THE ASSOCIATION OF BROMEGRASS MOSAIC VIRUS WITH PUCCINIA GRAMINIS TRITICI

by

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ABBREVIATIONS

BMV	Bromegrass mosaic virus
BMV-P	Bromegrass mosaic virus protein
BPB	Bromophenol blue
BSA	Bovine serum albumin
cv.	Cultivar
ds-RNA	Double stranded ribonucleic acid
Ei	Electrophoretic index
ELISA	Enzyme-linked immunosorbent assay
FITC	Fluorescein iso-thiocyanate
F/P ratio	Fluorescein-protein ratio
GAR	Goat anti-rabbit antiserum
IEM	Immunosorbent electron microscopy
IgG	Gamma globulin fraction of serum
O.D. 260nm, etc.	Optical density (absorbance) reading at indicated wavelength
PBS	Phosphate buffered saline
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PVX	Potato virus-X
p.v.i.	Post virus inoculation
SDS	Sodium dodecyl sulphate
ss-RNA	Single-stranded ribonucleic acid
TNV	Tobacco necrosis virus
Temed	NNN'N' tetramethylethyldiamine
VLP	Virus-like particle
WCuMV	Wild cucumber mosaic virus
110S, etc.	Sedimentation coefficient in Svedbergs
ex. Clipper	Extracted from Clipper

ERRATA

BMV-G_9 should read BMV-G9

T_4 " " T4

T_4-R " " T4-R

Concerning Figures 1, 3, 5 and 7:

The photographs were taken through a Zeiss dissecting microscope at a lens magnification of 4 X.

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CHAPTER I

LITERATURE REVIEW

A. VIRUS-FUNGUS ASSOCIATIONS

Viruses have been found to be associated with several fungi, belonging to each of the major taxonomic classes. Two types of virus-fungus associations have been discerned, namely (1) virus transmission by fungi and (2) viruses infecting fungi.

The economic importance of fungi is known since these microorganisms find wide application in the industrial, medical and agricultural fields. The discovery of virus associations with fungi have undoubtedly placed these eukaryotes in the realm for research by virologists and mycologists, internationally. At present the most rewarding research has been that carried out on viruses infecting fungi (fungal viruses or mycoviruses). Studies on transmission of viruses by fungi have so far not been well-documented. Those fungi concerning the transmission of plant viruses especially have been neglected, and one may ask the question whether this aspect was considered to be less important or whether the technical difficulties encountered could not be overcome.

Much is known about how viruses infect fungi in culture.

As yet it is not known how viruses might infect mycelium under natural conditions in fungi which may represent a reservoir for plant and animal viruses.

B. MYCOVIRUSES

Viruses that have been found to infect fungi have been placed in a category of their own, and are termed fungal viruses or mycoviruses (Lemke and Nash, 1974; Bozarth, 1972; Hollings, 1978). This was done to distinguish fungal viruses from the plant viruses that are transmitted by plant pathogenic fungi. Mycoviruses have been reported in many fungi and have been reviewed by Hollings and Stone (1971), Bozarth (1972), Lemke and Nash (1974), Saksena and Lemke (1978) and Hollings (1978).

For the subject of this thesis, it is important to have some knowledge of this type of virus-fungus association. To gain some perspective on this subject, a few of the most well-studied mycoviruses and the fungi they infect will be discussed briefly.

The most extensively studied system is the mycovirus of the Penicillium chrysogenum, the mold employed for the commercial production of penicillin. This study was largely developed from studies with two other species, Penicillium stoloniferum and Penicillium

fumiculosum. The anti-viral substances synthesised by Penicillium species have been associated with the presence of double stranded ribonucleic acid (ds-RNA) of viral origin in these species (Lemke and Nash, 1974).

The mycoviruses of Penicillium species have been found to be isometric particles 25-40 nm in diameter (Saksena and Lemke, 1978).

The killer systems, (i.e. production of a toxin lethal to sensitive strains) of Ustilago maydis and Saccharomyces cerevisae, controlled by killer factors which are cytoplasmic determinants, have been linked to the presence of a virus in the case of U. maydis (Wood and Bozarth, 1973) and a ds-RNA complex of possible viral origin in S. cerevisae (Berry and Bevan, 1972; Vodkin and Fink, 1973; Vodkin et al., 1974).

Virus particles have also been found in Aspergillus species (Banks et al., 1970; Hollings and Stone, 1971; Buck et al., 1973; Wood et al., 1974) and in strains of Schizophyllum commune (Koltin et al., 1973). In Aspergillus flavus and A. parasiticus, virus particles were found to be associated with non-aflatoxin producing strains and not with aflatoxin producing strains (McKenzie and Adler, 1972, cited in Lemke and Nash, 1974). The ability to form plaques in S. commune was found to be linked to the presence of a specific virus

(Koltin et al., 1973). The most well-characterised mycovirus system is the virus complex that causes watery stipe or die-back disease of cultivated mushroom, Agaricus bisporus. A virus complex of three types of mycoviruses have been found to be responsible for disease in A. bisporus (Hollings, 1962; Dieleman-van Zaayen and Temmink, 1968). Electron microscopic analysis of these three mycoviruses revealed hexagonal particles (34-36nm in diameter), isometric particles (25-27nm in diameter) and bacilliform particles (19x50nm in size). These mycoviruses are virulent and immensely pathogenic to this fungus. The viruses have been shown to be transmitted in A. bisporus by hyphal anastomosis. Non-pathogenic viruses have been found in two other mushroom species, Lentinus edodes (Ushiyama et al., 1977; 1980; Ushiyama and Nakai, 1980) and Boletus edulis (Huttinga et al., 1975).

In general, fungal viruses appear to be relatively a-virulent or latent, since apparently healthy mycelia often contain high titers of virus particles (Lemke and Nash, 1974). Thus far, mycoviruses have been shown to be mostly spherical in morphology and to contain ds-RNA as genomes. The ds-RNA mycoviruses from Penicillium have been found to be active inducers of interferon (Saksena and Lemke, 1978). Transmission of fungal viruses between strains of fungi have been shown to occur by heterokaryosis in Penicillium stoloniferum

(Lhoas, 1971), Ustilago maydis (Wood and Bozarth, 1973) and Gaeumannomyces graminis (Rawlinson et al., 1973), and by hyphal anastomosis in Agaricus bisporus (Gandy, 1960; Hollings, 1962).

Mycoviruses are important in medicine and industry because of their possible effects on the levels of metabolites and toxins produced by fungi, and the ds-RNA of these viruses have been found to be potent inducers of interferon.

C. FUNGAL VIRUSES AND PLANT PATHOLOGY

Virus-fungus relationships are important in plant pathology for two main reasons. Firstly, if the virus is pathogenic to the fungus then it could cause a decrease in the pathogenicity of the fungus, and would quite feasibly provide for a means of biological control. Secondly, if the virus is non-pathogenic towards the fungus but pathogenic to the higher plant, then the fungus plays an important rôle in the dissemination of the virus in nature, by acting as a vector for the transmission of the virus to other plants. In both cases the virulence of the virus determines the pathogenicity to either the fungus or the higher plant.

Mycoviruses have been identified in thirteen plant pathogenic fungi. These fungi are listed in Table 1 overleaf.

TABLE 1 : List of Plant Pathogenic Fungi in which Mycoviruses have been Observed.

FUNGUS	REFERENCE(S)
Colesporium solidaginis	Littlefield and Heath, 1979
Colletotrichum Lindemuthianum	Lemke and Nash, 1974
Diplocarpon rosae	Bozarth *et al*., 1972
Gaeumannomyces graminis	Rawlinson *et al*., 1973
Gonatobotrys species	Lemke and Nash, 1974
Helminthosporium victoriae	Bozarth, 1972
H. oryzae	Lemke and Nash, 1974
H. maydis	Bozarth *et al*., 1972
Puccinia graminis var. *tritici*	Rawlinson and Maclean, 1973
	Mussel *et al*., 1973
	(cited in Lemke and Nash, 1974)
P.helianthi	Littlefield and Heath, 1979
P. sorghi	Littlefield and Heath, 1979
Periconia circinata	Lemke and Nash, 1974
Piricularia oryzae	Lemke and Nash, 1974
Sclerotium cepivorum	Saksena and Lemke, 1978
Thanatephorus cucumeris	Bozarth, 1972
Thielaviopsis basicola	Bozarth and Goenaga, 1977
Uromyces phaseoli var. *typica*	Littlefield and Heath, 1979
U. phaseoli var. *vignae*	Yarwood and Hecht-Poinar, 1973
	McDonald and Heath, 1978

An interesting mycovirus complex is the one associated with Gaeumannomyces graminis which causes take-all disease of cereals. The presence of virus-like particles (VLP) (29nm in diameter) in this fungus has been reported to cause a decrease in the pathogenicity of this fungus (Lemaire et al., 1971, cited in Bozarth (1972)). This was a first report on a possible biological control of a fungus disease by a virus. However, conflicting results were found by Rawlinson et al., (1973) who suggested that a decrease in pathogenicity was not determined by the presence of a fungal virus. They identified latent viruses, 27nm and 35nm in diameter, in the strain they worked with. They found that strains containing both viruses were generally less pathogenic on wheat and strains containing only one of these viruses generally more pathogenic on wheat than strains lacking the viruses. Similar inconsistency between virus infection and reduced pathogenicity has been observed in studies on Helminthosporium victoriae (Bozarth et al., 1972), Piricularia oryzae (Saksena and Lemke, 1978) and Ustilago maydis (Wood and Bozarth, 1973).

Although the mycoviruses infecting plant pathogenic fungi have raised the possibility of using viruses as a biological control of fungal phytopathogenicity, the chances of using them in this regard are at present seemingly unviable and awaits further research.

The importance of fungal viruses in plant pathology is noted by their pathogenicity on certain higher fungi and the possible rôle they could play as a biological control system. Thus far, no virus is known which can infect and multiply in both a phytopathogenic fungus and a higher plant (Lemke and Nash, 1974).

Multiplication of tobacco mosaic virus in Pythium species during artificial infection has been reported (Saksena and Lemke, 1978). This, however, should be regarded at the present moment as an academic study only, having little bearing on a natural situation.

D. FUNGI AS VECTORS OF PLANT VIRUSES

1. HISTORY

The study of fungi as vectors of plant viruses is an old one that dates back to 1925. During the early observations no conclusive evidence existed for the transmission of plant viruses by plant pathogenic fungi. However, much speculation was made on this subject on the basis of observed fungal-virus associations. It was only in the 1960s that the first fungus vector of a plant virus was positively identified.

McKinney (1930 , cited in Teakle, 1969) was the first

to suggest that fungi might transmit viruses to higher plants. At that time, he stated: "It is possible that subterranean vectors such a nematodes, soil-borne insects, other animal forms, or even fungi, may carry the (wheat mosaic) virus." (Taken from Teakle, 1969). The first experiments carried out to prove that fungi could transmit viruses involved inoculations of plants with fungus spores or cultures originating from virus-infected plants (McKinney et al., 1925, as cited in Teakle, 1969; Johnson and Jones, 1943; Bawden and Kassanis, 1947). Later on, other workers tried to prove transmission by inoculating plants with a mixture of zoospores and virus (Teakle and Gold, 1963). These and other attempts all proved to be unsuccessful. Before 1955, much circumstantial evidence based on the simultaneous presence of fungi and viruses in the soil and on plants, led to the suggestions that certain disease complexes in plants were a result of dual infections by fungi and viruses transmitted by these fungi.

Linford and McKinney (1954) noted a high correlation between the presence of Polymyxa graminis and mosaic in wheat, but concluded that there was no evidence that the fungus was acting as a virus vector or a reservoir host. Fry (1958)(cited in Tomlinson and Garret, 1964) and Grogan et al., (1958) reported the constant association of Olpidium brassicae with lettuce big-vein disease. However, they considered O. brassicae not

to be a vector. Later Olpidium was shown to be closely associated with infection of plants by two other soil-borne viruses of tobacco stunt (Hidaka, 1960) and tobacco necrosis (Teakle, 1960). Confirmation of Olpidium brassicae as a vector for tobacco stunt agent (TSA), tobacco necrosis virus (TNV) and lettuce big-vein agent (BVA) came from studies by Hiruki (1965); Kassanis and MacFarlane (1964); and Campbell and Grogan (1963), Tomlinson and Garret (1964) and Lin et al., (1970), respectively. In 1966, Estes and Brakke showed that Polymyxa graminis was a vector and a reservoir for wheat mosaic virus.

Two other fungi, Synchytrium endobioticum and Spongospora subterranea have also been reported as vectors of Potato virus-X (PVX) (Nienhaus and Stille, 1965) and potato mop top virus (PMTV) (Jones and Harrison, 1969), respectively. Many other reports of fungi as possible vectors of plant viruses have been published and are reviewed in Section I.D.3.

The most well studied fungus vector - virus associations to date, mostly concerns the transmission of soil-borne viruses by free-living zoospores in the soil of very primitive fungi. The two major groups of fungi which are known to act as vectors for plant viruses are the Chytridiales Olpidium species and Synchytrium endobioticum) and the Plasmodiophorales (Polymyxa

graminis and Spongospora subterranea). Reports of fungal vectors belonging to other groups of fungi have largely been based on plant pathological observations.

2. FACTORS AFFECTING FUNGAL VECTOR STUDIES

Much difficulty has been encountered in establishing whether a particular fungus transmits a certain virus. The inability of proving fungi as vectors of plant viruses can largely be attributed to the following characteristics of this type of virus-vector relationship:

(i) Some of the fungus-transmitted viruses are either difficult or impossible to transmit mechanically, like lettuce big-vein agent.

(ii) Most of the viruses that have been reported to be fungus transmitted are soil-borne and so are their fungal vectors. The vast population of microorganisms in the soil makes it virtually impossible to identify a particular fungus as a vector of a particular virus.

(iii) The obligately parasitic nature of some fungal vectors has made it impossible to culture these fungi on artificial media. Thus far, no successful pure culture of a fungus vector has been obtained.

(iv) Since fungi cause disease on their own, it is difficult to decide whether the disease symptoms observed by fungal infection could be a result of a toxin produced by the fungus or a virus transmitted by the fungus.

(v) The fungus vectors usually harbour and transmit the viruses at a low concentration.

3. THE VIRUSES AND THEIR FUNGAL VECTORS

All the reported virus-fungal vector relationships are discussed under this heading. Table 2 overleaf contains a list of all the fungi and the respective viruses they transmit.

3.a. Olpidium brassicae

This fungus belonging to the order Chytridiales, is typified by its motile zoospores having one flagellum. The fungus is soil-borne, obligately parasitic and infects the roots of its hosts. Although the fungus itself is not highly pathogenic to its host, the viruses transmitted by it are highly virulent.

TABLE 2 : Fungal Vectors and Viruses Transmitted by Them.

FUNGUS	VIRUS	CHARACTERISTICS OF THE VECTOR RELATIONSHIP	REFERENCE(S)
Olpidium brassicae	Tobacco necrosis virus (TNV)	1. *in vitro* acquisition 2. Virus externally-borne on resting spore 3. internalisation of virus during encystment	Campbell and Fry (1960) Teakle (1960) Kassanis & MacFarlane (1964) Temmink *et al.*, (1973) Temmink (1970)
	Satellite virus (SV)	1. Same as for TNV 2. Different binding sites for TNV and SV on spore	Kassanis & MacFarlane (1968) Temmink *et al.*, (1973)
	Lettuce big-vein agent (BVA)	1. *in vivo* acquisition 2. Virus internally-borne in spores 3. No multiplication of virus in fungus	Campbell (1962) Campbell and Grogan (1963, 1964) Campbell and Fry (1966) Tomlinson and Garret (1962, 1964) Lin *et al.*, (1973)

Continued overleaf.

FUNGUS	VIRUS	CHARACTERISTICS	REFERENCES(S)
	Tobacco stunt agent (TSA)	Same as for BVA	Hidaka (1960) Hiruki (1965, 1972)
Olpidium radicale	Cucumber necrosis virus (CNV)	1. In vitro acquisition 2. Virus loosely bound to spore's surface.	Dias (1970) Temmink et al., (1970)
Synchytrium endobioticum	Potato virus-X (PVX)	1. In vivo acquisition 2. Not well-characterised	Nienhaus and Stille (1965)
Polymyxa graminis	Soil-borne wheat mosaic virus (SBWMV)	1. In vivo acquisition 2. Virus internally-borne in spores 3. External binding of virus to spore is not excluded 4. No multiplication of virus in fungus	Estes and Brakke (1966) Canova (1966) Brakke and Estes (1965, 1967) Rao and Brakke (1969) Rao (1969)
	Wheat spindle streak mosaic virus (WSSMV)	1. In vivo acquisition 2. Virus internally-borne (?) in spores	Slykhuis (1976) Slykhuis and Barr (1978)

Continued overleaf

FUNGUS	VIRUS	CHARACTERISTICS	REFERENCE(S)
	Oat mosaic virus (OMV)	1. Not well-characterised 2. Correlation between OMV symptoms and presence of fungal zoospores	Herbert and Panizo (1975)
	Barley yellow mosaic virus (BYMV) Wheat yellow mosaic virus (WYMV) Rice necrosis virus (RNV)	1. Not well-characterised 2. Only suggested to be fungus transmitted 3. For BYMV showed *in vivo* acquisition and possible internal carriage of virus	*Kusaba *et al.*, (1971) Inouye and Saito (1975) Inouye and Fujii (1977)
Polymyxa betae	Beet necrotic yellow vein virus (BNYVV)	1. *In vivo* acquisition 2. Virus internally-borne (?) in spores	*Tamada *et al.*, (1975) *Stocky *et al.*, (1977) *Vuittenez *et al.*, (1977)
Spongospora Subterranea	Potato mop top virus (PMTV)	1. Not well-characterised 2. Virus internally-borne (?) in spores	Calvert & Harrison(1966) Jones & Harrison (1967)

* Cited by others (see text)

Continued overleaf

FUNGUS	VIRUS	CHARACTERISTICS	REFERENCE(S)
Sphaerotheca lanestris and *Erysiphe graminis*	Tobacco mosaic virus (TMV)	1. *In vivo* acquisition (?) 2. Virus carried inside conidia	Yarwood (1971) Nienhaus (1971) Yarwood and Hecht-Poinar (1973)
Uromyces phaseoli var. *vignae*	Tobacco mosaic virus (TMV)	1. *In vivo* acquisition (?) 2. Virus carried inside spores (?)	Yarwood and Hecht-Poiner (1973) McDonald and Heath (1973)
Uromyces polygoni	Virus still unidentified	1. Transmission to *Chenopodium* sp. by conidia	Yarwood and Hecht-Poinar (1973)
Puccinia malvacearum, *P. striiformis*, *P. suaveolens*	Spherical virus-like particle (34nm in diameter)	1. Virus extracted from plants infected with these fungi	Lecoq *et al.*, (1974)
Puccinia graminis tritici	Bromegrass mosaic virus (BMV)	1. *In vivo* acquisition 2. Virus internally-borne (?) in uredospores	Von Wechmar (1980)

3.a.(i) Transmission of Lettuce Big-Vein Agent (BVA)

Since the BVA has not yet been characterised, it is not known whether it is a virus. A rod-shaped particle (244nm in length) has been associated with the roots of plants diseased with BVA (Chod et al., 1976, cited in Campbell (1978)). However, verification of this particle as BVA has not yet been received. It is known that BVA is not mechanically transmissible. Graft transmissibility has been demonstrated (Campbell et al., 1962; Tomlinson et al., 1962).

The constant association of O. brassicae with lettuce big-vein disease was noticed by Fry (1958 , cited in Tomlinson and Garret, 1964), and Grogan et al., (1958). It was only when the disease was shown to be graft transmissible (Campbell et al., 1962; Tomlinson et al., 1962) that O. brassicae was implicated as a vector for BVA. Confirmation of O. brassicae as a vector for lettuce big-vein agent came from studies by Campbell and Grogan (1963 and 1964), Tomlinson and Garret (1964) and Lin et al., (1970). The BVA was shown to be carried within resting spores of the fungus (Campbell, 1962; Campbell and Fry, 1966). Nonviruliferous cultures were found to acquire the agent from diseased plants (Tomlinson and Garret, 1962; Campbell and

Grogan, 1964) and transmit it to healthy plants. *In vitro* acquisition of BVA by *Olpidium brassicae* zoospores was shown not to occur (Campbell and Grogan, 1964). Multiplication of the BVA in the fungus does not seem to occur because viruliferous cultures have been found to be freed of BVA by passage on sugar beets (Campbell, 1962) and on plaintain (*Plantago major*) or *Veronica persica* (Tomlinson and Garret, 1964).

3.a.(ii) Transmission of Tobacco Necrosis Virus (TNV) and Satellite Virus (SV)

Tobacco necrosis virus (TNV) has been well-characterised, and consists of isometric particles about 26nm in diameter. The virus is soil-borne, mechanically transmissible and causes local lesions on many hosts. Many strains have been distinguished by host range studies, serology and the activation of different satellite virus strains. TNV is serologically unrelated to satellite virus (Kassanis, 1970).

Satellite virus (SV) is the smallest RNA-containing virus known (Kassanis, 1970). It has isometric particles about 17nm in diameter, and is dependent upon TNV for its multiplication.

The aim to establish whether *O. brassicae* was

a vector of TNV came from studies on the transmission of BVA and the close association of O. brassicae with plants infected with TNV (Teakle, 1960). Confirmation of the transmission of TNV by O. brassicae came from experiments done by Kassanis and MacFarlane (1964). Temmink et al., (1970) showed that single-sporangial cultures of O. brassicae could transmit TNV. The virus has been shown to be tightly bound to the zoospore body plasmalemma and axonemal sheath (Temmink et al., 1970; Temmink, 1971). The virus becomes internalised in the zoospore during encystment. Apparently, the virus on the axonemal sheath becomes internalised when the axoneme is withdrawn during encystment (Temmink, 1971). Release of the virus into the host cell occurs upon infection by the zoospore protoplast (Temmink, 1971). TNV has also been found to be carried externally on resting spores (Campbell and Fry, 1960). Experiments done on TNV transmission have concerned in vitro uptake of the virus by the zoospores, and multiplication of TNV within the zoospores is not evident. Necrosis of host cells by TNV infection causes the death of zoosporangia (Fry and Campbell, 1966). This phenomenon does not have any effect on the transmission of TNV since Kassanis and MacFarlane

(1965) have suggested that transmission of TNV by O. brassicae only requires the infection of host cells by the fungus. Satellite virus (SV) was found to be transmitted together with TNV after in vitro acquisition of a mixture of the two viruses by O. brassicae (Kassanis and MacFarlane, 1968). Electron microscopy of zoospores revealed that both SV and TNV were tightly bound to the zoospore plasmalemma (Temmink et al., 1970). It seems that specific binding sites for the two viruses, which are antigenically distinct, exist on the zoospore plasmalemma.

3.a.(iii) Transmission of Tobacco Stunt Agent (TSA)

TSA, like BVA, has not been fully characterised to be considered as a virus. Reports that spherical particles could be involved have been received, but much discrepancy as to the size of these particles exists (Campbell, 1978). TSA differs from BVA in that it is mechanically transmissible.

The association of O. brassicae with TSA was reported by Hidaka (1960 , cited in Grogan and Campbell, 1966). Confirmation of O. brassicae as a vector for TSA came from work done by

Hiruki (1965). He showed that the fungus lost the TSA upon passage on Cowpea (*Vigna sinensis*) seedlings. This TSA-free culture was found to acquire the TSA from roots of tobacco plants that were mechanically inoculated in the leaves with TSA. *In vitro* acquisition of the TSA by the zoospores of the fungus was found to be unsuccessful.

The TSA seems to be internally borne in the resting spores, since transmission was reported to occur after treatment of the spores (Hiruki, 1972, cited in Campbell, 1978). Multiplication of TSA within the fungus is not evident since the TSA was found to be lost by passage on cowpea (Hiruki, 1965). The transmission of TSA and BVA by *O. brassicae* is quite similar. The only major difference between the two disease agents is that the one is mechanically transmissible and the other not, but this should have no bearing on their vector relationships.

3.b. Transmission of Cucumber Necrosis Virus (CNV) by *Olpidium radicale*

Olpidium radicale has been shown to be identical to *O. cucurbitacearum* in all respects (Lange and Insunza, 1977). *O. radicale* displays the same characteristics as *O. brassicae*. Cucumber necrosis

virus (CNV) is an RNA-containing isometric virus with particles about 31 nm in diameter. The virus is soil-borne and is mechanically transmissible to many plants. Although exhibiting similar properties to TNV, CNV is serologically distinct from TNV (Dias and McKeen, 1972). CNV has been shown to be transmitted by O. radicale (Dias, 1970a). The virus is carried externally on the zoospores, and in vitro acquisition of virus occurs. Virus released from the roots adsorbs to zoospores after a 5-15 minute contact period, and enters the roots of the plants upon infection by zoospores (Dias, 1970b). Zoospores of O. radicale preferentially bound CNV to TNV in an in vitro acquisition system (Dias, 1970; Temmink et al., 1970). The transmission of CNV by O. radicale is similar to that of the O. brassicae-TNV system, only CNV seems to be more loosely bound to O. radicale than TNV is to O. brassicae (Temmink et al., 1970). The preferential binding of CNV to O. radicale is an example of the species specificity of fungal vectors.

3.c. Transmission of Potato Virus X (PVX) by Synchytrium Endobioticum

Synchytrium endobioticum is an obligate plant parasite belonging to the order Chytridiales. It has been extensively studied because of its severe

pathogenic effect on potatoes. The fungus is responsible for causing potato wart disease.

Potato virus X (PVX) is a flexuous rod-shaped RNA-containing virus of about 515nm in length (Bercks, 1970). PVX is transmitted mechanically quite easily. The virus spreads in the field by contact between healthy and virus-infected plants. The virus may also be soil-transmitted, since spread between plants whose only contact was below ground has been reported to occur (Roberts, 1950). Reports of transmission by grasshoppers were also published (Walters, 1952; Schmutterer, 1961 - both cited in Bercks, 1970). Since PVX spreads readily by contact in the field, the need for a vector is unnecessary. However, a vector for the virus could have been important in the dissemination of the virus prior to agriculture (Campbell, 1978).

Nienhaus and Stille (1965 , cited in Grogan and Campbell, 1966) presented evidence that Synchytrium endobioticum transmitted PVX. The virus was found to be transmitted by zoospores that were released from PVX infected plants. The zoospores were unable to transmit PVX when mixed with purified PVX in an in vitro uptake system. Apparently the virus is acquired by the fungus through an in vivo system only, and quite possibly carried internally in the

zoospore. The inability of the zoospores to transmit PVX by *in vitro* acquisition, places the PVX-*Synchytrium endobioticum* vector relationship with that of the BVA-, TSA-*Olpidium brassicae* relationship.

Only this one report on the transmission of PVX by a fungus exists and much research still needs to be done in order to provide more conclusive evidence for this vector relationship.

3.d. *Polymyxa graminis*

The fungus belongs to the order *Plasmodiophorales* and is typified by its motile zoospores having two flagellae. It is a soil-borne fungus and infects the roots of cereals. The fungus has been extensively studied because of its parasitic nature and worldwide distribution.

3.d.(i) Transmission of Soil-Borne Wheat Mosaic Virus (SBWMV)

Soil-borne wheat mosaic virus (SBWMV) is an RNA-containing rod-shaped virus of about 20nm x 110-160nm and 300nm. The virus is soil-borne and causes mosaic symptoms in wheat and barley.

The high correlation between the presence of *Polymyxa graminis* and mosaic in wheat was noted

by Linford and McKinney (1954). At that time these workers were unable to show that P. graminis was vector of SBWMV. Brakke and Estes (1965) and Brakke et al., (1965) reported a correlation between virus transmission and the fungus from infection of plants by root washings containing zoospores and virus. Polymyxa graminis was finally resolved as a vector for SBWMV (Estes and Brakke, 1966; Brakke and Estes, 1967).

The virus-vector relationship has been well-characterised. Transmission of SBWMV by spores stored for 3 years suggested that the resting spores harbour the virus (Rao , 1969). The virus is internally borne in resting spores and zoospores, or strongly bound and concealed on the spore's surface. Treatment of the spores with anti-SBWMV antiserum, acid, or alkali did not prevent transmission of the virus (Rao and Brakke, 1969). The virus is acquired from plants infected with SBWMV but not from an in vitro uptake system.

Release of the virus into healthy hosts occurs between 4-22 hours after inoculation with zoospores (Rao and Brakke, 1969). Multiplication of the virus in the fungus does not seem to occur because viruliferous cultures were freed of virus after three passages in clover (Canova, 1966).

3.d.(ii) Transmission of Flexuous Viruses of Cereals

The transmission of five flexuous, soil-borne viruses of cereals by Polymyxa graminis has been considered in a review by Campbell (1978). These viruses are discussed under the same heading because they have similar characteristics. The five viruses concerned are: Barley yellow mosaic virus (BYMV), wheat yellow mosaic virus (WYMV), Rice necrosis virus (RNV), Oat mosaic virus (OMV) and wheat spindle streak mosaic virus (WSSMV). All of these viruses have particles 13nm in diameter but varying lengths. BYMV, RNV and WYMV which are serologically related, have particles of two modal lengths, 275 nm and 550 nm (Inouye and Saito, 1975; Inouye and Fujii, 1977). OMV has particles 600-750nm in length (Herbert and Panizo, 1975) and WSSMV has particles 990-1975nm in length (Slykhuis, 1976). Correlations between Polymyxa graminis infection and the transmission of these viruses have been reported by Inouye and Saito (1975) and Inouye and Fujii (1977) for BYMV, WYMV and RNV; Herbert and Panizo (1975) for OMV; and Slykhuis (1976) for WSSMV.

Confirmation of Polymyxa graminis as a vector for WSSMV came from studies using unifungal cultures by Slykhuis and Barr (1978). The fungus

was shown to acquire the virus from plants that were mechanically inoculated. Apparently, the virus is internally borne since spores stored for 8 years still transmitted the virus.

A good correlation between OMV transmission and *P. graminis* infection was noted. Plants inoculated with virus infected root bits containing resting spores of *P. graminis* developed mosaic symptoms. Whereas, plants inoculated with virus-infected root bits from the same source plant but not containing resting spores, did not develop mosaic symptoms (Herbert and Panizo, 1975). Thus far, it has only been suggested that *Polymyxa graminis* is a vector for RNV, BYMV and WYMV (Inouye and Saito, 1975; Inouye and Fujii, 1977). Virus infection has been observed to occur whenever the resting spores of the fungus was present. Both *in vivo* acquisition by and internalisation of the virus in the resting spores seems evident for the proposed transmission of BYMV (Kusaba *et al*., 1971, cited by Inouye and Saito, 1975). Much evidence is still lacking for this type of vector relationship for these three viruses.

3.e. Transmission of Beet Necrotic Yellow Vein Virus (BNYVV) by Polymyxa betae

Polymyxa betae has a host range that is limited to Chenopodiaceae. The fungus infects sugar beet and causes stunting (Keskin, 1964, cited in Campbell, 1978). BNYVV has particles 20nm in diameter with lengths 60-105nm, 270nm and 390nm (Tamada, 1975). The virus has a narrow host range and is mechanically transmissible. The virus has been reported to be the cause of "rhizomania" disease of sugar beets (Tamada, 1975). A correlation between Polymyxa betae infection and the transmission of BNYVV has been reported (Tamada et al., 1975; Vuittenez et al., 1977, both cited in Campbell, 1978). Tamada et al., (1975) reported that non-viruliferous cultures were able to acquire the virus from infected plants. The observation of rod-shaped virus-like particles (VLP) in the zoospore and plasmodial thallus of the fungus (Tamada, 1975; Stocky et al., 1977, cited in Campbell, 1978) supports the theory that the virus is internally borne in the fungus.

3.f. Transmission of Potato Mop Top Virus (PMTV) by Spongospora subterranea

Spongospora subterranea is a soil-borne fungus belonging to the order Plasmodiophorales. The fungus is responsible for the formation of powdery scab

of potatoes. Potato mop top virus (PMTV) has been described as a rod-shaped virus having particles that are 20nm in width and 100-150nm and 250-300nm in length (Kassanis et al., 1972). The virus causes disease in potatoes and is considered to be soil-borne. PMTV is serologically related to soil-borne wheat mosaic virus (SBWMV) (Randles et al., 1976), and to TMV (Kassanis et al., 1972). Spongospora subterranea has been reported as a possible vector for PMTV (Calvert and Harrison, 1966), but evidence for this relationship is still lacking. The only other information on this fungus-virus relationship comes from the reported correlation between fungal and virus infections (Jones and Harrison, 1967). Evidently, PMTV was transmitted to healthy plants grown in soil containing virus-infected tubers with powdery scab, but not when grown in soil containing virus-free tubers with powdery scab. Apparently the virus is carried internally in the fungal resting spores since the virus was found to be transmitted by spores after dessication for one year.

3.g. Transmission of Tobacco Mosaic Virus (TMV) by Powdery Mildews

The powdery mildews belong to the order Erysiphales (Alexopoulus, 1962). These fungi are obligate

parasites and certain genera are known to destroy or to kill plants. Yarwood (1971) reported the transmission of TMV to Chenopodium quinoa by the powdery mildews, Sphaerotheca lanestris from oaks (Quercus agrifolia and Q. phellos) and Erysiphe graminis from barley (Hordeum vulgare). The reported transmission of TMV by conidia of these fungi was, however, explained as contamination or as the activation of a latent virus in Chenopodium.

Further evidence for the transmission of TMV by these two mildews came from studies done by Nienhaus (1971). He extracted infectious TMV directly from the washed conidia of these fungi. Besides excluding contamination by source plants of the fungi and activation of a latent virus in Chenopodium as an explanation for the results of Yarwood (1971), this study also indicates that TMV is internally borne in the conidia of these fungi. Detection of TMV in conidia and mildew infected barley tissue by electron microscopy, further supports the transmission of TMV by powdery mildews (Yarwood and Hecht-Poinar, 1973).

3.h. Transmission of Viruses by the Rust Fungi

The rust fungi are a vast group of parasites belonging to the order Uredinales (Alexopoulus, 1962).

These fungi cause disease in many agricultural crops and are of prime economic importance.

Yarwood and Hecht-Poinar (1973) were the first to demonstrate the possibility of virus transmission by these fungi. The authors reported the apparent association of TMV with several rusts. The detection of TMV in bean plants infected with Uromyces phaseoli (bean rust) and in germinated uredospores suggested to these authors that transmission of TMV by the fungus had occurred. They also observed that another rust, Uromyces polygoni (knotweed rust) was able to transmit a virus (still unidentified) to Chenopodium quinoa via uredospores.

The presence of rod-shaped (12 x 260nm and 10 x 660 nm) and spherical (35nm in diameter) virus particles have been reported in uredospore cultures of Uromyces phaseoli var. vignae (Cowpea rust) and in rusted leaf extracts by McDonald and Heath (1973). These authors made no suggestion of the possible transmission of these virus particles to the host plant, but considered these virus particles to be fungal viruses (Lemke and Nash, 1974; Bozarth, 1972). The verification of the presence of "TMV" rod-like particles in U. phaseoli by these authors could be regarded as a confirmation of the proposed transmission of TMV by U. phaseoli (Yarwood and Hecht-Poinar,

1973), since both sets of workers worked on the same fungal culture (Uromyces phaseoli var. vignae).

Lecoq et al., (1974) reported the extraction of spherical virus particles (34nm in diameter) sedimenting at 174S from plants infected with Puccinia malvacearum, P.striiformis and P. suaveolens. No virus particles were extracted from corresponding healthy plants. Since no attempt was made to detect the virus particles directly in the fungal mycelium or uredospores and teleutospores, this observation can hardly be described as a fungal virus system. The extraction of virus particles from fungus infected plants and not healthy plants, could be considered as being a situation of activation of a latent virus in these plants or possible transmission of the virus to these plants.

The possible transmission of Bromegrass mosaic virus (BMV) to healthy plants by Puccinia graminis tritici (stemrust of wheat) has been reported by Von Wechmar (1980). This author reported the detection of this virus within the uredospores. Apparently, the stemrust was able to acquire the virus from plants mechanically inoculated with BMV.

Rawlinson and Maclean (1973) reported the presence of a spherical virus-like particle (VLP), 38nm in

diameter with sedimentation coefficients of 110S and 58S in axenic cultures of Puccinia graminis tritici. Although the VLPs were detected in rust infected leaves as well, no correlation between the presence of the VLP in the fungus and transmission to wheat was thought to occur.

Several other species of rust fungi, namely Uromyces phaseoli var. typica, Puccinia helianthi, P. sorghi and Coleosporium solidaginis have been reported to have virus-like particles associated with them (Littlefield and Heath, 1979). Since mostly spherical virus particles have been discerned in most of the rust fungi, these particles may be ubiquitous in the Uredinales (Littlefield and Heath, 1979). These VLPs should be considered to be mycoviruses until such time that transmission to higher plants have been shown to occur.

3.i. Viruses Suspected of Having a Fungal Vector

Not much is known about the viruses that are suspected of having fungal vectors. No correlation between fungal infection and transmission of these viruses has been made. Their consideration for transmission by fungal vectors is merely based on their infection characteristics and comparison with other viruses having fungal vectors. Pea false leaf roll virus

(PFLRV) has been described as being seed-, aphid- and fungus-transmitted (Thottapilly and Schmutterer, 1968; Thottapilly, 1972, both cited in Campbell, 1978). The fungus Pythium ultimum has been reported by these authors as being a vector for PFLRV. This can be regarded only as a single observation with little merit (Campbell, 1978).

Red clover necrotic mosaic virus (RCNMV) which can be soil-borne (Hollings and Stone, 1977) has been suggested to be transmitted by Olpidium radicale (Lange and Insunza, 1977). The similarities between RCNMV and Cucumber necrosis virus (CNV) would provide for useful comparison between these two viruses if RCNMV is found to be transmitted by the same vector, O. radicale of CNV. The suggestion of a fungal vector for the agent of Freesia leaf necrosis which has been described as being soil-borne was made by Van Dorst (1975). Another virus, melon necrotic spot virus (MNSV) has been suspected of being soil-borne and having Olpidium as a vector (Komuro, 1971, as cited by Campbell, 1978). Sugar cane mosaic virus can be soil-borne under experimental conditions (Bond and Pirone, 1970). Whether a fungus vector can be involved in such a case is not known. It is quite possible that in such circumstances the virus could have been carried over to

a healthy plant by root contact (Campbell, 1978).

The chlorotic streak agent (CSA) causing chlorotic streak of sugar cane has not yet been resolved to being a virus or a mycoplasma-like agent. Transmission of the CSA by a fungus has been considered (Grogan and Campbell, 1966). It is difficult, however, to reconcile a vector relationship with this disease since stem cuttings which can be rooted serve as excellent sources of soil-borne inoculum (Campbell, 1978).

Viruses of the tomato bushy stunt virus (TBSV) group, namely TBSV, Petunia asteroid mosaic virus (PAMV) and Cymbidium ringspot virus (CyRSV) have no known vector, but the possibility of fungal vectors for these viruses could be important for their etiology (Campbell, 1978).

4. GENERAL OBSERVATIONS ON FUNGAL VECTORS

To date, two groups of fungi, the Chytridiales and Plasmodiophorales, have been shown to be the major fungal vectors of plant viruses. Reports of fungal vectors belonging to the orders Uredinales and Erysiphales have been published. All the fungal vectors are obligate parasites.

A high degree of vector specificity has been noticed for virus-fungal vector associations . Transmission of viruses by fungi is limited to one vector type (i.e. fungus only) and in some cases one fungal vector species is involved. A good example of the latter comes from the inability of Olpidium radicale, which transmits Cucumber necrosis virus (CNV), to acquire and transmit tobacco necrosis virus (TNV) (Temmink et al., 1970). The viruses involved infect and multiply in the shared plant host but no multiplication within the fungal vector is apparent.

Two modes of transmission of virus by the fungus are possible. In the first, the fungus can acquire the virus, which is usually soil-borne, from root exudates of virus infected plants in the soil or upon experimental mixing of spores and virus preparations. This is called in vitro acquisition, and the virus is usually externally bound to the spore. Internalisation of the virus can occur during encystment (Temmink, 1971). Transmission of the virus occurs during infection of healthy plants by the fungus. Secondly, the fungus acquires the virus from a virus-infected plant (in vivo acquisition) and transmits the virus to healthy plants upon infection. In this case, the virus is usually located within the fungal spore.

It has been proposed (Campbell, 1978) that two features

of fungal infection affect the capability of a fungus as a vector, namely (1) the existence of an ectoplast limited thallus to allow exchange of virus between the host and the fungus, and (2) the obligately parasitic nature of the fungus should be such that the host still functions normally in order to support virus multiplication.

CHAPTER II

INTRODUCTION

A. SOME CHARACTERISTICS OF BROMEGRASS MOSAIC VIRUS (BMV)

BMV is a spherical virus having particles 26nm in diameter with a sedimentation coefficient of 85S. The virus has a multipartitie genome (Lane, 1979) consisting of four pieces of ss-RNA with molecular weights of $1,1 \times 10^6$, $1,0 \times 10^6$, $0,75 \times 10^6$ and $0,3 \times 10^6$ daltons, designated RNA-1, RNA-2, RNA-3 and RNA-4, respectively. The four RNAs are encapsidated into three virions with slightly different bouyant densities. RNA-1 and RNA-2 are encapsidated individually, and RNA-3 and RNA-4 are encapsidated together. All three virions are required for infectivity.

BMV has distribution in central U.S.A., eastern Europe and South Africa (Lane, 1974). The strain BMV-G_9 which occurs in South Africa has been described by Von Wechmar and Van Regenmortel (1966). BMV-G_9 has been well-characterised serologically by Rybicki (1979).

BMV is easily transmitted by mechanical inoculation with sap. The virus can be transmitted by nematodes and beetles under laboratory conditions, but whether this occurs under natural conditions is not clear (Lane,

1981). The natural hosts of BMV are Bromus inermis and Agropyron distichum. Mechanical inoculation by man has been described as the most significant transmission mechanism for the virus (McKinney, 1953, cited in Lane, 1981). The virus has the potential of causing economically significant losses to crops should an appropriate vector appear (Lane, 1981). Indeed, BMV has been found to cause considerable damage in wheat in South Africa when transmitted by aphids (Von Wechmar and Rybicki, 1981).

In this thesis, the rôle that the fungus, Puccinia graminis tritici, serves as a vector for BMV is examined.

B. CHARACTERISTICS OF THE FUNGUS, PUCCINIA GRAMINIS TRITICI (STEMRUST OF WHEAT)

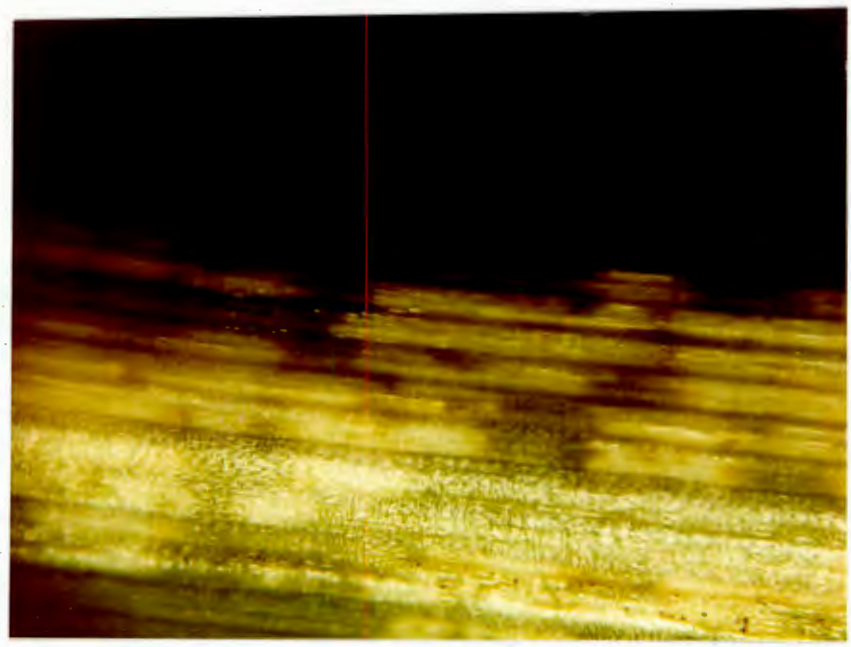
Puccinia graminis tritici belongs to the class Basidiomycetes, order Uredinales (Alexopoulus, 1962). This fungus is of major economic importance in the South African wheat growing industry. Another rust fungus, Puccinia graminis recondita, better known as leafrust of wheat, sometimes appears together with the stemrust fungus.

In South Africa, stemrust occurs in important grain growing areas such as the Orange Free State, Western Cape (Swartland) and South Western and Eastern Cape. In the Western Cape the stemrust fungus, Puccinia graminis

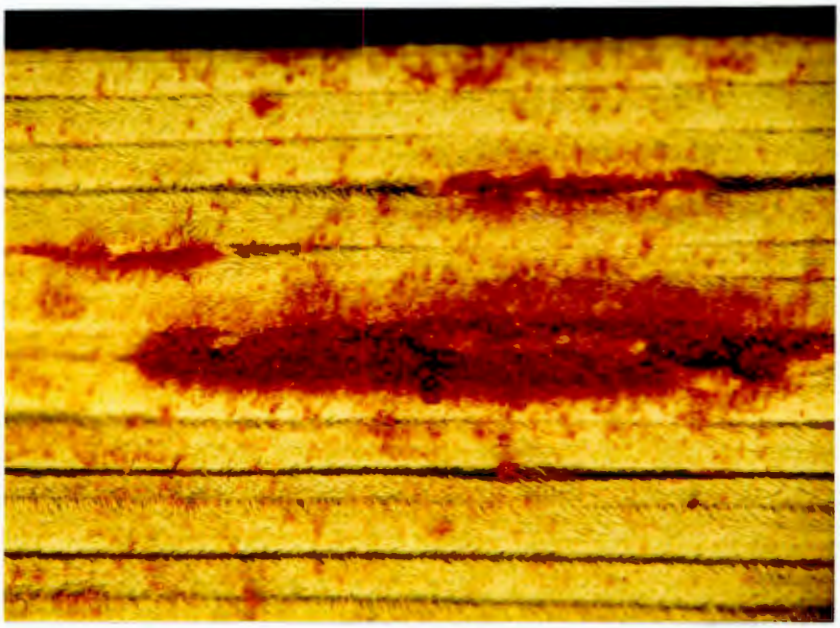
tritici, naturally infects Agropyron distichum on the coastal dunes. This plant is a natural host for BMV in South Africa. The simultaneous infection of Agropyron distichum with BMV and stemrust is illustrated in Figure 1.

The stemrust fungus has two life cycles, a sexual and an asexual one (Alexopoulus, 1962). The sexual life cycle of Puccinia graminis tritici is completed on barberry bush (Berberis vulgaris). Since this plant does not occur in South Africa, the fungus is limited to its asexual life cycle which is completed on graminaceous hosts.

The asexual life cycle is characterised by the infection of the grain plant by uredospores which represent the resting stage of the fungus. When the uredospores land on the leaf's surface, they germinate in the presence of sufficient moisture and produce hyphal threads that penetrate the plant tissue through the stomata. After penetration an active mycelium is developed within the plant tissue. The mycelium obtains nourishment from the host cells by means of haustoria. It has been postulated that the protoplasm of the haustorium in Puccinia graminis comes into intimate contact with the protoplasm of the host cell. This has been suggested to occur by oozing of the haustorial protoplasm through ultra-microscopic pores in the haustorial wall (Ehrlich



a



b

FIGURE 1: Dual infection of Agaropyron distichum with BMV and Puccinia graminis tritici (stemrust of wheat). Plants were collected from coastal dunes in the Western Cape.
(a) = mosaic symptoms caused by BMV infection on adaxial side of leaf.
(b) = stemrust pustules on abaxial side of leaf.

and Ehrlich, 1961, cited in Alexopoulus, 1962). The formation of new uredospores occurs in the uredium. The epidermis of the leaf bursts open as the number of uredospores increases in the upward expanding uredium. The formation of macroscopic pustules consisting of mature uredospores is seen within a few days after infection. The asexual cycle is repetitive and recurs several times in the spring and summer depending on the prevailing climatic conditions. The asexual cycle of Puccinia graminis tritici on wheat is represented diagrammatically in Figure 2.

C. OBJECTIVES AND REASON FOR THE STUDY INTO THE ASSOCIATION OF BMV WITH STEMRUST

The occurrence of infestation by stemrust on wheat crops in which BMV infection had reached epidemic proportions, in the Orange Free State, was first noticed by Von Wechmar in 1978 (Von Wechmar, 1980). Subsequently, the possible transmission of BMV by stemrust was investigated and reported (Von Wechmar, 1980).

The objective of this dissertation was the further investigation into the association of BMV with stemrust. The transmission of BMV by stemrust was investigated and consequently established. It was important to know whether the uredospores carried BMV inside or on their surfaces, since this has significant bearing on the

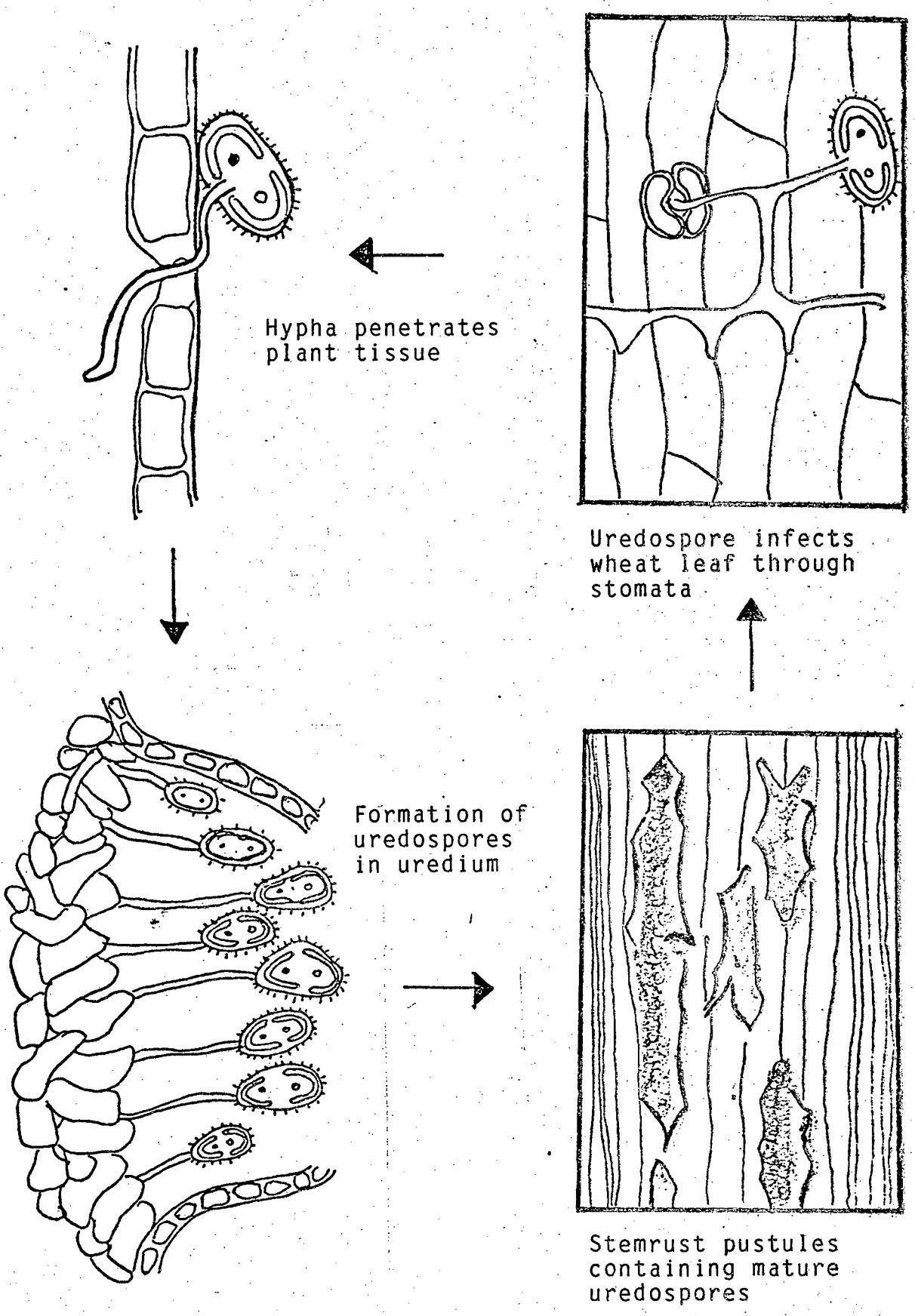


FIGURE 2: Asexual cycle of Puccinia graminis tritici on wheat.

(Redrawn from Agrios (1978)).

mode of transmission of BMV by the fungus. Techniques such as enzyme-linked immunosorbent assay (ELISA), fluorescent antibody binding studies and electron microscopy were employed for the detection of BMV in or on the uredospores.

In addition to this, the effect of BMV-infection of plants on stemrust infection was investigated. The results obtained for this case seemingly has application to natural field conditions occurring in the Orange Free State.

The stemrust-transmitted-BMV-isolates were partially characterised by electrophoresis and serology.

The results presented in this study are largely based on experiments in which the test virus was the BMV-Beth isolate (See III.B.1 on origin of viruses) and the test fungus was Puccinia graminis tritici (culture W138) (see III.C.1 on origin of fungal culture).

A report is also included of the transmission of BMV by Puccinia graminis recondita (leafrust of wheat). This is presented coincidentally with the reports of transmission of BMV by stemrust.

CHAPTER III

MATERIALS AND METHODS

A. BUFFERS AND REAGENTS

1. STANDARD SODIUM PHOSPHATE BUFFERS

Sodium phosphate buffers were made according to the specifications as in buffer No. 33A of Williams and Chase (1968). All the buffers presented below had a final molarity of 0,1 M, and were diluted with twice distilled water to give the desired molarity. The pH of the buffers were checked after dilution, and adjusted if necessary.

Stock Solutions

Solution A: NaH_2PO_4, 0,2 M
(Dissolve 27,6g of $NaH_2PO_4.H_2O$ in a final volume of 1 litre of distilled water).

Solution B: Na_2HPO_4, 0,2 M
(Dissolve 28,4g of Na_2HPO_4 in a final volume of 1 litre of distilled water).

1.a. 0,1 M Sodium Phosphate pH 6,0

Mix 438,5 ml of Solution A with 61,5 ml of Solution B.

Dilute to 1 litre with distilled water.

1.b. 0,1 M Sodium Phosphate, pH 6,5

Mix 342,5 ml of Solution A with 157,5 ml of Solution B.
Dilute to 1 litre with distilled water.

1.c. 0,1 M Sodium Phosphate, pH 7,0

Mix 195 ml of Solution A with 305 ml of Solution B.
Dilute to 1 litre with distilled water.

1.d. 0,1 M Sodium Phosphate, pH 7,2

Mix 140 ml of Solution A with 360 ml of Solution B.
Dilute to 1 litre with distilled water.

1.e. 0,1 M Sodium Phosphate, pH 7,4

Mix 95 ml of Solution A with 405 ml of Solution B.
Dilute to 1 litre with distilled water.

2. PHOSPHATE BUFFERED SALINE

Phosphate buffered saline (hereafter abbreviated in the text as PBS) was made at three pH values, namely pH 6,0, pH 7,0 and pH 7,4. The composition of these buffers was as follows:

2.a. PBS pH 6,0

Mix 1 part 0,15 M NaCl (8,7 g/litre) with 1 part 0,1 M Sodium phosphate, pH 6,0 (III.A.1.a).

2.b. PBS pH 7,0

Mix 1 part 0,15 M NaCl (8,7 g/litre) with 1 part 0,1 M Sodium phosphate, pH 7,0 (III.A.1.c).

2.c. PBS pH 7,4

Mix 1 part 0,15 M NaCl (8,7 g/litre) with 1 part 0,1 M Sodium phosphate, pH 7,4 (III.A.1.e).

3. VIRUS MULTIPLICATION, EXTRACTION AND STORAGE BUFFERS

3.a. 0,05 M Acetate buffers

These buffers were made from the method for buffer No. 1 of Williams and Chase (1968).

Stock Solutions

Solution A: Acetic acid, 0,2 M (11,55 glacial acetic acid/litre).

Solution B: Sodium acetate, 0,2 M. (Dissolve 27,2 g of $CH_3COONa.3H_2O$ in a final volume of 1 litre distilled water).

(i) 0,05 M Acetate, pH 4,0

Mix 410 ml of Solution A with 90 ml of Solution B.

Dilute to 2 litres with distilled water. This buffer was used for the extraction of the BMV strains, G_9 and Type.

(ii) 0,05 M Acetate, pH 5,0

Mix 148 ml of Solution A and with 352 ml of Solution B.

Dilute to 2 litres with distilled water. This buffer was used for the extraction of the BMV isolates, Beth and Palala.

Both of the buffers described above were used as storage buffers for the purified virus strains and isolates, as well as diluent for mechanical inoculation of BMV.

3.b. 0,1 μ Acetate/NaCl buffers

These buffers were made according to the specifications of Miller and Golder (1950). They were used for the extraction of Stemrust-transmitted-BMV.

Stock Solutions

Solution A: Sodium acetate, 2,0 N.

(Dissolve 272,16 g of $CH_3COONa.3H_2O$ in a final volume of 1 litre distilled water).

Solution B: Acetic acid, 3,5 M
(210 glacial acetic acid/litre).

Solution C: NaCl, 5,0 M
(Dissolve 292,2 g of NaCl in a final volume of 1 litre distilled water).

3.b.(i) 0,1 μ Acetate/NaCl pH 4,0

Mix 20,0 ml of Solution A with 33,7 ml of Solution B and 32,0 ml of Solution C.

Dilute to 2 litres with distilled water.

3.b.(ii) 0,1 μ Acetate, pH 5,0

Mix 20,0 ml of Solution A with 3,7 ml of Solution B and 32,0 ml of Solution C.

Dilute to 2 litres with distilled water.

3.c. 0,1 M Potassium phosphate, pH 7,0

This buffer was made according to the specifications as in buffer No. 27 of Williams and Chase (1968). This buffer was used extensively for the extraction of stemrust-transmitted-BMV.

Stock Solutions

Solution A: KH_2PO_4, 0,5 M
(Dissolve 68,04 g of KH_2PO_4 in a final volume of 1 litre distilled water).

Solution B: K_2HPO_4, 0,5 M
(Dissolve 87,09 g of K_2HPO_4 in a final volume of 1 litre distilled water).

The composition of the buffer was as follows:

Mix 78 ml of Solution A with 122 ml of Solution B. Dilute to 1 litre with distilled water.

4. DOUBLE-DIFFUSION GEL PRECIPITIN (OUCHTERLONY) BUFFERS

The following three buffers were used to make 0,7% solutions of agar for the test plates.

4.a. 0,05 M Acetate/NaCl buffer, pH 5,0

Mix 1 part 0,15 M NaCl (8,7 g/litre) with 1 part 0,1 M sodium acetate buffer, pH 5,0 (buffer in III.A.3.a.(ii) used at double strength).

4.b. Phosphate buffered saline, pH 7,0

PBS pH 7,0 (III.A.2.b) was used.

4.c.(i) 0,1 M Cacodylate buffer, pH 6,0 (Rybicki, 1979)

Dissolve 21,4 g sodium cacodylate (trihydrate M.W. 214) in 800 ml distilled water.
Adjust to pH 6,0 with 1 M HCl, and add distilled water to make 1 litre.

4.c.(ii) Cacodylate/NaCl buffer, pH 6,0

Mix 1 part 0,1 M cacodylate buffer, pH 6,0, with 1 part 0,15 M NaCl (8,7 g/litre).

5. GEL ELECTROPHORESIS BUFFERS

5.a. Polyacrylamide Gel Electrophoresis (PAGE) Buffers

These buffers were made according to the specifications of Laemmli (1970).

5.a.(i) Monomer solution

(30% acrylamide : 0,8% bisacrylamide)

Dissolve: 58,4 g acrylamide
4,0 g bisacrylamide
in a final volume of 500 ml water. Store at 4°C.

5.a.(ii) Resolving gel buffer

(1 M Tris/HCl pH 8,8)

Dissolve 60,6 g Tris (Merck Cat. No. 8382) in water. Adjust to pH 8,8 with HCl. Dilute to 500 ml with water. Store at 4°C.

5.a.(iii) Stacking gel buffer

(1 M Tris/HCl pH 6,8)

Dissolve 60 ,6 g Tris in water. Adjust to pH 6,8 with HCl. Dilute to 500 ml with water. Store at 4°C.

5.a.(iv) Bath buffer (10X)

Dissolve: 30,3 g Tris
141,0 g Glycine
10,0 g SDS

in a final volume of 1 litre water. Store at room temperature.

5.a.(v) Dissociation mixture

(10% SDS, 10% B-Mercaptoethanol, 15% glycerol, 0,01% bromophenol blue in 1 M Tris pH 6,8).

Mix and dissolve: 5 g SDS
5 ml B-Mercaptoethanol
4,5 ml Glycerol
6,3 ml 1 M Tris pH 6,8
2,5 ml 0,2% bromophenol blue.

Store at room temperature.

5.a.(vi) 10% SDS

(Sodium dodecyl sulphate)

Dissolve 50 g SDS in a final volume of 500 ml distilled water.

5.a.(vii) Initiator

(1,5% ammonium persulphate)

Dissolve 0,15g ammonium persulphate in a final volume of 10ml water. This solution is made up fresh each time.

5.a.(viii) Temed

(NNN'N' tetramethylethylenediamine)

5.a.(ix) Coomassie blue stain

The composition is as follows:

45% (v/v) methanol

10% (v/v) glacial acetic acid

0,2% (w/v) Coomassie blue.

(Procedure: Dissolve the dye (Coomassie blue) in a small volume of methanol and filter. Then add the rest of the methanol, water and acetic acid).

5.a.(x) Destaining solution

The composition is as follows:

25% (v/v) methanol

10% (v/v) glacial acetic acid

65% (v/v) distilled water.

5.b. Horizontal-Slab Agarose Gel Electrophoresis Buffers

The basic buffer was 0,01 M Sodium phosphate containing 1mM $MgCl_2$. The composition was as follows:

Mix: 200ml 0,1 M Sodium phosphate (III.A.1a-c,e)

2ml 1 M $MgCl_2$ (203,31g $MgCl_2.6H_2O$/litre)

Dilute to 2 litres with distilled water. The buffer was made at four pH values, namely pH 6,0, pH 6,5, pH 7,0 and pH 7,4.

The same buffer served as gel buffer and tank buffer for a particular experiment.

6. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) BUFFERS

6.a. Coating buffer

(0,05 M sodium carbonate, pH 9,6)

This buffer was made according to the specifications of Clark and Adams (1975).

Dissolve: 1,59g Na_2CO_3

2,93g $NaHCO_3$

0,2g NaN_3

in a final volume of 1 litre distilled water.

6.b. Antigen dilution buffer (PBS pH 6,0)

Phosphate buffered saline, pH 6,0 (III.A.2.a) was used.

6.c. Rinsing buffer (PBS-Tween)

Phosphate buffered saline pH6,0 containing 0,05% (v/v) Tween-20.

6.d. Post-coating and conjugate dilution buffer (PBS-T-BSA)

Phosphate buffered saline, pH 6,0 containing 0,05% (v/v) Tween-20 and 0,2% (w/v) Bovine serum albumin (BSA)

6.e. Substrate buffer (10% Diethanolamine)

This buffer was made according to the specifications of Clark and Adams (1975).

Mix: 97,0ml diethanolamine
800ml distilled water
0,2g sodium azide

Adjust to pH 9,8 with 1 M HCl. Add distilled water to give a final volume of 1 litre.

7. FLUORESCENT ANTIBODY STAINING BUFFERS

7.a. Conjugation buffer

(0,02 M sodium carbonate, pH 9,8)

This buffer was used for the conjugation of fluorescein iso-thiocyanate (FITC) to the gamma globulins. The stock buffer was made according to the specifications in buffer No. 19 of Williams and Chase (1968)

Stock Solutions

Solution A: Sodium acid carbonate, 1,0 M.
(Dissolve 84,0g $NaHCO_3$ in a final volume of 1 litre distilled water).

Solution B: Sodium carbonate, 1,0 M.
(Dissolve 106,0g Na_2CO_3 in a final volume of 1 litre distilled water).

Stock Buffer (1 M sodium carbonate, pH 9,8)

The composition was as follows:

Mix: 34,3ml of Solution A with 21,9ml of Solution B. Dilute to 1 litre with distilled water. To make 0,02 M sodium carbonate buffer pH 9,8, the stock buffer was diluted 1:50 with distilled water.

7.b. Rinsing buffer

Phosphate buffered saline, pH 7,0 (III.A.2.b).

7.c. Coating solution

0,1% (w/v) ovalbumin in distilled water.

7.d. Mounting fluid

90% (v/v) glycerol in 0,02 M carbonate buffer pH 9,8.

8. BUFFERS AND REAGENTS USED IN ELECTRON MICROSCOPY

8.a. Negative stains

(i) 2% uranyl acetate, pH 4,0

(ii) 2% phosphotungstic acid, pH 7,0.

8.b. Buffers and stains used for thin sectioning

8.b.(i) Palade's veronal-acetate buffered osmium tetroxide (OsO_4) (Palade, 1952)

Stock buffer

Dissolve: 14,7g sodium barbital (veronal)
9,7g sodium acetate
in a final volume of 500ml of distilled water.

Osmium tetroxide stock (OsO_4)

A 2% solution of OsO_4 is distilled water.

Working solution

Mix: 1,25ml stock buffer
1,25ml 0,1 N HCl

0,625ml distilled water

3,125ml 2% OsO_4

This solution is stored at 4°C.

8.b.(ii) Fixative

This consists of a 5% (w/v) solution of glutaraldehyde in 0,1 M sodium phosphate buffer, pH 7,2 (III.A.1.d). For convenient use a stock solution of 25% glutaraldehyde was always kept.

8.b.(iii) Stains

Saturated uranyl acetate (a 2% (w/v) uranyl acetate in distilled water). 0,2% (w/v) lead citrate in 0,1 N NaOH.

8.b.(iv) Spurr's epoxy resin

Mix: 26g anhydride hardener

4g flexibilizer

10g epoxy resin

0,4g accelerator

All the above reagents are obtainable in a Taab Spurr's epoxy resin kit.

B. PROPAGATION OF VIRUS STRAINS AND ISOLATES

1. ORIGIN OF THE VIRUSES

The virus strains BMV-Type and BMV-G_9 and other isolates used in the study were obtained from M.B. Von Wechmar (Department of Microbiology, U.C.T.). These strains have been well characterised (Lane, 1977; Von Wechmar and van Regenmortel, 1966 and 1968).

The virus isolates BMV-Palala and BMV-Beth are field isolates collected in the Orange Free State, South Africa. BMV-Palala originates directly from naturally infected field grown wheat cv Palala (hence the designation, BMV-Palala). BMV-Beth was obtained from naturally infected bromus plants (Bromus catharticus) collected at Bethlehem in the Orange Free State (hence the designation, BMV-Beth). Both of these viruses were highly infectious upon mechanical inoculation to wheat and barley seedlings. The virus isolate BMV-Beth was used extensively during the investigation. It is the same virus isolate M.B. von Wechmar (1980) used in her preliminary studies on the transmission of BMV by stemrust.

2. MULTIPLICATION, HARVESTING AND STORAGE

2.a. Hosts

The BMV strains and isolates were propagated in

either wheat (*Triticum aestivum* L.) *cvs*. Palala, Scheepers, Flameks and Betta or barley (*Hordeum vulgare* L.) *cvs*. Swanneck and Clipper.

Plants were grown in pots in steam sterilised soil at controlled plant growth room conditions of 24°C, 70% relative humidity and 14 hours daylight.

2.b. Inoculation and Multiplication

The virus strains and isolates were multiplied from lyophilized BMV infected leaf tissue. The inoculum was prepared by grinding the dried leaf tissue to a fine powder in a mortar and pestle, followed by addition of 0,05M sodium acetate buffer, pH 4,0 or pH 5,0 (III.A.3.a (i) and (ii)). Celite was added as an abrasive to puncture the leaf surface.

Wheat and barley seedlings (5-7 days old; fully developed two leaf stage) were inoculated by wiping the seedlings with cotton wool that was dipped in the inoculum. Special care was taken to inoculate the seedlings on both sides, especially at the stem base to ensure infection of emerging new leaves at the growth point. The plants were sprayed with water immediately after inoculation. The inoculated plants were grown under controlled plant growth room conditions (III.B.2.a).

For virus acquisition by the fungus, plants were inoculated with purified virus at 0,5 mg/ml concentration in 0,05 M sodium acetate containing celite.

2.c. Harvesting and Storage

Symptoms appeared in the form of mild mosaic usually 5 days after inoculation. At 7 days the plants were harvested at soil level.

Harvested plants were stored at 4°C in plastic bags if virus extraction followed soon afterwards. A third of the harvest from the first propagation was stored in two other ways:

(i) infected leaf tissue was lyophilized over self-indicating silica gel in a dessicator under vacuum, and stored in vials containing silica gel at 4°C. This served as a source stock of virus isolates. Virus stored in this fashion remains viable for long periods. BMV-G_9 stored in this way was viable after 15 years of storage as compared to BMV in buffer (pH 4-5) where infectivity decreased after several months. (Von Wechmar, unpublished results).

(ii) some of the infected plants were also placed in small plastic bags and stored at -20°C. (This was done to avoid the risk of mutation and to ensure the use of original stock

material for propagation). These plants were used routinely for further multiplication of the BMV isolates if required.

3. VIRUS EXTRACTION AND PURIFICATION

3.a. Centrifugation of Viruses

In the text, the terms "low speed" and "high speed" centrifugation refer to centrifugation at 10 000 rpm for 10 minutes, and 34 000 rpm for 100 minutes (or 50 000 rpm for 45 minutes), respectively. Low speed centrifugation was performed in the Sorvall RC2-B refrigerated centrifuge, using the Sorvall SS-34 and GSA rotors. High speed centrifugation was performed in the Beckman L3-50 and L5-65 ultra-centrifuges, using the Beckman type 35 and 50Ti rotors.

The relative centrifugal forces (RCF) exerted by the Sorvall SS-34 and GSA rotors at 10 000 rpm are 12062g and 16319g, respectively.

The RCF was 142 800g for the 35 rotor at 34000rpm and 226 600g for the 50Ti rotor at 50 000 rpm. Rate zonal centrifugation was done in a Beckman 27.1SW rotor which at 2 600 rpm has an RCF of 113 000g.

3.b. Extraction Procedure

The BMV infected plants were cut into approximately 2cm lengths. The cut leaves were homogenized in a Waring blender in the presence of 0,05 M sodium acetate buffer, pH 4,0 or pH 5,0 (III.A.3.a.(i) and (ii)). A leaf to buffer ratio of 1:2 (w/v) was used. The homogenizations were performed for brief periods to prevent excessive foaming and heating which causes loss of virus by degradation. The homogenate was filtered through cheesecloth to obtain a filtrate free of plant debris. The pH of the filtrate was adjusted to either pH 4,0 or pH 5,0, by dropwise addition of 10% (v/v) acetic acid. (Plant proteins and ribosomes start precipitating at about pH 5,0 and are irreversibly precipitated at about pH 4,5 (Gibbs and Harrison, 1976), whereas BMV which has an isoelectric point of about pH 7,0 remains in solution).

After low speed centrifugation of the BMV homogenate, the supernatant (usually clear-straw coloured) was decanted and subjected to a cycle of high speed centrifugation.

The virus pellet that was obtained was resuspended in 0,05 M sodium acetate buffer, pH 4,0 or pH 5,0. (The pellets were resuspended by pouring enough buffer into the tubes to cover the pellets, and

leaving them at 4°C overnight.) The resuspended pellets were centrifuged at 10 000 rpm for 10 minutes. This centrifugation step removed all denatured insoluble components, leaving the supernatant as a bire-fringent, purified and concentrated virus suspension. The purified virus was stored in sealed glass bottles at 4°C.

3.c. Further Purification

The abovementioned purified virus suspension was considered to be highly pure, and in some instances was used to inject rabbits in order to raise anti-serum directed against BMV. However, a BMV-G_9 purified virus suspension of a higher purity was prepared and was used for the production of a higher quality antiserum. Further purification of the purified virus suspension involved:

(i) rate zonal centrifugation on 10-40% sucrose gradients in a Beckman 27.1SW rotor at 26 000 rpm (113 000g) for 2 1/2 hours. The contents of the tubes were scanned by ultraviolet absorption at 254nm on an Isco fractionator. The BMV fraction was collected and dialized against 0,05 M sodium acetate buffer, pH 4,0 to remove the sucrose. This was followed by high speed centrifugation. The virus pellets were resuspended in a small volume

of 0,05 M sodium acetate buffer, pH 4,0.

(ii) The virus suspension was further purified by zone electrophoresis (III.F.) through a sucrose density gradient. The virus zone that was collected was dialised, centrifuged at high speed and resuspended in 0,05 M sodium acetate, pH 4,0.

3.d. Quantitation

The concentrations of purified virus were determined by using the ultraviolet absorption method. An appropriate dilution of the purified virus suspension was made in 0,05 M acetate buffer, pH 4,0 or pH 5,0, and was scanned in 1cm, 2ml volume quartz cuvettes in the range 220-310nm on a Beckman Model 25 spectrophotometer. The concentration of the virus in the suspension was determined by using the extinction coefficient of $E^{0,1\%}_{260nm} = 5,15$ (Lane, 1977).

The average yield of BMV was usually in the range of 1-3g/kg leaves.

C. PROPAGATION OF STEMRUST

1. ORIGIN OF CULTURE

The stemrust culture W138 used in this study was obtained

in the form of dessicator dried spores from J. Truter (Department of Agriculture and Fisheries, Winter Rainfall Region, Stellenbosch).

2. INOCULATION AND PROPAGATION

2.a. Hosts

Stemrust was routinely propagated on wheat (*Triticum aestivum* L.) *cvs*. Flameks, T_4 and Little Club.

The plants were sown and grown as before (see III.B.2.a).

2.b. Inoculation and Incubation

Wheat seedlings at the fully developed two-leaf stage were inoculated with a uredospore suspension at 2mg/ml. The uredospores (dessicator dried or freshly collected) were suspended in sterile distilled water containing 0,001% Tween-20 as a wetting agent, and this suspension was sprayed onto the plants using a small atomizer. The spray atomizer (Camag reagent spray Cat. No. 26100) used consisted of a 100ml volume conical flask with a manually operated air-driven spray attachment. After inoculation, the plants were incubated at 24°C in a moist chamber (40 x 40cm plastic box covered with plastic, sitting

on top of a 2cm deep tray filled with water) for 2-3 days, away from direct light. After incubation, the plants (still in the plastic box, the plastic covers having been removed 12 hours before) were placed under direct light and were grown at plant growth room conditions of 24°C, 70% humidity and 14 hour daylight.

At 3 days after inoculation, stemrust pustule development started and was noticed by the formation of white flecks on the leaf surface. Fully developed rust pustules occurred at about 4-5 days after inoculation. At this stage, the humidifiers were switched off to reduce the relative humidity. This was done in order to prevent germination of the uredospores on the leaf surface, since the viability of the harvested uredospores depended upon the uredospores being ungerminated at the time of harvesting.

2.c. Harvesting and Storage

Harvesting of the uredospores was done 14 days after inoculation when the stemrust pustules were fully developed. The uredospores were collected by gently tapping the leaves with a flame sterilised long, rounded spatula over a sterile, glass Petrie dish standing on a white sheet of newsprint. Uredospores falling onto the paper were also shaken into the Petrie dish. The collected uredospores

were placed in vials and dried overnight, with the vials open, in a dessicator containing dry self-indicating silica gel. The vials were sealed and stored at 4°C in a plastic box with sealing lid containing a few crystals of dry silica gel. Uredospores stored in this way were found to be viable after 3 months of storage. Long-term storage of uredospores involved storage of leaf tissue with stemrust pustules in plastic vials under liquid nitrogen in a cryostat. This was done to preserve the stock culture in case it was necessary to go back to original material.

D. ACQUISITION AND TRANSMISSION OR BMV BY STEMRUST

1. HOSTS

The following hosts were used:

(i) wheat (*Triticum aestivum* L.) *cvs* T_4, T_4-R (resistant to prevalent naturally occurring stemrust strains in South Africa) and Little Club.

(ii) barley (*Hordeum vulgare* L.) *cv* Clipper. The plants were grown under plant growth room conditions as before (III.B.2.a).

The wheat *cvs*. T_4 and Little Club were used for virus acquisition studies, and wheat *cv*. T_4-R and barley *cv*

Clipper were used in transmission experiments.

2. VIRUS ACQUISITION

Wheat seedlings at the two-leaf stage were mechanically inoculated with a purified preparation of BMV at 0,5mg/ml concentration (see III.B.2.b). At 5 days post virus inoculation, the plants were inoculated with a suspension of non-viruliferous stemrust uredospores at 2mg/ml (see III.C.2.b). The plants were incubated and grown as before (III.C.2.b). The stemrust uredospores (viruliferous uredospores) were harvested 16 days post inoculation with stemrust and stored as before (see III.C.2.c).

3. TRANSMISSION EXPERIMENTS

T_4-R wheat and Clipper barley seedlings (at the two-leaf stage) were inoculated with a suspension of viruliferous stemrust uredospores at 4mg/ml. Plants were usually inoculated with freshly collected viruliferous uredospores. The plants were incubated and grown as before (see III.C.2.b) for 16 days under plant growth room conditions. At this stage Clipper barley plants showed mature pustule development, whereas T_4-R wheat plants, resistant to stemrust infection, displayed no pustule development. The Clipper barley plants showed signs of yellowing and brown streaking, whereas T_4-R wheat plants displayed yellow tips and white flecks. After 16 days at plant

growth room conditions, the plants were sprayed with a fungicide and a systemic insecticide and were grown at 4°C under continuous light for 7 days. (Plants were sprayed with insecticide to keep away run-away aphids from maintenance colonies used by other workers in the laboratory). The entire plants were harvested, placed in plastic bags and stored at 4°C or processed immediately.

4. EXTRACTION OF THE STEMRUST-TRANSMITTED VIRUS

4.a. Extraction and Concentration

Initially, the same extraction procedure as used for the purification of the mechanically propagated BMV isolates was used (see III.B.3.b), only the buffers 0,1 μ acetate pH 4,0 and pH 5,0 were used during homogenisation. Since no virus was recoverable by this method a new extraction procedure for BMV was jointly developed with E.P. Rybicki and M.B. von Wechmar. This procedure was successfully used by these two workers for the extraction of aphid transmitted BMV and has been published (Rybicki and Von Wechmar, 1982).

Procedure

The infected plants were cut into approximately 2cm lengths and homogenised in a Waring blender in

the presence of 0,1 M potassium phosphate buffer pH 7,0 (III.A.3.c). A leaf to buffer ratio of 1:3 (w/v) was used. The homogenate was filtered through cheesecloth and subjected to one cycle of low speed centrifugation.

The supernatant was decanted and the virus precipitated by addition of 2,5% NaCl and 9% (w/v) P.E.G. (Polyethylene glycol, molecular weight 6 000d). The precipitate was collected by low speed centrifugation. The precipitate was resuspended in 0,1 M potassium phosphate buffer, pH 7,0, at a volume equivalent to one tenth of the original volume of clarified plant sap. The resuspended precipitate was clarified by low speed centrifugation. The supernatant was decanted and subjected to high speed centrifugation. The pellets were resuspended in 0,05 M sodium acetate pH 5,0, at a volume equivalent to 400 x less the volume of the original clarified sap.

The virus concentrate was further purified by rate zonal centrifugation on 10-40% linear sucrose gradients in a Beckman 27·1SW rotor at 26 000 rpm (113 000g) for 2 1/2 hours. The virus was collected by fractionation on an Isco fractionator and was dialized against 0,05 M sodium acetate pH 5,0 to remove the sucrose.

4.b. Quantitation

The concentration of the stemrust-transmitted-virus suspension was determined in two ways:

(i) The concentration of the virus in the virus concentrate before rate zonal centrifugation, was determined by Ouchterlony double diffusion tests using BMV at a known concentration as a standard.

(ii) The concentration of the virus in the rate zonal centrifugation fraction was determined by the ultraviolet absorption method using the extinction coefficient, $E^{0,1\%}_{260nm} = 5,15$.

E. ANTISERUM PRODUCTION

Purified virus at 1-2mg/ml concentration was mixed in a ratio of 1:1 (v/v) with Freund's incomplete adjuvant and emulsified by repeated uptake into a syringe. One millilitre of the emulsion was injected intramuscularly into rabbits at weekly intervals for three weeks. After that, the rabbits were boosted at four to six week intervals. Two weeks after the first injection, the rabbits were bled, from a marginal vein in the ear. Twenty to thirty ml of blood was collected at each bleeding. The blood was allowed to clot, and the serum

fraction decanted and centrifuged at low speed to remove residual blood cells.

Consecutive bleedings were packaged separately in labelled bottles. The antiserum was stored at either 4°C for immediate use, or at -20°C for long-term storage.

A total of six rabbits was used to produce antisera directed against purified BMV. Various other rabbits were injected in the same way with either uncrushed or crushed stemrust viruliferous uredospores. This was an attempt at indirectly detecting whether the stemrust uredospores carried virus. The viruliferous uredospore samples used for injection purposes were prepared as follows:

(i) Whole viruliferous uredospores were suspended in water.

(ii) Viruliferous uredospores were crushed in a mortar and pestle in the presence of water.

(iii) Viruliferous uredospores floated on water overnight to germinate (about 10-20% germination was observed) were crushed in a mortar and pestle.

Goat anti-rabbit (GAR) antiserum directed against rabbit gamma globulins and anti-BMV protein antiserum was obtained from antiserum stocks of the Microbiology

Department, U.C.T., Cape Town.

F. ZONE ELECTROPHORESIS

This technique used for the separation of plant viruses from plant proteins has been fully described by Van Regenmortel (1964, 1972) and Polson and Russel (1967).

The buffer used in this study was 0,1 μ acetate pH 5,0 (III.A.3.b.(ii)). The technique was used for the further purification of BMV-G_9 which was used to raise an antiserum free of antibodies reacting with host plant proteins (see III.B.3.c.(ii)).

G. THE DOUBLE-DIFFUSION GEL PRECIPITIN (OUCHTERLONY) TEST

This technique has been widely used for the serological investigation of spherical plant viruses.

In this study the Ouchterlony test was used for the determination of antiserum titre, and for the detection and determination of virus concentration in purified and crude stemrust-transmitted virus extracts.

The test plates consisted of 10cm plastic Petrie dishes containing 20ml of 0,7% (w/v) Bacto-Noble agar (Difco Laboratories, Detroit, Michigan, U.S.A.) gel with 0,1%

(w/v) sodium azide as preservative. The buffers used for the agar solution were PBS pH 7,0 and 0,05 M sodium acetate/NaCl pH 5,0 (III.A.4). Well patterns, hexagonally arranged holes (4mm in diameter, spaced 4mm apart) 4mm from a central hole of the same diameter, were cut into the agar gel using a gel punch. Each plate could accommodate four such patterns. The agar plugs were removed by suction on an aspirator.

For the assays, antiserum was always placed in the central well, and antigen dilutions in appropriate buffer in the surrounding wells. For the determination of the antiserum titre, serial two-fold dilutions of antiserum were placed in the central wells of eight or more hexagonal well-patterns, and serial two-fold dilutions of the homologous antigen in the surrounding wells of each pattern. In this way the optimal proportions for each antigen-antiserum combination were simultaneously determined. The titre of an antiserum was taken as the penultimate dilution of antiserum to give a visible precipitin reaction with its homologous antigen. For the detection of virus in purified or crude extracts of virus, one or two dilutions of antiserum were reacted against six serial two-fold dilutions of antigen.

For the determination of virus concentration in a virus suspension, one antiserum dilution was reacted against six serial two-fold dilutions of the test sample and

compared with reactions of BMV at a known concentration as determined by the ultraviolet absorption method.

In all the assays, the test plates were placed in a moist chamber for at least 18 hours at room temperature to allow the development of precipitin bands. In the case of weak reactions, the test plates were left for up to two days for the precipitin bands to reach maximum density.

The test plates were usually viewed for precipitin bands on a light box against a dark background. Prior to photographing, the plates were washed with water to remove all precipitates in the wells. The plates were subsequently flooded with saline to preserve the precipitin bands.

H. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The "sandwich" ELISA technique useful for small amounts of samples and its extreme sensitivity for virus detection (Voller et al., 1976; Clark and Adams, 1977) was considered to be an ideal assay technique for the detection of BMV in and on the stemrust uredospores.

1. PREPARATION OF ANTIBODIES

Three antisera namely antisera Nos. 1, 3 and 5 (see Table 4), having Ouchterlony gel diffusion titres of

1/128 and 1/512 respectively were used in the immunosorbent assays.

The procedure of Clark and Adams (1977) was used for the purification and enzyme-labelling of the gamma-globulin serum fraction.

1.a. Purification of Gamma-Globulin

One millilitre of antiserum was diluted with distilled water, and mixed 1:1 (v/v) with saturated ammonium sulphate by dropwise addition. The mixture was left at room temperature for 15 minutes. The precipitated globulin fraction was collected by low speed centrifugation. The precipitate was dissolved in 2ml half-strength PBS pH 7,4 (III.A.2.c) and dialized overnight at 4°C against 2 litres of half-strength PBS pH 7,4 to remove the excess ammonium sulphate. The gamma globulin fractions were purified by ion exchange chromatography by filtration through a column (15cm x 1cm) containing diethylaminoethyl (DEAE) cellulose (Whatman DE52 anion-exchange). The DEAE cellulose column was pre-equilibrated with half-strength PBS pH 7,4. The gamma globulin was washed through the DEAE cellulose column with half-strength PBS pH 7,4. One millilitre fractions were collected in Wasserman tubes and the effluent was monitored by U.V. absorption at 280nm.

The first protein fractions that were eluted were collected and pooled. The ultraviolet absorbance at 280nm of the pooled fractions was measured on a spectrophotometer, and the gamma globulin fraction was adjusted to a concentration of 1 mg/ml with half-strength PBS pH 7,4. The extinction coefficient of gamma globulin was taken as $E^{0,1\%}_{280nm} = 1,4$ (Clark and Adams, 1977).

1.b. Conjugation with Alkaline Phosphatase

The enzyme used for labelling of the gamma globulins was alkaline phosphatase from beef mucosa (Miles). The enzyme was obtained as a salt-free, freeze-dried powder and had a specific activity of 1 000 units per mg protein.

Two milligrams of enzyme was dissolved directly in 2ml of purified gamma globulin at a concentration of 1mg/ml. The mixture was dialysed overnight at 4°C against 2ℓ of half-strength PBS pH 7,4. The mixture was placed in a vial and glutaraldehyde (25% (w/v) solution from Fluka AG, Sweden) was added to give a final concentration of 0,06% by addition of 40 µℓ of 2,5% glutaraldehyde in half-strength PBS pH 7,4 to 2ml of the dialysed globulin-enzyme mixture. The new mixture was left at room temperature for four hours. This was followed by dialysis overnight at 4°C against

2ℓ of PBS pH 7,4, to remove unreacted glutaraldehyde. The enzyme conjugated gamma globulins were stored at 4°C in sealed vials in the presence of 0,5% (w/v) bovine serum albumin (BSA) which was added to stabilise the conjugates.

The enzyme conjugated gamma globulins were diluted in PBS-T-BSA buffer (III.A.6.c) before each assay.

2. ASSAY PROCEDURE

2.a. Equipment Used

Dynatech "Micro ELISA" flat-bottomed trays were used as test plates. Finn variable volume micropipettes (0-5µl, 50-250µl and 200-1000µl) and Gilson Repetman repeating, variable volume micropipettes (0-250µl and 200-1000µl) were used for all pipetting operations.

During the assay, the microtitre trays were placed in a moist plastic container with a lid and incubated at 37°C in a Memmert incubator. The optical absorbance of the hydrolysed substrate in the wells was recorded on a Titertek multiscan automatic read-out spectrophotometer (Type 310C).

2.b. Preparation of Antigens

Purified preparations of BMV and wild cucumber mosaic virus (WCuMV) were used as positive and negative controls, respectively. Serial two-fold or four-fold dilutions of the virus suspensions were made in PBS pH 6,0 buffer (III.A.2.a) starting at a concentration of 0,1mg/ml BMV and 0,2mg/ml (WCuMV) respectively.

Crude plant extracts were prepared by grinding plant tissue in 0,05 M sodium phosphate pH 7,0 followed by low speed centrifugation. (In some cases the partially clarified plant extracts were concentrated by ultracentrifugation). Serial two-fold dilutions of plant saps were made in PBS pH 6,0 buffer. Uredospore samples were prepared in three different ways:

(i) uredospores were washed by suspension in PBS pH 6,0 containing a few drops of 1% Tween-20 as wetting agent followed by mixing for fifteen minutes on a vortex mixer. The uredospores were separated from the washing fluid by centrifugation at 3 000 rpm on a BHG bench centrifuge. The washing fluid ("spore-wash") was kept and tested.

(ii) washed uredospores were crushed in the presence of PBS pH 6,0 in a mortar and pestle. About

20-30% of the spores were crushed in this fashion as observed by microscopic examination.

(iii) uredospores were germinated overnight on course woven silk fabric layered on water agar plates kept in a moist chamber, at room temperature. Ninety percent germination of uredospores was obtained in this fashion, as observed by microscopic observation. The germinated uredospores and hyphae were effectively crushed in a mortar and pestle.

Two-fold serial dilutions of all the prepared uredospore samples were made in PBS pH 6,0 buffer. The initial dilution of all spore samples approximated to a uredospore suspension of ± 50mg/ml.

2.c. Method for "Sandwich" ELISA

The wells were coated by the addition of 200µl of purified gamma globulin (usually at 5µg/ml concentration) diluted in coating buffer (III.A.6.a). The microtitre tray was incubated in a moist chamber at 37°C for 1 hour. The wells were emptied and washed by flooding the tray with PBS-Tween, pH 6,0 buffer (III.A.6.c).

Washing involved flooding the plate with rinsing buffer and then immediatly emptying it, followed by

two longer washes of three minutes each. The washing step was followed by flooding the tray with PBS-T-BSA buffer (III.A.6.d) and incubating at room temperature for 15 minutes. This was done in order to allow the BSA to coat the areas in the wells left uncoated by the gamma globulins, thus preventing non-specific adsorption of the antigens to the wells. The wells were drained and the trays were dried by gentle tapping on a paper towel.

200µl volumes of each dilution of sample in PBS pH 6,0 buffer were added in the appropriate rows of wells, and the tray was incubated for 1 hour at 37°C as before. The tray was washed and drained as before. 200µl volumes of the enzyme-conjugated gamma globulins diluted appropriately in PBS-T-BSA buffer were added to each well. The tray was incubated in a moist chamber at 4°C overnight for 18 hours.

(Before addition to the wells, the conjugate dilutions were cross-absorbed with an extract of non-viruliferous uredospores (as prepared in III.H.2.b.(ii)). This cross-absorption was done as follows:

400µl of uredospore extract was added to 20ml of diluted conjugate, incubated at 37°C for 20 minutes and centrifuged at low speed to remove spore components. The conjugates were cross-absorbed

in this fashion to prevent non-specific reactivity with uredospore components. Conjugates treated in this way were still found to react with high specificity with BMV and viruliferous uredospores, but had a low reactivity with non-viruliferous uredospores.

The wells were emptied, washed, drained and dried as before. 300µl of freshly prepared substrate solution (prepared by dissolving p-nitrophenol phosphate in substrate buffer (III.A.6.e.) to give a final concentration of 1mg/ml (w/v) was added to each well. The trays were left at room temperature for enzyme-substrate reaction to occur. After 45-50 minutes the absorbance of the hydrolysed substrate in the wells was read at 405nm in the Titertek Multiscan spectrophotometer.

2.d. Controls and Grid Titration

Controls that were used in the assays included BMV as a positive antigen control, WCuMV and healthy plant sap as negative antigen controls. (WCuMV, a tymovirus was selected as a negative control since the tymoviruses are morpholigically similar to the bromoviruses, of which BMV is a member (Matthews, 1970). These viruses have never been shown to cross-react serologically with the bromoviruses). Other controls included non-viruliferous uredospore samples for checking non-specific reactions with uredospore components. Another negative control

involved the coating of wells with gamma globulin of normal rabbit serum (NRS), instead of with anti-BMV gamma globulins. Reactions due to non-specific colour development were checked by the inclusion into each assay of a set of wells containing substrate solution only, PBS pH 6,0 buffer instead of antigen and the lowest dilution of sample only. The reactions obtained in these assays were observed to be highly specific of BMV - anti-BMV antibody reactions.

All the negative antigen controls were found to have an O.D. 405nm reading of not higher than 0,1 O.D. unit compared to average positive O.D. readings of 0,8 - 2,0 O.D. units.

The optimal proportions for the antisera used in the assays were determined for each particular antiserum. This involved grid titrations of purified BMV (serial four-fold dilutions) against coating concentrations of 0,1 µg/ml, 1µg/ml and 10µg/ml and conjugate dilutions of 1/250, 1/500 and 1/1000. The combination of coating IgG concentration and conjugate dilution that gave a wide range of O.D. 405nm readings (O.D. max of 1-2 O.D. units and O.D. min of 0,1 O.D. unit) for BMV reactions and a low background O.D. 405nm reading with controls was taken as the optimal proportions. It was found that a coating concentration of 5µg/ml was suitable for

all three of the antisera used, and for two of the antisera a 1/1000 dilution of conjugate was suitable whereas a 1/500 dilution of conjugate was essential for the other antiserum. The conjugate dilutions at optimal proportions listed above, was obtained using purified BMV as antigen. In assays involving uredospore samples, the conjugates were used at twice the concentration of optimal proportions in order to get a higher enhancement of colour reactions.

I. THE FLUORESCENT ANTIBODY STAINING TECHNIQUE

This technique has been applied in many fields of research in biology. Principles of the technique have been thoroughly reviewed by Holbrow and Johnson (1967) and Walker et al. (1971). In this study the technique was used for the detection of BMV on the surface of the uredospore. Use was made of fluorescein iso-thiocyanate (FITC) labelled antibodies. Both the indirect and direct method of staining was employed.

1. CONJUGATION OF GAMMA GLOBULINS

Purified gamma globulin fractions of antisera were prepared by the method of Clark and Adams (1970) (III.H.1.a). The gamma globulins were labelled with fluorescein iso-thiocyanate (FITC) by the dialysis method of Otsuki and Takebe (1969). Three antisera, namely goat anti-rabbit serum (GAR), anti-BMV serum (antiserum No. 3) and normal

rabbit serum (NRS) were labelled with FITC. Fluorescein iso-thiocyanate (Merck) was obtained as a dry powder.

The gamma globulin solution was adjusted to a concentration of 5mg/ml with 0,02 M sodium carbonate buffer, pH 9,8 (III.A.7.a). The extinction coefficient, $E^{0,1\%}_{280nm} = 1,4$ of Clark and Adams (1970) was used. 4ml of the gamma globulin solution was dialysed against 50ml of 0,006% (w/v) FITC solution in the same buffer. Dialysis was carried out at 4°C for 24 hours. The contents of the dialysis bag were passed through a column (15cm x 1cm) of superfine Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) to free the conjugated globulin from the uncoupled dye. The conjugated globulin was eluted by washing the column with 0,02 M sodium carbonate buffer, pH 9,8. One millilitre fractions of eluent were collected, and the optical densities at 495nm and 280nm were read on a Beckman Model 25 Spectrophotometer. The first fractions showing a high absorbance at both wavelengths were collected and pooled. These fractions corresponded to the first fractions that showed colour. The conjugate was dialysed overnight at 4°C against 0,02 M sodium carbonate buffer, pH 9,8.

The fluorescein-protein (F/P) ratio of the conjugates were calculated by using the equation,

$$F/P = 0,41 \times \frac{(FITC) \text{ in } \mu g/ml}{(IgG) \text{ in } mg/ml}$$

of Holbrow and Johnson (1967). The FITC concentration was calculated using the equation,

$$(FITC)_{\mu g/ml} = \frac{0.D.492nm - 1/2\ 0.D.320nm \times \text{dilution factor}}{0,2}$$

of Holbrow and Johnson (1967).

The F/P ratios of the conjugates were as follows:

(i) anti-BMV conjugate = 0,95
(ii) GAR conjugate = 1,98
(iii) NRS conjugate = 0,68

All these F/P ratios fell in the range of F/P ratios for conjugates that give rise to specific staining (Holbrow and Johnson, 1967).

2. STAINING PROCEDURE

2.a. The Indirect Method

New microscope slides (2,5cm x 7,5cm) were cleaned with commercial methanol. The slides were coated with a 0,1% solution of ovalbumin by brushing the slides with this solution and drying them in a stream of warm air. A drop of uredospores suspended in distilled water was placed on the slide. The spores were spread in chloroform vapour. This was

followed by fixation of the spores by flaming. The slides were washed in a stream of PBS pH 7,0 buffer (III.A.7.b). Unlabelled rabbit antibodies at a concentration of lmg/ml were added to the fixed uredospores with a Pasteur pipette, and the slides were incubated in a moist chamber at 37°C for 1 hour. The slides were washed twice in PBS pH 7,0 by agitation in a bath. The excess buffer was drained off and the slides were blotted lightly on paper towels. A 1/50 dilution of FITC conjugated GAR was added to the fixed uredospores with a Pasteur pipette, and the slides were incubated for 45 minutes at room temperature in the dark. Slides were washed and blotted as before. The stained uredospores were mounted in carbonated glycerol mounting fluid (III.A.7.d). The specimens were observed and photographed through an Olympus fluorescence microscope using blue violet exciter filters B_1 and B_2 and a barrier filter Y-53. The microscope was fitted with an Olympus C-35A camera.

2.b. The Direct Method

The uredospore specimens were fixed as in the indirect method (III.H.2.a). The incubation step with unlabelled antibodies was omitted, and the uredospores were directly stained with FITC conjugated anti-BMV antibodies and FITC conjugated normal rabbit serum. The slides were again incubated for 45 minutes

at room temperature in the dark. The specimens were washed, blotted and mounted as in the indirect method (III.I.2.a). Viewing and photography was done on the same Olympus fluorescence microscope using the same filter combinations as in the indirect method (III.I.2.a).

2.c. Controls

Controls that were done to check non-specific staining includedthe following:

(i) staining with FITC labelled GAR only and FITC labelled NRS.

(ii) incubation with normal rabbit serum (NRS) as primary antibody in the indirect method.

(iii) inhibition of adsorption of specific primary antibodies and staining with anti-BMV conjugate by the addition of BMV and/or anti-BMV IgG (unlabelled).

Some of these controls were not effective in limiting non-specific staining (See IV.F.2).

J. SLAB GEL ELECTROPHORESIS

1. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

The system described by Laemmli (1970) was used for the detection of BMV in plants inoculated with viruliferous uredospores. The buffers and solutions used for preparing the acrylamide gel and samples appear in Section (III.A.5.a). Electrophoresis was carried out in a Hoefer SE 600 vertical slab gel electrophoresis unit.

1.a. Procedure

1.a.(i) Preparation of Resolving Gel

A 12% polyacrylamide gel was prepared by mixing the following volumes of solutions:

Solution	Volume (ml)
Monomer solution	32,0
1 M Tris/HCI pH 8,8	30,0
Water	13,2
10% SDS	0,8
1,5% ammonium persulphate	4,0
Temed	0,02
	80,02

The vertical slab gel electrophoresis unit was assembled in the casting mode using glass plates (18cm x 16cm) and 1,5mm spacers. Twenty-five millilitres of the resolving gel solution was

immediately pipetted into the slab gel mould to give a gel of 1,5mm thickness and 100-110mm in length. The poured gel was carefully overlayed with distilled water using a Pasteur pipette. The gel was allowed to set at room temperature. Once the gel had set, the water was poured off by inversion of the casting unit onto paper towel. Next, the stacking gel was cast.

1.a.(ii) Preparation of the Stacking Gel

A 4,5% stacking gel was prepared by mixing the following volumes of solutions:

Solution	Volume (ml)
Monomer solution	2,25
1 M Tris/HCl pH 6,8	1,90
Water	9,20
10% SDS	0,15
100% glycerol	0,80
1,5% ammonium persulphate	0,70
Temed	0,02
	15,02

10ml of the stacking gel solution was immediately pipetted on top of the set resolving gel. A 12 slot comb was inserted and the gel was allowed to set at room temperature. Once the gel had set, the comb was carefully removed. The sample slots were

rinsed with distilled water and then with 1 M Tris/HCl pH 6,8.

1.a.(iii) Sample Preparation

Healthy and virus-infected leaf extracts were prepared by grinding freshly collected leaves in 0,05 M sodium phosphate buffer, pH 7,0 in a mortar and pestle. The extracts were partially clarified by low speed centrifugation. In some cases the clarified extracts were concentrated by ultracentrifugation in a Beckman airfuge at 19 psi for 15 minutes (sedimentation of all particles with sedimentation coefficient below 90S is facilitated at this speed).

A purified preparation of BMV was used as a standard.

Appropriate dilutions of samples were made in dissociation mixture (III.A.5.a.(v)). In the case of samples concentrated on the airfuge, the pellets were directly resuspended in dissociation mixture. The sample mixtures were then heated for 10 minutes in a boiling water bath.

1.a.(iv) Sample Application and Electrophoresis

Treated samples (10-25µl) were loaded into the wells with a 25µl Hamilton syringe, and electrophoresis

was performed at a constant current of 60 mA for 7 hours until the glycine ion front had reached about 1cm from the bottom of the gel. Electrophoresis was performed at 4°C with the water cooling system of the Hoefer slab gel unit in operation. During electrophoresis the upper buffer chamber was connected to the cathode and the lower buffer chamber to the anode.

1.a.(v) Staining and Destaining of the Gels

The gels were removed from the glass plate sandwiches, and stained overnight with a 0,2% (w/v) Coomassie blue stain (III.A.5.a.(ix)). The gels were destained in destaining solution (III.A.5.a.(x)) for 12-15 hours by diffusion. The stained gels were dried on a Hoefer SE 540 slab gel dryer for 3 hours. The dried gel was photographed.

2. HORIZONTAL-SLAB AGAROSE GEL ELECTROPHORESIS

This technique was previously used to compare electrophoretic mobilities of BMV strains (Rybicki, 1979) and Cauliflower mosaic virus (CaMV) isolates (D.H. du Plessis, 1981).

In this study, this technique was used in an attempt to compare the electrophoretic mobilities of purified

mechanically transferred BMV with stemrust transmitted-BMV isolates.

2.a. Procedure

A 1,5% solution of agarose (LGT agarose, Marine Colloids Incorporation; Miles distributors) was prepared by dissolving the agarose in 0,01 M sodium phosphate - 1 mM $MgCl_2$ buffer at pH 6,0, pH 6,5 pH 7,0 and pH 7,4 (III.A.5.b).

Glass slides (7,5cm x 7,5cm) were cleaned with commercial ethanol. The slides were coated with agarose, by brushing a thin layer of agarose over the surface of the slide with a brush, followed by drying in a stream of hot air. The coated slides were placed on a levelling stand and 9ml of agarose solution was poured onto each slide with a pipette. This gave a gel of 2mm thickness. Once the gel had set, wells (1-2mm in diameter) were cut into the gel using a 1,5mm punch attached to an aspirator. The slides were placed in a laboratory constructed electrophoresis apparatus (similar to the Beckman Durrum type unit) and lint wicks immersed in the tank buffer (0,01 M sodium phosphate/1 mM $MgCl_2$) were placed on each end of the gel overlapping by about 1 cm. A span of 5cm was allowed across the gel between the two wicks. The power was switched on and the voltage across the two wicks was adjusted to 25-30 V to give a

voltage of 5-6 V/cm across the gel. The power was switched off and 3-5µl of sample (BMV at 0,5 - 1 mg/ml) was applied to each well using a 10µl Hamilton syringe. A marker 0,05% bromophenol blue (BPB) was placed in a well at the edge of the wick connected to the cathode. The power was switched on and electrophoresis was carried out at 5-6 V/cm for 2 1/2 to 3 hours. (The time taken for the BPB marker to reach the other side of the wick was noted).

After electrophoresis the gels were rinsed with distilled water, covered with moist filter paper and pressed under paper towel under a 2 Kg weight for 10 minutes. The pressed gels were dried to a thin film in a stream of hot air. The dried gels were stained for 15 minutes with 0,2% (w/v) Coomassie blue staining solution, and destained in destaining solution (III.A.5.b.(ix) and (x)). The electrophoretic indices (Ei) were calculated as the distance of migration of the BPB relative to that of the virus, expressed as a ratio.

K. ELECTRON MICROSCOPY

1. NEGATIVE STAINING

Various extracts of uredospores and uredospore washings (III.H.2.b) in 0,05 M sodium acetate buffer pH 5,0 or

pH 6,0 were negatively stained with either 2% uranyl acetate pH 4,0 or 2% phosphotungstic acid pH 7,0.

1.a. Procedure

A drop of sample was placed on a carbon coated grid sitting on parafilm. The grid was covered with a Petrie dish and left for 15 minutes at room temperature. The grid was washed with a series of drops of distilled water from a Pasteur pipette. Grids were stained for 1 minute with 2% uranyl acetate pH 4,0 or 2% phosphotungstic acid pH 7,0. The grid was drained by touching the edge to a filter paper, and allowed to dry. The grid was viewed in a Zeiss 109 transmission electron microscope at 80 KV.

2. IMMUNOSORBENT ELECTRON MICROSCOPY

This technique also known as serologically specific electron microscopy (SSEM) or the Derrick method was first described by Derrick (1973) for the assay of plant viruses. The technique has been used extensively in the detection, quantitation and characterisation of plant viruses (Milne, 1975, 1977; Derrick & BrLansky, 1976). It has been used successfully by Derrick & BrLansky (1976) for the detection of seed-borne plant viruses, and for the detection of viruses in their aphid, nematode and fungal vectors (Roberts and Harrison, 1979 ; Roberts and Brown, 1980 and Delvecchio et al., 1979, respectively).

The technique described below is the short incubation method of Milne (1980).

The same uredospore samples used for negative staining were used. 5µl drops of diluted antiserum were placed on Parafilm in a humid Petrie dish. (Antiserum No. 3 at 1/64 dilution was used.) The grids were incubated face down on these drops for 5 minutes. The face of the grid was rinsed with 20 consecutive drops of 0,1 M sodium phosphate buffer, pH 7,0. The grid was drained by touching the edge to filter paper. The grid was not allowed to dry at this stage. The grid was then incubated face down for 15 minutes on a drop of the uredospore sample. Next, the grid was rinsed with 30 consecutive drops of distilled water from a Pasteur pipette. The face of the grid was stained with 2% uranyl acetate for 1 minute, and drained as before and allowed to dry. The grid was viewed in the electron microscope.

3. THIN SECTIONING OF UREDOSPORES

Uredospores floated on water in a vial overnight to germinate and swell, was pelleted by centrifugation at 3 000 rpm for 10 minutes in a BHG bench-top centrifuge. The uredospore pellet was fixed in 5% glutaraldehyde in 0,1 M sodium phosphate buffer, pH 7,2 (III.A.1.d.) by incubation for 1/2 to 4 hours at 4°C. The fixed preparation was washed in phosphate buffer overnight.

The uredospores were pelleted by centrifugation, and post-fixed in Palade's OsO_4 buffer (III.A.8.b.(i)) for 1 hour. This was followed by three rinses with distilled water. Uredospores were pelleted by centrifugation between washes. After washing the fixed uredospores were incubated at room temperature for 30 minutes in 0,5% uranyl acetate in 80% acetone. The sample was dehydrated in 96% acetone for 15 minutes, followed by three ten-minute rinses in 100% acetone. The dehydrated sample was embedded in Spurr's epoxy resin. This involved incubation for 1 1/2 hours in a solution 1:1 of Spurr's resin and 100% acetone, followed by two 1 hour incubations at 40°C with Spurr's resin. The samples were finally embedded in oven dried Beem capsules. The embedded samples were left in the Beem capsules in an oven at 40°C overnight to harden. Ultrathin sections were cut on an LKB ultramicrotome using a glass knife. The sections were floated on carbon coated grids and stained with saturated uranyl acetate in water for 3 minutes, washed with distilled water, followed by staining with 0,2% lead citrate in 0,1 N NaOH for 3 minutes. The grids were drained by touching the edge on blotting paper, and allowed by dry. The grid was viewed in the electron microscope.

CHAPTER IV

RESULTS

A. PROPAGATION OF STEMRUST

Initially, several wheat cultivars were screened in order to select the most suitable cultivar(s) for the routine propagation of stemrust. The degree of susceptibility of these cultivars to stemrust infection was evaluated by microscopic examination of the pustule size and the degree of stemrust infection per cluster of plants (± 20 seedlings per pot). The evaluations of susceptibility to stemrust for the various cultivars tested at that time appear in Table 3. The following criteria were used for these evaluations:

(a) highly susceptible - high incidence of stemrust pustules, pustules large in size.

(b) susceptible - low incidence of stemrust pustules, pustules were predominantly small in size.

(c) mildly susceptible - stemrust pustules were few in number and scattered. Besides being small, the pustules were often surrounded by necrotic haloes.

(d) resistent - no pustule formation occurred, only white flecks were observed.

TABLE 3: Susceptibility of various wheat cultivars to infection by stemrust. Four groups are distinguished according to the type of infection observed.

CULTIVAR	INFECTION TYPE
Flameks	highly susceptible
Little Club	"
Memnon	"
T_4	"
Zaragosa	"
Palala	"
Hellenic	Susceptible
SST2	"
SST3	"
Betta	Mildly susceptible
Elize	"
Inia	"
K_{20}	"
Liesbeeck	"
Scheepers	"
Zambesi	"
Belinda	"
Dipka	Resistant
SST3-R	"
T_4-R	"

It was decided that Stakman's key for race identification was not valid for observations of stemrust infection on wheat in the presence (and absence) of virus infection. Therefore, an arbitrary key (displayed above) was designed to evaluate stemrust infection, (see page 92).

The cultivars, Palala, T_4 and Zaragosa are highly susceptible to the stemrust strain under study as well as virus (BMV) infection (Von Wechmar, personal communication), and should therefore be considered to be unsuitable for commercial cropping. Little Club and the cultivars Flameks and Memnon which are also highly susceptible to stemrust are no longer of commercial interest. The cultivars, Betta and Scheepers which are mildly susceptible to stemrust infection, and T_4-R which is resistant to stemrust infection can be regarded as being safe to use in field crops, if stemrust infection is the only criterion that is considered. These three cultivars are, however, extremely susceptible to virus (BMV) infection (Von Wechmar, personal communication) and their use for field cropping should take into account their susceptibility to virus infection which could be devastating during epidemic virus conditions and availability of vectors. The types of stemrust pustules observed on Flameks, Palala, Little Club, Betta and Scheepers are presented in Figure 3.

An interesting feature of the pustule-type on Betta is that the pustule is surrounded by a necrotic halo. This

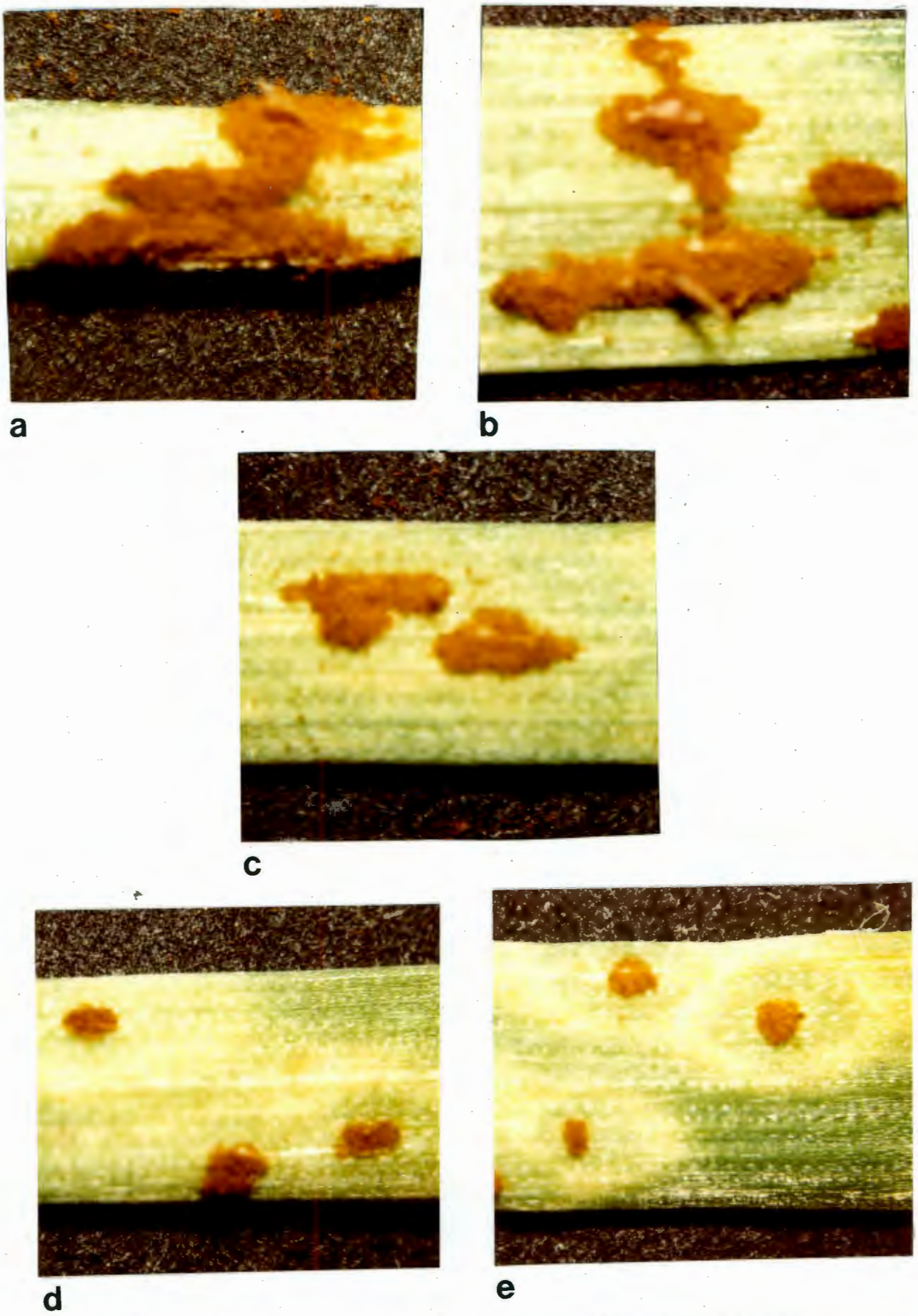


FIGURE 3: Pustule types on five wheat CVS inoculated with stemrust.
(a) = Little Club, (b) = Flameks,
(c) = Palala, (d) = Scheepers and
(e) = Betta

is an expression of a hypersensitive reaction to stemrust infection by this cultivar. The observations tabulated in Table 3 indicated that Flameks, Palala and T_4 in addition to the classic Little Club were good hosts for routine propagation of stemrust. These cultivars were chosen because (1) they were readily available and (2) because they are of commercial interest. At first stemrust (culture W138) was propagated on Flameks only. After the third propagation, various small sized pustules were observed besides the large stemrust pustules normally obtained on Flameks. Microscopic investigation of these small pustules indicated the presence of leafrust (Puccinia graminis recondita). The leafrust colony was separated from the stemrust by single pustule transfers on cultivars Benita and Belinda. These two cultivars were used for this purpose since they were the only seedlings available at that time. They were, however, found to be only mildly susceptible to stemrust infection but being susceptible to leafrust infection, they served as excellent hosts for this fungus. The separated leafrust uredospores were stored in vials at 4°C for later use (see III.C.2.c.).

A new collection of stemrust (culture W138) uredospores, which was uncontaminated by leafrust (seen after several propagations) was obtained from Stellenbosch (Mr. J. Truter, Plant Pathology, Winter rainfall region, Department of Agriculture and Fisheries, Stellenbosch).

In order to get a better idea regarding the yield of uredospores that was obtainable from a particular wheat cultivar, for the selection of a good propagation host, the yield of uredospores (expressed in mg uredospores/g leaves) was determined for seven cultivars. Little Club and the following cultivars were chosen for this selection test: three highly susceptible wheat cultivars namely Flameks, Palala and T_4; two mildly susceptible cultivars namely, Betta and Scheepers; and one resistant cultivar namely T_4-R. Betta and Scheepers were included in this test to see whether the yield of uredospores from these cultivars coincided with their observed mild susceptibility to stemrust. The cultivar T_4-R was included to offset the result obtained for cultivar T_4. It also served as a "neutral" spacer between the randomly arranged susceptible cultivars.

The seven cultivars (several pots of each) were randomly assorted and inoculated with a stemrust uredospore suspension at 2mg/ml (wt/vol). The plants were inoculated and incubated as described in Chapter III.C.2.b. The uredospores were harvested from each cultivar 14 days later, and the yield of stemrust uredospores was expressed as the number of milligrams of uredospores obtained per gram weight of leaves.

The results of this experiment are presented in Figure 4. The histograms represent average values of two determinations.

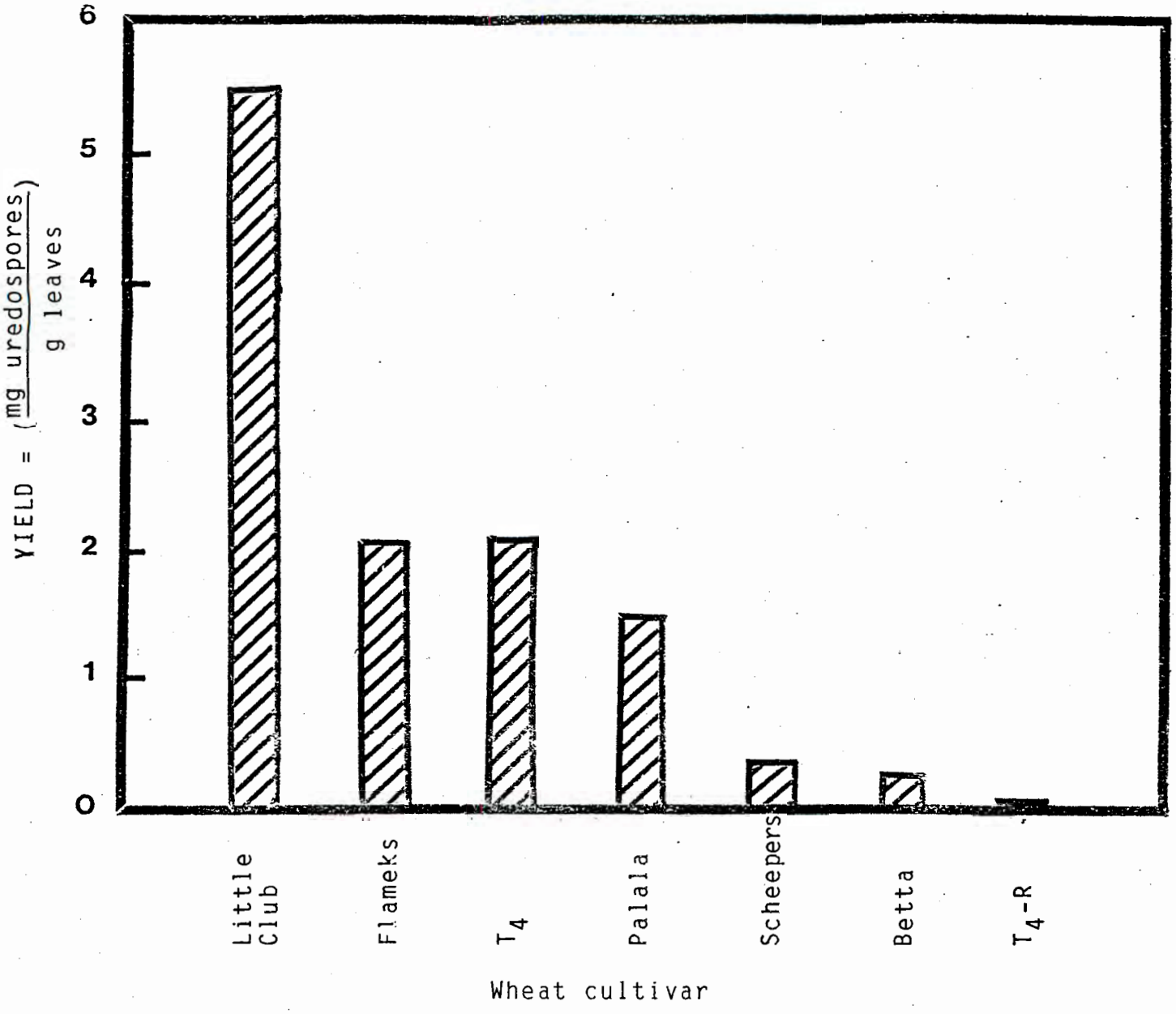


FIGURE 4: Yield of stemrust uredospores obtained from seven wheat cultivars

Since a total number of five pots of plants per cultivar was tested in each of the two experimental cases, it was felt that two determinations were a sufficient check for experimental error. Flameks, Little Club, Palala and T_4 all yielded high levels of uredospores, the order for the cultivars being Little Club $>$ Flameks and T_4 $>$ Palala. T_4-R as expected gave rise to an insignificant number of pustules and uredospores. Scheepers and Betta yielded a small amount of uredospores. The yield from Scheepers was higher than that of Betta. This is possibly due to the hypersensitivity of Betta to stemrust infection.

It was decided from this experiment to use Little Club, Flameks and T_4 routinely for the propagation of stemrust.

Initially, it was found that harvested uredospores stored in vials at 4°C (see III.C.2.c.) did not germinate efficiently when floated on water. This phenomenon was found to be largely due to the fact that uredospores in pustules on the leaf, were found to germinate on the leaf's surface prior to harvesting (see Figure 5). This occurred because of the high relative humidity (+ 70%) in the plant growth rooms that was maintained throughout the propagation of the fungus. Subsequently, the humidifier was switched off when the first pustules appeared on the leaf. By doing this, the germination of uredospores in situ on the leaf's surface was prevented and ungerminated uredospores were collected during harvesting.

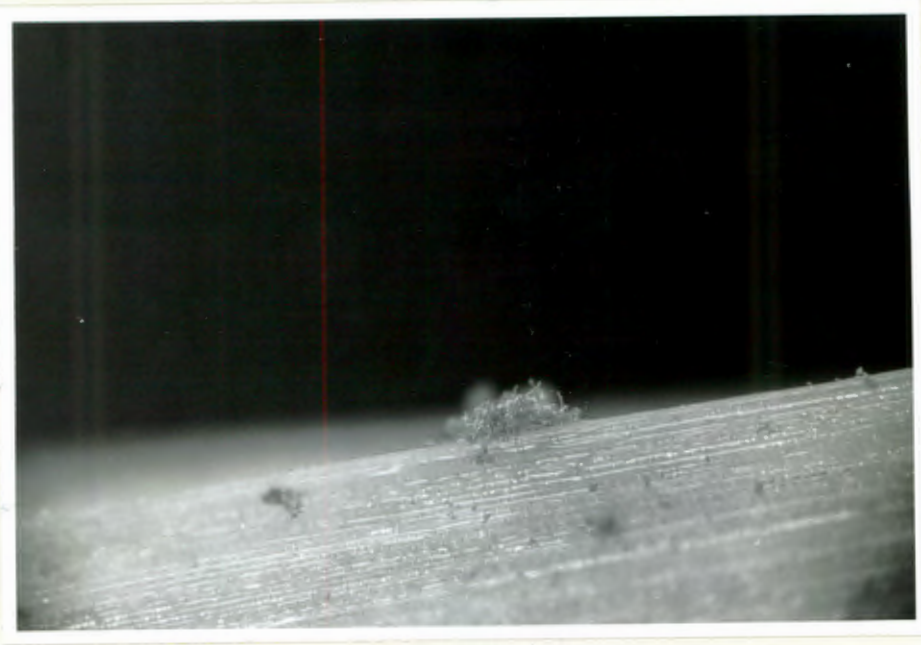


FIGURE 5: Stemrust pustule on leaf's surface showing germ tubes of germinated uredospores.

B. ACQUISITION OF THE VIRUS BY THE FUNGUS

The fungus acquired the virus by means of an *in vivo* acquisition system. In other words, the fungus acquired the virus upon infection on virus-infected plants that were mechanically inoculated with BMV. *In vivo* acquisition has been described for, amongst others, *Olpidium brassicae* with lettuce big-vein agent (BVA) (Tomlinson and Garret, 1962; Campbell and Grogan, 1964), *Synchytrium endobioticum* with potato virus-X (PVX) (Nienhaus and Stille, 1965) and *Polymyxa graminis*

with soil-borne wheat mosaic virus (SBWMV) and with wheat spindle streak mosaic virus (WSSMV) (Slykhuis and Barr, 1978).

In this study BMV at 0,5mg/ml concentration was mechanically inoculated onto wheat seedlings and five days later, after multiplication had occurred, the plants were inoculated with stemrust uredospores (2mg/ml (w/v) suspension in water). Uredospores were harvested 16 days later.

The same procedure was followed for the acquisition of virus by leafrust. At this stage it is best to distinguish between uredospores that had not been allowed to acquire virus and those that had acquired virus. Uredospores that were only propagated on and harvested from healthy plants are designated non-viruliferous uredospores. These spores have had no contact whatsoever with BMV inside the laboratory. Non-viruliferous uredospores that acquired virus by propagation on *BMV-infected plants are designated as viruliferous uredospores. *(Plants were mechanically inoculated with either BMV-Beth, BMV-Palala or BMV-G_9. BMV-Beth was used in the majority of cases.)

During several acquisition studies, it was noticed that as BMV multiplication increased in the plant, so the apparent susceptibility of the plant to stemrust decreased.

An experiment (the results of which appear in Figures 6

and 7) was set up in which wheat plants (cv. T_4) at the same stage of growth (i.e. seedlings at the fully-developed two-leaf stage) were inoculated simultaneously with BMV (Beth isolate) at a concentration of 0,5mg/ml. The virus inoculated plants were divided into five groups and were inoculated with a suspension of non-viruliferous uredospores at 2mg/ml (w/v) in water, at 2, 4, 6, 8 and 10 days post virus inoculation (p.v.i.), respectively. In each case, the uredospores were harvested at 16 days post-rust inoculation. Prior to harvesting, a representative leaf from each of the five groups of plants was selected and photographed. The yield of uredospores obtained from each group of plants was expressed as the number of milligrams of uredospores harvested per gram of plant leaves.

A graph of the uredospore yield versus number of days p.v.i. is presented in Figure 6. The points on the curve represent average values for three determinations. Since a total number of six pots of plants per group was tested in each ofthe three experimental cases, it was felt that three determinations provided sufficient check for experimental error.

From the Figures 6 and 7, it can be seen that the later the virus-infected plants were inoculated with stemrust (or alternatively, as the virus concentration increased in the plant), so the susceptibility of the virus-infected

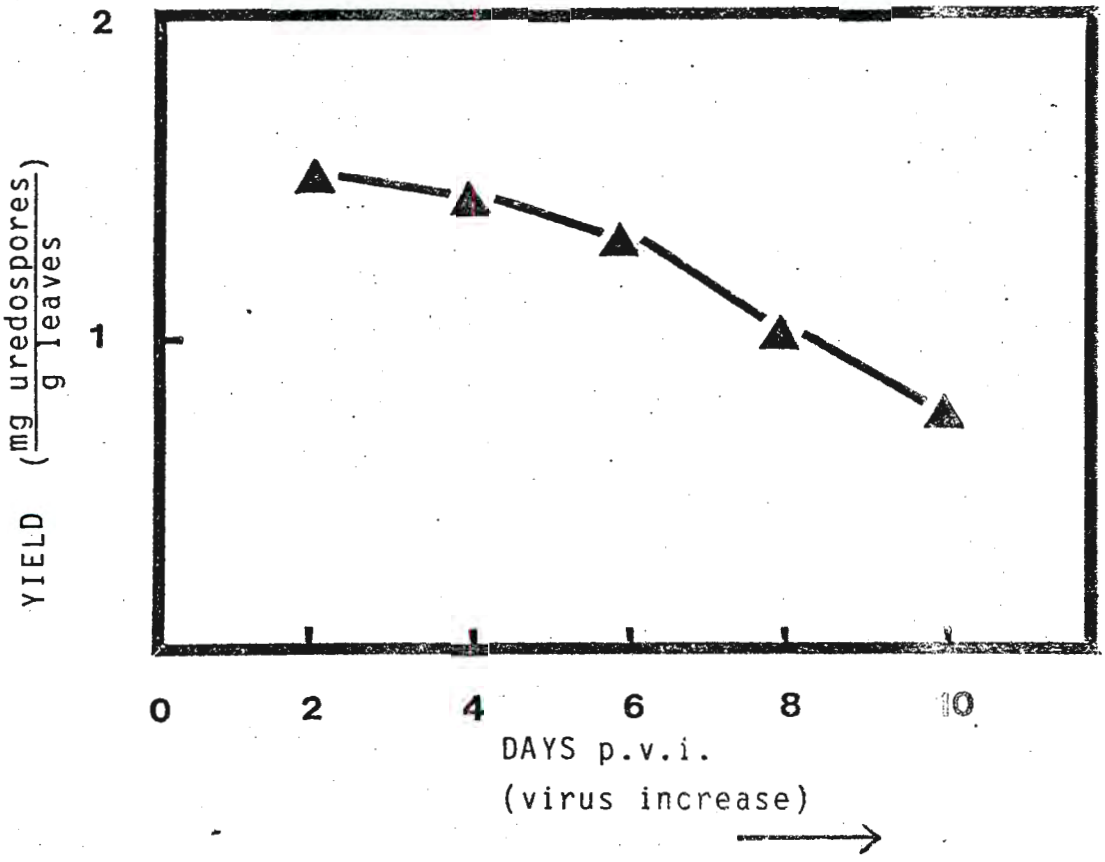


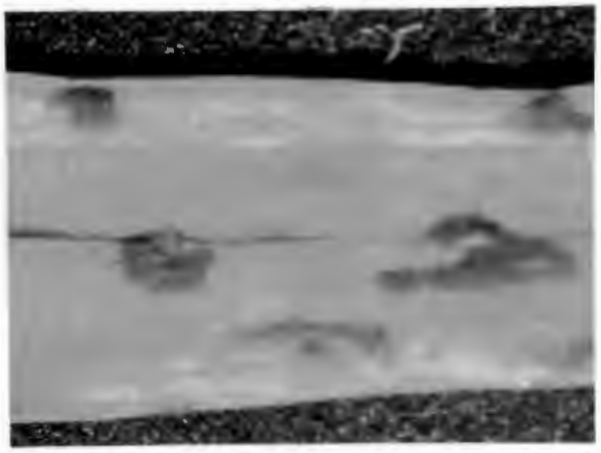
FIGURE 6: Effect of BNV-infection of wheat on the yield of uredospores.

plants to stemrust infection decreased. Thus, BMV-infected plants were less susceptible to stemrust.

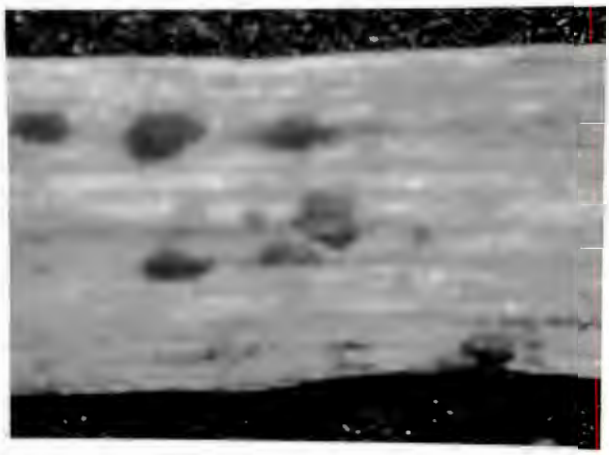
Since a good average yield of viruliferous uredospores was required for routine transmission studies, it was decided to inoculate BMV-infected plants at 5 days post virus inoculation with non-viruliferous uredospores. At this stage of inoculation, the susceptibility of the plants to stemrust infection was just starting to decrease (see Figure 6). It was postulated that inoculation prior to 5 days p.v.i. would not allow the virus enough time to build up in the plant tissue, thus limiting the possible uptake of virus by the fungus. Also, inoculation with stemrust after 5 days p.v.i. would give rise to a very low yield of viruliferous uredospores, since the plants were apparently less susceptible to stemrust infection at this stage of virus multiplication.

The pustule development on the leaf's surface for each particular group of plants was recorded photographically before harvesting of the uredospores. Representative leaf replicas from each group of plants are presented photographically in Figure 7. The results in Figure 7 again illustrate that as the concentration of BMV in the plants increased, so the susceptibility of the virus-infected plants to stemrust infection decreased. The decrease in stemrust infection is indicated by the pustule size, which decreased accordingly. Plants

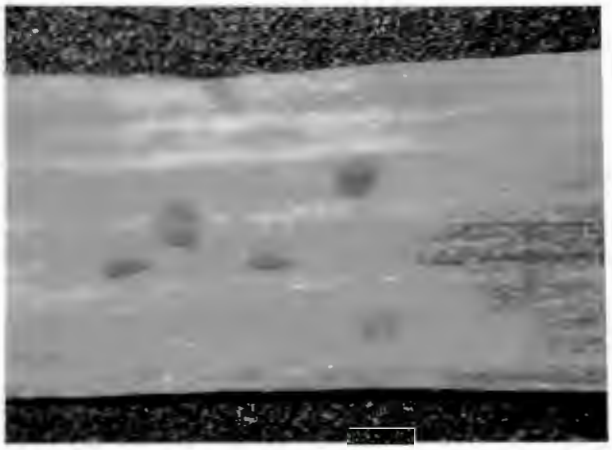
a



b



c



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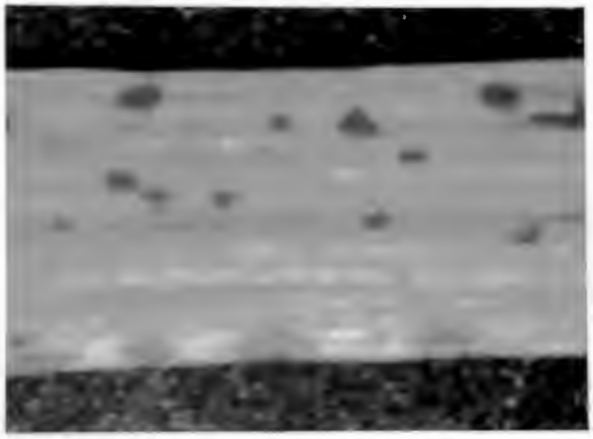

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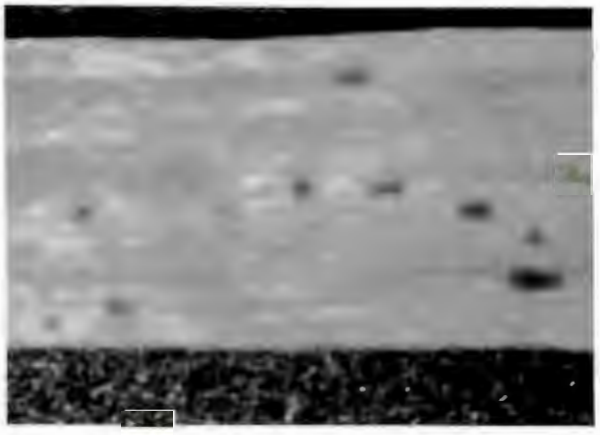


f

FIGURE 7: Effect of BMV-infection of T_4 wheat on the size of stemrust pustules. The susceptibility of wheat plants to stemrust was found to be decreased by the presence of BMV.

(a) = stemrust infection on healthy leaf

(b) - (f) = stemrust infection on BMV-infected leaves; uredospores were inoculated onto virus-infected plants at 2, 4, 6, 8, and 10 days post virus inoculation, respectively.

inoculated at 2 days p.v.i. with stemrust yielded pustules that were slightly smaller than those observed on healthy plants. At 8 and 10 days p.v.i. the pustules were significantly smaller. Although the pustules are the same size in these two cases, the yield of uredospores from plants inoculated with uredospores at 10 days p.v.i. was much lower than that for plants inoculated at 8 days p.v.i.

C. ANTISERA

In order to raise antibodies to BMV, rabbits were injected (III.E.) with purified virus preparations (III.B.3.b. and b.). Three types of virus preparations were used as immunogens, and pairs of rabbits were injected with the same immunogen. Virus preparations were used at two levels of purity, namely (i) purification by differential centrifugation only (III.B.3.b.) and (ii) further purification by sucrose rate zonal centrifugation and/or zone electrophoresis (III.B.3.c.(i) and (ii)).

The details for the antisera used (not used) and obtained are listed in Table 4.

The first type of immunogen produced an antiserum containing insignificant amounts of anti-host antibodies, since such antisera were found not to react with healthy

TABLE 4: Details of Antisera Produced and Obtained.

Antiserum	Immunogen used	Reciprocal titre	Reaction with healthy sap	
			Ouchterlony	ELISA
No. 1	BMV-G_9 (purified by zone electrophoresis or sucrose density gradient centrifugation)	128	R	NT
No. 2	"	32	NR	NT
No. 3	BMV-Type (purified by differential centrifugation)	512	NR	R
No. 4	"	128	NR	NT
No. 5	BMV (field isolate)(purified by differential centrifugation)	512	NR	R
No. 6	"	256	NR	NT
No. 7 (GAR) (goat anti-rabbit serum)	Rabbit gamma globulins	NT	N/A	N/A
No. 8 (anti-BMV-P)	BMV-Protein	NT	NT	NT
No. 9 (NRS) (Normal rabbit serum)	None	N/A	N/A	N/A
No. 10	Whole viruliferous uredospores	NR	N/A	N/A
No. 11	crushed viruliferous uredospores	NR	N/A	N/A
No. 12	germinated viruliferous uredospores	NR	N/A	N/A
No.13	Crushed, germinated viruliferous uredospores	NR	N/A	N/A

Abbreviations Used in Table 4:

R	=	reaction
NT	=	not tested
NR	=	no reaction
N/A	=	not applicable
GAR	=	goat anti-rabbit serum
NRS	=	normal rabbit serum

plant sap in Ouchterlony tests, and to form a single virus precipitin band with crude extracts of BMV. This is illustrated in Figures 8(a) and (b).

These antisera were, however, found to react slightly in an ELISA yielding an OD405nm absorption value of 0,05 units with a 1/32 dilution of healthy sap (see Figure 16).

The second type of immunogen was made to produce an antiserum largely free of anti-host antibodies. Early bleedings from such antisera were found not to react visibly with healthy sap in Ouchterlony tests (Figures 8(c) and (d)). However, antiserum No. 1 (directed against immunogen (ii)) was found to contain antibodies directed largely towards BMV-protein (BMV-P) from the tenth bleed onwards. This phenomenon can be explained by the disassembly of BMV in the immunogen by the physiological pH of the rabbit from which this antiserum was obtained. This antiserum was later found also to react with plant host proteins. Antiserum No. 1 was used initially during the investigation in ELISA (see Figure 19) and its use was discontinued after these findings. The reactions of antiserum No. 1 with BMV-P and plant host proteins appear in Figures 8(e) and (f).

Since the corresponding antiserum (antiserum No. 2) from the second rabbit of the pair immunised with immunogen

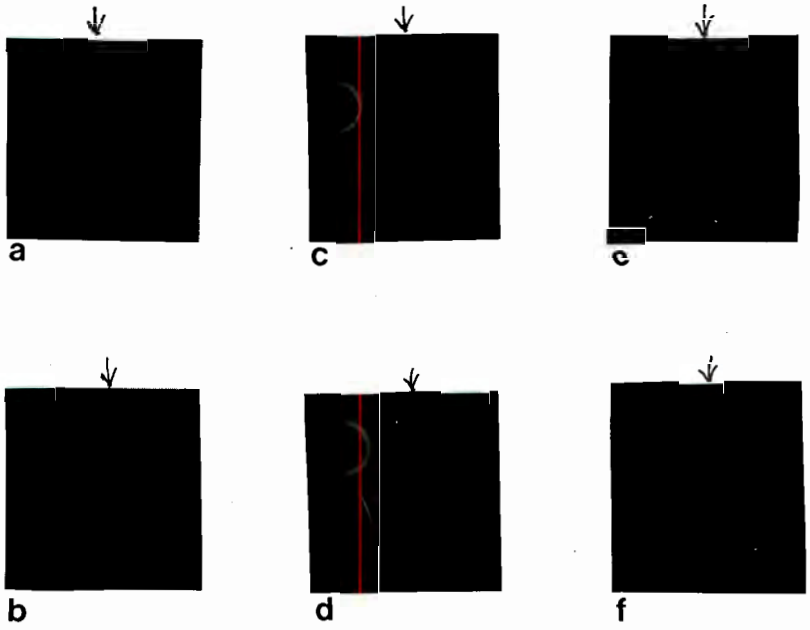


FIGURE 8: Precipitin bands formed by antisera Nos. 1, 2, 3 and 5 (see Table 4). Reactions of these four antisera with various antigens are shown. In all the cases antiserum was either at 1/2 or 1/4 dilution. BMV preparations were at a concentration of 0,5 mg/ml. In each case the arrows indicate the starting points.

Figure 8(a)

Gel buffer = Acetate/NaCl, pH 5,0. Antiserum No. 3 in central well. Arranged clockwise in surrounding wells are: (from arrow) BMV-infected sap, BMV-Type (purified by differential centrifugation), healthy sap, purified preparations of mechanically transferred, stemrust-transmitted-BMV isolates Palala, G_9 and Beth, respectively (purified by differential centrifugation).

Figure 8(b)

Gel buffer = Acetate/NaCl, pH 5,0
Antiserum No. 5 in central well.
Arrangement in surrounding wells is the same as in Figure 8(a) above.

Figure 8(c)

Gel buffer = Acetate/NaCl, pH 5,0
Antiserum No. 1 in central well. Arranged clockwise in surrounding wells are (from arrow): BMV-Beth (purified by differential centrifugation), BMV-Beth (sucrose density gradient sample), next 3 wells are empty, healthy sap.

Figure 8(d)

Gel buffer = Acetate/NaCl, pH 5,0
Antiserum No. 2 in central well.
Arranged clockwise in surrounding wells are (from arrow): BMV-Beth (sucrose density gradient sample), BMV-Beth (purified by differential centrifugation), empty well, next two wells are BMV-Beth (sucrose density gradient sample), healthy sap.

Figure 8(e)

Gel buffer = Acetate/NaCl, pH 5,0
Antiserum No. 1 (bleed ten) in central well.
Arranged clockwise in surrounding wells are (from arrow): mechanically transferred, stemrust-transmitted-BMV-G_9 (purified by differential centrifugation), BMV-G_9 (sucrose density gradient sample), BMV-P at 200 µg/ml and 100 µg/ml, respectively, crude extract of mechanically transferred, stemrust-transmitted-BMV-Beth, healthy sap extract.

Figure 8(f)

Gel buffer = cacodylate/NaCl, pH 6,0
Antiserum and arrangement is the same as in Figure 8(e).

(ii) had too low a titre (see Table 4) for enzyme or FITC conjugation* of gamma globulins, the antisera (antisera Nos. 3 and 4) from the other rabbits that were immunised with immunogen (i) were subsequently used extensively throughout the investigation. (*Conjugation of antibodies with enzyme (and presumably other compounds e.g. FITC) are known to lower the avidity of an antiserum to such an extent that it could become non-reactive if it has an initial low titre before conjugation (Koenig, 1978)).

The other rabbits injected with various uredospore samples (III.E.), in an attempt at indirectly detecting BMV in or/and on uredospores, were found not to react in Ouchterlony tests with BMV even though the test plates were incubated for several days at room temperature (see III.G.). The failure of these rabbits to produce anti-BMV antibodies can largely be attributed to a low concentration of BMV in the immunogen. It should be noted that very few BMV virions were observed in electron micrographs of uredospore samples (see IV.F.3 - Figures 25,26,27, and 28). It is known that spherical plant viruses generally illicit a low immunogenic response in rabbits compared to rod-shaped and elongated plant viruses, and that below a concentration of 0,01mg/ml a spherical virus like BMV is not sufficiently immunogenic to produce a detectable level on anti-BMV antibodies in the rabbit as assayed by precipitin

techniques (von Wechmar, personal communication). Since it was postulated and later shown (see IV.F.3) that the uredospores only carry a very small amount of virus, the possibility that the immunogenicity of the BMV in the uredospore samples, used as "potential BMV" - immunogens, must have been significantly below the threshold (i.e. concentration of less than 0,01mg/ml) for the production of detectable levels of antibodies.

The titers of the antisera were determined by the Ouchterlony gel-precipitin technique (see III.G.) and are expressed as reciprocals in Table 4.

D. TRANSMISSION STUDIES

1. HOSTS AND SYMPTOMS

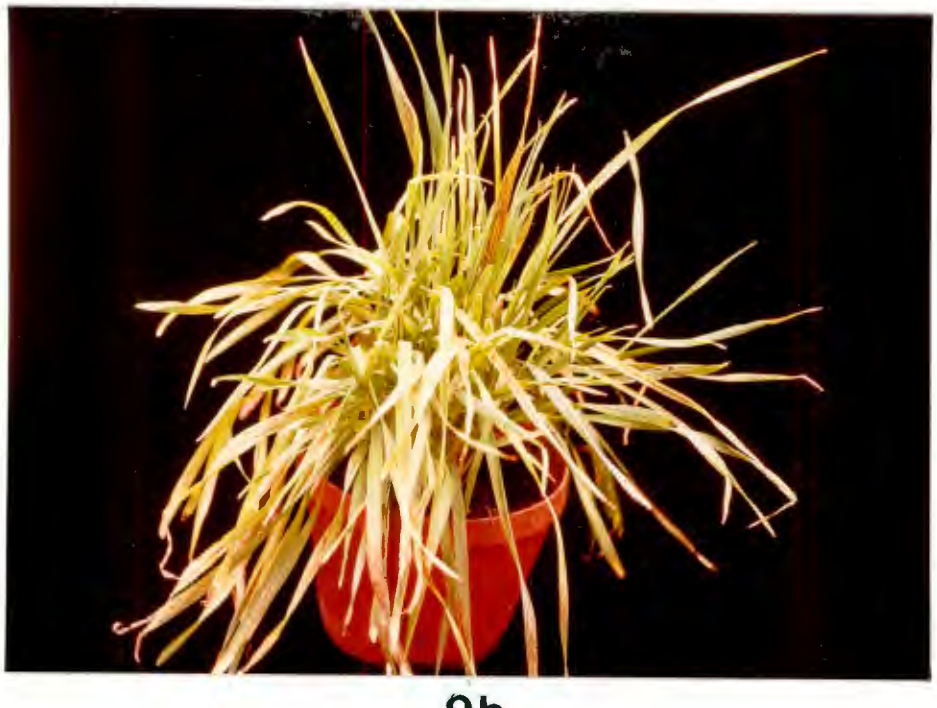
In transmission studies, healthy plants (wheat and barley seedlings) were inoculated with either viruliferous uredospores or non-viruliferous uredospores. A uredospore suspension of 4mg/ml in water (as opposed to 2mg/ml used for routine propagation of non-viruliferous uredospores - see III.C.2.b.) was used as inoculum. A higher density of uredospores was used as inoculum for transmission experiments in order to ensure maximum transmission of BMV by the uredospores. The stemrust (or leafrust) inoculated plants were grown at 24°C for 16 days, sprayed with

fungicide to stop the growth of the fungus, and then grown at 4°C under continuous light for 1 week (see III.D.3.). At the time of harvesting wheat plants showed symptoms of yellow tipping and barley plants showed symptoms of yellowing and brown streaking (see Figure 9). These symptoms were observed on plants inoculated with viruliferous uredospores. It is interesting to note that the plants did not show symptoms for typical BMV infection.

Mild mosaic symptoms are usually displayed by plants mechanically inoculated with BMV. When plants were inoculated with non-viruliferous uredospores, wheat plants developed similar symptoms as those inoculated with viruliferous uredospores and barley plants developed symptoms of mild yellowing with occasional brown streaking occurring in some plants.

To establish whether stemrust (or leafrust) transmitted BMV to the plants, the plants were processed for virus extraction and crude extracts were analysed by Ouchterlony tests, polyacrylamide gel electrophoresis (PAGE), ELISA and sucrose density gradient centrifugation. By employing these techniques it was possible to show that viruliferous stemrust (and leafrust) uredospores were able to transmit BMV. Non-viruliferous uredospores were not found to transmit BMV.

9a



9b

FIGURE 9(a): Symptoms produced on barley (Clipper) when inoculated with viruliferous stemrust uredospores. (Viruliferous leafrust caused the same symptoms). No characteristic symptoms of BMV infection were ever observed on Clipper infected in this manner although the virus could be extracted from these plants.

FIGURE 9(b): Symptoms produced on barley (cv. Comma) when mechanically inoculated with stemrust-transmitted-BMV-Beth Note the stunted growth, yellowing and brown streaking.

In transmission experiments the following were used: wheat (cvs T_4, T_4-R, Palala) and barley (cv Clipper). All these cultivars were found to be highly susceptible to BMV infection upon mechanical inoculation. They were therefore considered to be suitable hosts to establish whether the stemrust (or leafrust) fungus transmitted BMV.

T_4-R and Clipper proved to be interesting hosts for transmission experiments, because these hosts, although resistant to stemrust and leafrust infection, respectively still had the virus transmitted to them by these fungi. It was thus found that resistance to stemrust or leafrust did not prevent the fungus from transmitting the virus to such a plant. The implications of this finding are further described in Chapter V.

2. PURIFICATION OF STEMRUST-TRANSMITTED-BMV

To determine whether viruliferous uredospores of stemrust transmitted BMV to healthy plants, plants inoculated with these uredospores were processed for virus extraction.

Isolation of BMV from stemrust (or leafrust) infected plants was done at pH 4,0 and pH 5,0 by a method similar to that for the extraction of mechanically transmitted BMV (see III.B.3.b.). No virus was recoverable from

plants inoculated with viruliferous uredospores by these extraction procedures. Instead, the virus was lost during the early stages of extraction since the precipitates which consisted of plant debris, obtained in the clarification step were found to be infectious when mechanically inoculated onto healthy plants. No virus was detectable in the pellets obtained by ultra-centrifugation of clarified sap, since the resuspended pellets were neither infectious nor were they found to react in Ouchterlony tests with anti-BMV antiserum (see Figure 13(d)). Sap from the plants that became infected when inoculated with the plant debris was used for further propagation of the stemrust-transmitted-BMV. The virus was subsequently successfully extracted at pH 4,0 from these plants. Stemrust-transmitted-BMV isolates that were obtained in this way are called mechanically-transferred, stemrust-transmitted-BMV. This is done to distinguish such isolates from the stemrust-transmitted-BMV that was successfully extracted directly from viruliferous stemrust-infected plants. This nomenclature is necessary for clarification later on, since the two types of isolates have been found to exhibit different pH stabilities.

Following these findings it was postulated that the fungus selectively acquired and transmitted variants of the various BMV isolates. These variants were considered to be unstable at pH 4,0 and pH 5,0 and would

therefore account for the inability of extracting the stemrust-transmitted-BMV at pH 4,0 and pH 5,0. Stemrust-transmitted-BMV was later shown to be more stable at pH 7,0 (see IV.G.1. on the stability of stemrust-transmitted-BMV). With this aforesaid hypothesis in mind, a new purification procedure (III.D.4.) was followed which in the meantime had been adapted by other workers in the laboratory experiencing similar problems with BMV extraction.

The main features of this extraction procedure (fully described in III.D.4.) are:

(i) homogenisation of plant tissue in the presence of 0,1 M KPO_4, pH 7,0 buffer.

(ii) precipitation of the virus and clarification of plant sap by 9% PEG and 2,5% NaCl.

(iii) 400 x concentration by ultracentrifugation.

(iv) Analysis of concentrates by Ouchterlony tests, density gradient centrifugation and U.V. fractionation, electron microscopy and infectivity assay.

Both stemrust-transmitted-BMV and leafrust-transmitted-BMV (trial experiments for the latter) were successfully extracted, directly from viruliferous fungus-infected

FIGURE 10(a): Sucrose density gradient profile of a stemrust-transmitted-BMV-Beth concentrate extracted at pH 7,0. Sedimentation is from left to right.

FIGURE 10(b): Sucrose density gradient profile of a leafrust-transmitted-BMV concentrate extracted at pH 7,0. Sedimentation is from left to right.

plants, by working at the higher pH of 7,0. No virus was extracted from healthy plants inoculated with non-viruliferous uredospores.

Although T_4-R and Clipper are resistant to stemrust and leafrust infection, respectively and consequently did not support fungus growth, it was possible to extract virus from these plants when inoculated with viruliferous uredospores. Thus, to repeat the statement made earlier (IV.D.1), resistance in a plant to stemrust or leafrust infection did not prevent the fungus from transmitting BMV to such a plant. Analysis of the stemrust-transmitted-virus concentrates on sucrose density gradients by monitoring UV absorption at 254nm, revealed a single peak (Figure 10). This fraction was positively identified as BMV by scanning in the UV range 310-220nm, electron microscopy and Ouchterlony tests.

It was further shown that this fraction corresponded to similar fractions sedimenting at 72S (Rybicki and von Wechmar, 1982).

The UV absorption curve of the virus fraction was typically that of BMV, showing a peak at 260nm, and having a 260/280 ratio of 1,53 (Figure 11). Virus particles in this fraction adsorbed specifically to grids coated with anti-BMV antiserum No. 3 at 1/64 dilution. The electron micrograph (Figure 12) reveals spherical

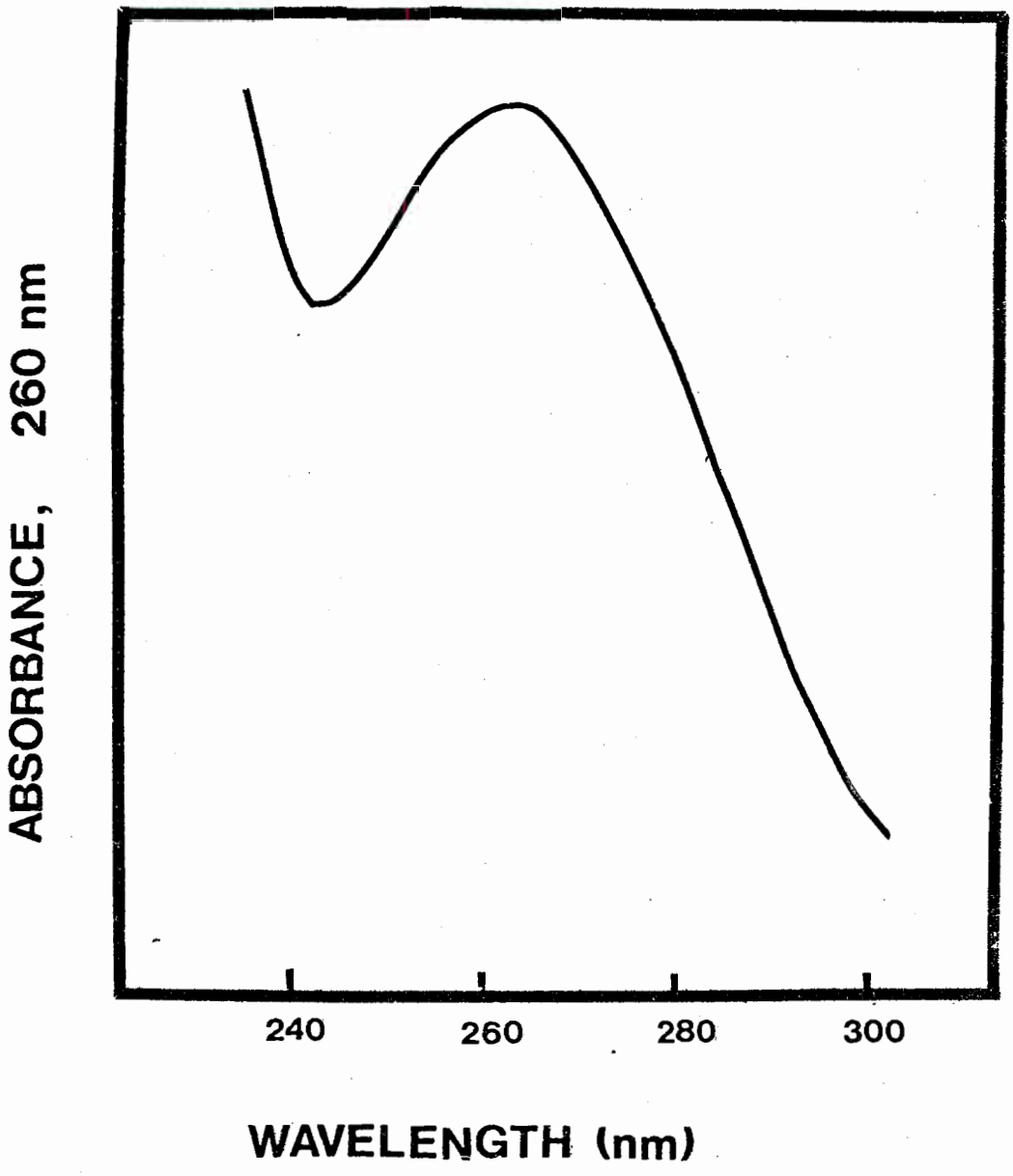


FIGURE 11: UV absorption curve of stemrust-transmitted-BMV in 0,1 M KPO_4, pH 7,0 buffer. Scan of fractionated sample shown in Figure 10(a) is shown.

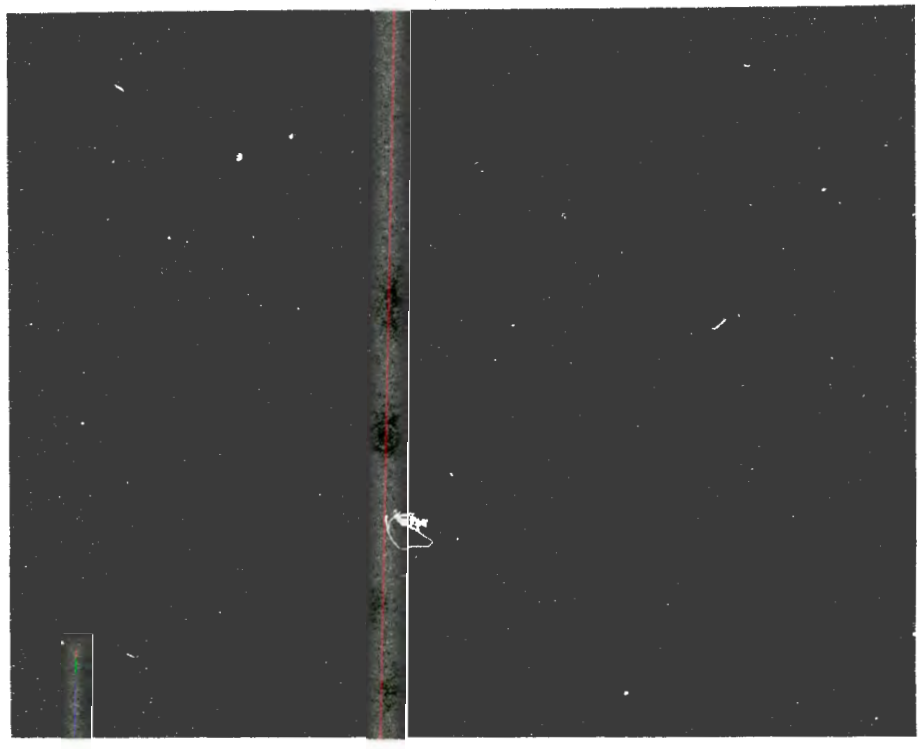


FIGURE 12: Electron micrograph of an IEM assay of stemrust-transmitted-BMV-Beth. Virus particles from a sucrose density gradient fraction are shown. The grids were coated with anti-BMV antiserum (No. 3)at 1/64 dilution. Bar = 100nm.

particles ± 30nm in size which correspond to BMV.

The sucrose density gradient fraction reacted with anti-BMV antiserum in Ouchterlony tests by forming a single precipitin band (Figures 13 a and b).

Stemrust-transmitted-BMV concentrates that were not analysed on sucrose gradients were similarly found to react with anti-BMV antiserum in Ouchterlony tests by forming a single precipitin band (Figures 13 c and e).

The concentration of the stemrust-transmitted-BMV in the fractionated and unfractionated concentrates was determined by UV absorption and by Ouchterlony tests, utilising the optimal proportions point with BMV at a known concentration as a standard (see Figure 14).

The yield of stemrust-transmitted-BMV was found to be of the order of 1-2mg/kg leaf tissue. This yield is a 100-fold less than that obtained for mechanically inoculated BMV (III.B.3.d.). No yield determination was made for leafrust-transmitted BMV since the small quantity of virus sample obtained for this test was used up for other experiments. Since only small quantities of stemrust and leafrust-transmitted-virus isolates were obtained by direct extraction of BMV from rust-infected plants, these isolates were increased by mechanically inoculation onto healthy plants.

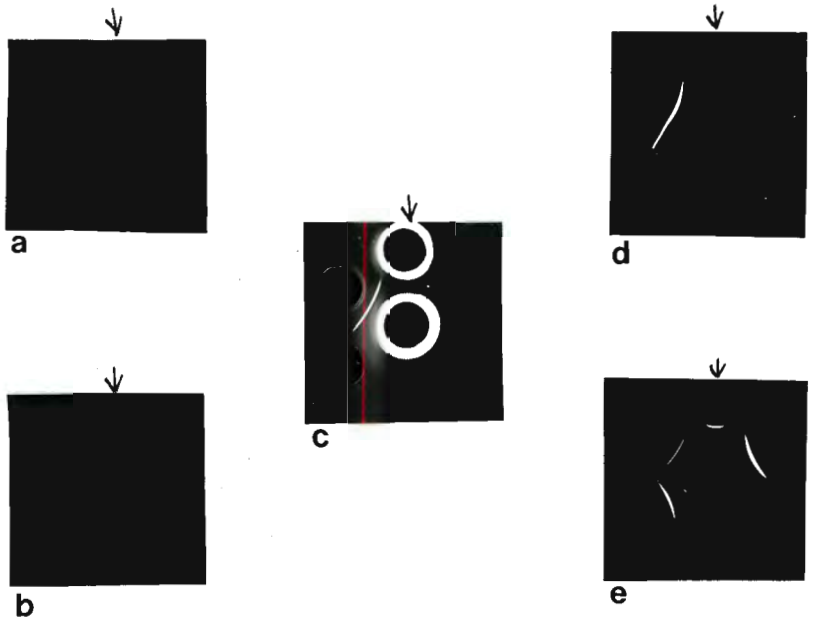


FIGURE 13: Gel-precipitin reactions of mechanically transferred, stemrust-transmitted-BMV, stemrust- and leafrust-transmitted-BMV (sucrose density gradient fractions and differential centrifugation concentrates), and resuspended high speed pellets from purification at pH 4,0 and pH5,0. In all cases shown, anti-BMV antiserum was used at either 1/2 or 1/4 dilution. In each case the arrows indicate the starting points.

Figure 13(a):

Gel buffer = Acetate/NaCl pH 5,0
Antiserum No. 3 in central well.
Arranged clockwise in surrounding wells are: (from arrow) stemrust-transmitted-BMV-Beth (sucrose density gradient fraction); BMV-Type; BMV-Beth infected plant sap; healthy sap; leafrust-transmitted-BMV (sucrose density gradient fraction); mechanically-transferred, stemrust-transmitted-BMV-Beth (purified by differential centrifugation).

Figure 13(b):

Gel buffer = Acetate/NaCl pH 5,0
Antiserum No. 5 in central well.
Arrangement in surrounding wells is the same as in Figure 13(a).

Figure 13(c):

Gel buffer = phosphate/NaCl, pH 7,0
Antiserum No. 3 in central well.
Arranged anti-clockwise in surrounding wells are: (from arrow) healthy sap; concentrate of stemrust-transmitted-BMV-Beth (purified by differential centrifugation only). Remaining wells are empty.

Figure 13(d)

Gel buffer = Acetate/NaCl pH 5,0
Antiserum No. 3 in central well.
Arranged clockwise in surrounding wells are: (from arrow) 'high speed' pellets of stemrust-transmitted-BMV-Beth extracted at pH 5,0 and 4,0, respectively; next two wells are 'high speed' pellets of stemrust-transmitted-BMV-G_9 extracted at pH 4,0 and 5,0, respectively; 'high speed' pellet of stemrust-transmitted-BMV-Palala extracted at pH 5,0; BMV-G_9 at 0,5 mg/ml

Figure 13(e):

Gel buffer = Acetate/NaCl pH 5,0
Antiserum No. 3 in central well.
Arranged clockwise in surrounding wells are: (from arrow) stemrust-transmitted-BMV-Palala; stemrust-transmitted-BMV-G_9; 2 empty wells; leafrust-transmitted-BMV; stemrust-transmitted-BMV-Beth. (All these isolates were mechanically transferred and then extracted at pH 4,0. So theyare all called mechanically transferred, stemrust- (or leafrust-) transmitted-BMV).

As a final note on purification of stemrust-transmitted-BMV, it is important to note that extraction of the virus at pH 7,0 yielded results only 60% of the time. However, this method of extraction was used regardless of this observation since it proved to be the most efficient of the methods tested.

Regarding Figure 14:

By comparison with precipitin bands in Figure 14(a) the stemrust-transmitted-BMV-Beth sample reacted optimally at 1/2 dilution (* in Figure 14(b)) and this corresponded to a 1/8 dilution (0,06mg/ml) (*in Figure 14(a)) of the standard. Therefore, the stemrust-transmitted-BMV-Beth isolate had a concentration of 0,12mg/ml.

3. DETECTION OF STEMRUST-TRANSMITTED BMV IN CRUDE EXTRACTS OF PLANTS

3.a. Polyacrylamide gel electrophoresis (PAGE)

Crude extracts of plant tissue (prepared as in III.J.1.a.(iii)) were analysed on 12% acrylamide gels. Purified BMV-G_9 was used as a standard in these runs. 20-25µl sample volumes were usually loaded onto the gels and electrophoresis was carried out at 60mA for 7-8 hours. The stained gels were dried and photographed.

Figure 15 shows the electrophoretic profiles of

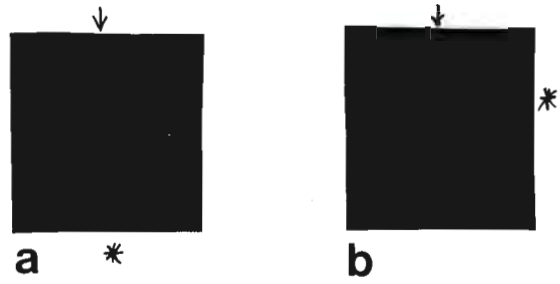


FIGURE 14: Determination of virus concentration in crude extract of stemrust-transmitted-BMV using BMV at a known concentration as a standard. In both cases, antiserum was at 1/16 dilution and antigens were diluted 2-fold. The arrows indicate the starting points. Asterisks indicate optimal proportions points.

Figure 14(a)

Gel buffer = Acetate/NaCl pH 5,0
Antiserum No. 3 in central well.
Arranged clockwise in surrounding wells are: (from arrow)
BMV-Beth at 0,5 mg/ml then followed by 5 serial 2-fold dilutions.

Figure 14(b)

Gel buffer = Acetate/NaCl pH 5,0
Antiserum No. 3 in central well.
Arranged clockwise in surrounding wells are: (from arrow)
stemrust-transmitted-BMV-Beth diluted 2-fold 5 times.

various plant saps. Although it was possible to detect BMV in crude extracts of plants mechanically inoculated with BMV, it was not possible to detect stemrust-transmitted-virus in crude leaf extracts, even after concentration in the airfuge (see lanes 3 and 4 in the electrophoregram in Figure 15). The inability to detect BMV in the crude leaf extracts of plants inoculated with viruliferous uredospores, can be attributed to the low concentration of BMV in these extracts. Since the yield of stemrust-transmitted-virus was found to be 1-2mg/Kg, by analogy, the maximum amount of BMV in crude leaf extracts that were loaded onto a gel amounts to approximately 0,1ng. (3g of leaves in 3ml buffer were used to prepare crude extracts and 25µl of this was loaded onto a gel). Such a low concentration of BMV is below the detection limit for proteins on polyacrylamide gels when stained with Coomassie blue. This, therefore, explains the failure of this technique at detecting stemrust-transmitted-BMV in crude leaf extracts.

3.b. Detection of stemrust-transmitted-BMV by ELISA

Since PAGE was unsuitable for the detection of stemrust-transmitted-BMV in leaf extracts, ELISA was investigated as an alternative. The double-antibody "sandwich" ELISA technique has been shown to be sensitive for the detection of plant viruses in plant tissue extracts (Clark and Adams, 1977).

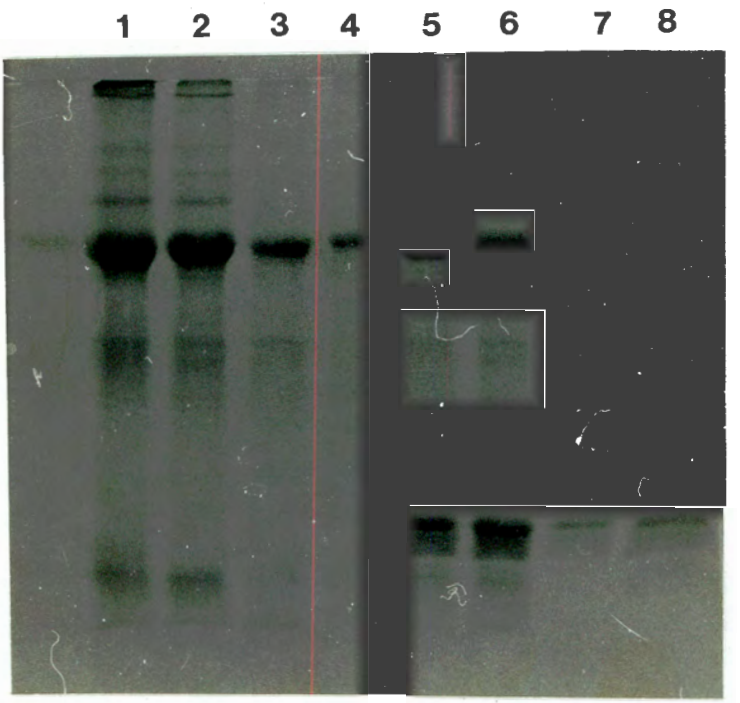


FIGURE 15: Electrophoregram of PAGE of crude leaf extracts on a 12% polyacrylamide gel. Electrophoresis was done at 60ma for 7 hours. Profiles for the samples are as follows: Lanes 1 and 2 = healthy plant sap; Lane 3 = crude extract of stemrust infected plants; Lane 4 = sample in Lane 3 concentrated; Lanes 5 and 6 = BMV-Beth infected sap; Lane 7 = BMV-Type; Lane 8 = BMV-Beth.

Crude extracts of plant tissue (infected and healthy) were prepared as in Section III.H.2.b.). These extracts were reacted with anti-BMV antiserum in ELISA.

With ELISA it was possible to detect stemrust-transmitted-BMV in crude leaf extracts. The reactivities of BMV-infected sap (mechanical inoculation), healthy sap and crude sap extracts of stemrust-transmitted-BMV appear in Figure 16.

The antiserum used (antiserum No. 3) was found to react slightly with healthy plant sap giving an O.D. value of 0,1 for a 1/16 dilution. This non-specific reaction with plant sap was, however, much lower than the reactions obtained with BMV-infected sap and stemrust-transmitted-BMV crude extracts. Thus, the reaction obtained with a crude extract of stemrust-transmitted-BMV was specific for BMV. As compared to the reaction obtained with BMV-infected sap (mechanically inoculated), this latter reaction is very weak. Since BMV-infected sap at 1/500 dilution exhibited an O.D. value of twice that obtained for a crude extract of stemrust-transmitted-BMV at 1/2 dilution.

This result indicates that the yield of BMV from

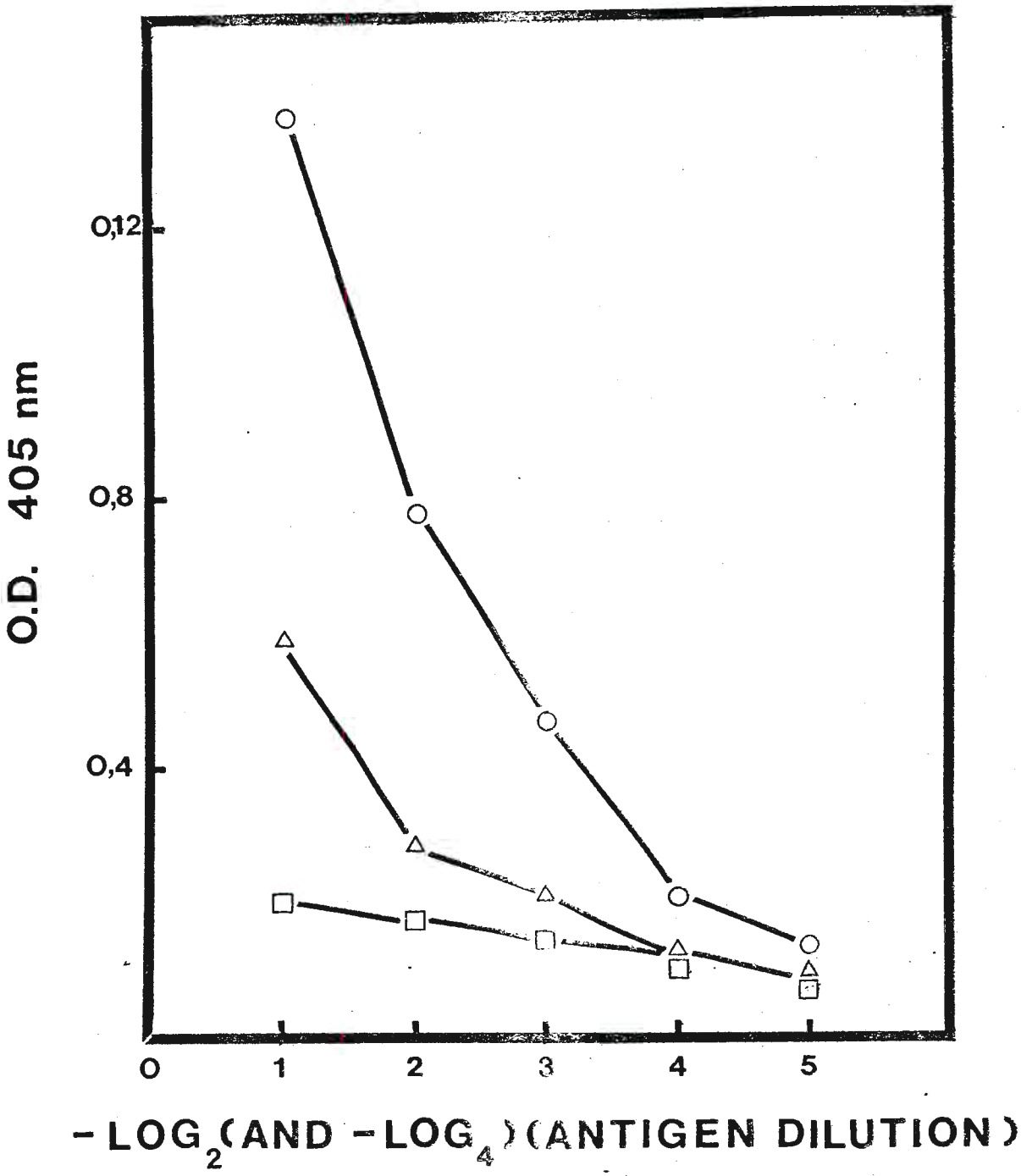


FIGURE 16: Reactions of BMV-Beth infected plant sap (○), sap from Clipper barley inoculated with viruliferous stemrust uredospores (△), and healthy plant sap (1:1 mixture of wheat and barley sap) (□) with anti-BMV antiserum No. 3. BMV-infected plant sap was initially at 1/500 dilution and then diluted 4-fold. The other two saps were diluted 2-fold, starting at 1/2. The plates were coated with anti-BMV IgG at 5µg/ml and the conjugate dilution was at 1/750.

plants when transmitted by stemrust, is much lower than that obtained from plants mechanically inoculated with BMV.

4. SIMULATED TRANSMISSION OF BMV IN THE ABSENCE OF UREDOSPORES AND IN VITRO ACQUISITION AND INHIBITION OF TRANSMISSION WITH ANTI-BMV ANTISERUM

4.a. Spray-inoculation with purified BMV

Spray-inoculation with purified BMV was an attempt at simulating transmission of BMV by viruliferous uredospores. In such an experiment, a purified preparation of BMV-Beth, at 0,5mg/ml concentration, was sprayed onto healthy plants, in the same manner as for inoculation of plants with viruliferous uredospores (see III.C.2.b.). Plants inoculated in such a fashion with purified BMV-Beth became infected.

It is possible that the mechanical force of the spray was suficiently strong to puncture surface cells to allow virus entry. This finding has some bearing on the mode of transmission of BMV by stemrust.
For instance, if the mechanical force of the spray punctures the surface cells, then the virus, if it is carried on the outside of the uredospores, enters

the plant through the punctures. It is very likely that plants growing naturally in the field are punctured by the effects of sand, mechanical contact between plants and insect feeding points.

4.b. In vitro acquisition

An attempt was made at allowing non-viruliferous uredospores to acquire BMV *in vitro* by mixing uredospores with a purified BMV preparation. The non-viruliferous uredospores were mixed with BMV-Beth at 0,1mg/ml concentration. The uredospores were then pelleted by centrifugation at 3 000 rpm for 5 minutes on an BHG bench-top centrifuge. The uredospore pellet was briefly washed with distilled water, then centrifuged. The washed uredospores were then resuspended in water and spray-inoculated onto plants. These plants were treated in the same fashion as plants inoculated with viruliferous uredospores that acquired BMV from BMV-infected plants. No virus was extracted from plants inoculated with non-viruliferous uredospores that were mixed with BMV. Thus, *in vitro* acquisition of BMV by uredospores did not occur. The inability to show *in vitro* acquisition of BMV by uredospores can probably be accounted to the short incubation period of three minutes of non-viruliferous uredospore-BMV mixtures. This short incubation period was necessary in order to prevent pre-germination of the uredospores during

the incubation step and the washing step. Indeed, germination of the uredospores during these steps could probably account for the low level of stemrust infection observed on plants inoculated with non-viruliferous uredospores that were mixed with BMV. In control experiments, non-viruliferous uredospores were mixed with PBS pH 6,0 buffer, instead of with BMV, and these uredospores were treated in the same manner as the test uredospores. Since a low level of infection was obtained with these uredospores as well, it is possible that either pre-germination of the uredospores or the effect of centrifugation on the uredospores rendered many of them non-viable. It is possible that disorientation of the protoplasmic contents of the uredospores occurred during centrifugation causing some of the uredospores to be non-viable.

4.c. Inhibition of transmission by antiserum

Viruliferous uredospores that had acquired BMV by in vivo acquisition were found not to transmit BMV when incubated with antiserum. In such experiments viruliferous stemrust uredospores were mixed with anti-BMV antiserum at 1/8 dilution. An incubation time of 3 minutes was allowed. The uredospore-antiserum mixture was centrifuged at 3 000 rpm for 5 minutes on a BHG bench-top centrifuge. The

uredospore pellet was briefly washed in distilled water, centrifuged, and the resulting uredospore pellet was resuspended in water and inoculated onto healthy plants. These plants were grown in the same fashion as for plants inoculated with viruliferous uredospores during transmission experiments. No virus was extracted from plants inoculated with uredospores that were mixed with antiserum. From this it was concluded that some virus was carried externally on the uredospores and that its transmission by the fungus was inhibited by inactivation of the BMV on the uredospore's surface by antiserum specific to BMV. However, this result is inconclusive since viruliferous uredospores that were incubated with normal rabbit serum also did not transmit BMV, since no virus was extracted from plants inoculated with these uredospores.

A low level of infection by stemrust was also observed in these inhibition experiments, and can be attributed to the same reasons associated with a low stemrust infection in in vitro acquisition experiments (see IV.D.4.b).

E. GERMINATION OF UREDOSPORES

The first attempts at germinating uredospores involved the floating of uredospores on water. By this method,

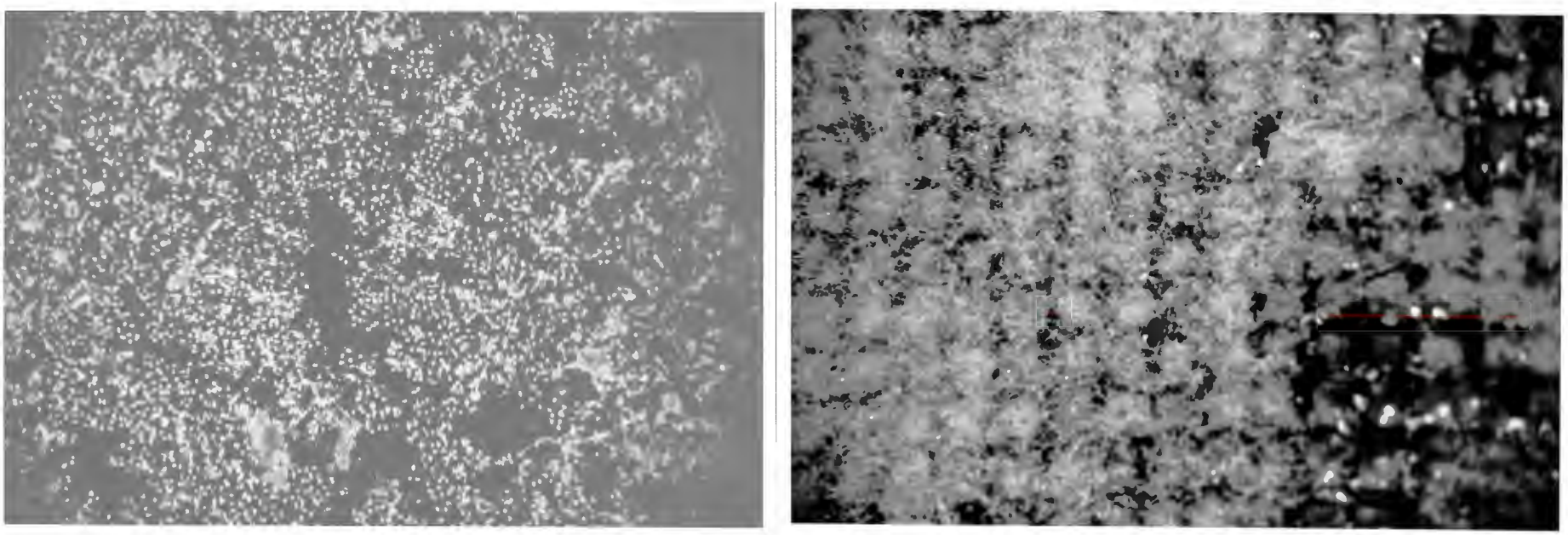
10-20% germination was observed by macroscopic examination (see Figure 17(a)). The low percentage germination obtained at that time was described to the pre-germination of uredospores on the leaf surface (see Figure 5). This pre-germination was later corrected by lowering the humidity of the plant growth rooms at the time of pustule development (see IV.A.). However, after this correction, freshly collected uredospores were found to germinate with an efficiency of only 50-60% when floated on water.

Uredospores that were stored at 4°C (see III.C.2.c.) for a month were found to germinate with an efficiency of only 30%. The inefficient germination obtained by this method was accounted to the observations described below. Clumping of uredospores during floating was a common occurrence. This clumping was observed to cause a remarkable decrease in germination since many uredospores were excluded from moisture in this way. Uredospores that floated freely were observed to germinate well. No germination occurred when the spores were submerged in water.

Subsequently, a new method of germination was developed. McDonald and Heath (1977) used collodion membrane for germinating the uredospores of cowpea rust fungus. A similar system, employing coarse-woven silk fabric to allow spores to fall through, and water agar plates was developed. Uredospores were evenly spread over

the surface of a silk membrane placed on top of 1% water agar and were incubated in a moist chamber at room temperature overnight. With this system 90-100% germination was obtained with both freshly collected uredospores and uredospores stored for one month and longer at 4°C (see Figure 17(b)).

In order to separate germ tubes and hyphae from the uredospore coats of germinated uredospores, the following method of germination was developed: uredospores were sprinkled onto 1% water agar plates, and were covered with silk fabric. A glass slide coated with ovalbumin was suspended about 1mm above this. The whole experimental system was placed in a humid box overnight to allow germination of the uredospores. Germ tubes and hyphae that grew through the silk fabric became lodged onto the underside of the glass slide. These hyphae and germ tubes were free from uredospore coat. The germ tubes and hyphae were crushed on the slide in the presence of a few drops of PBS pH 6,0 buffer using a glass rod. The hyphae and germ tubes were crushed by rolling the glass rod across the surface of the slide. These extacts of germ tubes and hyphae were used for the detection of BMV inside the uredospores in electron microscopy (see IV.F.3.).



a b

FIGURE 17 Germination of stemrust uredospores.

17(a): Germination obtained by floatation of uredospores on water.
17(b): Germination of uredospores on top of coarse woven silk on water agar.

F. DETECTION OF BMV IN AND ON STEMRUST UREDOSPORES

1. ENZYME-LINKED IMMUNOSORBENT ASSAY

The double antibody "sandwich" ELISA of Clark and Adams (1977) and Voller et al. (1976) was used for the detection of BMV in and on stemrust uredospores because of its extreme sensitivity for virus detection. The assay procedure that was used in this study is fully described in III.H.2.c.

In each case, the uredospores were used at an initial suspension of 50mg/ml in PBS pH 6,0 buffer and then diluted two-fold. Antigen controls in the assays included BMV-Beth as a positive control and WCuMV as a negative control. In another control gamma globulins of normal rabbit serum (NRS) was used to coat the wells instead of anti-BMV antibodies. Viruliferous uredospores reacted positively in these assays, whereas the reaction of non-viruliferous uredospores was negligible with a highest O.D. 405nm of 0,05 units.

The reactions with viruliferous uredospores were found to be highly specific for BMV since the same antisera did not react with WCuMV but did react with BMV-Beth (see Figure 18). When normal rabbit serum was used to coat the wells no reaction was obtained for any of the samples tested.

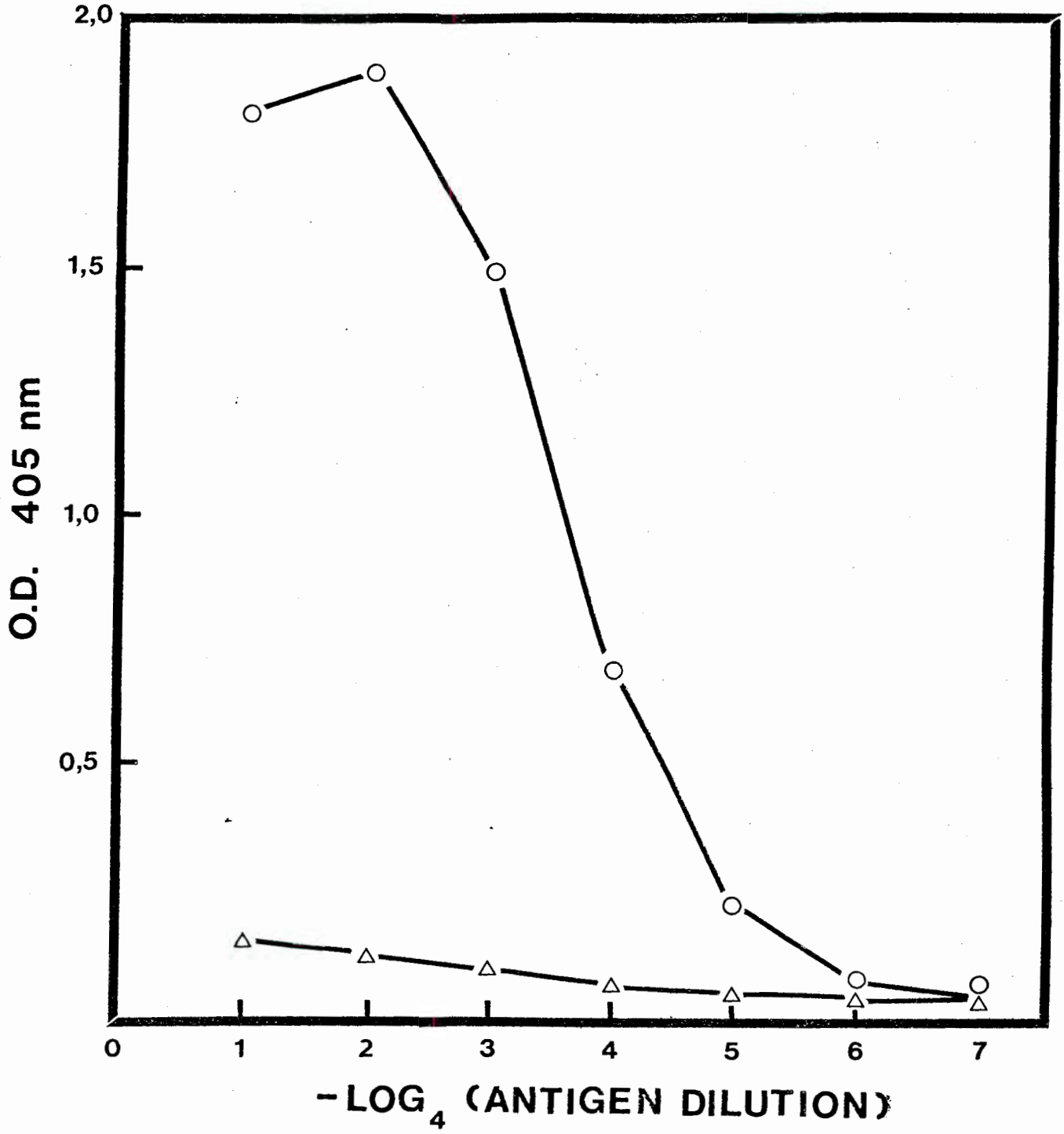


FIGURE 18: Reactivity of antiserum No. 3 with BMV-Beth and WCuMV in sandwich ELISA. (○) represents BMV-Beth and (△) WCuMV. Coating antibody concentration was 5µg/ml, and conjugate dilution was 1/1000. Viruses were at an initial concentration of 0,4mg/ml.

With the ELISA technique it was possible to demonstrate that the virus was carried both inside and on stemrust uredospores. The results obtained with three anti-BMV antisera are illustrated in Figures 19, 20 and 21.

Figure 19 depicts the reactivities of antiserum No. 1 with three uredospore samples. From this it can be seen that the viruliferous stemrust uredospores carried the virus predominantly on the outside of the uredospore since the "spore wash"-uredospore sample (1/1 dilution) yielded an O.D. 405nm reading twice that of the "crushed, washed"-uredospore sample at the same dilution, the reading being 0,2 and 0,1 O.D. units respectively. The higher reactivity obtained for the "crushed"-uredospore sample is the sum of the reactivities for the "spore-wash"-uredospore sample and the "crushed, washed"-uredospore sample. The reaction of this sample only serves to indicate that viruliferous stemrust uredospores carry BMV, and gives no indication of the location of the virus associated with these uredospores. Only slight reactions yielding a highest O.D. 405nm reading of 0,05 units was obtained for corresponding non-viruliferous uredospore samples. A background level reaction was obtained with the unrelated WCuMV, whereas BMV reacted positively in the same assay (not shown in Figure 19, see Figure 18). Since this antiserum was found to react non-specifically (see IV.C.) it was decided to discontinue its use and two other anti-BMV antisera were utilised in similar

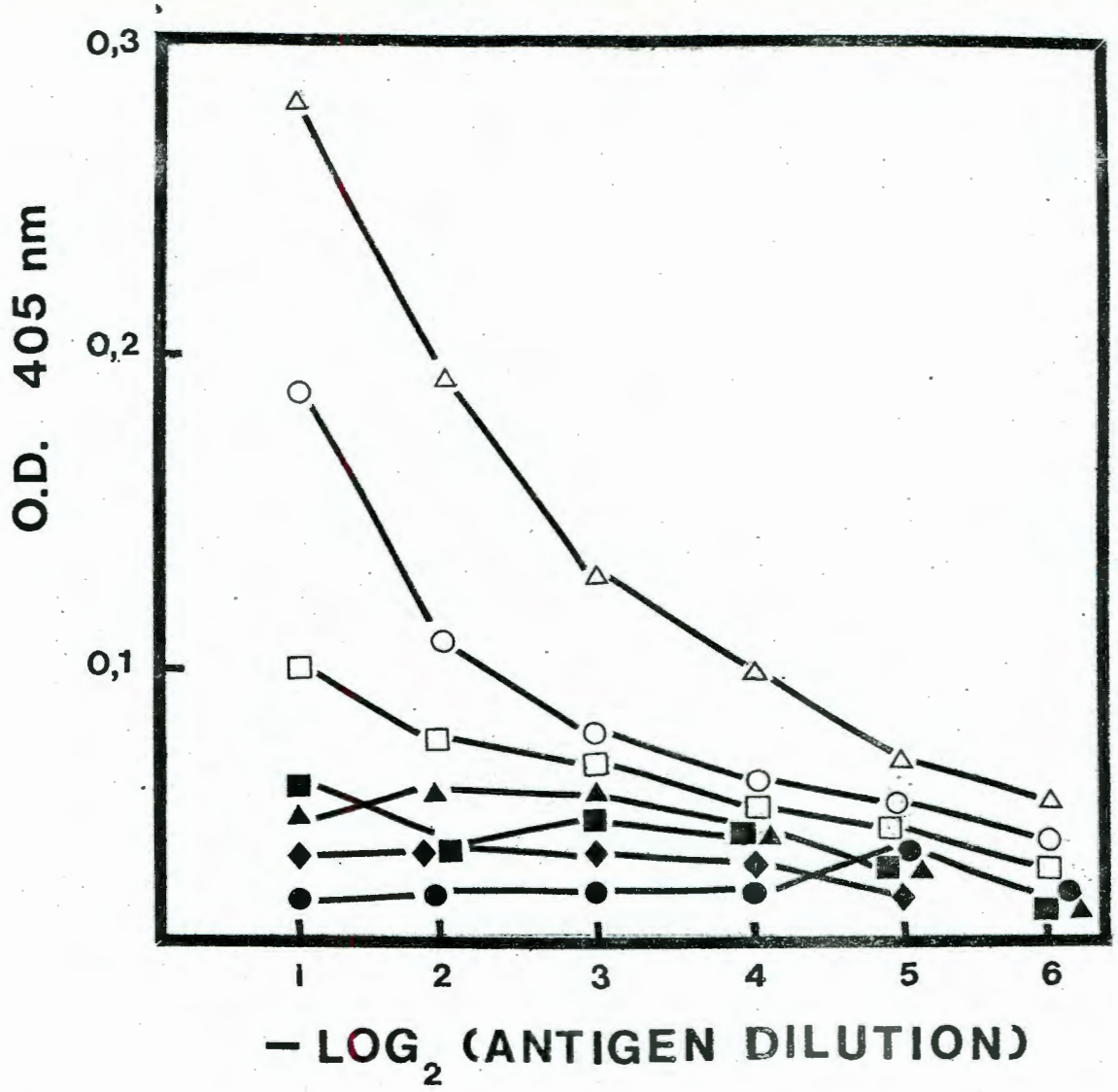


FIGURE 19: Reactivity of antiserum No. 1 with stemrust uredospore samples in sandwich ELISA.

(△) = crushed uredospores
(○) = spore-wash
(□) = crushed, washed uredospores

] viruliferous uredospores

Corresponding shaded symbols = corresponding spore samples for non-viruliferous uredospores

(◆) = WCuMV

Coating antibody concentration was 5 µg/ml and conjugate dilution was 1/500.

Uredospore samples were at an initial suspension of 50 mg/ml. WCuMV was at an initial concentration of 0,5 mg/ml.

assays. The other two antisera yielded similar results.

The results obtained with antisera Nos. 3 and 5 are illustrated in Figures 20 and 21 respectively. The results obtained with these two antisera indicate the presence of BMV inside of and on viruliferous uredospores.

With antiserum No. 3 (see Figure 20) the "spore-wash"-uredospore sample reacted to the same extent as the "crushed"-uredospore sample and the "crushed, germinated"-uredospore sample. The reactivities of the latter two uredospore samples only indicate that viruliferous uredospores carry BMV and gives no idea of the localisation of the virus. The high reactivity of the "spore-wash"-uredospore sample indicates that the virus is carried predominantly on the surface of viruliferous uredospores. The "crushed washed-germinated"-uredospore sample reacted to a lesser extent. This reaction indicates that some virus is carried internally by the uredospores. (Virus adhering to the outside of these uredospores have been removed by thorough washing of these uredospores prior to germination and crushing). Only a weak reaction was obtained with the "crushed, washed"-uredospore sample. This is due to the inability to efficiently rupture ungerminated uredospores.

Similar results were obtained with antiserum No. 5 (see Figure 21). Only, in this assay the "spore-wash"-

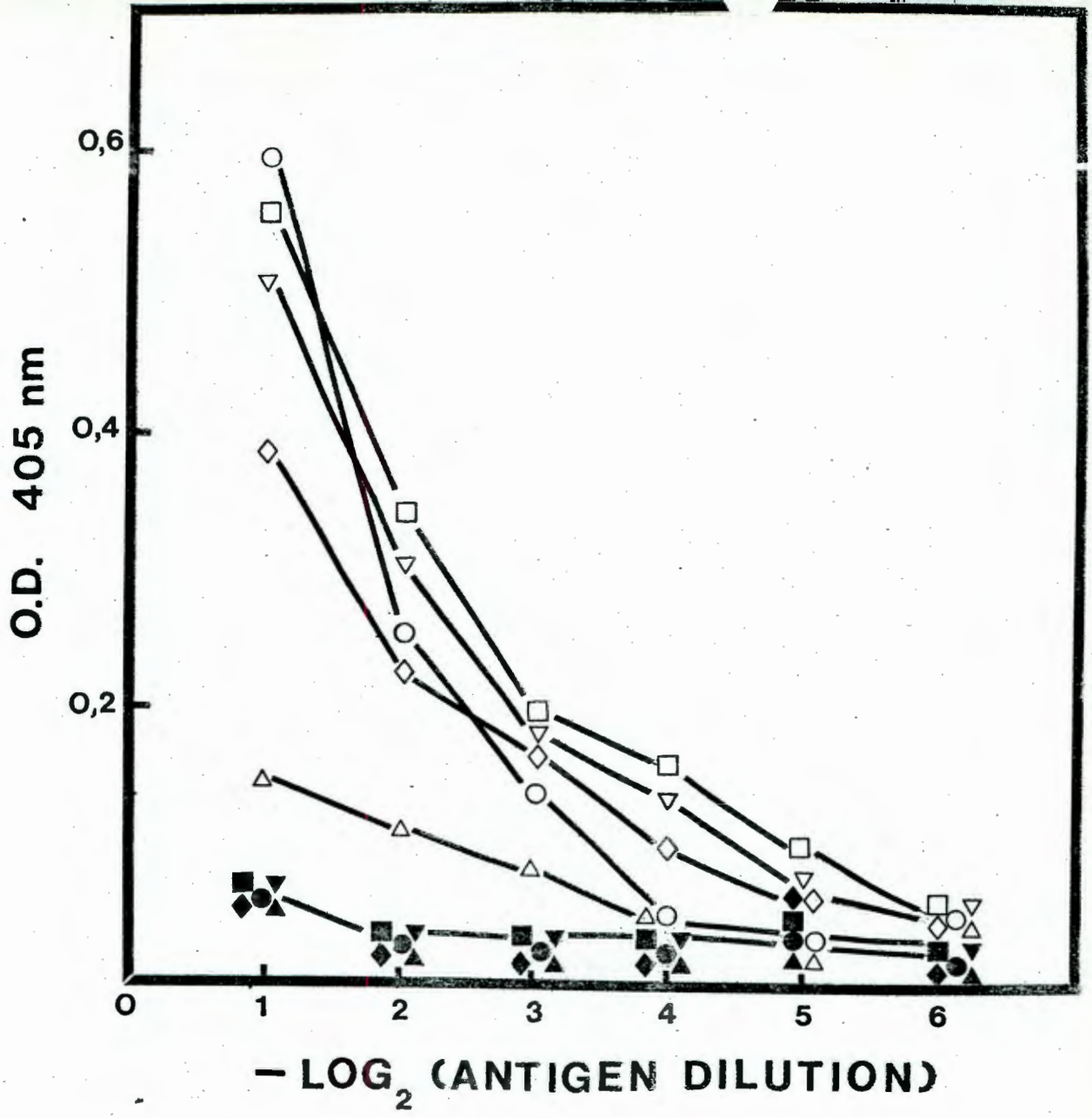


FIGURE 20: Reactions of stemrust uredospore samples with anti-BMV antiserum No. 3 in sandwich ELISA.

(○) = spore washings
(□) = crushed uredospores
(▽) = crushed, germinated uredospores
(◇) = crushed, washed-germinated uredospores
(△) = crushed, washed uredospores

viruliferous uredospores

Shaded symbols = corresponding spore samples for non-viruliferous uredospores. Coating antibody concentration was at 5 µg/ml and conjugate dilution was 1/150.

Uredospore samples were at an initial suspension of 50 mg/ml.

uredospore sample reacted to a lesser extent than the "crushed"- and "crushed, germinated"- uredospore samples, both of which displayed the same degree of reactivity. The "crushed, washed - germinated"-uredospore sample showed the same degree of reactivity as the "spore-wash" uredospore sample.

In both the assays performed with antisera Nos. 3 and 5, the corresponding non-viruliferous uredospore samples were virtually non-reactive, yielding a highest O.D. 405nm reading of 0,05 units. Thus, the non-viruliferous uredospores were shown to be BMV-free.

The results obtained with viruliferous uredospores indicate that non-viruliferous uredospores when propagated on BMV-infected plants were able to acquire the virus. In conclusion, it can be said that ELISA revealed a close association of BMV with viruliferous uredospores and that the virus is located both inside and on these uredospores. The reactions obtained in these assays were highly specific for BMV since the antisera reacted positively with a purified preparation of BMV-Beth and not with the unrelated WCuMV. The non-reactivity of non-viruliferous uredospores in these assays indicates that these uredospores were indeed BMV-free.

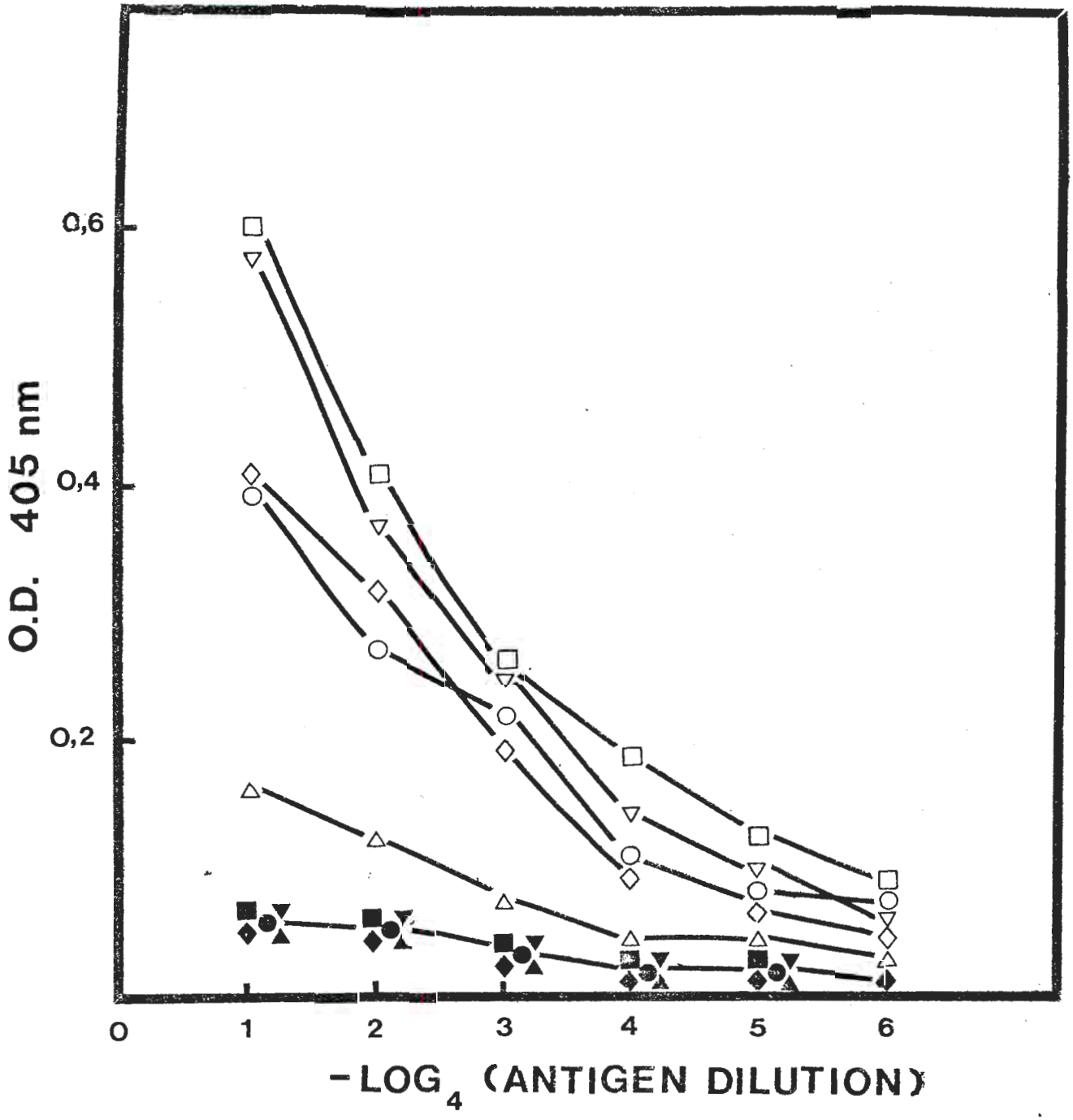


FIGURE 21: Reactions of stemrust uredospore samples with anti-BMV antiserum No. 5 in sandwich ELISA.

Symbols have the same meaning as those in Figure 20. The conditions of this assay were identical to those presented in the legend of Figure 20.

2. FLUORESCENT ANTIBODY BINDING STUDIES

This technique was used for the detection of BMV on the surface of stemrust uredospores. Two methods of staining were employed, namely the indirect and direct staining techniques (see III.I.2.a and b).

In the indirect method, the binding of unlabelled anti-BMV antibodies to BMV adsorbed to the uredospore coat was detected with FITC-labelled goat anti-rabbit antibodies (GAR). In the direct method, BMV adsorbed to the uredospore coat was directly detected by the binding of FITC-labelled anti-BMV antibodies. The stained uredospore specimens were observed for fluorescence under an Olympus fluorescent microscope and photographs of the specimens were taken using an Olympus C35-A camera attached to the microscope. In each case, an exposure time of four minutes was allowed.

The results obtained for the indirect staining procedure are illustrated in Figure 22.

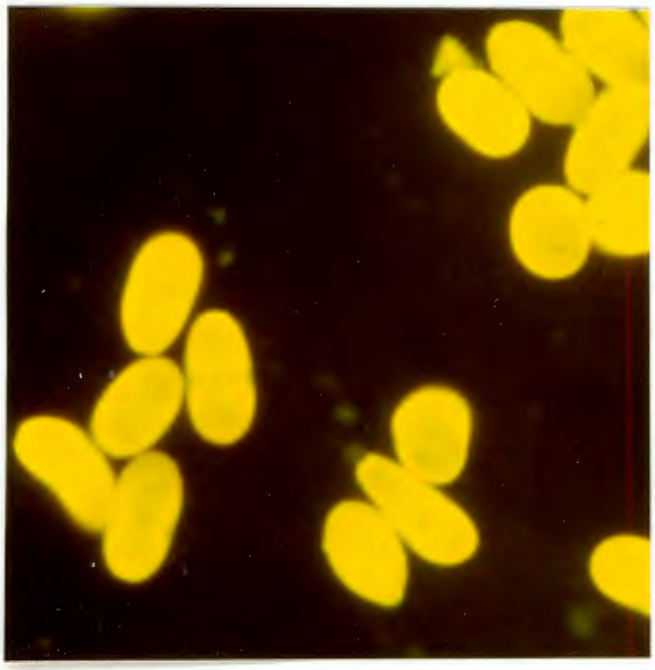
Figure 22(a) shows the intense fluorescence observed when viruliferous uredospores were incubated with unlabelled anti-BMV antibodies and then stained with FITC-labelled GAR. This fluorescence positively indicates the presence of BMV on the surface of these uredospores.

Figure 22(b) shows the lower degree of fluorescence

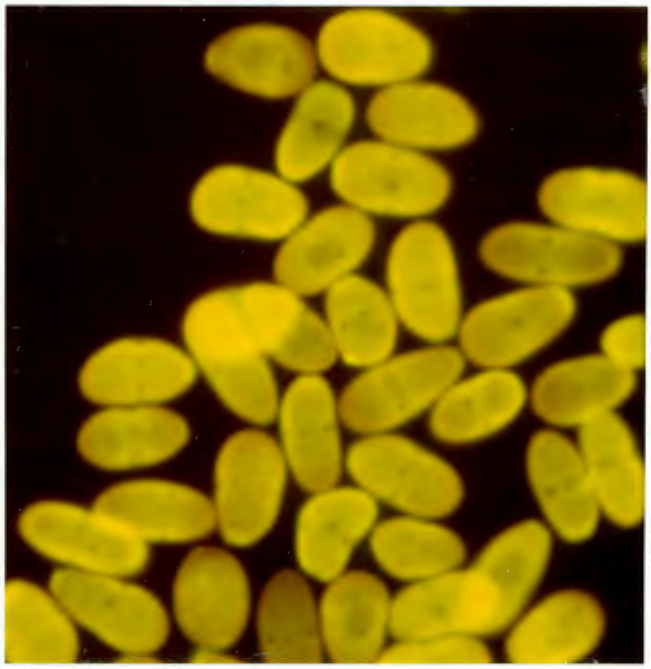
observed when non-viruliferous uredospores were incubated with unlabelled anti-BMV antibodies and then stained with FITC-labelled GAR. The low fluorescence observed on these uredospores is due to some non-specific staining. In ELISA these uredospores were found to react only with an insignificant O.D. 405nm reading of 0,05 units. The degree of fluorescence observed on viruliferous uredospores (see Figure 22(a)) is of a much greater magnitude than that observed on non-viruliferous uredospores, thus, the insignificant level of fluorescence observed on the latter uredospores cannot be regarded as evidence for the presence of BMV on these spores.

Figure 22(c) shows the decrease in fluorescence observed when viruliferous uredospores were incubated with normal rabbit serum instead of with anti-BMV antibodies, and then stained with FITC-labelled GAR. Again, the weak fluorescence observed in this case is also due to some non-specific staining. However, this result confirms the specificity of the fluorescence observed in Figure 22(a) since by comparison it can be seen that the fluorescence in Figure 22(a) is much higher than that in Figure 22(c).

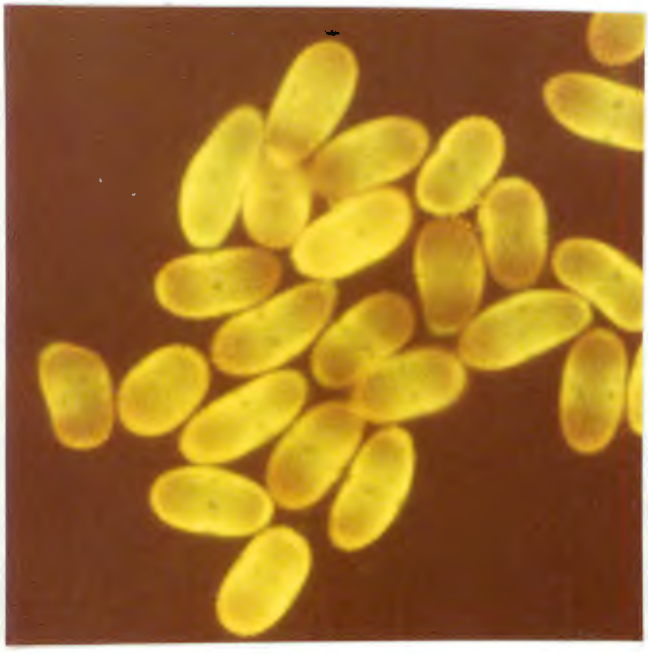
Figure 22(d) shows the result obtained when viruliferous uredospores were only stained with FITC-labelled GAR. An insignificant amount of fluorescence can be observed on some uredospores. Thus, no non-specific adsorption of FITC-labelled GAR occurred.



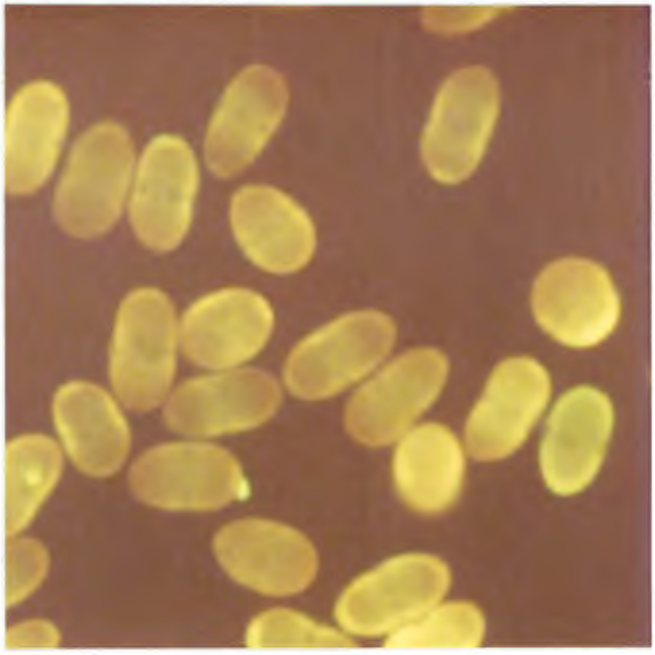
a



b



c



d

FIGURE 22: Fluorescence obtained with viruliferous stemrust uredospores by the indirect fluorescent staining procedure.

22(a): Specific antibodies + FITC-labelled GAR on viruliferous uredospores

22(b): Specific antibodies + FITC-labelled GAR on non-viruliferous uredospores

22(c): Normal rabbit serum (NRS) + FITC-labelled GAR on viruliferous uredospores

22(d): FITC-labelled GAR, only on viruliferous uredospores.

All the photographs were taken after an exposure time of 4 minutes.

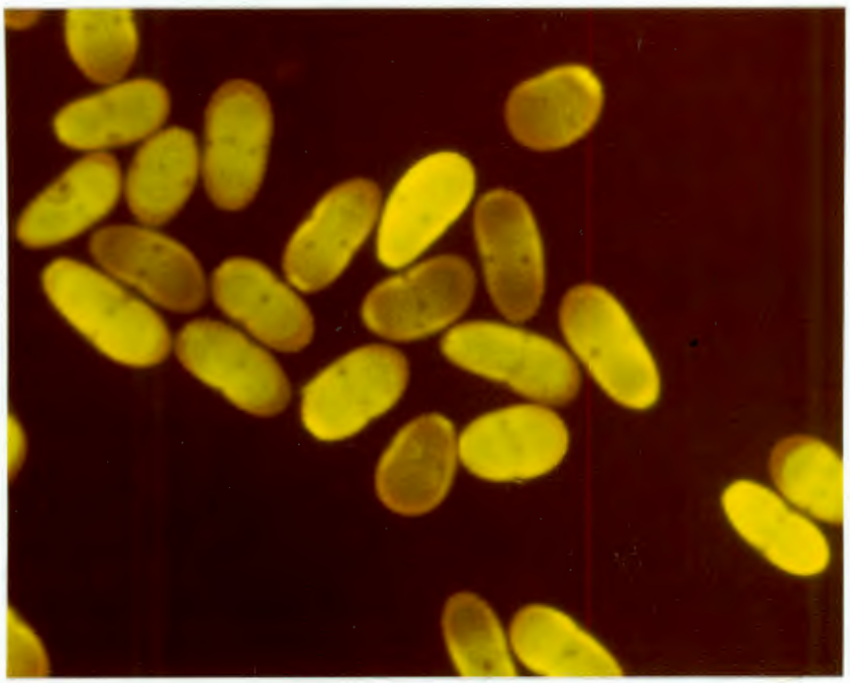
In order to confirm the results obtained with the indirect method, the direct method of staining was also used. The results obtained with this method are illustrated in Figure 23.

Figure 23(a) shows the fluorescence obtained when viruliferous uredospores were stained with FITC-labelled anti-BMV antibodies at 1/100 dilution. Only a few uredospores were observed to fluoresce brightly with the direct technique as opposed to the many brightly stained uredospores observed with the indirect technique (see Figure 22(a).

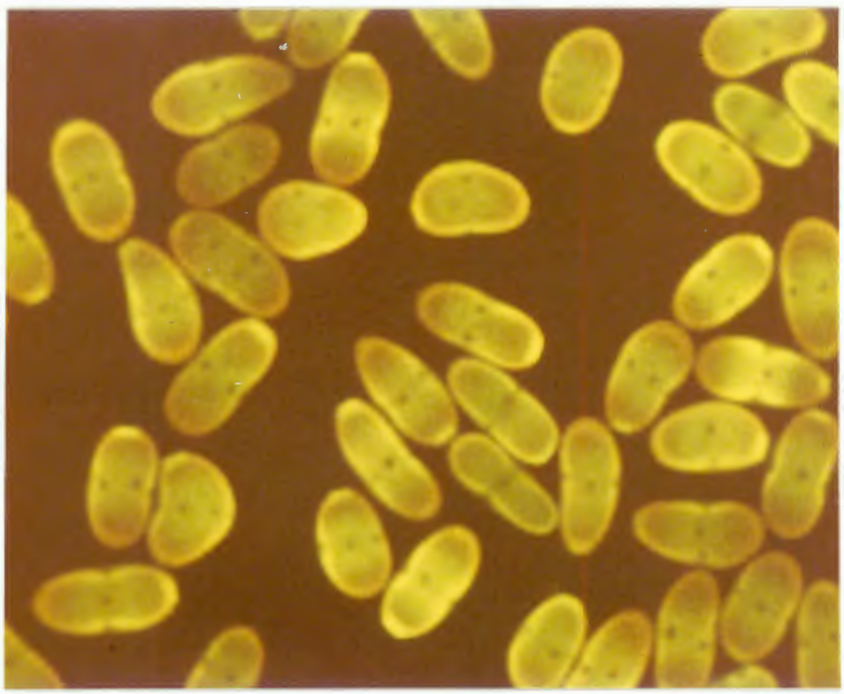
Figure 23(b) shows the decrease in fluorescence observed when the binding of FITC-labelled anti-BMV antibodies was inhibited by the prior incubation of viruliferous uredospores with unlabelled anti-BMV antibodies.

No fluorescence was observed when non-viruliferous uredospores were stained with FITC-labelled anti-BMV antibodies, and when viruliferous uredospores were stained with FITC-labelled normal rabbit serum.

The binding of FITC-labelled anti-BMV antibodies to viruliferous uredospores was totally inhibited when these antibodies were first incubated with purified BMV at 0,5mg/ml concentration, since no fluorescence was observed in this case.



a



b

FIGURE 23: Fluorescence obtained with viruliferous stemrust uredospores by the direct fluorescent staining procedure.

Figure 23(a):

Fluorescence obtained when viruliferous stemrust uredospores were stained with FITC-labelled anti-BMV antibodies at 1/100 dilution.

Figure 23(b)

Decrease in fluorescence observed on viruliferous stemrust uredospores by the inhibition of the binding of FITC-labelled anti-BMV antibodies by unlabelled anti-BMV antibodies.

In both instances, photographs were taken after an exposure time of 4 minutes.

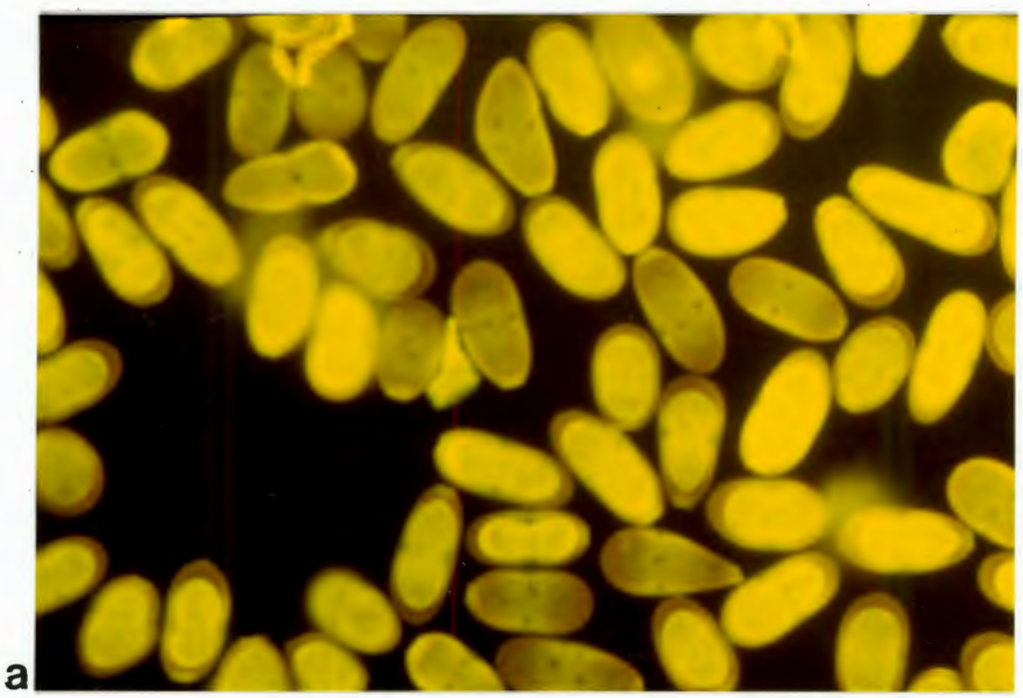
The results obtained for the latter three cases are not shown since the degree of fluorescence was too low to record photographically and technical difficulties prevented the making of prints from these negatives.

Figure 24(a) and (b) indicates the effects of overheating of uredospores during fixation. Haloes often appeared around the uredospores (Figure 24(a)) and in extreme cases the uredospore coat was ruptured (Figure 24(b)). Overheating during fixation gave rise to non-specific staining. This was overcome by briefly passing the specimen through a feable flame and not allowing the uredospores to be singed.

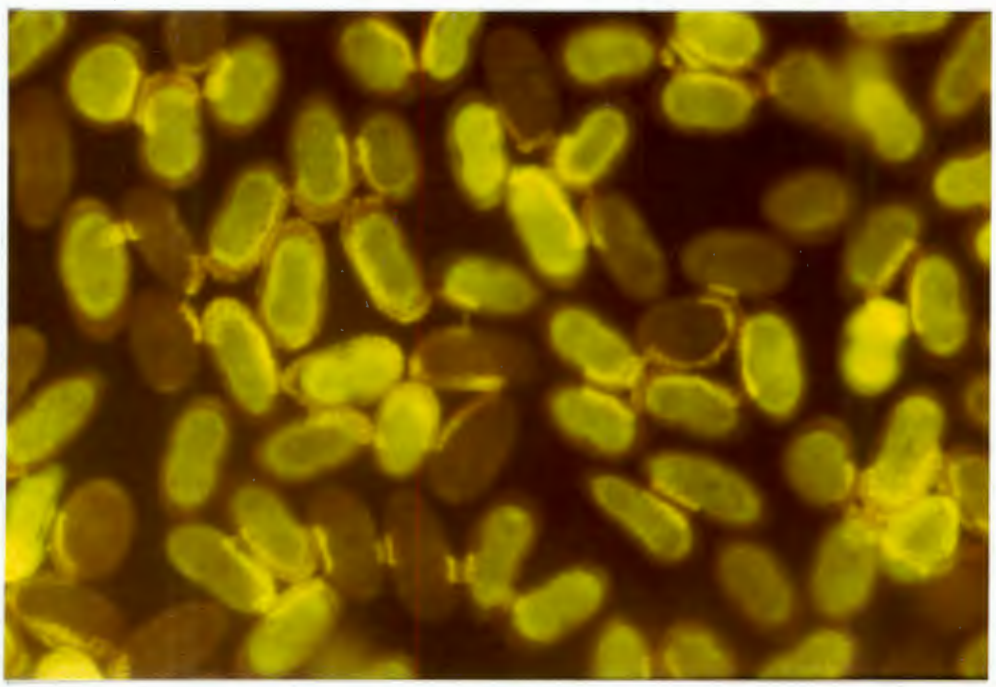
The fluorescent antibody staining technique thus positively indicated the presence of BMV on viruliferous uredospores. Similar results were obtained with both the indirect and the direct method of staining. The staining observed was specific for BMV since substitution of normal rabbit serum for anti-BMV antibodies yielded none or lesser fluorescence, and fluorescence was decreased by inhibition of the binding of anti-BMV antibodies by purified BMV and by unlabelled anti-BMV antibodies.

3. ELECTRON MICROSCOPY: I.E.M., THIN SECTIONING AND FERRITIN LABELLED ANTIBODY BINDING

All attempts at detecting BMV in stemrust uredospore samples with negative staining failed. Consequently,



a



b

FIGURE 24: Effects of overheating during fixation of stemrust uredospores for fluorescent antibody binding studies.
(a) - appearance of haloes around uredospores
(b) - disruption of spore coat.

immunosorbent electron microscopy (IEM) was used for the detection of BMV in or on stemrust uredospores. With this technique it was possible to demonstrate the presence of BMV on and inside viruliferous stemrust uredospores.

BMV particles were detected in uredospore washings, extracts of washed uredospores and in extracts of germinated uredospores. The particles observed in these samples are depicted in the electron micrographs which appear in Figures 25, 26 and 27.

The observation of BMV particles in extracts of germinated uredospores (Figure 25) indicates that viruliferous uredospores carry BMV. No BMV particles were observed in similar extracts of non-viruliferous uredospores. The presence of BMV on the outside of the viruliferous uredospores is indicated by the observation of BMV particles in washings of viruliferous uredospores (Figure 26).

Although the detection of BMV particles in an extract of washed viruliferous uredospores (Figure 27) indicates the presence of BMV inside these uredospores, this cannot be regarded as being conclusive since the particles observed could also be due to residual virus particles on the uredospore coat that were not removed by washing. An attempt was made at detecting BMV in extracts of germ tubes that were free of uredospore coat (see IV.E.), in order to find conclusive evidence for the internal

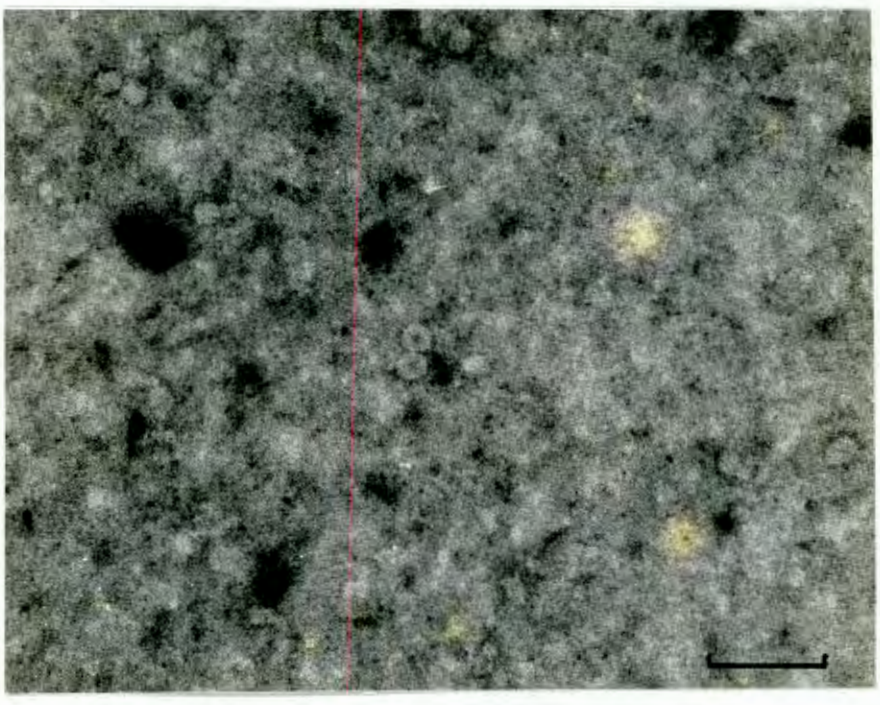


FIGURE 25: Electron micrograph of an IEM assay of a germinated, viruliferous uredospore extract. Grids were coated with anti-BMV antiserum No. 3 at 1/64 dilution. Bar = 100nm.

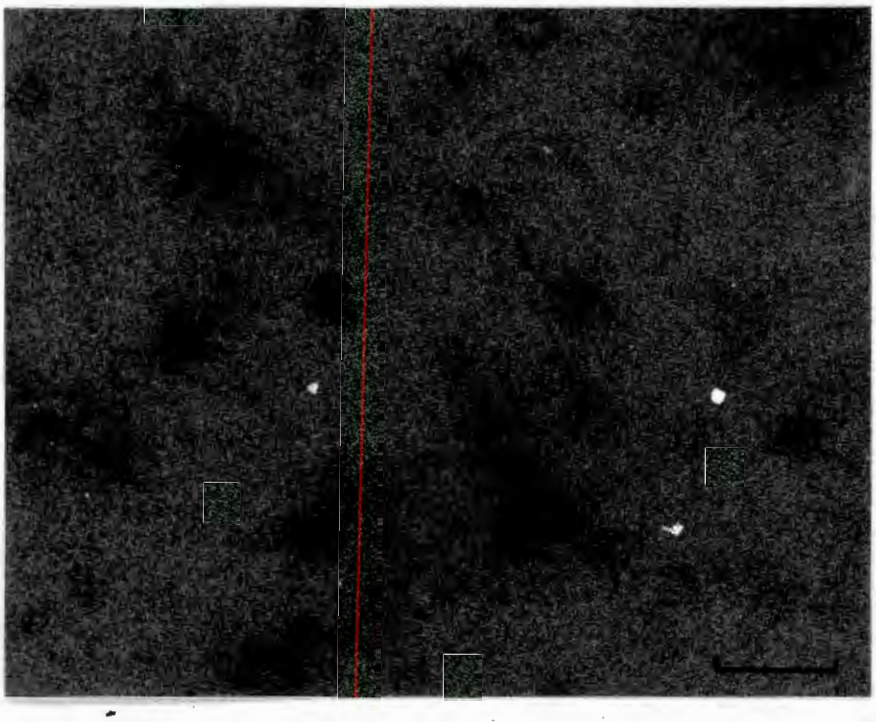


FIGURE 26: Electron micrograph of an IEM assay of washings of viruliferous uredospores. Grids were coated with anti-BMV antiserum No. 3 at 1/64 dilution. Bar = 100nm.

carriage of BMV by viruliferous stemrust uredospores. Such attempts failed and can largely be attributed to the technical difficulties encountered in collecting sufficient germ tubes free of uredospore coat.

In all the three cases in which BMV particles were detected in uredospore samples, only a few BMV particles were observed in each specimen. This explains why only a few particles can be observed in the electron micrographs in Figures 25, 26 and 27. Although only a few BMV particles were observed in uredospore samples,the IEM technique positively indicated that viruliferous uredospores carry BMV. Confirmation for the location of BMV on the outside of viruliferous stemrust uredospores comes from the detection of BMV particles in washings of such uredospores (Figure 26). The location of BMV within viruliferous uredospores by thin sectioning yielded inconclusive results. Virus-like particles similar in morphology to BMV could be observed in some thin sections of uredospores. These particles are indicated by the arrows in Figure 28. Only a few scattered BMV-like particles can be observed in this electron micrograph. It is difficult to concede that these virus-like particles are indeed BMV. Thus, from the results obtained with thin sectioning alone it is best not to conclude that the viruliferous stemrust uredospores harbour BMV within themselves. However, the results obtained with ELISA do indicate that this is possible.

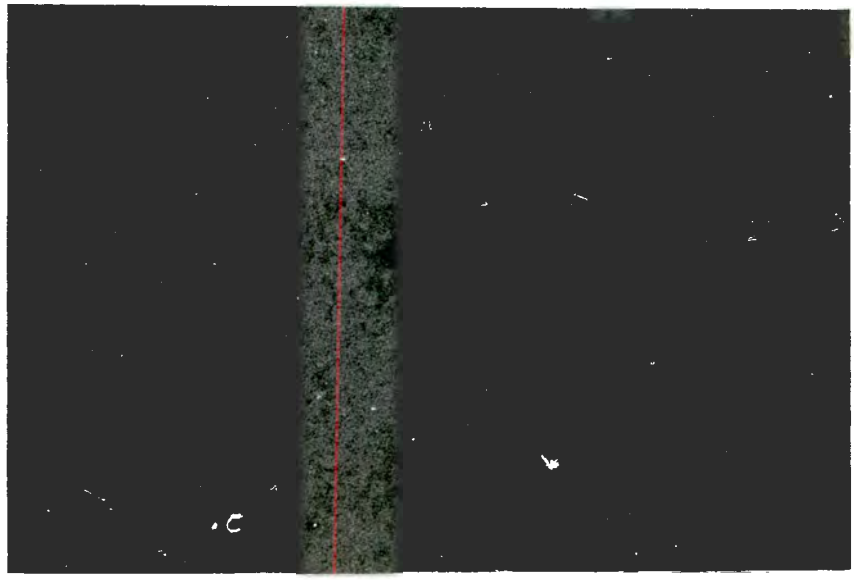


FIGURE 27: Electron micrograph of an IEM assay of an extract of washed viruliferous uredospores. Grids were coated with anti-BMV antiserum No. 3 at 1/64 dilution. Bar = 100nm.

Since thin sectioning of biological specimens is such a highly specialised technique, it is possible that the technique of thin sectioning employed in this study was not very efficient in illustrating the presence of BMV in the uredospores. Extensive and intricate thin sectioning techniques would be required for the localisation of BMV within the uredospores. This would be a study on its own and was beyond the scope of this project. Ferritin-labelled antibody binding studies were done by an adaption of the post-embedding technique described for the localisation of bacterial antigens by Walker et al. (1971). This technique is not described in Chapter III, Materials and Methods, since no positive results were obtained. An attempt was made at using ferritin-labelled antibodies for the localisation of BMV on the uredospore coat. In these experiments, viruliferous uredospores were mixed with anti-BMV antiserum No 3, washed and then incubated with ferritin-labelled goat anti-rabbit antibodies. The treated uredospores were thin sectioned, and the specimens were observed for the presence of ferritin granules adsorbed to the uredospore coat. In several such experiments, no ferritin deposits could be seen in association with the uredospore coat. The failure of these experiments to localise BMV on the uredospore coat was attributed to the possible removal of the antibodies from the uredospore coat during the processing of the specimens for thin sectioning.

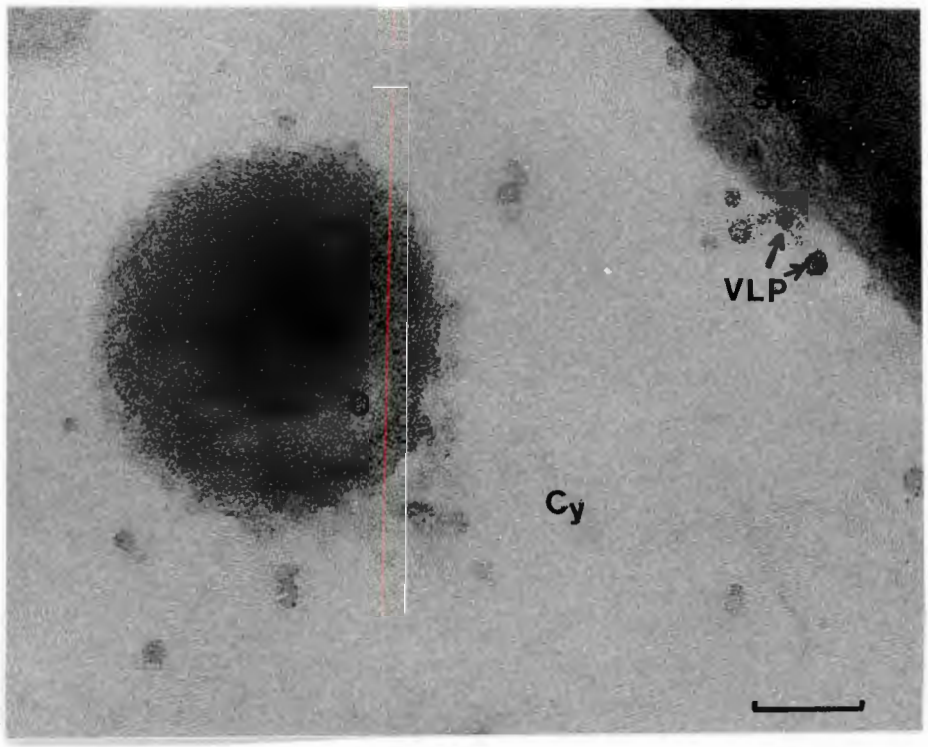


FIGURE 28: Detection of BMV-like virus particles in a thin section of viruliferous uredospores

O = organelle, Sc = uredospore coat, Cy = cytoplasm, VLP = virus-like particle. Bar = 150nm

G. CHARACTERISATION OF STEMRUST-TRANSMITTED-BMV

The stemrust-transmitted-BMV isolates were compared with regard to pH stability, electrophoretic migration and serological reactivity. This was done in order to determine whether these fungus-transmitted BMV isolates differed from mechanically transmitted BMV.

1. STABILITY OF STEMRUST-TRANSMITTED-BMV

The successful extraction of stemrust-transmitted-BMV at pH 7,0 (in contrast to traditional extraction) raised the possibility of the existence of a variant of BMV-Beth that was more stable at pH 7,0 than at pH 5,0 (see IV.D.2). This was done by checking the stability of the stemrust-transmitted-BMV by Ouchterlony tests performed at pH 7,0 and at pH 5,0. In these tests the stability of the stemrust-transmitted-BMV was determined from the nature of the precipitin band that is formed when it is reacted against antiserum No. 3 containing antibodies against intact virus and virus subunits.

The following criteria were used:

(a) a sharp precipitin band indicated stability at that pH.

(b) a broad diffused band and multiple precipitin bands indicated instability at that pH.

An interesting finding was that stemrust-transmitted-BMV-Beth was stable at both pH 5,0 and pH 7,0. The results of these experiments are illustrated in Figure 29. Stemrust-transmitted-BMV-Beth was shown to be stable at both pH 5,0 and pH 7,0. The same result was obtained for BMV-type, but in this case a sharper precipitin band can be observed. The stability of stemrust-transmitted-BMV-Beth at pH 7,0 explains why it was possible to extract BMV from plants inoculated with viruliferous uredospores, by working at pH 7,0.

The mechanically-transferred, stemrust-transmitted-BMV-Beth was found to be totally unstable at pH 7,0, but was stable at pH 5,0 (see Figure 29, well 6). This result indicates that the pH 7,0 stable variant is lost upon mechanical transfer of the stemrust-transmitted-BMV.

2. ENZYME-LINKED IMMUNOSORBENT ASSAY

The serological reactivities of stemrust-transmitted-BMV isolates, mechanically transmitted BMV-Beth, BMV-Palala, BMV-Type and BMV-G_9 were compared in ELISA. Two antisera were used in this investigation, namely anti-BMV antiserum No. 3 and anti-BMV protein antiserum No. 8. The assays were performed at pH 6,0. The results obtained with the anti-BMV antiserum (No. 3) appear in Figure 30. Since the curves are virtually inseparable from each other, it appears that all the

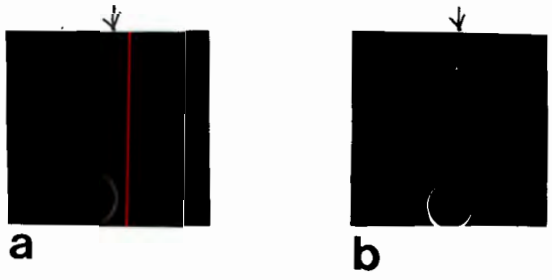


FIGURE 29: Stability of stemrust-transmitted BMV-Beth and mechanically transmitted BMV-Beth at pH 5,0 and pH 7,0 in gel precipitin tests. In both cases anti-BMV antiserum was at 1/4 dilution. The arrows indicate the starting points.

FIGURE 29(a): Gel buffer = Acetate/NaCl, pH 5,0
Antiserum No. 3 in central well.
Arranged clockwise (from arrow) in surrounding wells are: stemrust-transmitted-BMV-Beth, BMV-Type, BMV-Beth infected plant sap, healthy plant sap, mechanically transmitted BMV-Beth and leafrust-transmitted-BMV.

FIGURE 29(b): Gel buffer = phosphate/NaCl, pH 7,0
Antiserum No. 3 in central well.
Arranged clockwise (from arrow) in surrounding wells are: leafrust-transmitted-BMV, stemrust-transmitted-BMV-Beth, BMV-Type, BMV-Beth infected plant sap, healthy plant sap and mechanically transmitted BMV-Beth.

viruses are closely related to each other. According to the reactivities observed from the curves, it can be seen that BMV-G_9 is the most reactive of all. BMV-type, BMV-Palala and stemrust-transmitted-BMV-Beth appear as a group, and BMV-Beth and stemrust-transmitted-BMV-Palala appear together.

Thus, the stemrust-transmitted-BMV isolates differ serologically from their mechanically transmitted counterparts, being more reactive in one case and less reactive in the other. For instance, stemrust-transmitted-BMV-Beth is more reactive than mechanically transmitted BMV-Beth, whereas stemrust-transmitted-BMV-Palala is less reactive than mechanically transmitted BMV-Palala. The two stemrust-transmitted-BMV isolates differ significantly from each other, with stemrust-transmitted-BMV-Beth being the most reactive.

An unexpected observation in this assay was that the homologous reaction between BMV-type and its antiserum was of a lower order than that of some of the heterologous reactions. For example, the reaction of BMV-G_9 was greater than that of BMV-Type with its own antiserum.

The reactivities of the BMV isolates with anti-BMV protein antiserum (No. 8) appear in Figure 31. More discernable differences between the isolates were obtained using this antiserum. The order of reactivities

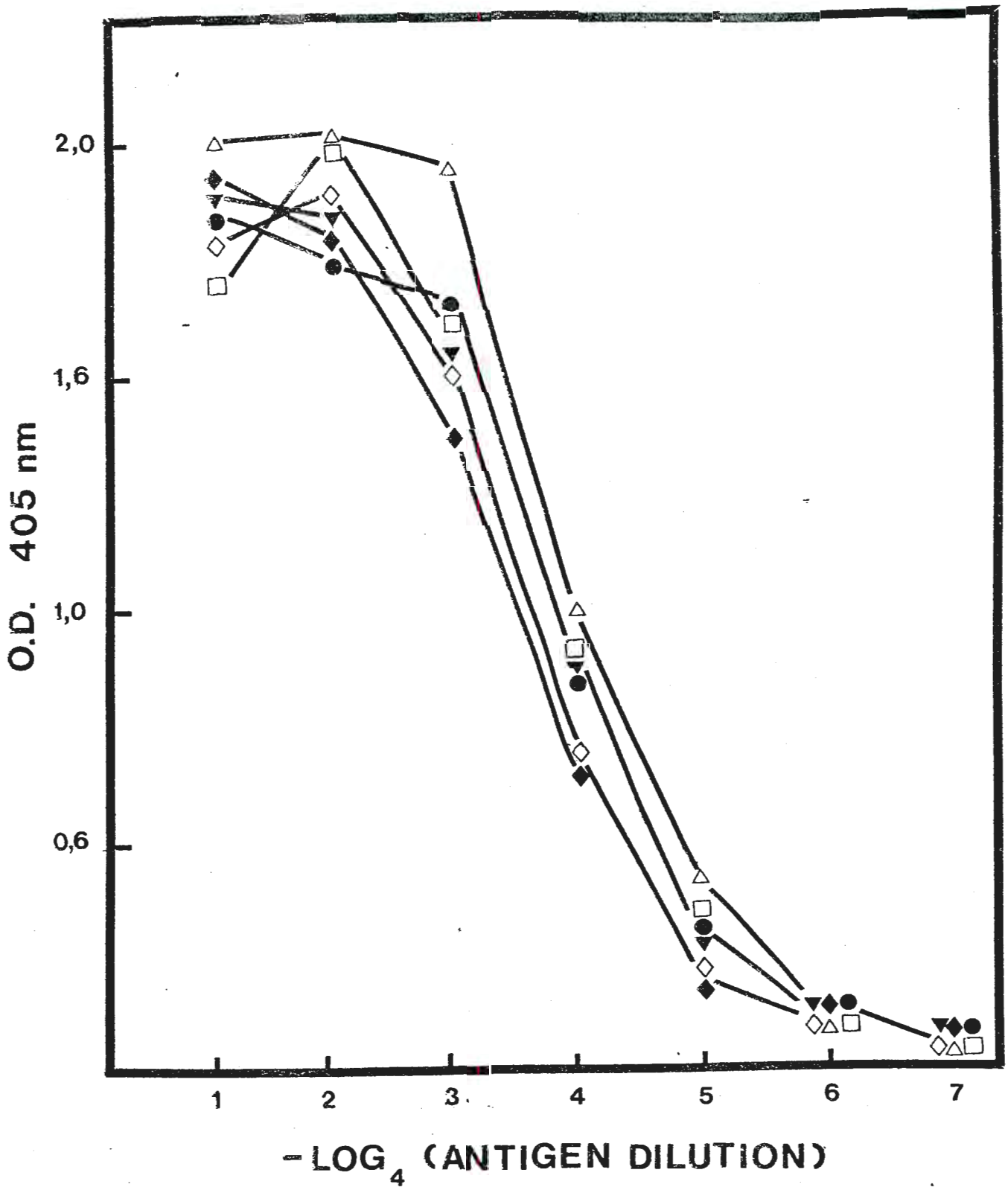

2,0
1,6
1,0
0,6
O.D. 405 nm
1
2
3
4
5
6
7
-LOG4 (ANTIGEN DILUTION)

FIGURE 30: Reactions of stemrust-transmitted-BMV and mechanically transmitted-BMV preparations with anti-BMV antiserum No. 3 in sandwich ELISA.

(△) = BMV-G_9
(□) = BMV-Palala
(Ō) = BMV-Beth
(●) = BMV-Type
(▼) = stemrust-transmitted-BMV-Beth
(◆) = stemrust-transmitted-BMV-Palala

Coating antibody concentration was at 5 µg/ml and conjugate dilution was at 1/1000. All the BMV preparations were at an initial concentration of 0,4 mg/ml.

are BMV-G_9 > stemrust-transmitted-BMV-Palala > stemrust-transmitted-BMV-Beth > BMV-Type, BMV-Palala and BMV-Beth. Again, it was possible to demonstrate that the stemrust-transmitted-BMV isolates -BMV-Palala and -BMV-Beth, differed significantly from their mechanically transmitted counterparts. In this assay, both the stemrust-transmitted-isolates were found to be more reactive than their mechanically-transmitted counterparts. Of the mechanically transmitted viruses, BMV-G_9 was most reactive and was clearly distinguishible from BMV-Type, BMV-Palala and BMV-Beth which appeared as a group.

The results obtained in both of the assays are based on the reaction of one antiserum in each case. Although it was possible to demonstrate differences between the stemrust-transmitted-BMV isolates and the mechanically transmitted BMV isolates, these results should have been supported by reactions with antisera from many rabbits. However, the production and the utilisation of many antisera for more stringent serological differentiation was not within the scope of this project.

3. HORIZONTAL-SLAB AGAROSE GEL ELECTROPHORESIS

Three stemrust-transmitted-BMV isolates were compared electrophoretically with respect to mechanically transmitted BMV-G_9, BMV-Type and BMV-Beth. This comparison was done at pH 6,0, pH 6,5 and pH 7,0.

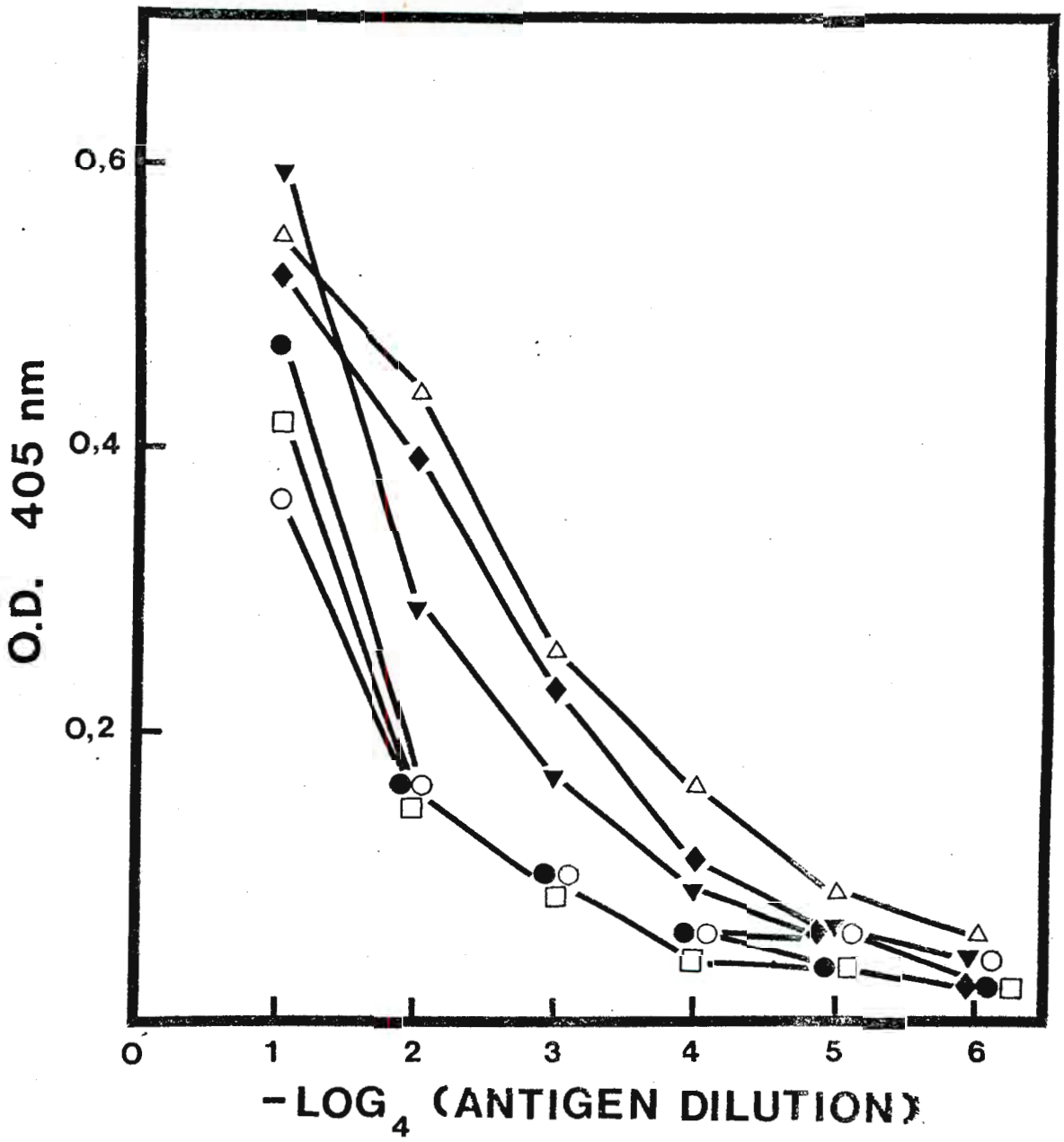


FIGURE 31: Reactions of stemrust-transmitted-BMV and mechanically transmitted-BMV preparations with anti-BMV protein antiserum No. 8 in sandwich ELISA.

Symbols have the same meaning as those in Figure 30. The conditions of this assay were identical to those presented in the legend of Figure 30.

The results of these experiments appear in Figure 32. In Figure 32(a), the electrophoretic mobilities at pH 6,0 are illustrated. All the viruses are electropositive. BMV-Type, BMV-Beth, stemrust-transmitted-BMV-Beth (extracted from Clipper, barley) and stemrust-transmitted-BMV-Palala have similar mobilities and have migrated the furthest towards the cathode. BMV-G_9 and stemrust-transmitted-BMV-Beth (extracted from T_4-R, wheat) exhibit slower mobilities. The mobilities of the latter two viruses appear to be identical.

Figure 32(b) illustrates the electrophoretic mobilities at pH 6,5. All the viruses, except BMV-G_9 are electropositive. BMV-G_9 clearly differs from the other viruses at this pH since it migrated towards the anode. BMV-Type, BMV-Beth and stemrust-transmitted-BMV-Beth (ex. Clipper) have similar mobilities and are the most electropositive. Stemrust-transmitted-BMV-Beth (ex. T_4-R) and stemrust-transmitted-BMV-Palala which have similar mobilities are less electropositive. Figure 32(c) illustrates the electrophoretic mobilities obtained at pH 7,0. At this pH all the viruses are electronegative. The order of the rates of migration of the viruses towards the anode, starting at the fastest, is BMV-G_9, stemrust-transmitted-BMV-Beth (ex. Clipper), BMV-Beth, BMV-Type and stemrust-transmitted-BMV-Beth (ex. T_4-R). Stemrust-transmitted-BMV-Palala was

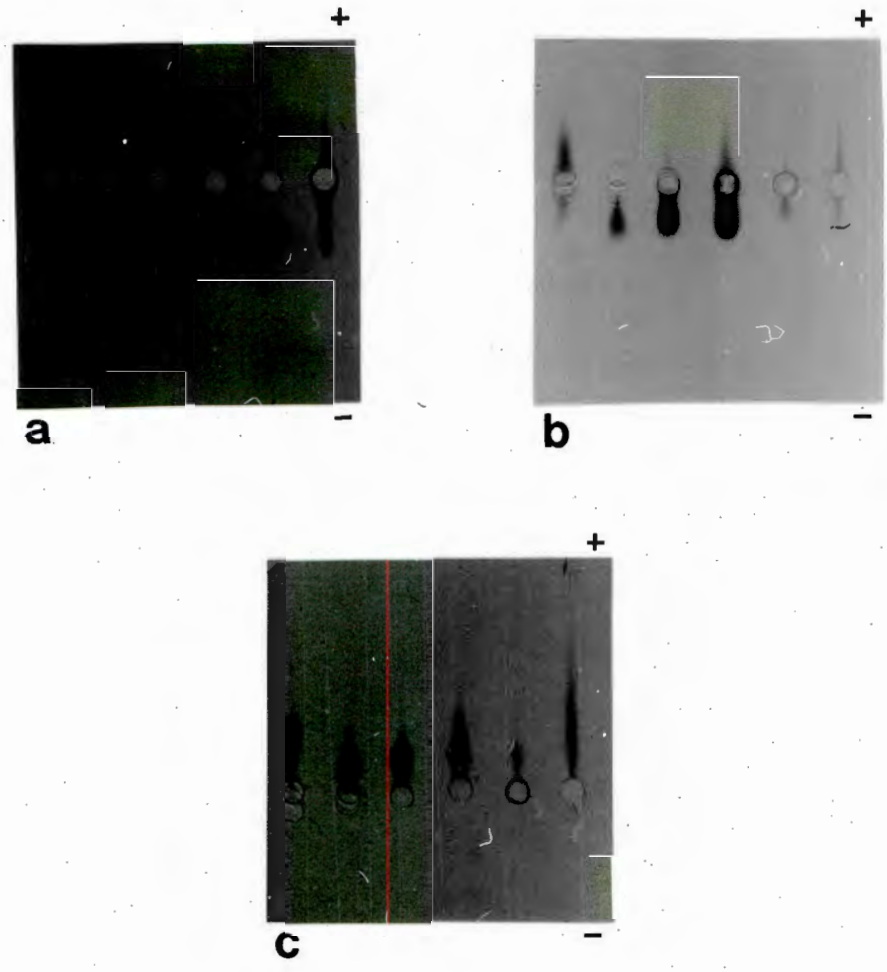


FIGURE 32: Electrophoresis of stemrust-transmitted-BMV and mechanically transmitted BMV.

(a), (b), (c) = electrophoresis at pH 6,0, pH 6,5 and pH 7,0 respectively.

In all three cases, electrophoresis was carried out at 5 V/cm for 2 1/2 hours. The sample distribution from left to right is: BMV-S_9, BMV-Type, BMV-Beth, stemrust-transmitted-BMV-Beth (extracted from Clipper), stemrust-transmitted-BMV-Beth (extracted from T_4-R) and stemrust-transmitted-BMV-Palala

unstable at this pH as only a protein streak can be observed.

All the viruses, except stemrust-transmitted-BMV-Palala appeared to be relatively stable at all three pH values since they largely migrated as discrete zones. Only, stemrust-transmitted-BMV-Palala appeared to be more unstable at pH 6,5 and pH 7,0 since protein streaks can be observed.

The electrophoretic mobilities of the viruses with respect to the migration of bromophenol blue (BPB) was determined in each case, and was expressed as a ratio. These electrophoretic indices (Ei) are presented in Table 5. The positive and negative signs associated with the electrophoretic indices indicate the migration of the viruses towards the cathode and anode respectively.

In the final analysis of these results, it can be seen that at pH 6,5 and pH 7,0 stemrust-transmitted-BMV-Beth and stemrust-transmitted-BMV-Palala differ electrophoretically from the mechanically transmitted BMV isolates.

TABLE 5: Comparison of mobilities of stemrust-transmitted-BMV isolates to mechanically transmitted BMV.

VIRUS	Ei values		
	pH 6,0	pH 6,5	pH 7,0
BMV-G_9	+ o,11	- 0,07	- 0,11
BMV-Type	+ 0,12	+ 0,08	- 0,06
BMV-Beth	+ 0,12	+ 0,08	- 0,065
stemrust-transmitted-BMV-Beth (ex. clipper)	+ 0,12	+ 0,09	- 0,08
stemrust-transmitted-BMV-Beth (ex. T_4-R)	+ 0,11	+ 0,06	- 0,07
stemrust-transmitted-BMV Palala	+ 0,12	+ 0,05	-

Ei = electrophoretic index, which is described as the mobility of the virus relative to that of bromophenol blue.

DISCUSSION

A. ACQUISITION

The susceptibility of BMV-infected plants to stemrust infection decreased as BMV multiplication in the plant increased. If stemrust inoculation was carried out after 5 days post virus inoculation (p.v.i.) a considerable decrease in the yield of uredospores from BMV-infected plants was observed (see Figure 6). Thus, in order to obtain a good average yield of viruliferous uredospores during routine acquisition studies, BMV-infected source plants were always inoculated with non-viruliferous uredospores at 5 days (p.v.i.). It was postulated that inoculation prior to 5 days p.v.i. would not allow the virus enough time to build up in the plant tissue, thus limiting the possible uptake of virus by the fungus.

In vivo acquisition of BMV by the rust fungus was shown to occur, since non-viruliferous uredospores acquired the virus by propagation on BMV-infected plants. *In vivo* acquisition has been described for, amongst others, *Olpidium brassicae* with Lettuce big-vein agent (BVA) (Tomlinson and Garret, 1962; Campbell and Grogan, 1964); *Synchytrium endobioticum* with Potato virus X (PVX) (Nienhaus and Stille, 1965) and *Polymyxa graminis* with soil-borne

wheat mosaic virus (WSSMV) (Slykhuis and Barr, 1978). The *in vivo* acquisition of BMV by stemrust appears to be similar to that of PVX by *Synchytrium endobioticum*.

Attempts at allowing non-viruliferous uredospores to acquire BMV *in vitro* by mixing uredospores with a purified BMV preparation failed (IV.D.4.b). No BMV was extracted from plants inoculated with uredospores treated in this manner. However, it cannot be concluded that *in vitro* acquisition of BMV by stemrust uredospores could not occur, since the short incubation period of non-viruliferous uredospores with purified BMV could have been insufficient to allow optimal association of BMV with these uredospores.

The brief washing step that followed after mixing could have removed most of the BMV that was not tightly bound to the uredospores. The short incubation period of 3 minutes was necessary in order to prevent pre-germination of the uredospores. This seemed to have occurred in any case since a low level of stemrust infection was observed on plants inoculated with non-viruliferous uredospores that were mixed with BMV. The possible disorientation of the protoplasmic components within the uredospores, caused by the centrifugal separation of uredospores from the virus suspension, could have rendered the uredospores non-viable. This in addition to the pre-germination of the uredospores during these *in vitro* acquisition experiments

could account for the low level of stemrust infection observed. Obviously, the experimental *in vitro* acquisition does not simulate the natural *in vivo* acquisition mechanism (see V.E. on modes of acquisition).

B. TRANSMISSION

1. SYMPTOMATOLOGY

Wheat plants inoculated with viruliferous uredospores showed symptoms of yellow leaf tips, and barley plants displayed a more severe overall yellowing and brown streaking (see Figure 9). The plants did not show mild mosaic symptoms which are usually displayed by plants mechanically inoculated with BMV. Thus, the presence of BMV in these plants could easily be overlooked.

The totally different expression of symptoms in plants infected with BMV by stemrust-transmission compared to that obtained by mechanical-transmission, can be regarded as the cumulative effect both stemrust and *stemrust transmitted-BMV* infection had on these plants. The growth of both of these pathogens in the same plant and the consequent exhaustion of the plant metabolism could give rise to the different symptom expression observed. The instantaneous infection of many host cells by BMV

during mechanical inoculation allows for rapid multiplication of the virus in individual plant cells, thus giving rise to mosaic symptoms.

It is postulated that BMV infection of host cells is a slow process during stemrust-transmission since the virus has to rely on the fungus and environmental factors for its penetration into the plant tissue. (This is further discussed under modes of transmission - V.E.). The low concentration of BMV in and on the viruliferous uredospores would also affect the rate of infection of BMV in the plant host during stemrust-transmission. These differences in the route of infection of the plant by BMV may account for the diferences in symptom expression.

2. EXTRACTION OF STEMRUST-TRANSMITTED-BMV AND ITS STABILITY

To determine whether viruliferous uredospores transmitted BMV to healthy plants, plants inoculated with these uredospores were processed for virus. It was impossible to extract BMV from plants that were inoculated with viruliferous uredospores by working at pH 4,0 and pH 5,0 which is the normal pH for BMV extraction. However, the fungus-transmitted-BMV was successfully extracted at pH 7,0. The presence of BMV in these purified and crude extracts were examined by infectivity assay, rate zonal centrifugation, electron microscopy, Ouchterlony

tests and ELISA. The yield of stemrust-transmitted-BMV was found to be 1-2 mg/Kg leaf tissue which for BMV is an extremely low yield.

The successful extraction of stemrust-transmitted-BMV from plants can be attributed to the finding that these virus isolates were stable at pH 7,0 (see IV.G.1). However, when stemrust-transmitted-BMV was mechanically transmitted, it was possible to extract this mechanically-transferred, stemrust-transmitted-BMV by working at pH 5,0. This is ascribed to the finding that the latter isolates were stable at pH 5,0 but unstable at pH 7,0 (see IV.G.1). It is necessary to realise that both pH 5,0 and pH 7,0 stable variants of BMV could co-exist in the mechanically inoculated BMV-infected source plants. Acquisition of BMV by stemrust from these plants could quite feasibly involve the selection of a variant that is more stable at pH 7,0. The acquisition of some variants that are stable at pH 5,0 is not excluded. Thus, it seems likely that the rust fungus selectively transmits a variant of BMV that is more stable at pH 7,0. It would therefore be expected that a purified preparation of stemrust-transmitted-BMV would contain pH 7,0 stable variants and some pH 5,0 stable variants with the variant that is more stable at pH 7,0 predominating.

Since the stemrust-transmitted-BMV has to multiply in plants that are infected with the fungus, it is possible

that the pH 7,0-stable variant of BMV is adapted to cope with the change in metabolism of the plant caused by infection with the fungus vector. During mechanical transmission of the stemrust-transmitted-BMV, the pH 5,0 stable variant, that is present in a lesser proportion, probably is more virulent than the pH 7,0 stable variant. The virulent nature of the pH 5,0 variant in this instance is probably due to the absence of biochemical changes in the plant which occur during fungus transmission. The pH 5,0 stable variant thus predominates during mechanical transmission. This possibly explains why mechanically-transferred, stemrust-transmitted-BMV is more stable at pH 5,0 than at pH 7,0.

The differences in pH stability of the isolates were further supported by comparison of their serological reactivity and electrophoretic mobility (see IV G) indicating that actual biochemical differences existed. This possibly explains the hypothesis that stemrust selectively acquires and transmits a variant of BMV that is different from that transmitted upon mechanical inoculation.

The inability to extract BMV from plants inoculated with non-viruliferous uredospores indicated that these uredospores did not carry BMV. Indeed, this was verified by techniques such as ELISA, fluorescent antibody binding studies and electron microscopy which revealed that no BMV was associated with non-viruliferous uredospores.

(IV.F.1., 2. and 3.). In addition to this, the activation of latent BMV within the plants by stemrust infection is excluded. Thus extraction of BMV from plants inoculated with viruliferous uredospores is definitely due to the transmission of BMV by these uredospores.

Attempts at inhibiting transmission of BMV by stemrust by inactivation with antiserum yielded inconclusive results(IV.D.3.c.).

C. THE EFFECT OF BMV INFECTION IN PLANTS ON SUBSEQUENT STEMRUST INFECTION

BMV infection was found to have an adverse effect on stemrust infection, resulting in a decrease of stemrust infection according to the length of virus establishment in the plant (see Figures 5 and 6 - IV.B.).

Dual infection of plants with both virus and fungi have been found to be synergistic in some cases (Nitzany, 1966; Raju *et al.*, 1969; Beute, 1970 and Nitzany *et al.*, 1974); antagonistic, in which viral infection caused protection against fungal infection (Latch and Potter, 1977 and McIntyre and Dodds, 1979); and in others shown to have no effect at all (Raju *et al.*, 1969; Latch and Potter, 1977).

The antagonistic interaction between BMV and stemrust

infection in wheat observed in this study appears to be similar to the interactions between ryegrass mosaic virus (RMV) and Puccinia coronata (Latch and Potter, 1977), and Tobacco mosaic virus (TMV) and Phytophthora parasitica var. nicotianae (McIntyre and Dodds, 1979). The induced protection of plants to fungus infection by prior virus infection is probably due to the triggering off of different protection mechanisms by a generalised stress response (McIntyre and Dodds, 1979).

The finding that BMV-infection had an adverse effect on stemrust infection has significant bearing on the occurrence of stemrust infection of plants in the Orange Free State. The low incidence of stemrust infection in the Orange Free State (von Wechmar, personal communication) could largely be due to the epidemic proportion of BMV in wheat in this region (von Wechmar and Rybicki, 1981). Since BMV-infection is well established in wheat and pasture grasses in this region before the advent of stemrust infection, it is highly likely that these BMV-infected wheat plants have an apparent induced protection against stemrust infection. The result would be a low visible incidence of stemrust infection. Since virus infection of plants can have either a synergistic or antagonistic effect on fungus infection, it is important that the possible presence of virus in plants is taken into account during the screening and evaluation of field trials for fungus infection generally.

D. DETECTION OF BMV IN AND ON STEMRUST UREDOSPORES

In order to get an idea of the mode of transmission of BMV by stemrust, it was important to discover the nature of the association of BMV with viruliferous uredospores.

The implementation of sensitive techniques such as ELISA, fluorescent antibody binding techniques and immunosorbent electron microscopy made it possible to detect the minute quantities of virus trapped in or on the uredospore.

1. ELISA RESULTS

ELISA revealed that viruliferous uredospores carried BMV internally and on the surface of the spore coat (IV.F.1). The reactions obtained with the "crushed"- and "crushed, germinated"-uredospore sample indicated that viruliferous uredospores carry BMV, but no deduction can be made as to the location of the virus associated with these uredospores. The nature of the carriage of BMV by viruliferous uredospores was revealed by the reactivities of the "spore-wash"-uredospore sample and the "crushed, washed-germinated"-uredospore sample. These reactivities respectively indicated that BMV was carried on and inside the uredospores. Since the reactions of these two samples were of the same order (best illustrated in Figure 21), it seems likely that a similar quantity of virus is carried inside and on the uredospores. However, no definite calibration as to the content of the virus

associated with viruliferous uredospores could be made.

The process of washing the uredospores effectively removed the BMV that was adsorbed to the spore coat, since the "crushed, washed"-uredospore sample was virtually non-reactive. The weak reaction that was obtained can be accounted for by the presence of BMV within the uredospores which were ruptured during crushing. Only 20% of intact uredospores were ruptured by crushing with a mortar and pestle (III.H.2.b). The low reactivity of the "crushed, washed"-uredospore sample when compared to the higher reactivity obtained with the "crushed, washed-germinated"-uredospore sample can be attributed to the inefficiency of the crushing procedure to rupture intact uredospores. However, germinated uredospores were ruptured most efficiently by crushing with a mortar and pestle (III.H.2.b). Thus, more spore contents and consequently more virus are released than from crushed, ungerminated uredospores. This explains the higher reactivity of the "crushed, washed-germinated"-uredospore sample compared to that of the "crushed,washed"-uredospore sample. Since the washing step effectively removed BMV from the surface of the uredospore, the reaction of the "crushed, washed-germinated"-uredospore sample can in all probability be attributed to the release of BMV carried inside the viruliferous uredospores.

The reactions of the uredospores samples were shown to be specific for BMV: no reaction was obtained when normal

rabbit serum was substituted for anti-BMV antiserum, and the same anti-BMV antibodies did not react with an unrelated virus, WCuMV, but did react positively with purified BMV in assays performed under identical conditions (see IV.F.1).

Non-viruliferous uredospore samples prepared in the same way as viruliferous uredospore samples were non-reactive in ELISA. This result provides confirmation that non-viruliferous uredospores used in this study were indeed BMV-free.

In conclusion, it can be said that ELISA revealed a close association of BMV with viruliferous uredospores, and that the virus was carried both inside and on the uredospores.

2. FLUORESCENT ANTIBODY BINDING STUDIES

The fluorescent antibody staining technique positively indicated the presence of BMV on the surface of viruliferous uredospores. Similar results were obtained with both the indirect and the direct staining technique. The fluorescence observed on viruliferous uredospores (Figures 22(a) and 23(a)) was specific for BMV since substitution of normal rabbit serum for anti-BMV antibodies yielded none or lesser fluorescence (Figure 22(c)). Fluorescence was also decreased by the inhibition of the

binding of anti-BMV antibodies by purified BMV and by unlabelled anti-BMV antibodies (Figure 23(b)).

Although some non-specific fluorescence was observed to occur with the indirect staining procedure, the results obtained with this method were confirmed by the direct staining procedure. No non-specific staining was observed to occur with the direct procedure. The result obtained when viruliferous uredospores were stained by the direct method, indicated that not all the uredospores collected off BMV-infected plants carried BMV on their spore coats (Figure 23(a)). Whether all these uredospores carried BMV inside remains an open question.

3. ELECTRON MICROSCOPY

Aliquots of the same uredospore samples used in ELISA were screened for the detection of BMV by immunosorbent electron microscopy, which gave further confirmation that BMV was both on and inside viruliferous uredospores. The observation of BMV particles in extracts of germinated uredospores substantiated the ELISA results that viruliferous uredospores carried BMV (Figure 25). No BMV particles were observed in extracts of non-viruliferous uredospores. Confirmation of the presence of BMV on the outside of viruliferous uredospores was indicated by the observation of BMV particles in washings of viruliferous uredospores (Figure 26). Although the detection of BMV in an extract

of washed viruliferous uredospores (Figure 27) indicated the presence of BMV inside the uredospores, this cannot be regarded as being conclusive since the observed BMV particles could also have resulted from residual virus particles on the spore coat that were not removed by the washing procedure. On the other hand, ELISA results showed that the washing procedure (III.H.2.b) effectively removed BMV that was adsorbed to the outside of viruliferous uredospores (V.E.1.). In all probability the detection of BMV by immunosorbent electron microscopy in this uredospore sample is due to the presence of BMV within viruliferous uredospores.

The high reactivity of viruliferous uredospores in ELISA and the intense fluorescence of these spores indicate that a substantial number of BMV particles were associated with these uredospores. The fact that only a few BMV particles could be observed in the electron microscope could be attributed to the very small sample aliquot that can be examined in this manner. The highest number of BMV particles were observed in spore washings of viruliferous uredospores. This finding is in agreement with the ELISA and fluorescent antibody staining result, on similar preparations, indicating external carriage of BMV on the uredospore coat.

Considerable difficulties were experienced with thin sectioning. The chitinous spore coat is tough and

escapes smooth sectioning, resulting in squashed specimen sections. Only a few scattered BMV-like particles could be observed in prepared specimens (see Figure 28).

It is difficult to concede that these particles are indeed BMV.

Spherical virus-like particles (VLPs (38mm in diameter) have been observed in thin sections of Puccinia graminis tritici grown in axenic culture (Rawlinson and MacLean, 1973) and were considered to be mycoviruses. The BMV-like particles observed in thin sections of uredospores do not appear to be identical in size to these VLPs.

E. MODES OF ACQUISITION AND TRANSMISSION

Acquisition: It has been shown that non-viruliferous uredospores of stemrust when grown on BMV-infected plants acquire BMV by an in vivo acquisition system, and that these viruliferous uredospores transmit BMV to healthy plants when these plants are infected with such uredospores.

In vivo acquisition of BMV by non-viruliferous uredospores could occur in a combination of two modes. Growth of the fungal mycelium in host tissue infected with BMV could allow for the uptake of BMV within the mycelium of the fungus. Ehrlich and Ehrlich (1961, cited in

Alexopoulus, 1962) have suggested that exchange between the contents of the protoplasm of the haustorium and the protoplasm of the host cell can occur via ultra-microscopic pores in the haustorial cell wall of the fungus. These authors have shown that a sheath of naked protoplasm exists between the haustorial cell wall and the host protoplasm. If this protoplasmic exchange between the plant cell and the fungus haustorium exists, it appears to be possible that BMV could be taken up into the haustorium if this fungal structure occurs within BMV-infected plant tissue. The BMV that is taken up by the haustorium can then be passed along with nutrients through the fungal mycelium where it eventially can be packaged into the uredospores.

It is unlikely that the BMV taken up in this manner would finally become lodged on the uredospore coat. Therefore, another mode of acquisition must operate in order to explain how uredospores carry BMV on their surfaces. This external carriage of BMV by the uredospores can probably occur at the time when the epidermis of the leaf is ruptured to release the mature uredospores. It is postulated that the rupturing of the epidermis causes rupturing of BMV-infected plant cells in the vicinity of the emerging uredospores. The leakage of BMV from these ruptured cells could allow for intimate contact between the uredospores and BMV. Since the uredospore coat is rough, it is possible that the BMV when it comes into

contact with the uredospore coat lodges itself within the crevices on the spore coat. The BMV is probably not tightly bound to the uredospore coat. This would explain why it is possible to remove BMV from the uredospore coat by washing of the uredospores in buffers containing weak detergent. Also, if this mode of acquisition is in operation for the external carriage of BMV by the uredospores, one would not expect all the uredospores that are collected off virus-infected plants to have BMV associated with their spore coats. The results obtained with the direct fluorescent staining method on viruliferous uredospores indicate that this is the case (see Figure 23(a)).

It has been shown that certain mechanically transmissible plant viruses (Southern bean mosaic virus and tobacco mosaic virus) are closely associated with the exine layer of pollen (Hamilton et al., 1976) derived from plants infected with these viruses. Thus, the association of BMV with the outside of the uredospore which like pollen has a rough surface is feasible.

The second mode of acquisition of BMV by the uredospores is not supported by the inability to show that non-viruliferous uredospores acquire BMV in vitro. Maybe, adsorption of BMV to the uredospore coat which occurs on the plant is governed by a number of factors. For instance, the BMV in fungus-virus-infected plants could

be chemically altered to a certain extent. It has been shown that stemrust-transmitted-BMV differs biochemically to mechanically transmitted BMV. These differences exhibited by stemrust-transmitted-BMV have been ascribed to the possible adaptation of the virus to conditions within stemrust-infected plants. It therefore seems likely that the binding of BMV to the spore coats of uredospores occurring on BMV-infected plants could depend on a slightly modified variant which is then possibly linked to the uredospore coat by chemical compounds occurring within infected leaf cells. Thus, if such a system for binding of BMV to uredospores occurs in the plant, then the absence of these binding factors in in vitro acquisition experiments explains the inconclusive results obtained with this experimental system.

Experiments in which BMV is chemically linked to uredospores was not designed, but it is possible that such experiments and the use of BMV-infected sap instead of purified BMV in in vitro acquisition experiments could effect in vitro acquisition of BMV by uredospores.

Transmission: The detection of BMV both in and on the uredospores provides for two possible modes of transmission of the virus by stemrust. The first mode of transmission concerns the fate of BMV carried inside the uredospores. It is possible that during infection of the plant with viruliferous uredospores, the BMV is carried along in

the fungal protoplasm of the infection hyphae that infect the host tissue. Thus, BMV occurring in the mycelium could enter the plant host cells by the reverse of the method for acquisition of the virus by the haustorium. A similar mode of transmission (and also this type of acquisition) has been postulated by Tomlinson and Garret (1964) for the transmission of lettuce big-vein agent (BVA) by Olpidium brassicae and by Hiruki (1965) for the transmission of tobacco stunt agent (TSA) by the same fungus. Campbell (1978) has also suggested that one of the chracteristics of a fungal vector should be "an ectoplast-limited thallus that allows exchange of virus between the fungus and the host cell".

The second mode of transmission involves a facultative mechanical entry into the plant of BMV carried on the surface of the uredospores. That this could happen was indicated by spray-inoculating plants with purified BMV suspensions. This inoculation method caused puncturing of host surface cells allowing BMV to enter (see IV.D.4.a). Puncturing of plant leaves can occur in naturally growing field plants by the effects of wind and sand, mechanical contact between plants and insect feeding points. Thus, it is quite possible that BMV carried on the surface of the uredospores enters the plants via punctures on the surface cells of the plant. Transmission of viruses associated with the outside of pollen has been suggested to occur via entry of these viruses through

abrasions on the leaf on which the pollen lands (Hamilton et al., 1976). It is unlikely that the BMV carried on the surface of the uredospore can enter the plant at the point of hyphal penetration, since the appresorium that is formed over the stomatal pore through which the hyphae penetrate, effectively plugs the stomata after the infection hyphae has entered the host tissue. Since the cell-walls of the infection hyphae are not continuous with the outer layer of the uredospore coat, carriage of the BMV on the outside of the uredospore coat into the plant tissue by these hyphae is not likely.

Both of the modes of transmission described above could occur jointly under natural conditions. The efficiency of transmission of BMV would be greatly affected by the mode of transport of the uredospores in nature, which could occur by winds or be insect-borne (e.g. aphids and their predators). The transmission of BMV on the outside of the uredospore can be considered to be similar, in many respects, to the transmission of viruses borne on the exine layer of pollen.

It has been found that T_4-R wheat and Clipper barley (resistant to stemrust and leafrust respectively), when inoculated with viruliferous uredospores still had BMV transmitted to them. This finding can be explained by the suggested mode of transmission describing entry of the virus carried on the outside of the uredospore, since

this mode of transmission does not rely on infection of the plant by the fungus. This situation has considerable implication since it means that both resistant and susceptible cultivars are vulnerable to the transmission of BMV by the rust fungus.

F. IMPLICATIONS OF THE TRANSMISSION OF BMV BY STEMRUST

The transmission of BMV by the stemrust fungus, which belongs to the Uredinales, is the first report of the transmission of a plant virus by a highly specialised fungal phytopathogen. This is a unique example, since all the reports on active transmission of plant pathogenic viruses by fungi deal with fungi belonging to the lower orders, Chytridiales and Plasmodiophorales.

The simultaneous infection of Agropyron distichum by stemrust and BMV (see Figure 1) indicates that a long-standing relationship between BMV and stemrust under natural conditions could exist. The finding that stemrust transmits BMV poses the possibility that the transmission of plant viruses by other fungi infecting virus-infected wheat or other plants could occur and these possible associations should be investigated. The availability of contemporary sensitive techniques makes this possible.

The transmission of BMV by stemrust in the laboratory

has important application to the field condition occurring in the Orange Free State (and possibly elsewhere) where BMV has been found to be epidemic. Since it has been shown that aphid-transmission of BMV is to a large extent responsible for the epidemic proportion of BMV in theOrange Free State (von Wechmar and Rybicki, 1981) one could ask the quesiton: Is stemrust also an important vector?

It is possible that the rust fungus initially transmits the virus to the plants and that the aphid vectors acquire BMV from these plants and rapidly spread the virus throughout the plant population. Simultaneous transmission of other viruses (Barley yellow dwarf virus, an aphid-borne picornavirus, Barley Stripe mosaic virus and sugarcane mosaic virus; Rybicki and von Wechmar, 1982, and von Wechmar, unpublished) by the aphid vectors could result in a synergistic reaction amongst the viruses during their simultaneous multiplication in the plant. This synergism could lead to an explosive multiplication of BMV, resulting in an epidemic situation as the numbers of aphid vectors and consequently the frequency of transmission of the viruses increase.

Since resistance to rust infection does not protect a plant from the transmission of BMV by the fungus uredospores, the choice of the suitability of a cultivar for cropping should not only take into account the

susceptibility of that cultivar to stemrust but also its susceptibility to virus infection.

It would be interesting to know whether the mycoviruses associated with rust fungi and several other plant pathogenic fungi (see Table 1) are possibly transmitted to their plant hosts. Also investigating plant pathogenic fungus-virus associations generally, may throw new light on disease patterns and epidemics.

CONCLUSIONS

The transmission of BMV by stemrust was established. It was possible to show that non-viruliferous uredospores when grown on BMV-infected plants acquired the virus *in vivo*. The resulting viruliferous uredospores were able to transmit the BMV to healthy plants, since BMV was extracted only from plants inoculated with these uredospores. It was also shown that leafrust could transmit BMV, but this situation was not closely examined. It is possible that this fungus-virus relationship is similar to that of BMV transmission by stemrust since these two fungi are closely related.

BMV has been shown to be intimately associated with viruliferous uredospores by techniques such as ELISA, fluorescent antibody binding studies and electron microscopy which revealed the presence of BMV in and on the uredospores.

Genetic resistance of a particular cultivar to the fungus did not prevent the transmission of the virus by the fungus.

Virus infection of plants was shown to have an adverse effect on stemrust infection. Plants visibly became

less susceptible to stemrust infection as the virus progressively became more established in the tissue.

It was shown that mechanically transmitted BMV had different stability characteristics to stemrust-transmitted-BMV.

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