by a thin region of chromatin and sacs filled with bacilliform and atypical virus-like particles (Figure 3.18a & b).

Figure 3.17:

Electron micrographs of thin sections of SYNV-infected *N. glutinosa* leaf 28 weeks post-inoculation, showing (a) whole cell components with nucleus contains unformed virus particles. (b) is a higher magnification of the nucleus part.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

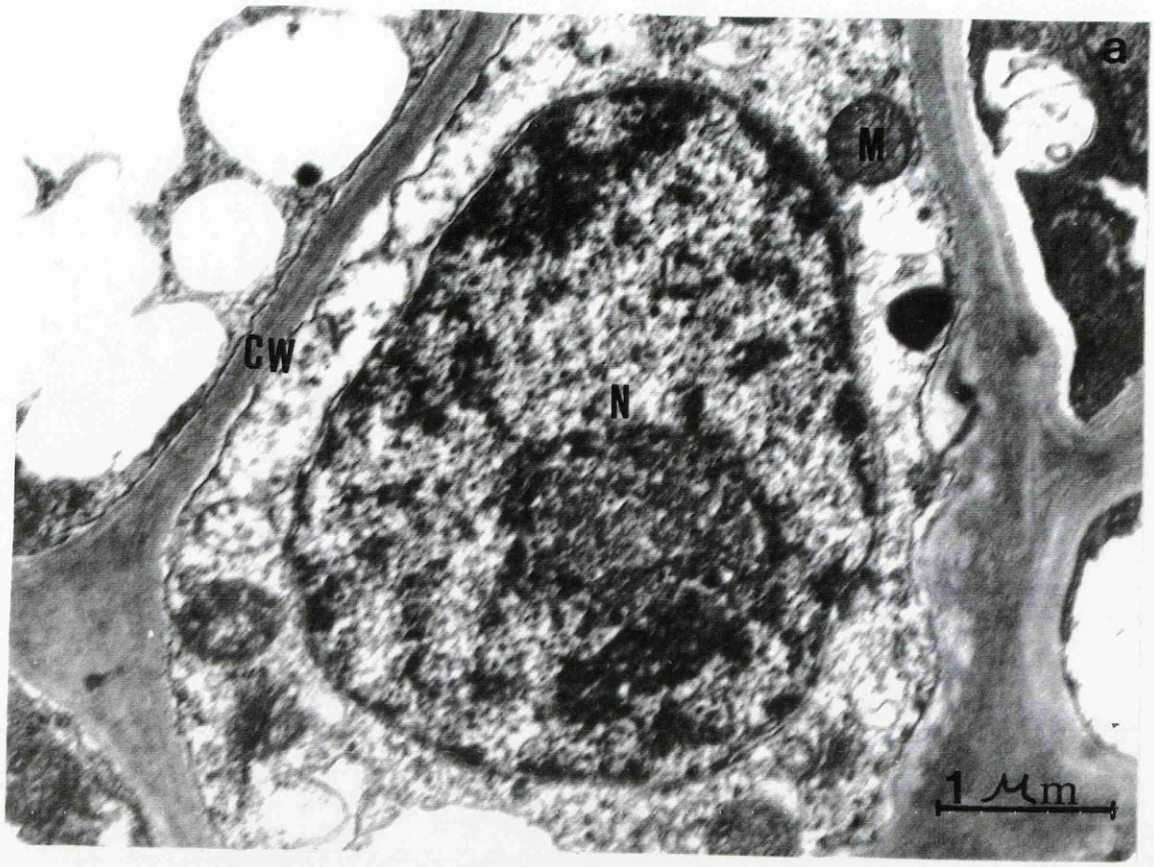
M = Mitochondrium.

CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
M
CW
N
1 µm

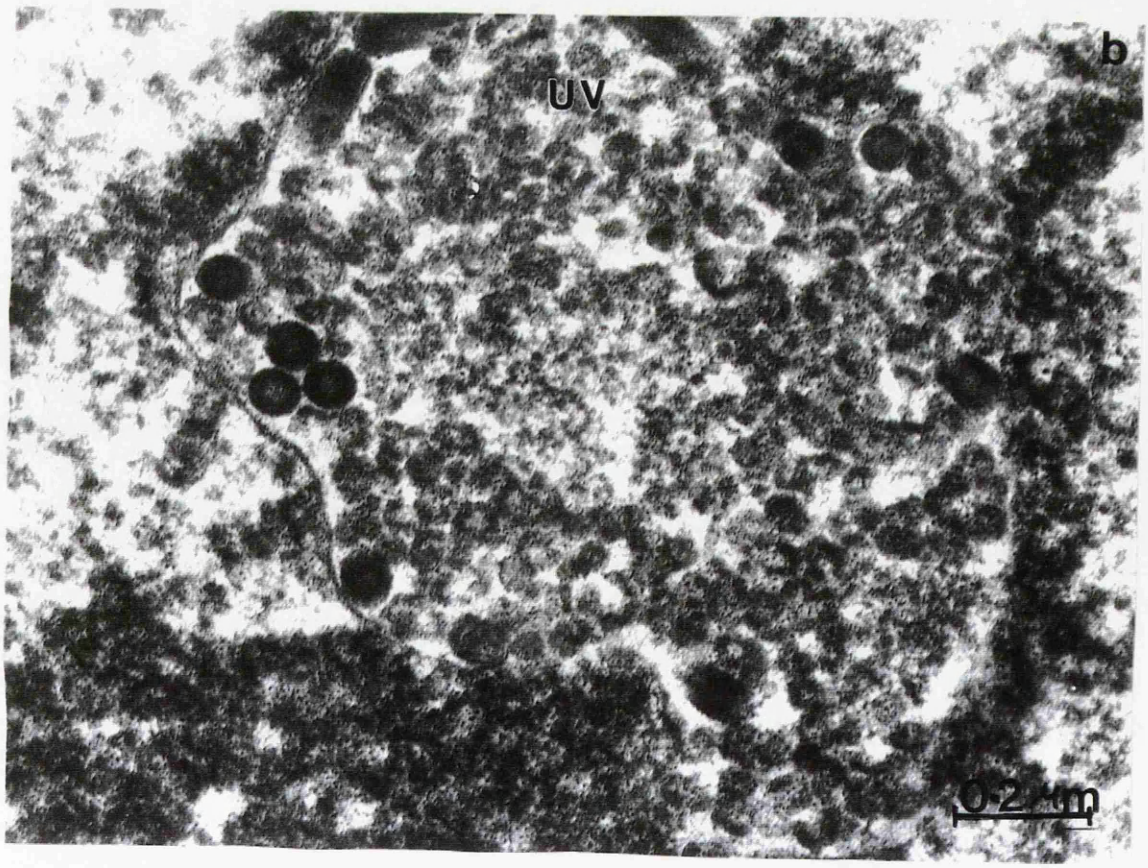

b
UV
0·2 µm

Figure 3.18:

Electron micrographs of thin sections of SYNV-infected *N. clevelandii* leaf 23 weeks post-inoculation, showing (a) three distinguishable areas in the nucleus plus a few virus particles. (b) higher magnification of the nuclear region containing short bacilliform and atyipcal "unformed virus" virus-like particles.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

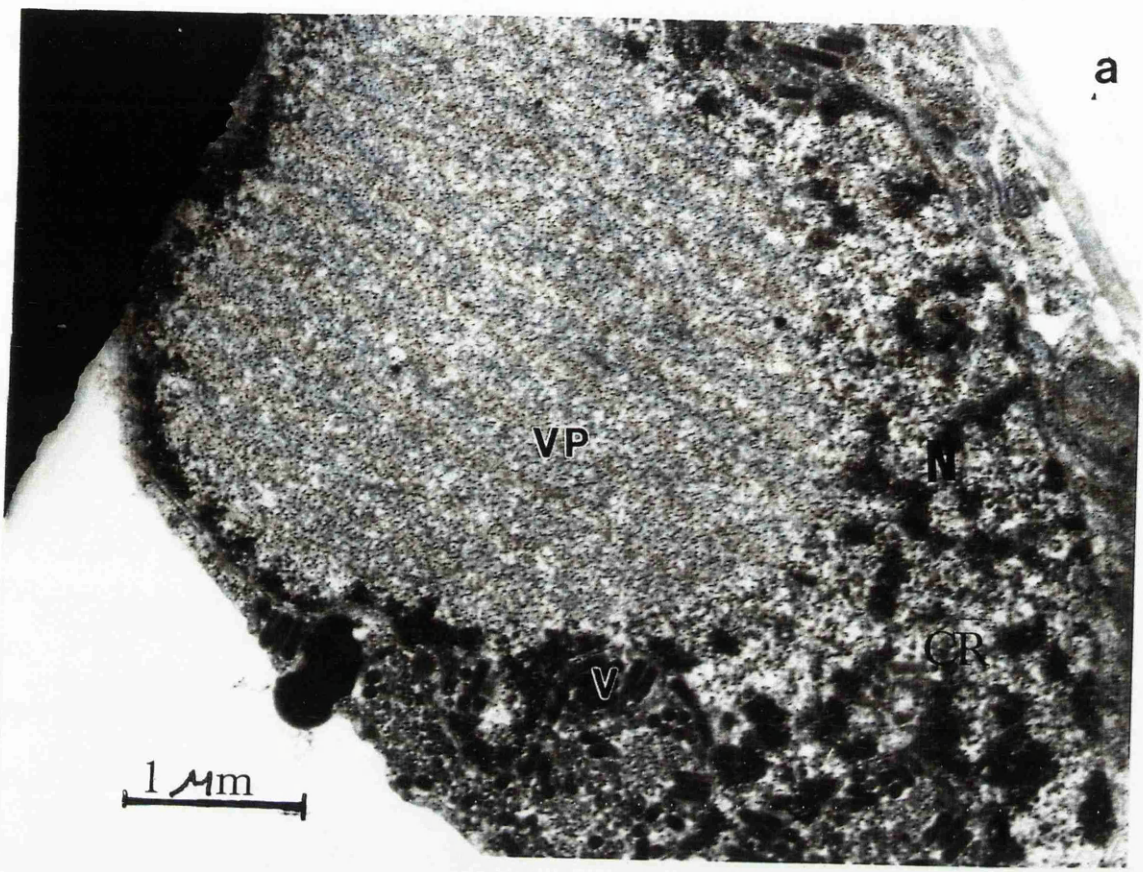
M = Mitochondrium.

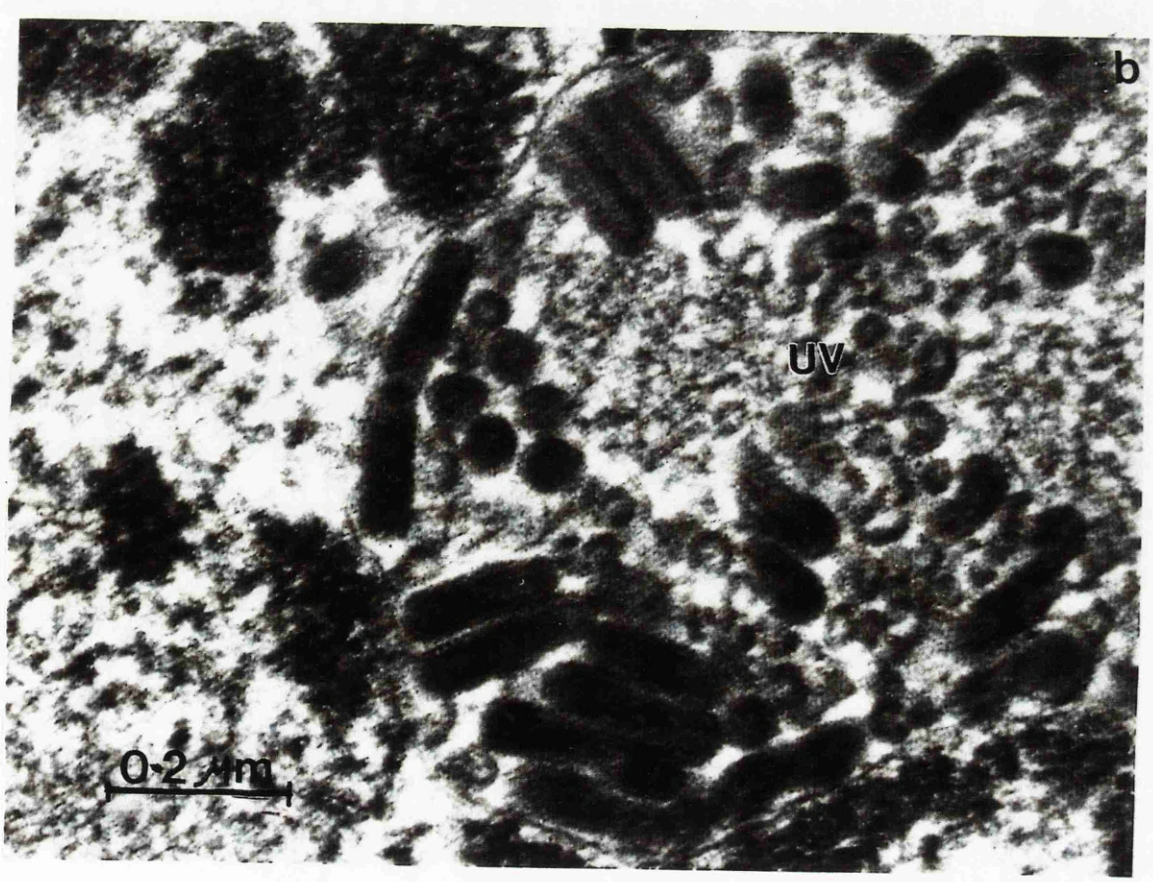
CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
VP
N
CR
V
1 µm


b
UV
0·2 µm

3.6- Changes in levels of individual virus structural proteins in SYNV-infected plants:

Total protein extracts were prepared from *N. glutinosa* plants infected with SYNV as described in sections (2.4.1 & 2.6.6). Samples were collected starting from 5 days post-inoculation and continuing up to 65 days. Unexpanded leaves from 5 days infected plants did not show symptoms, but later, the plants developed severe symptoms. Protein samples from infected *N. glutinosa*, healthy plants and purified SYNV were fractionated by polyacrylamide gel electrophoresis as described in section (2.4.1). After running the gel, proteins were blotted onto a nitrocellulose membrane as described in section (2.6.6) and probed for SYNV protein using anti-SYNV antiserum. Figure 3.19 shows a typical result. In order to compare levels of SYNV antigen, each lane in the gel was loaded with protein extracted from an equal mass of leaf tissue.

Anti-SYNV antibodies reacted strongly to the four major virus proteins in the lane containing purified virus. As early as 5 days post-inoculation SYNV proteins were detectable albeit at relatively low levels (Figure 3.19). As virus infection progressed from 15 days onwards, SYNV antibodies reacted more strongly to the protein samples but only three bands corresponding to the G, N, and M1 proteins were visible. The intensity of the three proteins bands gradually declined with increasing time after inoculation. No reaction to M2 protein was detectable in any of the samples. However, M2 appears to be a poor antigen since it reacted only faintly in lanes containing purified SYNV.

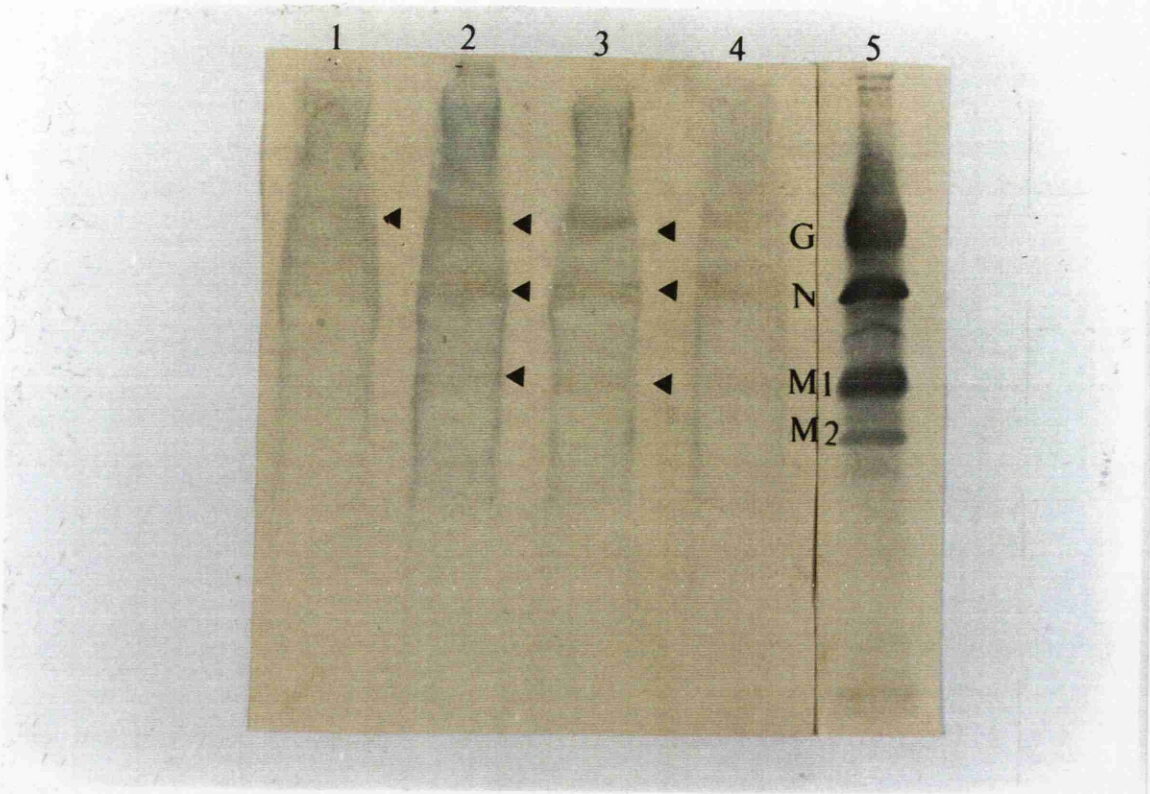


Figure 3.19:

Immunoblotting of SYNV-proteins extracted from *N. glutinosa* plant leaves at different times after inoculation. The four bands corresponding to G, N, M1 and M2 virus proteins are shown in lane (5) which contain purified virus. Lanes 1, 2, 3, & 4 contain total protein extracted 5, 15, 45, and 65 days after inoculation show the three bands of G, N, and M1.

3.7- Symptom expression in EMDV-infected plants:

The three *Nicotiana* species were inoculated with EMDV. *N. clevelandii* developed the most severe visible symptoms of any of the three. Symptoms included vein clearing, mottling and crinkling in unexpanded leaves and developed 2-3 weeks post-inoculation. Subsequently, leaves became chlorotic and underwent a severe necrosis of most of the lamella of the leaf. After the infection had spread throughout the whole plant, plant growth almost ceased, plants becoming severely stunted and eventually dying. The infected plants survived longer if inoculated at the 10 to 15 leaf stage rather than the 8 leaf stage.

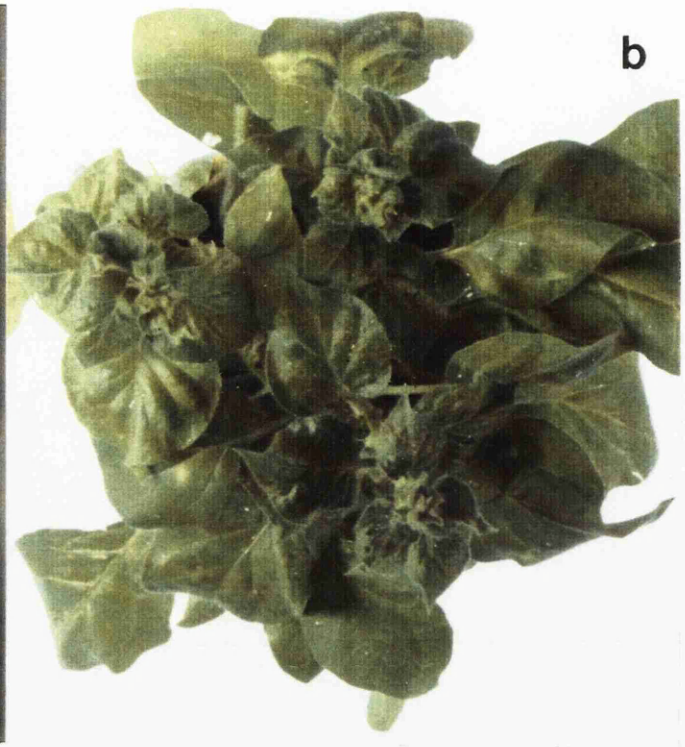
N. glutinosa plants infected with EMDV showed more tolerance to the virus infection than did *N. clevelandii*. The symptoms appeared 15-25 days post-inoculation under the greenhouse conditions, depending on the exact age of plants at the time of inoculation. The symptoms started with leaf cupping and vein clearing then following at later stages with yellowing of unexpanded leaves. Large chlorotic local lesions were visible on inoculated leaves. The infected plants were severely stunted and growth was very slow compared to controls. Infected plants rarely survived more than 6 weeks after the symptoms appeared. Those plants which survived produced small leaves with severe symptoms. The flowers of infected plant were usually very few in number and produced seeds which often failed to germinate.

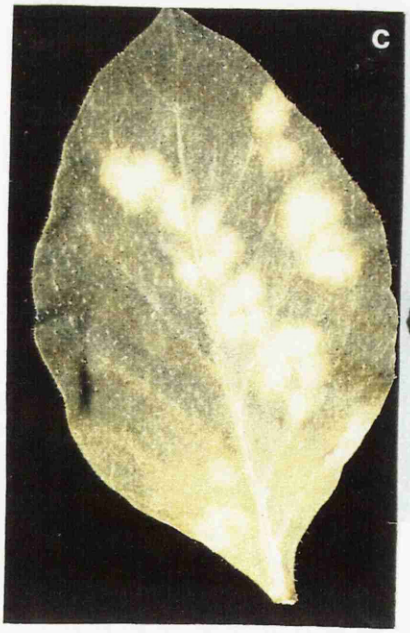
N. edwardsonii plants appeared to be the most tolerant to EMDV and were thus the most suitable host in which to study EMDV infection. Infected plants showed symptoms of vein-clearing, mottling and leaf cupping of the unexpanded leaves and were severely stunted. Plants, especially those infected early (8 leaf stage) (figures 3.20 a & b) often died after 5-6 weeks. If

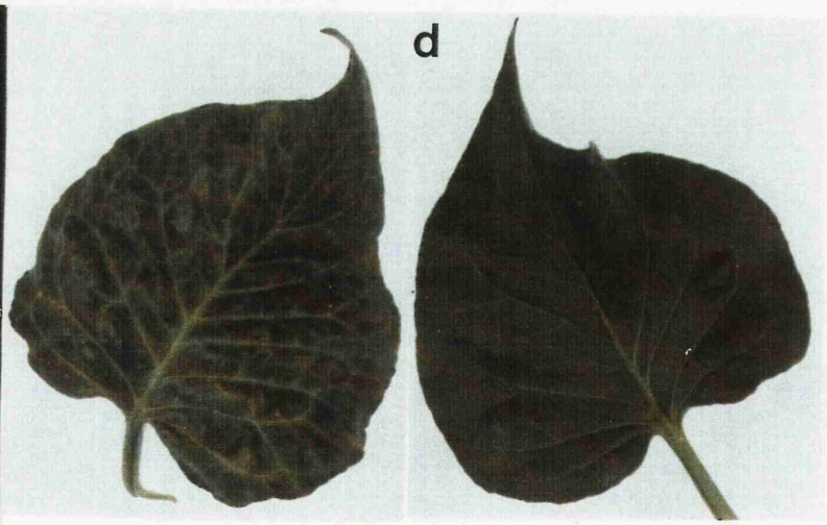
Figure 3.20:

(a)- *N. edwardsonii* plants infected with EMDV, showing; **(a)** severe stunting (right) infected plant, (left) healthy control plant. **(b)** symptoms of vein-clearing, leaf cupping and mottling of the leaves 4 weeks post-inoculation. **(c)** Inoculated leaf (>10 leaf stage) showing local lesions in expanded leaves 6 weeks post-inoculation. **(d)** (left) unexpanded leaf showing symptoms of vein-clearing, 4 weeks post-inoculation. (right) Control leaf.


a


b


c


d

plants were inoculated at the (>10 leaf stage), symptoms appeared 5-7 days longer than in plants infected young with obvious local lesions plus severe chlorosis. Vein clearing and mottling in unexpanded leaves appeared after about 6 weeks (figures 3.20c & 3.21b). In late inoculated plants which survived infection, unexpanded leaves developed severe mottling and vein clearing between 7 and 9 weeks post-inoculation and after 10 weeks, expanded and some unexpanded leaves began to develop large chlorotic lesions which subsequently turned necrotic. These chlorotic areas were very pronounced. Occasionally late inoculated plants did not show any sign of symptoms until 8 weeks after inoculation. These plants did not show typical systemic symptoms but developed chlorotic areas in one part of the leaf, other parts was remaining green (figure 3.21 a, c & d). These plants were almost normal in height compared to the control plants.

In general, *Nicotiana spp,* infected with EMDV showed much more severe symptoms compared to plants infected by SYNV. However, the three hosts responded to virus infection somewhat differently from each other. *N. edwardsonii* plants were most tolerant to the infection whereas *N. clevelandii* plants were the most susceptible. Table 3.2 summarised the main feature of EMDV symptoms in the three hosts.

Figure 3.21:

N. edwardsonii plants infected with EMDV. **(a)** which did not show vein-clearing symptoms, but showed chlorotic areas in unexpanded leaves 8 weeks post-inoculation. **(b)** infected with EMDV (>10 leaf stage) showing leaf cupping, mottling and vein-clearing in unexpanded leaves 6 weeks post-inoculation. Note chlorotic lesions in unexpanded leaves (arrowed). **(c)** and **(d)** symptoms in expanded and unexpanded leaves of plants inoculated at (>10 leaf stage). **(e)** Control leaf plant at the same age.


a
b

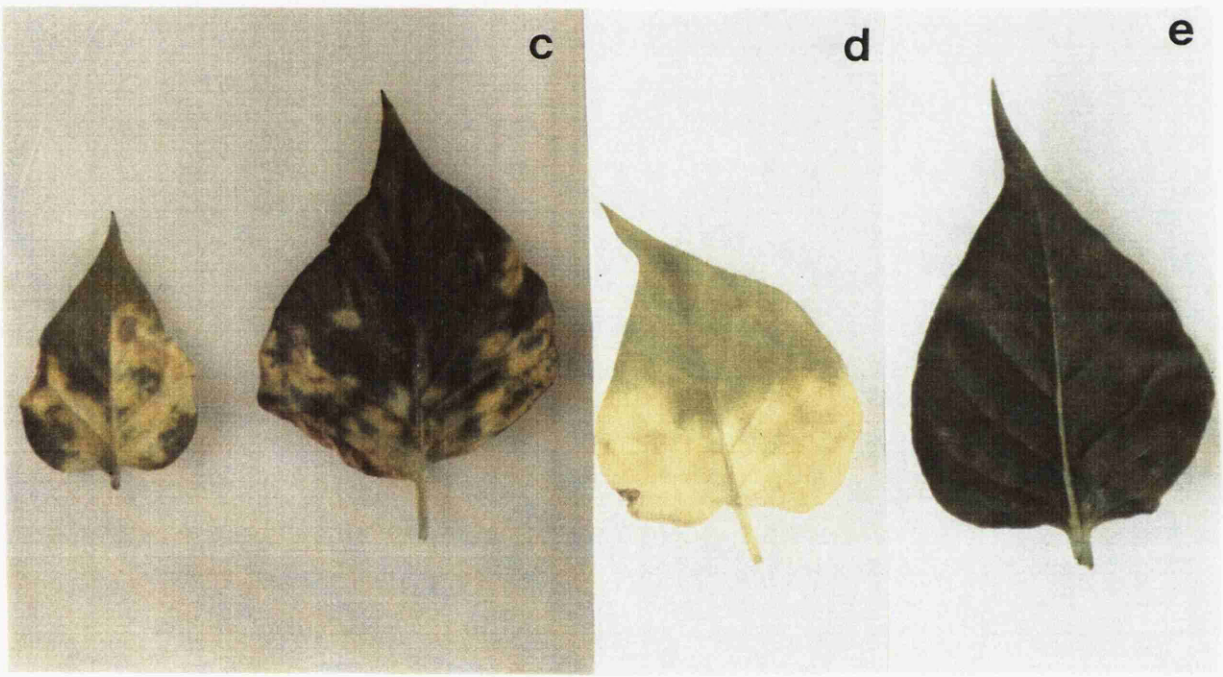

c
d
e

Table (3.2): Summary of EMDV symptoms in three hosts.

Hosts	Symptoms
N. glutinosa.	- Symptoms appeared after 15-25 days with leaf-cupping, mottling, vein-clearing and stunting. - Large areas of chlorosis developed at later stage of infection. - Local lesion are an obvious feature of inoculated leaves. - Severe stunting.
N. clevelandii.	- Symptoms appeared after 14-21 days with leaf-cupping, vein-clearing, mottling and plant stunting. - Large areas of chlorosis developed. These became necorotic at later stage of infection - Symptoms was very severe, plants severely stunted.
N. edwardsonii.	- Symptoms appeared after 15-25 days with leaf cupping, vein-clearing, mottling and severe stunting. - Plants inoculated in late stage, symptoms appeared 5-7 days later with local lesion and severe chlorotic lesion in expanded leaves. - Symptoms of late stage infection (after 7 weeks); severe mottling and vein-clearing in unexpanded leaves others expanded and some of unexpanded leaves developed large chlorotic areas turn to necrotic later.

3.8- Morphology, structure and intracellular location of EMDV virus particles:

3.8.1- Morphology and structure of EMDV:

Electron micrographs of ultrathin sections of EMDV-infected *N. edwardsonii*, *N. glutinosa* and *N. clevelandii* were examined at various times after inoculation. These revealed distinct morphological and structural features of the virus particles during the infection period. Sections examined were taken from the first appearance of symptoms 3-4 weeks after inoculation. Most of the cells observed contained numerous heavily stained, bacilliform or round elements representing virus particles in longitudinal and transverse section respectively. Virus particles were mostly located in massive inclusions at the periphery of the nucleus. These nuclear inclusion were often bounded by the lamellae of the nuclear envelope, which became widely separated. In addition, inclusions were also observed at a distance from the nucleus, encased in membrane possible originating from the nuclear membrane or endoplasmic reticulum. In many sections, virus particles were seen with their envelope continuous with the inner nuclear membrane as though they were budding from it.

Two morphologically distinct types of virus particle were observed within the same cells; bacilliform, with both ends rounded, and bullet-shaped with one rounded and one flat end. The former had rather constant dimensions (220-250 nm x 70-80 nm) and were invariably detached or in the process of detaching themselves from the cellular membranes, appearing free in the perinuclear spaces or in the cytoplasmic sacs. The bullet-shaped particles were usually shorter and variable in their size and in many instances still connected by their basal flat end to the nuclear membrane.

The fine structure of virus particles in cross section showed three concentric rings corresponding to the nucleocapsid and envelope with its

projections. The lucent core, perhaps reflects the helical organisation of the nucleocapsid in the intact particle (Fig. 3.22 a & b).

As the infection progressed, the virus particles assembled in large number in the perinuclear spaces and were observed scattered in the cytoplasm encased in membrane bounded sacs. Ten weeks post-inoculation, the total number of virus particles had decreased but large numbers of infected cells showed evidence of internal ultrastructural disruption and effects on individual organelles had become evident. Irrespective of the host and age of infection, virus particles were always present, usually in perinuclear inclusions or in membrane-bound sacs within the cytoplasm.

3.8.2- Cytology of EMDV-infected cells:

Infection by EMDV induced major cytological changes in systemically infected leaves of the three hosts studied in the first 3-4 weeks after inoculation. Over this period, the major changes in infected cells were confined to the nuclei, which showed in most cases, a drastic reduction in chromatin material which was distributed near the viral inclusions. A large swelling of the perinuclear space with virus particles accumulating there was also evident (Figure 3.22c). The nucleoli when seen, were apparently unchanged. Large areas of densely staining viroplasm developed in the nuclei and invaginations of the inner nuclear membrane were evident. Samples from leaves showing local lesions were examined and also revealed abnormalities. Three weeks after inoculation, local lesions appeared in inoculated leaves. These local lesions, when examined under the electron microscope, showed large numbers of virus particles in the perinuclear space. Some of them were also found in inclusions in the nucleus surrounded by a membrane originating from the inner nuclear membrane. After 3 weeks, cells of *N. glutinosa* contained large regions of viroplasm and some virions in the endoplasmic reticulum (Figures 3.22c and 3.23a). Chloroplasts and

Figure 3.22:

(a) and (b) Electron micrographs of thin sections showing the fine structure of EMDV virus particle in cross-section in *N. edwardsonii* leaves.

(c)- Electron micrographs of thin sections of EMDV in infected *N. glutinosa* leaf, showing local lesion 3 weeks post-inoculation.

Standard abbreviations:

N = Nucleus.

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

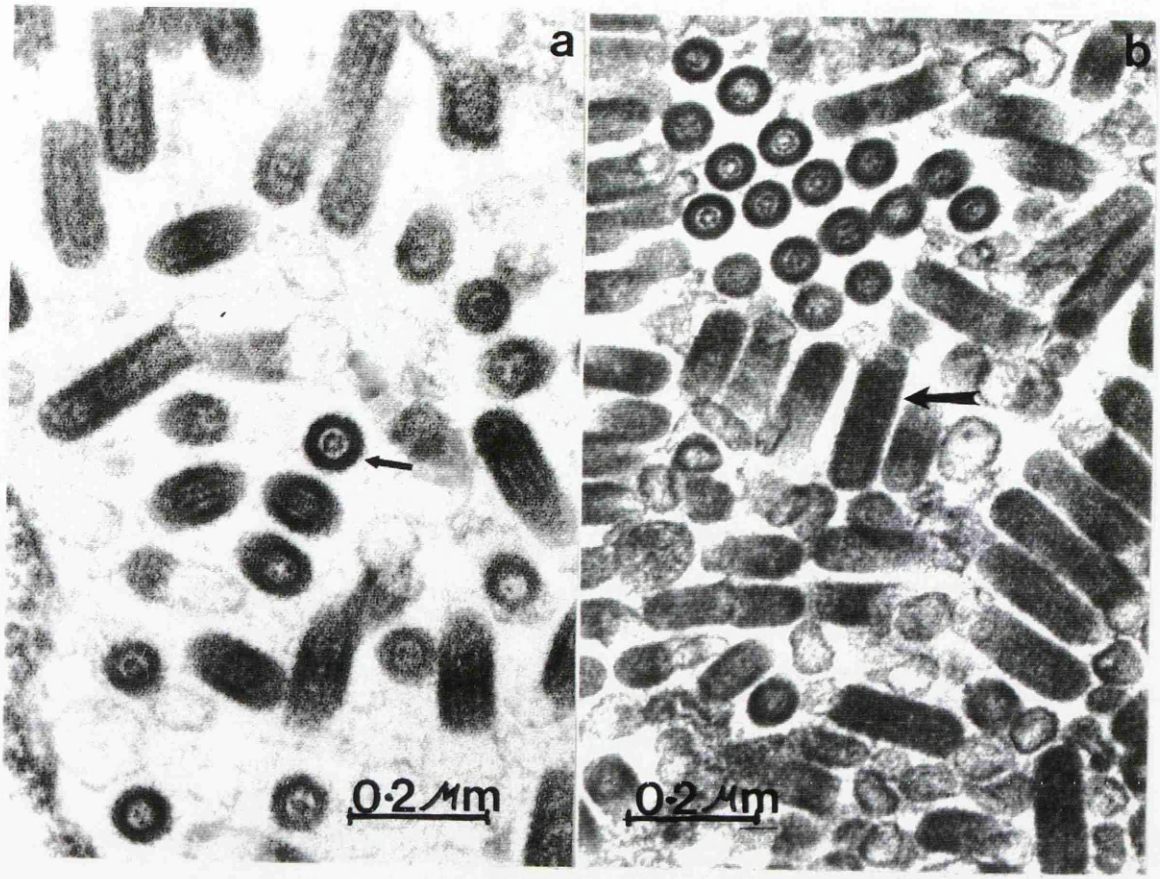
M = Mitochondrium.

CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
0·2 μm
b
0·2 μm

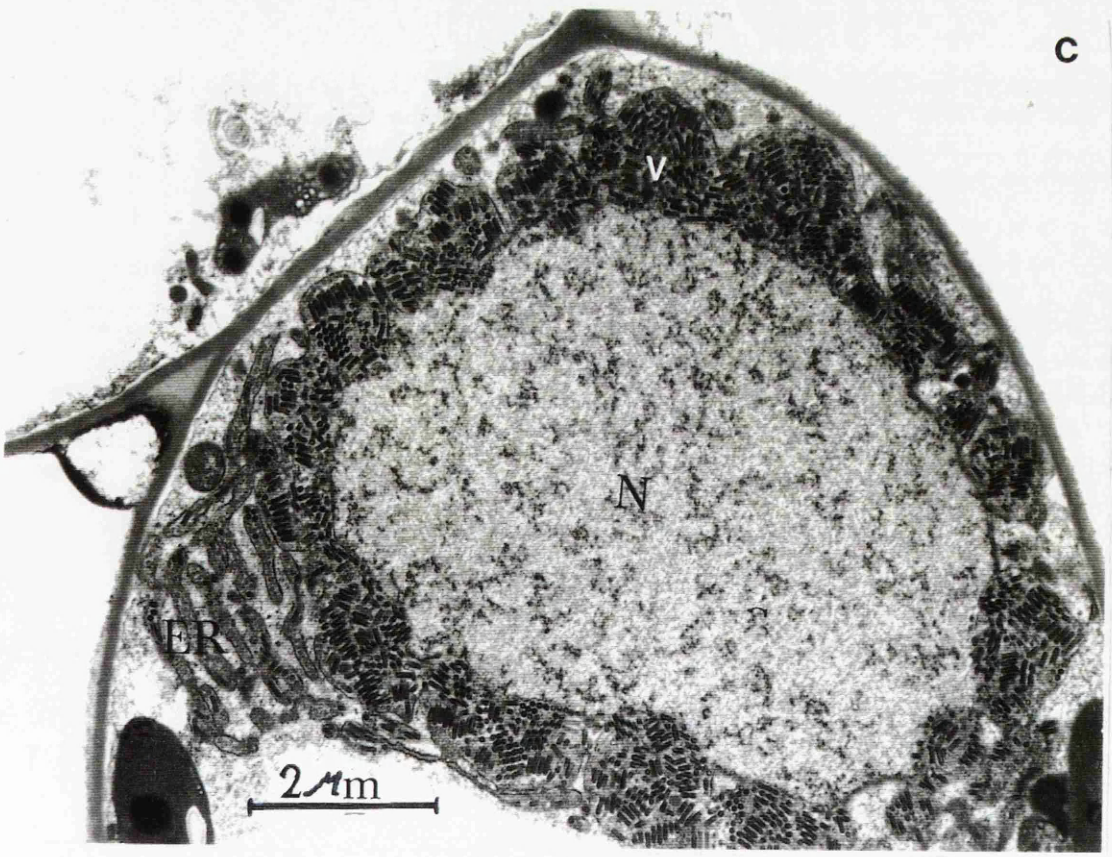

c
V
N
ER
2 μm

Figure 3.23:

(a)- Electron micrograph of a thin section of EMDV-infected *N. edwardsonii* leaf 6 weeks post-inoculation, showing a massive inclusions containing virus particles around the nucleus.

(b)- Electron micrograph of thin section of EMDV-infected *N. glutinosa* leaf 6 weeks post-inoculation, showing abnormal chloroplast and virus particles in membrane bound sacs.

Standard abbreviations:

N = Nucleus.

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

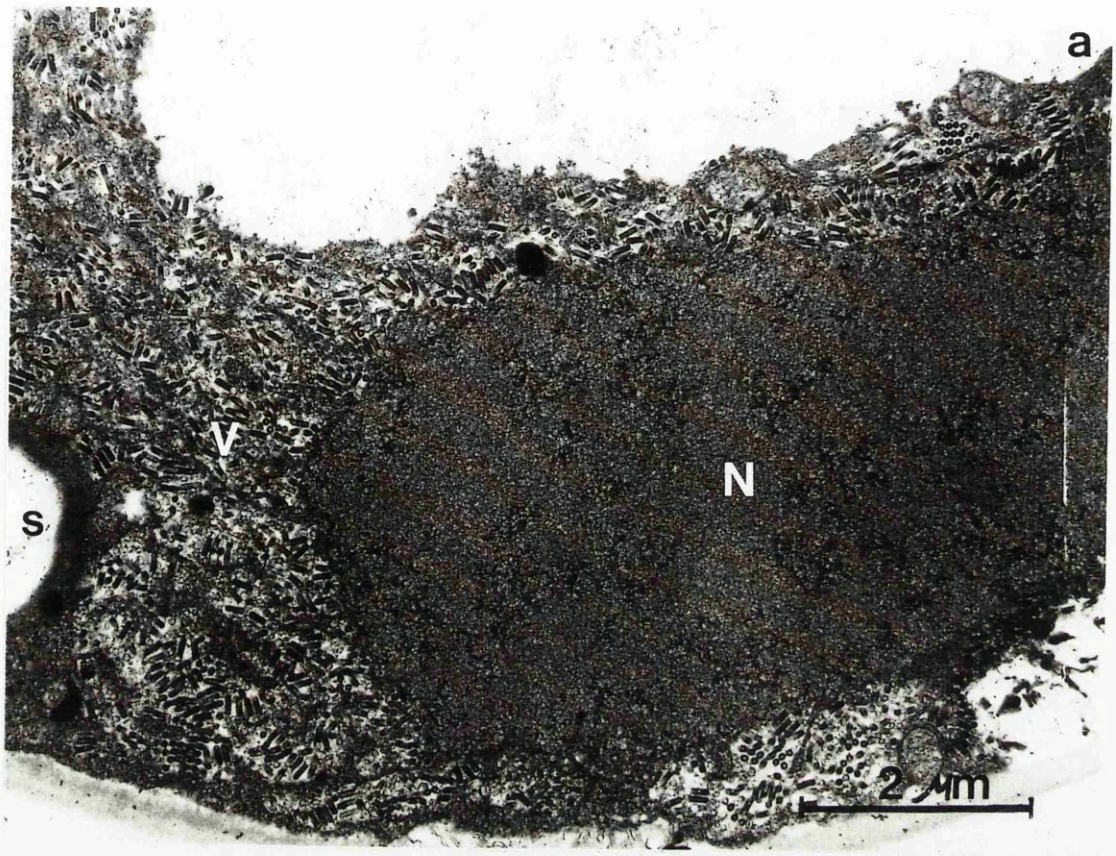
M = Mitochondrium.

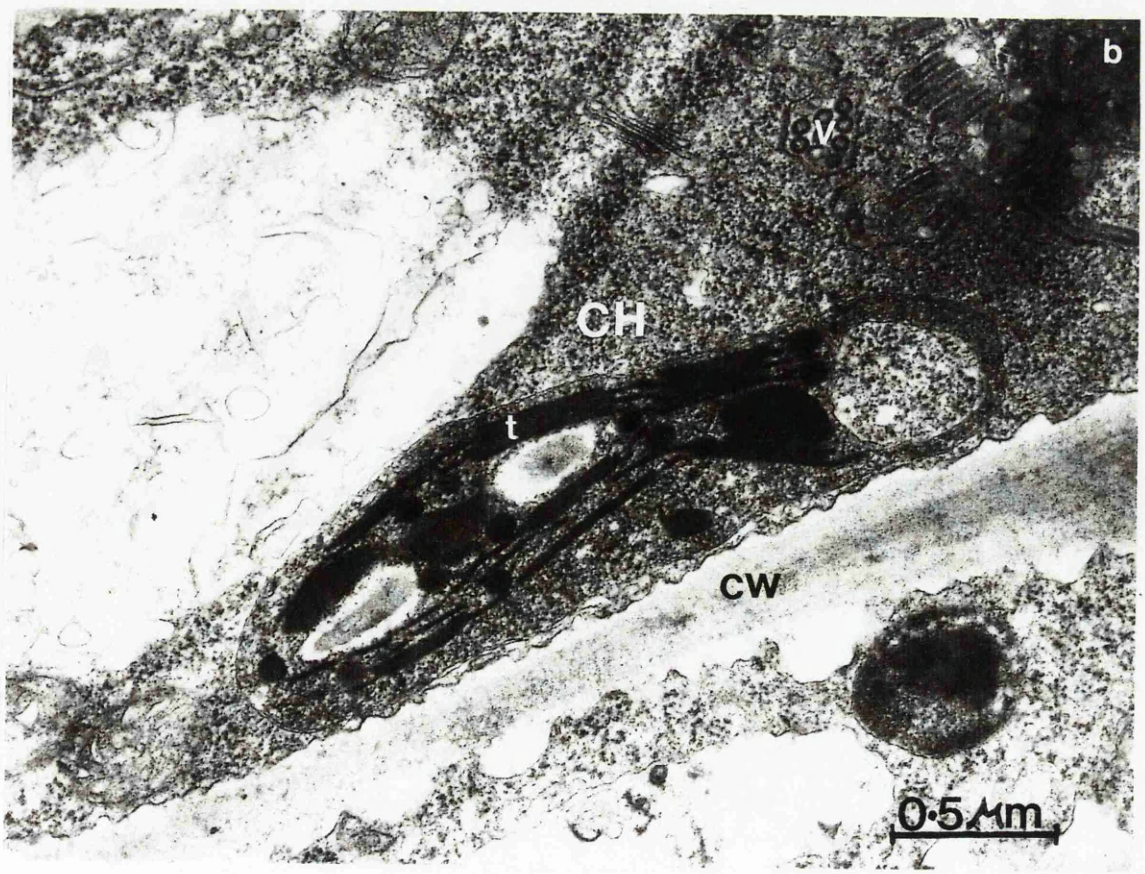
CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
V
N
S
2 µm


b
V
CH
t
CW
0·5 µm

mitochondria were apparently unaffected. At this stage of infection, virus particles were only observed in the cytoplasm in sections from inoculated leaves.

Five weeks after inoculation of *N. glutinosa*, infected cells started to show dramatic changes. Up to 5 weeks post-inoculation, nuclei were unchanged in their overall shape but contained large numbers of virus particles in the perinuclear space, developing areas of viroplasm. Chloroplasts showed morphological disturbances with developing starch and alteration in their shape (figure 3.23b). Beyond 6 weeks post-inoculation, (Figure 3.23a) large numbers of virus particles were still present within the nucleus. In chloroplasts, thylakoids were reduced in number and large areas of starch had developed within chloroplasts causing swelling. Some lipid bodies were also observed in the chloroplasts.

Only a few infected *N. edwardsonii* plants survived beyond 6 weeks after inoculation. In some cases plants inoculated at later stages of growth survived for a longer time.

Six weeks post-inoculation, systemically infected leaves *N. edwardsonii*, still contained nuclei with numerous virus particles. Chloroplasts contained a large areas of starch (Figure 3.23b). Between 8 and 12 weeks post-inoculation, abnormalities increased in infected *N. glutinosa* and *N. edwardsonii*. In *N. edwardsonii* infected leaf and calyx, chloroplasts developed substantial areas of inclusion bodies or electron dense material (Figure 3.24a &b). By 12 weeks post-inoculation, cytoplasmic invaginations in the nuclei were observed. These invaginations often contained other cytoplasmic components such as mitochondria (Figure 3.26b) and virus particles budding from the inner membrane of the nucleus. Similar abnormal changes in the nuclei and chloroplasts were observed in infected *N. glutinosa* at 14 weeks post-inoculation, although only a few infected plants survived and with a few small leaves left by this stage.

Figure 3.24:

(a)- Electron micrograph of a thin section of EMDV-infected *N. edwardsonii* calyx 9 weeks post-inoculation, showing abnormalities in chloroplasts and a large number of virus particles lodged in the nucleus.

(b)- Electron micrograph of a thin section of EMDV-infected *N. edwardsonii* leaf 9 weeks post-inoculation, showing abnormalities in the chloroplasts and sacs of virus particles scattered in the cytoplasm.

Standard abbreviations:

N = Nucleus

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

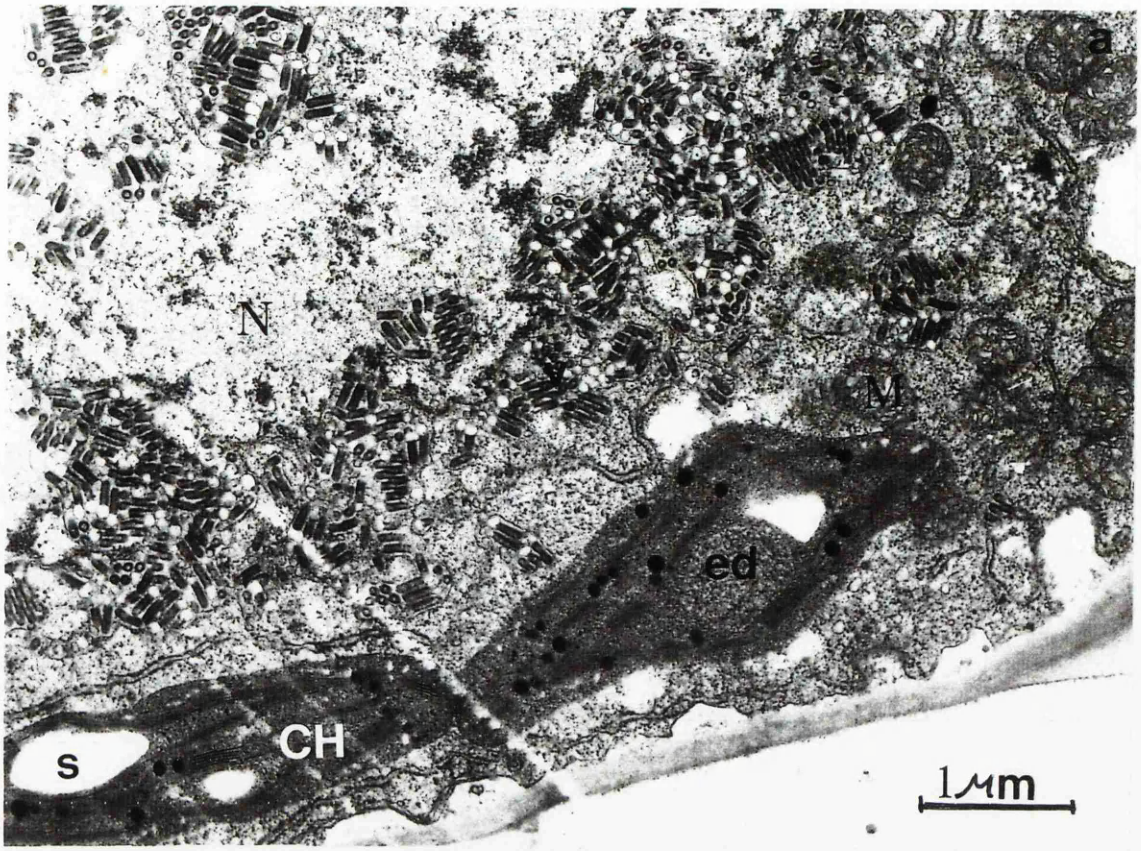
M = Mitochondrium.

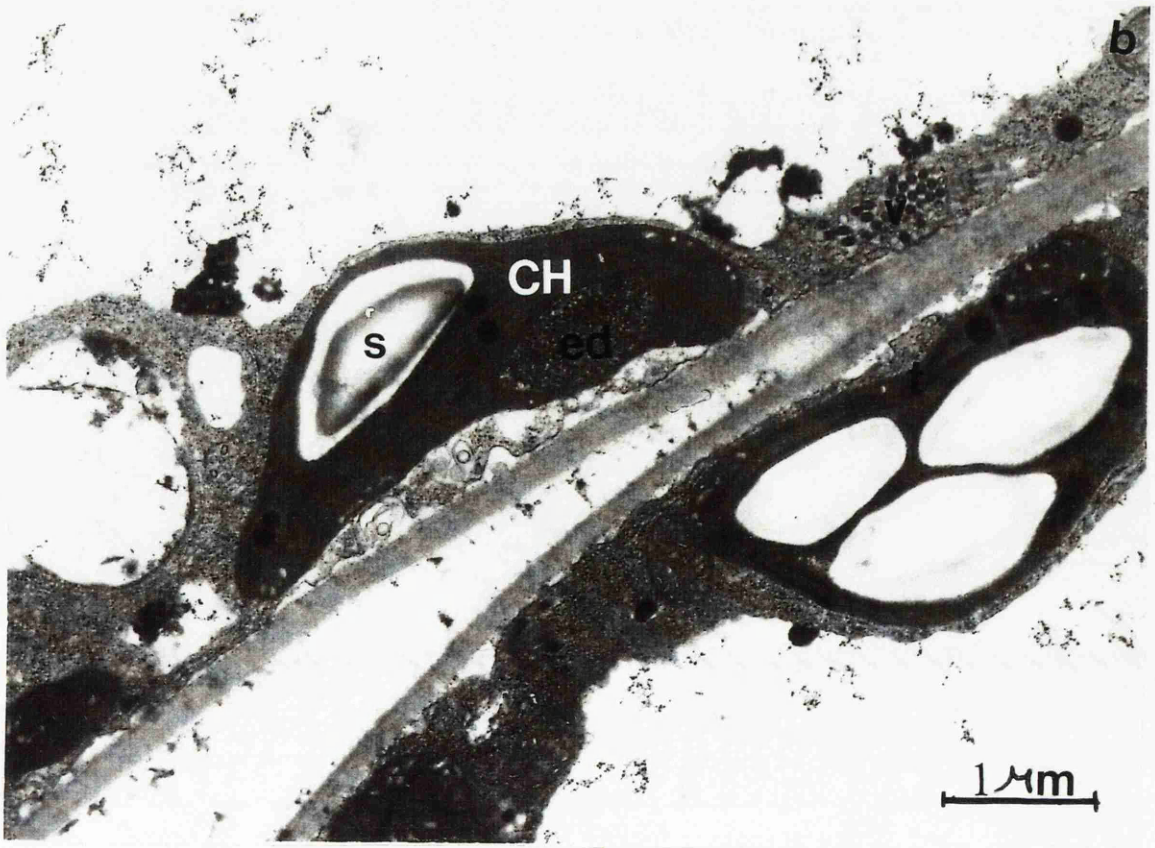
CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
N
M
ed
CH
S
1µm


b
V
CH
S
ed
1µm

In severely infected leaves of *N. edwardsonii* 10 weeks after inoculation, all cell components were severely affected, large areas of viroplasm were developed and nucleoli showed some lucent areas and altered shape (Figure 3.25). Some unexpanded leaves from infected plants at this age showed symptoms of chlorotic lesion (see figure 3.21), which in thin sections under the electron microscope contained a mixture of short bacilliform particles (160-180 nm) and a few other of standard length (220-250 nm) with both ends rounded (Figure 3.26a). These chlorotic leaves were inoculated onto healthy plants. The infected plants survived up to 24 weeks after inoculation and showed a mixture of systemic and chlorotic symptoms, different from those normally observed.

Cytopathological changes in *N. edwardsonii* plants, 12-20 weeks after inoculation were characterised by increasing abnormalities in the nucleus; by abnormalities in chloroplasts were as observed earlier in infection. Nuclei of infected leaf cells still contained many virus particles in the perinuclear space and the shape of the nucleoli were altered. Number of virus particles was slightly reduced compared to the earlier stages. Invaginations of the nucleus were extensive (figure 3.26b) showing virus particles budding from the inner membrane within the invagination. Similar of ultrastructural changes in the chloroplasts from infected leaves have been observed in chloroplasts from calyx cells (Figure 3.27).

A few infected *N. glutinosa* plants which survived 23 weeks after inoculation, were examined. Three distinct areas within nuclei could be recognised (Figure 3.28a); a large area of viroplasm a second region contained chromatin and surrounding the viroplasm and a third region which contained groups of virus particles in perinuclear inclusions. In some cells, the nuclei appeared to contain nucleocapsids or immature virus particles (Figure 3.28b). Within these cells, virus particles were consistently observed which were shorter (160-180 nm) than the standard virus (220-250 nm) but exhibited the normal features of bacilliform particles, with both ends rounded. In these cells,

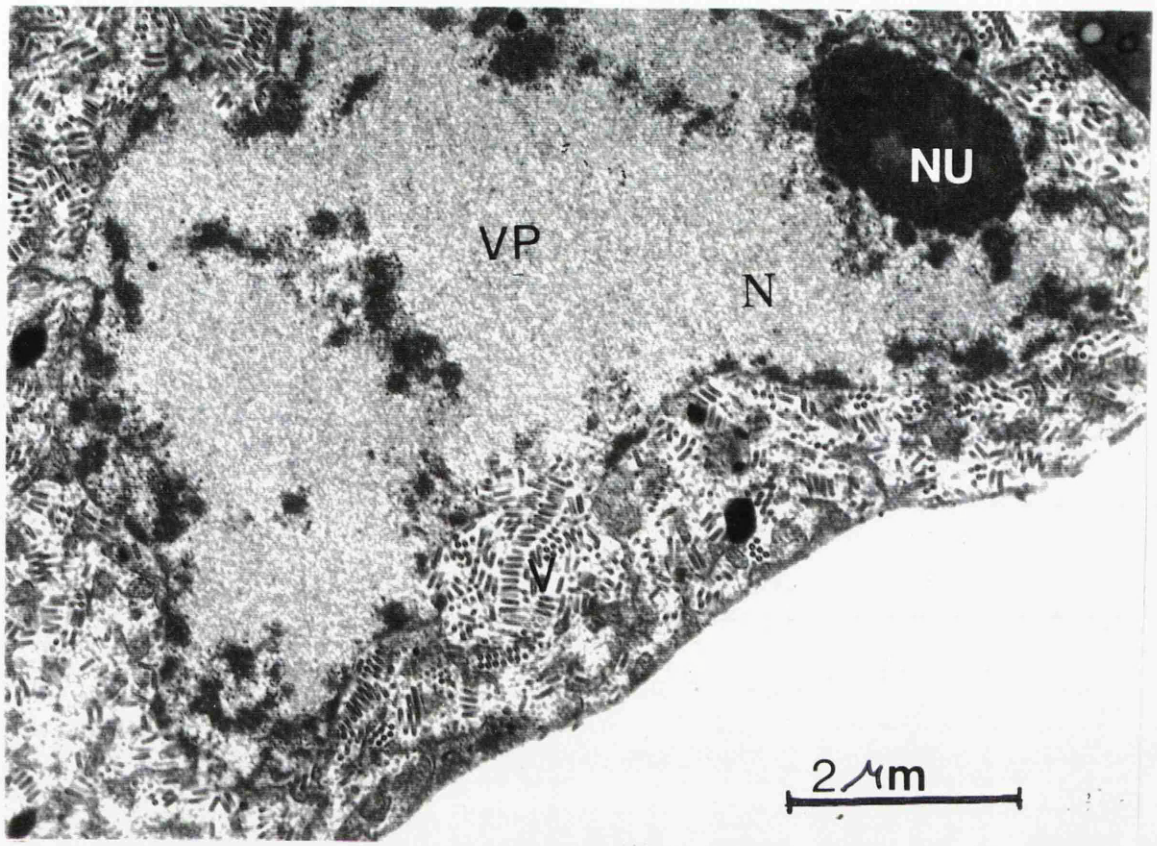


Figure 3.25:

Electron micrograph of a thin section of EMDV-severely infected *N. edwardsonii* leaf 10 weeks post-inoculation. Note a large area of viroplasm, altered shape of nucleoli and a large number of virus particles.

Standard abbreviations:

N = Nucleus. ed = Electron dense material. CH = Chloroplast. V = Virus. S = Starch. ER = Endoplasmic reticulum. W = Cell wall. t = Thylakoids. VP = Viroplasm. NU = Nucleolus. CR = Chromatin material. CY = Cytoplasm. M = Mitochondrium. CI = Cytoplasmic invagination. I = Inner membrane. O = Outer membrane. UV = Atypical virus-like particle.

Figure 3.26:

(a)- Electron micrograph of a thin section of an EMDV-infected *N. edwardsonii* unexpanded leaf 10 weeks post-inoculation, showing different lengths of virus particles as indicated by large arrow for standard virus and a small arrow for short virus particles.

(b)- Electron micrograph of a thin section of EMDV-infected *N. edwardsonii* leaf 12 weeks post-inoculation, showing the cytoplasmic invaginations in the nucleus.

Standard abbreviations:

N = Nucleus.

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

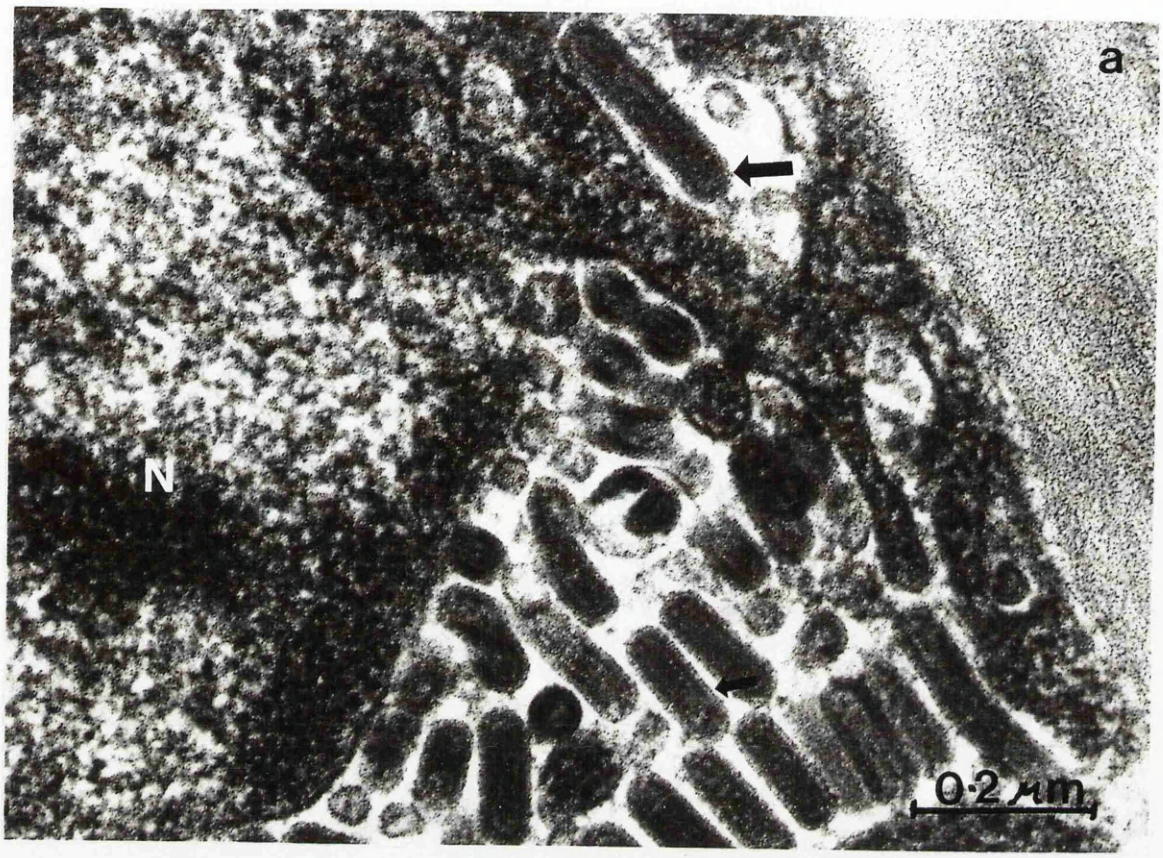
M = Mitochondrium.

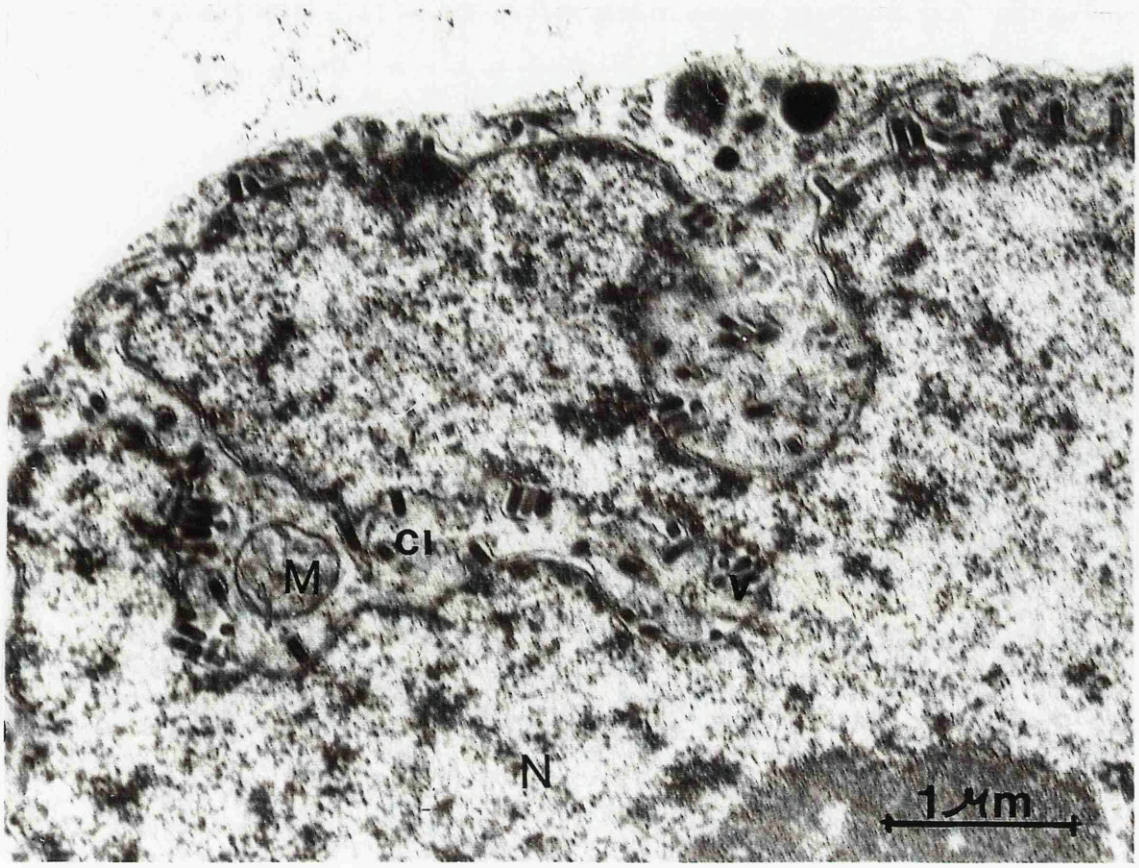
CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
N
0·2 µm


M
CI
V
N
1 µm

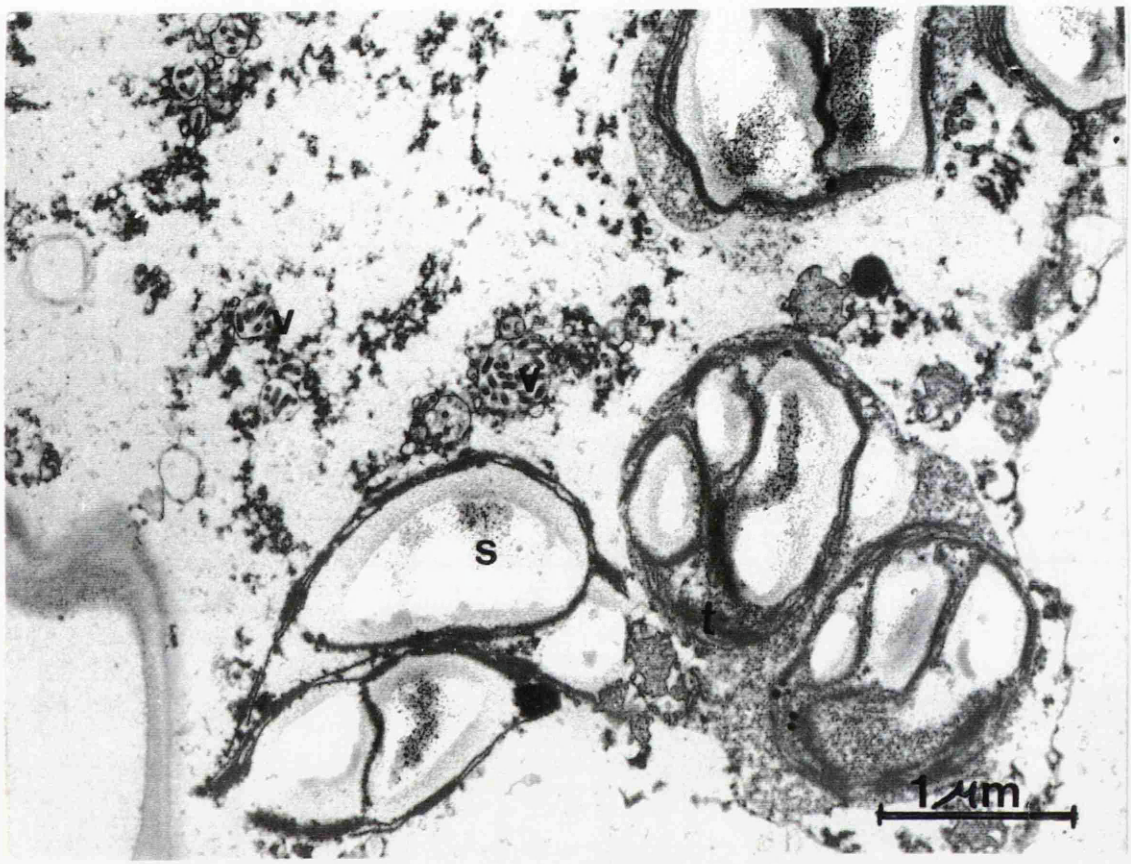


Figure 3.27:

Electron micrograph of a thin section of EMDV-infected *N. edwardsonii* calyx 14 weeks post-inoculation, showing a disrupted cell with abnormal chloroplasts.

Standard abbreviations:

N = Nucleus, ed = Electron dense material, CH = Chloroplast, V = Virus, S = Starch, ER = Endoplasmic reticulum, CW = Cell wall, t = Thylakoids, VP = Viroplasm, NU = Nucleolus, CR = Chromatin material, CY = Cytoplasm, M = Mitochondrium, CI = Cytoplasmic invagination, I = Inner membrane, O = Outer membrane, UV = Atypical virus-like particle.

Figure 3.28:

(a)- Electron micrograph of a thin section of EMDV-infected *N. glutinosa* leaf 23 weeks post-inoculation, Note the three distinct regions in the nucleus, abnormal chloroplasts and enlarged nucleoli with 2-3 lucent areas.

(b)- Electron micrograph of a nucleus of EMDV-infected *N. glutinosa* leaf cell 23 weeks post-inoculation, showing nucleocapsids and immature virus-like particles.

Standard abbreviations:

N = Nucleus.

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

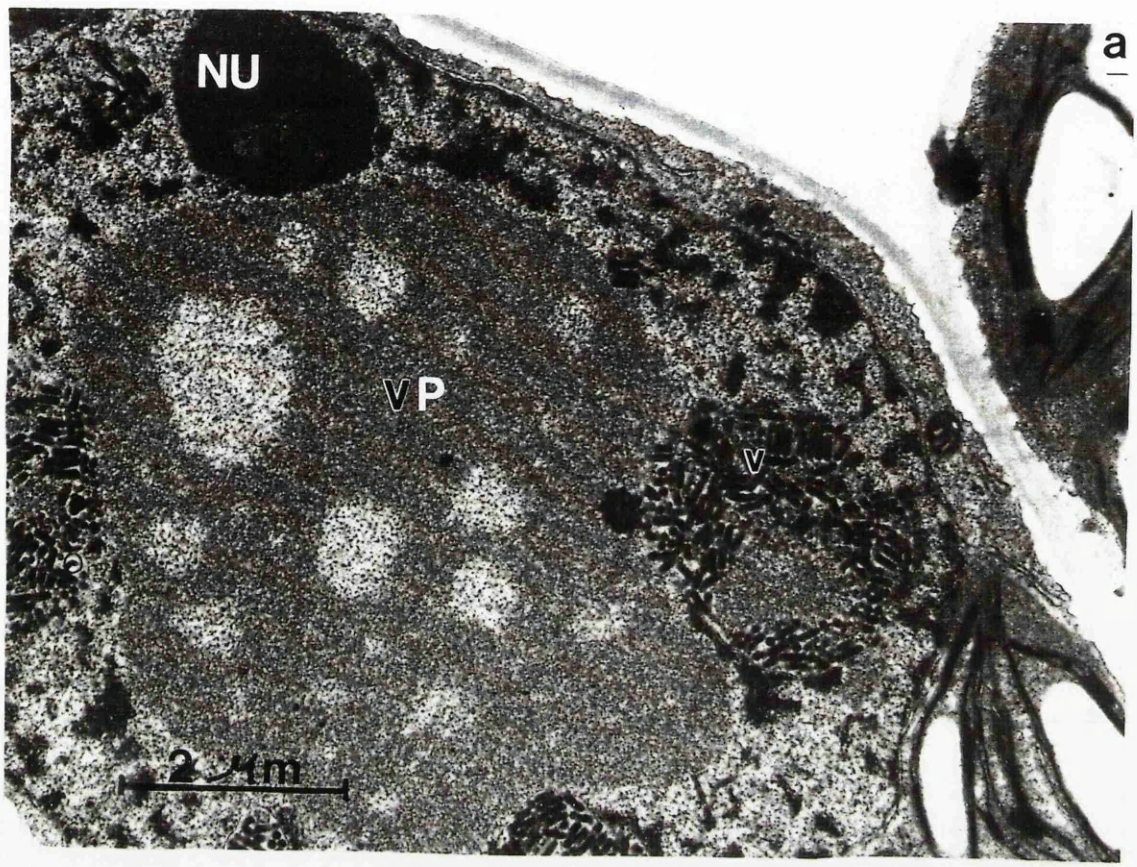
M = Mitochondrium.

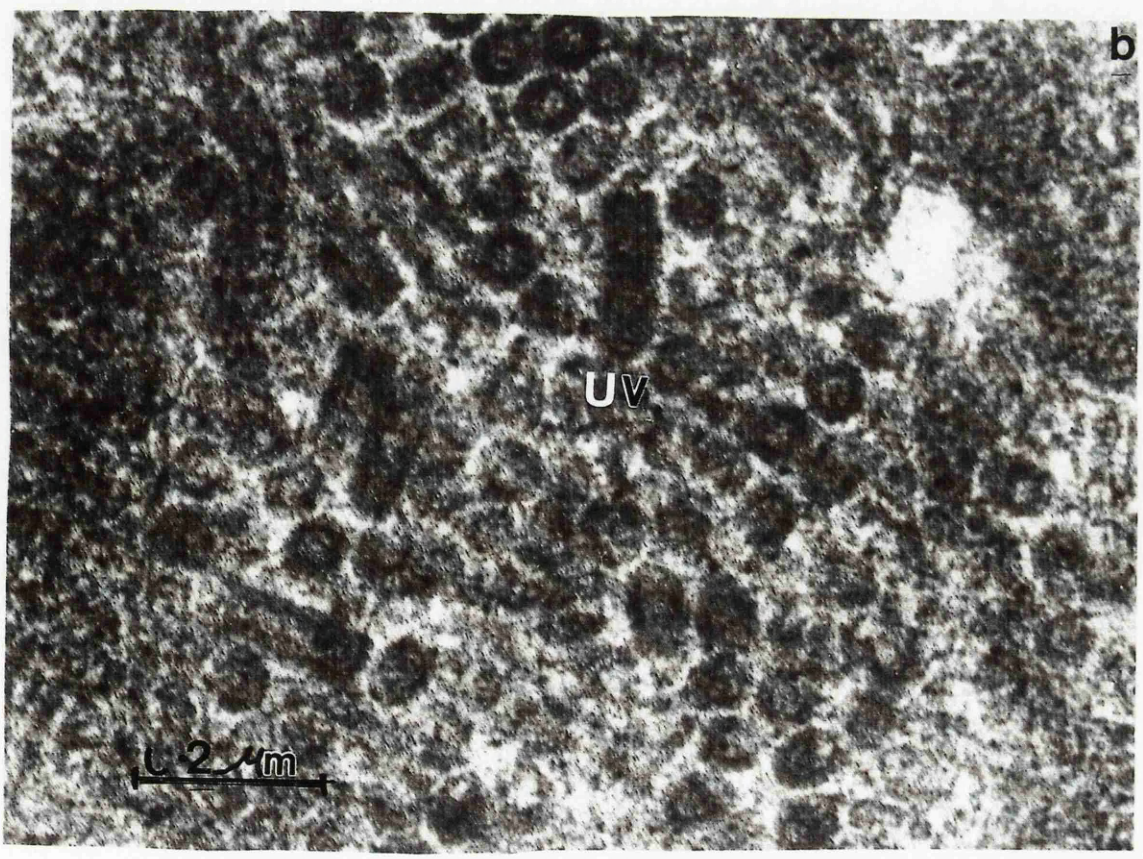
CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


NU
VP
V
a
2 µm


UV
b
2 µm

containing the virus particles of mixed length, the standard virus particles were less abundant than the shorter ones (Figure 3.29). The presence of particles of this type had been correlated with the development of DI particles in *N. edwardsonii* infected with SYNV (Ismail, *et al.*, 1987). These short particles may therefore represent similar DI particles of EMDV.

Electron microscope sections taken from infected *N. glutinosa* 5 and 23 weeks post-inoculation were immunogold labelled using anti-EMDV antibodies. The gold particles bound extensively to virus particles and to the viroplasm in both sections (figures 30 & 31). No major differences were detected in the amount or pattern of binding of gold particles between sections taken 5 and 23 weeks after inoculation. Fewer gold particles bound to sections than in comparable experiments using SYNV-infected plants and anti-SYNV serum (see figure 3.16). Since the overall level of EMDV antigen, as measured by ELISA in infected plants, is several times greater than SYNV antigen in comparable plants (see page 78), it presumably reflects a difference in the titres of the antisera rather than levels of antigen within the cells. No gold particles were seen associated with chromatin nor to the other regions of the infected cell. In control labelling sections from healthy *N. glutinosa* using anti-EMDV antibodies, no gold particles were observed binding to the cells.

N. edwardsonii plants inoculated with leaves showed chlorotic lesion symptoms responded to the infection by producing both kinds of chlorotic and a few leaves with vein-clearing and mottling systemic symptoms after 6-8 weeks. Ultrastructure of cells from vein clearing and mottling symptoms of infected leaves after 23 weeks showed no differences from the earlier observations.

Leaves of *N. edwardsonii* with chlorotic lesions (figure 3.21) were sectioned and examined under the electron microscope. In the green areas which showed no sign of symptoms, virus particles were not observed in the cells but

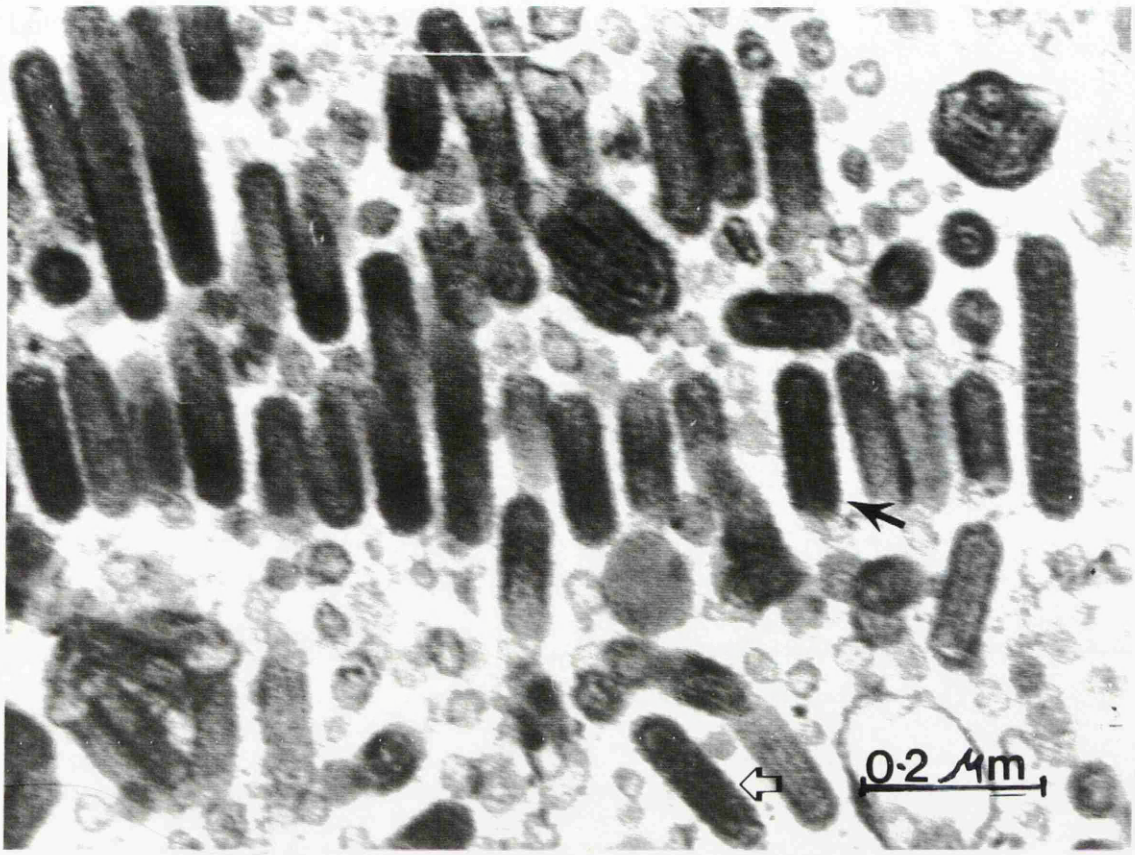


Figure 3.29:

Electron micrograph of a thin section of EMDV-infected *N. glutinosa* leaf 23 weeks post-inoculation, showing short particles (black arrow) as well as standard length virus particles (white arrow).

Standard abbreviations:

N = Nucleus, ed = Electron dense material, CH = Chloroplast, V = Virus, S = Starch, ER = Endoplasmic reticulum, CW = Cell wall, t = Thylakoids, VP = Viroplasm, NU = Nucleolus, CR = Chromatin material., CY = Cytoplasm., M = Mitochondrium., CI = Cytoplasmic invagination., I = Inner membrane., O = Outer membrane., UV = Atypical virus-like particle.

Figure 3.30:

Electron micrographs of immunogold labelled thin sections of EMDV-infected *N. glutinosa* leaf 5 weeks post-inoculation, showing gold particles bound to the sites of virus particle accumulation and to the viroplasm. (a) and (b) show the sections at high and low magnification.

Standard abbreviations:

N = Nucleus.

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

M = Mitochondrium.

CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.

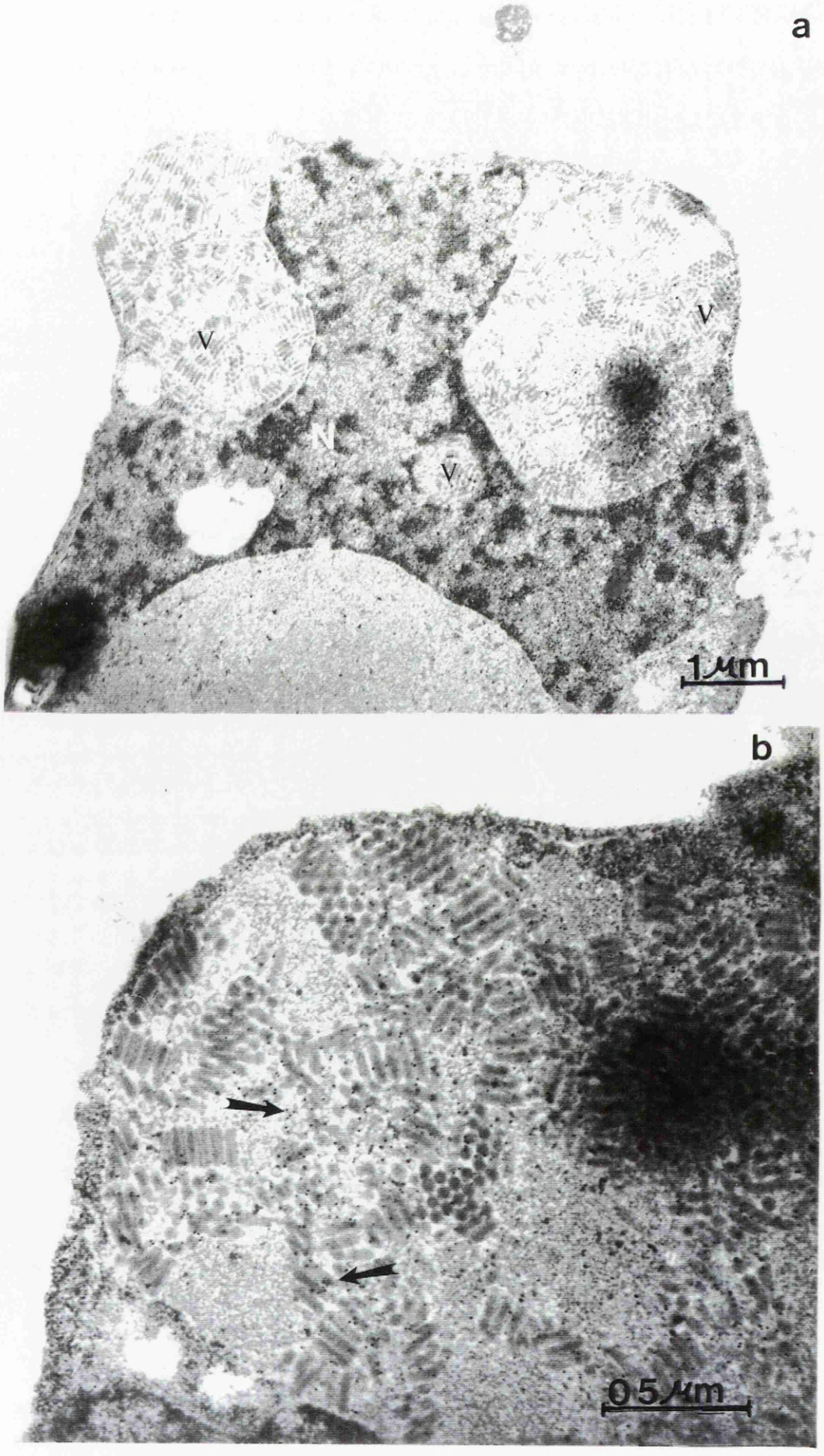

a
V
V
V
V
1 µm
b
0.5 µm

Figure 3.31:

Electron micrographs of immunogold labelled thin sections of EMDV-infected *N. glutinosa* leaf 23 weeks post-inoculation, with anti-EMDV antibodies (a) and (b) show the section high and low magnification.

Standard abbreviations:

N = Nucleus.

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

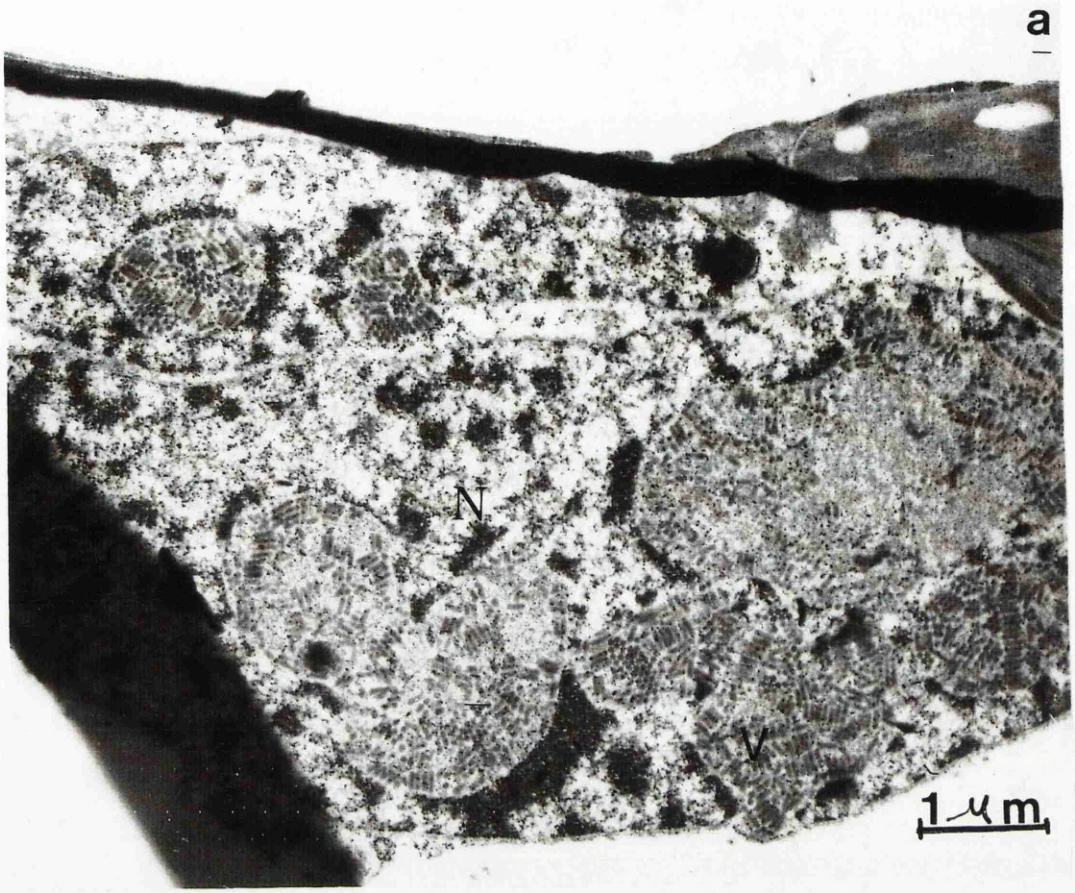
M = Mitochondrium.

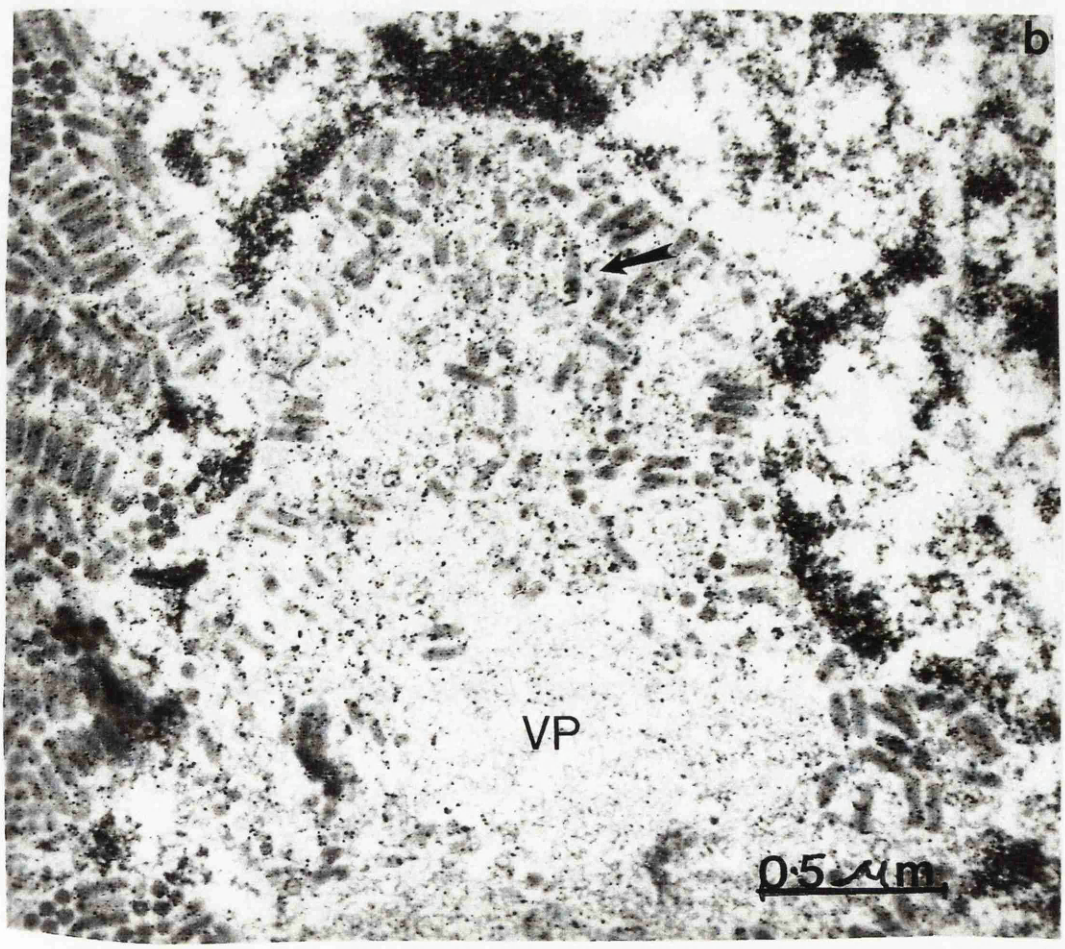
CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
N
V
1 μm


b
VP
0·5 μm

the cells revealed some abnormalities (Figure 3.32a). Thylakoids were disrupted and large areas of starch were seen in the centre of chloroplasts. Nuclei were deformed with some invaginations of the envelope and enlargement of nucleoli. The yellow parts of the infected leaf, contained very abnormal cells (Figure 3.32b). Nuclei were extensively disrupted, many virus particles were present in membrane bound sacs and chloroplasts were also severely disrupted.

Virus particles were mostly bacilliform in shape with both ends rounded but short in length (160-180 nm) although a few standard length particles (200-230 nm) were present.

3.9- Serological assays for EMDV-infected plants:

Protein samples from healthy and infected *N. edwardsonii* plants and purified EMDV were prepared and fractionated by polyacrylamide gel electrophoresis as described previously in section (2.4.1). Proteins were blotted onto a nitrocellulose membrane by the contact diffusion method or by electrophoretic transfer as described in section (2.6.6). Preliminary experiments were carried out by staining the gels with silver as described in section (2.4.2). Samples from infected plants, 4 and 6 weeks after inoculation and from healthy controls, purified EMDV and purified SYNV were run in parallel. Infected plants 4 and 6 weeks after inoculation showed additional bands co-migrating with the G and N proteins of purified EMDV, when compared to healthy controls. Bands co-migrating with M1 and M2 were not visible (Figure 3.33).

In western blots, anti-EMDV antibodies were reacted with protein samples from healthy plants and purified EMDV and SYNV. The antibodies reacted extensively to purified and partly purified EMDV. In contrast, the antibodies did not react either to purified SYNV nor to total proteins from healthy plants (Figure 3.34). Antibodies reacted strongly to the major virus proteins in the EMDV-infected plants. The G and N proteins reacted strongly 5 weeks post-inoculation with little changes in samples 7 and 10 weeks post-inoculation. In contrast

Figure 3.32:

Electron micrographs of thin sections of EMDV-infected *N. edwardsonii* unexpanded leaf 23 weeks post-inoculation showing chlorotic lesions. (a) Section in the green area showing abnormalities in the nucleus and chloroplast but no virus particles. (b) Section in the yellow chlorotic area, showing severely abnormal cells components and many virus particles.

Standard abbreviations:

N = Nucleus.

ed = Electron dense material.

CH = Chloroplast.

V = Virus.

S = Starch.

ER = Endoplasmic reticulum.

CW = Cell wall.

t = Thylakoids.

VP = Viroplasm.

NU = Nucleolus.

CR = Chromatin material.

CY = Cytoplasm.

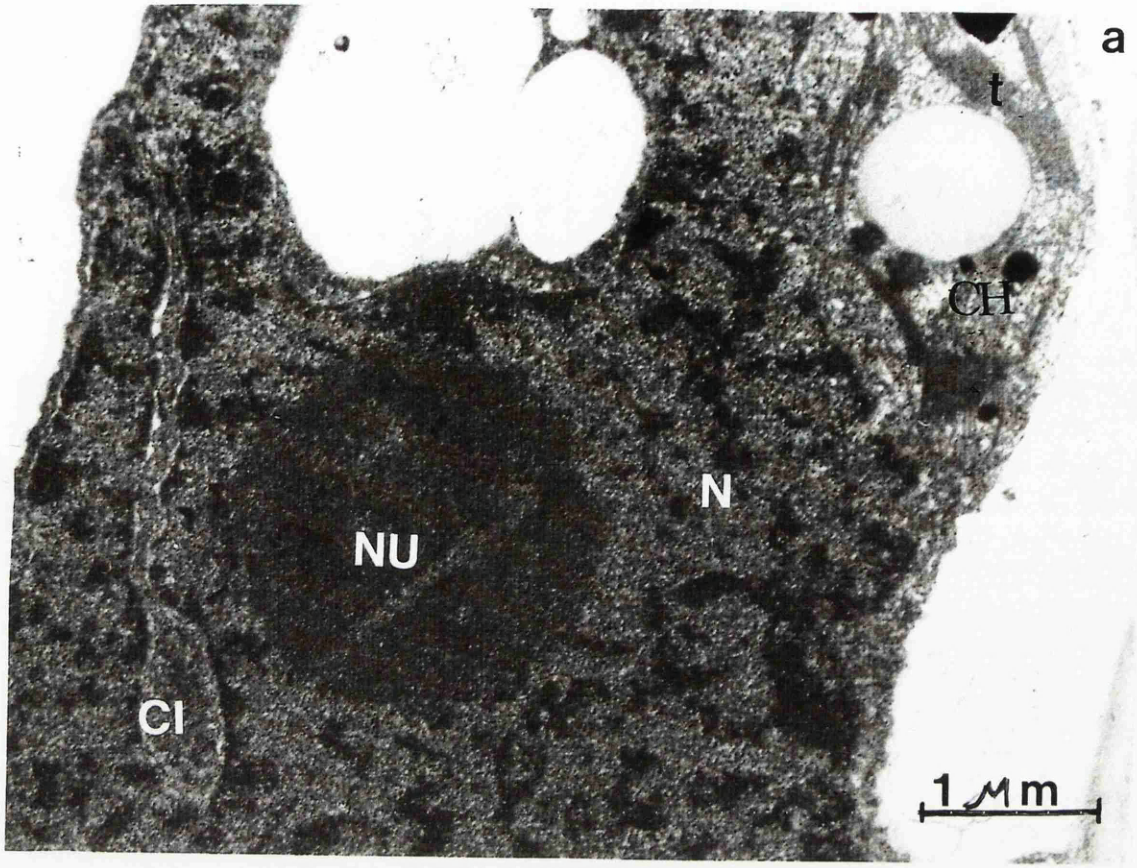
M = Mitochondrium.

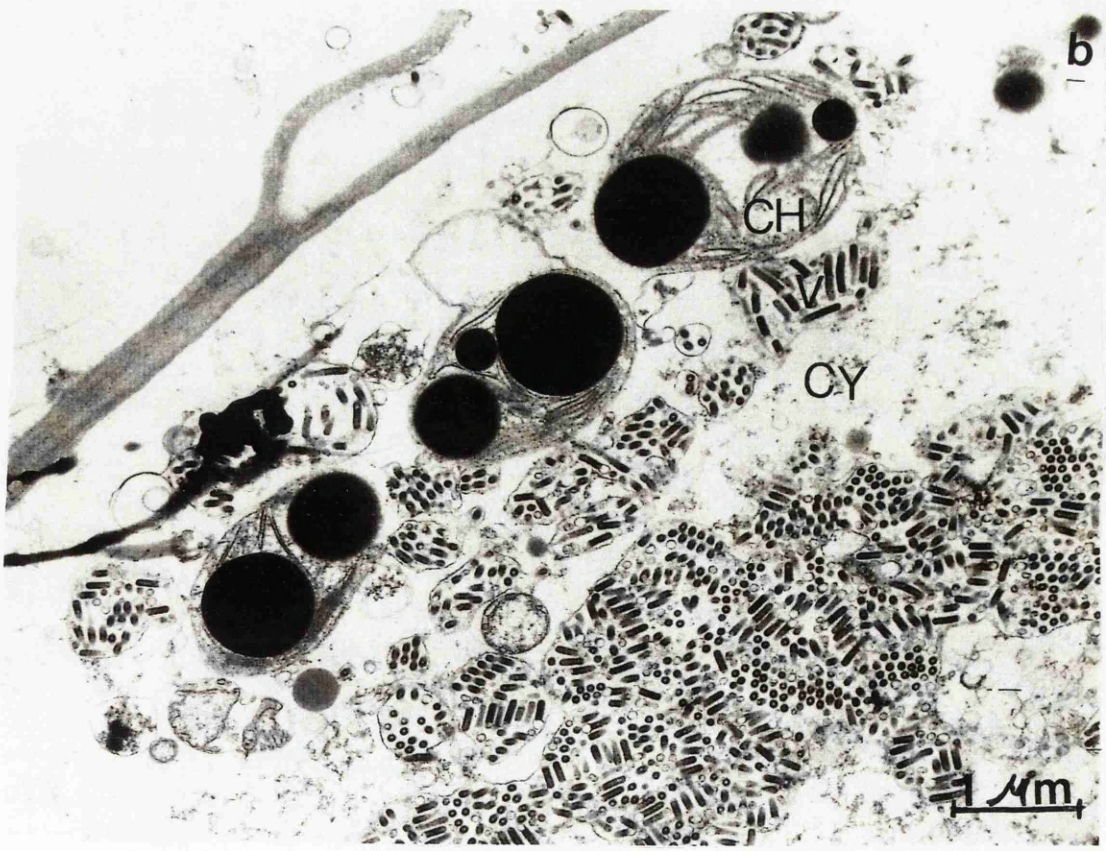
CI = Cytoplasmic invagination.

I = Inner membrane.

O = Outer membrane.

UV = Atypical virus-like particle.


a
t
CH
N
NU
Cl
1 µm


b
CH
CY
1 µm

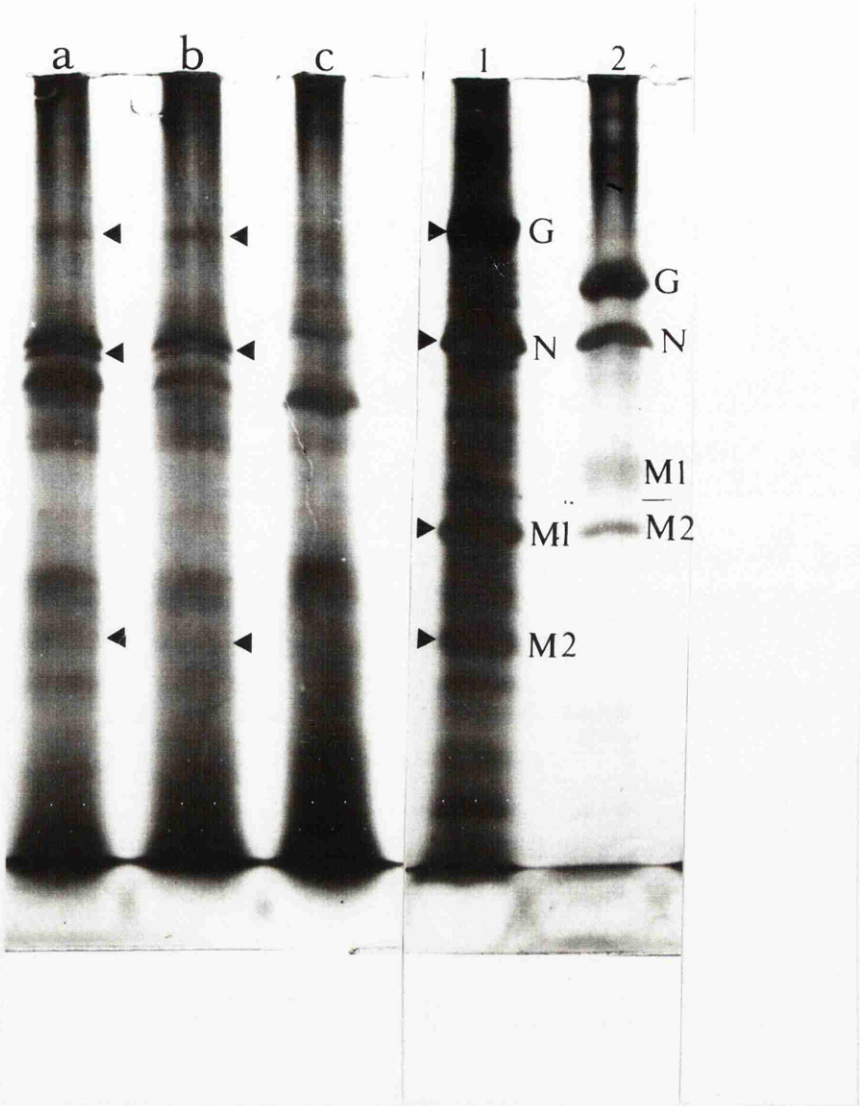


Figure 3.33:

SDS-Polyacrylamide gel of total proteins from EMDV-infected *N. edwardsonii* with silver. (a) Infected plants 4 weeks post-inoculation, (b) infected plants 6 weeks post-inoculation, (c) healthy control plants. (1) and (2) contain purified EMDV and purified SYNV respectively. The four bands of EMDV and SYNV proteins in lane (1) and (2) are labelled G, N, M1 and M2. Additional bands co-migrating with G and N in EMDV-infected plants are indicated by arrows.

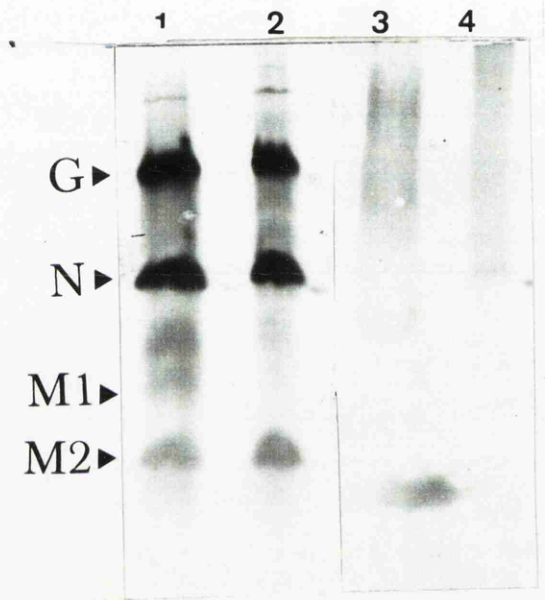


Figure 3.34:

Western blots of using anti-EMDV serum to probe proteins separated by SDS-Polyacrylamide gel electrophoresis. (1) partly purified EMDV, (2) purified EMDV, (3) SYNV, and (4) total proteins from healthy plants.

M1 protein was not detected in any of the samples (Figure 3.35). This could be due to the small amount of M1 protein in infected cells compared to the other proteins. Alternatively, this may reflect the poorer antigenicity of the M1 protein. Indeed in western blots to lanes containing purified EMDV, the M1 protein stained the most weakly of the four major proteins (figure 3.34). M2 protein bands were observed in all samples of infected plants, but stained less strongly than G and N proteins. This result may be contrasted with SYNV infected *N. glutinosa* in which the M1 protein was detectable in blots of total proteins from infected leaves but not the M2 protein.

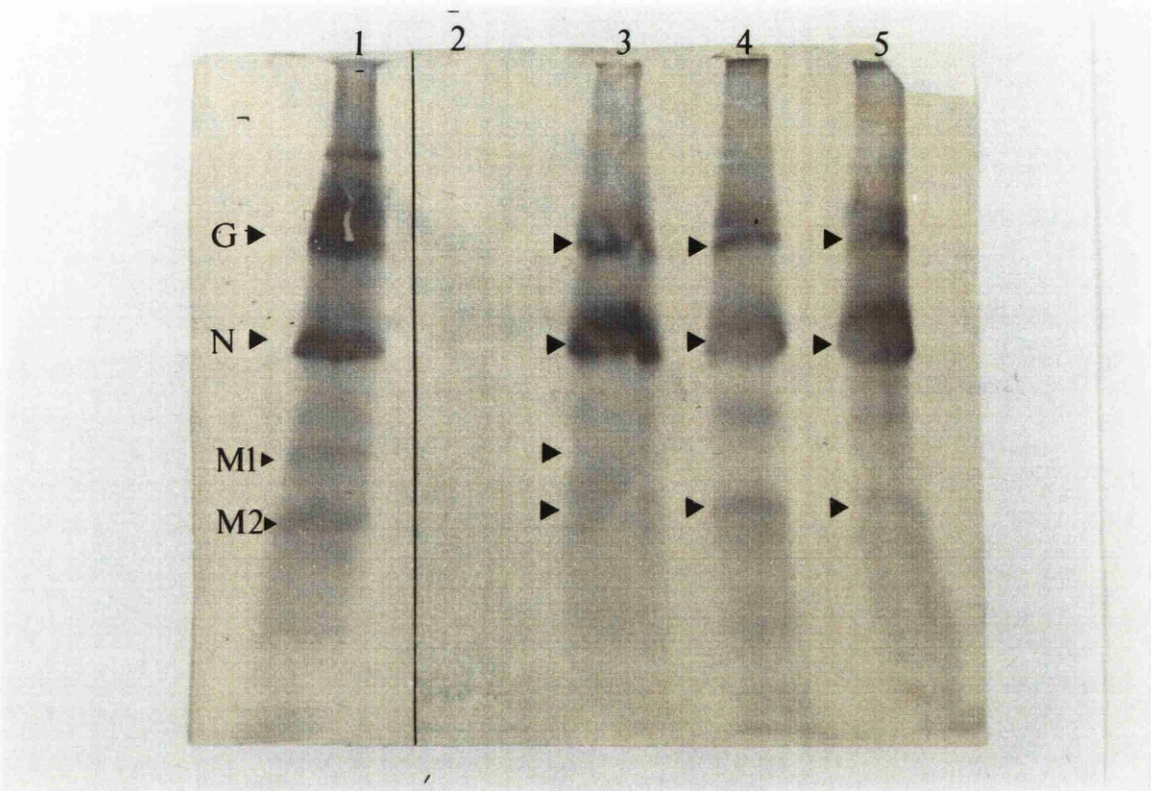


Figure 3.35:

Immunoblotting of total proteins from EMDV-infected *N. edwardsonii* leaves taken at various time after inoculation. Lane (1) purified EMDV, the bands corresponding to G, N. M1 and M2 proteins are indicated. (2) Healthy control plants. (3) Infected plants 5 weeks post-inoculation. (4) Infected plants 7 weeks post-inoculation. (5) Infected plants 10 weeks post-inoculation. Bands co-migrating with the four major proteins are arrowed.

Chapter Four

The effect of SYNV on photosynthesis rates

The effect of SYNV infection on photosynthesis rates

4.1- Introduction:

Photosynthesis is the process whereby light energy from the sun is converted to chemical energy and conserved in the form of ATP and NADPH, which can be used to drive the biosynthesis of organic molecules such as glucose and amino acids.

Reduction in photosynthetic rates frequently has been reported for virus-infected plants, in some cases where symptoms are mild, or apparently absent as well as in cases where chlorosis, mosaic or local lesion formation occurs. The results appear to depend on the virus strain, the type of host studied and the protocol of the experiments. The loss of the pigment that accompanies viral infections of plants is most commonly reported to cause a reduction or loss of photosynthetic activity in the host cell. Viral infections have also been reported to have no detrimental effect, or even a stimulating effect, on photosynthesis (Magyarosy, *et al.*, 1973; Smith and Neales, 1977), while some other viral infections have been reported to induce changes in carbon metabolism of photosynthesising cells, resulting in accumulation of various amino acids and organic acids (Bedbrook and Matthews, 1972; Magyarosy, *et al.*, 1973). On the other hand, early work with TMV suggested increased photosynthesis rates occurred in infected tobacco (Owen, 1957b; Zaitlin and Hesketh, 1965).

In view of the widespread interest expressed in increasing photosynthetic efficiency (Brown, *et al.*, 1975; Platt and Bassham, 1978) and the obligate involvement of chlorophyll in photosynthesis both as light-absorbing antenna and as a part of the reaction centre, several parameters related to photosynthesis have been studied in leaves from both the healthy and systemically SYNV-infected plants. Learning what alterations, if any, occur in the infected leaves was the aim of the experiments reported in this chapter, and to provide additional understanding about virus multiplication.

The changes in photosynthetic rates in healthy and SYNV-infected *N. edwardsonii* and *N. glutinosa* were examined by measuring net photosynthesis (Pn) at different light intensities during a period of infection. The efficiency of light capture and primary photochemistry in healthy and infected plants can be estimated by plotting the gross photosynthesis rate (Pg) against the corresponding incident light intensities.

The photosynthetic process involves a series of complex partial reactions whose operation depends upon the structural integrity of the chloroplast. Viruses may affect photosynthesis in a variety of ways and the present study was conducted to determine the mechanisms by which SYNV affects the photosynthetic rates in its host.

One of the methods of measuring photosynthesis rates is by monitoring CO_2 assimilation in leaves or whole plants. Usually this is achieved by infrared gas analysers (IRGAs), which are relatively expensive, but stable and sensitive instruments (Naidu *et. al.*, 1984). Polarographic oxygen evolution measurement is the other commonly used and relatively inexpensive and simple method, for evaluating CO_2-dependent oxygen evolution in leaf discs (Delieu and Walker, 1981). In this chapter experiments are reported where oxygen electrodes were used to measure photosynthesis rates of leaf discs from both healthy and infected plants. The apparatus and the procedures which were used are described in section (2.7.1). The data were analysed to determine the effects of SYNV on the maximum gross photosynthesis rates, the maximum net photosynthesis rates, dark respiration rates (Rd) and the quantum efficiencies of photosynthesis at low light intensity (α), and the ratio of physical (rp/rp + rx) to total diffusion resistance to CO_2 fixation (θ). Two models which describe the photosynthesis-light response curve, (Rabinowitch, 1951; Marshall and Biscoe, 1980), described in section (2.7.4.1) were used to estimate these important parameters.

4.2. Results:

In 1951 Rabinowitch proposed a number of alternative models to describe photosynthesis-light responses (PLRs). These were based on the biochemical reactions within the chloroplast, and involved required several assumptions to describe these complex reactions. The model proposed by Rabinowitch (1951) described the relationship between photosynthesis and irradiance in terms of a rectangular hyperbola, and subsequently this relationship has been widely used by many workers. One of the most popular reasons on the use of the oxygen electrode (figure 2.1) was based on this relationship (Walker, 1987). The rectangular hyperbola accounts only for the biochemical reactions within the chloroplast. Before fixation, CO_2 must diffuse through the leaf boundary layer, the stomata and the mesophyll layer (comprising intercellular layer, cell wall and intracellular fluid) which could present a significant effect on the overall estimation of photosynthesis rates. Recently, Marshall and Biscoe (1980) proposed a non-rectangular hyperbola model which describes more accurately the dependence of photosynthetic rate on irradiance and parameters related to physical and chemical processes. The model is more suitable and contains sufficient parameters to account for the major characteristics observed in a PLR.

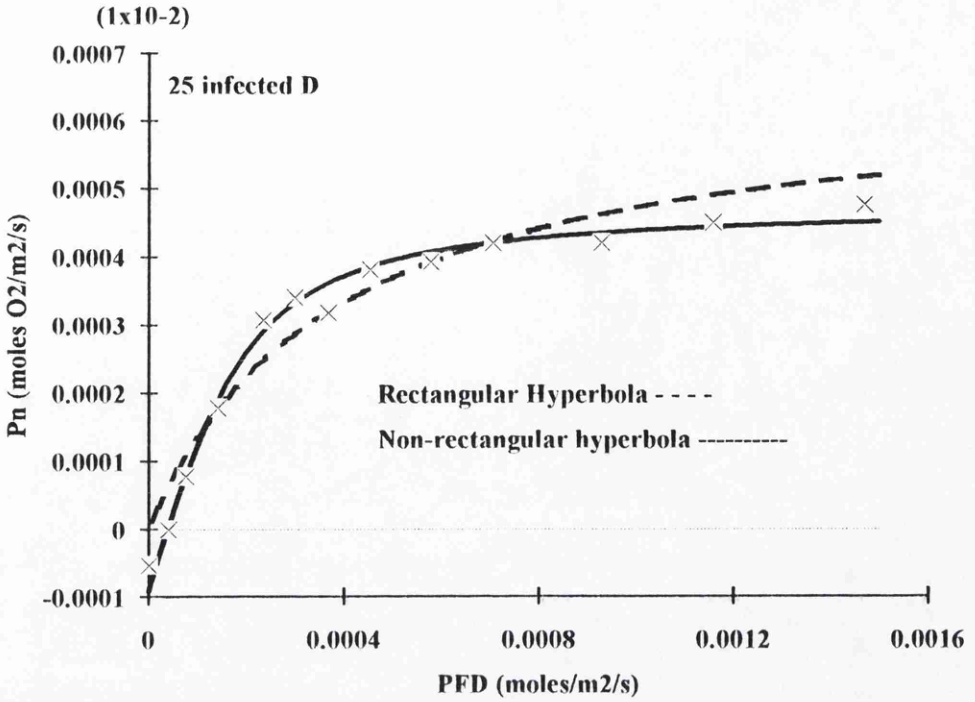
A difference between the goodness of fit of two models to the data, became clear when the data were analysed and plotted for each parameter and for each plant. For instance, at 25 days after inoculation in *N. edwardsonii*, control and infected plants (figure 4.1), it became clear that the rectangular hyperbola overestimated Pgmax (maximum gross photosynthesis) and Rd (dark respiration) and under-estimated photosynthesis at intermediate light levels. Further, the residual errors of the fit were consistently higher when the rectangular hyperbola model (4.299×10^{-6}) was used (c.f. non-rectangular hyperbola model 1.638×10^{-6} in control plant (D). Further differences are shown in appendix). Moreover, the non-rectangular model provides more information about the photosynthesis rates by estimating four parameters. Although, the Rabinowitch model consistently

gave a poorer fit to the data than the Marshall and Biscoe model, the results were broadly similar. Therefore, for simplicity the data analysed by the Marshall and Biscoe model are the only ones presented and discussed in this chapter. However, it is emphasised that both models were applied to all the data.

Photosynthesis assimilation measurements are based on the amount of oxygen released or consumed from control and infected leaf discs. Net photosynthesis rate (Pn) and dark respiration rate (Rd) were the main parameters measured during the experiment. Five healthy and five infected plants were monitored at each time interval post-infection as described in section (2.7.1).

Healthy and infected *N. edwardsonii* plants photosynthesis rates were measured at four intervals during the infection period. Basically the experiment was designed to measure the effects of SYNV on *N. edwardsonii* photosynthesis at four stages during infection which severity of symptom expression differed. The first stage, was where the infected plants first started to show symptoms (10 days post-inoculation), the second was where most of the symptoms had developed and the infection reached its peak (25 days post-inoculation), the third stage was where by the symptoms started to change to yellow mottled spots (30 days post-inoculation) and finally the fourth was where the whole infected plant showed yellow mottled spots at 40 days post-inoculation.

N. glutinosa plants infected with SYNV were measured as for *N. edwardsonii* except that only three measurements were taken during the infection period. The first measurements were taken 15 days after inoculation, when the symptoms had just started to show, the second measurements were taken during the acute phase of infection after 35 days after inoculation and finally, the third were taken 55 days after inoculation when the yellow mottled spots symptoms were very well established throughout the whole infected plant.



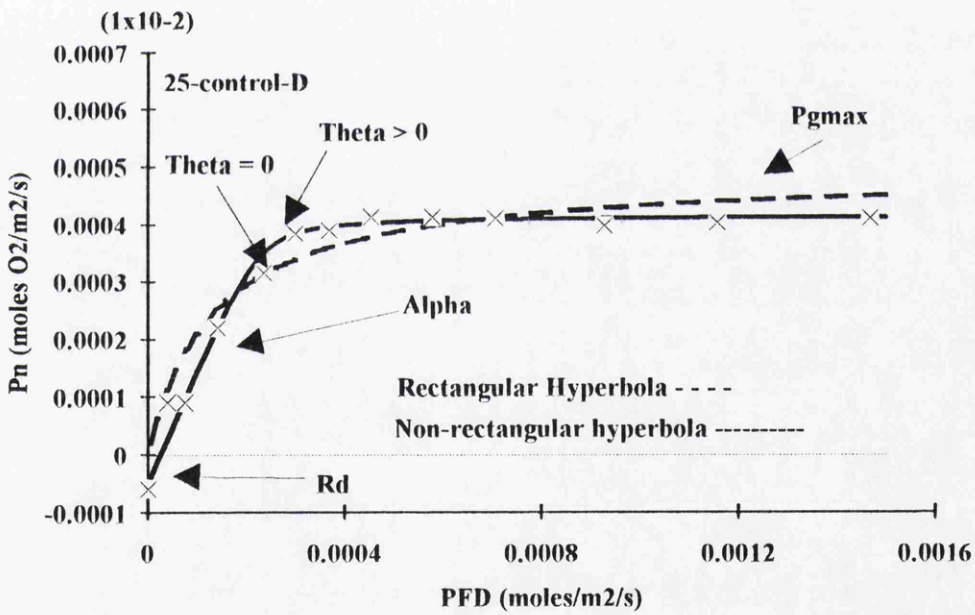


Figure 4.1:
N. edwardsonii (25 days):

Infected-D	Control-D
Rabinowich	Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
Pgmax= 6.500E-06	Pgmax= 4.907E-06
α = 1.704E-02	α = 3.646E-02
Error= 4.123E-06	Error= 4.299E-06
Marshall & Biscoe	Marshall & Biscoe
Rd= -9.117E-07	Rd= -5.018E-07
Pgmax= 4.954E-06	Pgmax= 4.158E-06
α = 2.473E-02	α = 1.965E-02
θ = 7.518E-01	θ = 9.750E-01
Error= 1.870E-06	Error= 1.638E-06

4.2.1- The effect of SYNV infection on photosynthesis in *N. edwardsonii* :

Ten to 14 days post-infection, *N. edwardsonii* started showing symptoms in new leaves, characterised by vein-clearing and yellowing. After 10 days, leaf discs (10 cm^2) with mosaic symptoms were cut from five infected plants and used to measure oxygen evolution as described in section (2.7.1). These procedures were used on the other batches of infected plants 25, 30, and 40 days post-inoculation. At each time of measurement, five healthy control plants of the same age as the infected plants were used and their photosynthesis rate was determined.

4.2.1.1- The effect SYNV infection on maximum net and gross photosynthesis rates of infected *N. edwardsonii*:

Net photosynthesis rates (Pn) were calculated per m^2 of leaf area at different light intensities. In the first 10 days after inoculation, infected *N. edwardsonii* showed no differences in net photosynthesis rates when compared to the net photosynthesis rates of control plants (figure 4.2a). At 25 days after inoculation, the net photosynthesis rates (Pn) increased in both healthy control and infected plants. Despite the establishment of systemic symptoms of vein-clearing in infected plants at this stage, Pn of infected plants was not significantly lower than that of the control plants (figure 4.2b). Although, infected plants appear to photosynthesize less than the control plants at all light intensities as shown in figure (4.2b), the decrease in photosynthesis in infected plants were statistically not significant at $p = 0.05$. Later than 25 days (30 and 40 days post-inoculation), the infected plants started to show yellow mottled spots. In general, the photosynthesis rates in both healthy and infected plants were lower, possibly as a results of senescence process. However, although the Pn of infected plants were consistents reduced more markedly than the control plants, these differences were not statistically significant at $p = 0.05$ (figure 4.3a). Forty days after inoculation, both healthy and infected plants leaves were at a stage of late

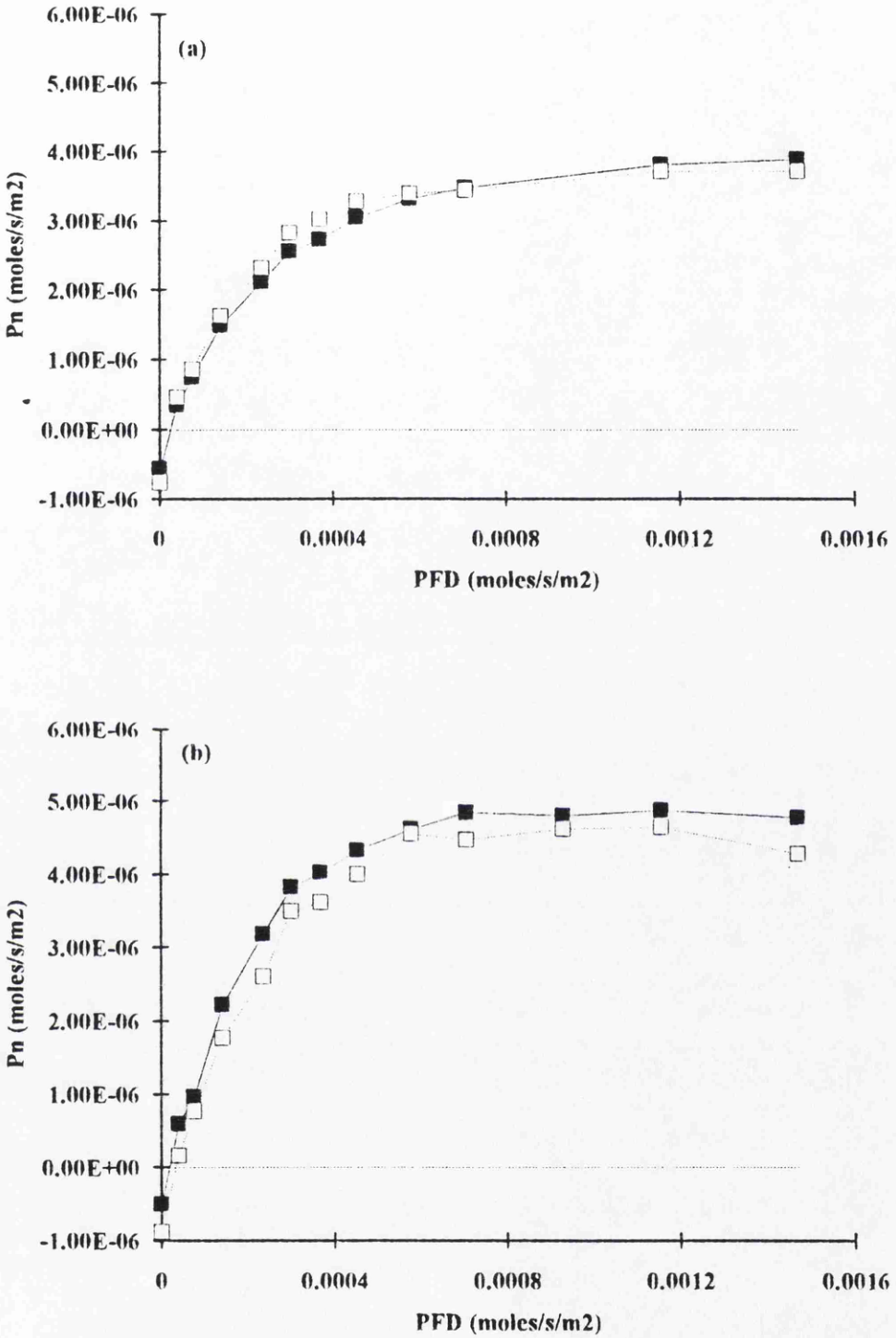


Figure 4.2: The effects of SYNV on net photosynthesis rates in *N. edwardsonii*. (a) 10 days after inoculation, (b) 25 days after inoculation (dark box, control, white box infected, LSD = 149x10-6; p = 0.05). Each data point represents the average of 5 sparate determinations

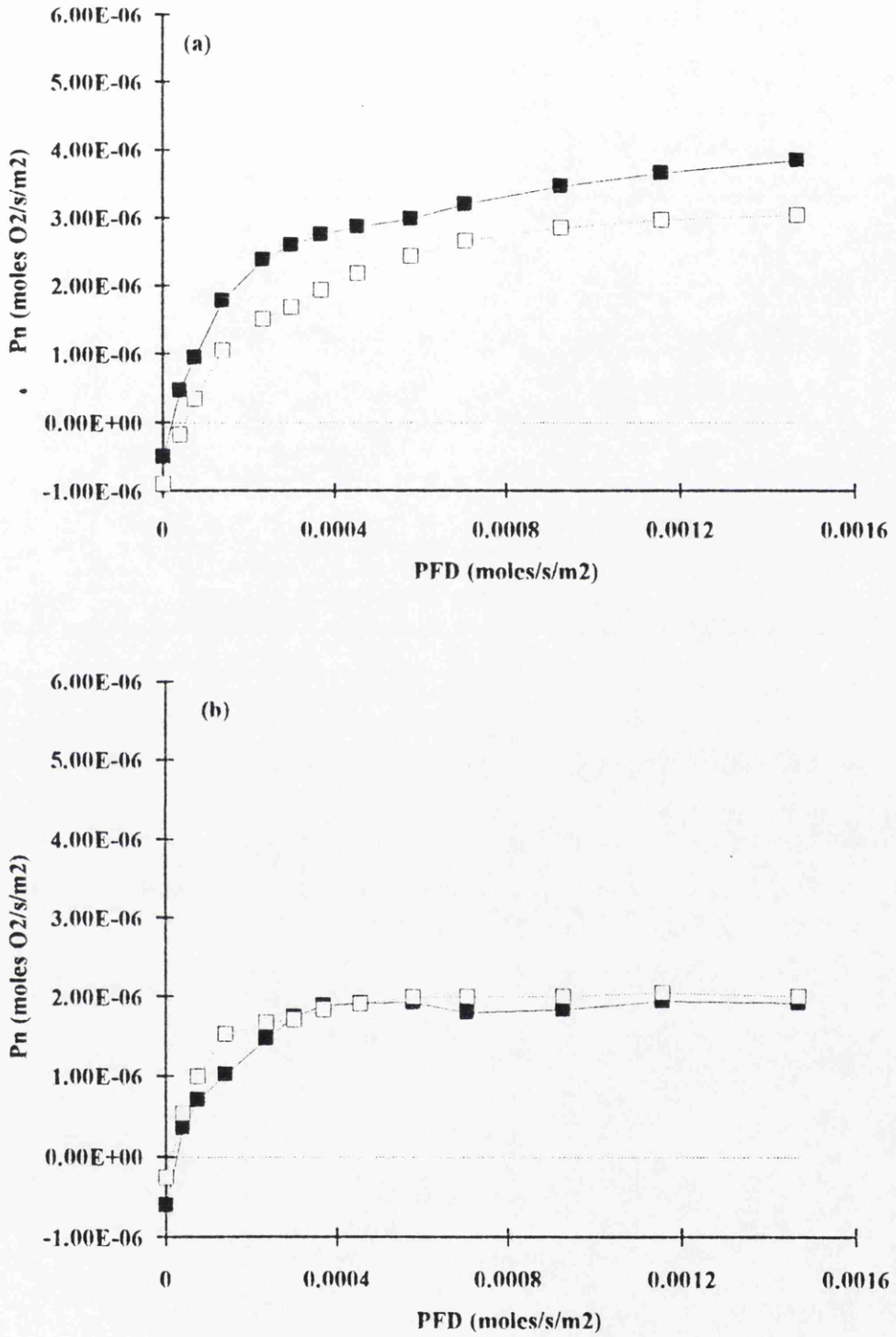


Figure 4.3: The effects of SYNV on net photosynthesis rates in *N. edwardsonii*. (a) 30 days after inoculation, (b) 40 days after inoculation (dark box, control, white box infected, LSD=149x10-6; p = 0.05). Each data point represents the average of 5 separate determinations.

senescence. At this stage, the differences between control and infected plants, did not change from the previous measurements at 30 days as shown in figure (4.3b). The net photosynthesis from both control and infected plants were decreased due to the senescence process.

4.2.1.2- Analysis of the data:

Using the Marshall and Biscoe model (non-rectangular hyperbola), the estimations of the maximum gross photosynthesis rate (Pgmax), maximum net photosynthesis rate, dark respiration rate, the ratio of physical resistance to the total diffusion resistance to CO_2, and the quantum efficiency of photosynthesis at low light intensity (α), were calculated and the data were analysed by an analysis of variance procedure using the statistical package MINITAB release 8 on an IBM-AT compatible computer.

The two-factor analysis of variance (infection and age) for the maximum gross photosynthesis rates showed neither significant differences between the infected and non-infected plants nor any interaction between infection and leaf age at the four stages of infection. However, leaf age itself, had a very significant effect at p= 0.01 on Pg (figure 4.4a). The maximum gross photosynthesis rates (Pgmax) of infected plants at 10 days (3.82 μ moles $O_2/s/m^2$) were less than the Pgmax of control plants (4.72 μ moles $O_2/s/m^2$), subsequently reaching the level of control plants at 25 days (5.48 μ moles $O_2/s/m^2$ and 5.38 μ moles $O_2/s/m^2$, respectively), but then declined at 30 days (3.58 μ moles $O_2/s/m^2$ and 4.70 μ moles $O_2/s/m^2$, respectively). The sharp decrease in Pgmax of infected plants presumably reflects the severity of infection between 25 and 30 days after inoculation. Pgmax also declined in control plants but less sharply than in infected plants. By 40 days, the differences between Pgmax of infected plants (2.12 μ moles $O_2/s/m^2$) and control plants (2.45 μ moles $O_2/s/m^2$) had narrowed as shown in figure (4.4a) possibly reflecting to the change in symptoms and the onset of the stage recovery.

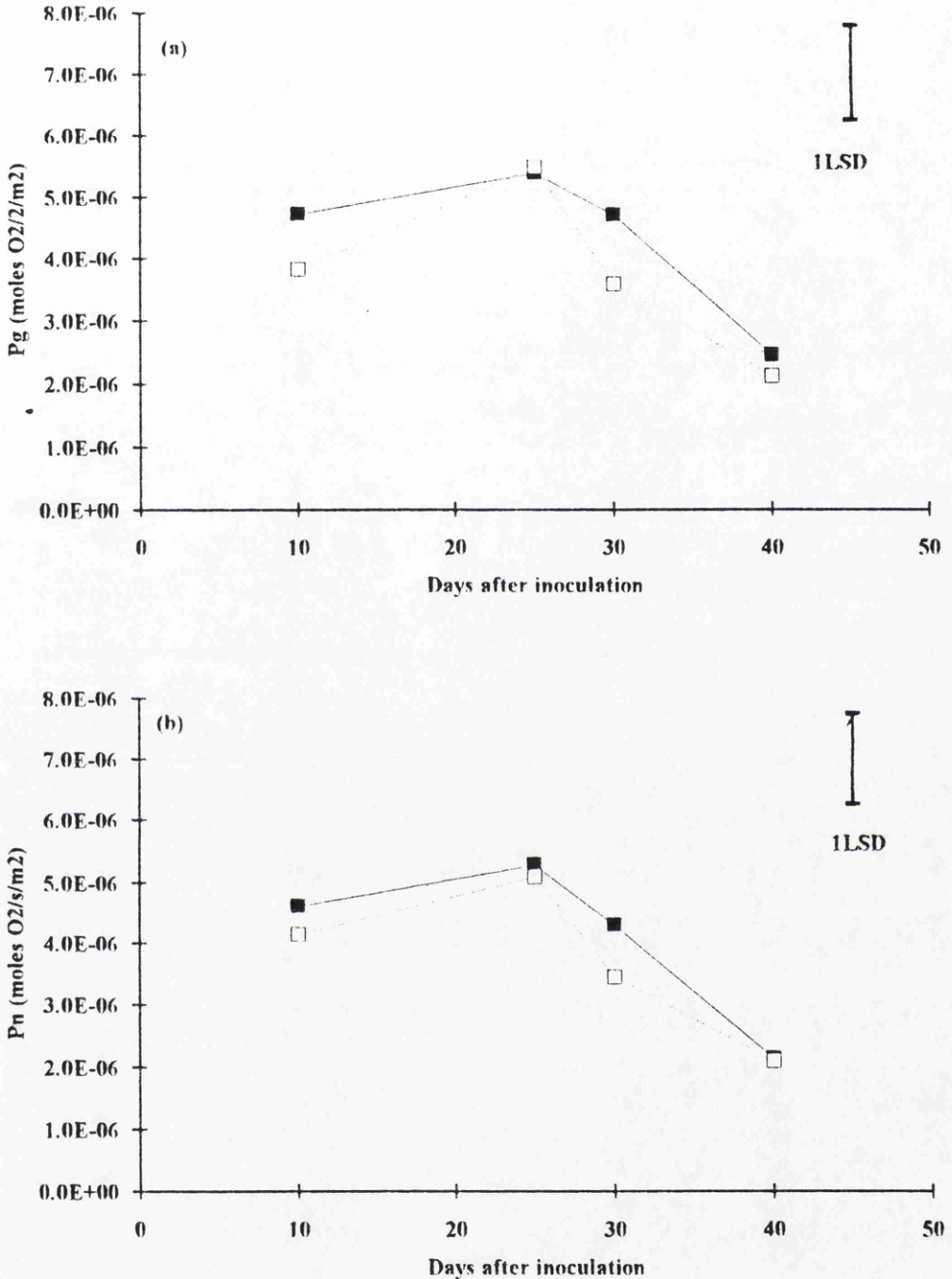


Figure 4.4:

(a)- The effect of SYNV on the maximum gross photosynthesis in *N. edwardsonii* at different times post-inoculation (dark box, control; empty box, infected; LSD= 1.53x10-6; p = 0.05; each data point represents the average of 5 separate determinations).

(b)- The effect of SYNV on the maximum net photosynthesis in *N. edwardsonii* at different times post-inoculation (dark box, control; empty box, infected; LSD= 1.49x10-6; p = 0.05; each data point represents the average of 5 separate determinations).

The maximum net photosynthesis rates (Pnmax), as estimated by equation 8 (section 2.7.3.1), were analysed and showed an almost similar pattern to the maximum gross photosynthesis at all four stages as shown in figure (4.4b).

4.2.1.3- The effect of SYNV infection on the quantum efficiency in *N. edwardsonii*:

The quantum efficiency of photosynthesis at low light intensity (α) was determined from the slope of the photosynthesis rate vs light intensities plots. The analysis of α showed no significant effects of the virus infection at all four stages (figure 4.5a). Analysis showed that the quantum efficiencies of photosynthesis increased during the first two stages (10 and 25 days) in infected plants while control plants were less but that their differences were not significant. By 30 days where the infection was at the highest severity, quantum efficiencies were less than the control plants and remained lower than the controls even at 40 days post-inoculation.

4.2.1.4- The effect of SYNV infection on the dark respiration rates of *N. edwardsonii*:

Dark respiration of infected and control plants were also estimated using the non-rectangular model. Analysis of the data (shown in figure 4.5b) shows that infected plants respiration rates at the first three stages (10, 25 and 30 days; -0.60, -0.96, & -0.74 μ moles $O_2/s/m^2$, respectively) were greater than in control plants at the same stages (10, 25 & 30 days; -0.43, -0.28, & -0.34 μ moles $O_2/s/m^2$, respectively) and thereafter, the respiration rates decreased, reaching its lowest at 40 days (-0.26 μ moles $O_2/s/m^2$). The differences in dark respiration rates between control and infected plants were significant at $p = 0.05$ only at 25 and 30 days. The dark respiration rates data obtained from the rectangular hyperbola model were under estimated when compared with the data from the non-rectangular model as shown in figure (4.1a & b). In fact, most if not all of

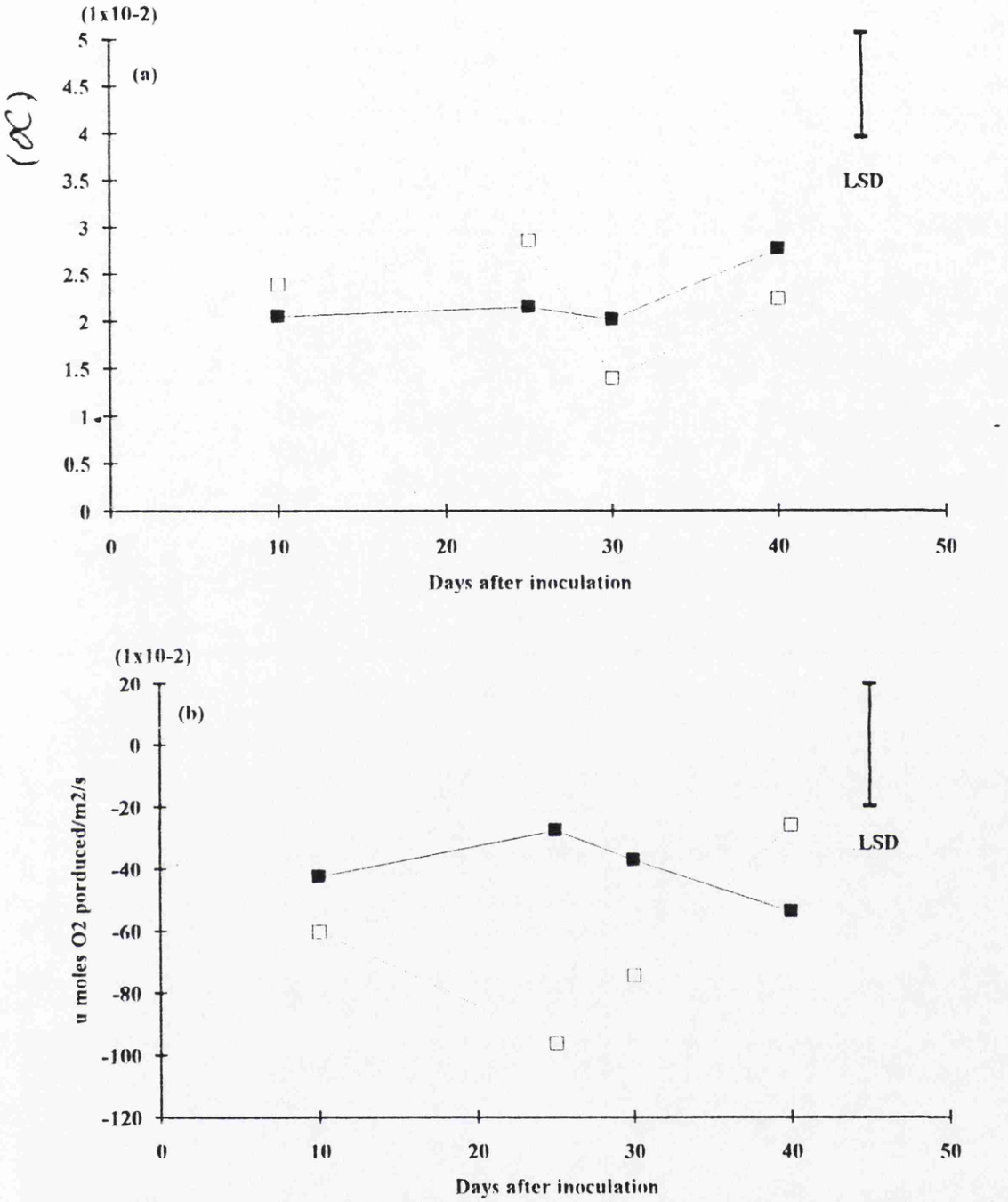


Figure 4.5:

(a)-The effect of SYNV on the quantum efficiency of photosynthesis at low light intensity (α) in infected *N. edwardsonii* plants at different times during the infection period (dark box, control; empty box, infected; LSD= 0.01; p = 0.05; each data point represents the average of 5 separate determinations).

(b)- The effect of SYNV on dark respiration in *N. edwardsonii* at different times post-inoculation (dark box, control; empty box, infected; LSD= 0.4x10-6; p = 0.05; each data point represents the average of 5 separate determinations).

the estimated dark respiration data for each measurement was estimated to be equal to zero.

4.2.1.5- The effect of SYNV infection on the physical and biochemical resistance to CO_2 diffusion *N. edwardsonii*:

One of the parameters which can be estimated in the non-rectangular model is the ratio of physical to total diffusion resistance to CO_2 (θ). This factor is not considered in the rectangular model. The value of theta (θ) is given by;

$$\theta = (r_p)/(r_p + r_x)$$

Where r_p = Physical resistance, r_x = Chemical or carboxylation resistance.

Analysis of θ, shown in figure (4.6) shows that although the resistance to CO_2 was slightly higher in infected plants at all four stages, these differences were not statistically significant at $p = 0.05$.

4.2.2- The effects of SYNV infection on chlorophyll levels in *N. edwardsonii*:

4.2.2.1- The effects of SYNV infection on total chlorophyll:

The amount of chlorophyll form each healthy control and infected leaf disc were extracted in 25 ml of methanol and the absorbance measured spectrophotometrically as described in section (2.7.2). The total chlorophyll was calculated by adding the amounts of chlorophyll *a* (chl*a*) and chlorophyll *b* (chl*b*) together.

Analysis of variance of the total chlorophyll data showed a very highly significant effect for both treatment and age of plants and also of the interaction between these two factors. Small differences between the amount of chlorophyll in healthy and infected plants were observed at 10 and 25 days after inoculation but these were not significantly different at $p = 0.05$. However, by 30 days, the levels in control plants declined very rapidly, reached its minimum at 40 days (206 g/m^2 of leaf) which made these measurements highly significant from the

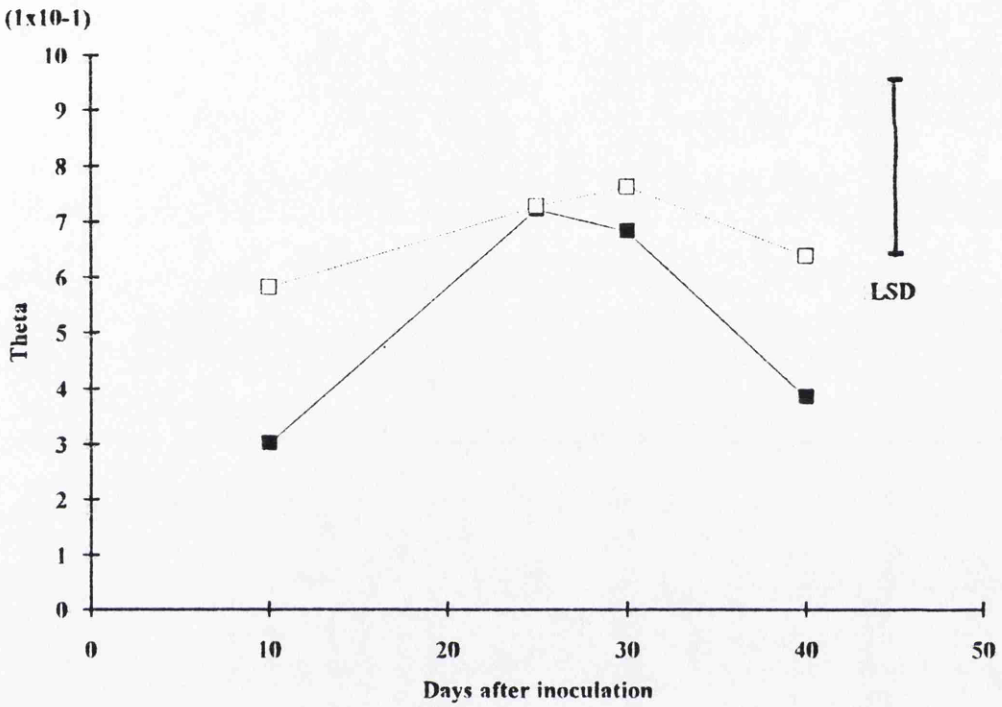


Figure 4.6: The effect of SYNV on (θ) in infected *N. edwardsonii* at different times during the infection period (dark box, control; empty box, infected; LSD= 0.312; p = 0.05; each data point represents the average of 5 separate determinations).

other three measurements at 10, 25 and 30 days after inoculation. On the other hand, no differences in the amount of chlorophyll in infected plants were observed between 10 and 25 days (329 and 283 g/m^2 of leaf, respectively) and after that, the amount decreased very sharply, reaching its lowest value at 40 days (105 g/m^2 of leaf) and was very highly significant at 30 and 40 days ($p = 0.05$) as shown in figure (4.7a).

The major differences in total chlorophyll content between the infected and control plants were evident during the period between 25 and 30 days after inoculation (283 and 129 g/m^2, respectively) as shown in table (4.1). Although the total chlorophyll levels in infected plants started to fall from 10 days after inoculation, the differences between healthy and infected plants were not significant at 10 and 25 days. The results indicate that the virus infection induces a very dramatic reduction in the chlorophyll levels in infected *N. edwardsonii*. Also as time and virus infection interaction was significant it suggested that SYNV infection accelerates senescence in *N. edwardsonii*.

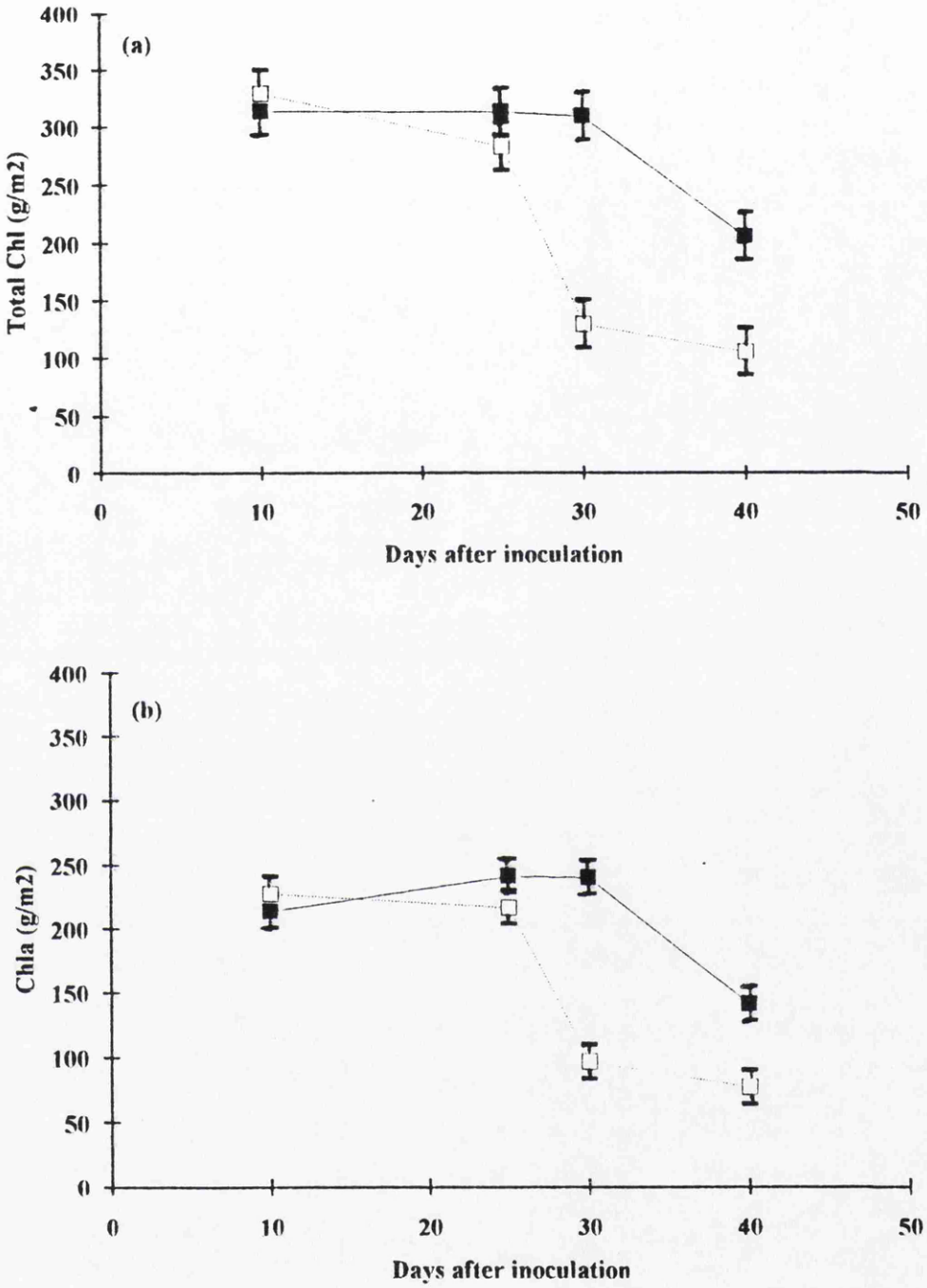


Figure 4.7:

(a)- The effects of SYNV on total chlorophyll of infected *N. edwardsonii* plants at different times after inoculation. (White box, infected, black box control. LSD = 41g/m2; p = 0.05. Each data represents the average of 5 determinations).

(b)- The effects of SYNV on chlorophyll *a* of infected *N. edwardsonii* plants at different times after inoculation. (White box, infected, black box control. LSD = 26 g/m2; p = 0.05. Each data represents the average of 5 determinations).

Table 4.1: The levels of chlorophyll in healthy and infected *N. edwardsonii.*

Stages	Total	%	Chl*a*	%	Chl*b*	%
10 days						
Control	314		213		100	
Infected	329	104	227	106	101	101
25 days						
Control	313		241		71	
Infected	283	90	216	89	66	92
30 days						
Control	323		240		83	
Infected	129	40	96	40	33	39
40 days						
Control	206		141		64	
Infected	105	57	77	54	28	44

All values are the mean of five measurements. (g/m^2 of leaf). Percentage are expressed as % of control.

4.2.2.2- The effects of SYNV in chlorophyll *a* and *b*:

Three major pigment-protein complexes can be identified in higher plant chloroplasts. The light harvesting chlorophyll *a/b* complex (LHC2b) contains approximately equal amount of chlorophyll *a* and *b* and acts solely as a light harvesting antenna transferring its energy mainly to photosystem II, but also to photosystem I. The other two complexes are the reaction centres PSI and PSII and contain mostly Chlorophyll *a* (Platt, *et. al.*, 1979).

The effects of SYNV on the amount of chlorophyll *a* in infected *N. edwardsonii* plants were very similar to effects on total chlorophyll. The amount of chlorophyll *a* started to decrease from 10 days after inoculation, reached its minimum at 40 days whereas in the controls, it started to increase at 10 days, reached a maximum at 25 to 30 days, after which declined sharply by 40 days, where the leaves started to senescence as shown in table (4.1). In the first two stages (10 and 25 days), the reduction in the amount of chlorophyll *a* in infected plants was not significant. The major differences were shown at 30 and 40 days which were very highly significant at $p = 0.01$ as shown in figure (4.7b).

The effect of SYNV on chlorophyll *b* was also highly significant in infected plants. The amount of chlorophyll *b* decreased very sharply after 10 days post-inoculation, reached its minimum at 40 days as shown in table (4.1), but this was not statistically significant until 30 days after inoculation. Significant differences between infected and control plants were shown only at 30 and 40 days after inoculation as shown in figure (4.8a).

The ratio of chlorophyll *a* to chlorophyll *b* shown in figure (4.8b), up to 30 days was not significantly different from the controls. However, at 40 days after inoculation, infected plants showed small but highly significant ($p = 0.01$) increases in the chlorophyll *a/b* ratio compared to controls. This suggests that chlorophyll *b* has been lost more rapidly than chlorophyll *a* in the older infected plants, and possibly reflects an increased loss of LHC2b in comparsion with reaction centres.

4.2.3- The effect of SYNV infection on photosynthesis in *N. glutinosa*:

Two to three weeks post-inoculation, *N. glutinosa* started to show visual symptoms of vein-clearing in unexpanded leaves. After 15 days, leaf discs (10 cm^2) with systemic symptoms were cut from five infected plants and used to measure oxygen evolution as described in section (2.7.1). These procedures were used on other batches of infected plants after 35 and 55 days post-inoculation, as well as five healthy control plants at the same age as infected plants.

4.2.3.1- The effect of SYNV infection on the maximum net and gross photosynthesis rates of *N. glutinosa*:

In the first 15 days post-inoculation, infected *N. glutinosa* showed no major difference from healthy plants in the net photosynthesis rate (Pn), suggesting that the infection was still in an early stage of development, as shown in figure (4.9a). Infected plants became more severely infected and at 35 days

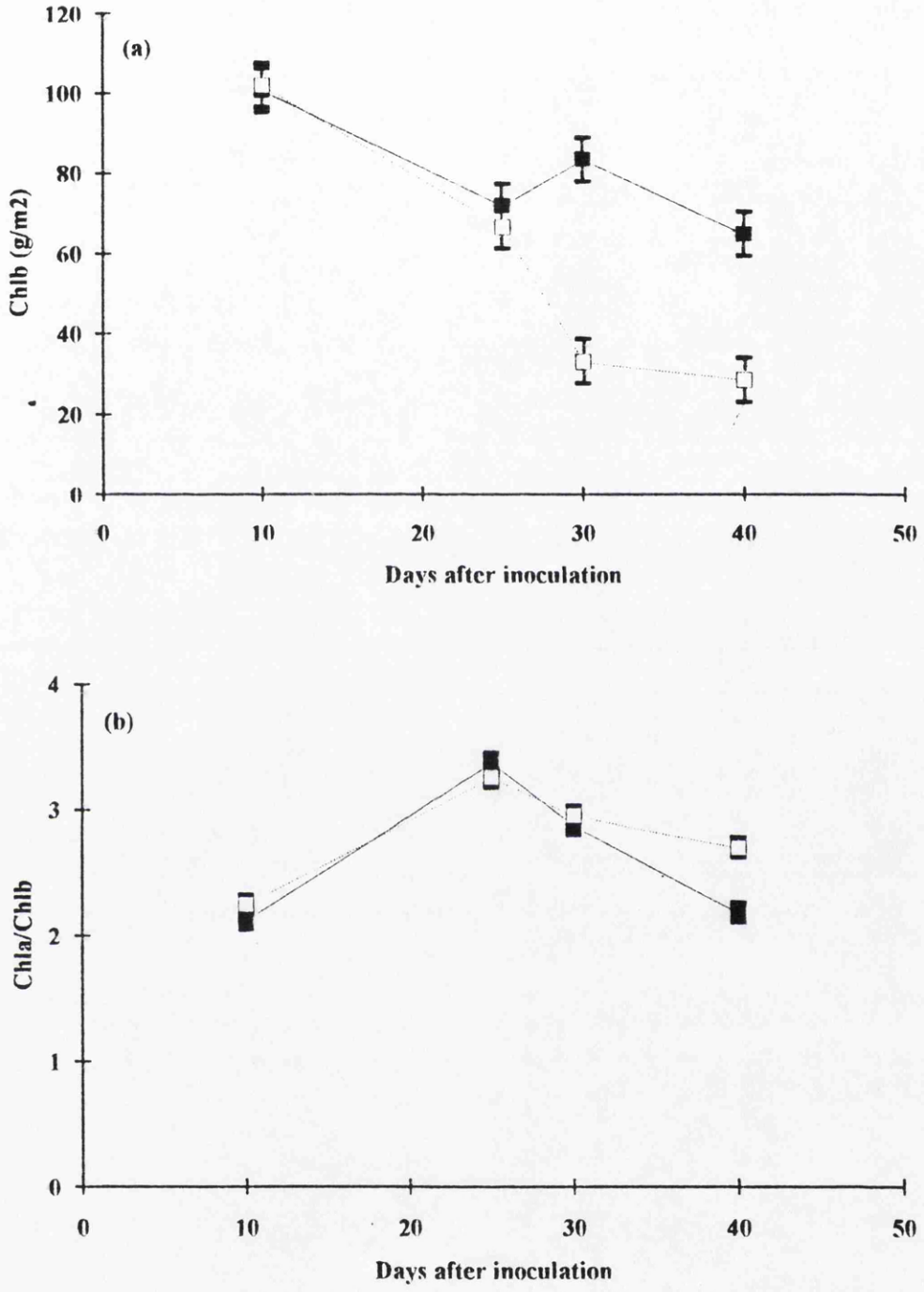


Figure 4.8:

(a)- The effects of SYNV on chlorophyll *b* of infected *N. edwardsonii* plants at different times after inoculation. (White box, infected, black box control. LSD = 11 g/m2; p = 0.05. Each data represents the average of 5 determinations).

(b)- The ratio of Chl*a*/Chl*b* of infected *N. edwardsonii* plants at different times after inoculation. (White box, infected, black box control. LSD = 0.16 g/m2; p = 0.05. Each data represents the average of 5 determinations).

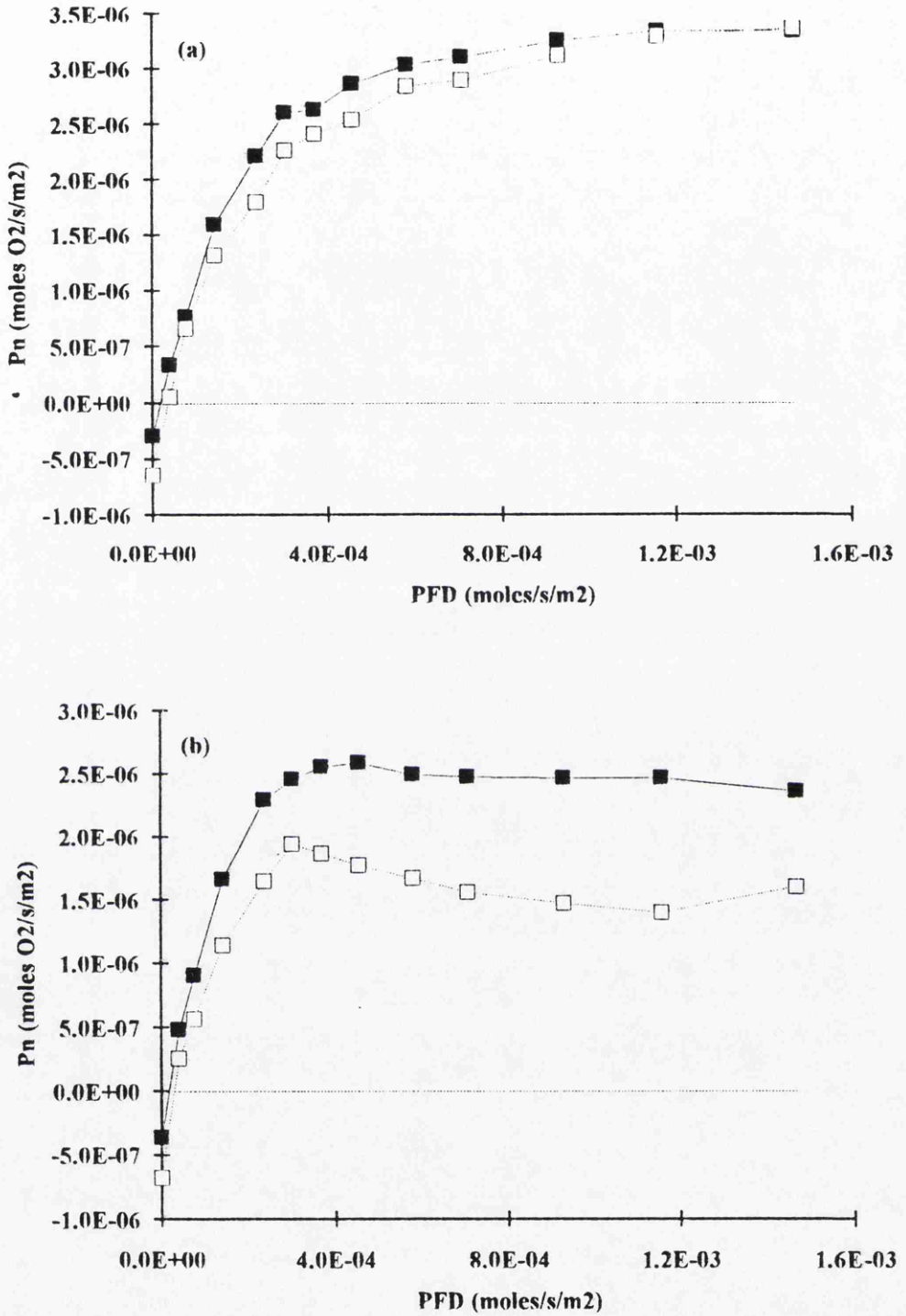


Figure 4.9: The effects of SYNV on net photosynthesis rates of infected *N. glutinosa*. (a) 15 days post-inoculation and (b) 35 days post-inoculation (dark box, control, white box, infected, LSD = 89x10-6; p = 0.05).Each data point represents the average of 5 separate determinations.

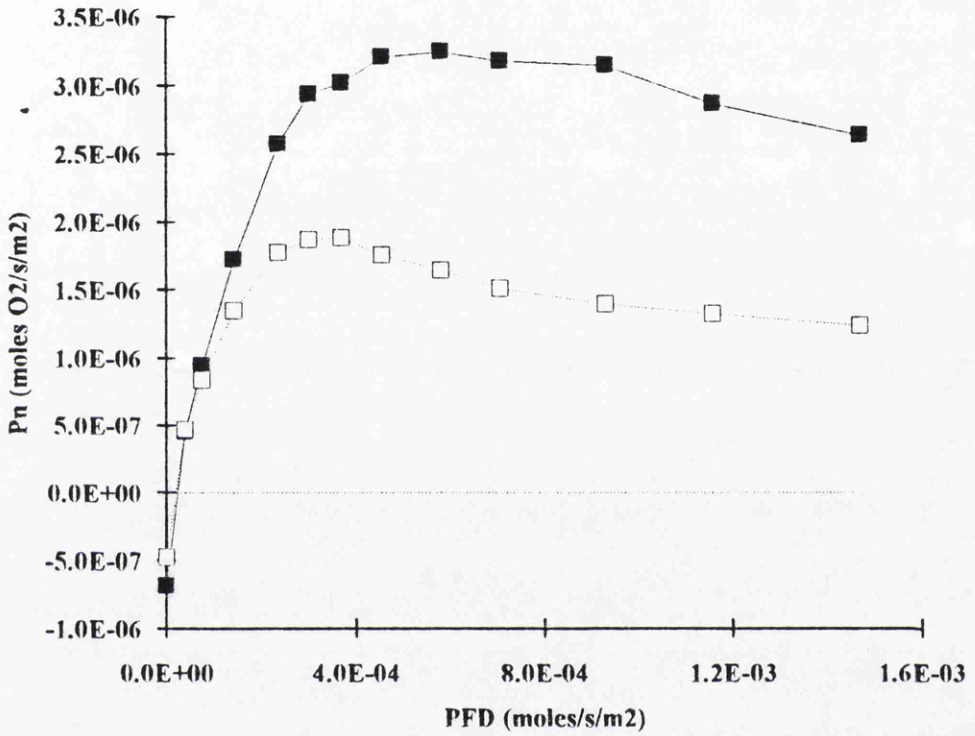


Figure 4.10: The effects of SYNV on net photosynthesis rates of infected *N. glutinosa* 55 days post-inoculation (dark box, control, white box, infected, LSD = 89x10-6; p = 0.05). Each data point represents the average of 5 separate determinations.

after inoculation, the differences in Pn between infected and control plants became very clear. Infection reduced Pn at high light intensities while at low light intensities, no differences from the controls were observed. However, high light intensities appeared to inhibit Pn at this stage which could be due to photoinhibition or photorespiration (4.9b). By 35 days after inoculation, infected plants started to show yellow mottled spots and chlorotic lesions in unexpanded leaves. These symptoms presisted in the plant for over a year. Pn measured at 55 days after inoculation, showed a further decrease in infected plants compared with the previous measurements, indicating that the virus infection had a very dramatic effect on the photosynthetic rates of *N. glutinosa* plants, particularly at high light intensities (figure 4.10).

The analysis of the data of maximum net (Pnmax) and maximum gross (Pgmax) photosynthesis rates of infected and control plants, (figures 4.11a & b), showed similar effect of virus infection on both parameters. Analysis revealed a significant effect of virus infection which became clear at the late stage of infection (35 & 55 days).

Similar results were obtained when the Rabinowitch model (rectangular hyperbola model) was used to estimate the maximum gross photosynthesis rates.

4.2.3.2- The effect of SYNV infection on the quantum efficiency in *N. glutinosa*:

Analysis of photosynthesis rates in *N. glutinosa* plants, showed that virus infection had no significant effects on the quantum efficiency of photosynthesis at any stages of infection (figure 4.12a). This can also be seen in figures 4.9 and 4.10 where the initial slopes at low light intensities show no major differences between control and infected plants. Although the virus infection caused no major changes of quantum efficiency, a slight increase (α) occurred with the increasing leaf age, an indication that the efficiency of light harvesting complexes involved in light capture were not significantly affected.

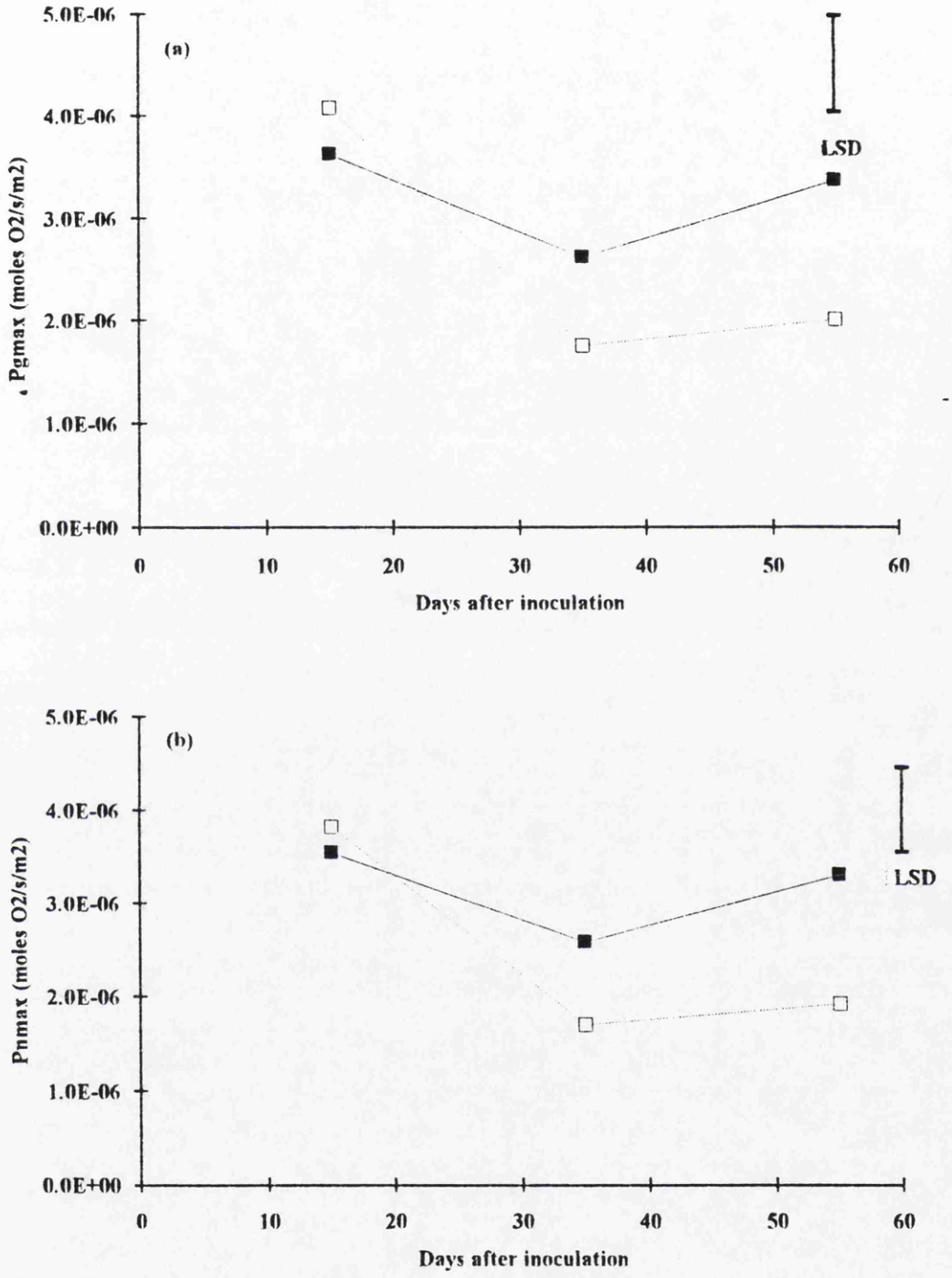


Figure 4.11:

(a)- Effects of SYNV on maximum gross photosynthesis of *N. glutinosa* (Dark box, control; empty box, infected; LSD = 0.95x10-6; each data point represents the average of 5 separate determinations).

(b)- Effects of SYNV on maximum net photosynthesis of *N. glutinosa* (Dark box, control; empty box, infected; LSD= 0.89x10-6; p = 0.05; each data point represents the average of 5 separate determinations).

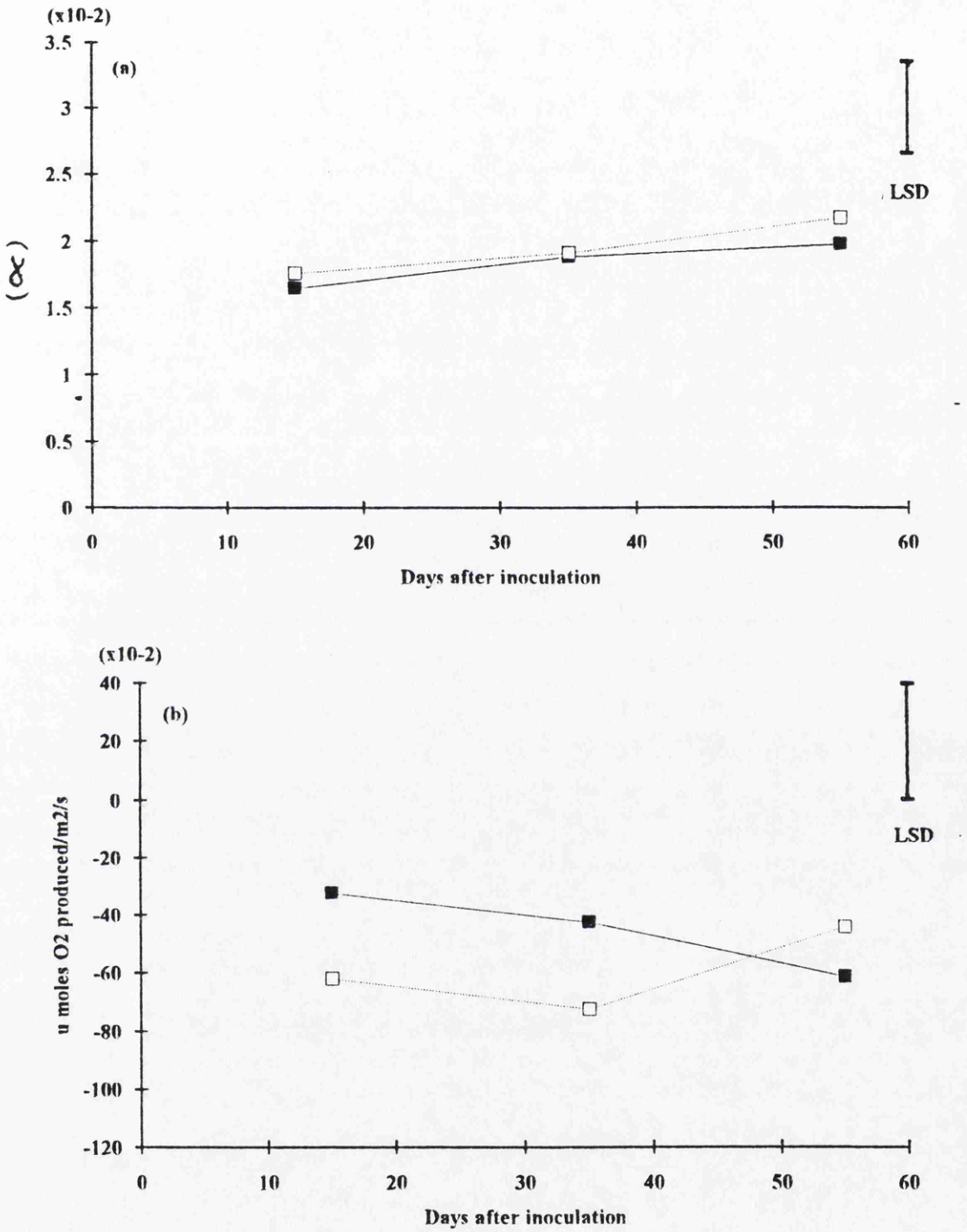


Figure 4.12:

(a)-Effects of SYNV on the quantum efficiency of photosynthesis at low light intensity (α) of infected *N. glutinosa* plants at different times during the infection period (dark box, control; empty box, infected; LSD = 0.07; p = 0.05; each data point represents the average of 5 separate determinations).

(b)- Effects of SYNV on the dark respiration rates (Rd) of *N.glutinosa* at different times post-inoculation (dark box, control; empty box, infected; LSD = 0.4x10-2; p = 0.05; each data point represents the average of 5 separate determinations).

4.2.3.3- The effect of SYNV infection on the dark respiration rates of *N. glutinosa*:

Dark respiration for each leaf of infected and healthy *N. glutinosa* plants was estimated and analysed using the non-rectangular model . Analysis of the data showed no significant differences between infected and healthy plants (figure 4.12b). No major differences in the dark respiration rate between healthy control and infected plants was monitored, is an indication that the mitochondria, where most of the dark respiration process occurs, were apparently unaffected by virus infection.

4.2.3.4- The effect of SYNV infection in the physical and biochemical resistance to CO_2 diffusion in *N. glutinosa*:

The data of the ratio of physical to total diffusion resistance to CO_2 (θ) in *N. glutinosa* plants, estimated by the non-rectangular model were analysed statistically but no significant differences between healthy and infected plants were observed (figure 4.13).

4.2.3.5- Conclusions on the observation on theta (θ) and Pgmax:

According to the Marshall and Biscoe (1980), Pgmax and theta (θ) can be calculated from:-

$$\mathbf{Pgmax} = \mathbf{C_a}/(\mathbf{r_p} + \mathbf{r_x})$$

$$\theta = (r_p)/(r_p + r_x)$$

Where $\mathbf{C_a}$ = CO_2 concentration in air, $\mathbf{r_p}$ = Physical resistance, $\mathbf{r_x}$ = Chemical or carboxylation resistance.

Three factors which influence gross photosynthesis rates and the ratio of physical to total resistance of CO_2 diffusion were the concentration of CO_2 in air (C_a), physical resistance (r_p) and biochemical resistance (r_x). Since CO_2 was provided by 600 µl 1M of sodium bicarbonate throughout the experiment, it can

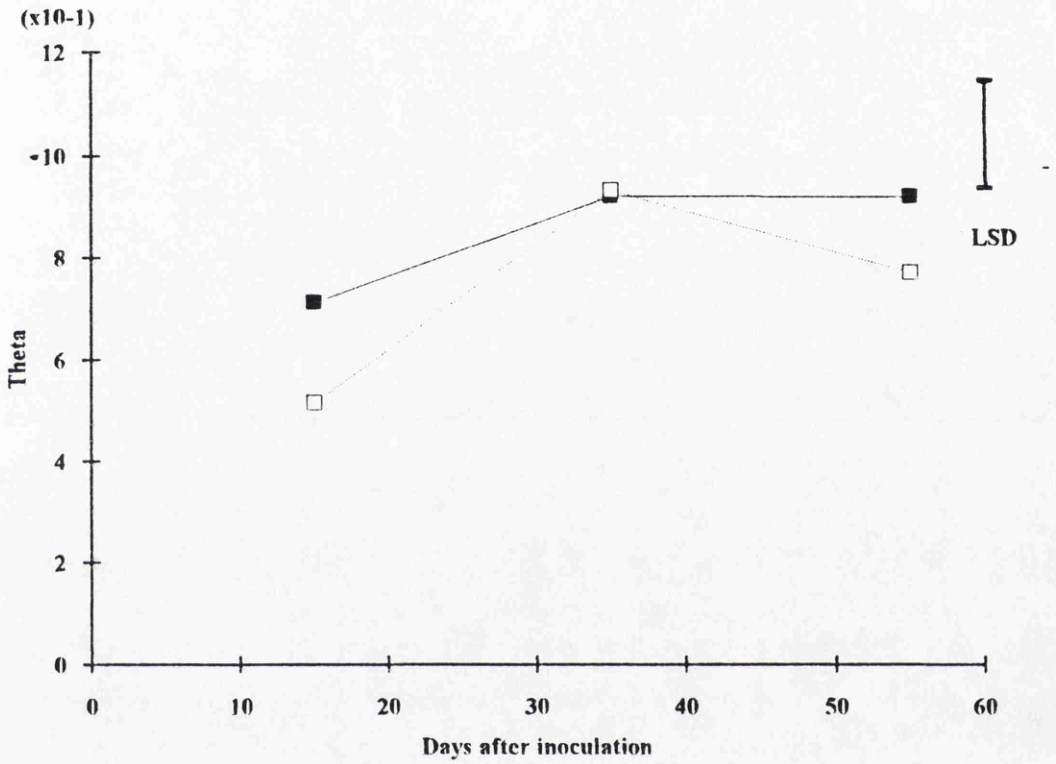


Figure 4.13: The effects of SYNV on (θ) in infected *N. glutinosa* at different times during the infection period (dark box, control; empty box, infected; LSD= 0.21; p = 0.05; each data point represents the average of 5 separate determinations).

be assumed that the concentration of CO_2 was saturating (~ 1%) and constant throughout the time of experiment (Delieu and Walker, 1981). Therefore (r_p) and (r_x) are the only two factors which should influence Pgmax and θ. Therefore, the effect of changes in rp and rx on θ can be predicted (table 4.2).

Table 4.2: Predicted effects of changes in rp and rx on Pgmax and θ:

Assumption	changes	Expected changes	
		Pgmax	θ
rp > rx	rp	√	-
	rx	-	-
rp ≅ rx	rp	√	√
	rx	√	√
rp < rx	rp	-	√
	rx	√	√

Where rp = physical resistance, rx = biochemical resistance, √ = significant changes,
(-) = insignificant changes.

The results presented in this chapter suggest a significant decrease in Pgmax of infected *N. glutinosa* only and no decrease in theta (θ), as shown in figures (4.11b & 4.13). Therefore, from table 4.3 it is concluded that the physical resistance to CO_2 diffusion (rp) was affected by SYNV infection, and further, rp>rx.

4.2.4- The effect of SYNV infection on chlorophyll levels in *N. glutinosa* :

4.2.4.1- The effect of SYNV infection in *N. glutinosa* total chlorophyll:

Chlorophyll levels were measured as described previously for *N. edwardsonii* (section 2.7.3).

Analysis of variance of the total chlorophyll data (table 4.3) showed a highly significant ($p = 0.01$) effect of virus infection. The reduction or loss of chlorophyll was apparent at 15 days after inoculation where total chlorophyll levels in infected plants were already less than in healthy plants of the same age. Later, the effects of virus infection became very clear, showing a large and significant differences 35 days after inoculation. The differences between control and infected plants was less marked at 55 days after inoculation, but still the effect was significant as shown in figure (4.14a). As with *N. edwardsonii,* it appears that virus infection may speed up the onset of senescence.

Table 4.3: The levels of chlorophyll in healthy and infected *N. glutinosa.*

Stages	Total	%	Chl*a*	%	Chl*b*	%
15 days						
Control	293		220		73	
Infected	261	89	197	89.5	64	87.7
35 days						
Control	319		236		83	
Infected	168	52.7	125	52.9	43	51.8
55 days						
Control	258		206		73	
Infected	188	72.9	136	66	52	71

All values are the mean of five measurements, (g/m^2 of leaf). Percentage are expressed as % of control.

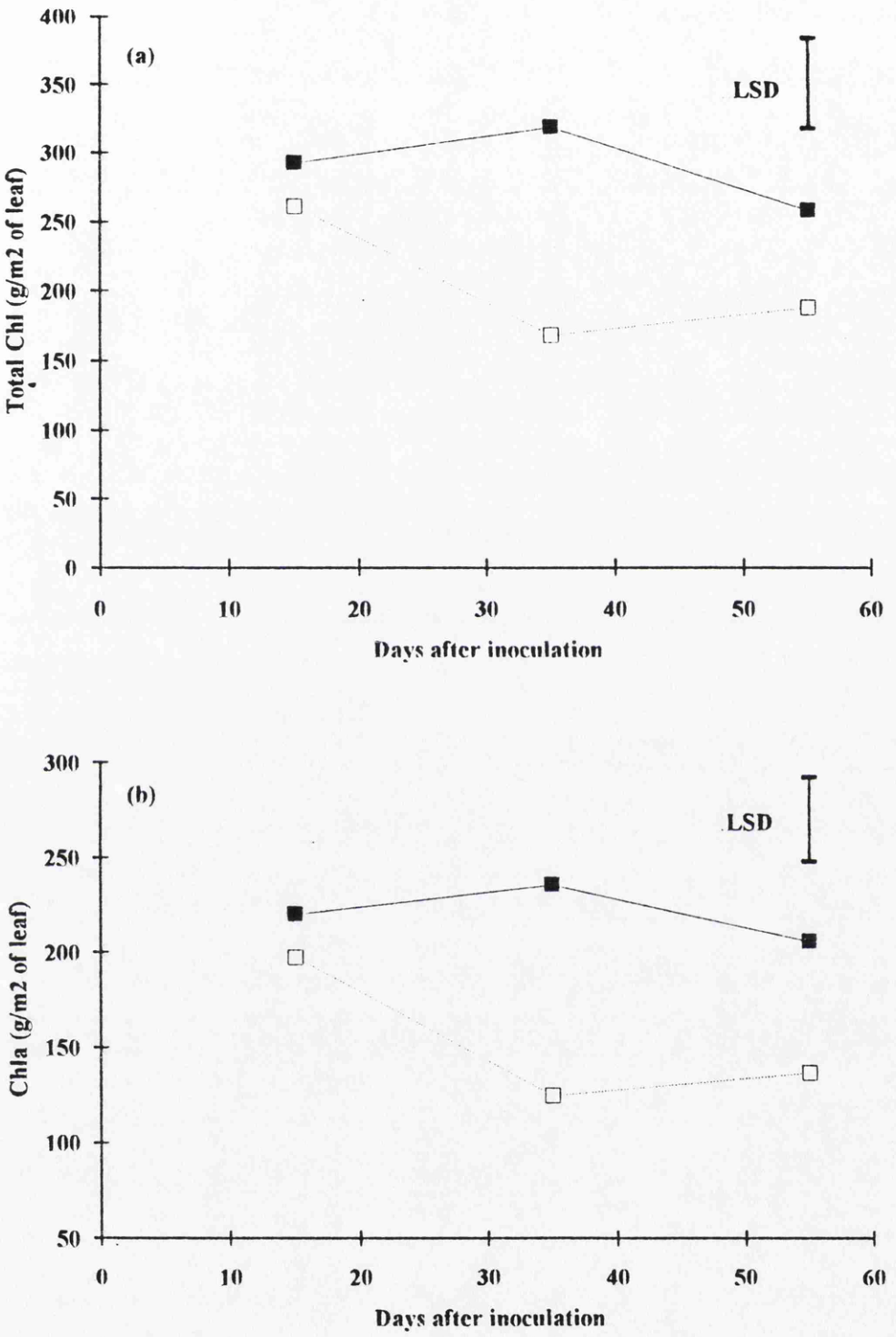


Figure 4.14: The effect of SYNV infection on total chlorophyll (a) (LSD = 65 gram of total Chl/m2 of leaf) and chlorophyll *a* (b) (LSD = 44 gram of Chl*a* /m of leaf) in *N. glutinosa* plants (Dark box, control; white box, infected; each data point represents the average of 5 separate determinations)

.2. .2- The effect of SYNV infection in *N. glutinosa* chlorophyll *a* and *b*:

The infection-induced decrease in chlorophyll *a* levels were significant from 35 days onwards, where very severe symptoms were apparent (figure 4.14b).

The effect of SYNV infection on chlorophyll *b* levels were as severe as those observed on chlorophyll *a*. The effect of infection was apparent 35 days after inoculation where the maximum differences between control and infected plants were evident, and was very highly significant ($p = 0.01$). These differences presisted to 55 days after inoculation (figure 4.15a).

The analysis of the effect of infection on chlorophyll *a* and *b* levels were both highly significant. To determine if these effects were more significant for chlorophyll *a* than chlorophyll *b*, further analysis was conducted on the ratio of chlorophyll *a* to chlorophyll *b*. The analysis showed a small apparent decrease in the ratio of both control and infected plants. The results suggest that infection produced a similar decrease in both chlorophyll species (figure 4.15b).

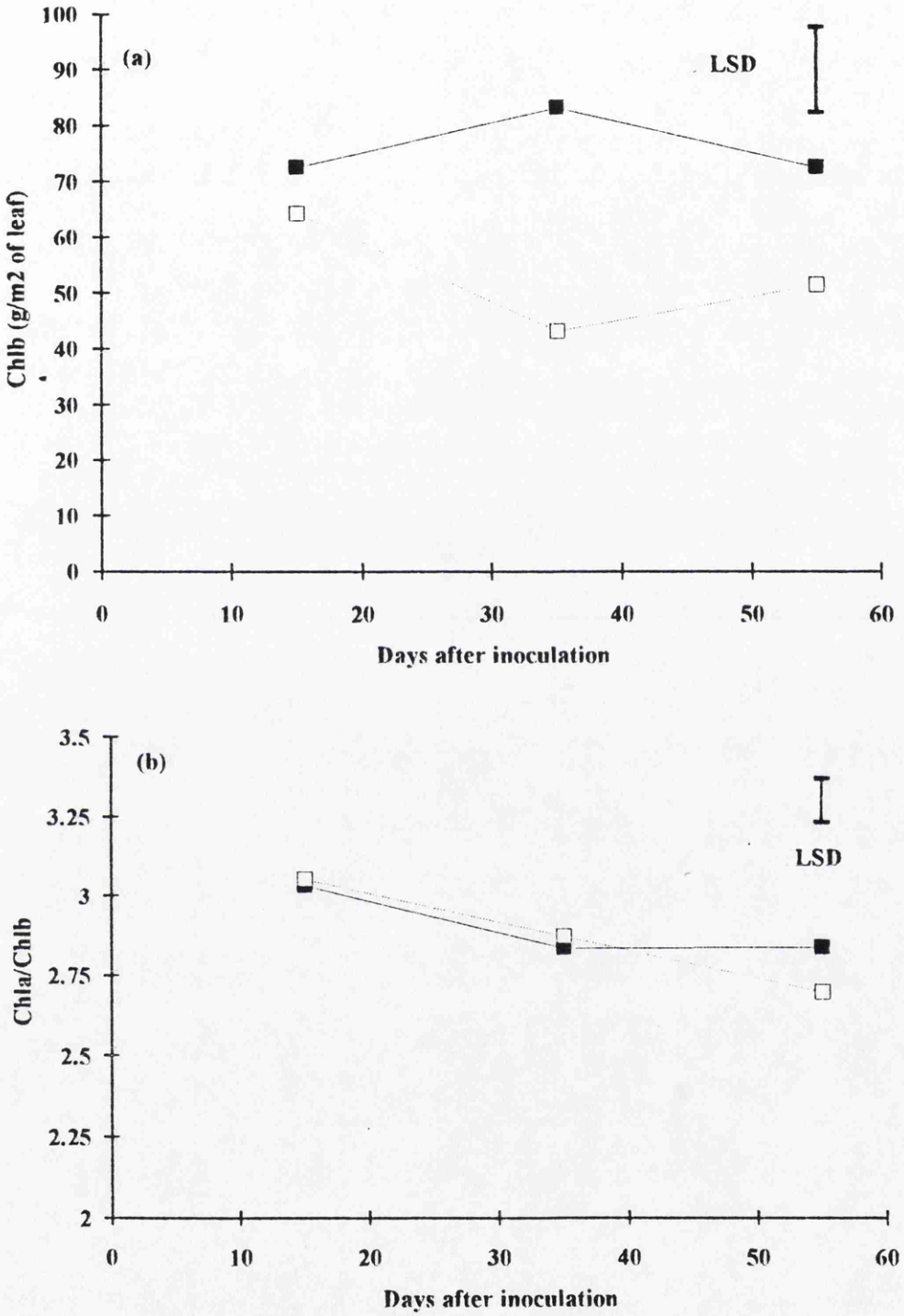


Figure 4.15: The effect of SYNV infection on chlorophyll *b* (a) (LSD = 15 gram Chl*b*/m2 of leaf) and in the ratio of chlorophyll *a* to *b* (b) (LSD = 0.14) in infected (white box) and healthy (dark box) *N. glutinosa* plants. (Each point data represents the average of 5 separate determinations.).

DISCUSSION

One important factor limiting studies of plant rhabdoviruses has been the difficulty of devising simple and reproducible purification protocols suitable for recovery of adequate virus of sufficient purity for biochemical analysis. The concentration of rhabdoviruses in infected plants has been reported to be lower than that of many other plant viruses (Francki, 1972; Jackson, *et. al.*, 1987), although the results of Ismail *et. al.* and those presented in this thesis suggest that SYNV and particularly EMDV can accumulate at moderately high levels in infected plants. Sonchus yellow net virus (SYNV) has been the most extensively studied plant rhabdovirus and was one of the first to be purified with a reasonable yield. SYNV was first purified by Jackson and Christie (1977) and the same method was used in this research for purification of SYNV. The method of Jackson and Christie was also used to purify EMDV since no previously published method had been reported to give satisfaction purity and yield. Using this method, EMDV was recovered in larger amount (18-27 μg/g leaf) than SYNV (12-16 μg/g leaf). The thickness of the celite pads used for filtration was the critical factor for both purity and quantity of the virus recovered at the end. The thickness of celite pad was also reported to be an important factor for the purification of SYNV (Jackson, *et. al.*, 1987).

In the case of EMDV, celite pads thicker than 2.50 mm seriously diminished yield of the virus, but pads less than 2.00 mm thick resulted in considerable contamination with chloroplast fragments. Attempts to purify EMDV by various methods have been reported. Russo and Martelli (1973) purified EMDV using a method similar to that described by Peters and Kitajima for Sowthistle yellow vein virus (SYVV) purification. EMDV was recovered in a reasonable amount but the infectivity of the virus was lost during the early stages of purification. Adam, *et. al.*, (1987) used partly purified EMDV in his studies. The infectivity of the virus was maintained but the virus preparation contained

large amounts of host contaminants. In the work described in this thesis using the Jackson and Christie (1977) method for EMDV purification, the purified virus maintained its infectivity even after storage in liquid nitrogen. Maintaining infectivity of the virus after storage in liquid nitrogen could be related to the use of magnesium acetate, manganese chloride and sodium sulphite in the maintenance buffer, the presence of which have been reported to conserve the envelope of SYNV during storage.

All the plant rhabdoviruses which have been investigated contain a number of structural polypeptides which can be separated by polyacrylamide-gel electrophoresis. Purified SYNV and EMDV proteins were separated by polyacrylamide gel electrophoresis; both showed four bands corresponding to the four major proteins. Both SYNV and EMDV virions contain what were originally believed to be two membrane matrix (M) proteins (M1 and M2) in addition to the glycosylated (G) protein and the nucleocapsid (N) protein. Both EMDV and SYNV have been assigned to the type II subgroup of plant rhabdoviruses (Peters, 1981; Russo and Martelli, 1973). This group appears to resemble the lyssovirus subgroup of animal rhabdoviruses (Sokol, *et. al.*, 1971). Studies on the location and function of the two membrane proteins of the type lyssovirus rabies, suggested that the M2 of rabies virus and M protein of VSV have a similar location associated with the viral envelope and an analogous function, while M1 protein of rabies virus appears to be similar to the VSV non-structural protein (NS) which is associated with the nucleocapsid and functions as part of the replicase complex (Jackson, *et. al.*, 1987). The G and M2 proteins of rabies virus are both localised in the surface of the infected cell while the NS and M1 proteins are located within the interior of infected cells (Cox, *et. al.*, 1981). This Cox's study, on the location of M proteins of rabies virus, led to reconsideration of the location and function of M1 and M2 proteins of type II plant rhabdoviruses. Recent work on SYNV M suggested that M2 protein was associated with nucleocapsid and therefore correlated with NS and M1 of VSV and rabies viruses

respectively (Jones and Jackson, 1990; Cox, *et. al.*, 1981). Moreover, the genes for M2 protein of SYNV, the M1 protein of rabies and the NS protein of VSV are all located adjacent to the N gene near the 3' end of the genome (Cox *et. at.*, 1981). This suggests that in SYNV, M2 fulfils a role similar to M1 in rabies and NS in VSV. In the light of these considerations, it well be necessary to reconsider the location and function of M1 and M2 of EMDV. It seems premature to assign M1 or M2 proteins of EMDV to either envelope membrane or to the nucleoprotein. It has been suggested that all rhabdoviruses with only one M protein are assembled and distributed in the cytoplasm whereas those which contain both M1 and M2 proteins are largely confined to the perinuclear space of the infected cells. In this study, both SYNV and EMDV virus particles were observed in abundance in the perinuclear space of infected plant cells this confirms that both viruses are indeed members of subgroup II of the plant rhabdoviruses.

Infectivity assays have shown that for several rhabdoviruses, virus levels in infected tissues initially increase, reach a maximum, and then decline (Jackson and Christie, 1977; Francki and Randles, 1980; Ismail, *et. al.*, 1987). Using ELISA to determine the virus levels in infected leaf hosts (*N. glutinosa* and *N. edwardsonii*), levels of SYNV virus antigen rose within infected *N. glutinosa* and *N. edwardsonii* unexpanded leaves between 5 and 15 days after inoculation. During this period, the virus appears to multiply and spread rapidly, as indicated by the concentration of levels of virus antigen, and reached its maximum at 15 days post-inoculation. After 15 days post-inoculation, most of the infected cells, if not all, contained large numbers of virus particles. This time period coincided with the increase of symptoms severity in unexpanded leaves and the appearance of typical symptoms of vein-clearing in all unexpanded leaves in both infected hosts. During this phase of rapid virus synthesis and spread (5-15 days), large areas of viroplasm, containing scattered patches of granular matrix were observed within infected cells. These areas of viroplasm appear to be involved in the

synthesis, replication or assembly of the nucleocapsids as in immunogold labelling experiments, a great many gold particles reacted strongly to such regions (Ismail, 1988).

Following the peak of virus concentration 15 days after inoculation, the level of virus antigen declined in unexpanded leaves in both hosts. This fall in virus antigen concentration in both hosts was accompanied by a reduction in numbers of virus particles per cell (as judged from electron microscope) and the beginning of a change in the location of virus particles observed in the electron microscope from nuclei to the membrane bound sacs in the cytoplasm. This change in location has been reported to suggest that the virus in the nucleus was either degraded or that virus inclusions "moved" into the cytoplasm. Type II rhabdoviruses are believed to assemble in the nucleus (Francki and Randles, 1980; Peters, 1981; Jackson, *et. al.*, 1987; Jackson and Christie, 1977; Van Beek, *et. al.*, 1985b) In this study, SYNV particles were consistently observed scattered in the cytoplasm within membrane-bound sacs during the later stage of infection. Similar observations were reported previously in *N. edwardsonii* plants infected with SYNV (Ismail, *et. al.*, 1987; Ismail, 1988) and with other type II plant rhabdoviruses, including WSMV (Sinha, 1971) and clover enation mosaic virus (Vela and Rubio-Huertos, 1974).

After 30 days post-inoculation, the virus levels in infected *N. edwardsonii* and *N. glutinosa* reached a plateau and declined only very slowly thereafter. The decline in SYNV antigen levels in various tissues of infected *N. edwardsonii* plants has been previously reported (Jackson and Christie, 1977; Ismail, *et. al.*, 1987). During this stage, the decline in virus concentration was accompanied by the appearance of yellow mottled spots in unexpanded leaves in *N. glutinosa* and a few *N. edwardsonii* plants whereas most *N. edwardsonii* plants started to show signs of recovery.

In general, *N. glutinosa* plants appeared to be the more susceptible host to SYNV infection. Not only did the virus replicate and accumulate to greater levels but the symptoms (especially stunting) were more severe than in *N. edwardsonii* (see figure 3.5a). Although virus antigen levels rose in a comparable manner in both *N. edwardsonii* and *N. glutinosa* over the period 5-15 days, antigen levels were consistently two to three fold greater in the latter host. Since electron microscopy studies indicated that in leaf tissue, at least, most cells became infected in both hosts, this suggests that *N. glutinosa* cells may support a higher level of SYNV replication than cells of *N. edwardsonii.* The greater severity of symptom expression in *N. glutinosa* might reflect a greater ability to support SYNV replication.

EMDV caused more severe infection in *N. edwardsonii* than did SYNV in the same host. The concentration of EMDV antigen in *N. edwardsonii* was much higher than SYNV and symptoms were much more severe. The concentration of virus antigen rose during the first two weeks and reached a maximum after 3 weeks at which time the infected plants showed severe symptoms. The maximum levels of virus concentration correlated with the appearance of large numbers of virions in almost all cells of infected leaves. After 4 weeks, the virus antigen levels dropped sharply reaching a plateau and continuing up to 24 weeks (168 days after inoculation). However, the levels of EMDV antigen were always greater than levels of SYNV in the same host. Possibly the sharp decline in virus antigen levels from 4 weeks on wards might reflect a difficulty in experiments. By 6 weeks post-inoculation, the most severely infected plants were dead or dying, surviving plants; sampled after this time therefore would have been the least severely infected at the earlier stage.

External symptoms, which occur as a host plant response to virus infection, are very varied and the factors controlling the nature and occurrence of symptoms include, type and strain of virus, type and variety of host plant,

physiology of the host, the presence of other viruses and pathogens and environmental and climatic conditions. The symptoms of plant rhabdoviruses in their hosts are varied, ranging from stunting to chlorotic spots, strips or streaks, mottling, vein-clearing and yellowing (Jackson, 1979). The severity of SYNV and EMDV symptoms depended to a great extent on the age of the plants at the time of inoculation. This phenomena has been observed and documented for other plant rhabdovirus such as rice transitory yellowing virus (RTYV) (Chiu, *et. al.*, 1968). In this study, the appearance of symptoms of SYNV and EMDV demonstrated very clearly that the severity of the symptoms depended not only on the type of the virus but differed between two closely related hosts. Since *N. edwardsonii* is a hybrid of *N. glutinosa* and *N. clevelandii*, it must share its genes with *N. glutinosa*. Nevertheless it supported less virus replication, showed little or no stunting and recovered better from SYNV infection. An increase in severity of symptoms has been reported to be associated with increased virus production (Matthews, 1991). Studies on photosynthetic function in the two hosts suggested further differences in the effects of SYNV infection. There are discussed in detail later. Although studies were not carried out in such detail comparing EMDV infection in *N. glutinosa* and *N. edwardsonii*, the latter host appeared to survive infection better, suggesting that it again may be more tolerant to this virus.

EMDV is a member of type group II of plant rhabdoviruses, first reported from southern Italy causing severe stunting to eggplant accompanied by pronounced mottling and crinkling of the leaves and general unfruitfulness (Martelli, 1969, 1973). The virus has not been considered to be of economic importance, but recent reports from different countries in the Mediterranean region and Middle East, demonstrated that the virus which is widespread and has several other hosts than eggplant may indeed be of economic importance (Lockhart, 1987; Martelli and Hamadi, 1986; El-Maataoui, *et. al.*, 1985; Danesh and Lockhart, 1989; Cherif and Martelli, 1985). EMDV is a vigorous virus compared to SYNV, causing severe symptoms in the hosts used in this study. The

severity of the symptoms were increased in infected plants with time in spite the decline in the virus antigen at later stage of infection. The increase of symptoms severity of EMDV were similar to the most of other plant rhabdoviruses studied. Study with potato yellow dwarf virus (PYDV), a type II plant rhabdovirus induces a leaf chlorosis, stunting and reduced tuber production. These symptoms are increased and persisted in infected plants (Falk, *et. al.*, 1981; Wagner, 1987). As with other plant rhabdoviruses, the severity of EMDV symptoms were dependent to a great extent on the age of the plants at the time of inoculation. This was observed with *N. edwardsonii* infected with EMDV, where if the plants inoculated at an early stage (4-6 leaves), the plants developed severe symptoms and failed to survive for more than 6 weeks whilst if the plants were inoculated at a stage of growth with more than 10 leaves, symptoms were mild and plants could survived for longer than 10 weeks.

The symptoms of EMDV were more severe than those caused by SYNV. Both caused vein-clearing, mottling of leaves in systemically infected leaves, stunting and often local lesions in inoculated leaves. The symptoms in the three hosts studied were similar in their appearance but differed in severity, with very severe symptoms in *N. clevelandii*, less in *N. glutinosa* and least in *N. edwardsonii* plants. However, the severity and the time of symptom appearance were heavily dependent on the age of the plants at the time of inoculation. It seems the differences in symptom expression in the three hosts depend on the physiology of the host. Similar observations on EMDV symptoms were reported for tomato, eggplant and *Nicotiana spp.* in the Mediterranean region (Martelli, 1969; Cherif and Martelli, 1985; El-Maataoui, *et. al.*, 1985; Martelli and Hamadi, 1986). Although, the symptoms observed in tomato grown in Morocco were slightly different from those observed in eggplant and it was thought at first that the virus was a new plant rhabdovirus, the virus was proven later to be a strain of EMDV (Adam, *et. al.*, 1987) and was later reported in Italy, causing the same symptoms in tomato (Castellano and Martelli, 1987). Recent reports on the

occurrence of EMDV, suggest that the virus induces different symptoms in different hosts and has a wide range of hosts including some varieties of potato where the virus has been reported in the field. It is believed to be passed on through infected tubers (Danesh and Lockhart, 1989).

The morphology and the structure of SYNV virus particles have been extensively studied and documented (Jackson and Christie, 1977; Jackson, *et. al.*, 1987; Ismail, *et. al.*, 1987; Ismail, 1988). The results in chapter 3 confirmed that SYNV has typical bacilliform particles, ranging from 200-220 nm. Mature virus particles were observed assembled in the perinuclear space in large numbers. Two shapes of virus particles were observed in the same cell, those with bacilliform shape rounded at both ends and which resemble the mature virus particles and bullet-shaped which were often seen still connected with the inner nuclear membrane by the base. These may be in the process of budding. Similar observation have been reported for SYNV and other type II plant rhabdoviruses (Ismail and Milner, 1988; Martelli and Castellano, 1970). In the late stages of infection (6 weeks), generation of short particles (160-180 nm) with two rounded ends were observed. These short particles have been reported to be defective interfering (DI) particles (Ismail and Milner, 1988), and they are discussed in detail later.

Two morphologically distinct types of EMDV virion were also observed. Bacilliform and bullet-shape virus particles were seen in sections taken at different time after inoculation from infected leaves. These virions often occurred in massive aggregates in the nuclei and accumulated in the perinuclear space. The bacilliform virions ranged from 230-250 nm and were relatively uniform, while the bullet-shape virions which were still connected to the inner nuclear membrane had varying lengths. The internal structure of the virions seemed similar to those described for other type II plant rhabdoviruses, (Christie and Jackson, 1977; Jackson, *et. al.*, 1987). Cross sections revealed two concentric

electron opaque rings separated by an electron clear gap and a lucent canal in the centre. These observations suggest that the outer ring represents the envelope and its outer projections while the inner ring represents the nucleoproteins. It has been suggested that the inner canal in the centre of the virion could contain the helical structure of RNA (Martelli and Castellano, 1970). Similar observations were reported with EMDV infected tomato and eggplants (Martelli and Castellano, 1970; Russo and Martelli, 1973). Differences in the measurement of virion sizes and some differences in internal structure of the virion could be due to the different procedures used to fix and stain the sections (Martelli and Castellano, 1970; Francki and Peters, 1981).

Cytopathological changes in infected plant cells caused by plant rhabdoviruses have been well documented (Francki and Randles, 1980). SYNV has received the most attention (Ismail, 1988; Ismail and Milner, 1988; Jones and Jackson, 1990). The involvement of the nucleus in the assembly and maturation of plant rhabdoviruses has also been well documented (Wolanski and Chambers, 1971; Jones and Jackson, 1990). SYNV, a type II plant rhabdovirus was assembled and matured in the nucleus. Ultrastructural changes in the nucleus caused by SYNV have been reported, starting 5 days after inoculation (Ismail, 1988). Similar changes were also observed in this study and included swelling of the nuclei, alterations of chromatin distribution and enlargement of the perinuclear space and these changes appeared from 3 weeks post-inoculation in *N. edwardsonii*. The ultrastructural changes continued with the progress of the infection and included the development of viroplasm regions in the nucleus and cytoplasmic invaginations into the nuclei. Similar affects were seen earlier (3 weeks after inoculation) in infected leaves showing local lesions from *N. glutinosa* and *N. clevelandii*. Cytoplasmic and nuclear invaginations have been reported in *N. edwardsonii* infected with SYNV (Ismail, 1988) and briefly in other rhabdovirus-infected plants, although, it has been suggested that they may occur generally in plant rhabdovirus infections (Martelli and Russo, 1977). In this

study, cytoplasmic invaginations occur in all three species infected with SYNV and in EMDV infected cells, suggesting that cytoplasmic invaginations are characteristic of rhabdovirus infection, regardless of the host. The mechanism of the development of the invaginations is not entirely clear, but evidently they involve the invagination of the nuclear envelope into the nucleus. Maturation and budding of the nucleocapsids through the inner nuclear membrane may then result in the separation of the inner and outer nuclear membrane and in filling of the invagination, forming an inclusion body. These steps can be seen in the electron micrographs in figures 3.12, 3.16 and in the case of EMDV figure 3.34. The enlargement of the perinuclear space has been widely reported in plants infected with type II plant rhabdoviruses, and is believed to take place as a result of the rapid multiplication and maturation of the virus (Francki, *et al.*, 1981; Jackson, *et. al.*, 1987). Alterations in chromatin distribution have been reported for several type II plant rhabdoviruses e.g. EMDV, SYNV and PYDV (Martelli and Russo, 1977; Lee and Peters, 1972; Lin, *et. al.*, 1987; Ismail and Milner, 1988; Ismail, 1988). Similar effects have been observed in this study; chromatin was distributed near the periphery of the nucleus with the interior filled with viroplasm. In addition, in cells from chronically infected plants, even where virus particles were not seen, changes in distribution of chromatin were still observed. These alterations were not seen in healthy control plants of the same age. Ultrastructural changes in both hosts became more striking and widespread over the 6 weeks following inoculation. Later in infection, virus particles were increasingly seen in the cytoplasm surrounded by membrane. It seems that the virus particles replicate and assemble in the perinuclear space and are transported to the cytoplasm at the same time as the cells show increasing ultrastructural changes in the nucleus and in other organelles. Similar observations have been reported in *N. edwardsonii* infected with SYNV (Ismail, 1988).

One of the most interesting points in *N. edwardsonii* plants infected with SYNV, was the observation of nucleocapsids or morphologically abnormal

particle in the nuclei during the late stage of infection (over 20 weeks after inoculation). Enveloped virus particles were not seen in all cells examined. These observations coincided with the appearance of the recovery stage from a severe symptoms. These results suggest that the virus was unable to assemble properly, possibly due to a build-up of "virus inhibitors" at the late stages of infection, a breakdown of virus *in situ,* or its inactivation.

Immunogold labelling using antibodies raised against total SYNV proteins, Ismail (1988) showed that, at the early stages of infection, large number of gold particles reacted strongly to the viroplasm region in the nucleus as well as to the virus particles. In results presented in chapter three, the same technique was used to label *N. edwardsonii* cells 20 weeks after inoculation. Unformed virus-like particles were seen in vesicles associated with the nucleus. These reacted very strongly to antibodies raised against whole virus and purified G-protein. Few gold particles were seen in the viroplasm. These results suggest that the vesicles contain a large amount of virus antigen despite their morphology, the virus-like particles present within these vesicles must be at least in part enveloped since they reacted to anti-G antibodies. The reactions of antigen to the viroplasm, may suggest that at the late stage of infection few virus proteins were still present in the viroplasm. Antibodies to whole virus proteins and to G protein did not react to the cytoplasm nor to any other organelles such as chloroplasts. These results suggest that G protein not associated with virions was not present in the cell in large amounts.

The development of vesicles or unformed virus-like particles were rare in infected *N. glutinosa* 20 weeks post-inoculation and in most cases were mixed with standard virus particles. This was the most cytopathological difference between the two hosts in their responce to the SYNV infection at the later stages of infection. The rapid development of changes in infected *N. glutinosa*, may related to the physiology of the plant.

By 4 weeks post-inoculation, both *N. edwardsonii* and *N. glutinosa* showed alterations in chloroplast morphology. Chloroplasts in infected cells became enlarged, developed large areas of starch and showed disrupted and reduced thylakoids. This was reflected in the decline in total chlorophyll in infected leaves, and as discussed below, in the effect on photosynthesis. Ultrastructural changes in chloroplasts of infected plants have been reported with rhabdoviruses as well as a variety of other plant viruses, such as turnip yellow mosaic virus (TYMV) (Hatta and Matthews, 1974), and tomato spotted wilt virus (Mohamed, 1973). Changes in the chloroplasts increased with the progress of the infection, starch took up a large proportion of the chloroplast and a further reduction in the number of thylakoids was evident. In the later stages of infection, chloroplasts developed electron dense materials in the centre. The contents and the mechanism of the development of these are not known, but it was clear that they were observed only in the infected cell after about 20 weeks post-inoculation. A study with *N. edwardsonii* plants infected with SYNV (Ismail, 1988) demonstrated that anti-SYNV antibodies produced against whole virus proteins bound strongly to the thylakoids and grana of the chloroplast. Moreover, western blots of chloroplasts purified from infected plants showed the presence of an immunologically reactive protein co-migrating with the SYNV-N protein. These studies suggest a possible direct involvement of virus protein most probably the N protein with the chloroplast. However, the identity of the electron dense material is still unclear and must be the subject of further investigation. Chloroplasts from *N. glutinosa* infected cells with SYNV showed similar changes to those of *N. edwardsonii*. However, these changes in *N. glutinosa* appeared earlier than in infected *N. edwardsonii*.

Infection by EMDV appears to induce severe cytological changes in cells from systemically infected leaves in the three *Nicotiana spp.* used in this study. From the results presented in chapter three, it became clear that the cytological changes in three hosts were different and that differences were apparent as early

as 3-4 weeks after inoculation. The major changes occurred in the nuclei, which showed in most cases, drastic reductions in chromatin material and large swellings of the perinuclear space due to virus accumulation. In the first 3-4 weeks after inoculation, other organelles appear unaffected. With the progress of the infection, after 6 weeks, other organelles started to show abnormalities in their structure and shape. Chloroplasts were disrupted with thylakoids reduced in number, and starch grain development. Similar observations have been reported with EMDV infected eggplant (Martelli and Castellano, 1970; Russo and Martelli, 1972). Local lesions were often observed in inoculated leaves of *N. glutinosa* and *N. edwardsonii*. The changes were more pronounced in *N. glutinosa* than in *N. edwardsonii*. These observations of symptom expression and cytopathological changes in infected *N. glutinosa* and *N. edwardsonii* plants, support the view that *N. glutinosa* is the more susceptible to EMDV infection and that this could be related to physiological differences between the two hosts.

Various factors may influence the development and severity of symptoms in virus infected plants. In addition to the genetical composition of the plant, the age of the host plant at the time of infection can be a critical factor in determining symptom expression. In general, the younger the plant, the more susceptible it is to virus infection, and very old leaves, or old plants are usually relatively resistant to infection. This was demonstrated with plants infected with both plant rhabdoviruses and other plant viruses such as cucumber mosaic virus (CMV) (Francki and Randles, 1980; Walkey and Pink, 1984; Walkey, 1985). A similar effect is observed with EMDV infected *N. edwardsonii* and *N. glutinosa*. In plants inoculated at the 4-6 leaf stage, the EMDV infection developed was very severe and infected plants would not survive for longer than 6-7 weeks after inoculation. Late infected plants (>10 leaves in plant) survived for more than 20 weeks and further examination of the infected cells and the behaviour of the virus were possible. It has been suggested that one of the reasons for the greater tolerance of plants to infection when inoculated at late stage, is that the virus is

completely dependent upon the host cells for its multiplication, and in older leaves the transport of assimilates and metabolism, is slower than in younger leaves (Walkey, 1985).

By 6 weeks after inoculation, abnormalities in infected cells were very evident. Virus particles were seen in the cytoplasm, and the chloroplasts developed a substantial area of electron-dense material in addition to starch accumulation and reduction of the grana. However, fewer virus particles were generally seen in most infected cells. The reduction in virus particle number was correlated with the decrease in virus concentration in infected leaves. These have been observed with other plant rhabdoviruses and may be the case with all plant rhabdoviruses (Ismail, *et. al.*, 1987; Ismail and Milner, 1988; Jackson, *et. al.*, 1987). Cytoplasmic invaginations were also evident in infected cells. These invaginations were often seen in expanded leaves showing no symptoms 9 weeks post-inoculation and in green areas from unexpanded leaves showing chlorotic lesions from late infected plants. The mechanism(s) of cytoplasmic invaginations are not known but they have been observed in SYNV-infected plants (chapter 3). Previous studies on cytopathological effects of EMDV have not considered the various stages during the infection period. Similarities in the cytopathological changes and to some extend in the appearance of symptoms in the early stages of infection (3-4 weeks) between EMDV and SYNV in infected cells are clear.

Immunogold labelled sections taken from EMDV-infected *N. glutinosa* 5 weeks after inoculation, and using anti-EMDV serum showed that the only regions where the gold particles bound were virus particles in the perinuclear space areas of viroplasm within the nucleus. No gold particles were observed elsewhere within the infected cells. In particular, in contrast to the results reported for SYNV infected plants by Ismail (1988) no antigen could be detected associated with chloroplast or the plasma membrane. These results suggest that the site of synthesis of virus proteins is within the nuclei and that nucleocapsids

then bud into the perinuclear space. This was clear in sections (figure 3.30a & b) where gold particles can be seen ot be almost equally distributed between the virus particles and the pernuclear inclusion of viroplasm. Similar pattern of binding were seen in sections of EMDV-infected *N. glutinosa* 23 weeks post-inoculation although fewer gold particles were bound to the viroplasm; presumably the synthesis of virus antigen at this late stage of infection was reduced. This was also supported by the fewer numbers of virus particles observed in the infected nuclei (as judged by electron microscopy) compared to the earlier stages of infection. Even at late stages of infection, virus particles and virus antigen consistently appeared in the perinuclear space and in the viroplasms within nuclei but not elsewhere.

In general, fewer gold particles bound to sections from EMDV-infected *N. glutinosa* 5 and 23 weeks post-inoculation compared to immunogold labelled sections from SYNV-infected plants. The overall level of EMDV antigen, as measured by ELISA in infected plants was several times greater than SYNV antigen in comparable plants (see figure 3.3) but presumably reflects differences in the titres of the antisera rather than levels of antigen within the cells.

Ismail (1988) had previously shown that proteins from infected *N. edwardsonii* (75 days after inoculation) co-migrated with two of the structural proteins of SYNV (G & N) and the third antigenic band of molecular weight 41kD could be detected in chronically infected plants. Experiments designed to determine the levels of virus proteins in infected *N. glutinosa* at different times during the infection period were carried out and in contrast to *N. edwardsonii,* three major proteins were detectable in infected leaves from 5 days up to 65 days after inoculation. The results indicated that levels of the major structural proteins reached a maximum and then declined gradually with increasing time after inoculation. In contrast, Ismail (1988) showed that in *N. edwardsonii*, levels of M1 and M2 proteins declined much more rapidly than levels of G and N proteins.

This is a further evidence of differences in the response of the two hosts to SYNV infection. It is also of interest that at 15 days post-inoculation, the virus infection had not reached its maximum acute phase, as redlected in symptom expression and cytopathological changes. Possibly at 15 days, large amounts of unassembled proteins were present.

Similar experiments to determine the levels of EMDV proteins were carried out. The results, presented in chapter three, confirm that purified EMDV is clearly distinct virus from purified SYNV. The purity of the virus preparation was clear by the reaction of antiserum to the individual proteins of EMDV but not to the total proteins of controls. Immunoblotting has been used successfully to distinguish EMDV from other plant rhabdoviruses such as PYDV and also to identify tomato vein-yellowing virus (TVYV) as a strain of EMDV (Adam, *et. al.*, 1987). The levels of EMDV proteins detectable on western blots rose and fell as anticipated from the ELISA results. The individual virus proteins reacted to varying degrees. G and N gave strong bands whereas M2 reacted poorly and M1, although detectable in lanes containing purified virus was almost undetectable in total protein samples. These results may reflect differences in the antigenicity of the various proteins and their relative abundance in virions. However, like SYNV-infected *N. glutinosa* and in contrast to SYNV-infected *N. edwardsonii* (Ismail, 1988), the levels of G, N and M2 relative to each other did not obviously differ in samples taken early and late in infection.

Repeated passage of RNA viruses between hosts or cell cultures often results in the generation of defective or incomplete virus particles which have the same protein components as standard virus but their genomes differ from those of the original virus as a result of deletions (Ismail and Milner, 1988; Resende, *et. al.*, 1991). Typical defective interfering (DI) RNAs exhibiting the properties defined by Huang (1973) have been described for only a few plant viruses, tomato bushy stunt virus (Hillman, *et. al.*, 1987; Morris and Knorr, 1990; Knorr,

et. al., 1991), turnip crinkle virus (Li, *et. al.*, 1989; Cascone, *et. al.*, 1990), cymbidium ringspot virus (Burgyan, *et. al.*, 1989, 1991) and from the rhabdoviruses; potato yellow dwarf virus (PYDV) (Adam, *et. al.*, 1983) and SYNV (Ismail and Milner, 1988). SYNV was reported to generate defective interfering (DI) particles in the late stages of infection (over 3 months) in chronically infected *N. edwardsonii* (Ismail and Milner, 1988). In this study, the virus in infected *N. edwardsonii* and *N. clevelandii* plants was observed to generate short particles which have all the characteristics of DI particles. *N. clevelandii* plants infected with SYNV and containing short particles were investigated by Dr. M. McElwee. Sequencing studies of the genome of both short and standard particles showed that particles had a deletion of their genomic RNA. The results suggest that DI particles were produced in the late stages of infection (over 6 weeks after inoculation) and their numbers increased with age of infected plants It is also became clear that the appearance of DI particles was associated with the change in symptoms. Whether the change in symptoms was directly caused by the generation of DI particles is unclear. Similar observations have been reported with other plant viruses such as tomato spotted wilt virus (TSWV) (Resende, *et. al.*, 1991). However, in *N. glutinosa*, the appearance of short particles was very rare compared to the other two hosts. Possibly the ability of this host to support greater replication mitigates against the generation of DI particles.

In EMDV infected of both *N. glutinosa* and *N. edwardsonii* plants showing chlorotic lesions, short bacilliform particles were seen 7 weeks post-inoculation. Although similar observations have been reported in *N. edwardsonii* chronically infected with SYNV and later documented as DI particles (Ismail and Milner, 1988; McElwee and Milner, unpublished results), it is premature to conclude that the short particles of EMDV are DI particles, based solely on the length and the correlation with the appearance of chlorotic symptoms.

Several reports have demonstrated that virus infection reduces photosynthesis rates; many of these refer to the effects of virus infection on herbaceous plants. These include barley yellow dwarf virus in barley (Jensen, 1968), and in wheat (Jensen, 1972), maize dwarf mosaic virus in maize (Tu and Ford, 1968), sugar beet yellows virus in sugar beets (Hall and Looms, 1972a, b), and tomato aspermy virus in tomato (Hunter and Pest, 1973). All these authors found reduction in photosynthesis in infected leaves, which Matthews (1970) suggested was a secondary consequence of infection, occurring sometime after virus multiplication, when symptom expression was well advanced.

In this study, two models have been used which describe photosynthesis rates at different light intensities, the rectangular hyperbola of Rabinowitch (1951) and the non-rectangular hyperbola of Marshall and Biscoe (1980). Although, the rectangular model is very well known and widely used for the description of photosynthesis rates by oxygen electrodes, the non-rectangular hyperbola model gave a consistently better fit to the data. The residual errors of the fitted data of most, if not all, cases (total of 70 leaf-discs) were consistently higher when the rectangular model was used. Furthermore, the non-rectangular model describes more parameters of photosynthesis rates than the rectangular model as described in chapter two (section 2.7.3.1).

The results presented in chapter four show the effects of SYNV infection on photosynthetic rates of *N. edwardsonii* and *N. glutinosa*, and suggests that the effect of virus infection depends to a great extent, on the host and the severity of the symptoms during the infection period.

In *N. edwardsonii*, the amount of chlorophyll in infected plants declined rapidly after 30 days post-inoculation. Infection appeared to accelerate the normal ageing process which is observed in the control plants. To establish whether the effect of virus infection was primarily on chlorophyll *a* or in *b*, measurements were taken for each chlorophyll species at the same time as the

measurements of photosynthesis rates. The results presented in chapter four, suggest that the effects of infection were similar for total chlorophyll, chlorophyll *a* and chlorophyll *b*. However, the major effect of infection appears to result in an increase in the chlorophyll *a/b* ratio at 40 days post-inoculation suggesting the accelerated loss of some reaction centres over LHC. However, as stated above these differences were not detected by direct measurement of Pgmax.

The decrease in the chlorophyll levels in infected plants might be expected to produce parallel decreases in gross photosynthesis rates. However, Pg of infected plants at high (Pgmax) light intensities and quantum efficiency (α) were not significantly different from controls. The former can be explained by considering that chlorophyll degradation reflects a loss of the light-harvesting complexes, but not the reaction centres. Therefore, at saturating light levels the rate of oxygen evolution of control and infected plants is similar. However, this would result in a lower efficiency of net photosynthesis (Pn) at low light levels (i.e. α) of infected plants, and this was not observed. Several workers have found difficulty in measuring differences in α determined from the sun and shade plants (Professor S. Long, University of Essex, personal communication; Dr. P. Dominy, University of Glasgow, personal communication). Apparently, gross losses in the light-harvesting capacity has to occur before significant changes in α can be observed.

Similar results on the effect of SYNV infection on chlorophyll levels of infected *N. glutinosa* plants were obtained. Infection was found to reduce total chlorophyll, chlorophyll *a* and chlorophyll *b* in parallel after 15 days post-inoculation. At first sight these results may suggest a direct correlation between the decline in chlorophyll levels and the decrease in the rate of net and gross photosynthesis rates in virus-infected *N. glutinosa* leaves. However, as for *N. edwardsonii*, no parallel changes in α were observed, and for *N. glutinosa*, and this suggests a loss of both LHC and reaction centres (i.e. a decrease in α and

Pgmax). Although infection did cause a decrease in Pgmax in *N. glutinosa*, no changes in α were observed (see above).

In the case of *N. edwardsonii*, infection does not significantly affect the gross or net photosynthesis rates. No difference in gross photosynthesis rates of infected plants suggests that chloroplast function is unaffected by infection. The analysis of the data of the quantum efficiency (α) of photosynthesis at low light intensities, which is an indication of the status of the light-harvesting complex (LHC), suggests that the light harvesting capacity was unaffected by infection. Similar results were reported for the effect of a strain of tomato bushy stunt virus and an isolate of cucumber mosaic virus on the chlorophyll content and photosynthetic rate of infected piggyback plant (*Tolmiea menziesii*; Platt, *et. al.*, 1979). The authors reported that although virus infection has a great effect on the levels of chlorophyll in infected plants, the photosynthetic rate of infected leaves was not significantly different from that of control plant leaves

In contrast, the photosynthesis rate of SYNV-infected *N. glutinosa* was significantly decrease compared to the rates of uninfected controls at 35 and 55 days post-inoculation. This decrease in photosynthetic rate was correlated with the severity of the vein-clearing symptoms. The effect of infection was very clear at saturating light intensities whereas, no significant differences of the quantum efficiency of photosynthesis (α) were observed. Further, it would appear that these infection-induced reductions are not associated with the light harvesting capacity of the photosynthetic apparatus, despite significant loss of chlorophyll, and presumably reflect changes in the efficiency of electron transport or photophosphorylation. Similar results were reported in a study on peanut green mosaic virus infected peanut leaves (Naidu, *et. al.*, 1984). The authors found that net photosynthesis was reduced over a range of light intensities, with the maximum reduction occurring at the highest light intensities. They concluded that

infection directly inhibited photosystem II as well reduced chlorophyll levels in the chloroplasts.

Conversely, the only major effect of SYNV infection *N. edwardsonii* net photosynthetic rates was due to increases in dark respiration rates (consumption of oxygen) of infected plants. The effect of SYNV infection on the dark respiration of *N. edwardsonii* appeared only in the acute phase of infection (maximum symptom expression at 25, 30 days after inoculation) which suggests that the mitochondria of infected plants, where the bulk of dark respiration occurs, were stimulated. Mitochondria from SYNV-infected *N. edwardsonii*, have been reported to show abnormalities, clumping, and loss of most of their cristae and some of their mitochondrial matrix (Ismail, 1988). However, at 40 days after inoculation, the infected plants had recovered and respiration rates decreased so that no significant differences with controls were apparent. This recovery of the respiration of infected plants correlated well with the disappearance of vein-clearing symptoms. An increase in dark respiration has been documented for many other virus-infected plants (Kosuge and Kimpel, 1981; Smith and Neales, 1977).

In contrast, the dark respiration rates of SYNV-infected *N. glutinosa* were not significantly different at any time post-inoculation, and further, it did not change with plant age. Similar results were reported on the effect of virus infection on the photosynthetic properties of peach leaves infected with viruses causing peach rosette and decline disease, where the dark respiration rate of young leaves was increased by virus infection, although the increase was not significant (Smith and Neales, 1977).

Virus infection could affect the activity of biochemical and physical resistance to CO_2 diffusion. Physical diffusion resistance, includes the boundary layer, stomata and mesophyll resistance all of which have a great effect on the photosynthesis rates occurring in the chloroplasts. Plant virus infection other than plant rhabdoviruses have been reported to increase the resistance to CO_2 diffusion (Smith and Neales, 1977; Jensen, 1971; Hall and Loomis, 1972). However, in this study, SYNV infection showed no significant effects on the physical or biochemical resistance (i.e. θ) to CO_2 fixation in infected *N. edwardsonii* and *N. glutinosa* plants.

As mentioned previously in chapter four, two factors influence both the gross photosynthesis rate (Pgmax) and theta (θ); these are the physical resistance (rp) and biochemical resistance (rx). Therefore, three possibilities could cause changes in Pgmax and θ (see table 4.2). The results suggest that SYNV infection may affect the physical resistance in infected *N. edwardsonii* and *N. glutinosa* plants.

It is difficult to reconcile some of the observations on photosynthesis made in this study with other published work (Delieu and Walker, 1981). The non-rectangular hyperbola model (Marshall and Biscoe, 1980) suggests that θ is a measure of the physical and biochemical resistance to CO_2 fixation. However, according to Delieu and Walker (1981), Walker, (1987), the CO_2 concentration in an O_2 electrode (1%) is sufficiently high that the physical resistance (rp) = 0. If this were true, $\theta = 0$ and the non-rectangular hyperbola model should degenerate into the rectangular hyperbola of Rabinowitch (1951) (see chapter two). However, in all of the experiments reported in this study, it was found that ($0<\theta<1$), suggesting that rp was significant and raising the possibility that the samples were CO_2-limited at high light. Alternatively, it is possible that 1% CO_2 does saturate photosynthesis of leaf discs in the O_2-electrode but that θ does not

reflect rp and rx (Leverenz, 1987 & 1988). This point is unresolved and awaits further clarification.

❖❖❖ ⌘ ❖❖❖

REFERENCES

Adam, G. (1982): Plant virus studies in insect vector cell culture, in: Vector in virus biology, edited by M. A. Mayo and K. A. Harrapp, pp. 37-62, Prathear, London.

Adam, G., Chagas, C. M. and Lesemann, D. E. (1987): Comparison of the three plant rhabdoviruses isolates by two different serological techniques. *Journal of Phytopathology.* **120 (1)**: 31-43.

Adam, G. and Gaedigk, K. (1986): Inhibition of PYDV infection in vector cell monolayers by lysosomotropic agents. *Journal of General Vrology.* 67: 2775-2780.

Adam, G. and Gaedigk, K. and Mundry, K. W. (1983): Alterations of plant rhabdoviruses during successive mechanical transfers. *Zeitschrift fur Pflanzenkrankheiten und Pflonzenschutz.* **90**: 28-35.

Adam, G. and Hsu, H. T. (1984): Comparison of structural proteins from two potato yellow dwarf viruses. *Journal of General Virology.* **65**: 991-994.

Ahmed, M. E., Sinha, R. C. and Hochester, R. M. (1970): Purification and some morphological characters of wheat striate mosaic virus. *Virology,* **41**: 768-771.

Almeida, J, D., Hawatson, A, F., Pinteric, L. and Fenje, P. (1962): Electron microscope observations on rabies virus by negative staining. *Virology.* **18:**147-

AL-musa, A. M. and Lockhart, B. (1990): Occurrence of eggplant mottled dwarf virus in Jordan. *Journal of Phytopathology.* (Berl). **128** (4): 283-287.

Amheiter, H. Davis, N. L., Wertz, G., Schubert, M. and Lazzarini, R. A. (1985): Role of the nucleocapsid protein in regulating vesicular stomatitis virus RNA synthesis. *Cell,* **41**: 259-267.

Atabekov, J. G. and Dorokhov, Yu, L. (1984): Plant virus-specific transport function and resistance of plants to viruses. *Advances in Virus Research.* Vol. **29**: 313-364.

Atabekov, J. G. and Morozov, S. Y. (1979): Translation of plant virus messenger RNAs. *Advances in Virus Research.* Vol. **25**: 1-91.

Bedbrook, J. R and Matthews, R. E. F. (1972): Changes in proportions of the early products of photosynthetic carbon fixation by TYMV infection. *Virology* **48**: 255-258

Bedbrook, J, R, and Matthews, R. E. F. (1973): Changes in the flow of early products of photosynthetic carbon fixation associated with the replication of TYMV. *Virology,* **53**: 84-91.

Beijerinck, M, W. (1898): Over een contagium vivum fluidum als oorzaak van de vlekziekte der tabaksbladen. *Versl Gewone Vergad Wis Natuurk. A fd. K. Akad. Wet.* Amsterdam. 7: 229-235.

Bell, J. C., and Prevec, L. (1985): Phosphorylation sites on phosphoprotein NS of vesicular stomatitis virus. *Journal of Virology.* **54**: 697-702.

Belle-Isle, H. D. and Emerson, S. U. (1982): Use of hybrid infectivity assay to analyse primary transcription of temperature-sensitivemutant of New Jersey serotype of vesicular stomatitis virus. *Journal of Virology,* **43**: 37-40.

Bellett, A. J. D. and Cooper, P. D. (1959): Some properties of the transmissible interfering component of VSV preparations. *Journal of General Microbiology.* **21**: 498.

Bergmann, J. E., Tokuyasu, K. T. and Singer, J. S. (1981):Passage of an integral membrane protein, the vesicular stomatitis virus glycoprotein, through the Golgi apparatus in route to the plasma membrane. *Proceedings of the National Academy of Sciences, U.S.A.* **78**: 1746-1750.

Black, L. M. (1969): Insect tissue cultures as tools in plant virus research. *Annual Review of Phytopathology.* 7: 73-100.

Black, L. M. (1970): Potato yellow dwarf virus. *CMI/AAB Descriptions of Plant Viruses.* No. **35**.

Black, L. M. (1979): Vector cell monolayers and plant viruses. *Advance in Virus Research.* **25**: 192-271.

Blackman, F. F. (1905): *Annual of Botany.* **19**: 281-295.

Blumberg, B. M., Giorgi, C. and Kolakofsky, D. (1983): N protein of vesicular stomatitis virus selectively encapsidates leader RNA *in vitro*. *Cell,* **32**: 559-567.

Boakye, D. B., and Randles, J. W. (1974): Epidemiology of LNYV in south Australia III. Virus transmission promoters and vector feeding behaviours on host and non-host plants. *Australian Journal of Agriculture Research.* **25**: 791-802.

Bowen, B., Steinberg, J., Laemmli, U. K., and Weintraub, H. (1980): The detection of DNA-binding proteins by protein blotting. *Nucleic Acids Research.* **8**: 1-20.

Bradford, M. M. (1976): A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry.* **72**: 248-254.

Bradfute. O, E. and Tsai, J. H. (1983): Identification of maize mosaic virus in Florida, *Plant Disease.* **67**:1339-1342.

Brewbaker, J. L. (1981): Resistance to maize mosaic virus, in:Virus and Viruslike Diseases of Maize in the United States. Edited by D. T. Gordon., J. K. Knoke, and G. E. Scott. pp. 145-151., *South. Coop. Ser. Bull.* **247.**

Brown, D. T. and Riedel, B. (1977): Morphogenesis of vesicular stomatitis virus: Electron microscope observations with freeze-fracture techniques. *Journal of Virology,* **21**: 601-609.

Brown, F. (1987): The family rhabdoviridae, general description and taxonomy, in: The rhabdoviruses, edited by R. R. Wagner, pp. 1-8, Plenum Press. New York, London.

Brown, F., Bishop, D. H. L., Crick, J., Francki, R. I. B., Holland, J. J., Hull, R., Johnson, K., Martelli, G., Murphy, F. A., Obijeski, J. F., Peters, D., Pringle, C. R., Reichmann, M. E., Schneider, L. G., Shape, R. E., Simpson, D. I. H., Summers, D. F., and Wagner, R. R. (1979): Rhabdoviridae. *Intervirology.* **12**: 1-

Buchanan, B, B,. Hutcheson, S. W., Magyarosy, A. C., and Moutalbini, P. (1981): Photosynthesis in healthy and diseased plants. In effects of disease on the

physiology of the growing plant. Edited by. P. G. Ayres. society for experimental biology. seminar series II. pp. 13-28. Cambridge University Press.

Burnette, W. N. (1981): "Western blotting" Electrophoretic transfer of protein from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. *Analytical Biochemistry.* **112**: 195-203.

Burgyan, J., Grieco, F. and Russo, M. (1989): A defective interfering RNA molecule in Cymbidium ringspot virus infection. *Journal of General Virology.* **70**: 235-239.

Burgyan, J., Rubino, L. and Russo, M. (1991): *De novo* generation of Cymbidium ringspot virus defective interfering RNA. *Journal of General Virology.* **72**: 505-509.

Cartwright, B., and Brown, F. (1972): Serological relationships between different strains of vesicular stomatitis virus. *Journal of General Virology.* **16**: 391-397.

Cascone, P. J., Carpenter, C. D., Li, X. H., and Simon, A. E. (1990): Recombination between satellite RNAs of turnip crinkle virus. *EMBO Journal.* **9**: 1709-1715.

Castellano, M. A. and Martelli, G. P. (1987): Tomato vein yellowing in Italy, a disease caused by EMDV. *Phytopatholgia Mediterranea.* **26**: 46-50.

Chen, M. J. and Shikata, E. (1968): Electron microscopy of virus-like particles associated with transitory yellowing virus-infected rice plants in Taiwan. *Plant Protection Bulletin. (Taiwan).* **10**: 19-28.

Chen, M. J. and Shikata, E. (1971): Morphology and intracellular localisation of RTYV. *Virology.* **46**: 786-796.

Cherif, C., and Martelli, G. P. (1985): Outbreaks and new records: Tunisia. Mottled dwarf of eggplant. *FAO Plant Protection Bulletin.* **33**: 166-167.

Chiu, R-J., and Jean, J. H. (1969): Leafhopper transmission of transitory yellowing of rice, in: The Virus Diseases of the Rice Plant, *Proceedings of the*

International Symptosium of Rice Research Institute. 1967. Edited by R-J. Chen, pp. 131-137, Johns Hopkins Press, Baltimore.

Chiu, R-J., and Jean, J. H. Chen, M. H. and Lo, T. C. (1968): Transmission of transitory yellowing virus of rice by two leafhoppers. *Phytopathology,* **58**: 740-747.

Choi, T. J. (1993): Moecular analysis of the genome of SYNV. *Ph.D dissertation, University of California at Berkeley.*

Choi, T. J. and Jackson, A. O. (1993): Sequence analysis of the trailer region of SYNV genomic RNA. *In press.*

Christie, S. R. (1969): A *Nicotiana* hybrid developed as a host for plant viruses. *Plant Disease Reporter.* **53**: 939-941.

Christie, S. R., Christie, R. G. and Edwardson, J. R. (1974): Transmission of bacilliform virus of sowthistle and *Bidens pilosa. Phytopathology.* **64**: 840-845.

Chu, P. W. G., and Francki, R. I. B. (1982): Detection of LNYV by an ELISA in plant host and the insect vector. *Annual Applied Biology.* **100**: 149-156.

Clark, M. F., Lister, R. M., and Bar-Joseph, M. (1986): ELISA techniques. *Methods in Enzymology.* **118**: 742-766.

Clinton, G. M., and Huang, A. S. (1981): Distribution of phosphoserine, phosphothreonine and phosphotyrosine in proteins of vesicular stomatitis virus. *Virology.* **108**: 510-514.

Compans, R. W. and Klenk, H. D. (1979): Viral membranes, in: Comprehensive virology. Edited by H. Fraenkel-conrat and R. R. Wagner, Vol. **13**, pp. 293-405. Plenum Press. New York.

Conti, M. and Plumb, R. T. (1977): Barley yellow striate mosaic virus in the salivary glands of its planthopper vector *Laodelphax striatellus. Journal of General Virology.* **34**: 107-114.

Cox, J. H., Weiland, F., Dietzschold, B., and Schneider, L. G. (1981): Revaluation of the structural proteins M1 and M2 of the rabies virus, in: the replication of negative strand viruses. Edited by D. H. L. Bishop and R. W. Compans, pp. 639-645, Elsevier/ North Holland, New York.

Culver, J. N., Lindbeck, Alwyn G. C., and Dawson, W. O. (1991): Virus-host interactions: Induction of chlorotic and necrotic responses in plant by Tobamoviruses. *Annual Review of Phytopathology.* **29**: 193-217.

Dale, J. L., and Peters, D. (1981): Protein composition of the virions of five plant rhabdoviruses. *Intervirology.* **16**: 86-94.

Danesh, D., and Lockhart, B. E. L. (1989): Eggplant mottled dwarf virus in potato in Iran. *Plant Disease.* **73**: 856-858.

Dawson, W. O., Bubrick, P., and Grantham, G. L. (1988): Modifications of TMV coat protein gene affecting replication, movement and symptomatology. *Phytopathology.* **78**: 783-789.

De, B. P., and Banerjee, A. K. (1984): Specific intractions of veiscular stomatitis virus L and Ls proteins with heterologous genome ribonuclroprotein template lead to mRNA synthesis *in vitr.* Journal of Virology, **51**: 628-634.

De, B. P., and Banerjee, A. K. (1985): Requirements and functions of the vesicular stomatitis virus L and NS proteins in the transcription process *in vitro. Biochemistry and Biophysics Research Communication.* **126**: 40-49.

De, B. P., Thornton, G. J. B., Luk, D. and Banerjee, A. K. (1982): Purified matrix protein of vesicular stomatitis virus blocks viral transcription *in vitro. Proceedings of the National Academy of Sciences, U.S.A.* **79**: 7137-7141.

Delieu, T., and Walker, D. A. (1981): Polarographic measurement of photosynthetic oxygen evolution by leaf discs. *New Phytiologist.* **89**: 165-178.

Doi, Y., Chang, M. U. and Yora, K. (1977): Orchid fleck virus, *CMI/AAB Description of Plant Viruses,* No. **183**.

Dubois-Dalcq, M., Holmes, K. V. and Rentier, B. (1984): Assembly of rhabdoviriodae. In assembly of enveloped RNA viruses. Springer-Veriag Vien, New York.

Dubois-Dalcq, M., Narayan, O. and Friffin, D. E. (1979): Cell surface changes associated with mutation of visna virus in antibody-treated cell surface. *Virology,* **92**: 353-366.

El-Maataoui, M., Lockhart, B. E. L. and Lesemann, D. E.. (1985): Biological, serological, and cytopathological properties of tomato vein-yellowing virus, a rhabdovirus occurring in tomato in Marocco. *Phytopathology*, **75**: 109-115.

Esau, K. (1968): Viruses in plant hosts: Form, distribution, and pathologic effects, pp. 225. Madison; University Wisconsin Press.

Etchison, J. R., and Summers, D. F., (1979): Structure, synthesis, and function of the vesicular stomatitis virus glycoprotein, in : Rhabdoviruses, Vol. I Edited by D. H. L. Bishop, pp. 151-160. CRC Press, Boca Raton, Florida.

Falk, B. W., Purcifull, D. E., and Christie, S. R. (1986): Natural occurrence of SYNV in Florida lettuce. *Plant Disease*. **70**: 591-593.

Falk, B. W., and Weathers, L. G. (1983): Comparison of potato yellow dwarf virus serotypes. *Phytopathology*. **73**: 81-85.

Falk, B. W., Weathers, L. G., and Greer, F. C. (1981): Identification of PYDV occurring naturally in California. *Plant Disease*. **65**: 81-83.

Faulkner, G. P. and Lazzarini, R. A. (1980): Homologous interference by defective virus particles, in: Rhabdoviruses, Vol. 2. pp. 163-176. Edited by D. H. L. Bishop. Boca Raton. CRC Press.

Francki, R. I. B. (1972): Purification of viruses in: Principles and techniques in plant virology. Edited by Kado, C. I. and Agrawal, H. O., pp 295-335. Van Nostrand Reinhold, New York.

Francki, R. I. B. (1973): Plant rhabdoviruses. *Advanced in Virus Research.* **18**: 257-345.

Francki, R. I. B., Kitajima, E. W., and Peters, D. (1981): Rhabdoviruses, in: Handbook of plant virus infection and comparative diagnosis. Edited by E. Kurstak, pp 455-489. El. sevier, North Holland, Amsterdam.

Francki, R. I. B., and Randles, J. W. (1975): Composition of the plant rhabdovirus lettuce necrotic yellows virus in relation to its biological properties, in: Negative strand viruses. Edited by B. W. J. Maby and R. D. Barry, pp. 223-242, Academic Press, London.

Francki, R. I. B., and Randles, J. W. (1980): Rhabdoviruses infecting plants in: Rhabdoviruses, Vol. III. edited by D. H. L. Bishop, pp. 135-165. CRC. Press. Boca Raton Florida.

Frazier, C. L., and Shope, R. E. (1979): Serologic relationships of animal rhabdoviruses, in: Rhabdoviruses, Vol. I edited by D. H. L. Bishop, pp. 43. CRC Press, Boca Raton, Florida.

Gaedigk, K., Adam, G., and Mundry, K. W. (1986): The spike protein of potato yellow dwarf virus and its functional role in the infection of insect vector cells. *Journal of General Virology*, **67**: 2763-2773.

Gallione, C. J., Greene, J. R., Iverson, L. E. and Rose, J. K. (1981): Nucleotide sequences of the mRNA encoding the vesicular stomatitis virus N and NS proteins. *Journal of Virology*, **39**: 529-535.

Ganett, A. L., and Shalla, T. A. (1970): Discrepancies in the intracellular behaviour of three strain of TMV, two of which are serologically indistinguishable. *Phytopathology*. **60**: 419-425.

Garrett, R. G., and O'Loughlin, G. T. (1977): Broccoli necrotic yellows virus in cauliflower and the aphid *Brevicoryne brassicae*. *Virology*, **76**: 653-663.

Gill, C. C. (1974): Inclusions and wall deposits in cells of plants infected with oat necrotic mottle virus. *Can. Journal of Botany*. **52**: 621-626.

Greber, R. S. and Gowanlock, D. H. (1979): Cereal chlorotic mottle virus purification , serology and electron microscopy in plant and insect tissues. *Australian Journal of Biology Sciences*, **32**: 399-408.

Goldberg, K. B., Modrell, B., Hillman, B. I., Heaton, L. A., Choi, T. J. and Jackson, A. O. (1991): Structure of glycoprotein gene of SYNV, a plant rhabdovirus. *Virology*. **185**: 32-38.

Goodman, R. N., Kiraly, Z. and Wood, K. R. (1986): The biochemistry and physiology of plant disease. Columbia, MO: Univ. Missouri Press.

Hall, A. E., and Loomis, R. S. (1972a): Photosynthesis and respiration by healthy and beet yellows virus-infected sugar beets (*Beta vulgaris*). *Crop Science*. **12**: 566-572.

Hall, A. E., and Loomis, R. S. (1972b): An explaination for the difference in photosynthetic capabilities of healthy and beet yellows virus-infected sugar beets (*Beta vulgaris*. L.). *Plant Physiology,* **50**: 576-580.

Harmison, G. G., Meier, E. and Schubert, M. (1984): The polymerase gene of VSV, in: Nonsegmented Negative Strand Viruses. Edited by D. H. L. Bishop and R. W. Compans. pp. 35-40, Academic Press, New York.

Harris, K. F. (1979): Leafhoppers and aphids as biological vectors: Vector-virus relationships, in: Leafhopper vectors and plant disease agents. (K. Maramorosch and K. F. Harris, eds.), pp. 217-308, Academic Press, New York.

Harrison B. D., and Roberts, I. M. (1968): Association of tobacco rattle virus with mitochondria. *Journal of General Virology,* **3**: 121-124.

Harwood, J. L. (1980): Plant acyl lipids, structure, distribution and analysis, in: The biochemistry of plants, a comprehensive treatise, Vol. 4, (P. K. Stumpf. ed.), pp. 1-55. Academic Press. New York, London.

Hatta, T., Nakamoto, T., Takagi, Y., and Ushiyama, R. (1971): Cytological abnormalities of mitochondria induced by infection with cucumber green mottle mosaic virus. *Virology.* **45**: 292-297.

Hatta, T., and Ushiyama, R. (1973): Mitochondria vesiculation associated with cucumber green mottle mosaic virus-infected plants. *Journal of General Virology.* **21**: 9-

Hawkes, R., Nidgy, E. and Gordon, J. (1982): A dot-immunobinding assay for monoclonal and other antibodies. *Analytical Biochemistry.* **119**: 142-147.

Heaton, L. A., Hillman, B. I., Hunter, B. G., Zuidema, D., and Jackson, A. O. (1989): The physical map of SYNV genome, a plant rhabdovirus with six genes and conserved gene-junction sequences. *Proceedings of the National Academy of Science. USA.* **86**: 8665-8668.

Heaton, L. A., Zuidema, D., and Jackson, A. O. (1987): Structure of M2 protein gene of SYNV. *Virology.* **161**: 234-241.

Heggeness, M. H., Scheid, A. and Choppin, P. W. (1980): Conformation of the helical nucleocapsids of paramyxoviruses and VSV: Reversible coilling and

uncoilling induced by changes in salt concentration. *Proceedings of the National Academy of Sciences, U.S.A.* 77: 2631-2635.

Heyward, J. T., Holloway, B. P., Cohen, P. and Obijeski, J. F. (1979): Rhabdovirus nucleocapsid, in: Rhabdoviruses, Vol.1. Edited by D. H. L. Bishop. pp. 137-149, CRC Press, Boca Raton, Florida.

Hill, V. M., Marmell, L. and Summers, D. F. (1981): *In vitro* replication and assembly of VSV nucleocapsid. *Virology,* **113**: 109-118.

Hillman, B. I., Carrington, J. C., and Morris, T. J. (1987): A defective interfering RNA that contains a mosaic of plant virus genome. *Cell.* **51**: 421-433.

Hillman, B. I., Heaton, L. A., Hunter, B. G., Modrell, B., and Jackson, A. O. (1990): Structure of the gene encoding the M1 protein of SYNV. *Virology.* **179**: 201-207.

Hirki, C., and Tu, J. C. (1972): Light and electron microscopy of potato virus M lesions and marginal tissue in red kidney bean. *Phytopathology.* **62**: 77-85.

Holland, J. J. (1987): In: The rhabdoviruses. Edited by R. R. Wagner. pp297-360. Plenum Press, New York.

Holt, C. A., Hodgson, R. A. J., Coker, F. A., Beachy, R. N., Nelson, R. S. (1990): Characterisation of the mosaiced strain of TMV: identification of the region responsible for symptom attenuation by analysis of an infectious cDNA clone. *Molecular Plant Microbiology Interaction.* **3**: 417-443.

Howatson, A. F. (1970): Vesicular stomatitis and related viruses. *Advance of Virus Research.* **16**: 195-

Huang, A. S. (1973): Defective-interfering viruses. *Annual Review of Microbiology.* **27**: 101-117.

Huang, A. S. and Baltimore, D. (1977): Defective interfereing animal viruses, in: Comprehensive virology, Vol. 10. Edited by H. Fraenkd-Conrat and R. R. Wagner. pp. 73-116. Plenum Press. New York.

Huang, A. S. and Wagner, R. R. (1966): Defective T particles of VSV. 2. Biologic role in homologous interferance. *Virology.* **30**: 173.

Hull, R. (1976): The structure of tubular viruses. *Advances in Virus Research.* **20**:1-32.

Hull, R. (1989): The movement of viruses in plants. *Annual Review of Phytopathology.* **27**: 213-240.

Hummer, K. (1971): Bullet-shaped viruses, in: Comparative virology, (K. Maramorosch and D. Kurstak. eds.), pp. 361-386. Academic Press, New York, London.

Hunt, D. M., Mellon, M. G., and Emerson, S. U. (1979): Viral transcriptase, in : Rhabdoviruses, Vol. I, (D. H. L. Bishop, ed.), pp. 169-183. CRC Press, Boca Raton, Florida.

Hunter, C. S., and Peat, W. E. (1973): The effect of tomato aspermy virus on photosynthesis in the young tomato plant. *Physiological Plant Pathology.* **3**: 517-524.

Hsu, H. T., and Black, L. M. (1973): Inoculation of vector cell monolayers with potato yellow dwarf virus. *Virology.* **52**: 187-198.

Hsu, C-H., Kingsbury, D. W. and Murti, K. G. (1977):Assembly of vesicular stomatitis nucleocapsids *in vitro*; a kinetic analysis. *Journal of Virology,* **32**: 304-313.

Hsu, C-H., Morgan, E. M., and Kingsbury, D. W. (1982): Site-specific phosphorylation regulates the transcriptase activity of vesicular stomatitis virus. *Journal of Virology.* **43**: 104-112.

Ismail, I. D. (1988): Studies on a *Nicotiana* hybrid infected with a plant rhabdovirus. *Ph.D. thesis. Glasgow University, Botany Department.*

Ismail, I. D., and Milner, J. J. (1988): Isolation of defective interfering particles of Sonchus yellow net virus from chronically infected plants. *Journal of General Virology.* **69**: 999-1006.

Ismail, I. D., Hamilton, I. D., Robertson, I., and Milner, J. J. (1987): Movement and intracellular location of SYNV within infected *Nicotiana edwardsonii. Journal of General Virology* . **68**: 2429-2438.

Ivanovski, D. (1892): Uber die mosaikkrankheit der tabakspflanze. Bulletin No. 3: 65-70. St Petersbourgh: Imperial Academic of Sciences.

Ivanovski, D. (1903): Uber die Mossaikkrankheit der Tabakspflanze. *Zeitschrift fur flanzenkrankheiten.* **13**: 1-41.

Jackson, A. O. and Christie, S. R. (1977): Purification and some physicochemical properties of Sonchus yellow net virus. *Virology.* **77**: 344-355.

Jackson, A. O. (1978): Partial characterisation of the structural protein of SYNV. *Virology.* **87**: 172-181.

Jackson, A. O., Milbrath, G. M., and Jedlinski, H. (1981): Rhabdovirus diseases of the gramineae, in: Virus and virus-like diseases of maize in the United states. Edited by D. T. Gordon, J. K. Knoke, and G. E. Scott, pp. 51-76. *South coop. ser. bulletin.* **247.**

Jackson, A. O., Francki, R. I. B., and Zuidema, D. (1987): Biology, structure, and replication of plant rhabdoviruses, in: The rhabdoviruses. Edited by R. R. Wagner, pp. 427-508. Pleuum Press.

Jacobs, B. L., and Penhoel, E. E. (1982): Assembly of vesicular stomatitis virus: Distribution of the glycoprotein on the surface of infected cells. *Journal of Virology,* **44**: 1047-1055.

Jensen, S. G. (1972): Metabolism and carbohydrate compsition in barley yellow dwarf virus-infected wheat. *Phytopathology.* **62**: 587-592.

Johnson, D. C. and Schlesinger, M. J. (1980): vesicular stomatitis virus and sindbis virus glycoprotein transport to the cell surface is inhibited by ionophores. *Virology,* **103**: 407-424.

Jones, R. W., and Jackson, A. O. (1990): Replication of Sonchus yellow net virus in infected protoplasts. *Virology.* **179**: 815-820.

Kelley, J. M., Emerson, S. U. and Wagner, R. R. (1972): The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody. *Journal of Virology.* **10**: 1231.

Kim, K. S., Shock, T. L., and Goodman, R. M. (1978): Infection of *Phaseolus vulgaris* by bean golden mosaic virus. Ultrastructural aspects. *Virology.* **89**: 22-33.

Kitajima, E. W., and Costa, A. S. (1969): Association of ringspot virus (Brazilian tobacco rattle virus) with mitochondria. *Journal of General Virology.* 4: 177-181.

Kitajima, E. W., and Lovisolo, O. (1972): Mitochondria aggregates in *Diatura* leaf cells infected with henbane mosaic virus. *Journal of General Virology.* **16**: 265-271.

Knipe, D. M., Baltimare, D. and Lodish, H. F. (1977): Separate pathways of maturation of the major structural protein of vesicular stomatitis virus. *Journal of Virology,* **21**: 1128-1139.

Knorr, D. A., Mullin, R. H., Hearne, P. Q, and Morris, A. T. (1991): *De novo* generation of defective interfering RNAs of tomato bushy stunt virus by high multiplicity passage. *Virology*, **181**: 193-202.

Knudson, D. L., and MacLeod, R. (1972): The protein of PYDV. *Virology.* **47**: 285-295.

Knudson, D. L. (1973): Rhabdoviruses. *Journal of General Virology.* **20**: 105-130.

Konig, G. J. (1899): Die Flecken-Oder fur mosaikkiankheit des Hollandischen tobaks. *Zeitschrift Pflanzenkrankheiten.* 9: 65-80.

Kosuge, T. and Kimpel, J. A. (1981): Energy use and metabolic regulation in plant-pathogen interactions. In effects of disease on the physiology of the growing plant. Edited by P. G. Ayres. pp. 29-45, Cambridge university Press. Cambridge.

Kurstak, E. (1981): Hand book of plant virus infections. Edited by Kurstak, E., Elsevier. North Holland, London.

Laemmli, U. K. (1970): Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680-685.

Lastra, J. R. and Esparza, J. (1976): Multiplication of vesicular stomatitis virus in the leafhopper *Peregrinus maidis*, a vector of a plant rhabdovirus. *Journal of General Virology*, **32**: 139-142.

Lee, P. E. (1970): Developmental stages of wheat striate mosaic virus. *Journal of Ultrastructural Research*, **31**: 282-290.

Leonard, D. A. and Zaitlin, M. (1982): The temperature-sensitive strain of tobacco mosaic virus defective in cell-to-cell movement generate an altered virus-coded protein. *Virology*. **117**: 416-424.

Leverenz, J. W. (1987): Chlorophyll content at the light response curve of shade-adapted conifer needles. *Physiologia Plantarum*, **71**: 20-29.

Leverenz, J. W. (1988): The effect of illumination sequence, CO_2 concentration, temperature and acclimation on the convexity of the photosynthetic light response curve. *Physiologia Plantarum*, **74**: 332-341.

Li, X. H., Heaton, L. A., Morris, T. J. and Simon, A. E. (1989): Turnip crinkle virus defective interfering RNAs intensify viral symptoms and are generated *de novo*. *Proceedings of the National Academy of Sciences, USA*, **86**: 9173-9177.

Lin, M. T. and Campbell, R. N. (1972): Characterisation of broccoli necrotic yellows virus. *Virology*. **48:** 30-40.

Linstead, P. J., Hills, G. J., Plaskitt, K. A., Wilson, I. G., Harker, C. L. and Maule, A. J. (1988): The subcellular location of the gene I product of cauliflower mosaic virus is consistent with a function associated with the virus spread. *Journal of General Virology*, **69**: 1809-1818.

Lockhart, B. E. L. (1987): Evidence for identity of plant rhabdoviruses causing vein-yellowing diseases of tomato and *Hibiscus rosa-sinensis*. *Plant Disease*, **71**: 731-733.

Lodish, H. F. and Rothman, J. E. (1980): The assembly of cell membranes, in: Molecular Biology of Living Cells, pp. 135-153. Freeman, San Francisco.

Lwoff, A. (1957): The concept of virus. *Journal of General Microbiology*, **17**: 239-253.

Mackinney, G. (1941): Absorption of light by chlorophyll solutions. *Journal of Biological Chemistry*, **140**: 315-322.

MacLeod, R. (1967): The preparation of plant viruses for use as antigens, in: Methods in immunology and immunochemistry. Vol. 1. Edited by C. A. Williams and M. W. Chase. pp.102-115, Academic Press, New York.

MacLeod, R. (1968): An interpretation of the observed polymorphism of potato yellow dwarf virus. *Virology*, **34**: 771-777.

Magyarosy, A. C., Buchanan, B. B. and Schurmann, P. (1973): Effect of a systemic virus infection on chloroplast function and structure. *Virology*, **55**: 426-438.

Marks, M. D., Kennedy-Morrow, J. and Lesnaw, J. A. (1985): Assignment of the temperature-sensitive lesion in the replication mutant A1 of vesicular stomatitis virus to the N gene. *Journal of Virology*. **53**: 44-51.

Marshall, B. and Bisoce, P. V. (1980): A model for C_3 leaves describing the dependence of net photosynthesis on irradiance. I- Derivation. *Journal of Experimental Botany*, **31**: No.120, 29-39.

Marshall, B. and Bisoce, P. V. (1980): A model for C_3 leaves describing the dependence of net photosynthesis on irradiance. II- Application to the analysis of flag leaf photosynthesis. *Journal of Experimental Botany*, **31**: No.120, 41-48.

Martelli, G. P. (1969): Baclliform particles associated with mottled dwarf of eggplant (*Solanum melongena*. L.). *Journal of General Virology*, **5**: 319-320.

Martelli, G. P. and Castellano, M. A. (1970): Electron microscopy of EMDV. *Phytopatholgia Mediterranea*. **9**: 39-49.

Martelli, G. P. and Cherif, C. (1987): Eggplant dwarf virus associated with vein yellowing of honeysuckle. *Journal of Phytopathology*, **119**: 32-41.

Martelli, G. P. and Hamadi, A. (1986): Occurrence of eggplant mottled dwarf virus in Algeria. *Plant Pathology*, **35**: 595-597.

Martelli, G. P. and Russo, M. (1973): Eggplant mottled dwarf virus, *CMI/AAB Descriptions of Plant Viruses*, No. **115.**

Martelli, G. P. and Russo, M. (1977): Rhabdoviruses of plants. In the atlas of insect and plant viruses. Edited by Maramorosch, K. pp. 181-214. Academic Press, New York.

Martelli, G. P., Yilmaz, M. A. and Baloglu, S. (1984): Ultrastructure observations on virus-diseased plant from Western Turkey. *Phytopatholgia Mediterranea.* **23**: 9-14.

Matthews, R. E. F. (1979): Classification and nomenclature of viruses. 3th report of the International Committee on Taxonomy of Viruses. *Intervirology*, **12**: 131-296.

Matthews, R. E. F. (1980): Host plant resposes to virus infection, in: Comprehensive virology, Vol. **16**: 297-359. Edited by H. Fraenkel. convat and R. R. Wagner. Plenum Press, New York.

Matthews, R. E. F. (1982): Classification and nomenclature of viruses. 4th report of the International Committee on Taxonomy of Viruses. *Intervirology*, **15**: 64-179.

Matthews, R. E. F. (1991): Plant virology, third edition, Academic Press, New York.

Mayer, A. (1886): Ueber die mosaikkrankheit des tabaks. *Landwirtsch Vers Stu.* **32**: 451-467.

Mazliak, P. (1977): Glyco- and phospholipids of blomembranes in higher plants. In lipids and lipid polymers in higher plants. Edited by M. Tevini and H. Lichtenthaler, pp. 48-74. Springer-Verlag, New York.

McKay, M. B. and Warner, M. F. (1933): Historical sketch of tulip mosaic or breaking. The oldest known plant virus disease. *National Horticultural Magazine*, **3**: 179-216.

McLean, G. D. and Francki, R. I. B. (1967): Purification of lettuce necrotic yellows virus by column chromatography on calcium phosphate gel. *Virology.* **31**: 585-591.

McMullen, C. R., Gardner, W. S. and Myers, G. A. (1977): Ultrstructure of cell-wall thickenings and paramural bodies induced by barley stripe mosaic virus. *Phytopathology*, 67: 462-467.

McSharry, J. J. (1979): Viral membrane protein structure and function. In Rhabdoviruses. Vol I. Edited by D. H. L. Bishop, pp. 161-168. CRC Press, Boca Raton, Florida.

McSharry, J. J. and Wagner, R. R. (1971): Lipid composition of purified vesicular stomatitis viruses. *Journal of Virology*. 7: 59-70.

Milner, J. J., Hakkaart, M. J. J. and Jackson, A. O. (1979): Subcellular distribution of RNA sequences complementary to SYNV RNA. *Virology*, **98**: 497-501.

Milner, J. J. and Jackson, A. O. (1979): Sequences complementarity of SYNV RNA with RNA isolated from the polysomes of infected tobacco. *Virology*, **97**: 90-99.

Milner, J. J. and Jackson, A. O. (1983): Characterisation of viral complementary RNA associated with polysomes from tobacco infected with SYNV. *Journal of General Virology*, **64**: 2479-2483.

Mohamed, N. (1973): Some effects of systemic infection by tomato spotted wilt virus on chloroplasts of *Nicotiana tabacum* leaves. *Physiological Plant Pathology*, **2**: 247-258.

Morgan, E. M. and Kingsbury, D. W. (1981): Association of transcriptase and RNA methyltransferase activities of vesicular stomatitis virus with the L-protien, in: The Replication of Negative Strand Viruses. Edited by D. H. L. Bishop and R. W. Compans. pp. 817-820, Elsevier/North-Holland, New York.

Morrison, T. G. (1980): Rhabdoviral assembly and intracellular processing of viral components, in: Rhabdoviruses, Vol. 11. Edited by D. H. L. Bishop. pp. 95-114.CRC Press, Boca Raton. New York and London.

Morris, T. J. and Knorr, D. A. (1990): Defective interfering viruses associated with plant virus infection. In new aspects of positive-strand RNA viruses. Edited

by M. A. Brinton and F. X. Heinz, pp. 123-127. Washington, D. C. American society for microbiology.

Morrissey, J. H. (1981): Sliver stain for proteins in polyacrylamide gels: A modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry*, 117: 307-310.

Naidu, R. A., Krishnan, M., Ramanujam, P., Gnanam, A. and Nayudu, M. V. (1984a): Studies on peanut green mosaic virus infected peanut (*Arrachis hypogaea* L.) leaves. I- Photosynthesis and photochemical reactions. *Physiological Plant Pathology*, **25**: 180-190.

Naidu, R. A., Krishnan, M., Ramanujam, P., Nayudu, M. V. and Gnanam, A. (1984b): Studies on peanut green mosaic virus infected peanut (*Arrachis hypogaea* L.) leaves. II- Chlorophyll-protein complexes and polypeptide composition of thylakoid membranes. *Physiological Plant Pathology*, **25**: 191-198.

Nelson, R. (1932): Investigations in the mosaic disease of bean. Michigan agriculture experiment station section botany. *Technical bulletin*, No. **118**.

Odenwald, W., Arnheiter, Dubois-Dalcq, H. M. and Lazzarini, R. (1984): The nucleocapsid coiling of VSV at the inner surface of the plasmid membranes; immunolocalization of the matrix protein. In molecular biology of negative strand viruses. Edited by D. H. L. Bishop and R. W. Compans, Academic Press, New York.

O'Loughlin, G. T. and Chambers, T. C. (1967): The systemic infection of an aphid by a plant virus. *Virology*, **33**: 262-271.

Orenstein, J., Johnson, L., Shelton, E. and Lazzarini, R. A. (1976): The shape of vesicular stomatitis virus. *Virology*, **71**: 291-

Patton, J. T., Davis, N. L., and Wertz, G. W. (1984): Role of vesicular stomatitis virus proteins in RNA replication. In nonsegmented negative strand viruses. Edited by D. H. L. Bishop and R. W. Compans, pp. 147-152. Academic Press, New York.

Patzer, E. J., Wagner, R. R. and Dubovi, E. J. (1979): Viral membranes: Model systems for studing biological membranes. *CRC Crit. Review of Biochemistry.* 6: 165.

Perrault, J., Clinton, G. M. and McClure, M. A. (1983): RNP template of vesicular stomatitis virus regulates transcription and replication functions. *Cell* 35: 175-185.

Peters, D. (1981): Plant rhabdovirus group. *CMI/AAB Descriptions of Plant Viruses.* No. 244.

Peters, D. and Black, L. M. (1970): Infection of primary cultures of aphid cells with a plant virus. *Virology,* 40: 847-853.

Peters, D. and Kitajima, E. W. (1970): Purification and electron microscopy of sowthistle yellow vein virus. *Virology,* 41: 135-150.

Pirie, N. W. (1962): Prespective in biology and medicine. 5: 446.

Platt, S. G. and Bassham, J. A. (1978): Photosynthesis and increased production of proteins. In nutritional improvement of food and feed proteins. Edited by M. Friedman, pp. 195-243. Plenum Press, New York and London.

Platt, S. G., Henriques, F. and Rand, L. (1979): Effect of virus infection on the chlorophyll content, photosynthetic rate and carbon metabolism of *Tolmiea menziesii. Physiological Plant Pathology,* 16: 351-365.

Rabinowitch, E. I. (1951): In: Photosynthesis and related processes. *Intersciences.* New York. Vol. 2 (1), pp. 831-1191.

Randles, J. W. (1980): Transmission and epidemiology of lettuce necrotic yellows virus. In current topics in vector research, vol. I. Edited by C. F. Harris, pp. 169-187. Praeger publishers, New York.

Randles, J. W. and Coleman, D. F. (1970): Loss of ribosomes in *Nicotiana glutinosa* L. infected with lettuce necrotic yellows virus. *Virology,* 41: 459-464.

Randles, J. W. and Coleman, D. F. (1972): Changes in polysomes in *Nicotiana glutinosa* L. leaves infected with LNYV. *Physioogical Plant Pathology,* 2: 247-258.

Randles, J. W. and Francki, R. I. B. (1972): Infectious nucleocapsid particles of LNYV with RNA-dependent RNA polymerase activity. *Virology*, **50**: 297-300.

Reinero, A. and Beachy, R. N. (1986): Association of TMV coat protein with chloroplast membranes in virus-infected leaves. *Plant Molecular Biology*, **6**: 291-301.

Reinero, A. and Beachy, R. N. (1989): Reduced photosystem II activity and accumulation of viral coat protein in chloroplasts of leaves infected with TMV. *Plant Physiology*, **89**: 111-116.

Repik, P. (1979): Adsorption, penetration, uncoating and *in vitro* mRNA transcription process. In rhabdoviruses, vol.II. Edited by D. H. L. Bishop, pp. 1-33. CRC Press, Boca Raton, Florida.

Resende, R. D., Haan, P., Avila, A. C., Kitajima, E. W., Kormelink, R., Goldbach, R. and Peters, D. (1991): Generation of envelope and defective interfering RNA mutants of tomato spotted wilt virus by mechanical passage. *Journal of General Virology*, **72**: 2375-2383.

Rezain, M. A., Heaton, L. A., Pedersen, K., Milner, J. J. and Jackson, A. O. (1983): Size and compexity of polyadenylated RNAs induced in tobacco infected with SYNV. *Virology*, **131**: 221-229.

Rose, J. K. and Bergmann, J. E. (1982): Expression from cloned cDNA of cell surface and secreted forms of the vesicular stomatitis virus glycoprotein in eucaryotic cells. *Cell*, **30**: 753-762.

Roux, L., Simon, A. E. and Holland, J. J. (1991): Effects of defective interferance viruses on virus replication and pathogenesis *in vitro* and *in vivo*. *Advances in Virus Research*. Vol. **40**: 181-211.

Russo, M. and Martelli, G. P. (1972): Ultrastructural observations on tomato bushy stunt virus in plant cells. *Virology*, **49**: 122-129.

Russo, M. and Martelli, G. P. (1973): A study of the structure of eggplant mottled dwarf virus. *Virology*, **52**: 39-48.

Sanger, H. L. (1068):Characteristics of TRV; evidence that its two particles are functionally defective and mutually complementing. *Molecular of General Genetics*, **101**: 346-364.

Schoelz, J. E. and Zaitlin, M. (1989): TMV RNA enters chloroplasts in *vivo*. *Proceedings of the National Academy of Science, U.S.A.* **86**: 4496-4500.

Selstam, E. and Jackson, A. O. (1983): Lipid composition of SYNV. *Journal of General Virology*, **64**: 1607-1613.

Shalla, T. A. (1968): Virus particles in chloroplasts of plant infected with the U5 strain of TMV. *Virology*, **35**: 194- 203.

Shikata, E. and Maramorosch, K. (1966): Electron microscopy of pea enation mosaic virus in plant cell nuclei. *Virology*, **30**: 439-454.

Siegel, A., Zaitlin, M. and Sehgal, O. M. (1962): The isolation of defective TMV strains. *Proceedings of the National Academy of Science, U.S.A.* **48**: 1845-1851.

Sinha, R. C., Harwalkar, V. R. and Behki, R. M. (1976): Chemical composition and some properties of wheat striate mosaic virus. *Phytopathology. Z.* **87**: 314-323.

Smith, P. R. and Neales, T. F. (1977): Analysis of the effect of virus infection on photosynthetic properties of peach leaves. *Australian Journal of Plant Physiology*, 4: 723-732.

Sokol, F., Stancek, D. and Koprowski, H. (1971): Structural proteins of rabies virus. *Journal of General Virology*, 7: 241-249.

Stubbs, L. L. and Gragan, R. G. (1963): Necrotic yellows: A newly recognised virus disease of lettuce. *Australian Journal of Agricultural Research*, **14**: 439-459.

Sugimura, Y. and Ushiyama, R. (1975): Cucumber green mottle mosaic virus infection and its bearing on cytological alterations in tobacco mesophyll protoplasts. *Journal of General Virology*, **29**: 93-98.

Sulzinski, M. A. and Zaitlin, M. (1982): TMV replication in resistant and susceptible plants: In some resisttant species virus is confined to a small number of initially infected cells. *Virology.* **121**: 12-19.

Sylvester, E. S. and Richardson, J. (1970): Infection of *Heperomyzus lactucae* by sowthistle yellow vein virus. *Virology,* **42**: 1023-1042.

Sylvester, E. S. (1973): Reduction of excrtion, reproduction and survival in *Heperomyzus lactucae* fed on plant infected isolate of sowthistle yellow vein virus. *Virology,* **56**: 632-635.

Thornley, J. H. M. (1976): In: Mathematical models in plant physiology. Edited by J. F. Sukliffe and P. Mahiberg. pp. 92-110. Academic Press. London.

Tomenius, K., Clapham, D. and Meshi, T. (1987): Localisation by immunoglod cytochemistry of virus-coded 30K protein in plasmodesmata of leaves infected with TMV. *Virology,* **160**: 363-371.

Towbin, H., Staehelin, T. and Gordan, J. (1979): Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences. U.S.A.* **76**: 4350-4354.

Tu, J. C. and Ford, R. E. (1968): Effect of maize dwarf mosaic virus infection on respiration and photosynthesis of corn. *Phytopathology,* **58**: 282-284.

Van Beek, N. A. M., Lohuis, D., Dijkstra, J. and Peters, D. (1985): Morphogenesis of SYNV in cowpea protoplasts. *Journal of Ultrastructure Research,* **90**: 294-303.

Van Beek, N. A. M., Lohuis, D., Dijkstra, J. and Peters, D. (1985): Morphogenesis of festuca leaf streak virus in cowpea protoplasts. *Journal of General Virology,* **66**: 2485-2489

Van Beek, N. A. M., Derksen, A. C. G. and Dijkstra, J. (1985): Synthesis of SYNV protein in infected cowpea protoplasts. *Journal of General Virology,* **67**: 1701-1709.

Vela, A. and Rubio-Huertos, M. (1974): Bacilliform particles within infected cells of *Trifolium incarnatum. Phytopathologische Zeitschrift.* **97**: 343-351.

Volk, W. A., Snyder, R. M., Benjamin, D. C. and Wagner, R. R. (1982): Monoclonal antibodies to the glycoprotein of VSV: Comparative nrutralising activity. *Journal of Virology.* **42**: 220.

Wagner, R. R. (1975): Reproduction of rhabdoviruses in: Comprehensive virology. Vol. 4, pp. 1-93. Edited by H. Fraenkel. convat and R. R. Wagner. Plenum Press, New York.

Wagner, R. R. (1987): Rhabdovirus biology and infection ,an overview, in: The rhabdoviruses. Edited by R. R. Wagner, pp. 9-74. Plenum Press, New York and London.

Wagner, R. R., Prevec, L., Brown, F., Summers, D. F., Sokol, F. and McLeod, R. (1972): Classification of rhabdovirus proteins: A proposal. *Journal of Virology*, **10**: 1228-1230.

Wagner, R. R., Thomas, J. R. and McGowan, J. J. (1984): Rhabdovirus cytopathology: effects on cellular macromolecular synthesis, in: Comprehensive virology. Vol. 19, pp. 223-295. Edited by H. Fraenkel. convat and R. R. Wagner. Plenum Press, New York.

Walker, D. (1987): In: The use of oxygen electrode and fluorescence probes in simple measurements of photosynthesis. Edited by D. Walker, pp. part A, 2. Oxygraphics Ltd.

Walkey, D. G. A. and Pink, D. A. C. (1984): Resistance in vegetable marrow and other *Cucurbita spp.* to two British strains of cucumber mosaic virus. *Journal of Agricultural Science*, **102**: 197-205.

Walkey, D. G. A. (1985): In: Applied plant virology. Edited by D. G. A. Walkey, pp. 1-18. William Heinemann. Ltd.

Weintraub, M. and Ragetli, H. W. J. (1970): Electron microscopy of the bean and cowpea strains of southern bean mosaic virus within leaf cells. *Journal of Ultrastructure Research*, **32**: 167-189.

Wetzel, T., Dietzgen, R. G. and Dale, J. L. (1993): Genome organisation of lettuce necrotic yellows rhabdovirus. *IXth International Congress of Virology, Glasgow, Abstract, P60-27, pp.326.*

Wildy, P. (1971): Classification and nomenclature of viruses. First report of the International Committee on Nomenclature of Viruses. *Monogr. Virology*, **5**: 1-81.

Wolanski, B. S. (1969): Electron microscopy of lettuce necrotic yellows virus. *PhD. Thesis*, University of Melbourne.

Wolanski, B. S. and Chambers, T. C. (1971): The multiplication of lettuce necrotic yellows virus. *Virology*, **44**: 582-591.

Wolanski, B. S., Francki, R. I. B. and Chambers, T. C. (1967): Structure of lettuce necrotic yellows virus. I. Electron microscopy of negatively stained preparations. *Virology*, **33**: 287-296.

Wolanski, B. S. and Francki, R. I. B. (1969): Structure of lettuce necrotic yellows virus. II. Electron microscopy studies on the effects of pH of phosphotungstic acid stain on morphology of the virus. *Virology*, **37**: 437-447.

Woods, A. F. (1909): Observations on the mosaic disease of tobacco. U. S. Department of Agriculture Bureau of Plant Industry. *Bulletin No.* **18.** Washington. D. C.; Government Printing Office.

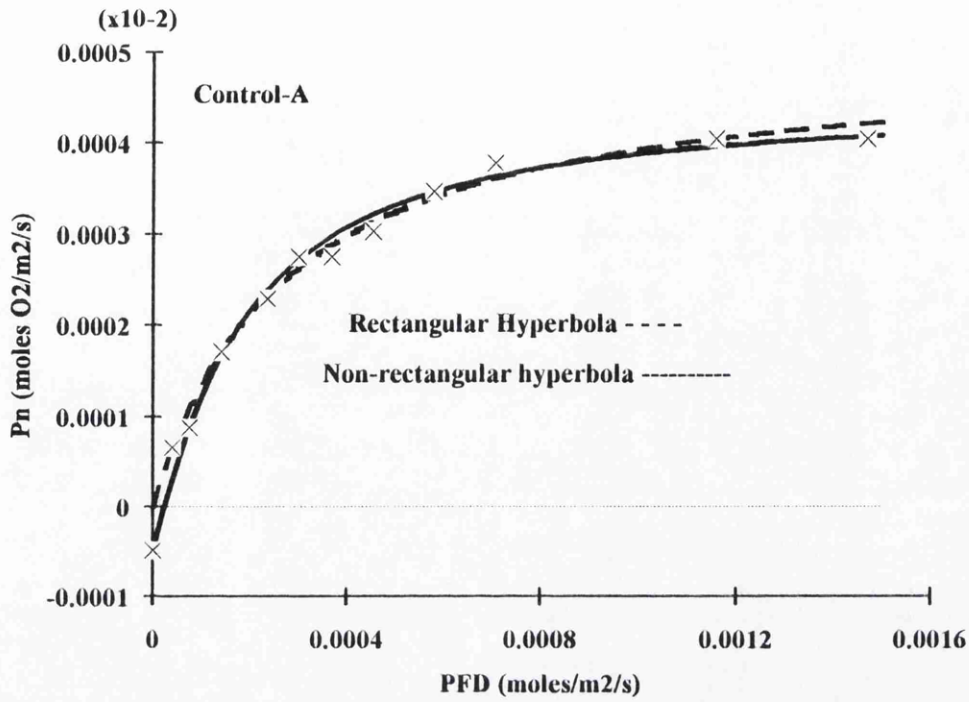
Zaitlin, M. (1979): How viruses and viroids induce disease. In plant disease. Edited by J. G. Horfall and E. B. Cowling. Academic Press, New York. Vol. **4**: 257-271.

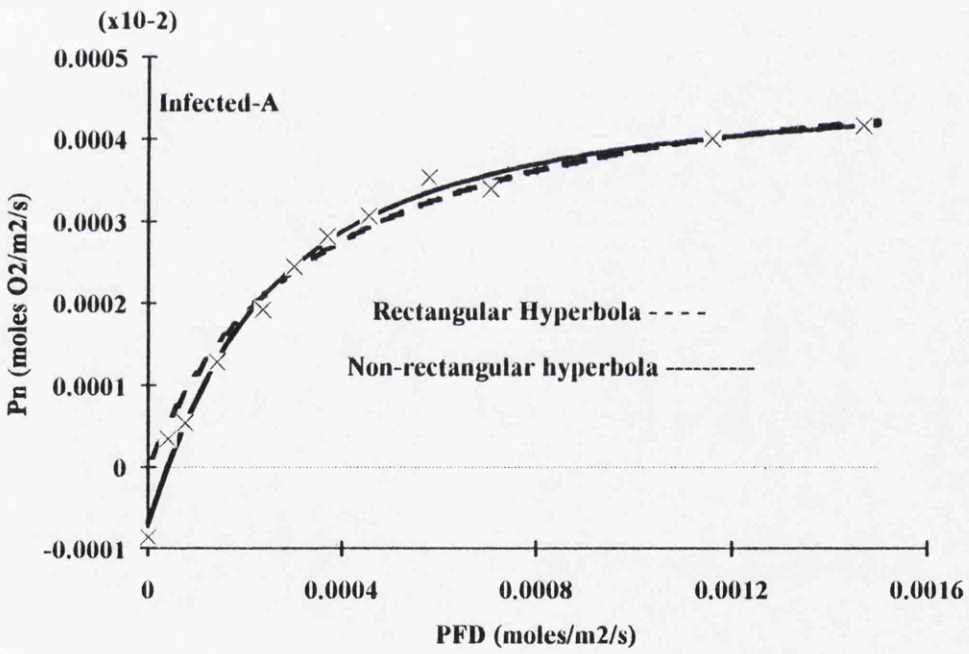
Zaitlin, M. and Hull, R. (1987): Plant virus-host interactions. *Annual Review of Plant Physiology*, **38**: 291-315.

Zakowski, J. J. and Wagner, R. R. (1980): Localisation of the membrane-associated proteins in VSV by use of hydrophobic membrane probes and cross-linking reagents. *Journal of Virology*. **36**:93.

Ziemecki, A. and Peters, D. (1976): The protein of sowthistle yellow vein virus: Characterisation and location. *Journal of General Virology*, **32**: 369-381.

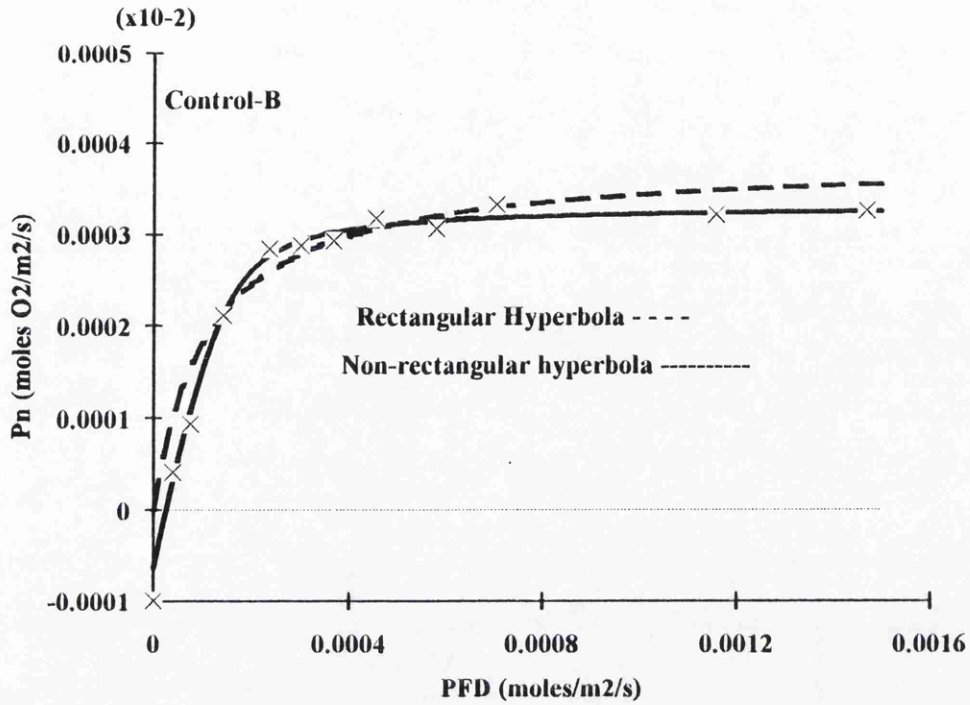
Zuidema, D., Heaton, L. A. and Jackson, A. O. (1987): Structure of the nucleocapsid protein gene of SYNV. *Virology*, **159**: 373-380.

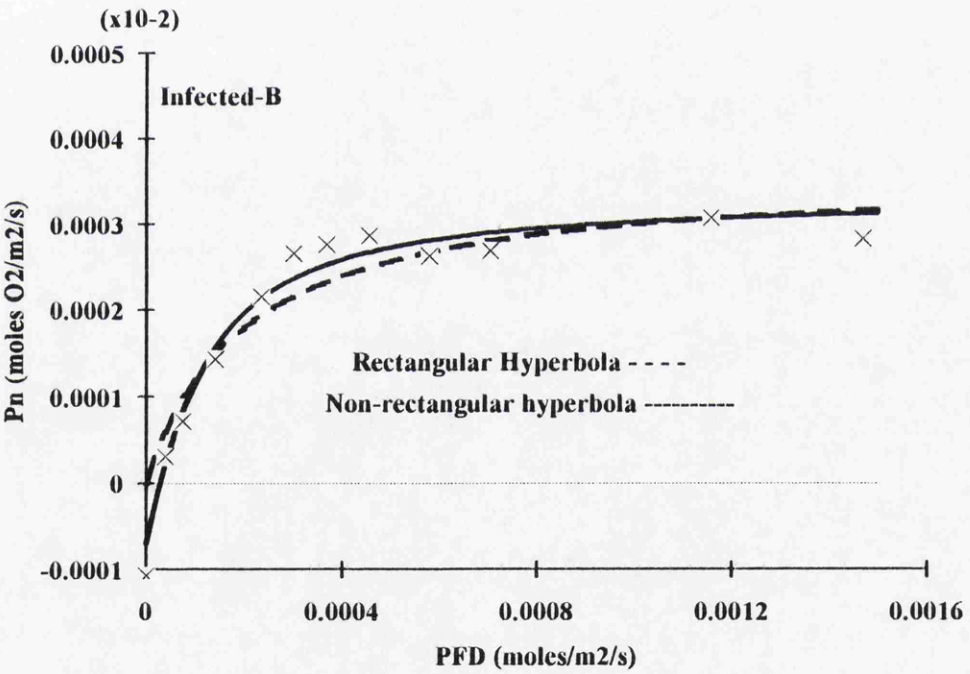




N. edwardsonii (10 days):

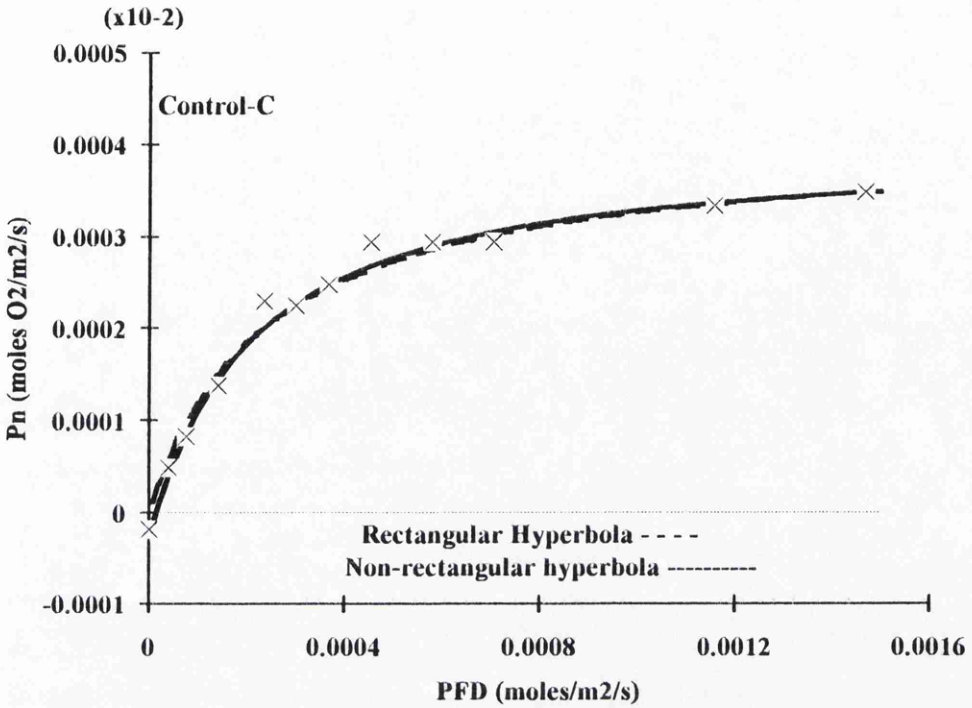
Control-A Rabinowich	Infected-A Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 4.975E-06	P max= 5.237E-06
α = 1.855E-02	α = 1.469E-02
Error= 1.498E-06	Error= 2.542E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -4.895E-07	Rd= -6.798E-07
Pmax= 4.814E-06	Pmax= 5.161E-06
α = 2.244E-02	α = 1.875E-02
θ = 3.906E-01	θ = 4.181E-01
Error= 1.202E-06	Error= 1.153E-06

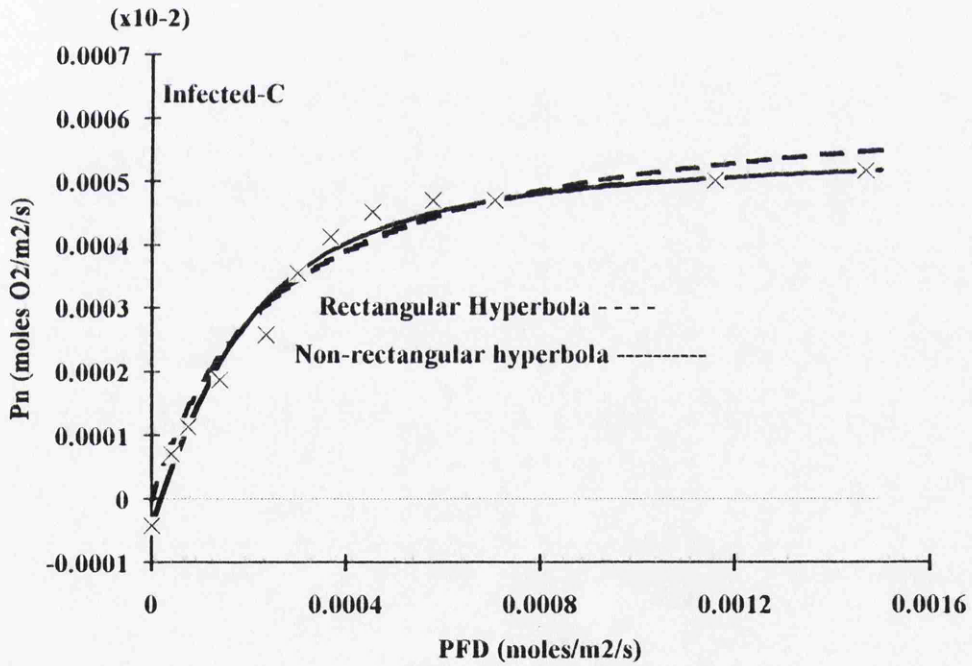




N. edwardsonii (10 days):

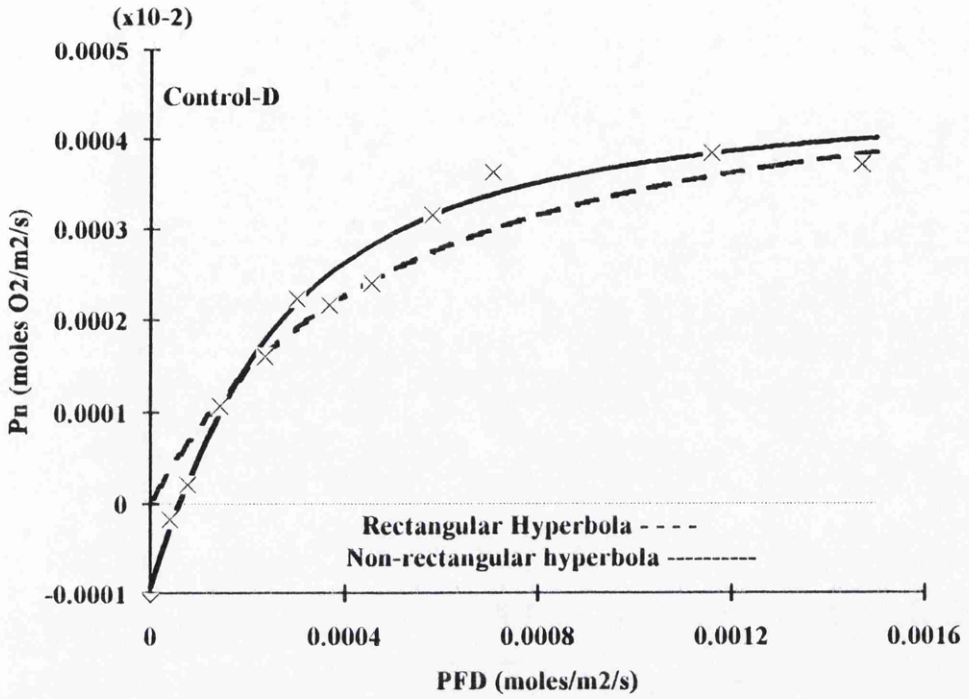
Control-B Rabinowich	Infected-B Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 3.806E-06	P max= 3.610E-06
α = 3.447E-02	α = 1.783E-03
Error= 3.283E-07	Error= 3.701E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -6.229E-07	Rd= -6.890E-07
Pmax= 3.359E-06	Pmax= 3.673E-06
α = 2.429E-02	α = 6.030E-03
θ = 8.931E-01	θ = 5.099E-01
Error= 1.171E-06	Error= 2.146E-06

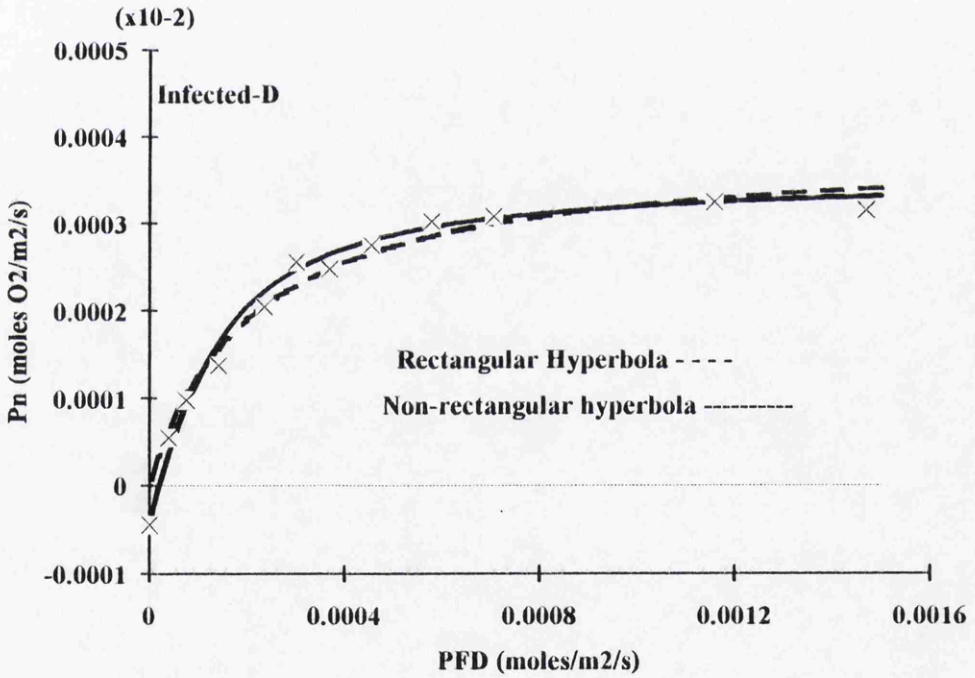




N. edwardsonii (10 days):

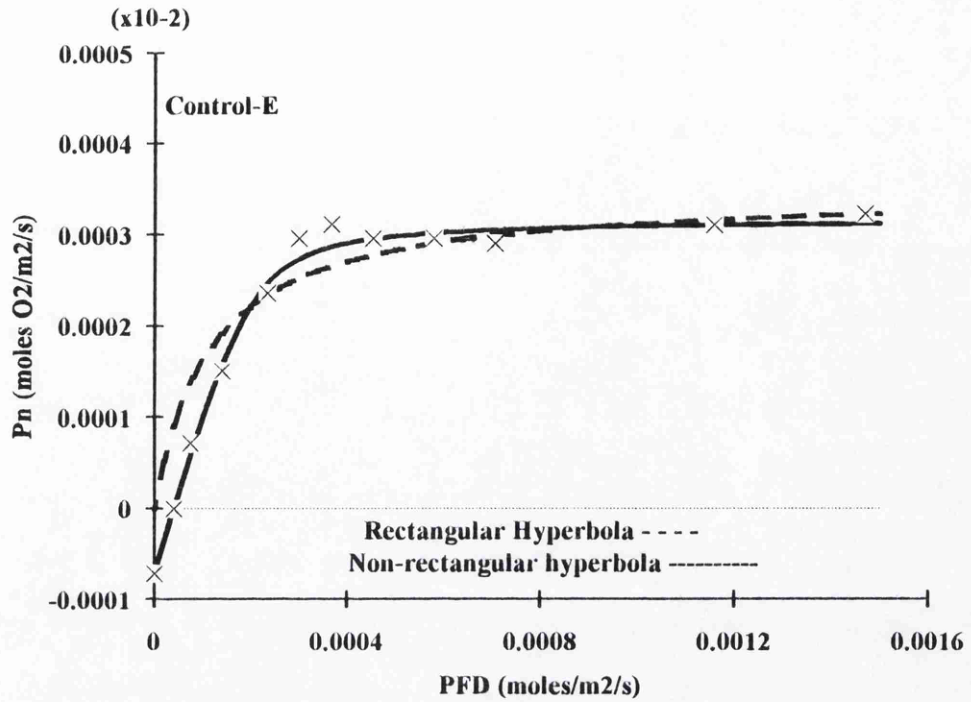
Control-C Rabinowich	Infected-C Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 4.046E-06	P max= 6.464E-06
α = 1.689E-02	α = 2.450E-03
Error= 1.321E-06	Error= 3.413E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -2.553E-07	Rd= -4.182E-07
Pmax= 4.172E-06	Pmax= 5.755E-06
α = 1.869E-02	α = 2.332E-03
θ = 1.778E-01	θ = 6.159E-01
Error= 1.001E-06	Error= 1.815E-06

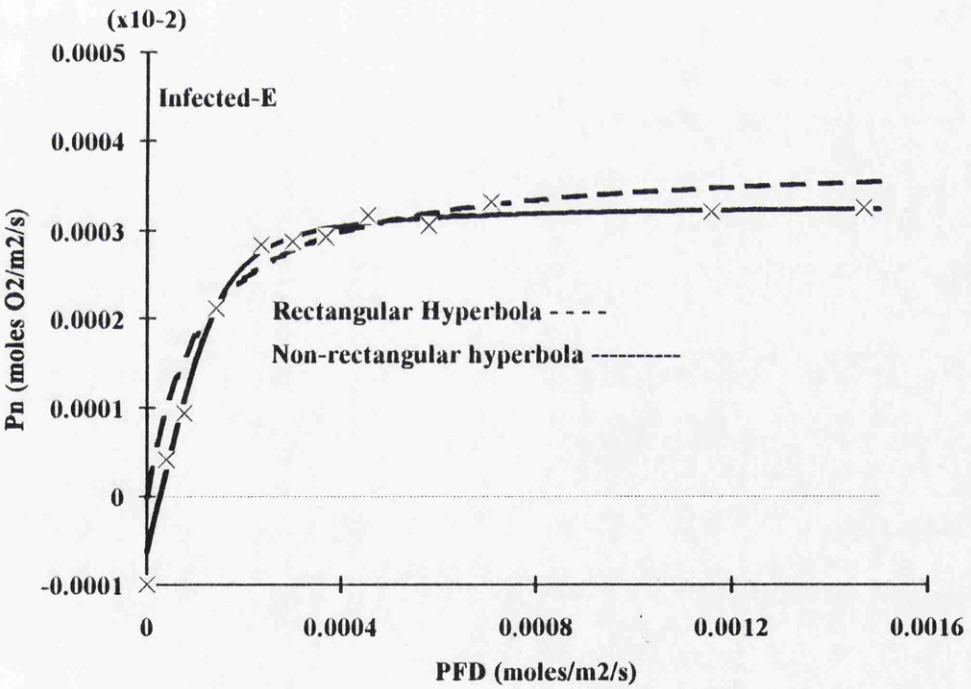




N. edwardsonii (10 days):

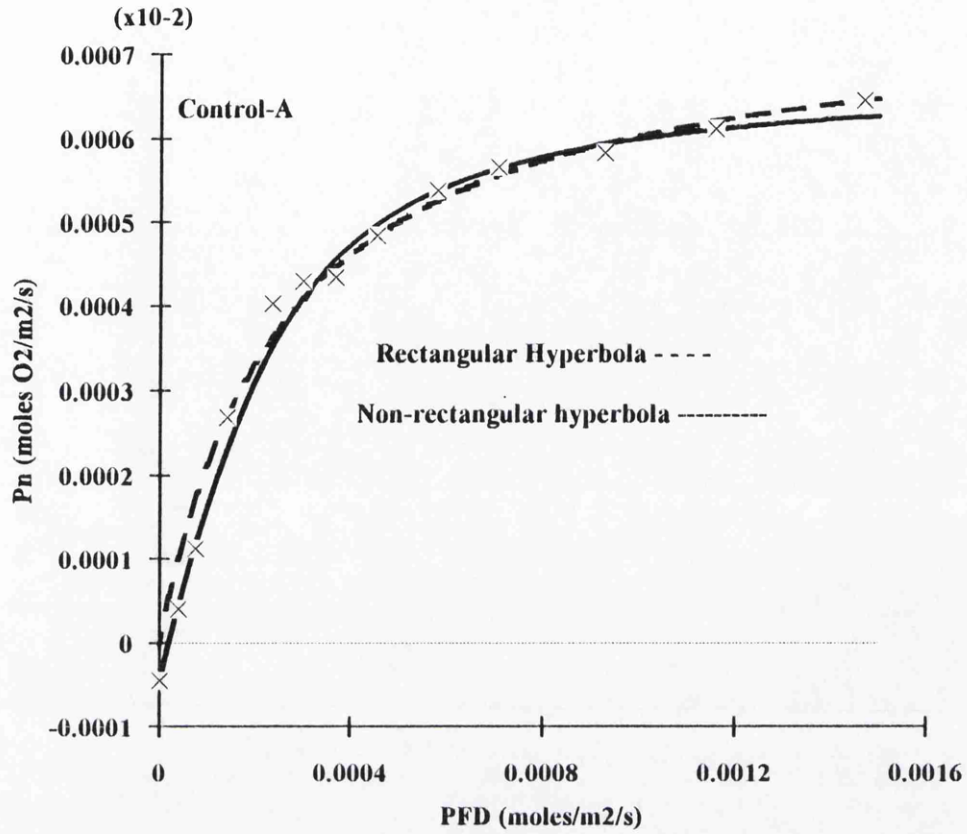
Control-D	Infected-D
Rabinowich	Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 5.179E-06	P max= 3.882E-06
α = 1.009E-02	α = 1.863E-03
Error= 3.935E-06	Error= 1.682E-06
Marshall & Biscoe	Marshall & Biscoe
Rd= -9.320E-07	Rd= -4.260E-07
Pmax= 5.129E-06	Pmax= 3.737E-06
α = 1.745E-02	α = 2.208E-03
θ = 4.668E-01	θ = 5.295E-01
Error= 1.898E-06	Error= 1.279E-06

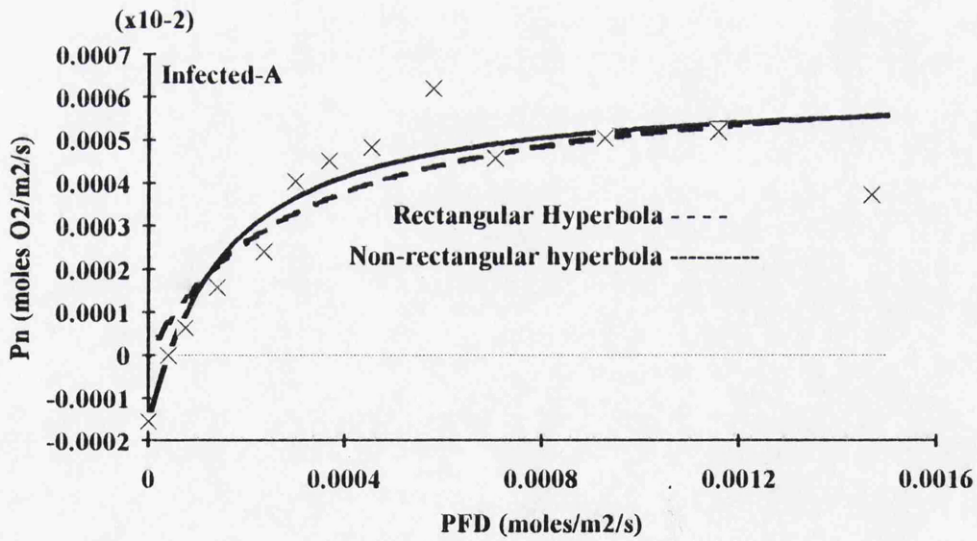




N. edwardsonii (10 days):

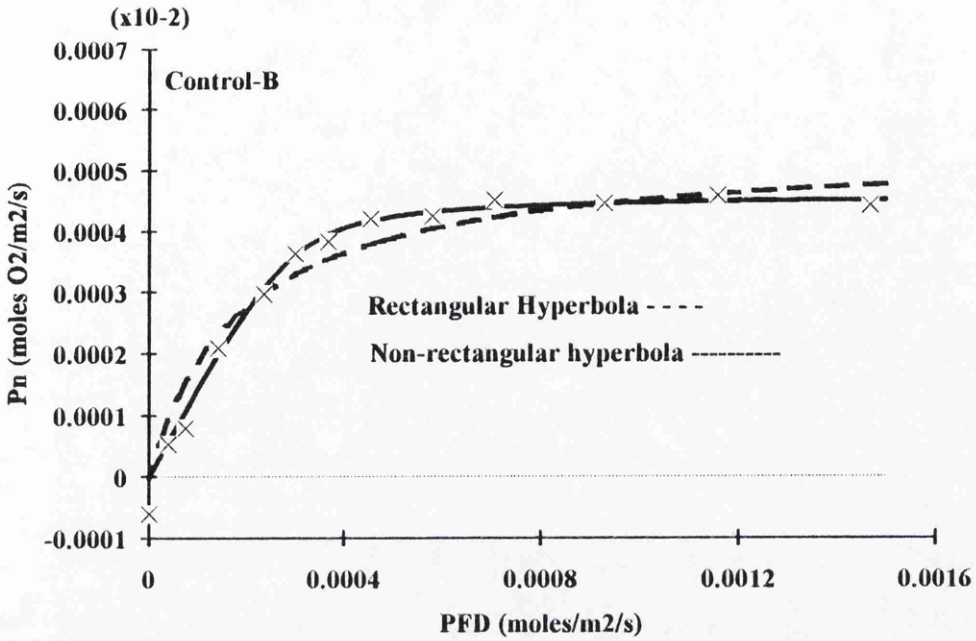
Control-E Rabinowich	Infected-E Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 3.477E-06	P max= 3.806E-06
α = 3.048E-02	α = 3.447E-03
Error= 3.990E-06	Error= 3.383E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -6.969E-07	Rd= -6.229E-07
Pmax= 3.213E-06	Pmax= 3.359E-06
α = 1.764E-02	α = 2.429E-03
θ = 9.320E-01	θ = 8.931E-01
Error= 1.125E-06	Error= 1.171E-06

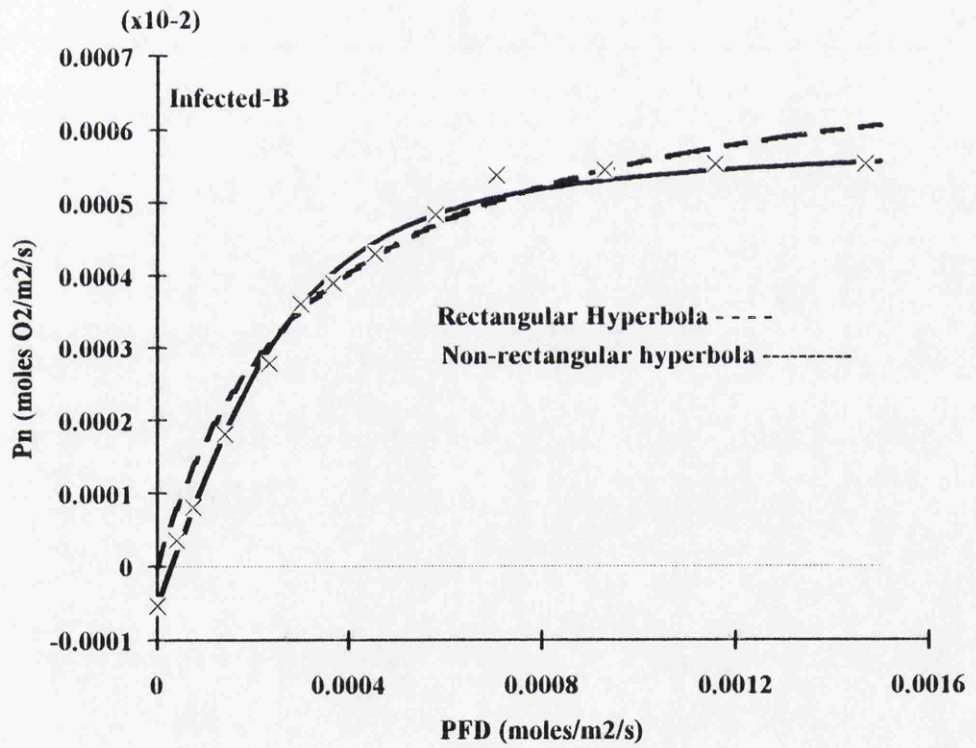




N. edwardsonii (25 days):

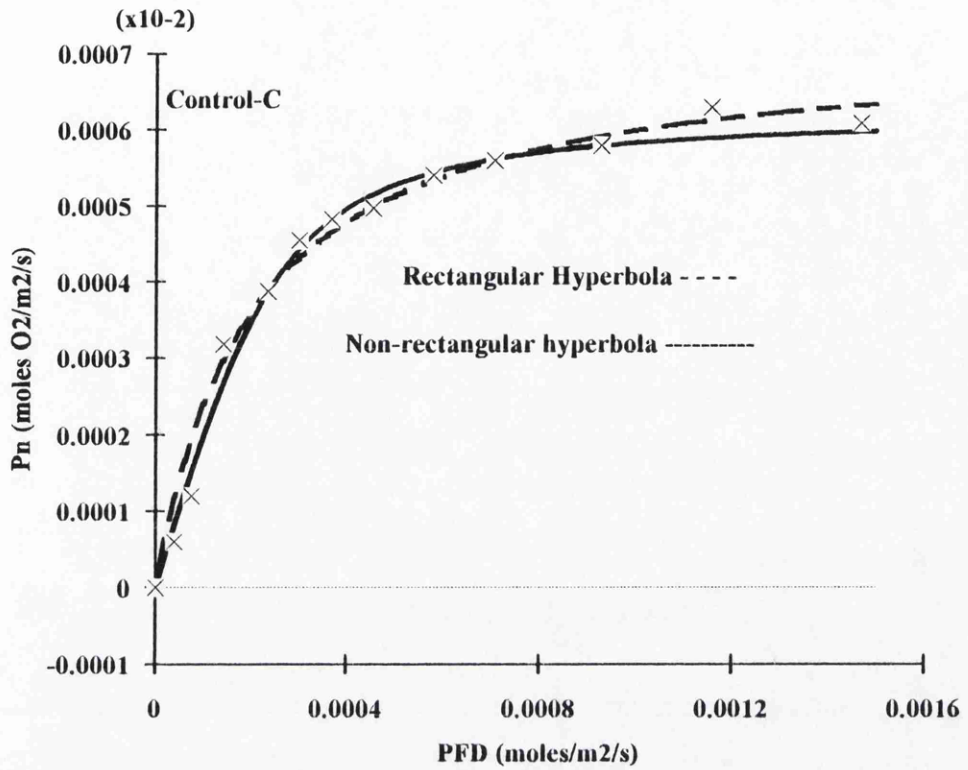
Control-A Rabinowich	Infected-A Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 7.578E-06	P max= 6.746E-06
α = 2.957E-03	α = 2.160E-03
Error= 2.823E-06	Error= 1.029E-05
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -4.399E-07	Rd= -1.548E-06
Pmax= 6.906E-06	Pmax= 7.474E-06
α = 2.325E-03	α = 4.610E-03
θ = 6.874E-01	θ = 1.900E-01
Error= 1.846E-06	Error= 7.097E-06

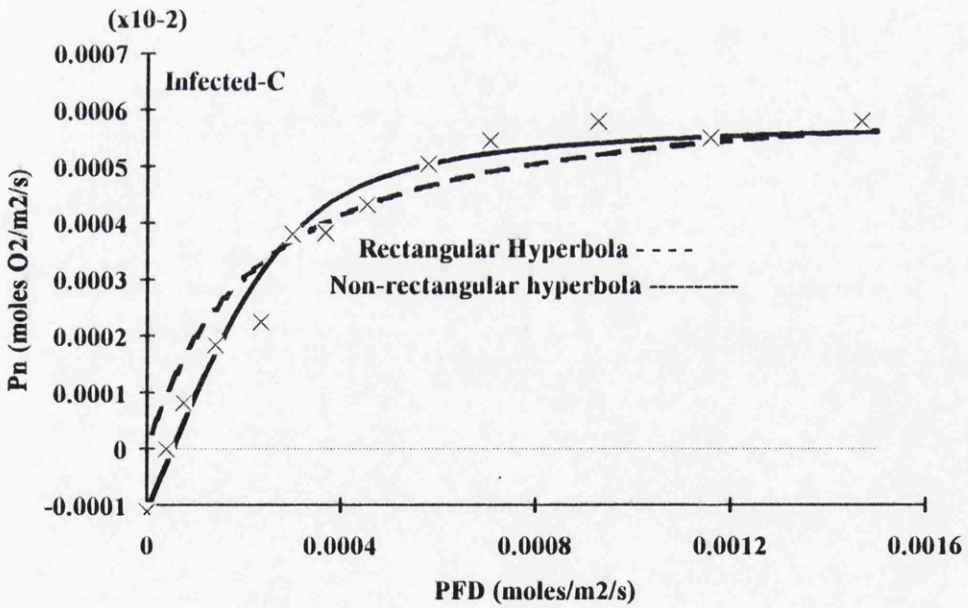




N. edwardsonii (25 days):

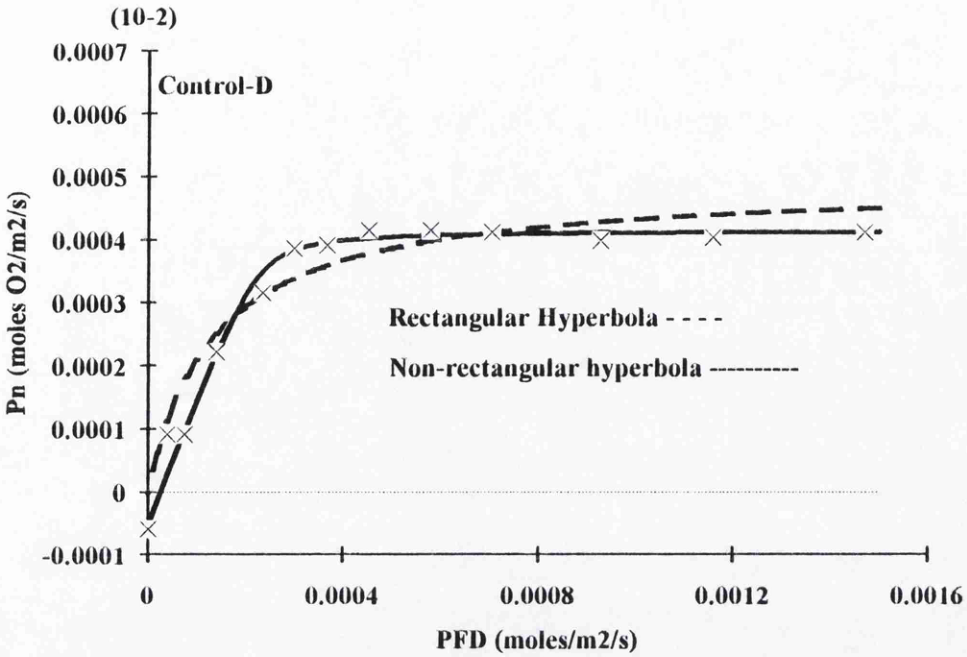
Control-B Rabinowich	Infected-B Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 5.374E-06	P max= 7.411E-06
α = 2.838E-02	α = 2.210E-02
Error= 3.806E-06	Error= 3.568E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= 0.000E+00	Rd= -5.377E-07
Pmax= 4.586E-06	Pmax= 6.000E-06
α = 1.451E-02	α = 1.854E-02
θ = 9.444E-01	θ = 8.047E-01
Error= 1.683E-06	Error= 1.289E-06

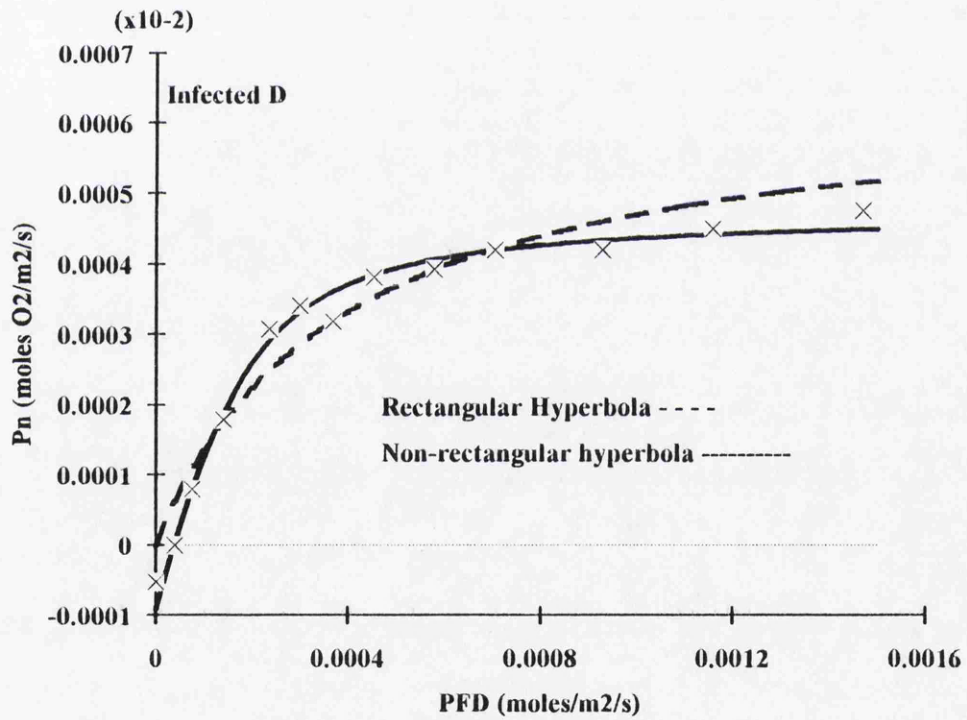




N. edwardsonii (25 days):

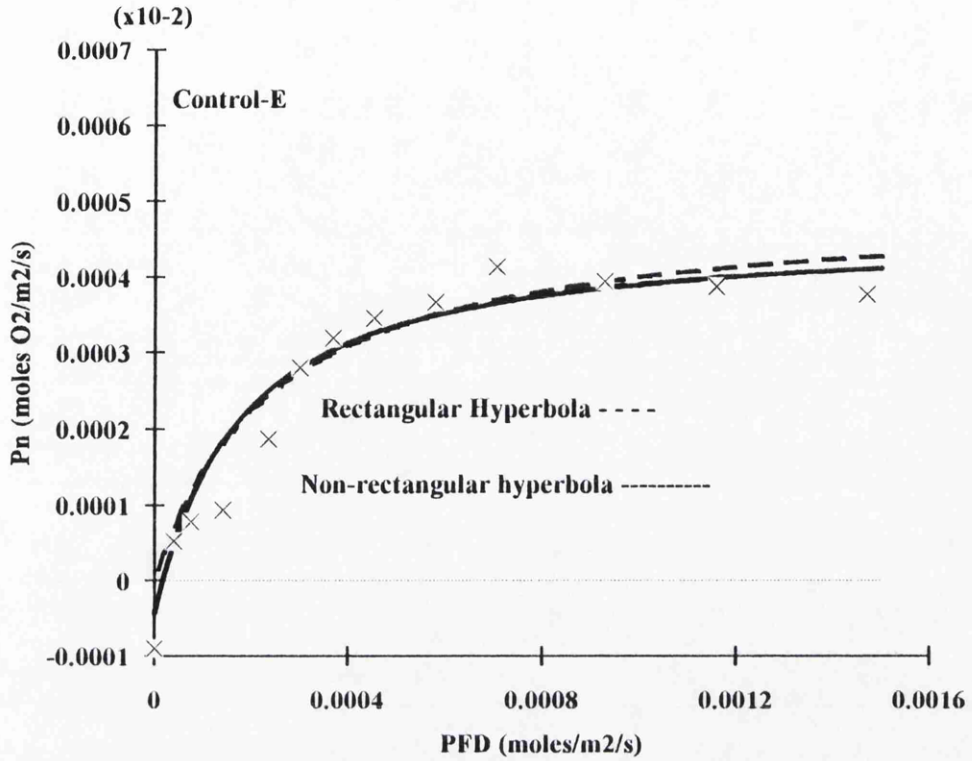
Control-C Rabinowich	Infected-C Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 7.192E-06	P max= 6.500E-06
α = 3.592E-03	α = 2.841E-03
Error= 2.582E-06	Error= 6.604E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= 0.000E+00	Rd= -1.106E-06
Pmax= 6.241E-06	Pmax= 6.000E-06
α = 2.165E-03	α = 2.176E-03
θ = 8.100E-01	θ = 8.637E-01
Error= 1.963E-06	Error= 3.300E-06

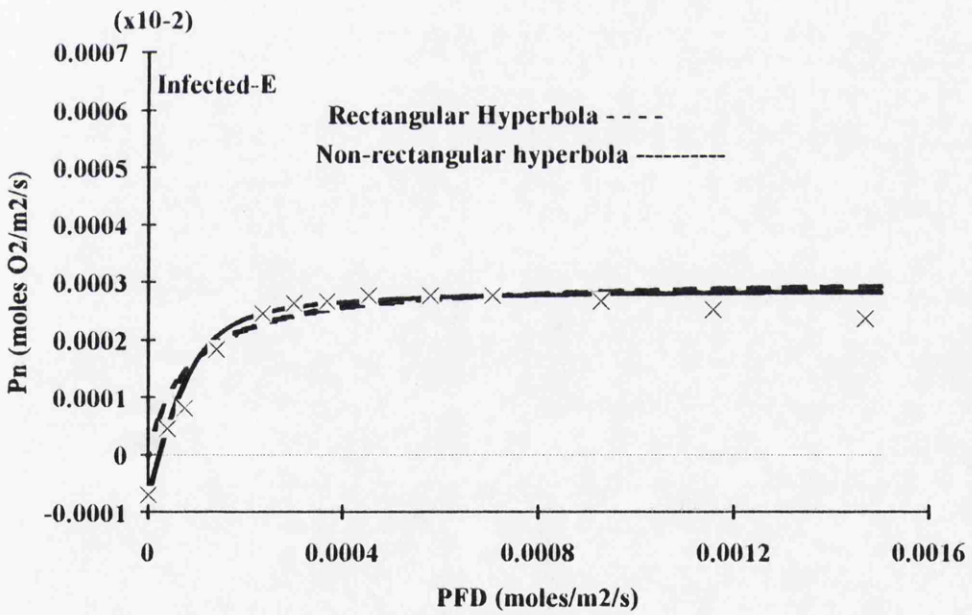




N. edwardsonii (25 days):

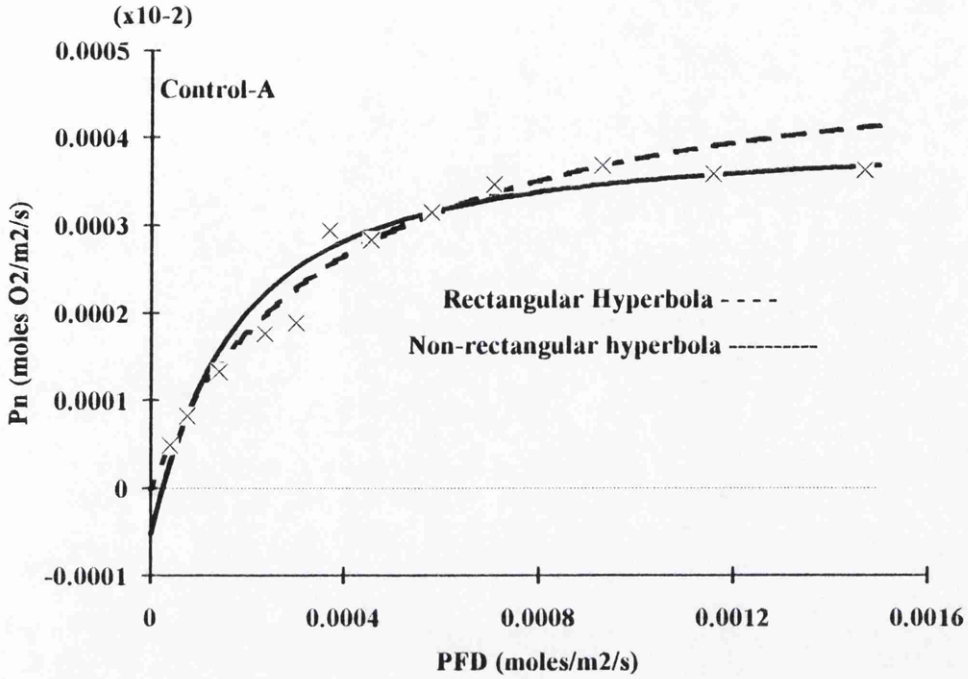
Control-D Rabinowich	Infected-D Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 6.500E-06	P max= 4.907E-06
α = 1.704E-02	α = 3.646E-02
Error= 4.123E-06	Error= 4.299E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -9.117E-07	Rd= -5.018E-07
Pmax= 4.954E-06	Pmax= 4.158E-06
α = 2.473E-02	α = 1.965E-02
θ = 7.518E-01	θ = 9.750E-01
Error= 1.870E-06	Error= 1.638E-06

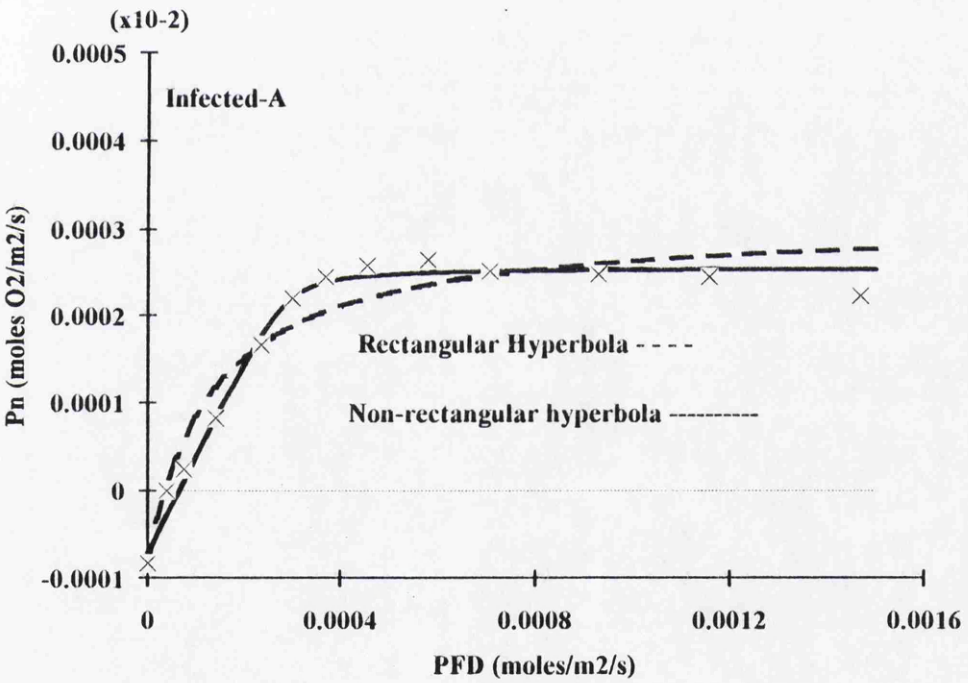




N. edwardsonii (25 days):

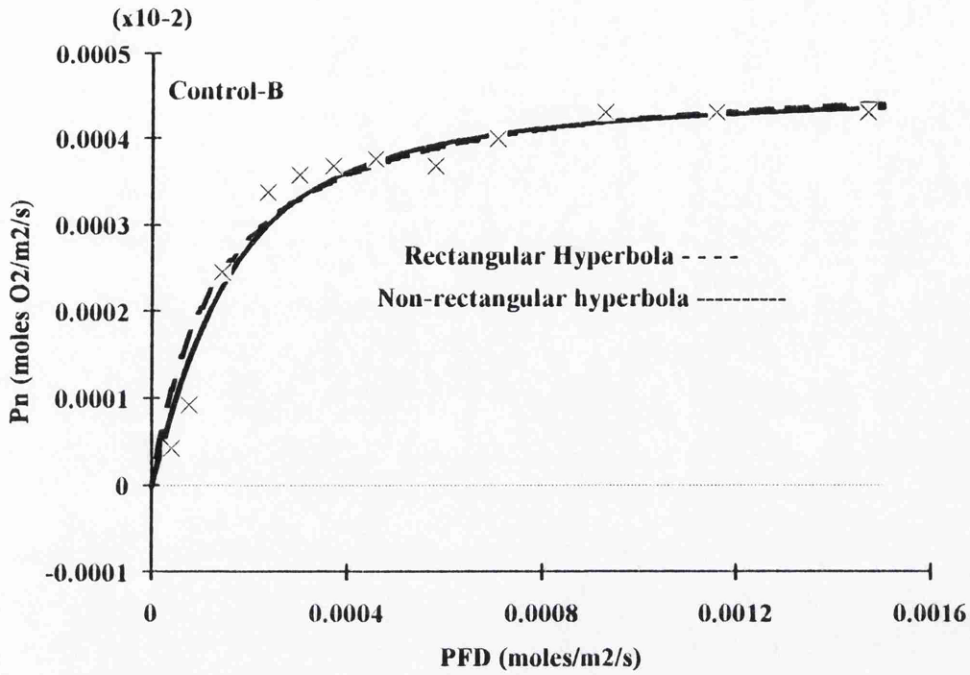
Control-E Rabinowich	Infected-E Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 4.971E-06	P max= 3.097E-06
α = 2.043E-03	α = 3.658E-03
Error= 4.734E-06	Error= 3.810E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -4.353E-07	Rd= -6.995E-07
Pmax= 5.000E-06	Pmax= 3.009E-06
α = 2.817E-03	α = 3.177E-03
θ = 1.020E-01	θ = 8.170E-01
Error= 3.885E-06	Error= 1.748E-06

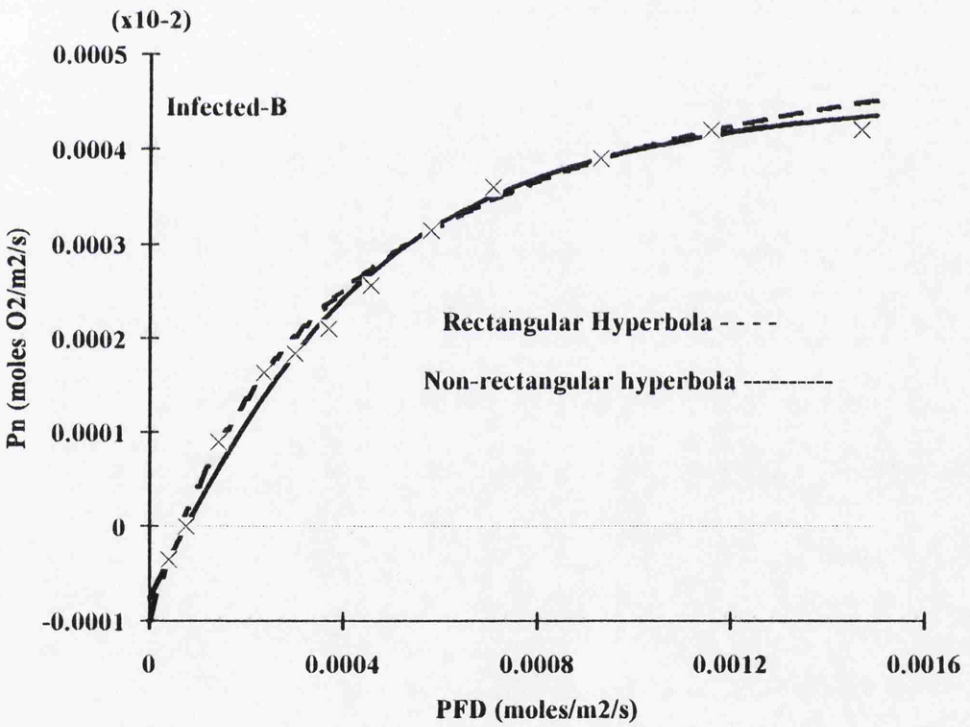




N. edwardsonii (30 days):

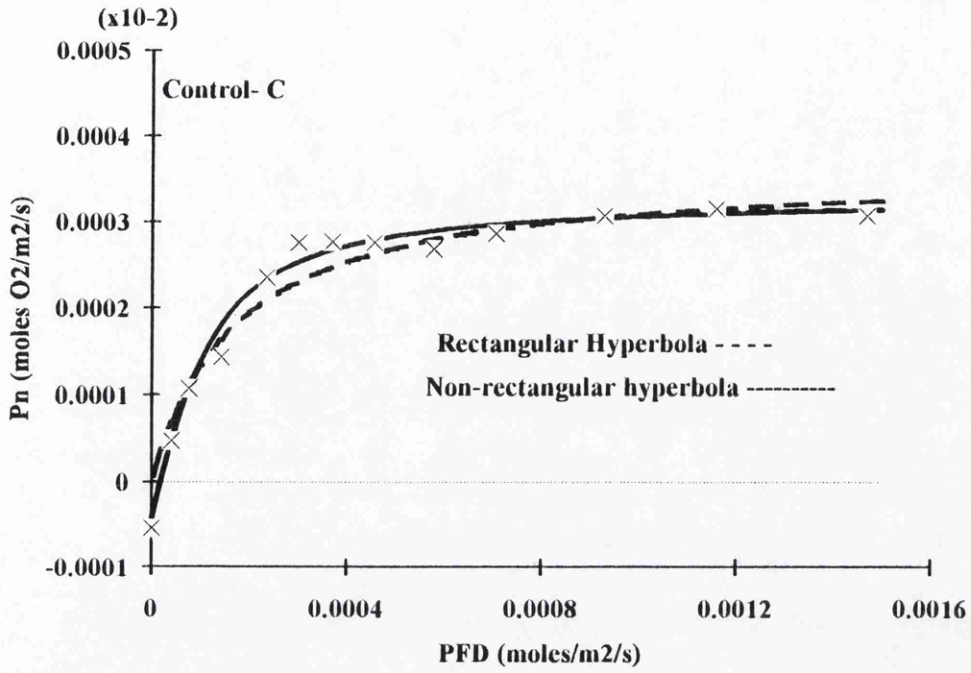
Control-A Rabinowich	Infected-A Rabinowich
Rd= 0.00E+00	Rd= -8.26E-07
P max= 5.182E-06	P max= 3.928E-06
α = 1.358E-02	α = 2.912E-03
Error= 2.052E-06	Error= 3.094E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -5.149E-07	Rd= -7.256E-07
Pmax= 4.365E-06	Pmax= 2.566E-06
α = 2.244E-02	α = 1.118E-03
θ = 3.806E-01	θ = 9.832E-01
Error= 2.285E-06	Error= 1.388E-06

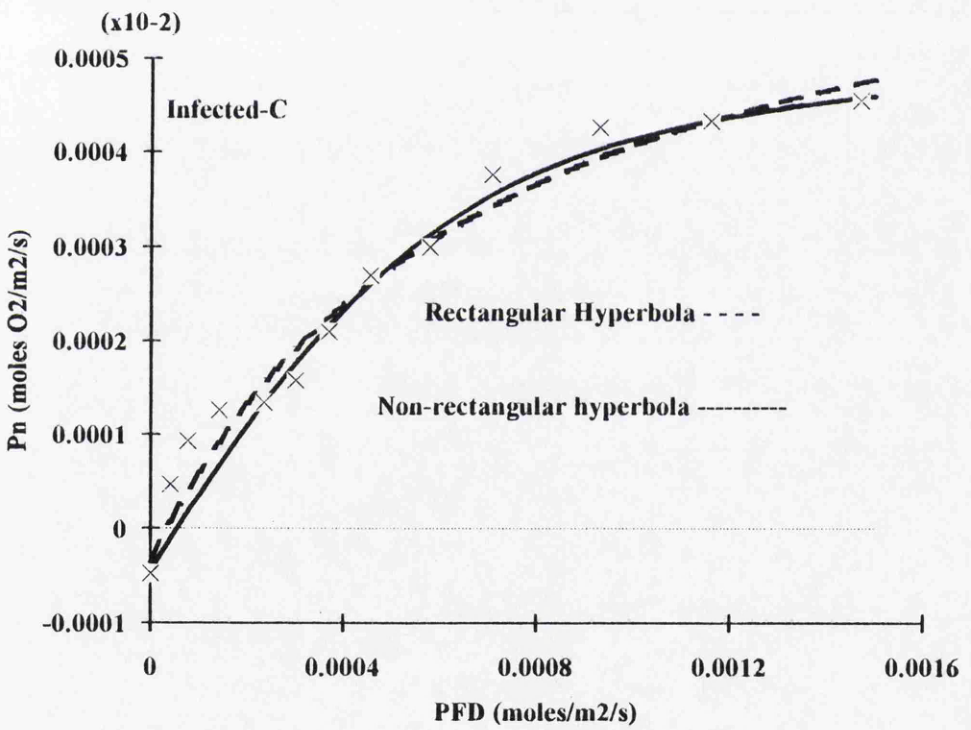




N. edwardsonii (30 days):

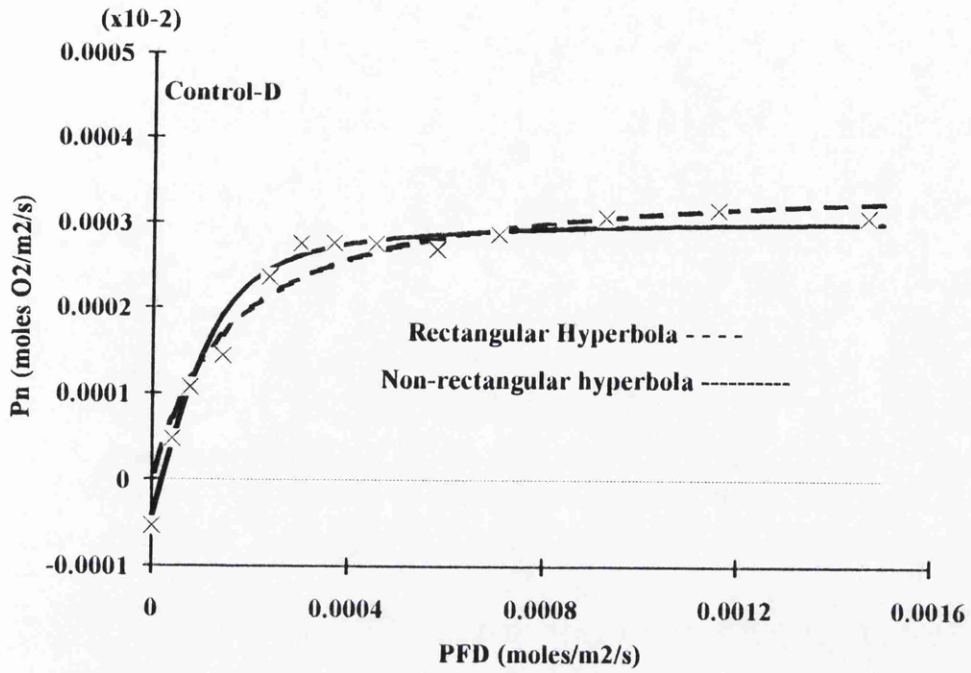
Control-B Rabinowich	Infected-B Rabinowich
Rd= 0.00E+00	Rd= -9.92E-07
P max= 4.806E-06	P max= 6.932E-06
α = 3.537E-02	α = 1.780E-03
Error= 2.759E-06	Error= 1.465E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= 0.000E+00	Rd= -7.568E-07
Pmax= 4.622E-06	Pmax= 5.162E-06
α = 2.244E-02	α = 1.045E-02
θ = 5.906E-01	θ = 7.510E-01
Error= 2.398E-06	Error= 1.568E-06

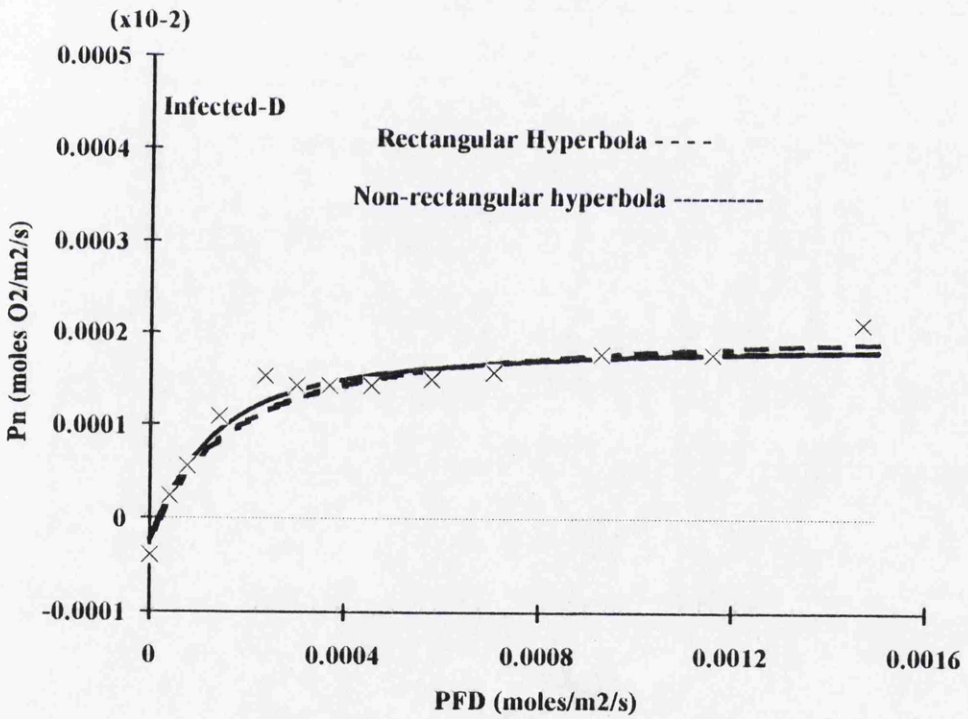




N. edwardsonii (30 days):

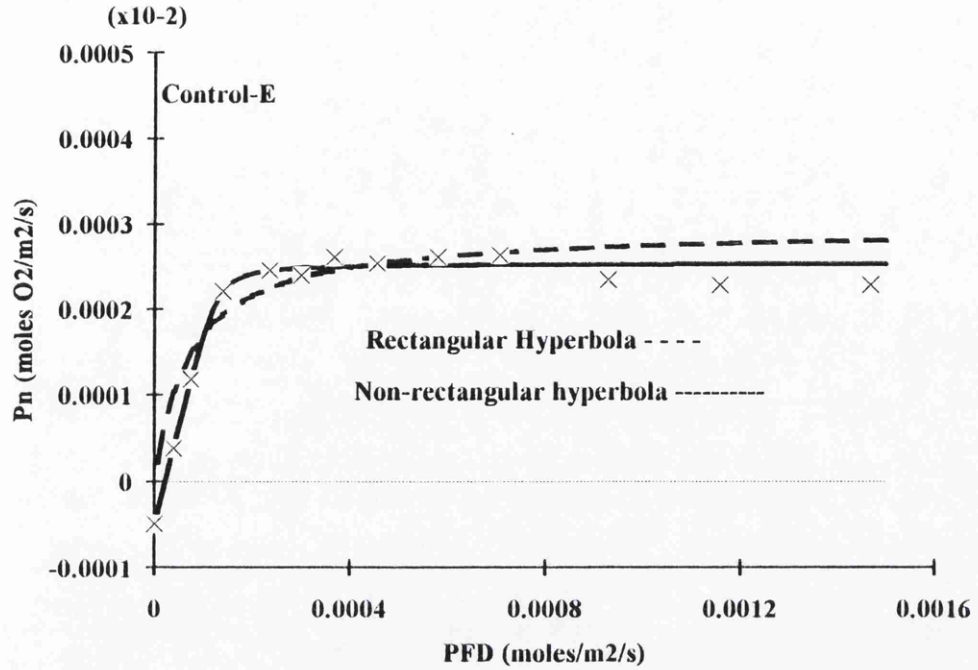
Control-C Rabinowich	Infected-C Rabinowich
Rd= 0.00E+00	Rd= -3.40E-07
P max= 3.623E-06	P max= 7.487E-06
α = 2.406E-03	α = 1.076E-03
Error= 1.909E-06	Error= 3.311E-05
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -4.257E-07	Rd= -4.321E-07
Pmax= 3.460E-06	Pmax= 5.367E-06
α = 2.536E-04	α = 8.195E-03
θ = 6.014E-01	θ = 8.033E-01
Error= 1.214E-06	Error= 2.911E-06

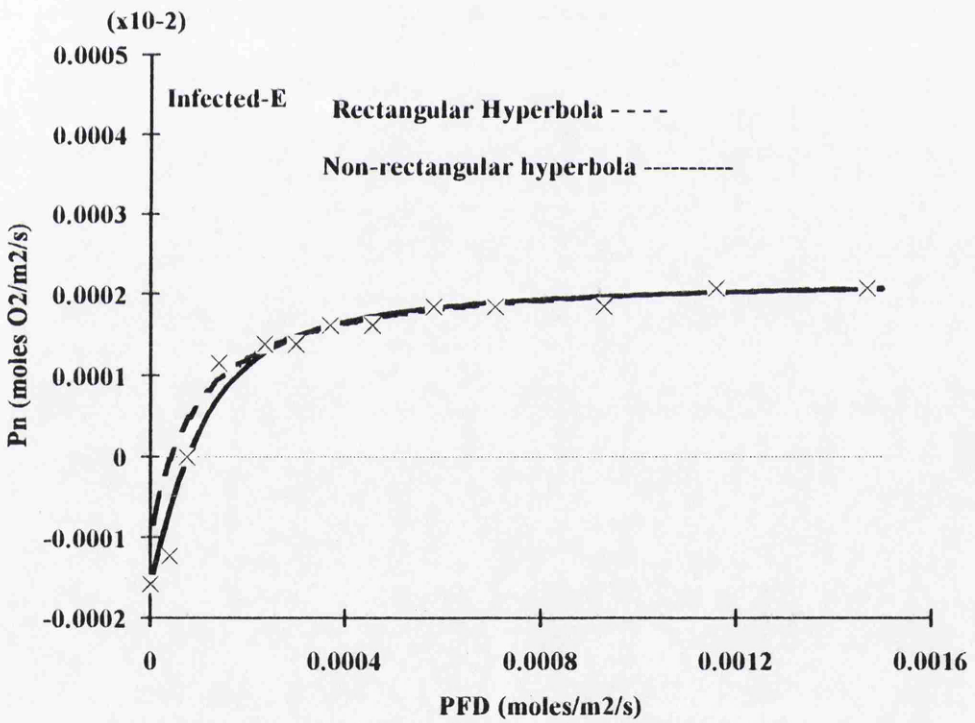




N. edwardsonii (30 days):

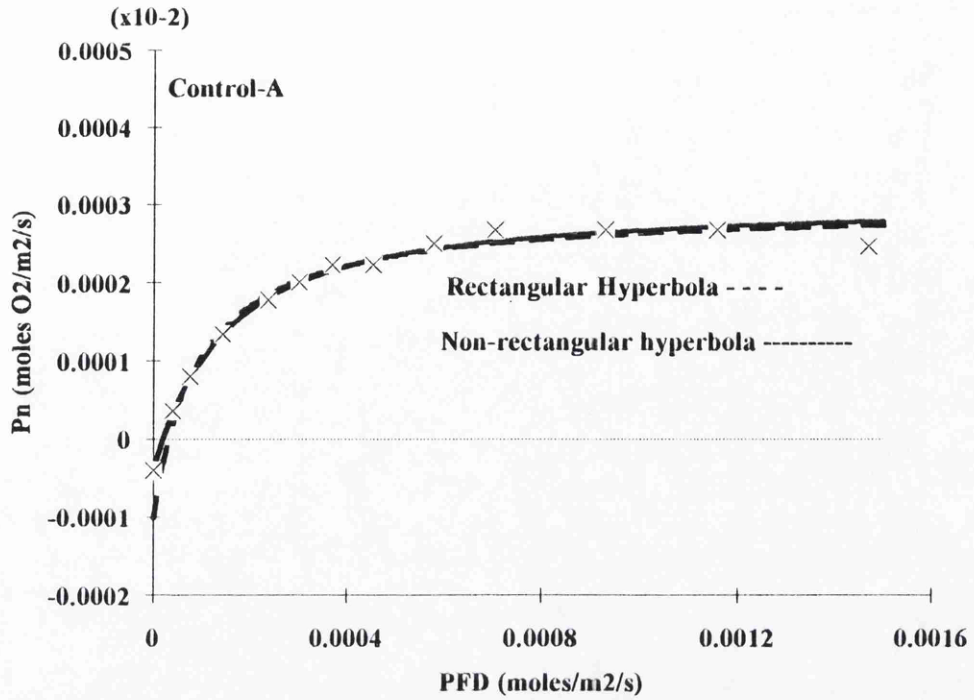
Control-D Rabinowich	Infected-D Rabinowich
Rd= 0.00E+00	Rd= -2.67E-07
P max= 3.589E-06	P max= 2.400E-06
α = 2.252E-02	α = 1.435E-03
Error= 1.918E-06	Error= 1.649E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -4.341E-07	Rd= -2.267E-07
Pmax= 3.154E-06	Pmax= 2.050E-06
α = 2.296E-02	α = 1.380E-03
θ = 8.100E-01	θ = 4.477E-01
Error= 1.440E-06	Error= 1.358E-06

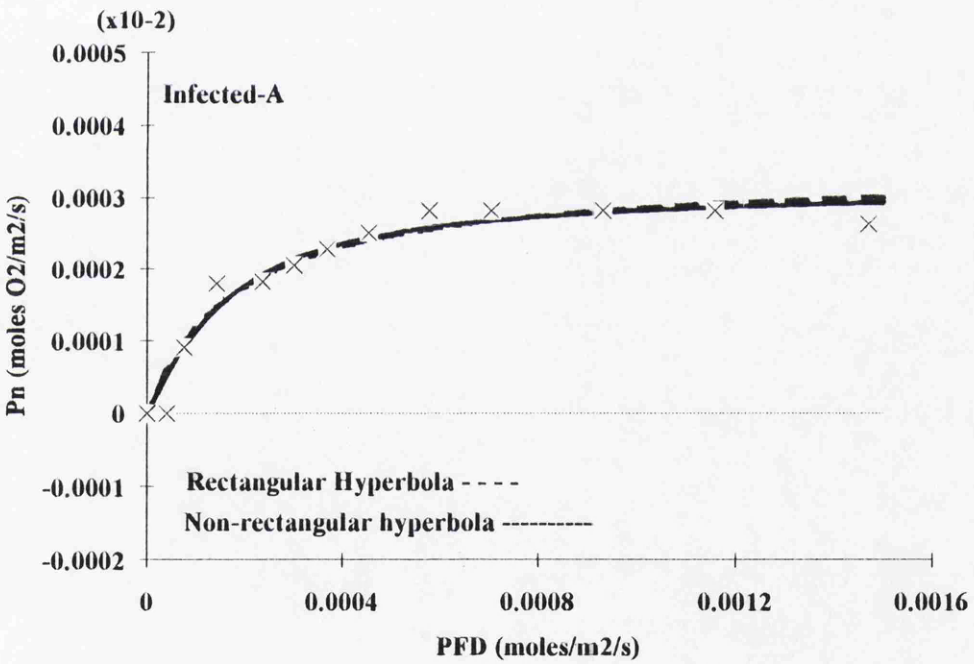




N. edwardsonii (30 days):

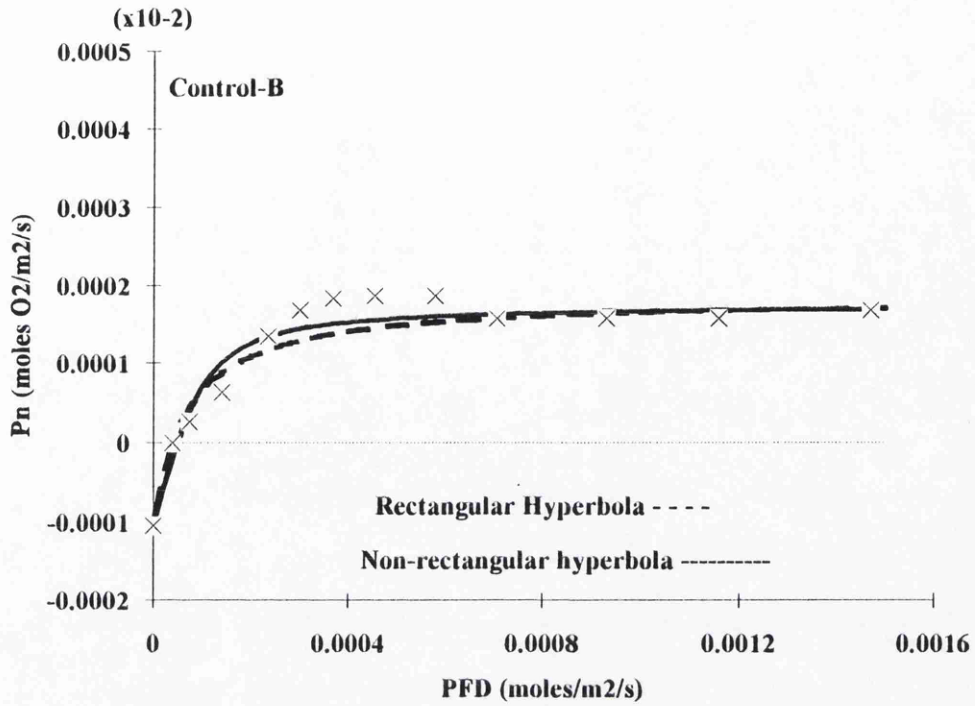
Control-E Rabinowich	Infected-E Rabinowich
Rd= 0.00E+00	Rd= -1.00E-06
P max= 2.952E-06	P max= 3.275E-06
α = 3.999E-03	α = 3.402E-03
Error= 3.071E-06	Error= 2.916E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -4.997E-07	Rd= -1.580E-06
Pmax= 2.551E-06	Pmax= 2.776E-06
α = 2.296E-02	α = 2.580E-03
θ = 9.763E-01	θ = 6.363E-01
Error= 1.133E-06	Error= 1.596E-06

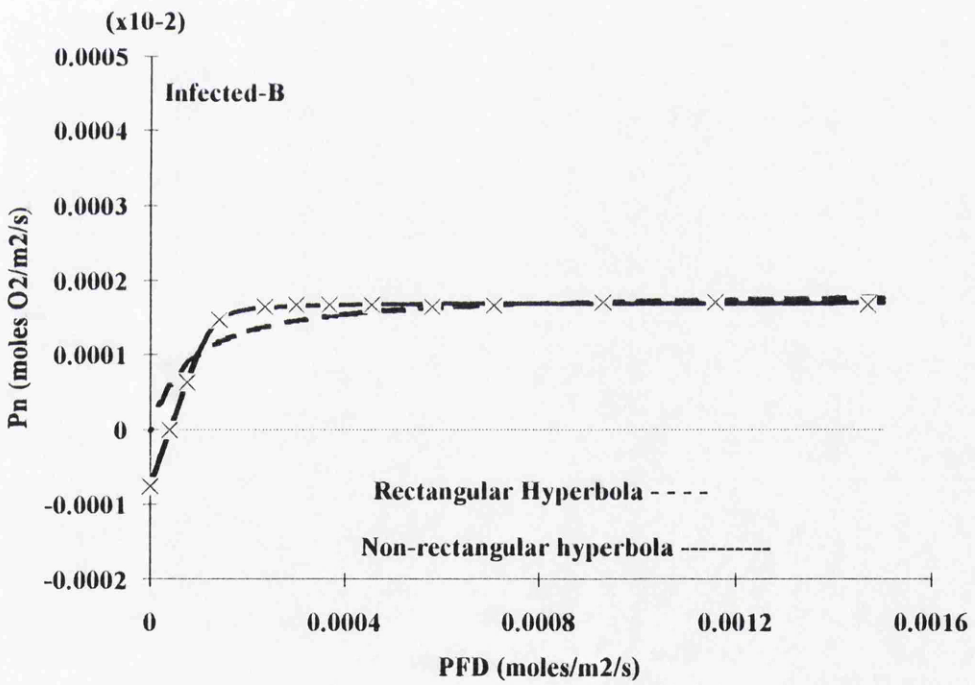




N. edwardsonii (40 days):

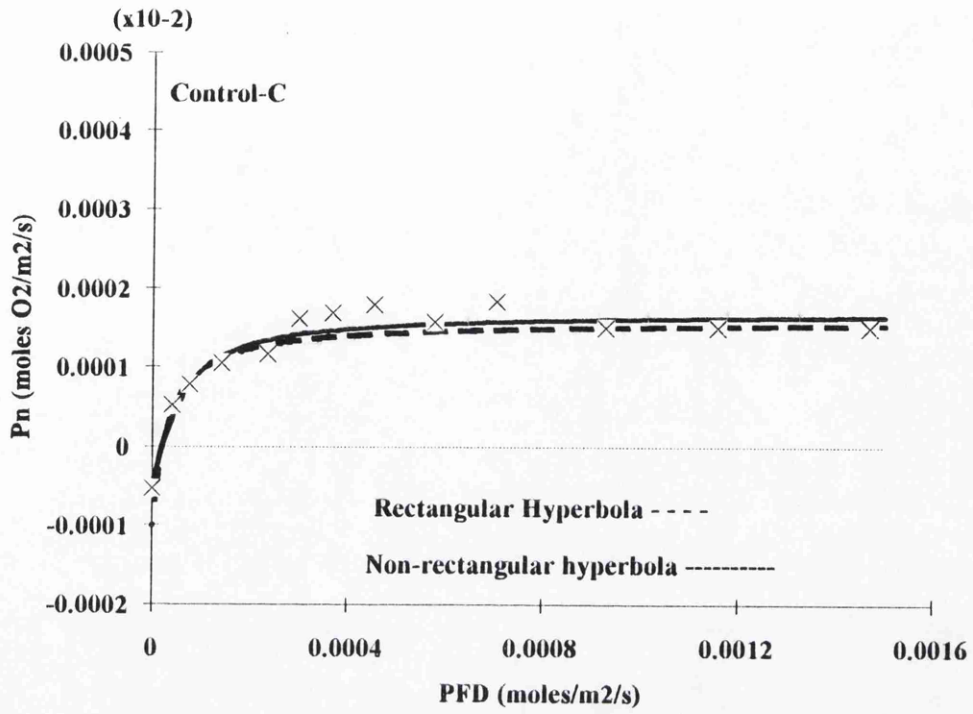
Control-A Rabinowich	Infected-A Rabinowich
Rd= -1.00E-06	Rd= 0.00E+00
P max= 0.000E+00	P max= 3.382E-06
α = 4.287E-02	α = 1.839E-02
Error= 1.656E-06	Error= 2.107E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -3.810E-07	Rd= 0.000E+00
Pmax= 3.312E-06	Pmax= 3.163E-06
α = 2.099E-02	α = 1.463E-02
θ = 2.923E-01	θ = 4.939E-01
Error= 8.557E-07	Error= 1.884E-06

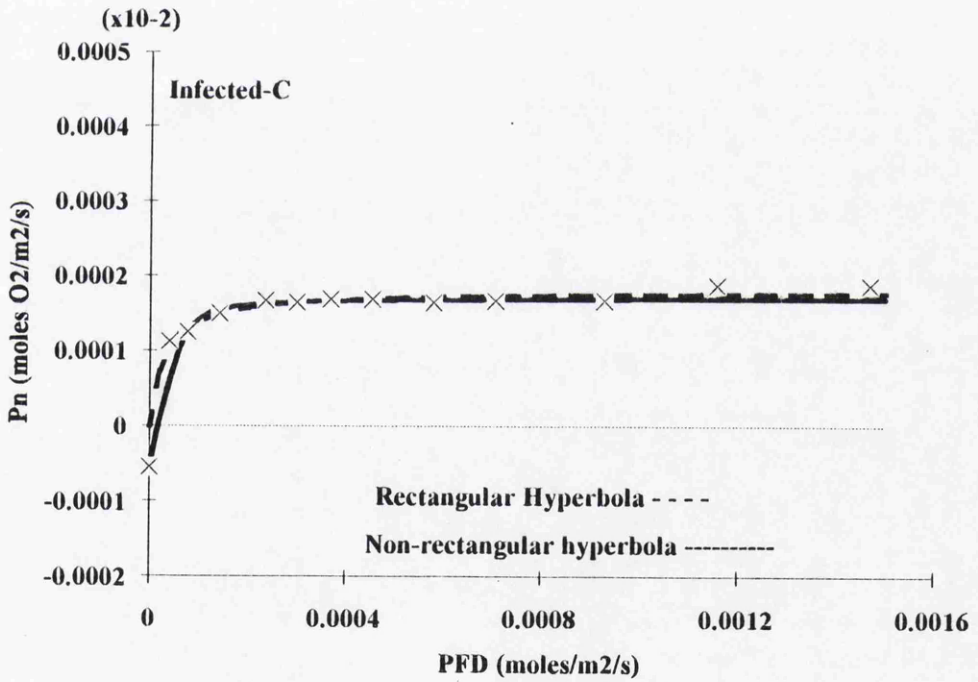




N. edwardfsonii (40 days):

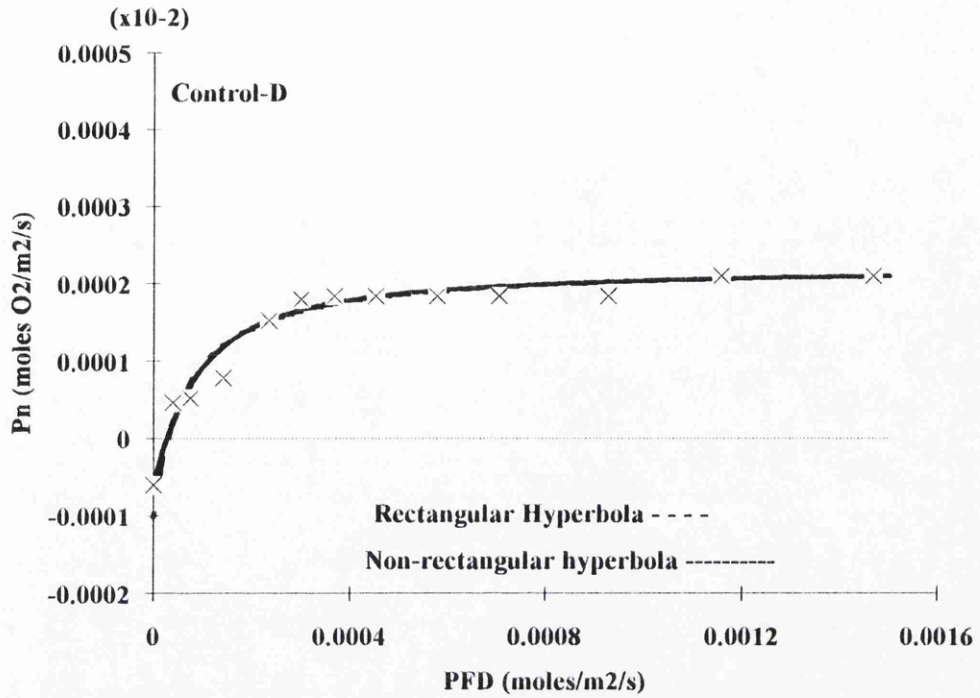
Control-B Rabinowich	Infected-B Rabinowich
Rd= -1.00E-06	Rd= 0.00E+00
P max= 2.842E-06	P max= 1.873E-06
α = 3.892E-02	α = 2.185E-02
Error= 2.453E-06	Error= 2.783E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -1.050E-06	Rd= -7.527E-07
Pmax= 1.990E-06	Pmax= 1.716E-06
α = 2.411E-02	α = 1.886E-02
θ = 7.680E-01	θ = 9.787E-01
Error= 2.101E-06	Error= 1.515E-06

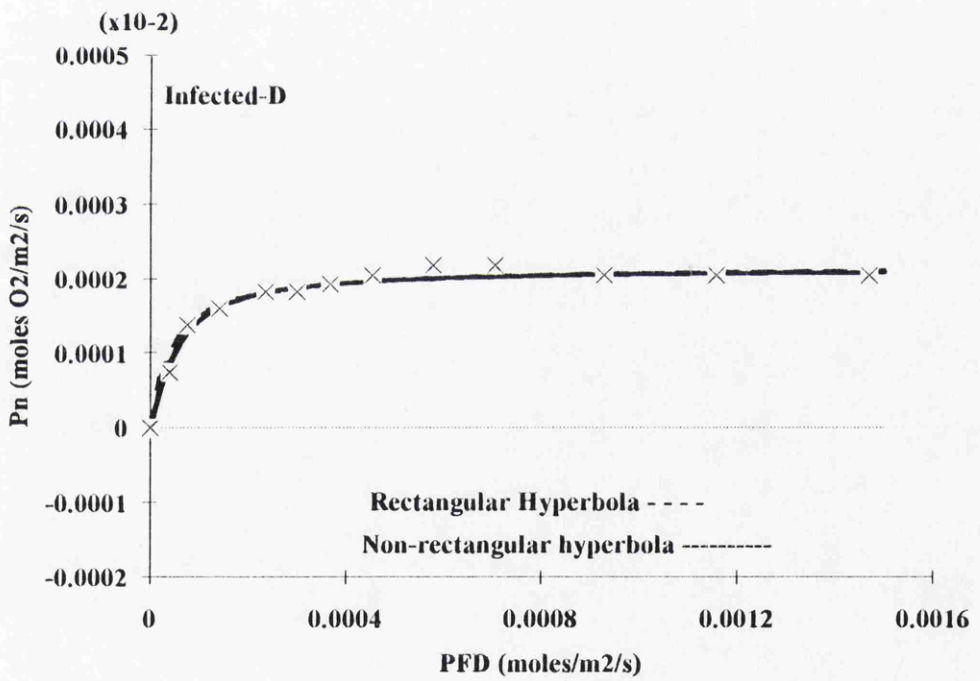




N. edwardsonii (40 days):

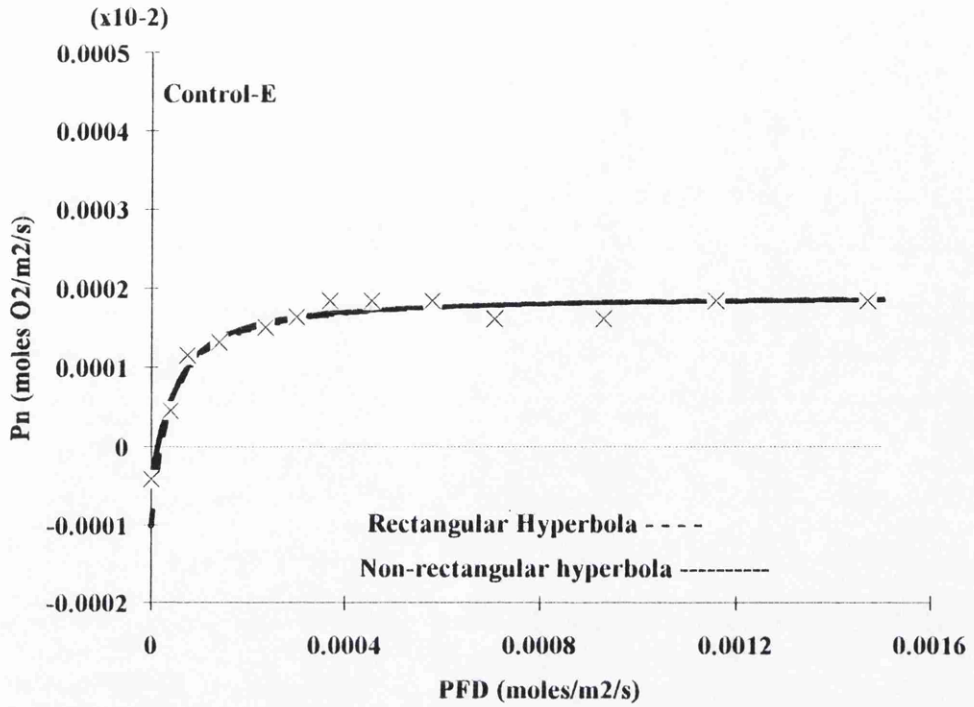
Control-C Rabinowich	Infected-C Rabinowich
Rd= -1.00E-06	Rd= 0.00E+00
P max= 2.586E-06	P max= 1.811E-06
α = 8.180E-02	α = 6.054E-02
Error= 2.271E-06	Error= 1.223E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -5.251E-07	Rd= -5.377E-07
Pmax= 2.005E-06	Pmax= 1.753E-06
α = 3.194E-02	α = 3.165E-02
θ = 4.232E-01	θ = 9.170E-01
Error= 1.780E-06	Error= 1.086E-06

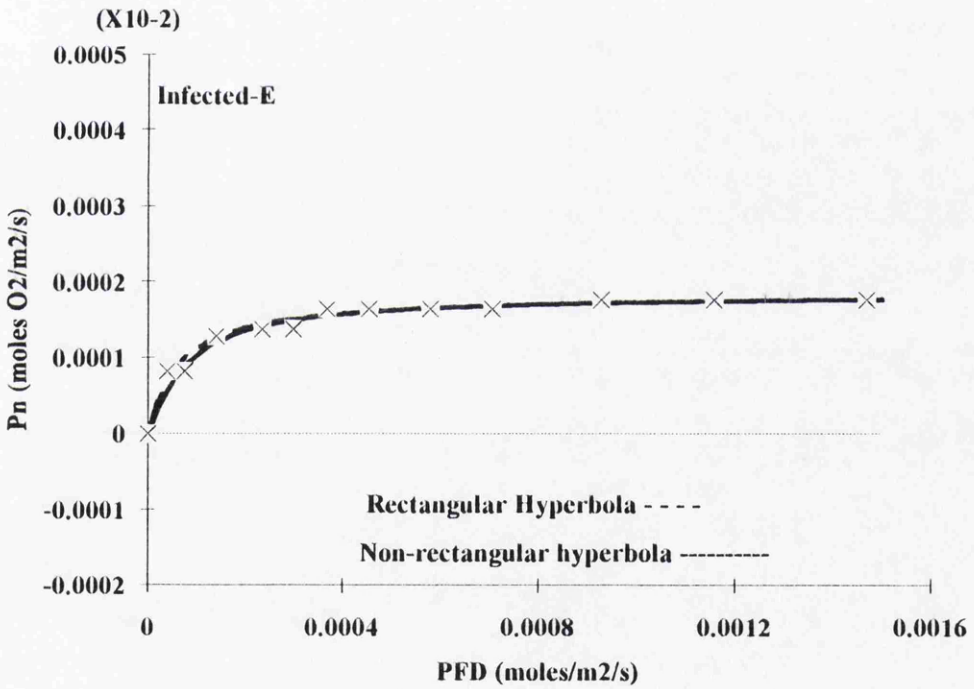




N. edwardsonii (40 days):

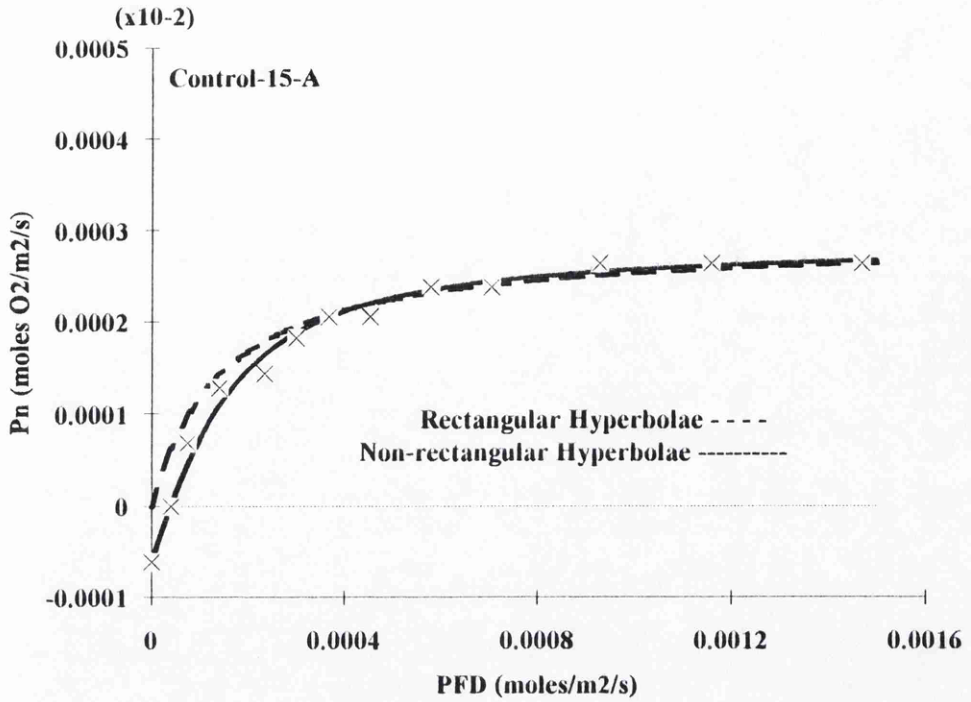
Control-D Rabinowich	Infected-D Rabinowich
Rd= -1.00E-06	Rd= 0.00E+00
P max= 3.242E-06	P max= 2.175E-06
α = 4.837E-02	α = 4.710E-02
Error= 1.937E-06	Error= 9.037E-07
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -6.002E-07	Rd= 0.000E+00
Pmax= 2.588E-06	Pmax= 2.146E-06
α = 2.570E-02	α = 2.818E-02
θ = 3.970E-01	θ = 5.167E-01
Error= 1.464E-06	Error= 7.876E-07

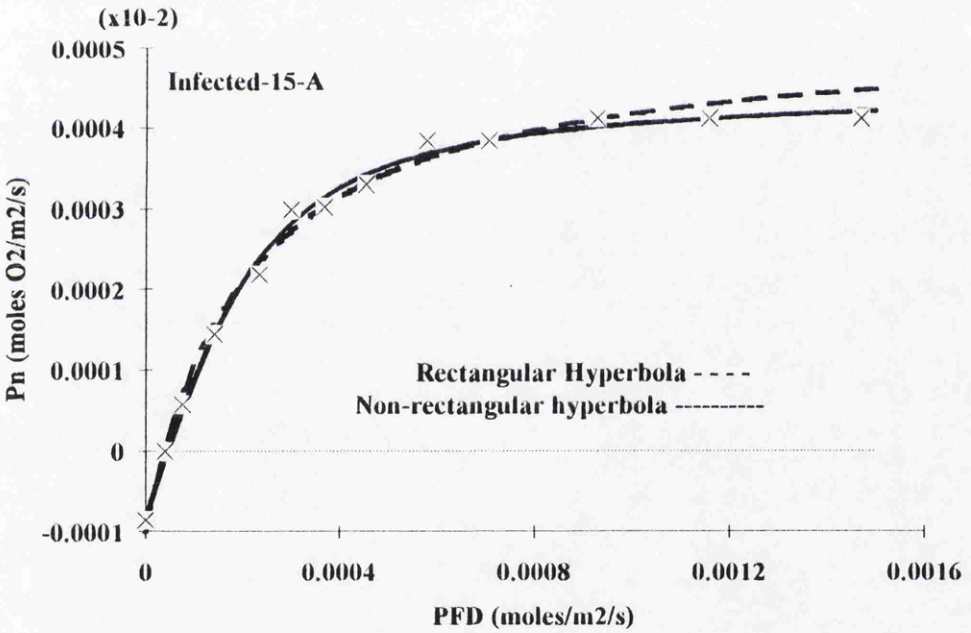




N. edwardsonii (40 ddays):

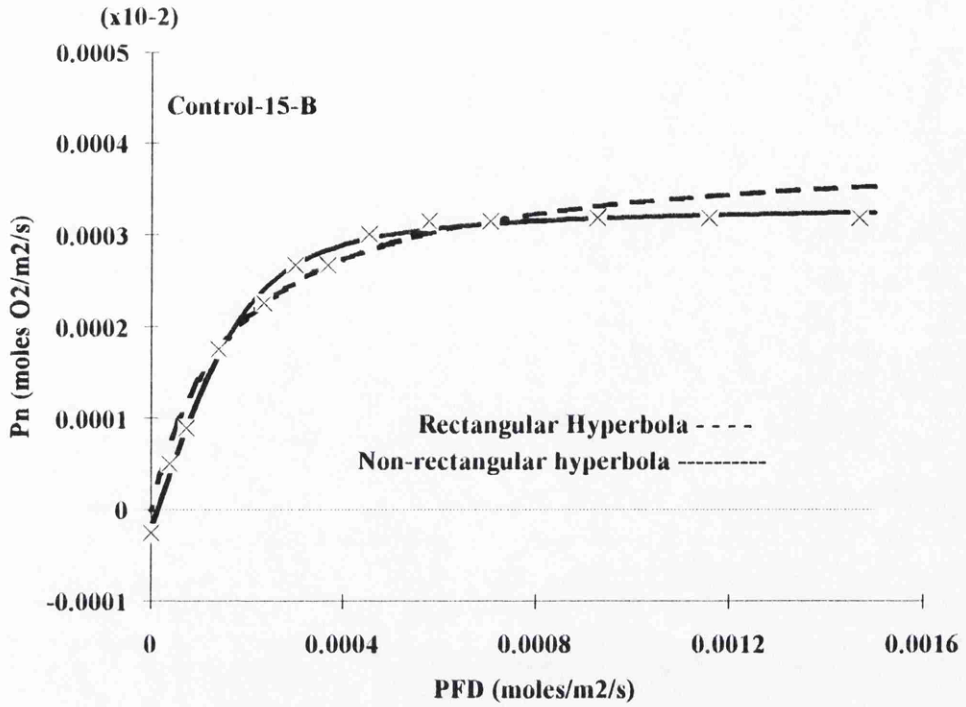
Control-E Rabinowich	Infected-E Rabinowich
Rd= -1.00E-06	Rd= 0.00E+00
P max= 2.932E-06	P max= 1.839E-06
α = 8.122E-02	α = 2.773E-02
Error= 1.696E-06	Error= 7.503E-07
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -4.106E-07	Rd= 0.000E+00
Pmax= 2.139E-06	Pmax= 1.835E-06
α = 6.238E-02	α = 1.812E-02
θ = 4.360E-01	θ = 3.910E-01
Error= 1.130E-06	Error= 7.991E-07

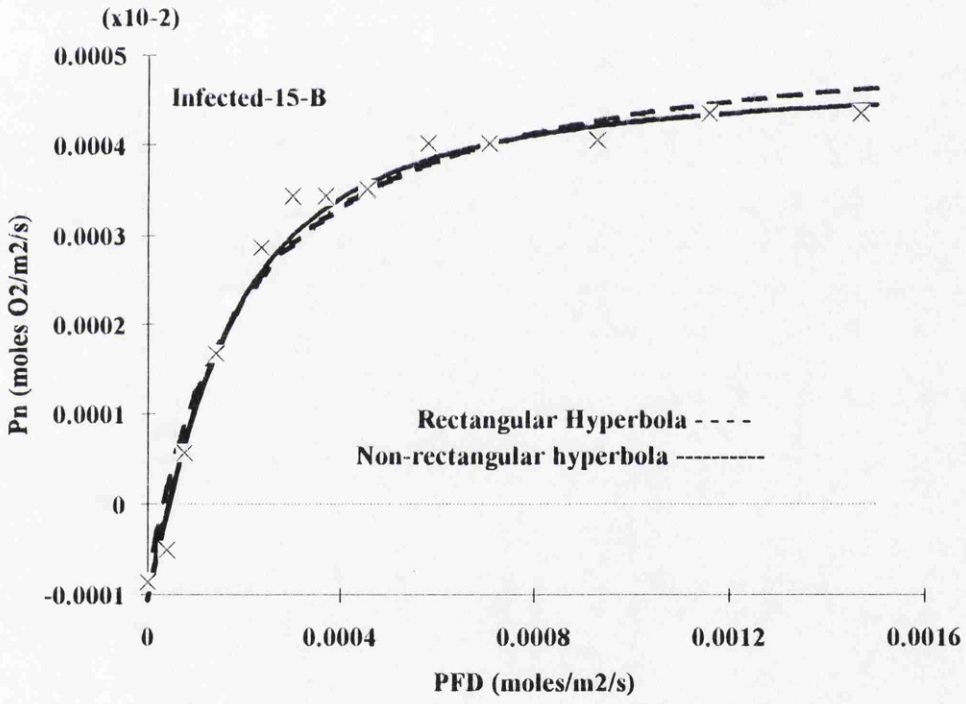




N. glutinosa (15 days):

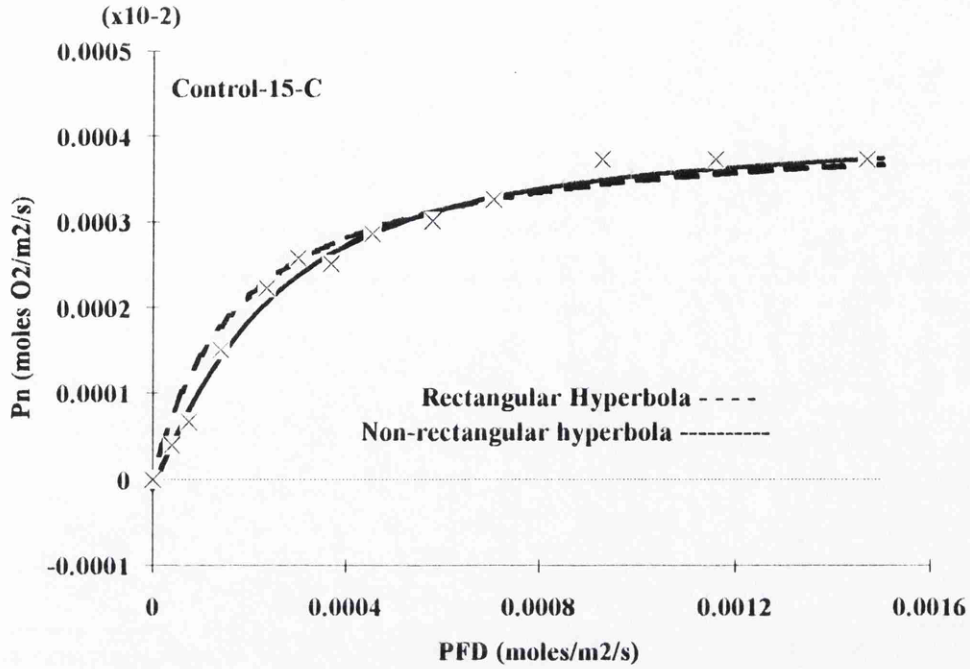
Control-A Rabinowich	Infected-A Rabinowich
Rd= 0.00E+00	Rd= -1.00E-06
P max= 4.770E-06	P max= 6.219E-06
α = 1.994E-02	α = 3.091E-02
Error= 3.363E-06	Error= 1.626E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -8.236E-07	Rd= -8.389E-07
Pmax= 4.252E-06	Pmax= 4.729E-06
α = 2.035E-02	α = 1.948E-02
θ 7.680E-01	θ 7.267E-01
Error= 1.047E-06	Error= 1.172E-06

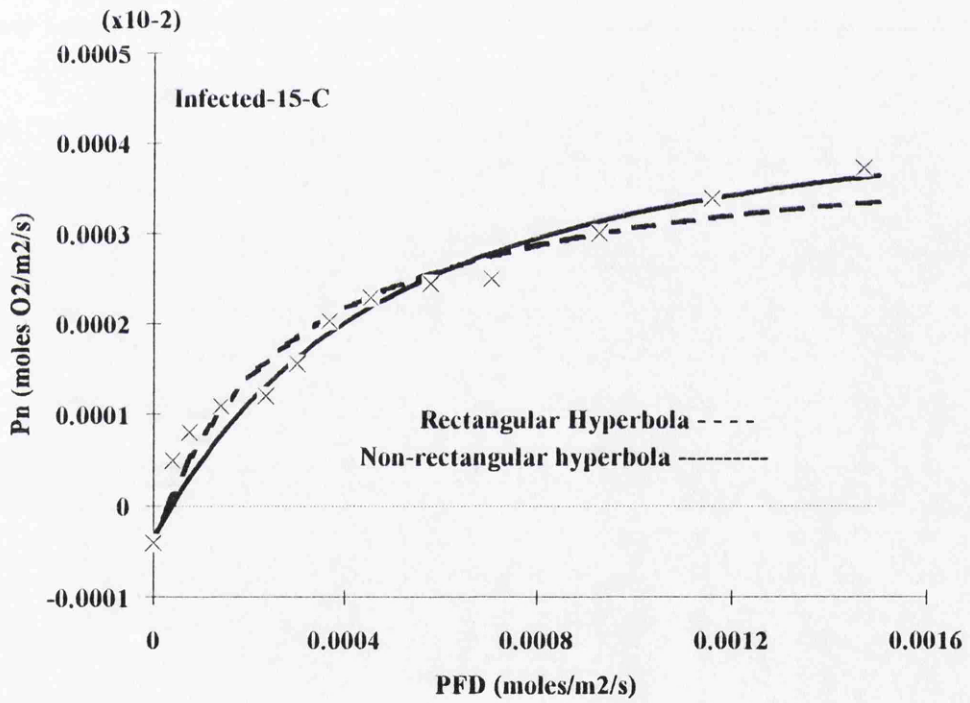




N. glutinosa (15 days):

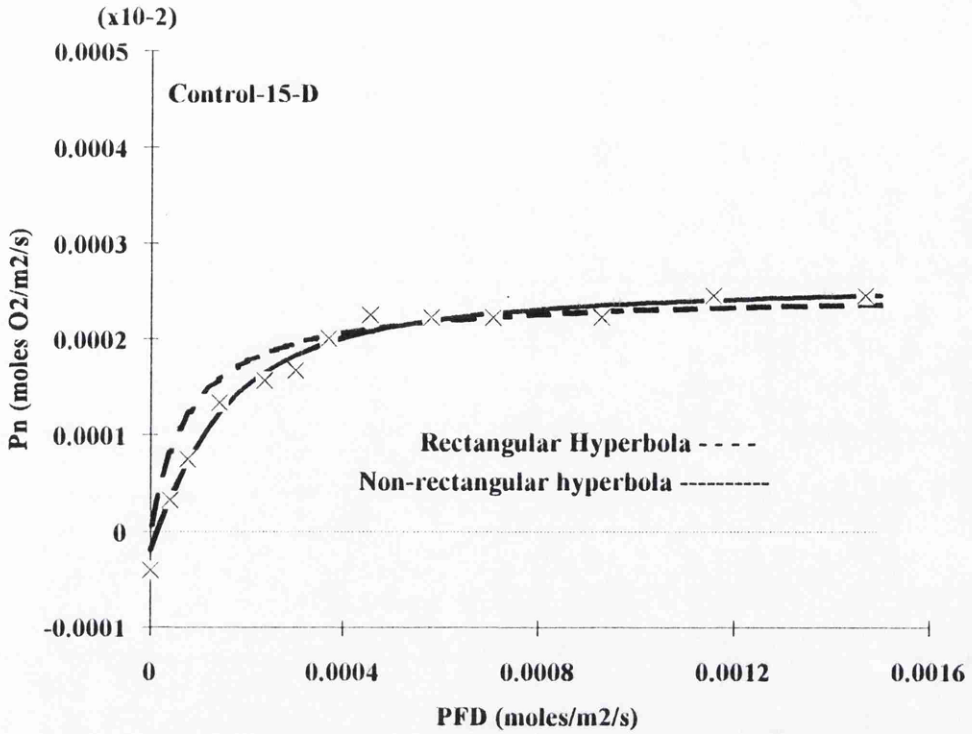
Control-B Rabinowich	Infected-B Rabinowich
Rd= 0.00E+00	Rd= -8.60E-07
P max= 3.944E-06	P max= 6.208E-06
α = 2.234E-02	α = 3.186E-02
Error= 1.989E-06	Error= 3.038E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -2.160E-07	Rd= -1.061E-06
Pmax= 3.359E-06	Pmax= 5.306E-06
α = 1.569E-02	α = 2.683E-02
θ = 8.589E-01	θ = 5.579E-01
Error= 7.932E-07	Error= 2.042E-06

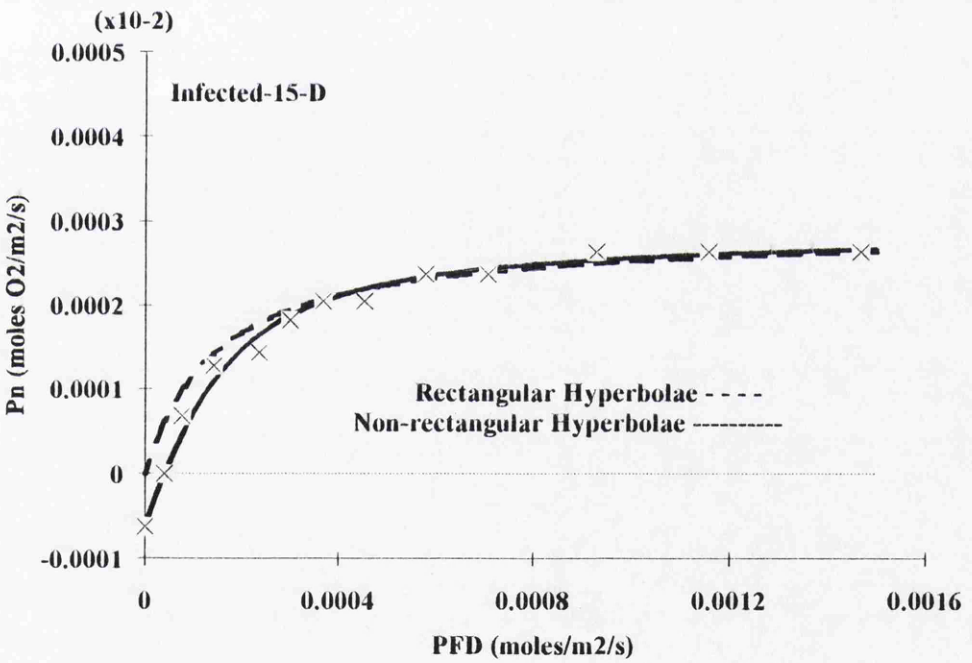




N. glutinosa (15 days):

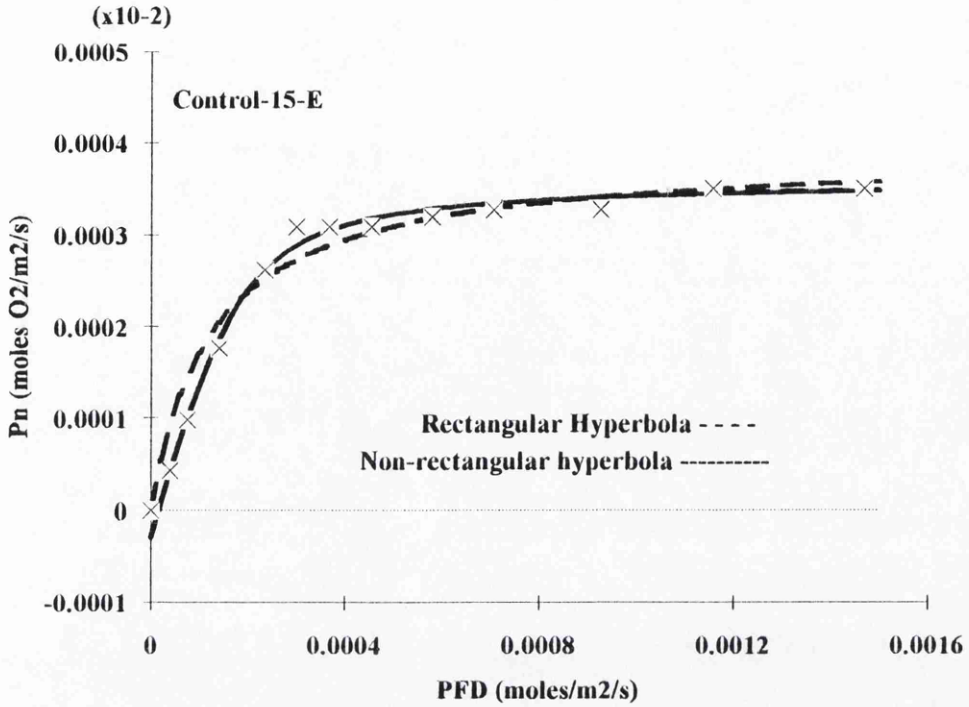
Control-C Rabinowich	Infected-C Rabinowich
Rd= 0.00E+00	Rd= -4.01E-07
P max= 4.114E-06	P max= 4.506E-06
α = 2.233E-02	α = 1.500E-02
Error= 2.183E-06	Error= 2.425E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -1.056E-07	Rd= -3.458E-07
Pmax= 4.202E-06	Pmax= 4.969E-06
α = 1.367E-02	α = 9.448E-03
θ = 5.309E-01	θ = 3.080E-01
Error= 1.312E-06	Error= 2.378E-06

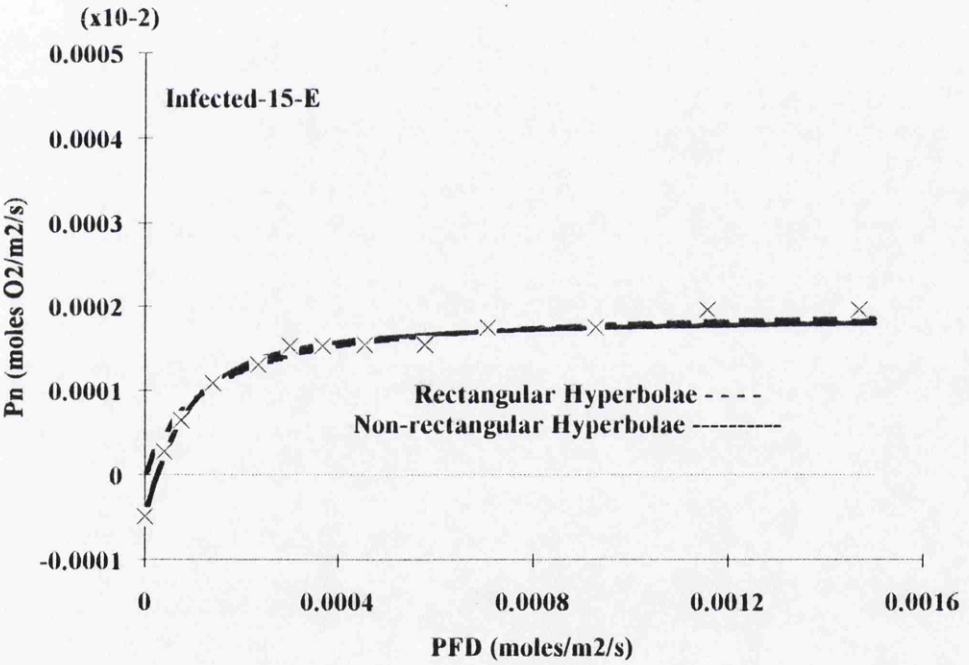




N. glutinosa (15 days):

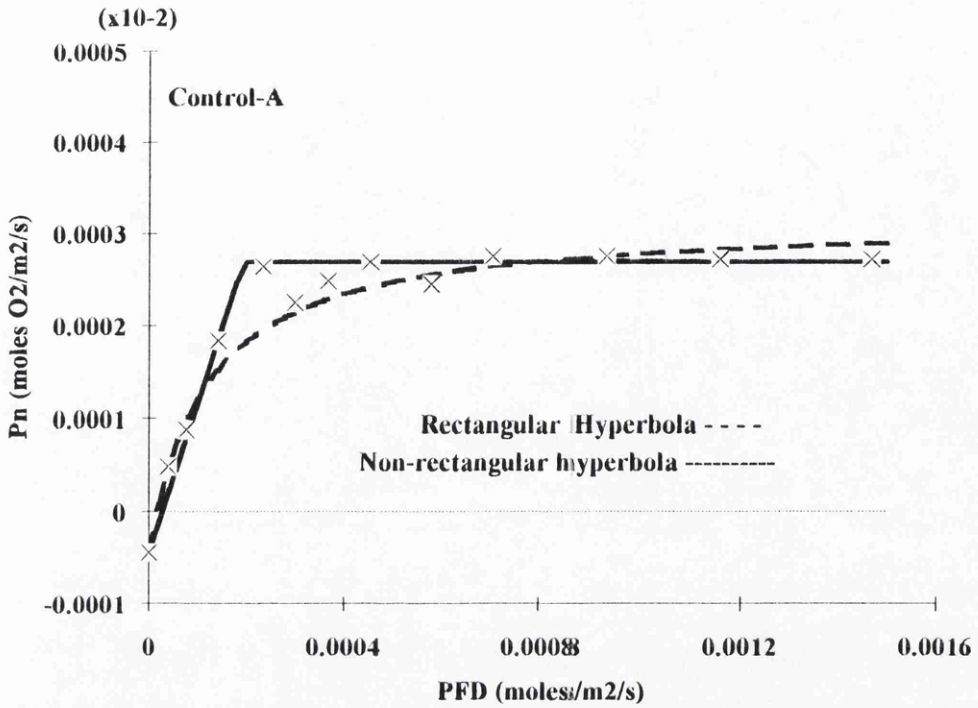
Control-D Rabinowich	Infected-D Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 2.484E-06	P max= 2.902E-06
α = 3.092E-02	α = 1.986E-02
Error= 2.661E-06	Error= 2.605E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -1.857E-07	Rd= -6.158E-07
Pmax= 2.703E-06	Pmax= 3.133E-06
α = 1.446E-02	α = 1.698E-02
θ = 5.543E-01	θ = 5.857E-01
Error= 1.078E-06	Error= 1.072E-06

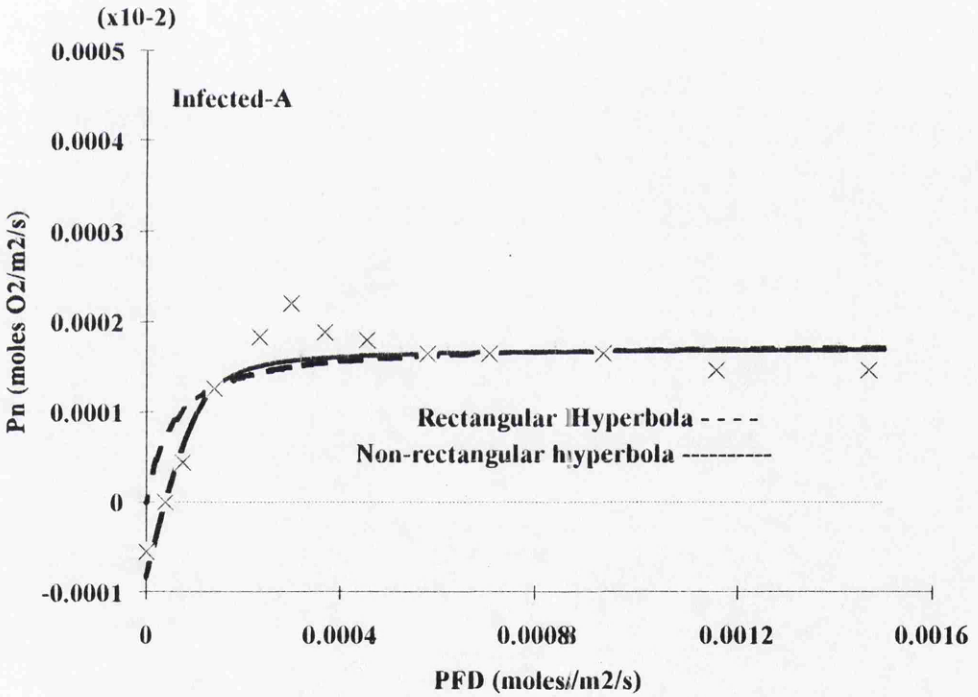




N. glutinosa (15 days):

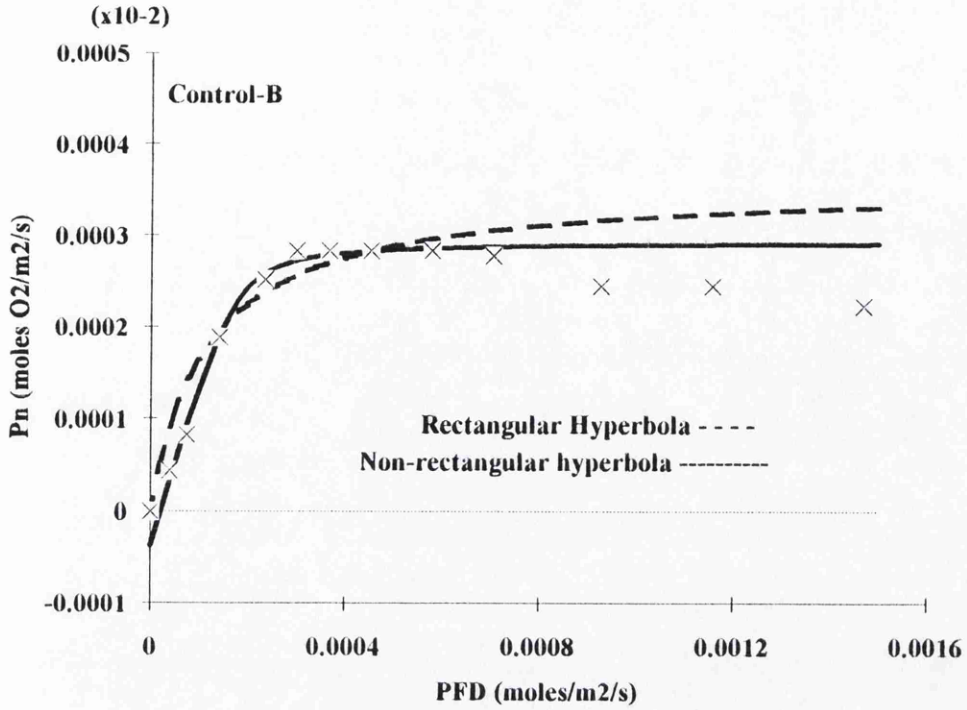
Control-E Rabinowich	Infected-E Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 3.868E-06	P max= 2.017E-06
α = 3.092E-02	α = 1.587E-02
Error= 2.183E-06	Error= 1.404E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -2.832E-07	Rd= -4.909E-07
Pmax= 3.608E-06	Pmax= 2.112E-06
α = 1.819E-02	α = 2.203E-02
θ = 8.459E-01	θ = 5.218E-01
Error= 1.009E-06	Error= 7.688E-07

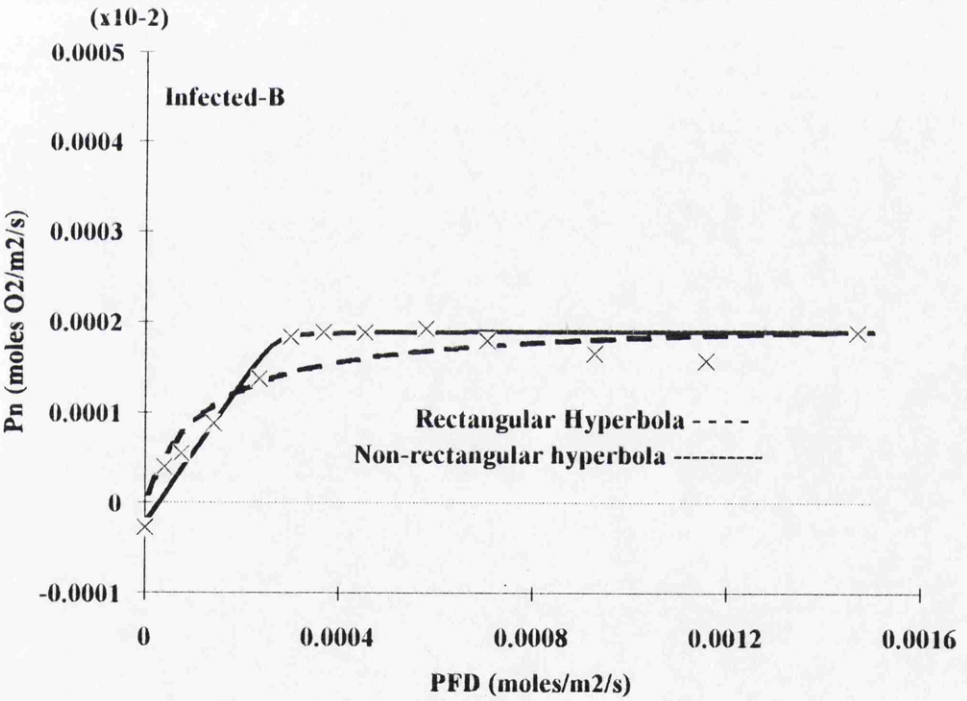




N. glutinosa (35 days):

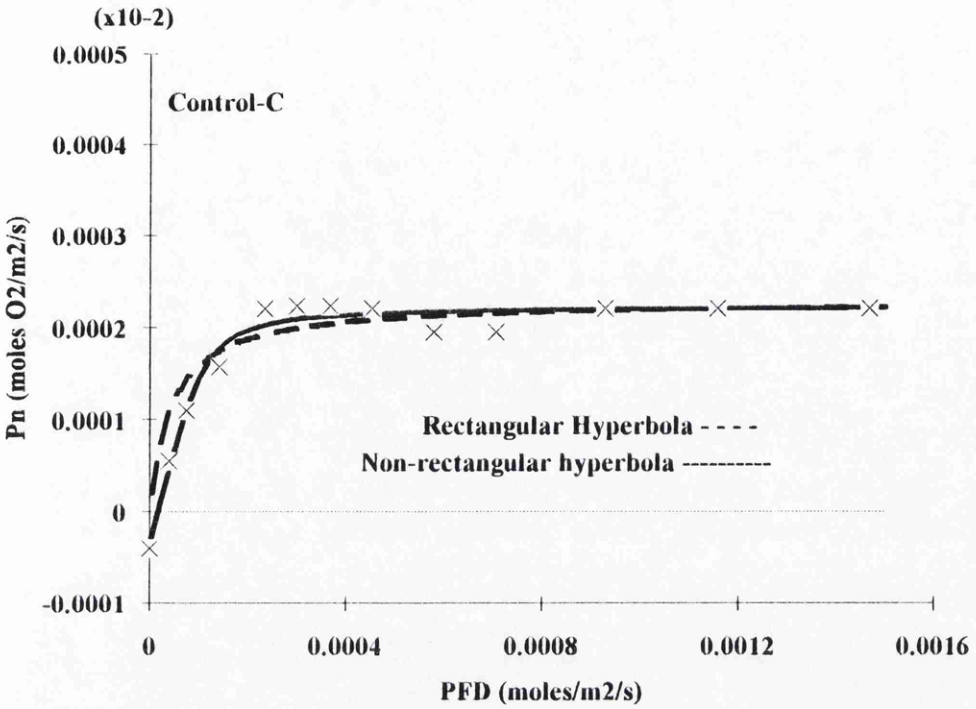
Control-A Rabinowich	Infected-A Rabinowich
Rd= -4.41E-07	Rd= 0.00E+00
P max= 3.602E-06	P max= 1.785E-06
α = 3.046E-02	α = 3.142E-02
Error= 2.189E-06	Error= 4.072E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -4.406E-07	Rd= -8.317E-07
Pmax= 2.693E-06	Pmax= 1.796E-06
α = 1.616E-02	α = 2.200E-02
θ = 1.000E+00	θ = 9.000E-01
Error= 1.516E-06	Error= 2.131E-06

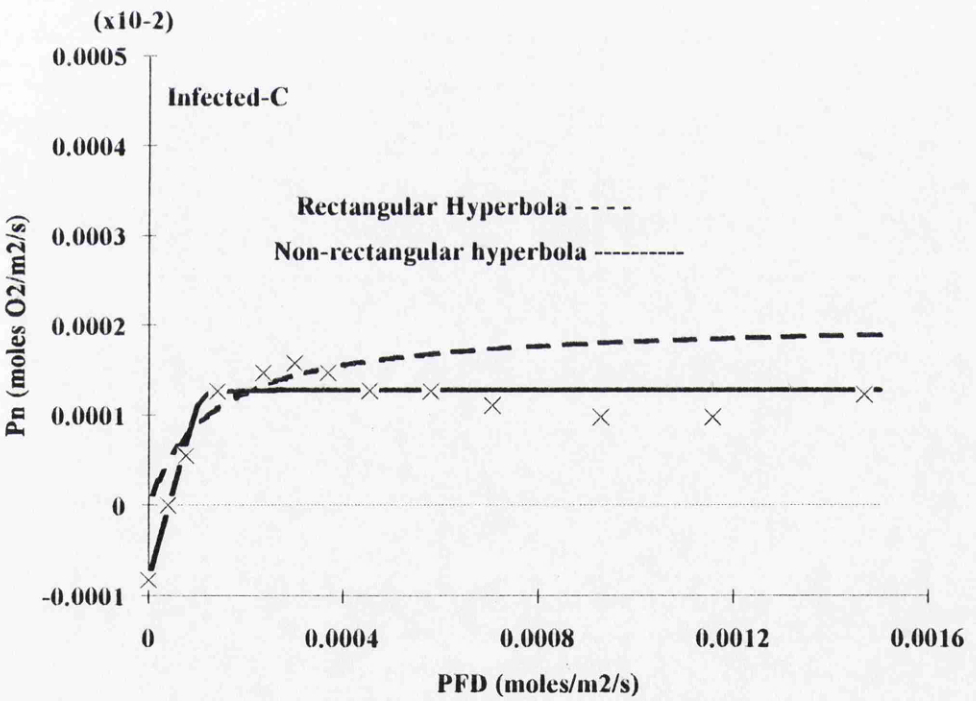




N. glutinosa (35 days):

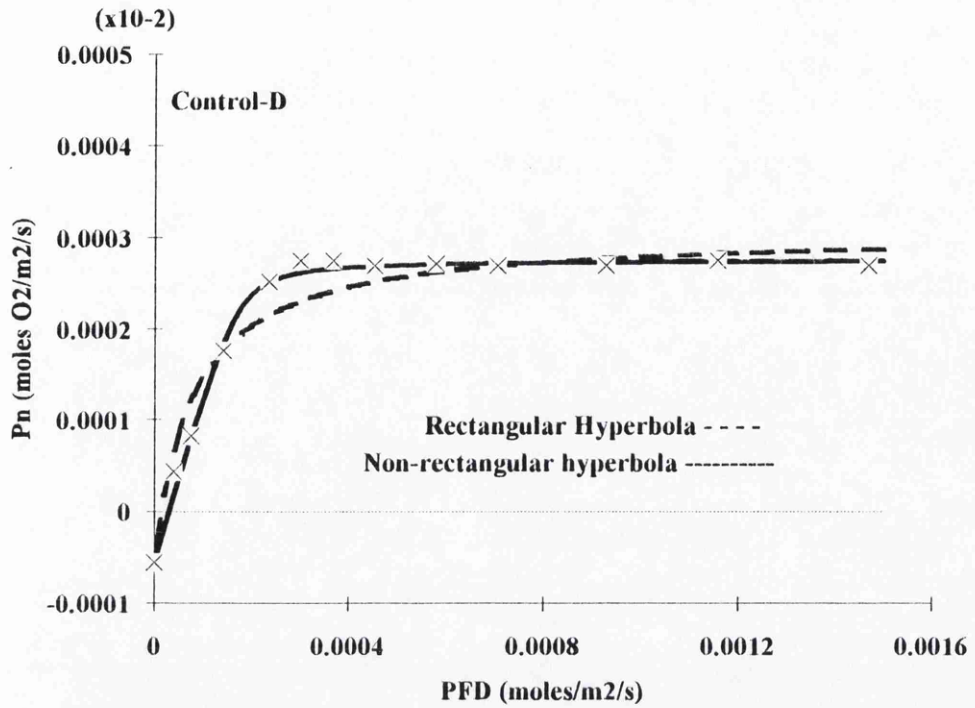
Control-B Rabinowich	Infected-B Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 3.563E-06	P max= 2.054E-06
α = 3.046E-02	α = 1.593E-02
Error= 2.032E-06	Error= 2.592E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -3.701E-07	Rd= -2.256E-07
Pmax= 2.948E-06	Pmax= 1.895E-06
α = 1.763E-02	α = 7.861E-03
θ = 9.538E-01	θ = 9.960E-01
Error= 9.239E-07	Error= 1.473E-06

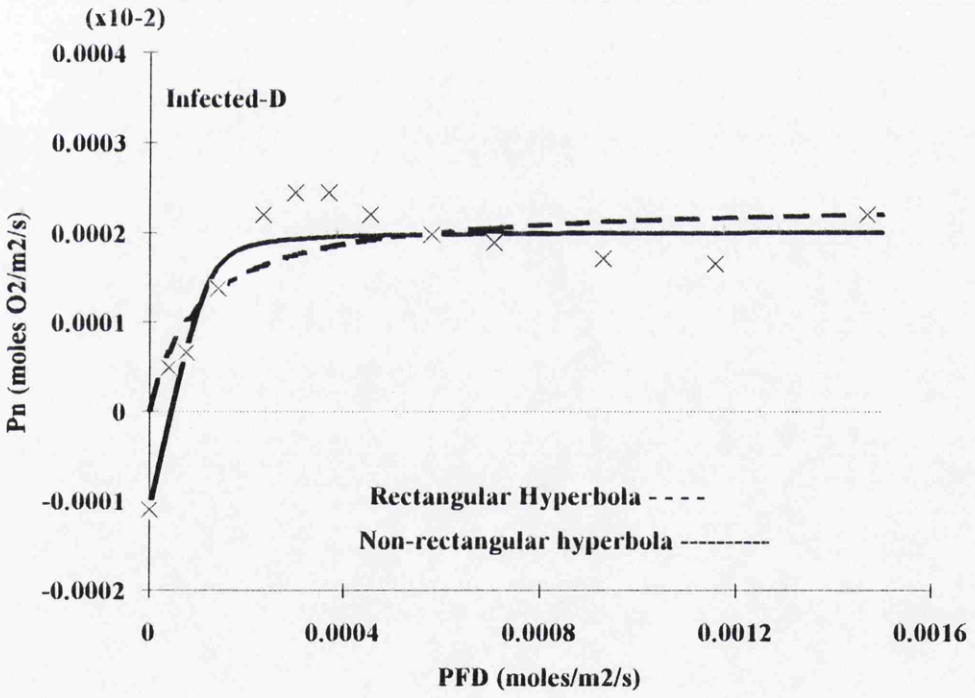




N. glutinosa (35 days):

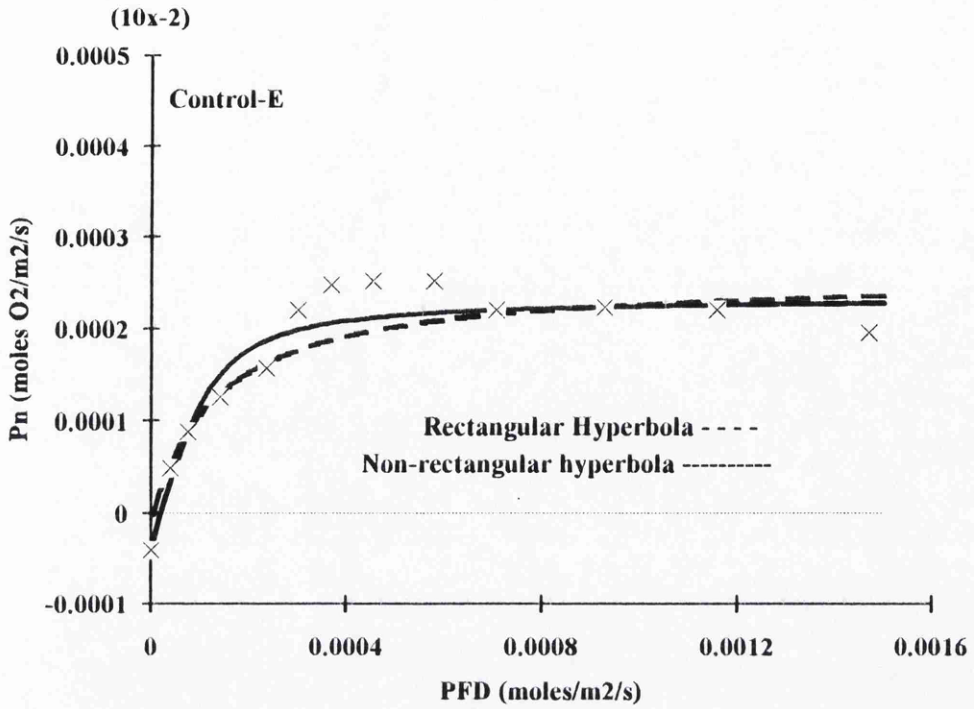
Control-C	Infected-C
Rabinowich	Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 2.286E-06	P max= 2.054E-06
α = 5.238E-02	α = 1.593E-02
Error= 2.674E-06	Error= 5.781E-06
Marshall & Biscoe	Marshall & Biscoe
Rd= -3.725E-07	Rd= -8.262E-07
Pmax= 2.266E-06	Pmax= 1.281E-06
α = 2.202E-02	α = 1.999E-02
θ = 9.056E-01	θ = 9.967E-01
Error= 1.241E-06	Error= 1.638E-06

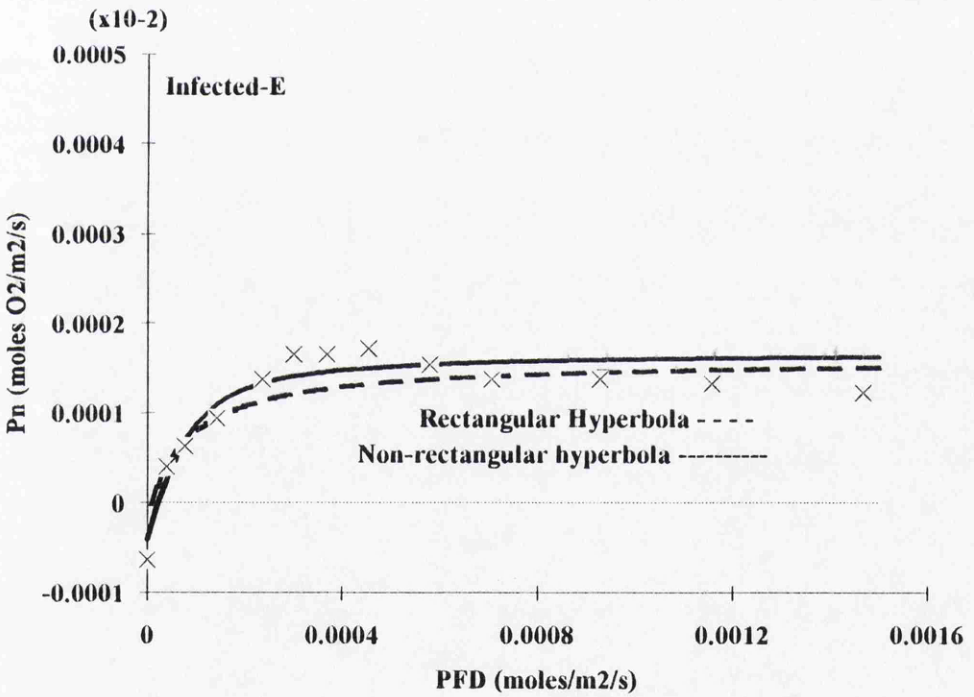




N. glutinosa (35 days):

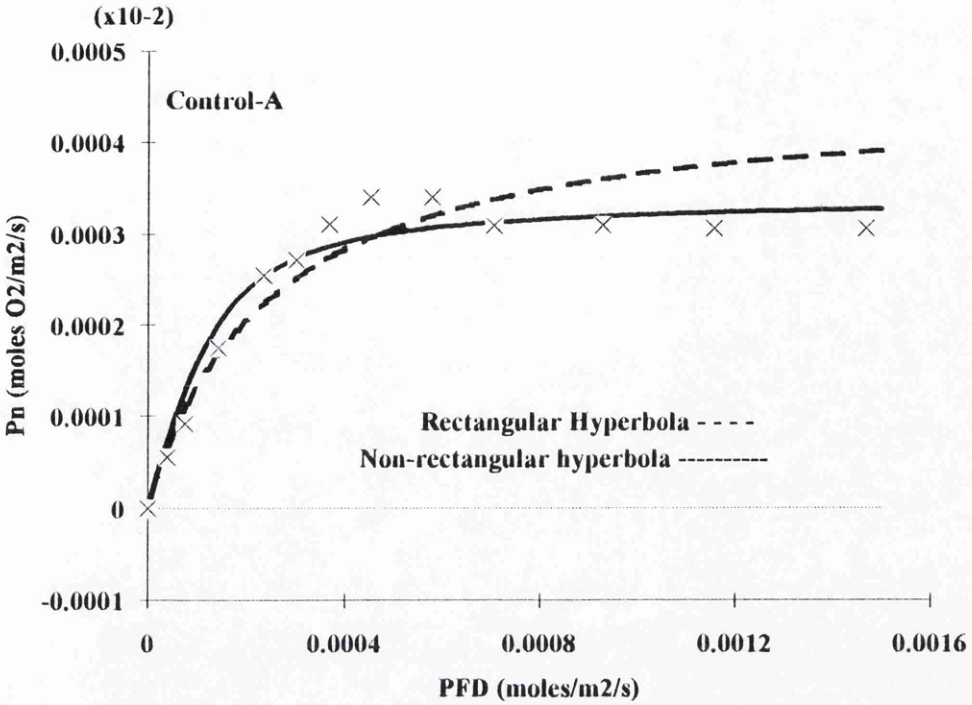
Control-D Rabinowich	Infected-D Rabinowich
Rd= -5.51E-07	Rd= 0.00E+00
P max= 3.605E-06	P max= 2.357E-06
α = 4.582E-02	α = 2.302E-02
Error= 2.348E-06	Error= 4.868E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -5.499E-07	Rd= -1.097E-06
Pmax= 2.774E-06	Pmax= 2.058E-06
α = 1.824E-02	α = 2.456E-02
θ = 9.690E-01	θ = 9.617E-01
Error= 7.378E-07	Error= 3.168E-06

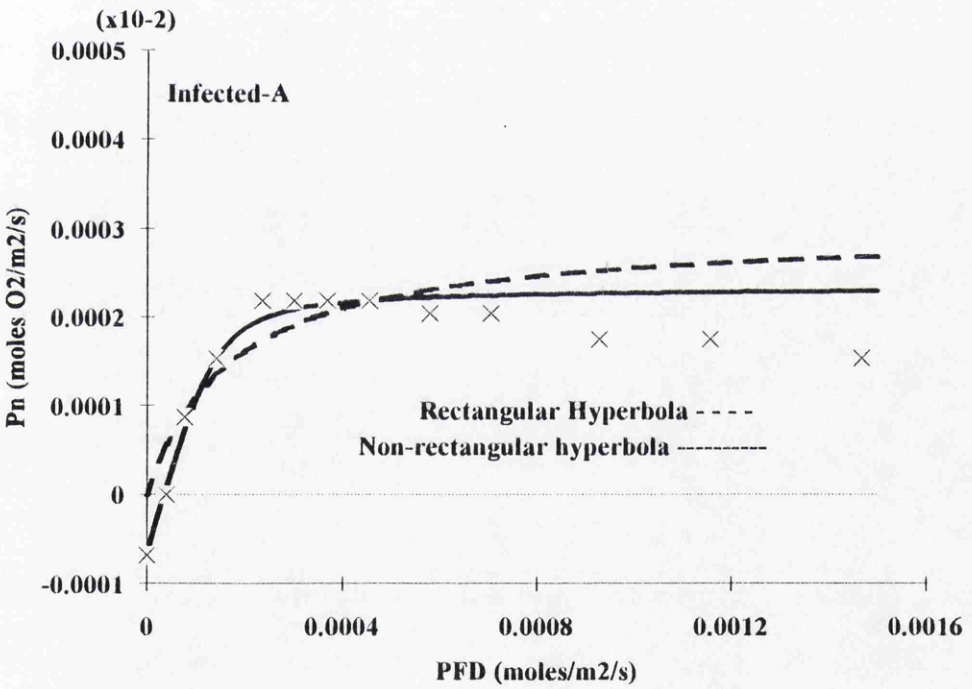




N. glutinosa (35 days):

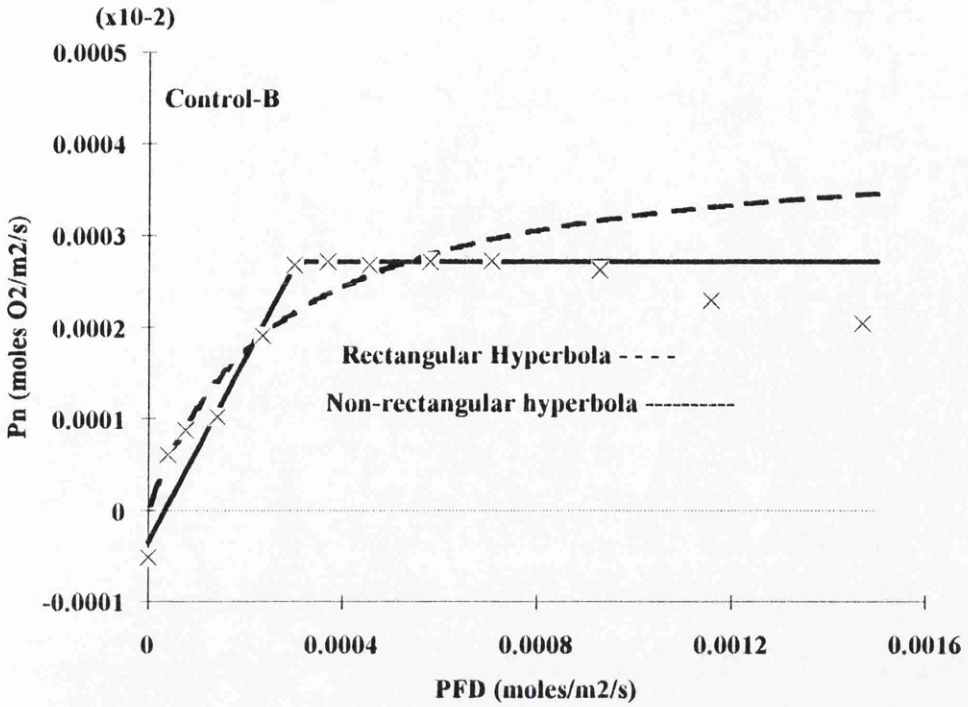
Control-E Rabinowich	Infected-E Rabinowich
Rd= -1.09E-07	Rd= -1.63E-07
P max= 2.673E-06	P max= 1.762E-06
α = 2.091E-02	α = 2.091E-02
Error= 2.975E-06	Error= 2.678E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -3.926E-07	Rd= -4.037E-07
Pmax= 2.417E-06	Pmax= 1.801E-06
α = 2.024E-02	α = 1.926E-02
θ = 7.791E-01	θ = 6.831E-01
Error= 2.118E-06	Error= 1.941E-06

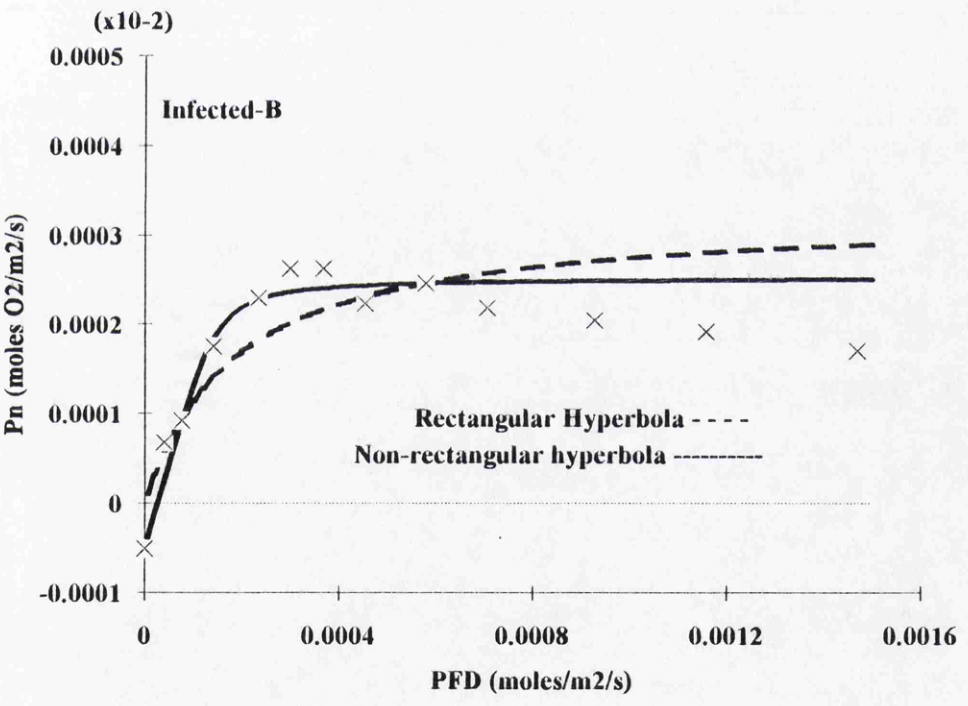




N. glutinosa (55 days):

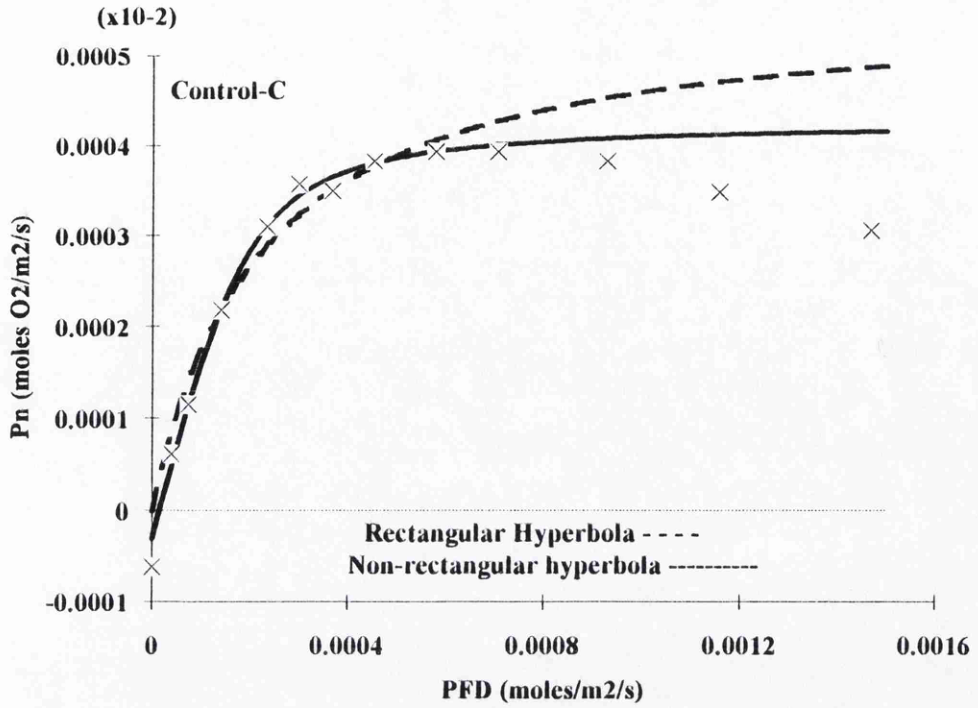
Control-A Rabinowich	Infected-A Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 4.527E-06	P max= 2.992E-06
α = 1.889E-02	α = 1.754E-02
Error= 2.124E-06	Error= 2.991E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= 0.000E+00	Rd= -6.741E-07
Pmax= 3.383E-06	Pmax= 2.392E-06
α = 2.000E-02	α = 2.000E-02
θ = 7.200E-01	θ = 9.000E-01
Error= 1.856E-06	Error= 1.005E-06

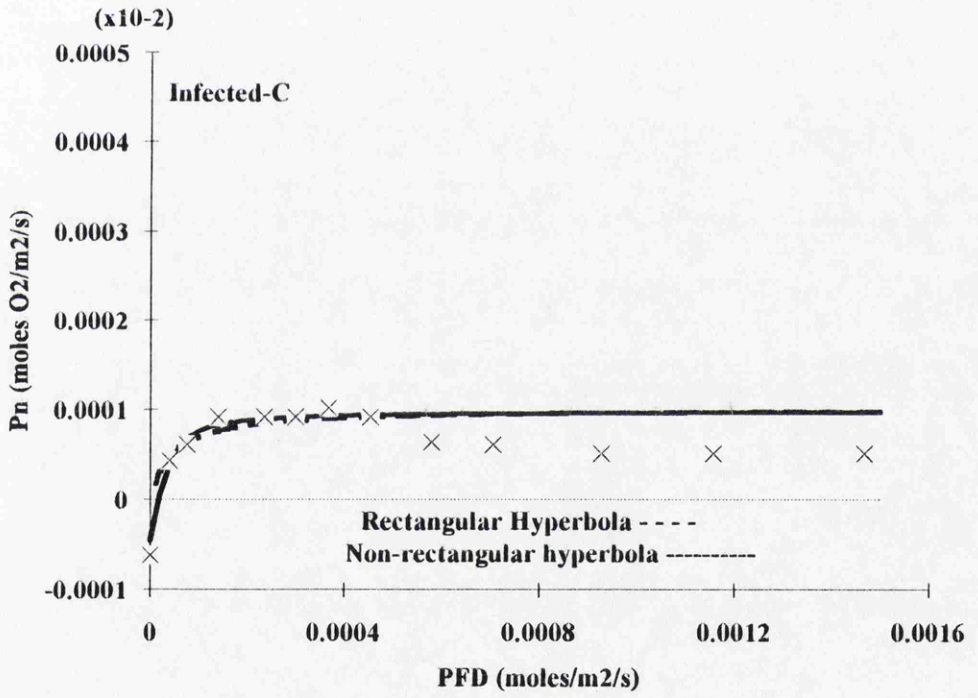




N. glutinosa (55 days):

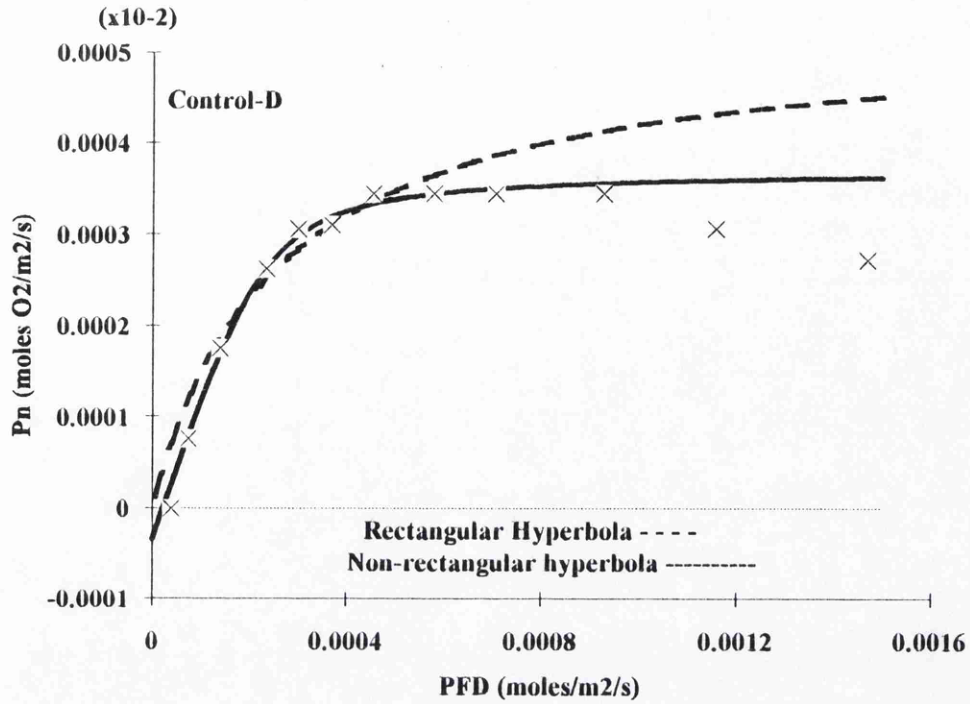
Control-B Rabinowich	Infected-B Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 4.063E-06	P max= 3.254E-06
α = 1.540E-02	α = 1.754E-02
Error= 2.066E-06	Error= 3.005E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -3.403E-07	Rd= -5.102E-07
Pmax= 2.717E-06	Pmax= 2.554E-06
α = 1.005E-02	α = 2.000E-02
θ = 1.000E+00	θ = 9.412E-01
Error= 1.394E-06	Error= 1.484E-06

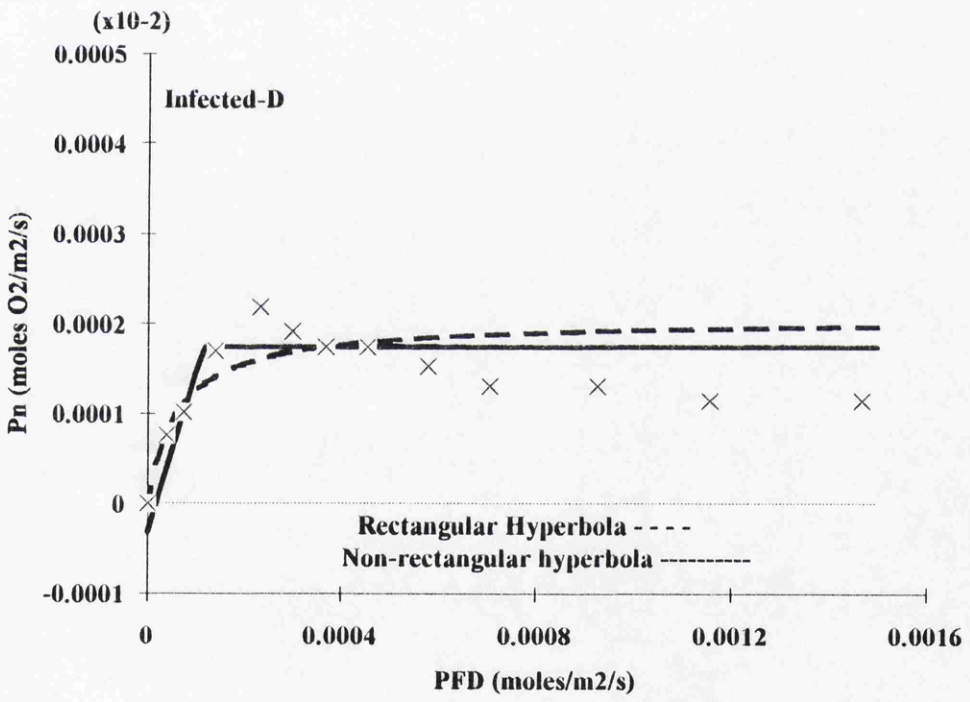




N. glutinosa (55 days):

Control-C Rabinowich	Infected-C Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 5.610E-06	P max= 1.009E-06
α = 2.544E-02	α = 2.262E-02
Error= 1.866E-06	Error= 1.685E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -3.044E-07	Rd= -4.527E-07
Pmax= 4.311E-06	Pmax= 1.098E-06
α = 2.061E-02	α = 2.926E-02
θ = 8.585E-01	θ = 7.466E-01
Error= 7.482E-07	Error= 1.135E-06





N. glutinosa (55 days):

Control-D Rabinowich	Infected-D Rabinowich
Rd= 0.00E+00	Rd= 0.00E+00
P max= 5.278E-06	P max= 2.054E-06
α = 2.043E-02	α = 3.211E-02
Error= 3.395E-06	Error= 1.561E-06
Marshall & Biscoe	**Marshall & Biscoe**
Rd= -3.417E-07	Rd= -3.115E-07
Pmax= 3.716E-06	Pmax= 1.750E-06
α = 1.580E-02	α = 1.772E-02
θ = 9.100E-01	θ = 1.000E+00
Error= 1.837E-06	Error= 1.555E-06